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Vicariance biogeography in the Pleistocene and speciation in North American wood warblers: A test of Mengel's model

(glacial cycles/mitochondrial DNA phylogeny/*Dendroica*)

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ABSTRACT **It is widely believed that habitat fragmentation during the Pleistocene initiated speciation events in many songbird genera. One such vicariance model for avian speciation in the Pleistocene was developed by R. M. Mengel for North American birds. This model suggests that the first Pleistocene glacial advance reduced the area of an extensive, eastern North American deciduous forest, forcing adaptation by some species to boreal forest. This, in turn, facilitated the development of transcontinental range expansions during interglacials. Subsequent glacial advances repeatedly fragmented the ranges of these species into eastern and western populations; western isolates speciated to form the multispecies groups observed among various North American birds. We used mtDNA restriction site data to reconstruct the phylogeny of the black-throated green warbler complex—the group that Mengel considered the best fit to his model. Contrary to Mengel's model, the phylogeny indicates that not all western endemics were derived from an eastern ancestor. Instead, our results imply a mix, wherein some western endemics were budded off an eastern source, as Mengel posits, while others probably resulted from intermontane isolations in the west.**

Next to the worldwide effects of continental drift, refugia generated by the climatic and glacial cycles of the Quaternary have inspired more discussion than any other set of vicariant events. For example, Pleistocene habitat refugia are commonly invoked to explain the formation and present-day distributions of closely related, but largely allopatric, phylogenetic species (as defined in refs. 1–4). For Africa (5, 6), Australia (7), and South America (8, 9), the principal models have been of forest refugia established during Pleistocene drought cycles. For North America, both ice-free eastern and western forest refugia, separated by the continental ice shields, and small arid land refugia, created by large reductions in the extent of arid land habitats during glacial maxima, are hypothesized to explain the origin of most closely related species of North American birds (10–14).

A particularly explicit vicariance model for the differentiation of North American birds was proposed by Mengel (10, 11), who predicted both sequences and approximate dates of differentiation for several species groups of North American birds. For each group in question, the story begins with an ancestral species adapted to the extensive deciduous forest of eastern North America at the end of the Pliocene; the model then follows the consequences of range changes created by four major glacial advances (figure 4 in ref. 10). Mengel argues that the first glacial advance forced the (eastern) ancestral species to adapt to the northern coniferous forest following the extensive reduction in eastern deciduous forest by the continental ice sheet. During subsequent interglacials, this adaptation to boreal forest enabled the ancestral species to spread westward across the continent, into the transcontinental band of northern forest developing in the wake of the receding ice sheet. Mengel then argued that succeeding glacial advances divided the transcontinental, boreal forest population of the eastern ancestor into two allopatric daughter populations: a large and ecologically generalized eastern population and a western population that became adapted to western montane environments (usually coniferous forest). Evidence suggests that these western vicars typically did not colonize the eastern deciduous forest during the following interglacials (10, 11). Note that Mengel's analysis was developed under the then-accepted paradigm of four Pleistocene glacial advances, but more recent oxygen isotope data, reviewed by Porter (15), now suggest that there have been considerably more than four major glacial advances during this epoch.

Mengel's (10, 11) glacial speciation model implies a specific phylogenetic branching pattern for avian clades having at least two endemic forms in western North America. Of all songbird groups, Mengel considered the five congeners of the *Dendroica virens* (black-throated green warbler) complex of wood warblers the best fit to his model. As a test of the model, we estimated both the branching pattern and times of divergence for four species in the *D. virens* group and also for the well-marked eastern and western subspecies (phylogenetic species) of the yellow-rumped warbler (hereafter referred to as *Dendroica coronata* and *Dendroica auduboni*, respectively), and *Dendroica magnolia* (magnolia warbler), which Mengel (10) considered their nearest relative. Unfortunately, the rarity and protected status of *Dendroica chrysoparia* (golden-cheeked warbler), the fifth member of the *D. virens* species complex, precluded obtaining specimens for this study. We were, however, able to include individuals from northwestern and southwestern populations of *Dendroica nigrescens* (black-throated grey warbler). This member of the *D. virens* group is presently considered monotypic, although some investigators have thought it should be broken into two races (16, 17). Finally, we included *Dendroica pensylvanica* (chestnut-sided warbler) as an outgroup for the focal species.

