The Auk 116(1):274–277, 1999

Speciation in North American Chickadees: II. Geography of mtDNA Haplotypes in *Poecile carolinensis*

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The Carolina Chickadee (*Poecile carolinensis*) of southeastern North America comprises two geographically structured, monophyletic clusters of mitochondrial DNA (mtDNA) haplotypes (Gill et al. 1993). Here, we report the mtDNA identities of specimens of *P. carolinensis* from new localities in southwestern Alabama west across southern Mississippi. The molecular identities of the new specimens define a clinal, not parapatric, transition from eastern haplotypes to western haplotypes. The results also document the first genetic cline structure between morphologically undifferentiated populations of a bird species.

Methods.—Earlier specimens from a coarse sampling transect from Texas to Georgia (collected in 1990) revealed that the divergent *P. carolinensis* haplotypes respectively occupy extensive ranges east and west of the Mobile Bay / Tombigbee River system of southwestern Alabama. These samples also focused attention on the Mobile Basin / Tombigbee River as the place of contact and possibly parapatric transition from eastern to western haplotypes (Gill et al. 1993).

To define the extent of coexistence of haplotypes and to test whether the contact was truly parapatric or a more gradual step cline, we collected additional specimens in February 1993 and February 1995, mostly from 10 new localities west from the Tombigbee River in southwestern Alabama and across the state of Mississippi (Fig. 1). We froze heart and liver samples within 30 min of collection. Specimens were preserved as traditional study skins and tissue vouchers in the collections of the Academy of Natural Sciences of Philadelphia.

Sequence compositions of eastern versus western haplotypes differ by an estimated 2.7%, which is about one-half the divergence (5.2%) between *P. carolinensis* and the Black-capped Chickadee (*Poecile atricapillus*). These unpublished divergence estimates are based on comparisons of 1,068 bp of the cytochrome-*b* gene from mitochondrial DNA (F. Gill, B. Slikas, and F. Sheldon unpubl. data). Earlier estimates of divergence based on calculations from RFLP comparisons were 2.4% for shared restriction fragments and 3.4% for shared restriction sites inferred from shared restriction fragments (Gill et al. 1993).

We screened samples collected in 1990 and 1993 for alternative haplotypes by means of restriction-enzyme digestion of whole, purified mtDNA using two diagnostic enzymes: *Hind* III and *Pvu* II (Gill et al. 1993). The 1995 samples were screened by restriction-enzyme digestion of a PCR product. In this latter method, we searched cytochrome-*b* sequences of eastern and western *P. carolinensis* for restriction sites unique to each sequence type. DNA was extracted from the 1995 samples using the Chelex protocol (Walsh et al. 1991). A 5-mL aliquot of the Chelex extract was used as template in a 50-mL PCR mix, using modified versions of published primers (Kocher et al. 1989) to amplify a 1-kb portion of the mitochondrial cytochrome-*b* gene. The primers used were L14990 (5′ CCA TCC AAC ATC TCA GCA TGA TGA AA 3′) and H16065 (5′ GGA GTC TTC AGT CTC TGG TTT ACA AGA C 3′). Application of different restriction enzymes identified two diagnostic sites: an *Xmn* I site present in eastern *P. carolinensis* sequences and absent in the western sequences, and a *BssK* I site present in western *P. carolinensis* and absent in eastern sequences. The PCR-based method was tested at the outset on individuals that had been typed by the previous protocol (using restriction enzyme digestion of whole mtDNA); the two methods gave identical typings. Then we cut PCR product from each 1995 specimen with both diagnostic restriction enzymes to identify the mitochondrial sequence as eastern or western; all samples were unambiguously typed by this method.

Results.—No morphological correlates of the *P. carolinensis* haplotype distinction are known. As discussed previously (Gill et al. 1993), the distributions of the eastern and western haplotypes do not correspond to the poorly defined morphological subspecies of *Poecile carolinensis*. Furthermore, specimens of eastern and western haplotypes were identical in the average measurements of four standard characters: wing length, tail length, exposed culmen length, and tarsus length (D. Agro unpubl. data). Detailed comparisons of vocalizations remain to be accomplished, but alert to the possibility of behavioral differences, we could discern no differences in vocalizations in the field.

