The Auk 126(1):198–210, 2009

Printed in USA.

GEOGRAPHIC VARIATION IN THE MITOCHONDRIAL CONTROL REGION OF BLACK-THROATED BLUE WARBLERS (*DENDROICA CAERULESCENS*)

WENDY E. GRUS,[1,3] GARY R. GRAVES,[2,4] AND TRAVIS C. GLENN[1,5]

[1]*Savannah River Ecology Laboratory, University of Georgia, Drawer E, Aiken, South Carolina 29802, USA; and*
[2]*Department of Vertebrate Zoology, MRC-116, National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, Washington, D.C. 20013, USA*

ABSTRACT.—We investigated the genetic population structure of the Black-throated Blue Warbler (*Dendroica caerulescens*), a Nearctic–Neotropic migrant passerine that breeds in cool mixed deciduous–coniferous forests in eastern North America. A cline in plumage color in breeding populations in the central Appalachian Mountains suggests either a contact zone between two formerly allopatric populations or the presence of a strong contemporary selection gradient. Analysis of 333 base pairs of the mitochondrial control region from 287 individuals sampled from 14 populations revealed relatively high haplotype diversity, low nucleotide diversity, and limited but significant phylogeographic structure across the breeding range (analysis of molecular variance [AMOVA], variation among populations = 2.7%; $P < 0.01$) and between northern and southern population groups (AMOVA, variation among groups = 2.9%; $P < 0.01$). Genetic differentiation among populations did not conform to an isolation-by-distance model. Nucleotide diversity was generally highest in the central Appalachians and lower in geographically peripheral populations. Populations from the northwestern periphery of the breeding range in Michigan had the lowest haplotype diversity and were genetically distinct from populations in the southern Appalachians. The star-shaped haplotype network, extensive sharing of common haplotypes among populations, and the haphazard distribution of rare haplotypes are most likely attributable to the combined effects of postglacial expansion from a single refugium (12,000–84,000 years ago) and long-distance dispersal events. The existence of a cline in plumage color, in the face of inferred recent gene flow, suggests that a strong selection gradient is operating, perhaps related to the migratory divide postulated from stable-isotope data. *Received 28 September 2007, accepted 9 October 2008.*

Key words: Appalachian Mountains, Black-throated Blue Warbler, *Dendroica caerulescens*, genetic structure, glaciation, mitochondrial DNA, plumage color, stable isotopes.

Variación Geográfica en la Región Control Mitocondrial de *Dendroica caerulescens*

RESUMEN.—Investigamos la estructura genética poblacional de *Dendroica caerulescens*, un paseriforme que migra entre el Neártico y el Neotrópico y que se reproduce en áreas frescas de bosques mixtos de coníferas en el este de Norte América. Una clina en la coloración del plumaje en las poblaciones reproductivas de las montañas Apalaches sugiere que existe una zona de contacto entre dos poblaciones que fueron alopátricas, o la presencia de un fuerte gradiente de selección contemporánea. El análisis de 333 pares de bases de la región control mitocondrial de 287 individuos muestreados en 14 poblaciones, reveló una diversidad de haplotipos relativamente alta, una baja diversidad de nucleótidos y una estructura filogeográfica escasa pero significativa en el área de distribución reproductiva (análisis de varianza molecular [AMOVA], variación entre poblaciones = 2.7%; $P < 0.01$) y entre los grupos formados por las poblaciones del norte y del sur (AMOVA, variación entre grupos = 2.9%; $P < 0.01$). La diferenciación genética entre poblaciones no se ajustó al modelo de aislamiento por distancia. La diversidad de nucleótidos fue generalmente más alta en el centro de las Apalaches y menor en las poblaciones periféricas. Las poblaciones de la periferia en el noroeste del área de distribución reproductiva en Michigan tuvieron la menor diversidad de haplotipos y fueron genéticamente diferentes a las de las montañas Apalaches del sur. La red de haplotipos con forma de estrella, la gran cantidad de haplotipos comunes compartidos entre poblaciones y la distribución al azar de haplotipos raros, son atribuibles probablemente al efecto combinado de la expansión postglacial a partir de un único refugio (12,000–84,000 años atrás) y eventos de dispersión de gran distancia. La existencia de una clina en la coloración del plumaje a pesar del flujo genético reciente inferido, sugiere que está operando un fuerte gradiente de selección, quizás relacionado a la división migratoria postulada con datos de isótopos estables.

[3]Present address: Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, C3-168, P.O. Box 19024, Seattle Washington 98109, USA.
[4]Address correspondence to this author. E-mail: gravesg@si.edu. Grus and Graves contributed equally.
[5]Present address: Department of Environmental Health Science, University of Georgia, Athens, Georgia 30602, USA.

The Auk, Vol. 126, Number 1, pages 198–210. ISSN 0004-8038, electronic ISSN 1938-4254. DOI: 10.1525/auk.2009.07159

Pleistocene glacial cycles were important drivers of phyletic diversification, genetic reorganization, and lineage extinction in plants and animals. Contemporary populations of Holarctic vertebrates often exhibit low haplotype diversity in formerly glaciated regions (Sage and Wolff 1986) or clines of decreasing haplotype diversity along transects from unglaciated to recently glaciated landscapes (Hayes and Harrison 1992, Hewitt 1996, Merilä et al. 1997, Milá et al. 2000). Such spatial patterns can be caused by the rapid expansion of bottlenecked populations from glacial refugia (Rogers and Harpending 1992, Rogers 1995) or by range-wide selective sweeps in which one or more favored haplotype spreads across a species' geographic range (Maruyama and Birky 1991). Weak phylogeographic structure observed in formerly glaciated regions may result from the consequences of postglacial range expansion or from ongoing demographic processes. In birds, for example, migratory behavior and high natal dispersal may act to spread mitochondrial haplotypes among distant populations (Zink 1996). Discriminating between the ancient effects of postglacial population expansion and current demographic processes in mobile organisms is, therefore, a major challenge for phylogeographic hypothesis-testing.

Here, we examine variation in the mitochondrial DNA (mtDNA) control region of Black-throated Blue Warbler (*Dendroica caerulescens*; hereafter "warbler"), a Nearctic–Neotropic migratory songbird whose breeding range is centered in cool mixed deciduous–coniferous forests of eastern North America (Holmes 1994). The highest breeding densities occur in mesic forests (700–1,400 m above sea level) in the Appalachian Mountains (Wilcove 1988, Graves 1997b, Haney et al. 2001). More than two dozen avian species (representing 10 families) share a similar breeding distribution in the Appalachians, occurring southward at higher altitudes to at least the Great Smoky Mountains (~36°N latitude). Taxonomists have applied trinomials to many Appalachian populations (American Ornithologists' Union 1957), but morphological differentiation is subtly clinal in most taxa (Zink and Remsen 1986). The warbler is unique among Appalachian species in exhibiting geographic variation in plumage color that is distinctive enough to be quantified in the field (Graves 1997a). Breeding populations in the southern Appalachians (*Dendroica caerulescens cairnsi*) have significantly darker plumage than those breeding on glaciated landscapes from Pennsylvania northward (*D. c. caerulescens*). Rudiments of this geographic pattern were recognized more than a century ago (Coues 1897, Ridgway 1902). Recent systematic collections (G. R. Graves unpubl. data) have revealed the existence of a cline in plumage color between southern Virginia (37°N) and the Susquehanna River (41°N), which lies near the southernmost limit of the Wisconsinan glaciation (18,000 years ago) in the Appalachians (Williams et al. 2000). This phenotypic pattern could represent a contact zone between two formerly allopatric populations or signal the presence of a strong selection gradient in the Appalachians (Mayr 1963).

Stable-isotope analysis of feather keratins provides inferential support for a north–south subdivision of warbler populations (Chamberlain et al. 1997, Rubenstein et al. 2002). Hydrogen isotope signatures suggest that "blue-backed" populations from the glaciated portion of the species' breeding range winter in the western Caribbean, whereas "black-backed" populations breeding in the central and southern Appalachians appear to winter in the eastern Caribbean (Rubenstein et al. 2002). Such migratory divides in passerine birds are often associated with partial barriers to gene flow and population differentiation (Bensch et al. 1999, Chamberlain et al. 2000, Ruegg and Smith 2002, Bearhop et al. 2005).