We estimated phylogenetic relationships in these warblers by analysis of restriction enzyme cleavage sites in mtDNA. Phylogenies generated with mtDNA data offer two methodological refinements useful for tests of historical biogeographic hypotheses like Mengel's. First, morphologically monotypic species with ranges that cross one or more centers of endemism, such as the northwestern and southwestern populations of *D. nigrescens*, may exhibit molecular divergence. Thus, mtDNA data can increase the amount of information available to biogeographers (18, 19). Second, to the extent that the mtDNA molecular clock is reliable, branch points in molecular phylogenies can be dated (20, 21), permitting at least a crude assessment of whether branching

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times are consistent with geologically dated vicariant events (18). Thus, the mtDNA data permitted us to assess whether both the branching pattern and the general timing of speciation events in the *D. virens* species complex conformed to Mengel's Pleistocene glacial speciation model.

MATERIALS AND METHODS

A total of 35 individuals representing eight species of *Dendroica* were assayed with a minimum of 14 restriction endonucleases. The mtDNA restriction fragment patterns for the five *D. coronata* included in this study were produced by Kessler (22), using procedures similar to those described below. All other specimens were analyzed in our laboratory, and the skins and tissues from these birds have been deposited in the University of Washington Burke Museum.

About 1 g of pectoral muscle from each bird was placed in STEN buffer (100 mM NaCl/10 mM Trizma base/100 mM EDTA, pH 7.5) within 2 hr of collection and held at 4°C until processed. Usually within a week, samples were homogenized in fresh STEN buffer and purified mtDNA was isolated by CsCl density-gradient centrifugation (23); 1- to 5-μl aliquots of each individual's mtDNA (total mtDNA sample volume per individual ranged from 200 to 500 μl) was digested with each restriction enzyme, and the resulting fragments were end-labeled with [α-^{32}P]dXTP(s) (24). Fragments were sized by gel electrophoresis and visualized by autoradiography.

Intra- and interspecific differences in the fragment patterns generated by each enzyme were readily explained by inferring single gains or losses of restriction sites. We were thus able to deduce restriction site maps for all 14 enzymes without physically mapping the molecules (25). By comparison to the *D. auduboni* analyzed in our laboratory, we were also able to infer mtDNA maps from Kessler's restriction fragment patterns for *D. coronata* (22). Finally, we calculated the percentage of sites shared between each pair of mtDNA haplotypes, and we used these data to estimate nucleotide sequence divergence values (d_{xy}; ref. 26) and standard errors (27).

We used Wagner parsimony criteria in PAUP v. 3.0 (28) to generate a set of trees for the mtDNA restriction site data. One of the shortest trees was exported to MACCLADE (29) so that a number of branch swaps could be executed and tree lengths and consistency indices could be calculated. The level of support for branching patterns was determined by bootstrapping 500 trees in PAUP via branch-and-bound searches with 100 bootstrap replications in each of five runs. We also used the RESTML program in PHYLIP v. 3.3 (30) to test statistically whether one of the most parsimonious mtDNA trees for the *D. virens* group differed from the user-defined tree predicted from Mengel's (10) model.

RESULTS

Species-specific mtDNA haplotypes were observed for each of the eight warbler species surveyed in this study. The two most closely related warbler species assayed were *Dendroica townsendi* and *Dendroica occidentalis*; this species pair could be distinguished by a minimum of three restriction endonuclease sites (a d_{xy} value of 0.0062). It is worth noting that we have consistently observed these mtDNA differences in larger population samples of *D. occidentalis* (n = 27) and *D. townsendi* (n = 38), which have been assayed for two of the three diagnostic enzymes studied (unpublished data). On average, 49 restriction sites representing 1.7% of the ≈16.8 kilobase pairs of the warbler mtDNA molecule were assayed per individual. Of the 94 restriction sites examined, 27 were shared by all 35 warblers surveyed (n = 30, this study; n = 5, ref. 22). (Tables that provide collection locales, haplotype designations, a restriction site presence/absence matrix, and all pair-wise d_{xy} values and their standard errors will be sent upon request.)

