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Phylogeographic population structure of Red-winged Blackbirds assessed by mitochondrial DNA

(intraspecific phylogeny/restriction sites/genetic distance/gene flow)

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ABSTRACT **A continent-wide survey of restriction-site variation in mitochondrial DNA (mtDNA) of the Red-winged Blackbird (*Agelaius phoeniceus*) was conducted to assess the magnitude of phylogeographic population structure in an avian species. A total of 34 mtDNA genotypes was observed among the 127 specimens assayed by 18 restriction endonucleases. Nonetheless, population differentiation was minor, as indicated by (*i*) small genetic distances in terms of base substitutions per nucleotide site between mtDNA genotypes (maximum $P \approx 0.008$) and by (*ii*) the widespread geographic distributions of particular mtDNA clones and phylogenetic arrays of clones. Extensive morphological differentiation among redwing populations apparently has occurred in the context of relatively little phylogenetic separation. A comparison between mtDNA data sets for Red-winged Blackbirds and deermice (*Peromyscus maniculatus*) also sampled from across North America shows that intraspecific population structures of these two species differ dramatically. The lower phylogeographic differentiation in redwings is probably due to historically higher levels of gene flow.**

Geographic patterns of nucleotide sequence divergence in mitochondrial DNA (mtDNA) have been described for conspecific populations within a number of vertebrate species (for reviews see refs. 1–4). Typically, intraspecific mtDNA polymorphism is extensive and geographically partitioned. For example, in a survey of deermice from across North America, 61 mtDNA genotypes (clones or haplotypes) were observed, and these were grouped into at least five distinct phylogenetic assemblages (clades) of molecules that characterized populations occupying separate regions of the continent (5). The list of species known to exhibit a qualitatively similar pattern of substantial phylogeographic population structure includes other small mammals, amphibians, reptiles, freshwater fishes, and the horseshoe crab (2). However, a few widely distributed species—most notably some marine fishes (for review see ref. 6), house mice (7), and, to an argued extent, humans (8, 9)—appear to show much less geographic differentiation in terms of mtDNA phylogeny.

It has been proposed (2, 10) that a major factor influencing mtDNA phylogeography is the historical pattern of gene flow (11), which is a joint function of (*i*) the intrinsic dispersal capabilities of a species and (*ii*) extrinsic (zoogeographic) impediments to movement. Under this interpretation, populations within species such as the assayed terrestrial mammals or freshwater fishes have distinct mtDNA structure due to limited realized gene flow over recent evolutionary time, while surveyed marine species that lack substantial geographic structure in mtDNA have had high historical interconnectedness, presumably due to considerable adult or larval dispersal in the relatively continuous marine environment.

Birds constitute another group of potentially highly mobile animals in which phylogeographic differentiation in mtDNA might commonly prove to be comparatively minor. In this paper, we present the first broad-scale geographic survey of mtDNA in an avian species. The Red-winged Blackbird (*Agelaius phoeniceus*) is an abundant species native to North America, with a nesting range extending from Alaska to Newfoundland and south to Cuba and Costa Rica. Northern populations are migratory, while most southwestern and Middle American populations are sedentary. We have chosen the redwing for this initial avian survey because, despite previous intense scrutiny, there remain significant grounds for dispute concerning the magnitude of population structure in this species. First, adult redwings exhibit considerable geographic variation in morphology (12–15), yet experimental transplants of eggs between nests show that a significant proportion of the regional differences in nestling development is nongenetic (16). Second, at least 23 subspecies have been recognized (17, 18), yet the magnitude of allozyme divergence is very small (Tom Gavin, personal communication). Third, redwings are moderately nest-site philopatric, usually nesting within 50 km of the hatching site (19), yet they have obvious dispersal capabilities, and populations occupy suitable nesting sites all across North and Middle America. We will show that mtDNA results can contribute to these deliberations by providing some knowledge of the historical, phylogenetic context of redwing population structure.