Other lines of evidence, however, predict that gene flow among breeding populations of warblers may be substantial. The settlement patterns of yearlings and older males at fine spatial scales appear to be driven by heterogeneity in habitat quality and by the despotism of older philopatric males (Holmes 1994, Holmes et al. 1996). A survey of 23 breeding populations distributed throughout the core range revealed that the proportion of yearling males in local populations increased as total relative abundance decreased northward and westward from the Appalachian Mountains (Graves 1997b). This implies that yearlings from high-density source areas in the Appalachians may disperse to low-quality habitats that support lower population densities near the northern and western margins of the breeding range (Graves 1997b). These factors may result in the spread of haplotypes and weak or imperceptible phylogeographic structure among contemporary breeding populations. The hypothesis that male natal dispersal is prevalent at local and perhaps regional spatial scales is supported by carbon isotope data from an altitudinal gradient in the Appalachians that indicated that yearling males rarely returned to breed in the altitudinal zone where they were hatched (Graves et al. 2002).

The phylogeography of malarial parasites provides further indirect evidence of gene flow among breeding populations of warblers (Fallon et al. 2006). Analyses of malarial infections in 32 populations of warblers sampled across the species' breeding range showed that lineages of *Plasmodium* and *Haemoproteus* are geographically widespread and did not provide site-specific information. The wide distribution of malarial parasites is thought to reflect natal dispersal of their hosts as well as mixing of breeding populations on the wintering grounds.

Finally, Davis et al. (2006) investigated geographic variation of the mtDNA control region and microsatellite markers in warblers from four breeding populations, three located north and one south of the Last Glacial Maximum. These analyses revealed significant genetic variation within populations but weak phylogeographic structure overall, and little differentiation between southern and northern populations. The strength of Davis et al.'s (2006) conclusion about differentiation between northern and southern populations, however, is limited by the number of populations sampled, particularly in the southern Appalachians, where phylogeographic structure may be more pronounced.

We sought to build on the work of Davis et al. (2006) with a more extensive analysis of geographic variation in the mtDNA control region of the warbler based on 287 specimens from 14 breeding localities. We addressed two fundamental questions: (1) Do haplotypes exhibit geographic variation among breeding populations? And (2) did glaciation history affect phylogeography?

Methods

Sample collection.—Because nucleotide and haplotype diversity may exhibit clinal variation from unglaciated to glaciated landscapes, we sampled breeding populations at strategic geographic intervals both north and south of the Last Glacial Maximum (Fig. 1): Cooper Creek, Georgia (34.75°N, 84.03°W; coordinates converted

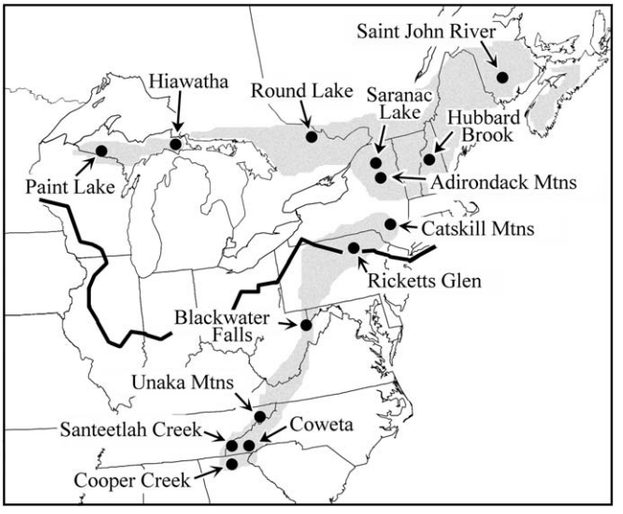

FIG. 1. Distribution of sampling localities within the core breeding range (shaded gray) of Black-throated Blue Warbler in eastern North America (Fallon et al. 2006). The thick black line indicates the glacial front at the Wisconsinan maximum, ~18,000 years ago.

to decimals; $n = 12$ individuals); Santeetlah Creek, North Carolina (35.33°N, 84.02°W; $n = 29$); Unaka Mountains, Tennessee (36.13°N, 82.33°W; $n = 16$); Blackwater Falls, West Virginia (39.13°N, 79.30°W; $n = 8$); Ricketts Glen, Pennsylvania (41.32°N, 76.33°W; $n = 10$); Catskill Mountains, New York (41.92°N, 74.50°W; $n = 21$); Adirondack Mountains, New York (43.70°N, 74.72°W; $n = 19$); Round Lake, Ontario (45.67°N, 77.50°W; $n = 15$); Saint John River, New Brunswick (45.83°N, 67.00°W; $n = 14$); and Paint Lake, Michigan (46.37°N, 88.93°W; $n = 18$) (Graves 1997b, Fallon et al. 2006). Population samples included both yearling (first breeding season) and older males (second or later breeding season), which can be distinguished by several plumage characters (Graves 1997a). To ensure that neither migrating nor wandering postbreeding birds were collected, males were collected during the peak of the breeding season (10–29 June, 1988–2000; Graves 2004). Specimens were packaged and frozen whole in liquid nitrogen immediately after collecting. Voucher specimens were deposited in the research collections of the National Museum of Natural History, Smithsonian Institution, Washington, D.C.

We combined our data with an overlapping section of mtDNA control-region sequence previously published for breeding populations of the warbler (Davis et al. 2006): Coweta, North Carolina (35.11°N, 83.52°W; 14 males, 7 females); Hubbard Brook, New Hampshire (43.95°N, 71.72°W; 26 males, 31 females); Saranac Lake, New York (44.15°N, 74.80°W; 9 males, 1 female); and Hiawatha National Forest, Michigan (46.08°N, 84.70°W; 27 males, 10 females). Davis et al. (2006) did not collect voucher specimens or specify collection dates for blood sampled from the North Carolina, Michigan, and New York populations.

DNA sequencing.—Detailed laboratory protocols are available online (see Acknowledgments). Briefly, genomic DNA was isolated from striated muscle samples by digesting 0.1 g of muscle in 900 μL of standard proteinase K digestion buffer (Sambrook et al. 1989). For the populations from the Adirondack and Catskill mountains, Ricketts Glen, and Santeetlah Creek, digestion was followed by a guanidine thiocyanate with diatomaceous-earth extraction protocol (Carter and Milton 1993). For the other six populations, the digestion was followed by a phenol chloroform extraction (Sambrook et al. 1989). The genomic DNA was quantified on 1% agarose gels and diluted with TLE (10mM Tris pH 8, 0.2 mM EDTA).

A 333-base pair (bp) section of domain 1 of the mtDNA control region was amplified with polymerase chain reaction (PCR) primers L5 (5′TTCTTGCTTTAAGGGTATGT) and H4 (5′TCAATAGATAACCATGTCCT) (Milot et al. 2000) for 162 individuals. Amplification was done in 25-μL volumes with final reaction concentrations of 2.0 mM $MgCl_2$, 150 μM of each dNTP, 0.25 μM of each primer, 1 unit of Taq polymerase, and 20 ng of DNA. The PCR amplicons were generated in Eppendorf gradient Mastercyclers using a touchdown protocol (Don et al. 1991) with an initial annealing temperature of 60°C and an ending annealing temperature of 50°C. Amplifications were confirmed on 1.0% agarose gels containing ethidium bromide.

Sequences from both DNA strands were determined directly from PCR products using Big-Dye terminator chemistry and an ABI 377-96 automated sequencer (Applied Biosystems, Foster City, California). All cycle-sequencing reactions were carried out in 10-μL volumes with 0.33 μM primer, 50 ng PCR product (1.0–2.5 μL of unpurified PCR product), and Big-Dye Terminators using ABI specifications except that the terminator mix was diluted 1:3 with 5× automated sequencing dilution buffer (10 mM $MgCl_2$, 400 mM Tris-HCl pH 9.0). Control-region sequences have been deposited in GenBank (accession numbers EU147998–EU148058).