Except for *D. pensylvanica* and *D. magnolia*, for which we analyzed only single specimens, we found intraspecific mtDNA polymorphisms in all species. The small number of birds collected for each species permitted only a superficial look at geographic patterns of mtDNA haplotype distribution within species. Within *D. nigrescens*, for example, birds collected in the northwestern United States (n = 6), differed from *D. nigrescens* collected in the southwestern United States (n = 5) by a minimum of three restriction sites, a d_{xy} value of 0.0077. A similar degree of mtDNA differentiation was observed between the individuals of *D. virens* collected in Michigan and the two specimens of *D. virens* collected in western Pennsylvania. It should be noted, however, that the two *D. virens* collected from the same locale in Pennsylvania also differ by at least three restriction sites from one another. Only for *D. nigrescens* was there bootstrap support >55% for mtDNA haplotype groupings within species.

A Wagner parsimony analysis of the 67 variable restriction sites revealed 36 minimum mutation-length trees of 95 steps and a consistency index of 0.71. All trees identified the *D. virens* species complex as a monophyletic assemblage and also distinguished Arizona *D. nigrescens* from Washington *D. nigrescens*. A strict consensus tree summarizing the 36 rival, minimum-length trees did not reveal mtDNA haplotype relationships within *D. virens*, within the Arizona group of *D. nigrescens*, or within the *D. coronata*/*D. auduboni* clade. Most of the differences between the mtDNA tree pictured in Fig. 1, and the 36 minimum-length trees identified by parsimony analysis, were branch rearrangements of mtDNA haplotypes within species. The *D. coronata*/*D. auduboni* clade was consistently recognized, but the relationship of this clade to *D. pensylvanica*, *D. magnolia*, and the *D. virens* clade was unclear—specifically, a tree length of 95 obtained either with *D. pensylvanica* or with the *D. coronata*/*D. auduboni*/*D. magnolia* clade as the sister group to the *D. virens* clade. There was, however, no strong support for *D. magnolia* grouping with *D. coronata*/*D. auduboni*. The four mtDNA haplotypes comprising the *D. coronata*/*D. auduboni*/*D. magnolia* assemblage were grouped together in only 56% of the bootstrapped trees. Furthermore, the length of the tree increased by only a single step if *D. magnolia* and *D. pensylvanica* were grouped, instead of *D. magnolia* joining *D. coronata* and *D. auduboni*. This last tree, pictured in Fig. 1, best accommodated both the Wagner parsimony analysis and the mtDNA distance data (i.e., mean d_{xy} values used to position the nodes under the assumption of a constant mtDNA nucleotide substitution rate).

Fig. 2 contrasts the mtDNA relationships within the *D. virens* group with the phylogeny predicted by Mengel's model (10). The trees shown are simplified, but the analyses described below were based on all 13 mtDNA haplotypes assayed in the *D. virens* species complex. Considering only the mutations along branches in the *D. virens* clade (rooted to *D. pensylvanica*, *D. magnolia*, *D. coronata*, *D. auduboni*) gave a minimum length tree of 40 steps with a consistency index of 0.80. When the mtDNA restriction site data were forced onto the Mengel phylogeny, a tree of 46 steps with a consistency index of 0.70 resulted. The only difference between the mtDNA tree and the Mengel-defined tree was with regard to the relationship of *D. virens*, *D. townsendi*, and *D. occidentalis* (Fig. 2). Mengel's hypothesis considered *D. virens* the sister taxa to *D. townsendi*, whereas the mtDNA phylogeny placed *D. occidentalis* as the sister species to *D. townsendi*. The relationship of mtDNA haplotypes within species was held constant in all trees compared.

