POPULATION STRUCTURE AND GENE FLOW IN THE CHIPPING SPARROW AND A HYPOTHESIS FOR EVOLUTION IN THE GENUS *SPIZELLA*

ROBERT M. ZINK[1] AND DONNA L. DITTMANN[1]

ABSTRACT.—We studied restriction site variation in the mitochondrial DNA (mtDNA) of 55 Chipping Sparrows (*Spizella passerina*) taken from widely dispersed points in their breeding range. A total of 21 haplotypes was observed, and on average, individuals differed little in percent haplotype divergence (0.12%). There was no detectable geographic variation in haplotypes, despite the sampling of three named subspecies. Single-generation dispersal distance was estimated from the mtDNA data at 5.4 km. One phylogenetic hypothesis for six species in the genus (excluding Timberline Sparrow [*S. taverneri*] and Worthen's Sparrow [*S. wortheni*]) suggested that Black-chinned Sparrow (*S. atrogularis*) and Field Sparrow (*S. pusilla*) were sister taxa, followed in sequence by Chipping, Brewer's and Clay-colored sparrows (*S. pallida*), with the American Tree Sparrow (*S. arborea*) most distant. Another hypothesis grouped Chipping and Brewer's sparrows. *Received 24 Aug. 1992, accepted 3 Feb. 1993.*

An important step in the evolutionary process is the origin of geographic patterns of genetic variation. Patterns of genetic variation form the basis for inferences about evolutionary processes that lead to the origin and maintenance of geographic variation and speciation. In the last 20 years, a number of molecular techniques, including protein electrophoresis and restriction site analysis of mitochondrial DNA (mtDNA; Avise et al. 1987), have been used to describe genetic variation within and among avian populations.

For north temperate birds, protein electrophoresis revealed few alleles that varied geographically (Barrowclough 1983). From this result, many researchers inferred that avian populations were essentially panmictic, and that gene flow was substantial (Barrowclough 1983, Barrowclough and Johnson 1988). Studies of mtDNA opened a new era because of several advantageous aspects of mtDNA evolution (Moritz et al. 1987). In particular, mtDNA appears to evolve faster than most types of nuclear DNA (including that which encodes allozymes), providing an enhanced probability of detecting geographic variation. Indeed, mtDNA studies reveal an array of population structures (Ball et al. 1988, Avise and Nelson 1989, Zink 1991, Avise and Ball 1991, Moore et al. 1991, Shields and Wilson 1987a, Ball and Avise 1992, Zink and Dittmann, in press). For instance, Avise and Nelson (1989) found two geographic groups of mtDNA lineages in the Seaside Sparrow (*Ammodramus maritimus*). Zink (1991,

[1] Museum of Natural Science, Louisiana State Univ., Baton Rouge, Louisiana 70803. (Present address RMZ: Bell Museum of Natural History, Ecology Bldg., Univ. of Minnesota, St. Paul, Minnesota 55108.)

in press) found significant mtDNA variation in the Fox Sparrow (*Passerella iliaca*), whereas in an allozyme survey no geographic differentiation was detected (Zink 1986). In the Song Sparrow (*Melospiza melodia*), which exhibits extensive geographic variation in morphology, there was considerable mtDNA variation but essentially no geographic structure (Zink and Dittmann, in press).

To date, mtDNA studies of geographic variation in birds have tended to involve species that exhibit geographic variation in morphology. Consequently, we know little about the genetic structure of species that exhibit little or no geographic variation in external phenotype. Because it is often assumed that geographic variation in external phenotypes reflects underlying genetic structure, it is appropriate to examine the geographically uniform species. Such comparisons will clarify the relationship between morphological and genetic variation. In this paper, we describe a survey of mtDNA restriction site variation in the Chipping Sparrow (*Spizella passerina*), a widespread migratory North American passerine bird (Fig. 1). Taxonomic variation in the Chipping Sparrow in North America (north of Mexico) is limited to three weakly demarcated subspecies (AOU 1957). Our samples were taken from regions as far apart as North Carolina and the Yukon. We estimate the degree of population subdivision, level of gene flow, and the correspondence of mtDNA variation and subspecies limits. To put our study in a phylogenetic perspective, we also provide an estimate of the relationships of species in the genus, with the exception of the Timberline Sparrow (*S. taverneri*) and Worthen's Sparrow (*S. wortheni*), species for which we lacked tissue samples.