Data analysis.—Sequences were edited using SEQUENCHER, version 3.1.1 or 4.0.1 (Gene Codes, Ann Arbor, Michigan). To confirm the mitochondrial origin of PCR products, we compared Black-throated Blue Warbler sequence with those from Yellow Warbler (*D. petechia*; Milot et al. 2000) in GenBank and computed transition:transversion (Ti:Tv) ratios to compare with those of other bird species. The Ti:Tv ratio for Black-throated Blue Warbler (9.6 for our data alone, 6.4 for the combined data) is similar to the average ratio for neutral mtDNA sites in birds (Belle et al. 2005). We also note the absence of heterozygotes, which would be expected in nuclear copies of mitochondrial genes. We examined the likely relationships between haplotypes with an unrooted minimum spanning network constructed in TCS1.18 (Clement et al. 2000). To investigate possible phylogeographic structure among the populations, we used ARLEQUIN, version 3.11 (see Acknowledgments), to calculate an analysis of molecular variance (AMOVA; Excoffier et al. 1992), which determines the amount of haplotype variation within populations, among populations in a group, and between groups of populations. We conducted AMOVA on our data and those of Davis et al. (2006) to ensure that the two data sets did not differ systematically. We pooled the data in subsequent analyses because they appeared to be complementary. Different groupings of populations were then investigated to determine whether genetic structure existed to match phenotypic and migratory differences between the populations. We categorized populations, *a priori*, into three geographic pools: northern (south to the Adirondack Mountains), central (Catskill Mountains to Ricketts Glen), and southern (Blackwater Falls southward). Four different

AMOVA tests were performed: (1) no geographic pools; (2) three geographic pools—northern, central, and southern; (3) two geographic pools—central combined with northern; and (4) two geographic pools—central combined with southern.

We conducted additional spatial analyses of molecular variance (SAMOVA), using both geographic and genetic distances among populations to maximally differentiate groups of breeding populations (Dupanloup et al. 2002). Spatial analyses of molecular variance require the number of groups to be predefined, but group membership is determined through the analysis. We ran separate SAMOVAs for two and three groups. The relationship between genetic and geographic distances among populations was assessed with Mantel tests (Bohonak 2002). All *P* values are two-tailed.

To investigate Holocene demographic history, we used ARLEQUIN to calculate haplotype (h) and nucleotide diversity (π) for each population, to perform Tajima's test (Tajima 1989) and Fu and Li's test (Fu and Li 1993) for neutrality, and to calculate a mismatch distribution of pairwise differences between haplotypes. We also calculated *FS* and R^2 (Ramos-Onsins and Rozas 2002) in DNASP (Rozas et al. 2003) to evaluate recent demographic processes.

We estimated the time since the most recent common ancestor (T_{mrca}) with two different methods. First, we estimated the number of generations since T_{mrca} using the quick calculation of $\tau = 2\ \mu t$, where t is the number of years since coalescence (generation time is one year) and μ is the mutation rate (Rogers and Harpending 1992, Schneider and Excoffier 1999), which we modeled at 0.2–0.7 substitutions site^{-1} MA^{-1} (Avise and Walker 1998). We then used ARLEQUIN to estimate τ and 95% confidence intervals (CI) of τ under a model of pure demographic expansion. We also used BEAST, version 1.4.5 (Drummond and Rambaut 2007), to estimate T_{mrca} using parameters similar to those used by Davis et al. (2006), except that we used a general time reversible (GTR) model with gamma rate distribution and invariable sites, a total chain length of 60 million sampling every 10,000 to achieve an effective sample size of >340 for T_{mrca}, and only one substitution rate in the simulations (0.3 substitutions site^{-1} MA^{-1}; complete parameters can be obtained from T. C. Glenn).

We used Chao's (1984) equation to obtain a nonparametric estimate of the true number of haplotypes (H') present in contemporary populations of the warbler based on the number of rare haplotypes present in the breeding sample,

$$H' = H_{\text{observed}} + \left(\frac{a^2}{2^b}\right)$$

where H_{observed} represents the observed number of haplotypes in a sample, a is the number of observed haplotypes that are represented by only a single individual in the sample (i.e., singletons), and b is the number of observed haplotypes represented by exactly two individuals in that sample (i.e., doubletons). A 95% CI for the true number of haplotypes in a sample was calculated with Chao's (1987) variance equation:

$$\text{var}\, H' = b\left[\left(\frac{a/b}{4}\right)^4 + (a/b)^3 + \left(\frac{a/b}{4}\right)^2\right]$$

RESULTS

Haplotype diversity and distribution.—For the combined data set, the 333-bp section of the mtDNA control region exhibited 64 variable sites (19%) and 94 unique haplotypes among the 287 typed individuals (Fig. 2). Most haplotypes differed by only a few base-pair changes (Fig. 3). The K81uf + I + G model (Hasegawa et al. 1985, Yang 1993, Gu et al. 1995) was selected by the hierarchical likelihood ratio test of MODELTEST, version 3.04 (Posada and Crandall 1998), as the best fit for the control-region data. Parameters estimated for this model were as follows: transition rate = 59.7; transversion rate 1 (A→T, C→G) = 8.3; transversion rate 2 (A→C, G→T) = 1.0 (for an average transition–transversion [Ti:Tv] ratio = 6.4); gamma shape parameter = 0.96; base frequencies A = 0.31, C = 0.31, G = 0.18, and T = 0.20; and proportion of invariable sites = 0.64.

The minimum spanning network of haplotypes was highly reticulated and organized around two principal and two subsidiary foci (Fig. 3). Most haplotypes were represented by singletons (67 of 94), 21 haplotypes were shared by 2–4 individuals, 2 haplotypes were shared by 7 individuals, and 4 common haplotypes were shared by 16–65 individuals (Fig. 2). The four most common haplotypes, which accounted for 55% of all individuals, were widely distributed geographically (Fig. 3). Most of the rarer haplotypes differed from common haplotypes by one or two nucleotides; related haplotypes often occurred in widely separated populations (Fig. 4). Pairwise nucleotide diversity (π = 0.00424–0.00812) was highest in Pennsylvania near the center of the breeding range and lowest in northern populations on glaciated landscapes and at the southern extreme of the breeding range in Georgia (Table 1 and Figs. 1 and 5A). The number of haplotypes detected per population was significantly correlated with sample size ($r^2 = 0.83$; $P = 0.0001$). Haplotype diversity (overall $h = 0.90$) varied from 0.79 (Paint Lake) to 0.96 (Blackwater Falls and Catskill Mountains) (Table 1 and Fig. 5B). Nucleotide and haplotype diversity were uncorrelated ($P = 0.15$), and neither diversity index was correlated with latitude ($P > 0.30$).

Population history.—Warblers exhibited significant among-population variation (AMOVA, 2.02%, $P = 0.004$) that was inconsistent with the predictions of an isolation-by-distance model (Mantel test, $r = 0.08$, $P = 0.27$; Fig 5C). Among-population variation was also significant when populations were pooled into two regional groups (highest support for the central and southern populations lumped; AMOVA, 2.86%, $P < 0.01$) or three regional groups (northern, central, and southern; AMOVA, 2.30%, $P < 0.02$). The two-group SAMOVA clustered the central and southern populations, except that Georgia was grouped with the northern populations (3.97% variation among groups, $P < 0.001$; 0.01% variation among populations within groups; 96.02% variation within populations).