The Mengel and mtDNA phylogenies (Fig. 2) were also analyzed using the user-tree option in the RESTML program

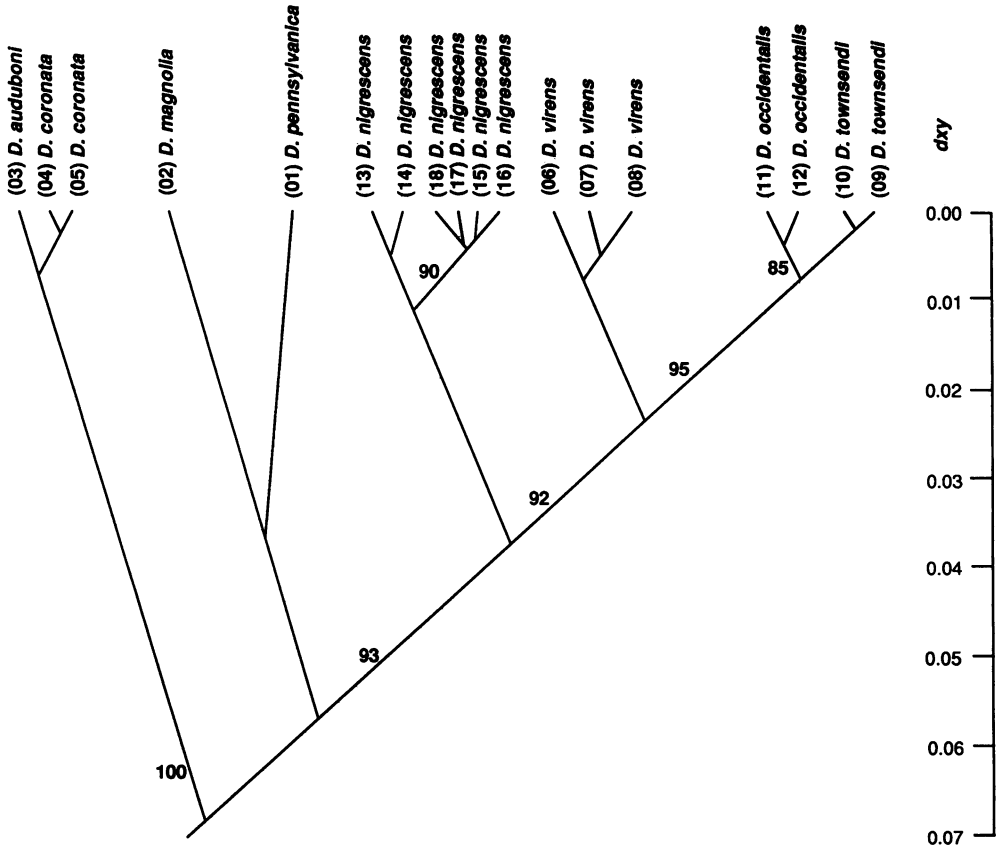

FIG. 1. Wagner parsimony tree based on mtDNA restriction sites (length, 96; consistency index, 0.70). Tree is rooted at the *D. coronata/D. auduboni* clade. Nodes were positioned using mean d_{xy} values under the assumption of a constant rate of molecular evolution. Numbers at nodes indicate the proportion of times that a clade was distinguished in the bootstrap analysis (only proportions >80% are shown).

distributed in PHYLIP v. 3.3 (30). This option permitted a statistical test of the mean and variance of log-likelihood differences between the two trees. In the present example, the two trees tested differed by only one branch swap, and thus the log-likelihood test using 1 degree of freedom (representing the single branch swap) seems appropriate (30). Mengel's tree was significantly worse than the mtDNA tree.

DISCUSSION

Of all North American birds, Mengel (10, 11) considered the *D. virens* complex of five species the best fit for his vicariance speciation model. Most have middle to high latitude ranges and *D. virens*, which represents the eastern "source" lineage in this group, currently inhabits both deciduous and coniferous forests. Mengel's fundamental predictions are (*i*) western endemics were proliferated by the successive midcontinental cleaving of the transcontinental range that the more eurytypic eastern source lineage developed by exploiting boreal forest during interglacial periods; and (*ii*) all such speciation events were concurrent with major glacial advances.