MATERIALS AND METHODS

A total of 127 Red-winged Blackbirds was collected during the 1986 nesting season from the following numbered breeding locales in the United States (14 states), Canada, and Mexico. Locales: 1, Edmonton, Alberta, Canada (subspecies *arctolegus*; $n = 4$); 2, El Carmen, Puebla, Mexico (*gubernator*; $n = 7$) and Coatetelco, Moreles, Mexico (*nelsoni*; $n = 5$); 3, Adams, Stevens, Grant, and Skagit Counties, WA (*nevadensis* and *caurinus*; $n = 19$); 4, Glenn County, CA (*caurinus*; $n = 4$); 5, Pima County, AZ (*sonoriensis*; $n = 3$); 6, Weld County, CO (*fortis*; $n = 8$); 7, Tom Green County, TX (*fortis*; $n = 4$); 8, San Petricio County, TX (*megapotamus*; $n = 4$); 9, Evangeline Parish, LA (*littoralis*; $n = 3$); 10, West Baton Rouge Parish, LA (*littoralis*; $n = 6$); 11, Leon County, FL (*mearnsi*; $n = 12$); 12, Barnwell County, SC (*phoeniceus*; $n = 9$); 13, Clarke County GA (*phoeniceus*; $n = 11$); 14, Pope County, IL (*phoeniceus*; $n = 6$); 15, Champaign County, IL (*phoeni-*

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ceus; n = 5); 16, Hennepin County, MN (*arctolegus; n* = 7); 17, Sandusky County, OH (*phoeniceus; n* = 6); 18, Somerset County, PA (*phoeniceus; n* = 1); 19, Gennesee County, NY (*phoeniceus; n* = 3). Heart, liver, and/or pectoral muscle samples were stored in cold MSB/Ca^{2+}/EDTA buffer (20) for periods of <7 days, until mtDNA was isolated by CsCl density gradient centrifugation (20). The mtDNA was digested with each of 18 informative restriction enzymes (see Table 1), and the fragments were radioactively end-labeled and electrophoretically separated according to molecular weight in 1% agarose gels (20). No attempt was made to score fragments <0.4 kilobases long. Each distinct digestion pattern was given an alphabetic character label, and the composite genotype for each individual was recorded. Data were summarized by using various statistical measures of nucleotide sequence divergence and diversity described in refs. 21 and 22.

RESULTS

From comparisons of various fragment profiles against a size standard (a 1-kilobase "ladder" purchased from Bethesda Research Laboratories), we estimate the mtDNA molecule in redwings to be ≈16.8 kilobases long. No mtDNA size variants were seen, and each individual appeared to be homoplasmic for a particular mtDNA genotype.

Among the 127 individuals sampled, we observed a total of 75 restriction sites, with an average of 63 sites (representing 378 nucleotides or 2.2% of the mtDNA genome) scored per individual. Altogether, 29 sites were polymorphic, i.e., present in some redwings and absent in others. The set of mtDNA digestion profiles produced by each endonuclease was interpretable in terms of restriction site maps whose interrelationships could be deduced by parsimony criteria. The simple structure of the data precluded the need for formal mapping of sites (as would normally be done through double-digestion procedures) yet allowed all data treatments to be based on mtDNA "site" rather than "fragment" information (22).

For each of the 18 enzymes employed, there was a common digestion pattern (labeled "C") that occurred in >50% of the individuals surveyed. Except for two enzymes (*Cla* I and *Bcl* I), the C pattern was observed at all 19 collection locales. The *Cla* I C pattern was not seen in the Evangeline Parish, LA sample, nor was the *Bcl* I genotype observed in Somerset County, PA, but these absences may simply be due to the small sample sizes at these two locations (*n* = 3 and 1, respectively).

By compiling the data across restriction enzymes, a composite haplotype was assigned to each individual. The total of 34 distinct mtDNA clones and their locales of collection are listed in Table 1. The most common genotype (genotype 1) was observed in 15 of the 19 geographic locations. Several other genotypes (genotypes 3, 17, 21, 26, and 28) also appeared in collections that were widely separated geographically.

By using the approach of Avise *et al.* (23), we constructed a composite parsimony network for the 34 mtDNA clones (Fig. 1). The network shown evokes a total of 38 character state changes, and, to retain branches of unitary length, three hypothetical genotypes (H1, H2 and H3) are required that are not actually observed. The network presented in Fig. 1 is not guaranteed to be the shortest possible [since we lack the computing facilities to evaluate all of the $>1.1 \times 10^{44}$ possible trees (22)], and in fact we have found other networks of the same length that differ in the peripheral placement of single clones found in single birds. Nonetheless, these networks all share the same basic structure, major features of which are discussed below.