When individual populations were compared in a pairwise fashion, 22 combinations exhibited significant F_{st} values ($P < 0.05$). After *P* values were adjusted for the number of simultaneous observations (0.05/91 = 0.00055), six pairwise combinations of populations showed significant F_{st} values. The Hiawatha population was significantly different from the Catskill Mountains population and three others from the southern Appalachians—Santeetlah Creek, Coweta, and the Unaka Mountains. The Paint River population

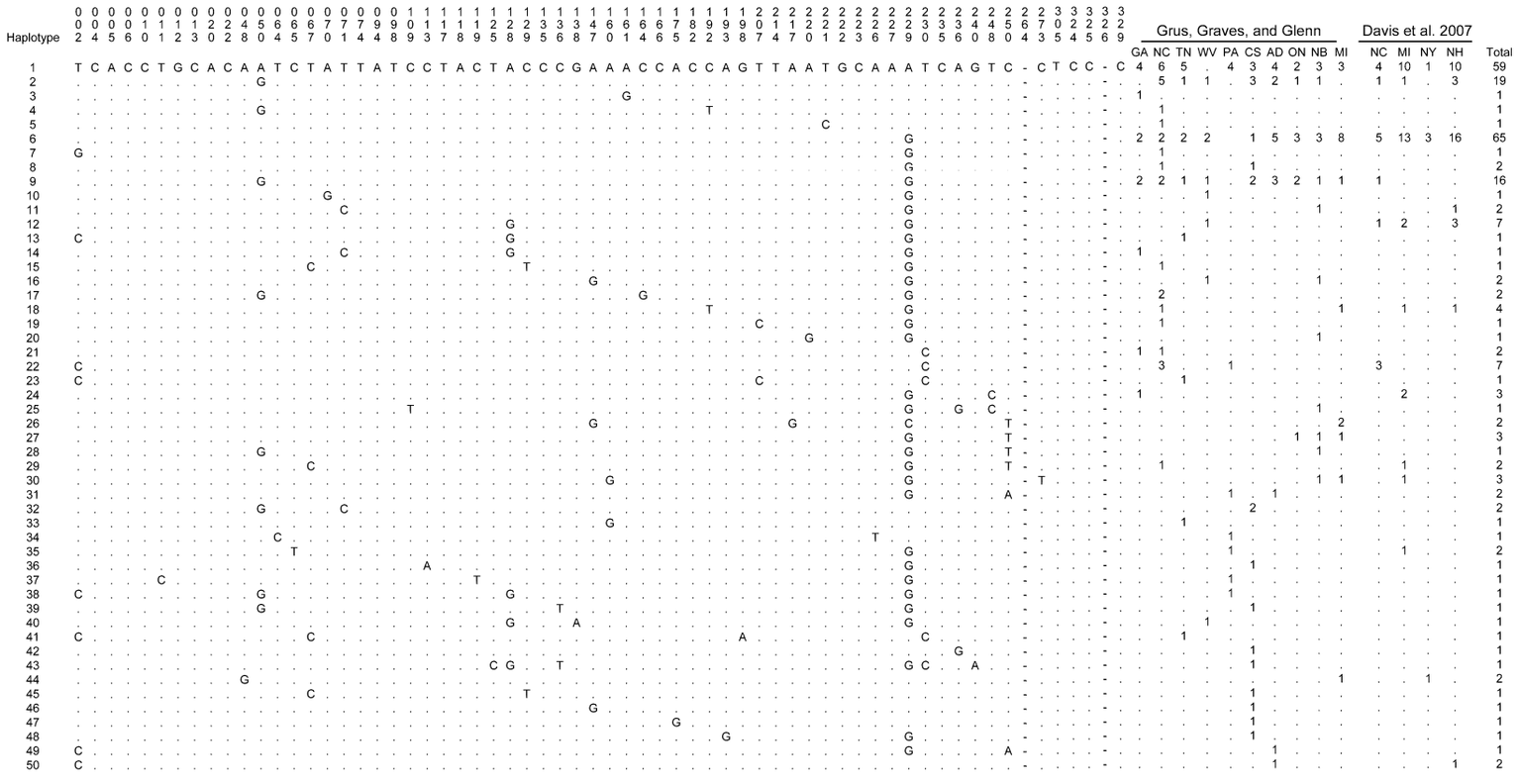

FIG. 2. Variable base positions in the mtDNA control region and frequency of haplotypes in Black-throated Blue Warbler (see text for details of base positions and population labels). *Figure 2 is continued on the next page.*

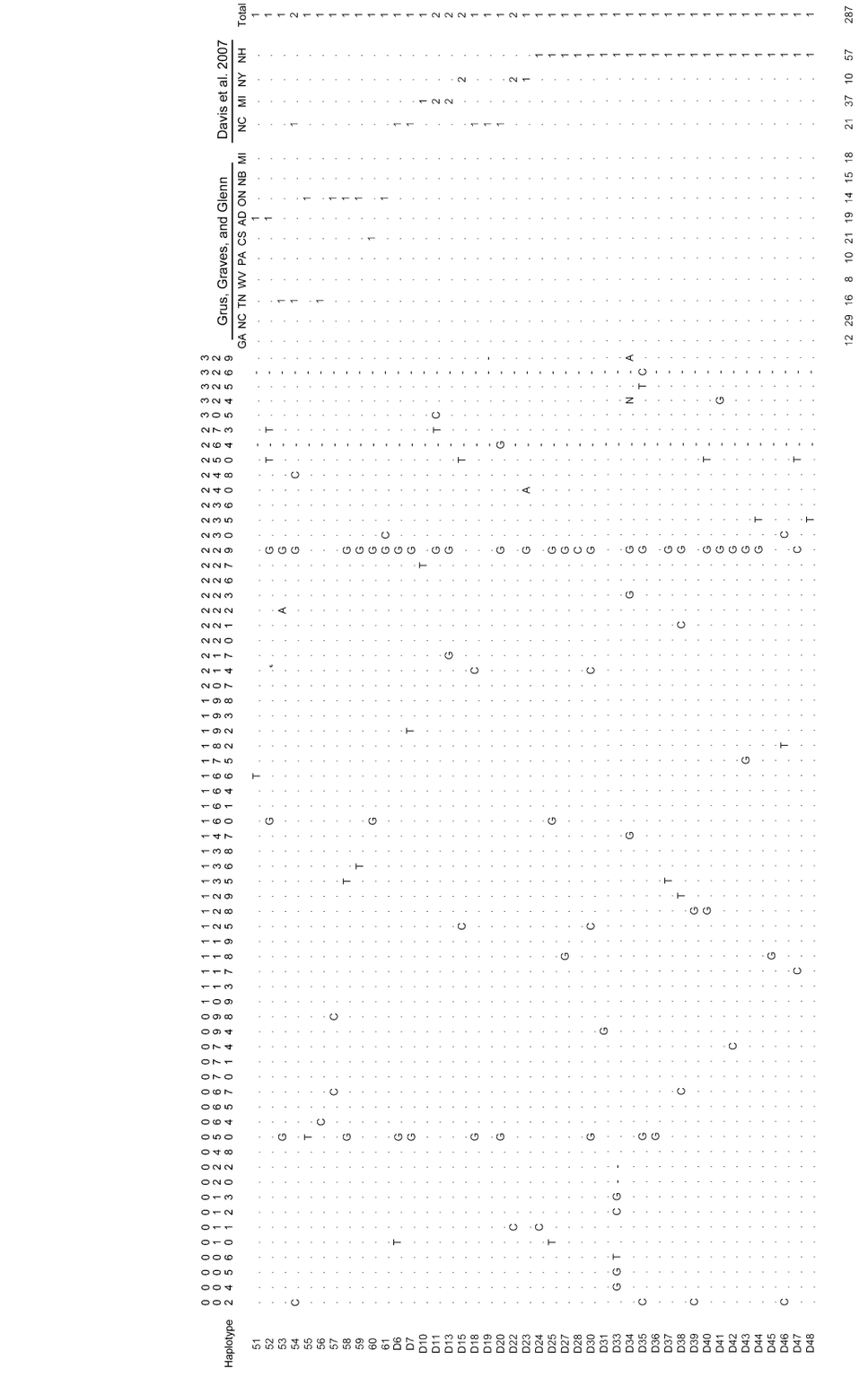

FIG. 2. *Continued.* Variable base positions in the mtDNA control region and frequency of haplotypes in Black-throated Blue Warbler (see text for details of base positions and population labels).