METHODS

Samples (Table 1) were collected during the breeding season from 13 sites (Fig. 1). From each of the 55 specimens, samples of liver, heart, and pectoral muscle were frozen in liquid nitrogen. Upon return to the laboratory, mtDNA was purified from tissues following procedures in Lansman et al. (1981) and Dowling et al. (1990). Purified mtDNA from each individual was digested with 20 restriction endonucleases (see Appendix I), end-labeled with ^{35}S, and electrophoresed in 0.7% to 1.1% agarose gels. Fragments were visualized with autoradiography. A letter was assigned to each distinct restriction fragment profile. The combination of an individual's letters for all endonucleases constitutes its composite haplotype. Each individual was scored for the presence or absence of each restriction fragment for each endonuclease. Fragment profiles produced by each endonuclease differed from each other by one or two restriction sites, making inference of the presence or absence of restriction sites straightforward. The matrix of restriction sites was used to estimate the percent nucleotide divergence (p) among haplotypes following Nei and Li (1979). A computer program written by J. E. Neigel was used to estimate single generation dispersal distance (Neigel et al. 1991). The method requires a phenogram based on mtDNA genetic distances (p-values), generation length (we assumed that two years was reasonable), geographic distance between each pair of localities, and an estimate of mtDNA evolutionary rate, for which we used one percentage per lineage per million years (Shields and Wilson 1987b). The procedure involves
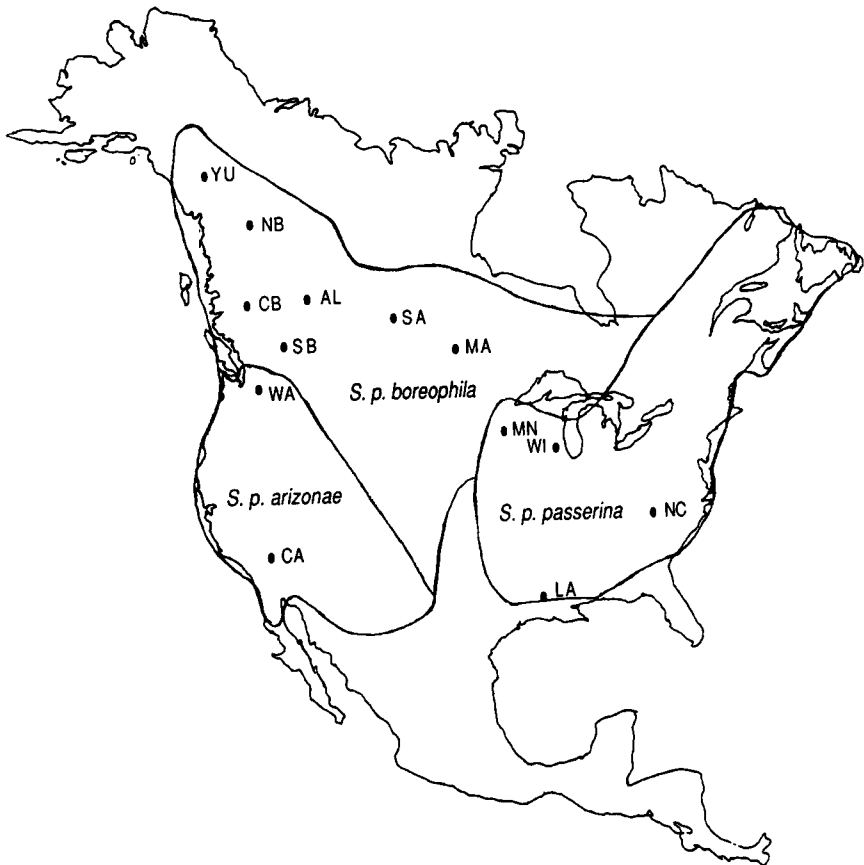
FIG. 1. Approximate breeding distribution of the three subspecies of the Chipping Sparrow, and location and codes of collecting sites (see Table 1).

dividing the phenogram into 10 equal sections based on level of divergence (beginning at p = 0.0), and estimating the dispersal distance for each section. It is expected (J. E. Neigel, pers. comm.) that the youngest lineage classes (i.e., the first few sections in the phenogram) will provide the best estimate of dispersal distance because they likely have not been limited or confined by geographic barriers to gene flow (therefore the relationship between genetic and geographic distance is monotonic). The matrix of restriction sites was analyzed by the computer program HENNIG86 (Farris 1989) to infer a phylogenetic network using the principle of maximum parsimony. The matrix of p-values was analyzed using the FITCH routine in the computer program PHYLIP (Felsenstein 1987), which produces a distance tree following the method of Fitch and Margoliash (1967). Also, a UPGMA phenogram was produced from the matrix of p-values (Sneath and Sokal 1973). For the phylogenetic analysis of species, only the pattern of restriction fragments (see Appendix 2) was analyzed because we lacked financial resources to map restriction sites; see Zink and Avise (1990) for justification. One individual per species was used. Both HENNIG86 and PHYLIP were used to