At the time that Mengel was writing, the chronology of the Pleistocene was based on four major glacial advances in North America occurring over the past 2 million years. Newer Pleistocene chronologies, however, based on data from $^{18}O/^{16}O$ isotope ratios in marine sediments, suggest that at least 7–10 major advances have occurred within the past 700,000 to 1 million years (see ref. 15; Fig. 1). Therefore, any critique and refinement of Mengel's model should take account of improved estimates for the number and timing of major glacial advances. Even with an improved understanding of Pleistocene glacial patterns, it will be difficult to match the timing of glacial advances to cladogenetic events because of errors associated with estimates of earth history chronology and estimates of sequence divergence. Only the primate mtDNA molecular clock has been calibrated (20), and we really don't know how useful this calibration is for songbirds. Thus, although it is straightforward to assess Mengel's hypothesis of relationship in the *D. virens* group with our data, the temporal prediction—that all splits occurred within the past 2 million years—is more difficult to evaluate.

Our mtDNA phylogeny (Fig. 2) provides two contradictions and two confirmations of the phylogenetic branching pattern predicted by Mengel's hypothesis for the *D. virens* complex. As suggested by Mengel (10), *D. nigrescens* does represent the most basal branch from the *D. virens* tree (although use of the primate mtDNA clock indicates that this event occurred before the first glacial advance; Fig. 2). However, southwestern *D. nigrescens* and northwestern *D. nigrescens* are sister taxa that represent an apparently late Pleistocene split rather than successive western isolates, as an extension of Mengel's model would predict. Moreover, although the mtDNA clade containing *D. townsendi* also joins *D. virens* as Mengel posited, *D. occidentalis* and *D. townsendi* are western sister taxa, rather than budding off from *D. virens* as sequential western isolates.

Refinements to Mengel's Model. Mengel thought he had only four glacial advances to work with. Thus, four western derivatives is one too many for his model because no western vicar is budded off in the first glacial advance. For this reason, Mengel argued that *D. chrysoparia*, which breeds only on the Edwards plateau of Texas, was isolated from *D. virens* in the present interglacial separation of the Edwards plateau from eastern forest. Adding *D. chrysoparia* to the *D. virens* mtDNA phylogeny would reveal whether it is an old derivative, as now "allowed" by Mengel's model, or whether it has more recently split from one of the other branches in this clade.