Table 1. Designations and geographic distributions of mtDNA genotypes observed in Red-winged Blackbirds

Clone	Haplotype*	Locale(s) of collection†	No. of birds
1	CCCCCCCCCCCCCCCCCC	1–4, 6, 8, 10–17, 19	47
2	CCCCCCCCCCCCCCCCCCB	11	1
3	CCCCCCCCCCCCCCCCBCC	4, 13, 17	5
4	CCCCCCCCCCCCCCCCDCC	14	1
5	CCCCCCCCCCCCCCCCGCC	2	1
6	CCCCCCCCCCCCCCACCCC	6	1
7	CCCCCCCCCCCCCDCCECC	14	1
8	CCCCCCCCCCACCCCCCCC	1	1
9	CCCCCCCCCCBCCCCCCCC	3	1
10	CCCCCCCCCCECCCCCCCC	5	2
11	CCCCCCCCCBCCCCCCACC	13	1
12	CCCCCCCCCDCCCCCCCCC	6	2
13	CCCCCCCCCDCCCCCCCBC	6	1
14	CCCCCCCCDCCCCCCCCCC	7	1
15	CCCCCCCBDCCCCCCCCCC	2	4
16	CCCCCCCBDCCCCCCCFCC	2	1
17	CCCCCCCDCCCCCCCCCCC	7–10, 12, 14–17, 19	18
18	CCCCCCCDCCCCCCCCCCB	13	1
19	CCCCCCCDCCCCCCCCCBC	16	1
20	CCCCCCCDCCCCCCCCDCA	13	1
21	CCCCCCCDCCCCCCBCCCC	6, 11–14, 17, 19	9
22	CCCCCCCDCCCBCCCCCCC	7	1
23	CCCDCCCCBCCCCCCCCCC	10	1
24	CCACCCBCCCCCCCCCCCC	2	2
25	CCACCCBCCCCCCCCCCBC	2	1
26	CCBCCCCCCCCCCCCCCCC	3, 4, 11, 17, 18	10
27	CCBCCCCCCCCCCCCCCCB	11	1
28	CCBCCCCCCCCCCCDCCCC	3, 8	3
29	CCBCCCCCCCCCCCDCCCB	11	1
30	CCBCCCCCCDCCCCCCCB	11	1
31	CCDCCCCCCCCCCCCCCCC	11	1
32	CCDCCCBCCCCCCCCCCCC	3	2
33	CCECCCCCCCCCCCCCCCC	12	1
34	DCCCCCCCCCCCCCCCCCC	5	1

*Letters, from left to right, refer to mtDNA genotypes (genotypes A–D) revealed by digestion with the following enzymes. Position: 1, *Ava* I; 2, *Bam*HI; 3, *Bcl* I; 4, *Bgl* I; 5, *Bgl* II; 6, *Bst*EII; 7, *Cla* I; 8, *Eco*RI; 9, *Hinc*II; 10, *Hind*III; 11, *Kpn* I; 12, *Nde* I; 13, *Pst* I; 14, *Pvu* II; 15, *Sst* II; 16, *Stu* I; 17, *Sac* I; 18, *Xba* I.

†Numbers refer to locales given in *Materials and Methods*.

Fig. 1 also shows the number of assayed individuals in the clonal network. Clone 1, observed in 47 specimens from 15 locales across the continent, occupies a central position in the network from which 15 other, less common genotypes are interrelated by gain or loss of a single restriction site. The next most common genotype, clone 17 observed in Texas, Louisiana, South Carolina, Illinois, Minnesota, Ohio, and New York, forms the hub of a subsidiary portion of the network from which five rarer clones (genotypes 18–22) appear related by single, independent restriction site losses. The third most common genotype, clone 26 observed in Washington, California, Florida, Ohio, and Pennsylvania, also can be hypothesized as a likely progenitor of another group of related genotypes (genotypes 27–30). Overall, the structure of the composite network suggests that most of the mtDNA clones can, for purposes of discussion, be tentatively considered as belonging to one of the following four groups: group A (clone 1 and its immediate relatives except for clones 17 and 26), group B (clones thought to be derived from H1), group C (clone 17 and its presumed derivatives), and group D (clone 26 and its immediate relatives) (Fig. 1).