TABLE 1

DISTRIBUTION OF SAMPLES (SEE FIG. 1) AND COMPOSITE HAPLOTYPES FOUND AT EACH SAMPLE SITE[a]

Geographic site (code)	N	Haplotype (s)
1. North Carolina (NC)	1	1
2. Wisconsin (WI)	2	1 (2)
3. Minnesota (MN)	2	1 (2)
4. Louisiana (LA)	3	1 (2), 2
5. Manitoba (MA)	8	1 (4), 3, 7, 15, 21
6. Saskatchewan (SA)	7	1 (3), 4, 5, 16, 17
7. Alberta (AL)	3	1, 6, 7
8. Yukon (YU)	2	1 (2)
9. Northern British Columbia (NB)	7	1 (4), 8, 18, 19
10. Central British Columbia (CB)	3	1, 7, 10
11. Southeastern British Columbia (SB)	5	1 (3), 20, 21
12. Washington (WA)	3	1 (2), 9
13. Southern California (CA)	9	1 (5), 11, 12, 13, 14

[a] Numbers of individuals with particular haplotype in parentheses. Samples 1–4 are from *S. p. passerina*, 5–11 from *S. p. boreophila*, and 12–13 from *S. p. arizonae*.

analyze the fragment matrix as described above. We also used the bootstrap procedure (Felsenstein 1985) in PHYLIP to evaluate the strength of the phylogenetic pattern obtained; because not all restriction fragments are independent, we do not interpret bootstrap values statistically but rather as a description of the data (Zink and Avise 1990).

RESULTS

Chipping Sparrow. — Based on restriction fragment profiles that yielded mtDNA fragments between 1000 and 8000 base pairs, we estimated the size of the mtDNA genome at 16,500. Five restriction endonucleases produced only a single pattern: *Ava* I, *Kpn* I, *Nci* I, *Sac* II, and *Stu* I. The other 15 endonucleases produced from two to four patterns (Appendix I). A total of 141 restriction sites was found. A total of 21 haplotypes was observed among the 55 Chipping Sparrows studied. Apart from haplotype 1, only haplotype 7 was identified in more than one individual (Table 1). Haplotype 1 occurred in every geographic sample and in 32 of the 55 individuals. Most haplotypes differed by one or two restriction sites. The average *p*-value among the 21 haplotypes was 0.28% ± 0.12 (SD; N = 210), and the range was from 0.06 to 0.62%. Among the 55 individuals, the average *p*-value was 0.12% ± 0.12 (SD), and the range was from zero to 0.62% (N = 1485). The distribution (Fig. 2) of *p*-values among all pairs of haplotypes reveals that most individuals have very similar mtDNA genomes. The topology of the UPGMA phenogram depends on the input order of haplotypes (because of identical values in the distance matrix),
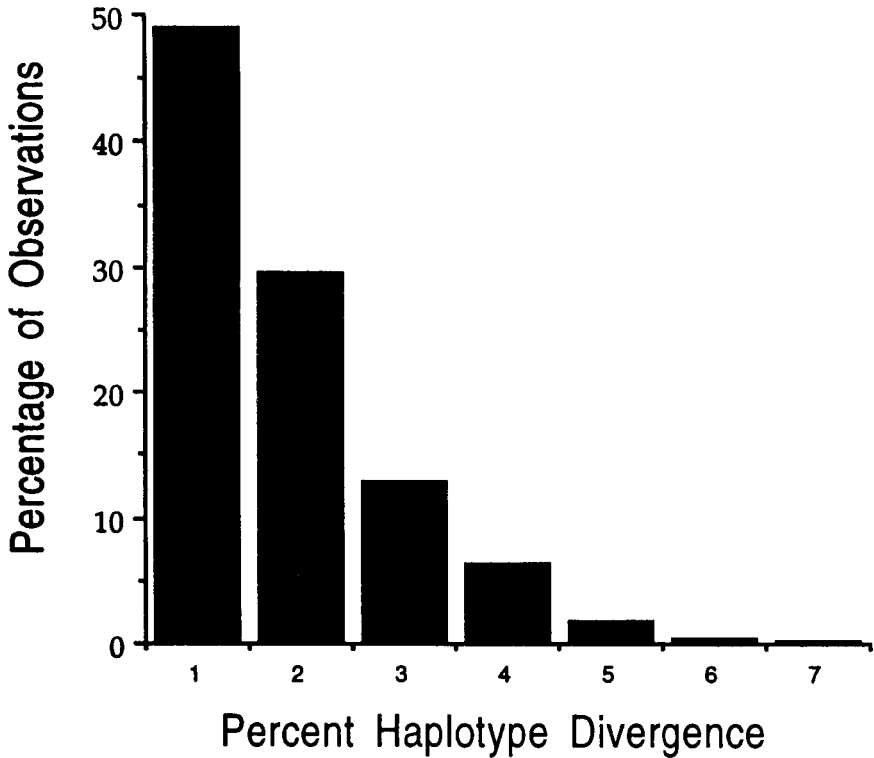
FIG. 2.   Distribution of pairwise haplotype distances among all individuals.