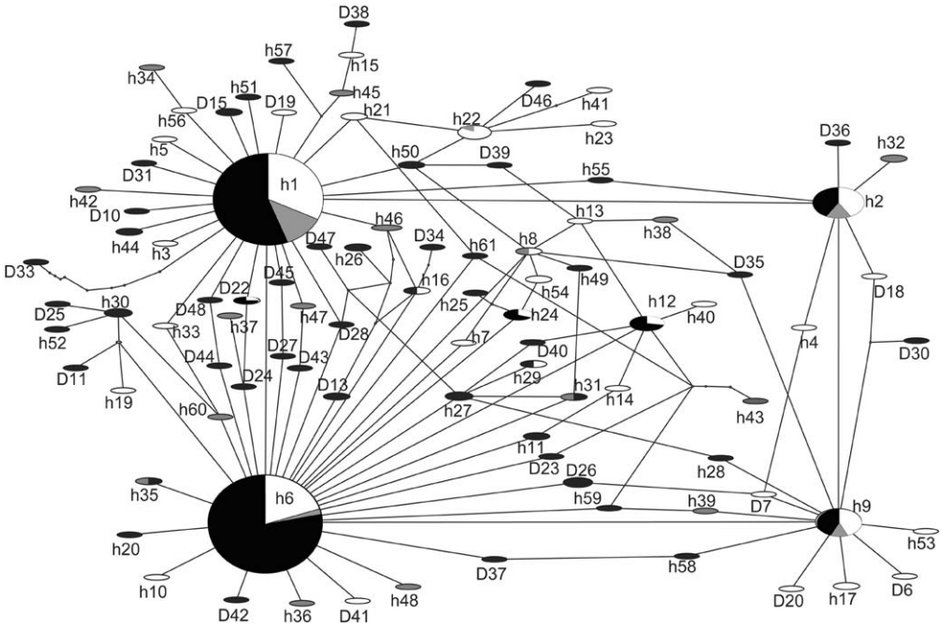

FIG. 3. Minimum spanning network for haplotypes of Black-throated Blue Warbler. Each oval represents a haplotype listed in Figure 2. Oval size is proportional to sample size. Missing haplotypes are indicated by small circles, and each branch indicates one nucleotide difference.

from Michigan also differed from populations in the Catskill and Unaka mountains. Tests for evidence of population expansion gave conflicting results. The subtly bimodal distribution of pairwise nucleotide differences (Fig. 6) suggests that warbler populations are in equilibrium and that a sudden-population-expansion model can be rejected (raggedness index = 0.05, $P = 0.18$; sudden expansion sum of squared deviation = 0.004, $P < 0.001$; Rogers and Harpending 1992). By contrast, Rozas et al.'s (2003) statistics reject the constant-population-size model ($FS = 159.41$, $P < 1 \times 10^{-6}$; $R^2 = 0.018$, $P < 0.01$). The rejection of neutrality hypotheses by Fu and Li's (1993) D^* statistic ($D^* = -4.62$, $P < 0.02$) and Tajima's (1989) D ($D = -2.25$, $P < 0.01$) provides additional evidence for population expansion. Although the strong rejection of neutrality could have other explanations besides population expansion, those explanations, such as selective sweeps (Maruyama and Birky 1991, Moyer et al. 2005) due to the linked coding regions of mtDNA, are rarely observed in mtDNA of animal populations (Gerber et al. 2001). The star-shaped topology of the haplotype tree is consistent with expectations of a recent range expansion (Avise and Walker 1998).

Under the model of pure demographic expansion, we estimated a mean $\tau = 2.07$ (95% CI: 0.74–2.83), yielding the number of years since T_{mrca} ($\tau = 2\,\mu t$) of 10,000 (95% CI: 3,700–14,000 years ago),

TABLE 1. Genetic variation in breeding populations of Black-throated Blue Warblers.

Population	Sample size	Number of haplotypes	Nucleotide diversity (π)	Haplotype diversity (h)
Cooper Creek, Georgia	12	7	5.081	0.8788
Coweta, North Carolina	21	12	6.359	0.9095
Santeetlah Creek, North Carolina	29	15	6.846	0.9236
Unaka Mountains, Tennessee	16	11	7.427	0.9083
Blackwater Falls, West Virginia	8	7	5.611	0.9643
Ricketts Glen, Pennsylvania	10	7	8.124	0.8667
Catskill Mountains, New York	21	15	7.884	0.9619
Adirondack Mountains, New York	19	9	5.230	0.8830
Hubbard Brook, New Hampshire	57	29	7.153	0.8929
Saranac Lake, New York	10	6	4.968	0.8889
Round Lake, Ontario	15	10	5.710	0.9451
Saint John River, New Brunswick	14	11	6.272	0.9429
Hiawatha NF, Michigan	37	12	4.237	0.8093
Paint Lake, Michigan	18	8	5.416	0.7908

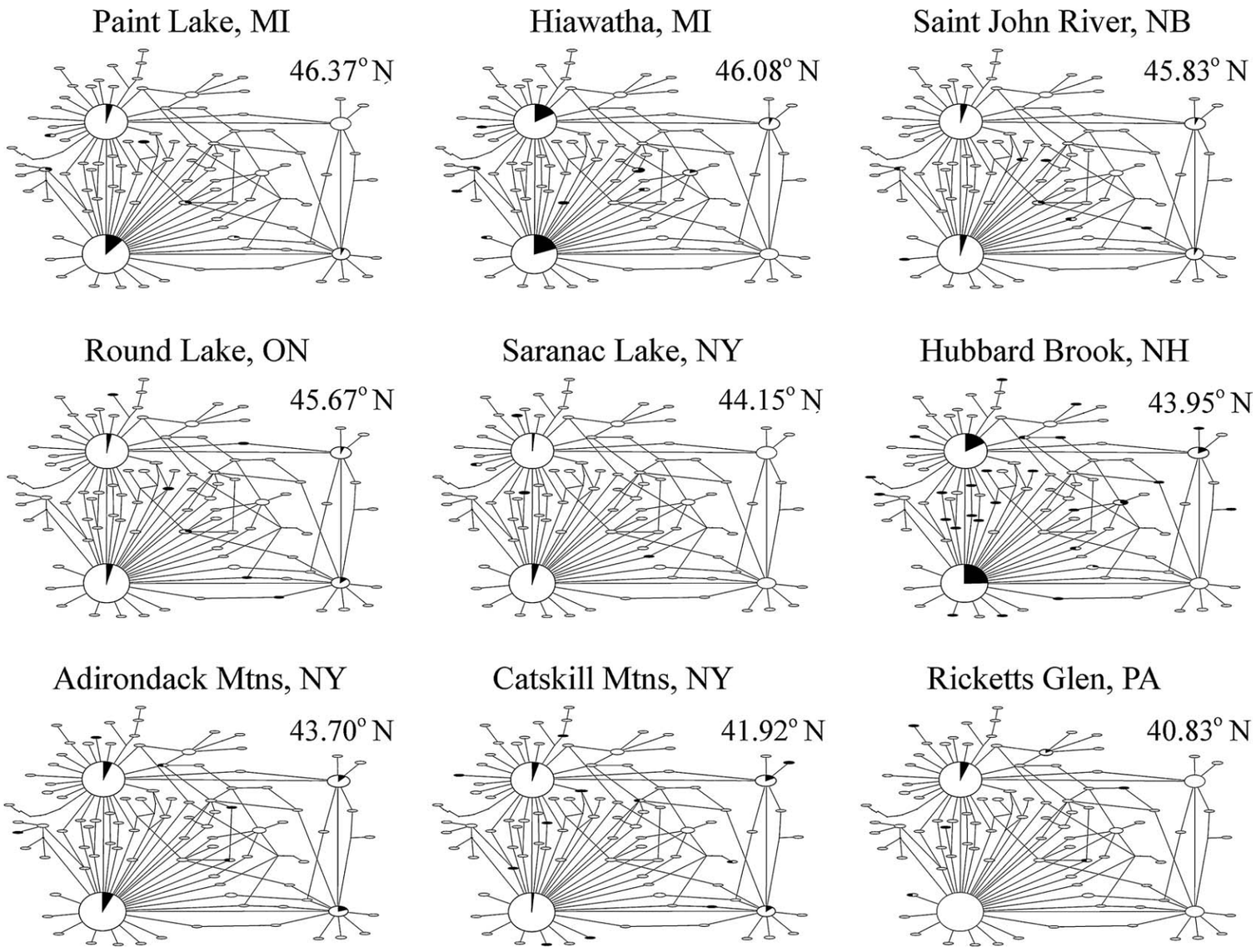

FIG. 4. Minimum spanning network for each population of Black-throated Blue Warbler. The network from Figure 3 is shown for each population, highlighting the haplotypes found in each population. *Figure 4 is continued on the next page.*