Fig. 2 plots the geographic distributions of representatives of these four clonal groupings. At any locale, the presence (in one or more individuals) of a member of a clonal group is

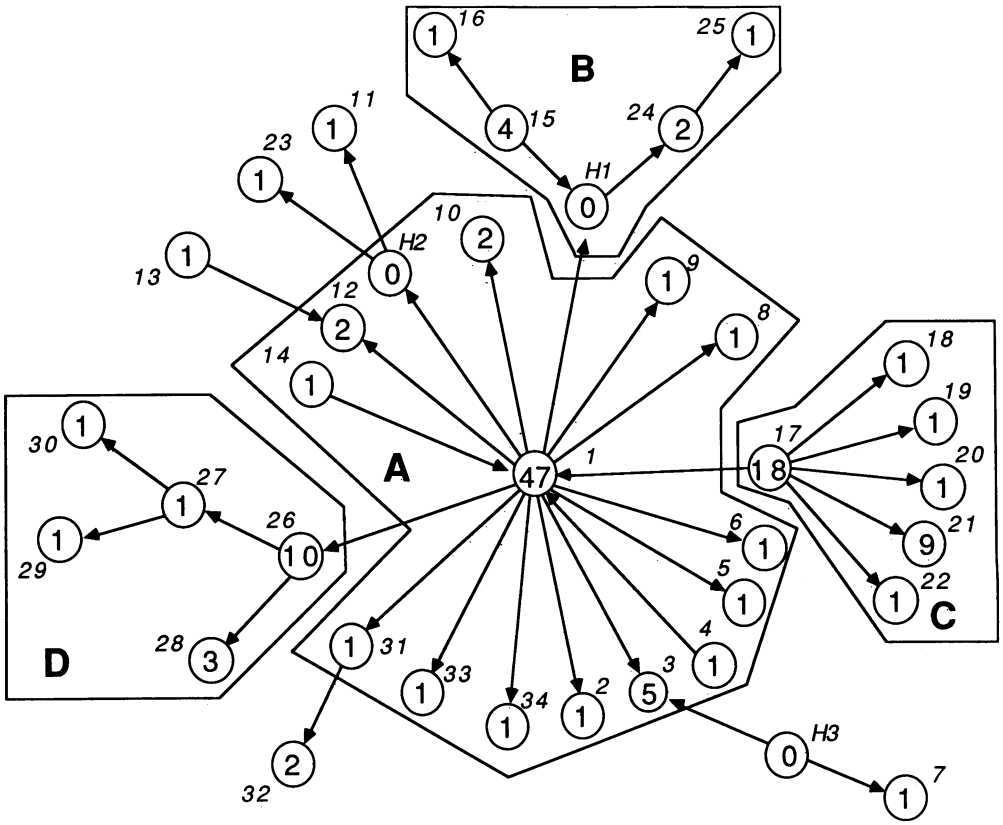

FIG. 1. Parsimony network summarizing possible relationships among the 34 composite mtDNA genotypes. Each arrow represents the direction of a single restriction site loss. Numbers outside circles indicate the mtDNA clonal designations as given in Table 1. Numbers inside circles indicate the abundances of various mtDNA clones among the 127 surveyed redwings. The goodness-of-fit or homoplasy index (24) is $F = 0.021$, indicating that the total number of mutation steps implied by the network (1768 steps) is slightly greater than the number actually observed (1732 steps).

indicated by a filled circle, and the absence is indicated by an empty circle (a convention adopted because of the small sample sizes at some sites). Representatives of clonal group A were found at all sampled sites, except for Pennsylvania and Louisiana. The eight individuals in clonal group B were restricted to the Mexican sites; members of group C were absent from the 43 individuals in the arc of more westerly locales (Canada, Washington, California, Arizona, and Mexico) but were otherwise present throughout the continent; and representatives of group D occurred at six widely scattered geographic sites (Washington, California, Ohio, Pennsylvania, Texas, and Florida).

By using a presence–absence matrix of restriction sites across clones, we also estimated numbers of base substitutions per nucleotide (P) between all pairs of mtDNA genotypes. In the entire study, the largest genetic distance (which was observed between several pairs of clones such as clone 7 versus clone 16 and clone 25 versus clone 30) involved six restriction-site changes and $P \approx 0.008$, a value very small in comparison to maximum conspecific mtDNA divergence in many other vertebrate species (2). Nonetheless, the small distance values were used to generate a UPGMA cluster phenogram (22) (data not shown, but which is available on request) that proved to reinforce the impression of the more salient mtDNA groupings noted above.