but in none was the phenogram geographically informative. For example, in one phenogram (not shown), a cluster contained haplotypes 9 (Washington), 12 (southern California), and 17 (Saskatchewan). The parsimony analysis found over 100 equally most-parsimonious trees (length 33, consistency index 0.84, retention index 0.58). The cladograms were geographically uninformative (as was the strict consensus). For example, a most parsimonious cladogram (Fig. 3) showed that the five haplotypes from southern California (1, 11, 12, 13, 14) were not sister taxa and in fact were distributed throughout the tree. In no instance did the cladogram show sister haplotypes being from the same sample. The estimate of gene flow is 5.4 km/generation (based on the youngest class of lineages).

Evolution of *Spizella* Species.—A total of 198 restriction fragments was scored for the six *Spizella* species and the outgroup, the Dark-eyed Junco (*Junco hyemalis*) (Appendix II); the Chipping Sparrow haplotype 1 was used to represent that species. MtDNA data reveal considerable differentiation among species (Table 2), with p-values ranging from 0.036
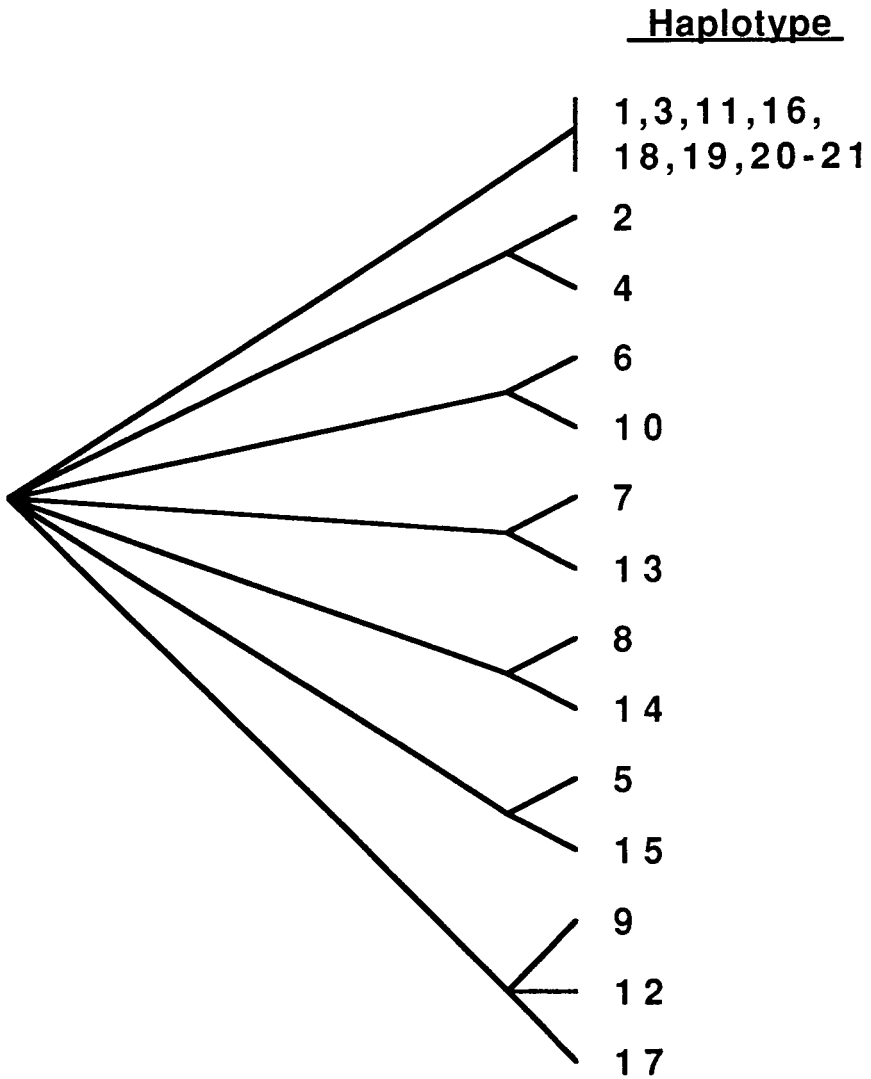
Haplotype