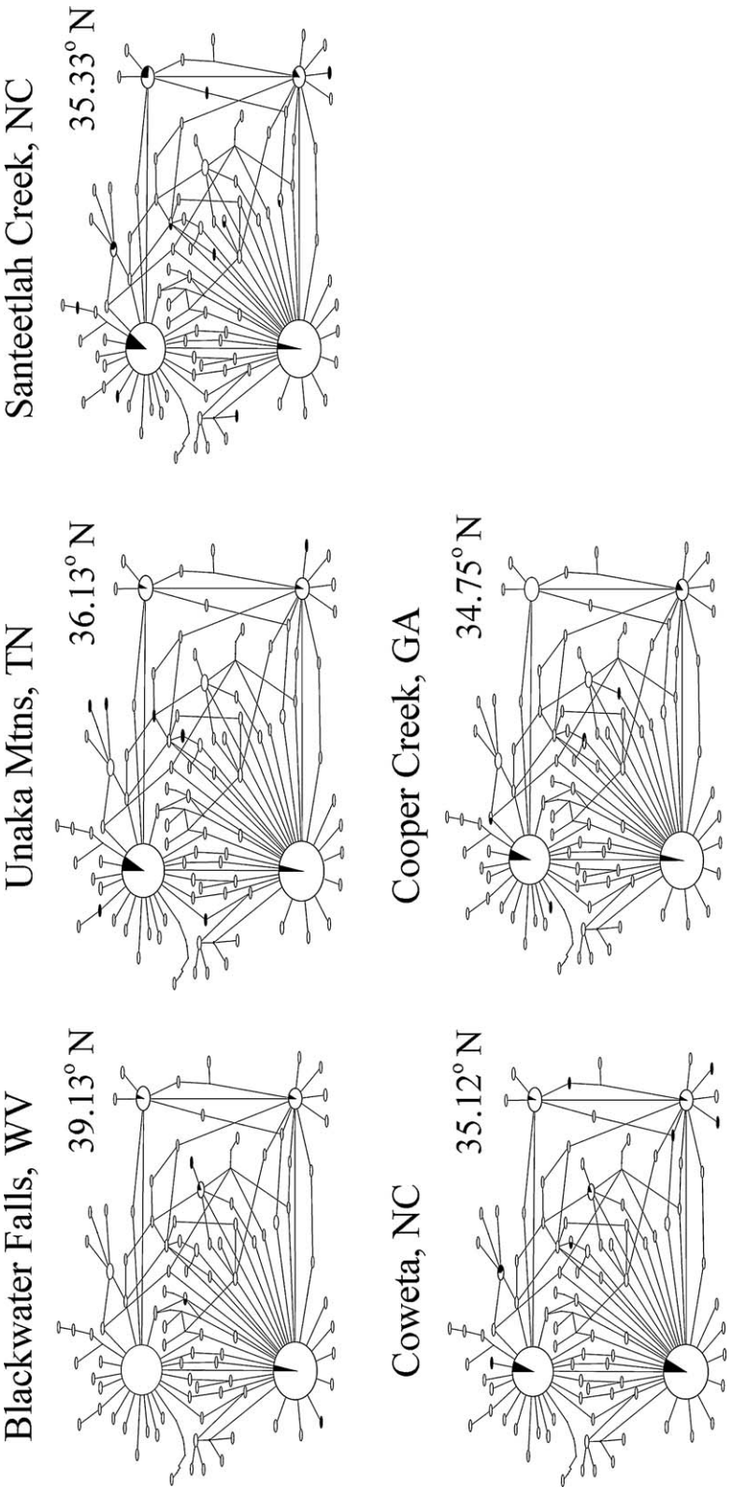

FIG. 4. *Continued.* Minimum spanning network for each population of Black-throated Blue Warbler. The network from Figure 3 is shown for each population, highlighting the haplotypes found in each population.

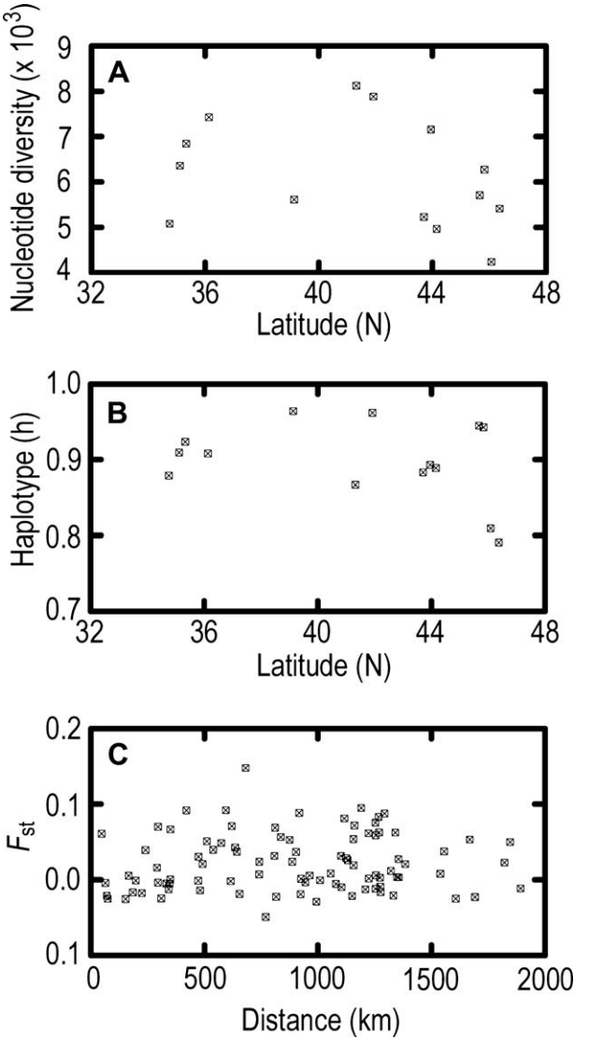

FIG. 5. Relationship of (A) nucleotide diversity and (B) haplotype diversity with latitude and (C) pairwise F_{st} values with geographic distance.

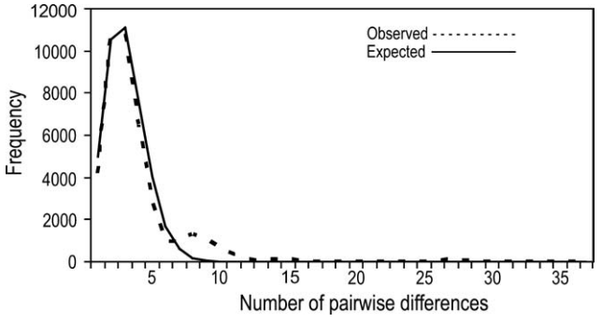

FIG. 6. Mismatch distribution for pairwise differences in Black-throated Blue Warbler haplotypes. The broken line represents the pairwise mismatch distribution. The expected distribution under the sudden-expansion model (Ti:Tv = 6.4) is represented by the solid line.

which suggests that the warbler was restricted to a single refugium near the end of the Pleistocene. The more complex coalescence simulations using BEAST yielded a median T_{mrca} of 45,000 years ago (95% CI: 12,000–84,000 years ago). If a slower mutation rate of 0.076 mutations per site per million years is assumed (cf. Davis et al. 2006), the T_{mrca} is estimated to be about four times older.

DISCUSSION

Breeding populations of Black-throated Blue Warblers exhibit relatively high haplotype diversity, low nucleotide diversity (cf. Milá et al. 2000, Milot et al. 2000), and subtle phylogeographic structure. We observed 94 haplotypes (67 singletons and 17 doubletons) in 287 individuals (0.33 haplotypes individual^{-1}) sampled from 14 populations. A nonparametric estimate (Chao 1984, 1987) of the lower bound of the true number of haplotypes for the pooled sample was estimated as 226 (95% CI: 160–292 haplotypes). The lower bound of the true number of haplotypes estimated from the four populations sampled by Davis et al. (2006) was 162 (95% CI: 86–238 haplotypes). The higher estimate of haplotype diversity from the combined data set indicated that analyses based on fewer geographic localities substantially underestimated the true number of haplotypes present in contemporary populations. The combined power obtained from sampling both more individuals and more localities was also important in revealing variation among population groups.