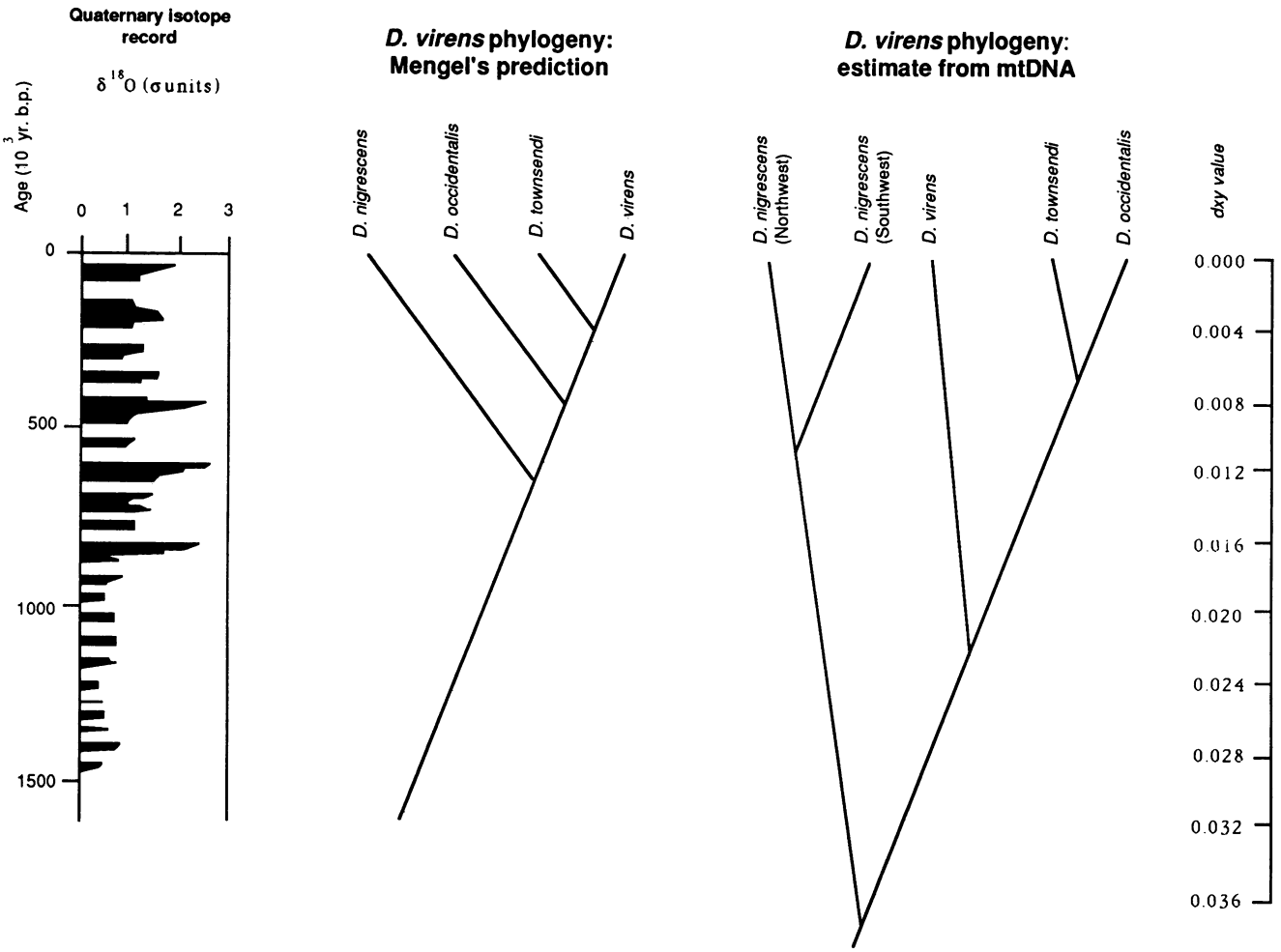

FIG. 2. Both the Mengel and mtDNA phylogenies for the *D. virens* species complex, plotted against the $\delta^{18}O$ oxygen isotope record (modified from ref. 15) and mtDNA sequence divergence values (d_{xy}). Peaks in $\delta^{18}O$ record correspond to peak glacial periods. b.p., Before present. d_{xy} values are plotted on the same time scale as the $\delta^{18}O$ record, assuming that mtDNA sequence divergence occurs at a mean rate of 2% per million years (20). The mtDNA tree was rooted to an outgroup consisting of *D. pensylvanica, D. magnolia, D. coronata,* and *D. auduboni.*

The data also suggest a vicariant hypothesis that could supplement Mengel's model: intermontane species pairs in songbirds developed in Coast Range and Rocky Mountain isolations during the Pleistocene. Specifically, the roughly contemporaneous western differentiation within *D. nigrescens* and between *D. occidentalis* and *D. townsendi* indicates that intermontane divergence in western North America may be a vicariance pattern worthy of more general investigation (see also ref. 32).

Our investigation of a single species group is far from sufficient to assess the general success or failure of Mengel's model. Despite the doubts cast by our data, this model of vicariance speciation is compelling enough to deserve testing with other avian species complexes. For example, the oxygen isotope data suggest that Mengel's speciation model could be evaluated for species complexes with larger numbers of western endemics, such as the *Empidonax* flycatchers. The *Vermivora ruficapilla* (Nashville warbler) species group would also make an interesting follow-up to the *D. virens* group investigated here. Four species in the *V. ruficapilla* group have distributions nearly identical to those of the *D. virens* group, even though they occupy different habitats (coniferous/deciduous forest edge) and are placed in a different genus.