FIG. 3. Most parsimonious cladogram of 21 Chipping Sparrow haplotypes. For geographic location of haplotypes, see Table 1.

(Chipping Sparrow vs Brewer's Sparrow [*Spizella breweri*]) to 0.151 (American Tree Sparrow [*S. arborea*] vs Field Sparrow [*S. pusilla*]); the average among *Spizella* species was 0.078 ± 0.041 (SD). The Fitch-Margoliash (F-M) tree (Fig. 4B) portrays the American Tree Sparrow as basal in *Spizella*, with Clay-colored Sparrow (*S. pallida*), Chipping Spar-
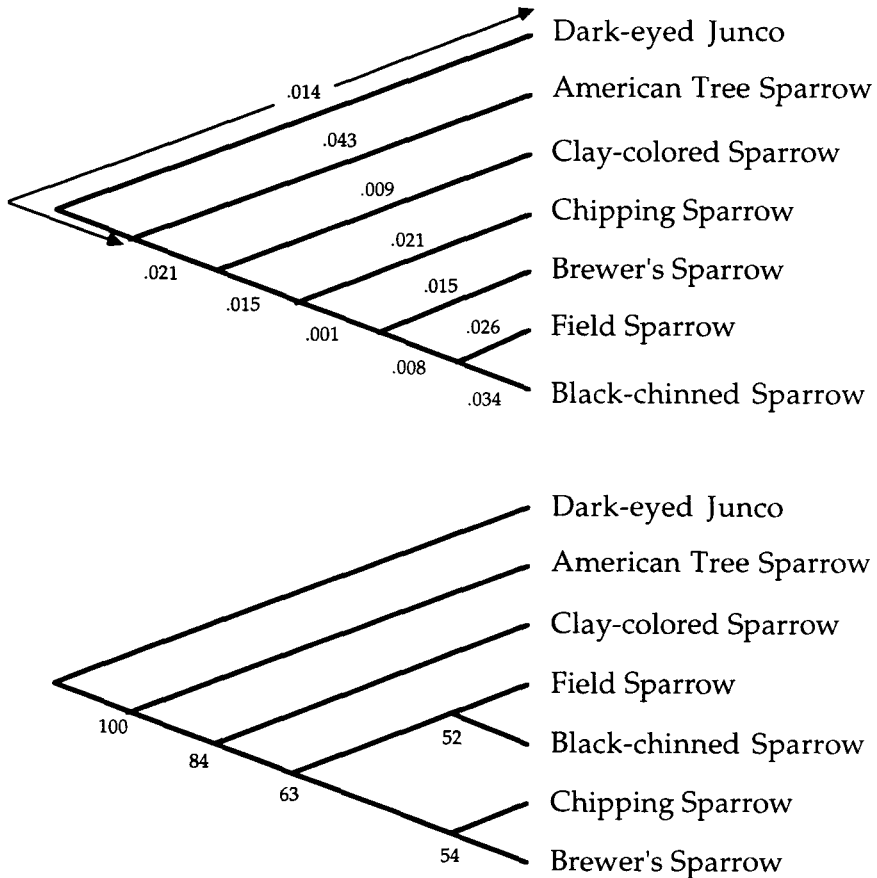
FIG. 4. A (bottom). Single most parsimonious cladogram of species of *Spizella*. Numbers indicate times out of 100 bootstrap replicate trees that that node occurred. B (top). Fitch–Margoliash tree; branch lengths in units of *p*.