It is clear that most variation (>96%) in the mtDNA control region in warblers occurs within breeding populations. Limited but significant differentiation was observed between southern "black-backed" populations (*D. caerulescens cairnsi*) and the northern "blue-backed" populations (*D. c. caerulescens*). The two westernmost populations in Michigan were genetically distinct from populations in the Catskill Mountains and in the southern Appalachians of Tennessee and North Carolina, which indicates that populations from the southern and western edges of the breeding range may have diverged significantly from one another. On the other hand, the haphazard distribution of rare haplotypes (e.g., doubletons) among widely separated locations (Fig. 2) and the distribution of malarial parasite lineages among warbler populations (Fallon et al. 2006) suggests that long-distance dispersal events occur with some regularity. Because 83% of the sampled individuals were male, and because natal dispersal in songbirds is invariably female-biased (Greenwood 1980, Clarke et al. 1997), our analyses more likely represent an optimistic than a conservative estimate of genetic differentiation among warbler populations.

Nucleotide diversity was highest in populations near the center of the breeding range and tended to be lower in northern populations on glaciated landscapes and in Georgia at the southern terminus of the species' breeding range. Haplotype diversity was lowest in Michigan near the western periphery of the breeding range. Collectively, these data suggest that breeding populations from the central part of the breeding range, from West Virginia to New York, are more genetically diverse. Lower genetic diversity in peripheral populations is, presumably, a consequence of geographic isolation and smaller effective population sizes. Alternatively,

higher genetic diversity in the central portion of the breeding range may represent a contact zone between two formerly allopatric populations that originated in separate Pleistocene refugia. However, coalescence analysis suggests that warbler populations were restricted to a single refugium between 12,000 and 84,000 years ago ($\mu = 0.3$), an interpretation consistent with the star-shaped topology of the haplotype tree. This implies that geographic variation in plumage color and migratory behavior observed in contemporary breeding populations originated no earlier than the last glacial period. Davis et al. (2006) drew a similar conclusion but estimated that T_{mrca} ranged from 25,400–72,100 years ago ($\mu = 0.3$) to 98,000–284,000 years ago ($\mu = 0.076$).

"Blue-backed" populations, which occupy the whole of the glaciated breeding range, are separated from the "black-backed" populations of the unglaciated southern Appalachians by a cline in plumage melanism extending from southern Virginia (37°N) to the Susquehanna River in Pennsylvania (41°N) (G. R. Graves unpubl. data). This phenotypic variation, which is believed to be genetically controlled, is exhibited in yearling and older males. Although it is possible that geographic variation in plumage color existed in refugial populations, we suspect that the observed pattern is a relatively recent phenomenon associated with sexual selection and a strong selective factor, perhaps related to the migratory divide postulated from stable-isotope data (Rubenstein et al. 2002). Although the genetic differences are small and relatively recent in origin, our data suggest that warblers are more appropriately managed as two conservation units rather than a single unit.

Although there is no direct evidence that breeding populations segregate on the wintering grounds, stable-isotope studies suggest that populations breeding on glaciated landscapes winter primarily in Cuba and Jamaica, whereas populations from the southern Appalachians winter largely in Hispaniola and Puerto Rico (Rubenstein et al. 2002). Migratory behaviors of songbirds can have a strong genetic component that may evolve rapidly (Berthold and Querner 1981; Helbig 1991, 1996; Berthold 2003; Bearhop et al. 2005), and genetic differentiation is often associated with migratory divides in the breeding range (Bearhop et al. 2005). The extent to which the hypothesized migratory divide in warbler breeding populations coincides with the position of the cline in plumage color is unknown. However, selection for wintering destination is a plausible mechanism for the maintenance of geographic variation in plumage color in the presence of recent (postglacial) or, possibly, ongoing gene flow. Under such selection, offspring from hybridization between individuals that winter on different islands in the Greater Antilles may be genetically programmed to end their migrations over open water in the Caribbean. In any event, genetic factors that encode plumage color and migratory behavior in warblers are not likely to be reflected in the selectively neutral mtDNA control region or the coding regions to which it is linked. New, massively parallel DNA sequencing technologies will facilitate powerful investigations of the genetic basis of phenotypic variation in birds. Future research into the mechanisms that generated and maintain the phenotypic variation among warbler populations will make use of the large number of individuals and populations that have already been sampled (Graves 2004, Fallon et al. 2006).

Acknowledgments

We thank P. Angle, J. Dean, C. Dove, C. Gebhard, C. Milensky, J. Ososky, A. Ross, and B. Schmidt for preparing specimens and several anonymous reviewers for comments on the manuscript. Scientific licenses to sample populations were issued by the Canadian Wildlife Service (Atlantic and Ontario Region), U.S. Fish and Wildlife Service, U.S. Department of Agriculture Forest Service , Georgia Department of Natural Resources, Michigan Department of Natural Resources, New York Department of Environmental Conservation, North Carolina Wildlife Resources Commission, Pennsylvania Game Commission, Tennessee Wildlife Resources Agency, West Virginia Department of Natural Resources, and Wisconsin Department of Natural Resources. M. Schable and C. Outz helped in laboratory analyses. Funding was provided by the Alexander Wetmore Fund, the Research Opportunities Fund, the Biodiversity Surveys and Inventory Program of the National Museum of Natural History, the Scholarly Studies Program, the Smithsonian Migratory Bird Center (all Smithsonian Institution), National Science Foundation Award 9732138 to the Savannah River Ecology Laboratory Research Experience for Undergraduates program, and U.S. Department of Energy (contract numbers DE-FC-09-96SR18546 and DE-FC09-07SR22506) to the University of Georgia's Savannah River Ecology Laboratory. For detailed laboratory protocols, see http://www.uga.edu/srel/DNA_Lab/protocols.htm. ARLEQUIN is available at lgb.unige.ch/arlequin/.

Literature Cited

American Ornithologists' Union. 1957. Check-list of North American birds, 5th ed. American Ornithologists' Union, Baltimore, Maryland.

Avise, J. C., and D. Walker. 1998. Pleistocene phylogeographic effects on avian populations and the speciation process. Proceedings of the Royal Society of London, Series B 265:457–463.

Bearhop, S., W. Fiedler, R. W. Furness, S. C. Votier, S. Waldron, J. Newton, G. J. Bowen, P. Berthold, and K. Farnsworth. 2005. Assortative mating as a mechanism for rapid evolution of a migratory divide. Science 310:502–504.

Belle, E. M. S., G. Piganeau, M. Gardner, and A. Eyre-Walker. 2005. An investigation of the variation in the transition bias among various animal mitochondrial DNA. Gene 355:58–66.

Bensch, S., T. Andersson, and S. Åkesson. 1999. Morphological and molecular variation across a migratory divide in Willow Warblers, *Phylloscopus trochilus*. Evolution 53:1925–1935.

Berthold, P. 2003. Genetic basis and evolutionary aspects of bird migration. Advances in the Study of Behavior 33:175–229.

Berthold, P., and U. Querner. 1981. Genetic basis of migratory behavior in European warblers. Science 212:77–79.

Bohonak, A. J. 2002. IBD (isolation by distance): A program for analyses of isolation by distance. Journal of Heredity 93:153–154.

Carter, M. J., and I. D. Milton. 1993. An inexpensive and simple method for DNA purifications on silica particles. Nucleic Acids Research 21:1044.

Chamberlain, C. P., S. Bensch, X. Feng, S. Åkesson, and T. Andersson. 2000. Stable isotopes examined across a migratory divide in Scandinavian willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) reflect their African

winter quarters. Proceedings of the Royal Society of London, Series B 267:43–48.

CHAMBERLAIN, C. P., J. D. BLUM, R. T. HOLMES, X. FENG, T. W. SHERRY, AND G. R. GRAVES. 1997. The use of isotope tracers for identifying populations of migratory birds. Oecologia 109: 132–141.

CHAO, A. 1984. Non-parametric estimation of the number of classes in a population. Scandinavian Journal of Statistics 11:265–270.