Molecular Phylogenies and Pleistocene Speciation Models. In the absence of reasonable methods for establishing divergence times, many biogeographers have simply assumed that many closely related species of birds were of Pleistocene origin (10, 12–14). As a check on this assumption we have assembled (Table 1) data on minimum and maximum estimates of mtDNA sequence divergence for 18 genera of North American birds. Using the primate calibration of the mtDNA clock (20), species pairs with sequence divergence values in excess of 0.04 would be considered to have split before the Pleistocene. On the one hand, the relevant d_{xy} value may actually lie closer to 0.02 if, as the $^{18}O/^{16}O$ data (15) indicate, the dramatic climatic fluctuations associated with the Pleistocene and the series of range fragmentations that these cycles may have initiated were most evident during the past 1 million years. On the other hand, the mtDNA clock for birds may tick faster than that for primates, which would permit larger d_{xy} values to be consistent with Pleistocene speciation events.

Several of the genera included in Table 1 were originally chosen for study because their relationships were close and equivocal, so the overview provided by our summary may be biased by an overrepresentation of young taxa. Still, two general points emerge. First, Pleistocene speciation appears to be a reasonable scenario for many species of birds, thus confirming earlier analyses based on the fossil record by Brodkorb (45) and Selander (13). Second, biogeographic analyses of speciation would benefit from having branching points sorted by age. The majority of genera listed in Table 1 includes species pairs with mtDNA sequence divergence values considerably in excess of 0.04. If indeed these pairs

Table 1. Minimum and maximum estimates of mtDNA sequence divergence between pairs of species and/or named subspecies in 18 genera of North American birds

Taxon	*n*	Min *D*	Max *D*	Ref.
Branta canadensis (Canada goose)	10	0.001	0.013	33
Anas (dabbling duck)	36	0.004	0.088	34
Athya (pochard duck)	6	0.025	0.043	34
Rallus (rail)	1	0.006	0.006	35
Limnodromus (dowitcher)	1	0.082	0.082	35
Parus bicolor (titmouse)	1	0.004	0.004	35
Parus (chickadee)	3	0.040	0.090	36
Dendroica (wood warbler)	39	0.006	0.076	This study; 37
Passerella iliaca (fox sparrow)	1	0.009	0.009	19
Melospiza (song sparrow and relatives)	3	0.026	0.030	37
Sturnella (meadowlark)	1	0.034	0.034	38
Agelaius phoeniceus (red-winged blackbird)	55	0.000	0.008	39
Quiscalus (grackle)	6	0.015	0.042	40
Icterus (oriole)	1	0.037	0.037	38
Pipilo (towhee)	10	0.025	0.098	41
Zonotrichia (crowned sparrow)	15	0.011	0.049	42
Phalaropus (phalarope)	3	0.055	0.083	43
Calidris (sandpiper)	3	0.093	0.124	43
Ammodramus (grassland sparrow)	21	0.021	0.109	44

n, Number of species and/or named subspecies pairs compared in the study. For example, 11 named subspecies of *A. phoeniceus*, permitting 55 different pair-wise comparisons, were assayed in the study by Ball *et al.* (39). Methods for calculating *D* (estimated mtDNA sequence divergence) values varied across studies, but all methods used give reasonably equivalent results for levels of mtDNA sequence divergence reported here.

diverged before the Pleistocene, then including any of them in studies of Pleistocene barriers leading to avian differentiation would obfuscate rather than elucidate real patterns.

Whether vicariance events during the North American Pleistocene can explain as much avian speciation and biogeography as traditionally believed remains to be resolved. Such a resolution must await not only further molecular data on the patterns and timing of divergence events in bird groups thought to have fragmented during the Pleistocene but also better palynological evidence proving the existence of refugia appropriate to the clades in question. Moreover, it will be important to include Australian and tropical African and South American birds in such studies. Speciation in Pleistocene forest refugia has been the dominant model for explaining present-day species composition and distribution in the Amazonian and tropical African avifaunas, but molecular data bearing on timing of divergence of tropical taxa are limited (see table 3 of ref. 46). These refuge models have recently been criticized on the grounds of poor palynological records (31, 47) and the consistency of extant species ranges with other hypotheses (48). As this study illustrates, molecular data can be used effectively to test both the branching patterns and the timing of divergences predicted from such models.

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