row, and Brewer's Sparrow successively more derived relative to the Field Sparrow and Black-chinned Sparrow (*S. atrogularis*), the latter of which were sister species. Branch lengths on the F-M tree suggest rate hetero-geneity, especially concerning the American Tree Sparrow and Clay-col-ored Sparrow. A single most parsimonious tree (Fig. 4A; length 237, consistency index 0.75, retention index 0.42) was found, which differs from the F-M tree in showing Chipping Sparrow and Brewer's Sparrow as sister taxa. Bootstrap analysis reveals the same tree topology (Fig. 4A), and shows that the strength of support for this pattern in the fragment data matrix is not high. To evaluate the topology produced by the F-M

TABLE 2
MATRIX OF *P*-VALUES AMONG SPECIES OF *SPIZELLA*

1. Dark-eyed Junco	0.000						
2. Chipping Sparrow	0.065	0.000					
3. Black-chinned Sparrow	0.081	0.061	0.000				
4. American Tree Sparrow	0.056	0.132	0.096	0.000			
5. Clay-colored Sparrow	0.051	0.043	0.141	0.062	0.000		
6. Field Sparrow	0.121	0.058	0.061	0.151	0.049	0.000	
7. Brewer's Sparrow	0.056	0.036	0.062	0.131	0.039	0.046	0.000

procedure, we input it into HENNIG86, and found its length to be 238 (consistency index 0.75, retention index 0.41), only one step longer than the shortest tree. Therefore, we would not interpret the differences between the two topologies with confidence.

DISCUSSION

Geography of mtDNA variation in the Chipping Sparrow.—The average number of haplotypes per individual (21/55 or 0.38%) is equivalent to that found in other passerine birds (Ball et al. 1988, Zink et al. 1991b, Zink 1991) and indicates that genetic variation exists in the mtDNA genome. Although a large part of the range was sampled, Chipping Sparrows exhibit no geographic differentiation in mtDNA restriction sites. Most individuals possess the same haplotype (1) or one at most a few steps removed from it. The mtDNA population structure thus consists of a group of closely related haplotypes that are not geographically structured. Thus, the three named subspecies (Fig. 1) have no support from the mtDNA data. However, absence of geographic variation does not mean that there is not genetic differentiation in other parts of the genome, such as that which might underlay the phenotypic variation partitioned taxonomically into subspecies.

The maximum distance (or depth) among extant haplotypes detected in our study, $p = 0.6\%$, is much less than the distance among the Chipping Sparrow and its relatives (minimum $p = 3.6\%$; Table 2). The extant haplotypes in the Chipping Sparrow thus trace to a common maternal ancestor that lived well after the separation of the Chipping Sparrow from other *Spizella*. The depth of the haplotype tree in the Chipping Sparrow is similar to that in some avian species (e.g., Red-winged Blackbird [*Agelaius phoeniceus*]; Ball et al. 1988), but "shallower" than that in others (e.g., Song Sparrow; Zink and Dittmann, in press). It is possible that a bottleneck culled haplotype lineages at a time equal to the accumulation of 0.6% sequence divergence. Stochastic lineage sorting could produce

such a distribution of haplotype lineages (Neigel et al. 1991). Given the calibration of mtDNA of 1% per lineage per million years (Shields and Wilson 1987b), the common (maternal) ancestor of extant haplotypes in the Chipping Sparrow existed about 300,000 years ago.

The mtDNA data also are consistent with the possibility that the range of the Chipping Sparrow recently expanded, as one would predict from the current distribution of Chipping Sparrows and Pleistocene glaciers (Pielou 1991). Rogers and Harpending (1992) suggest that a distribution of individual haplotype distances like that in Figure 2 suggests recent population expansion. The lack of geographic variation in mtDNA re-striction sites parallels several other avian species that also span recently unglaciated regions (Ball et al. 1988, Zink et al. 1991b, Moore et al. 1991). Recent range expansion and insufficient time *in situ* could explain lack of geographic variation in mtDNA. Across the same area as that surveyed here, Zink (in press) found four distinct groups of mtDNA haplotype lineages in the Fox Sparrow (*Passerella iliaca*). It is apparent, therefore, that currently codistributed species need not have had the same histories.