CHAO, A. 1987. Estimating the population size for capture–recapture data with unequal catchability. Biometrics 43:783–791.

CLARKE, A. L., B.-E. SÆTHER, AND E. RØSKAFT. 1997. Sex biases in avian dispersal: A reappraisal. Oikos 79:429–438.

CLEMENT, M., D. POSADA, AND K. A. CRANDALL. 2000. TCS: A computer program to estimate gene genealogies. Molecular Ecology 9:1657–1659.

COUES, E. 1897. Characters of *Dendroica cærulescens cairnsi*. Auk 14:96–97.

DAVIS, L. A., E. H. ROALSON, K. L. CORNELL, K. MCCLANAHAN, AND M. S. WEBSTER. 2006. Genetic divergence and migration patterns in a North American passerine bird: Implications for evolution and conservation. Molecular Ecology 15:2141–2152.

DON, R. H., P. T. COX, B. J. WAINWRIGHT, K. BAKER, AND J. S. MATTICK. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research 19:4008.

DRUMMOND, A. J., AND A. RAMBAUT. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. [Online.] BMC Evolutionary Biology 7:214, doi:10.1186/1471-2148-7-214.

DUPANLOUP, I., S. SCHNEIDER, AND L. EXCOFFIER. 2002. A simulated annealing approach to define the genetic structure of populations. Molecular Ecology 11:2571–2581.

EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131:479–491.

FALLON, S. M., R. C. FLEISCHER, AND G. R. GRAVES. 2006. Malarial parasites as geographic markers in migratory birds? Biology Letters 2:213–216.

FU, Y.-X., AND W.-H. LI. 1993. Statistical tests of neutrality of mutations. Genetics 133:693–709.

GERBER, A. S., R. LOGGINS, S. KUMAR, AND T. E. DOWLING. 2001. Does nonneutral evolution shape observed patterns of DNA variation in animal mitochondrial genomes? Annual Review of Genetics 35:539–566.

GRAVES, G. R. 1997a. Age determination of free-living male Black-throated Blue Warblers during the breeding season. Journal of Field Ornithology 68:443–449.

GRAVES, G. R. 1997b. Geographic clines of age ratios of Black-throated Blue Warblers (*Dendroica caerulescens*). Ecology 78: 2524–2531.

GRAVES, G. R. 2004. Testicular volume and asymmetry are age-dependent in Black-throated Blue Warblers (*Dendroica caerulescens*). Auk 121:473–485.

GRAVES, G. R., C. S. ROMANEK, AND A. RODRIGUEZ NAVARRO. 2002. Stable isotope signature of philopatry and dispersal in a migratory songbird. Proceedings of the National Academy of Sciences USA 99:8096–8100.

GREENWOOD, P. J. 1980. Mating systems, philopatry and dispersal in birds and mammals. Animal Behaviour 28:1140–1162.

GU, X., Y.-X. FU, AND W.-H. LI. 1995. Maximum likelihood estimation of the heterogeneity of substitution rate among nucleotide sites. Molecular Biology and Evolution 12:546–557.

HANEY, J. C., D. S. LEE, AND M. WILBERT. 2001. A half-century comparison of breeding birds in the southern Appalachians. Condor 103:268–277.

HASEGAWA, M., H. KISHINO, AND T. YANO. 1985. Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160–174.

HAYES, J. P., AND R. G. HARRISON. 1992. Variation in mitochondrial DNA and the biogeographic history of woodrats (*Neotoma*) of the eastern United States. Systematic Biology 41:331–344.

HELBIG, A. J. 1991. SE- and SW-migrating Blackcap (*Sylvia atricapilla*) populations in central Europe: Orientation of birds in the contact zone. Journal of Evolutionary Biology 4:657–670.

HELBIG, A. J. 1996. Genetic basis, mode of inheritance and evolutionary changes of migratory directions in Palearctic warblers (Aves: Sylviidae). Journal of Experimental Biology 199:49–55.

HEWITT, G. M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society 58:247–276.

HOLMES, R. T. 1994. Black-throated Blue Warbler (*Dendroica caerulescens*). *In* The Birds of North America, no. 87 (A. Poole and F. Gill, Eds.). Academy of Natural Sciences, Philadelphia, and American Ornithologists' Union, Washington, D.C.

HOLMES, R. T., P. P. MARRA, AND T. W. SHERRY. 1996. Habitat-specific demography of breeding Black-throated Blue Warblers (*Dendroica caerulescens*): Implications for population dynamics. Journal of Animal Ecology 65:183–195.

MARUYAMA, T., AND C. W. BIRKY, JR. 1991. Effects of periodic selection on gene diversity in organelle genomes and other systems without recombination. Genetics 127:449–451.

MAYR, E. 1963. Animal Species and Evolution. Belknap Press of Harvard University Press, Cambridge, Massachusetts.

MERILÄ, J., M. BJÖRKLUND, AND A. J. BAKER. 1997. Historical demography and present day population structure of the greenfinch, *Carduelis chloris*—An analysis of mtDNA control-region sequences. Evolution 51:946–956.

MILÁ, B., D. J. GIRMAN, M. KIMURA, AND T. B. SMITH. 2000. Genetic evidence for the effect of a postglacial population expansion on the phylogeography of a North American songbird. Proceedings of the Royal Society of London, Series B 267:1033–1040.

MILOT, E., H. L. GIBBS, AND K. A. HOBSON. 2000. Phylogeography and genetic structure of northern populations of the Yellow Warbler (*Dendroica petechia*). Molecular Ecology 9:667–681.

MOYER, G. R., K. O. WINEMILLER, M. V. MCPHEE, AND T. F. TURNER. 2005. Historical demography, selection, and coalescence of mitochondrial and nuclear genes in *Prochilodus* species of northern South America. Evolution 59:599–610.

POSADA, D., AND K. A. CRANDALL. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14:817–818.

RAMOS-ONSINS, S. E., AND J. ROZAS. 2002. Statistical properties of new neutrality tests against population growth. Molecular Biology and Evolution 19:2092–2100.

RIDGWAY, R. 1902. The birds of North and Middle America, part 2. Bulletin of the U.S. National Museum, no. 50.

ROGERS, A. R. 1995. Genetic evidence for a Pleistocene population explosion. Evolution 49:608–615.

Rogers, A. R., and H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Molecular Biology and Evolution 9:552–569.

Rozas, J., J. C. Sánchez-DelBarrio, X. Messeguer, and R. Rozas. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497.

Rubenstein, D. R., C. P. Chamberlain, R. T. Holmes, M. P. Ayres, J. R. Waldbauer, G. R. Graves, and N. C. Tuross. 2002. Linking breeding and wintering ranges of a migratory songbird using stable isotopes. Science 295:1062–1065.

Ruegg, K. C., and T. B. Smith. 2002. Not as the crow flies: A historical explanation for circuitous migration in Swainson's Thrush (*Catharus ustulatus*). Proceedings of the Royal Society of London, Series B 269:1375–1381.

Sage, R. D., and J. O. Wolff. 1986. Pleistocene glaciations, fluctuating ranges, and low genetic variability in a large mammal (*Ovis dalli*). Evolution 40:1092–1095.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schneider, S., and L. Excoffier. 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: Application to human mitochondrial DNA. Genetics 152:1079–1089.

Tajima, F. 1989. Statistical methods for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Wilcove, D. S. 1988. Changes in the avifauna of the Great Smoky Mountains: 1947–1983. Wilson Bulletin 100:256–271.

Williams, J. W., T. Webb III, P. H. Richard, and P. Newby. 2000. Late Quaternary biomes of Canada and the eastern United States. Journal of Biogeography 27:585–607.

Yang, Z. 1993. Maximum-likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. Molecular Biology and Evolution 10:1396–1401.

Zink, R. M. 1996. Comparative phylogeography in North American birds. Evolution 50:308–317.

Zink, R. M., and J. V. Remsen, Jr. 1986. Evolutionary processes and patterns of geographic variation in birds. Pages 1–69 *in* Current Ornithology, vol. 4 (R. F. Johnston, Ed.). Plenum Press, New York.

Associate Editor: J. Klicka