DISCUSSION

Two distinct aspects of the mtDNA data demonstrate that Red-winged Blackbirds from nesting locales across North America exhibit limited phylogeographic population structure: (*i*) estimated genetic distances in terms of base substitutions per nucleotide site between mtDNA genotypes were invariably small (maximum $P \approx 0.008$); and (*ii*) many of the mtDNA genotypes and phylogenetic assemblages of genotypes were widely distributed geographically, and the single most common mtDNA clone was present in populations throughout the continent. Thus the mtDNA phylogenetic network (Fig. 1) is characterized by few mutational steps between most pairs of genotypes and little geographic orientation to the clonal branches. Nonetheless, there is evidence for mild phylogeographic structure in redwings, since some clonal assemblages apparently have restricted geographic distributions (most notably assemblage B seen only in Mexico and assemblage C absent in the western locales). It is interesting to note that the populations exhibiting the greatest (though still limited) evidence for mtDNA differentiation involved the nonmigratory redwings in Mexico, where 8 of 12 assayed individuals belonged to the distinctive clonal assemblage B.

To underscore the conservative pattern of phylogeographic diversity observed in the mtDNA of Red-winged Blackbirds, it is instructive to compare results with mtDNA data from the deermouse *Peromyscus maniculatus*, the only other North American species to have been surveyed so extensively (5). In the deermouse study, 135 animals from 35 locales in Canada, Mexico, and across the United States were scored for an average of ≈41 restriction sites per individual. A summary and comparison of results to those for redwings are presented in Table 2.

In striking contrast to the redwing data, the 61 mtDNA genotypes observed in deermice grouped into at least five highly distinct phylogenetic assemblages that were also strongly patterned geographically. Some of the mtDNA

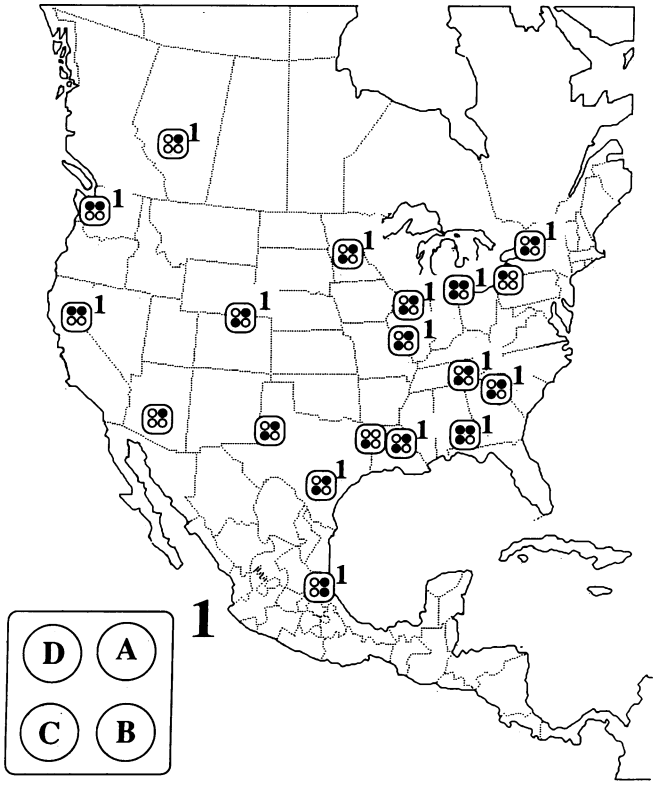

FIG. 2. Geographic distribution (presence, solid circle; absence, open circle) of members of each of the four mtDNA phylogenetic clonal assemblages identified in Fig. 1. Also indicated (by superscript 1) is the presence in any locale of mtDNA clone 1.

genotypes differed from one another by >20 assayed mutations steps. On average, pairs of mice drawn at random from North America exhibit a mtDNA genetic distance of $\bar{P} \approx 0.031$, >15 times greater than the value ($\bar{P} \approx 0.002$) for redwings. Even within any of the five geographic (and phylogenetic) subregions of *P. maniculatus*, genetic distances remained 3–8 times larger than for redwings continent-wide (Table 2). Furthermore, in the deermice there were no mtDNA clonal types shared by any of the major geographic subdivisions of the species, and particular genotypes within regions almost always appeared to have restricted geographic distributions (5).