The occurrence of sister haplotypes in different samples (Fig. 3) is potential evidence of gene flow (Slatkin and Maddison 1989). The dis-persal distance of 5.4 km/generation is consistent with those computed for other birds, both with and without geographic variation (Neigel and Avise, unpubl. data). It is as yet unknown whether this magnitude of gene flow prevents establishment of mtDNA geographic variation, as is pre-dicted for the immigration of a single individual per generation (for se-lectively neutral traits; Wright 1978). To examine the robustness of the dispersal estimate, we compared the estimated dispersal distances at each of the 10 levels to those based on random permutations of the haplotype distributions. At most levels, the actual observed value was greater than random values, suggesting that the Chipping Sparrow lineages have achieved an equilibrium between dispersal and genetic drift (Neigel and Avise, unpubl. data). The estimate of 5.4 km, however, was less than that obtained from the randomized estimate for its class, which lends confi-dence to that estimate. Nonetheless, mark-recapture or dispersal estimates from other genetic markers should be used to test the mtDNA estimate.

The question of why the Chipping Sparrow lacks significant geographic variation in phenotypes, despite occupying a large range, is unanswered. Many factors contribute to the evolution of geographic variation (Zink 1986). Perhaps limitation to one habitat, or selective regime, such as open pine woods and edges, prevents development of geographic variation (Miller 1956). However, the sheer latitudinal area occupied must place individuals in different temperature and humidity regimes (James 1970; Aldrich and James 1991). Possibly the seemingly continuous distribution

of Chipping Sparrows allows for sufficient gene exchange to prevent dif-
ferentiation. Another potential reason for why a species might lack geo-
graphic variability concerns its age. A relatively young species might not
have had enough time to respond to different environmental conditions
and evolve geographic differences. The Chipping Sparrow is differentiated
from its nearest relative by at least 3.6% estimated sequence divergence
(Table 2). This value is about average for avian passerine congeneric
species in the temperate zone (Zink et al. 1991a). Of course, degree of
geographic variation might be related to the time since a species has been
widespread, which might be unrelated to the distance to its nearest con-
gener. Nevertheless, the Chipping Sparrow does not appear particularly
"young," and other reasons for the lack of geographic variation should
be sought. Substantial gene flow and recent population expansion are
possibilities.

Evolutionary relationships in Spizella. — Because the mtDNA genome
is inherited as a single linkage unit, it represents a single "gene tree." Our
mtDNA trees (Fig. 4A, B) therefore represent preliminary hypotheses of
Spizella phylogeny. It seems clear that the American Tree Sparrow is very
distant from other congeners, as noted by Mayr and Short (1970). The
mtDNA genetic distance from American Tree Sparrow to the other taxa
averages 0.105, whereas the same figure for the Dark-eyed Junco is 0.072
(Table 2). Although we lack the taxa to conclude if *Spizella* is monophy-
letic, this should be tested. Both trees also suggest that the Clay-colored
Sparrow is relatively basal. We assume that Worthen's Sparrow is the
sister taxon to the Field Sparrow (Mayr and Short 1970), and that Tim-
berline Sparrow is the sister taxon to Brewer's Sparrow (Sibley and Monroe
1990); these propositions should be tested.

Mayr and Short (1970) stated that the Clay-colored, Brewer's and Chip-
ping sparrows formed a species group (the "*passerina*" complex), which
presumably means that they are monophyletic. This result was not ob-
tained in either the F-M or parsimony trees (Fig. 4A, B); Mayr and Short's
hypothesis requires 260 steps relative to the 237 in the shortest parsimony
tree. Mayr and Short's (1970) grouping of taxa apparently results from
the taxonomic concept of the "grade" as opposed to that of the "clade"
(Wiley 1981). That is, these species share ancestral features, ones not
necessarily indicative of phylogenetic affinity.

Mayr and Short (1970) stated that the Black-chinned Sparrow might
be related to the *S. passerina* complex; our data do not support this
proposition. The proposed sister taxon relationship between Black-chinned
Sparrow and Field Sparrow (Fig. 4A, B) is consistent with the possession
of a pink bill, a potential synapomorphy. Other morphological characters
should be analyzed as well. In addition, their songs are similar (Robbins

et al. 1983), although this needs quantitative analysis. The species's distributions also suggest sister-taxon status, each being almost parapatric with the other. Within the genus, the Black-chinned Sparrow is phenotypically most differentiated, at least in plumage color and pattern. The mtDNA genetic distances (Fig. 4B) from the hypothesized most recent common ancestor to the Field and Black-chinned sparrows suggest that these two species have undergone relatively rapid evolution, which is consistent with plumage evolution in the Black-chinned Sparrow but not the Field Sparrow. The amount of anagenesis in the Black-chinned Sparrow (0.34) is even greater than that in the Field Sparrow (0.26; Fig. 4B), suggesting an accelerated rate in the former. These two species should be compared to determine if differences in demography or potential for sexual selection might explain the apparent difference in magnitude and rate of phenotypic evolution. That is, because the unique features of the Black-chinned Sparrow apparently originated since it last shared a common ancestor with its sister taxon, evolutionary causes might be inferred from its current demography or current biotic interactions (Brooks and Mc-Lennan 1991).

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APPENDIX I

LETTER CODES FOR RESTRICTION FRAGMENT PROFILES FOR COMPOSITE HAPLOTYPES OBSERVED IN CHIPPING SPARROWS AND RELATED SPECIES[a]

Haplo-type num-ber	Letter codes																			
1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
2	·	·	·	·	·	·	·	·	·	B	·	·	·	·	·	·	·	·	·	·
3	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
4	·	·	·	·	·	B	·	·	·	B	·	·	·	·	·	·	·	·	·	·
5	·	C	·	B	·	·	·	·	·	·	·	·	B	·	·	·	·	·	·	·
6	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	B	·	·
7	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·
8	·	·	·	B	·	·	·	B	·	·	·	·	·	·	·	·	·	·	·	·
9	·	·	·	·	·	·	B	·	·	·	·	·	·	·	·	B	·	·	·	·
10	·	B	·	C	·	·	·	·	·	·	B	·	·	·	·	·	·	·	·	·
11	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
12	·	·	·	·	·	·	B	·	·	·	·	·	·	·	·	·	·	·	·	·
13	·	·	·	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	·
14	·	·	·	·	·	·	B	B	D	·	·	·	·	·	·	·	·	·	·	·
15	·	·	·	B	·	·	·	·	B	·	·	·	·	·	·	·	·	·	·	·
16	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	B
17	·	·	·	B	·	·	B	·	·	·	·	·	·	·	·	·	·	·	·	·
18	·	·	·	D	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
19	·	·	·	·	B	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
20	·	D	·	·	·	·	·	·	·	·	B	·	·	·	·	·	·	·	·	·
21	·	E	F	·	·	·	·	·	·	·	·	·	D	·	·	·	·	·	·	·

[a] A "dot" signifes the "A" pattern. Sequence of restriction endonucleases: *Ava* I, *Ava* II, *Bam*H I, *Ban* II, *Bcl* I, *Bgl* I, *Bgl* II, *Eco*R I, *Hinc* II, *Hind* III, *Hinf* I, *Kpn* I, *Mbo* I, *Msp* I, *Nci* I, *Nde* I, *Pvu* II, *Stu* I, *Sac* II, and *Xba* I.

APPENDIX II
PRESENCE/ABSENCE MATRIX OF RESTRICTION FRAGMENTS IN SPECIES OF SPIZELLA AND DARK-EYED JUNCO[a]

Dark-eyed Junco

0100001001010010010011000000000000000000001100100000100000100100100101010000001100010110010001010000
1100110000000011101000101100010100000101000000001000100000000010011100011010001000101000001000100
0001

Chipping Sparrow

1111000000000010001000101000000000000011111111000010001100000000010110101111100000000001111110000000
0001110000000000100000110101010110111010000001000110011111011111000000100010001000010110010000001
1001

Black-chinned Sparrow

1000000000000100110000101000000000110000000010100000100010010011000011100110000000000011010101000000
100000000000010001011001100000000000000000000000000000000001111000100000010000000100011100100000000
0111

American Tree Sparrow

0000111000000001010010010000000001000001000000001000000101101101001010100000011101001100101011 0000
1000000011000000111001000000000100000000000011010100000000100001000111100000001110011000001010000
0000

Clay-colored Sparrow

0100000111000010001011000000000100000000000000001000101000001001001010100010011000000100101000000100
1000000000110000100010100011110100000010100000000110000000001011100000100010001000010110010000010
0001

Field Sparrow

1100001000000000101000000011110000000000000010100000001100000100100111110000001001000110000100000011
0010000000001000100010100011110100000010100000000100001000101100110000100011000010010101000000000
0001

Brewer's Sparrow

0100001000101010001000101000000000000111100100100001010000010000010101000000010011111101011000000
00000100000000100100010100011011100000010000100100101000100011110001000000010001000001011000101000
0001

ᵃ Sequence of restriction endonucleases used (number of fragments): *Ava* I (14), *Ava* II (26), *Bam*H I (10), *Ban* II (22), *Bcl* I (12), *Bgl* I (16), *Eco*R I (13), *Hinc* II (21), *Hind* III (22), *Nci* I (14), *Nde* I (15), and *Pvu* II (13).