What accounts for the circumscribed mtDNA diversity in redwings, and the contrasting phylogeographic pattern compared to deermice? One possibility is that rate of molecular (including mtDNA) evolution is decelerated in birds (see refs. 25–30 for background concerning this widely addressed hypothesis). However, a severe restriction on accumulation of mtDNA mutations in redwings is hard to reconcile with the large number of mtDNA clonal types observed. Furthermore, from an analysis of interspecific divergence in geese, Shields and Wilson (32) suggest that the mean rate of mtDNA sequence divergence in birds is not far from the conventional estimate of 2% per million years reported for mammals and other vertebrates (4, 33). If this conventional rate also applies to Red-wing Blackbirds, then mean and maximum times since separation of assayed mtDNA genotypes in the species are about 100,000 and 400,000 years ago, respectively. It is difficult to know whether these estimates are reasonable—but they are not compatible with expected distances under a neutral model unless redwing breeding population sizes have historically been much smaller (at least intermittently) than they are at the present time (39).

A second possibility is that the Red-winged Blackbird is a demographically "young" species, whose extensive range represents the rapid colonization of the continent from some refugial area, for example in Central America. Certainly, much of the current northern range of redwings (and deermice) has been habitable only since the retreat of the Wisconsin glacier beginning some 18,000 years ago. Perhaps the greater mtDNA sequence diversity in the North American deermouse reflects the effects of population expansions from multiple (rather than a single) Pleistocene refugia where long-term population separations had allowed the accumulation of greater mtDNA sequence differences (31, 34). However, a putative difference in number of Pleistocene refugia is probably not the total explanation for the contrast in phylogeographic pattern between redwings and deermice, because even within a phylogeographic assemblage, deermice exhibit much higher mtDNA nucleotide diversity than do redwings (Table 2).

A third possibility, closely intertwined with the second, relates to the fact that Red-winged Blackbirds have far greater dispersal capabilities and realized gene flow than do deermice. Although most redwings (≈88%) nest within ≈100 km of hatching site, the remainder disperse farther, and there is one record of a bird banded as a nestling that was recovered during the reproductive period >1000 km from banding site (19). By comparison, the maximum reported distance for dispersal in deermice is ≈3 km (35), and typical movement of individuals is far less (36–38).

Overall, the limited mtDNA phylogeographic population structure of redwings compared to deermice (and many other terrestrial and freshwater vertebrates assayed) is probably attributable to a greater level of dispersal and gene flow,

Table 2. Comparison of mtDNA polymorphism in Red-winged Blackbirds (*A. phoeniceus*) and deermice (*P. maniculatus*) sampled from across North America

Distribution	Individuals surveyed, no.	Restriction sites scored, no. per individual	mtDNA genotypes, no.	Genotypic diversity*	Nucleotide diversity†
Continent-wide					
Red-winged Blackbirds	127	63	34	0.804	0.002
Deermice	135	41	61	0.967	0.031
Phylogeographic subsets‡ of deermice					
Central United States	70	42	27	0.906	0.008
Southern California	26	41	14	0.905	0.006
Eastern United States	31	39	15	0.926	0.015
Minnesota	3	43	2	0.533	0.008
Texas/Mexico	5	41	3	0.711	0.009

*$1 - \Sigma f_i^2$ where f_i is the frequency of the ith mtDNA genotype.
†$[n/(n-1)] \Sigma p_{ij} f_i f_j$, where p_{ij} is the estimated mean number of base substitutions per nucleotide site between mtDNA genotypes i and j.
‡See ref. 5.

mediated by the capacity for flight. This gene flow has historical as well as contemporary components, such that in comparison to deermice, the redwing is a genetically highly interconnected species without major phylogenetic subdivisions. However, this conclusion should not be taken to imply that redwings across North America are panmictic on a generation-to-generation scale. Even occasional long distance migration may be sufficient to prevent substantial genetic differentiation by genetic drift, and as noted by Slatkin (11), "Roughly speaking, an average of one individual or more exchanged between two populations will prevent different neutral alleles at the same locus from being nearly fixed in two populations."

Results from the mtDNA analysis suggest that the morphological and subspecific differentiation of Red-winged Blackbird populations has apparently occurred against a backdrop of little phylogenetic separation. Two major possibilities exist: (*i*) the morphological differences are primarily ecophenotypic and not based entirely on genetic differences [as is suggested by the transplantation experiments of James (16)], or (*ii*) genes responsible for morphological differences evolve so rapidly that geographic differentiation has arisen over a time scale too short to have been readily detectable by the mtDNA assays. Resolution of these possibilities will require that secure knowledge be gained concerning the genetic basis of the morphological variation in redwings.

Results of this study contribute to an emerging view in which the magnitude of mtDNA phylogenetic structure among conspecific vertebrate populations appears to be negatively correlated with species' dispersal capabilities.

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