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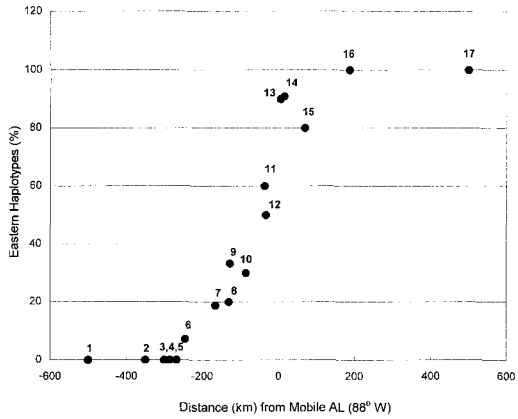

FIG. 1. Clinal variation in frequency of eastern mtDNA haplotypes in *Poecile carolinensis* in Alabama and Mississippi, centered on the longitude (88°W) of Mobile, Alabama. Localities to the west of Mobile are designated as negative distances, and localities to the east as positive distances. Localities (state, county or parish [Louisiana only], lat/long) followed by sample sizes in parentheses as follows: 1, Texas, Tom Greene, 31°08'N, 100°30'W (6); 2, Louisiana, Madison, 32°15'N, 91°05'W (3); 3, Louisiana, Iberville, 30°20'N, 91°12'W (12); 4, Mississippi, Issaquena, 32°38'N, 90°50'W (8); *5, Mississippi, Franklin, 31°25'N, 91°10'W (10); *6, Mississippi, Pike, 31°10'N, 90°30'W (14); *7, Mississippi, Marion, 31°15'N, 89°48'W (16); 8, Mississippi, Scott, 31°11'N, 89°20'W (5); *9, Mississippi, Forrest, 30°59'N, 89°16'W (9); *10, Mississippi, Perry, 31°05'N, 88°48'W (10); *11, Alabama, Mobile, 31°10'N, 88°20'W (10); *12, Alabama, Choctaw, 32°06'N, 88°21'W (10); *13, Alabama, Sumter, 32°35'N, 87°56'W (10); *14, Alabama, Baldwin, 31°08'N, 87°50'W (16); *15, Alabama, Bibb and Perry, 32°45'N, 87°08'W (10); 16, Alabama, Talladega, 33°18'N, 86°02'W (5); 17, Georgia, Greene, 33°30'N, 83°00'W (11). Localities marked with an asterisk represent new localities or additional specimens; other localities reported previously by Gill et al. (1993).

Individual *P. carolinensis* with eastern or western haplotypes co-occur at localities from Pike County, Mississippi (90°W), east to Perry County, Alabama (87°W), a distance of 355 km. This step cline is bordered by a sample with only western haplotypes from the Homochito National Forest in Franklin County, Mississippi (91°W), and a sample with only eastern haplotypes from the Talladega National Forest in Talledega County, Alabama (86°W). The small sample sizes for these flanking localities do not preclude low frequencies of alternative haplotypes. No eastern haplotypes, however, were present in an additional 32 *P. carolinensis* examined from localities west of 91°W, or in an additional 28 *P. carolinensis* from localities east of the Appalachians (Gill et al. 1993).

The zone of overlap itself comprises about 15% of the species' longitudinal span (degrees) from eastern Texas (100°W) to the Atlantic coast of Georgia (81°W). The distance between localities with 25% eastern haplotypes (projected for Lamar County, Mississippi) and 75% eastern haplotypes (Tombigbee River, Alabama) is roughly 140 km. Eastern haplotypes increase steadily in frequency (20% per 100 km) eastward from Pike County, Mississippi, and then sharply at the Mobile Bay/Tombigbee River system of southwestern Alabama (longitude 88°W; Fig. 1). The two easternmost localities with western haplotypes (Bibb and Perry counties, Alabama) fall between the two major branches of the Tombigbee River and thus fall within a past northeastern extension of the old Mobile Basin.

Birds with each haplotype consorted as flockmates. To examine whether alternative haplotypes sorted sexually, we examined 10 apparently mated pairs of *P. carolinensis* at four localities within the zone of overlap between haplotypes, namely Mobile County, Alabama; Choctaw County, Alabama; Forrest County, Mississippi; and Marion County, Mississippi. The proportions of western haplotypes at these four localities were 0.40, 0.50, 0.67, and 0.81, respectively. We considered the pairs to be mated because they were isolated pairs of opposite sex when collected in February 1993 and February 1995. During these times, the chickadees were singing on territory and highly responsive to song playbacks. *Poecile carolinensis* is a permanent resident that pairs early and defends breeding territories early in the year in the deep south. Six of the 10 pairs were mixed pairs, i.e. pairs that comprised individuals with alternative east/west haplotypes. These data are inadequate to test pair compositions against expected associations at each locality, but the mixed-pair compositions mitigate against the possibility of strong assortative mating between sympatric species.

Discussion.—Our primary goal was to sharpen the geographic resolution of the contact zone between eastern and western haplotype groups of *P. carolinensis*, because our initial data suggested that the divergent haplotypes occupied parapatric distributions separated by the Mobile Basin/Tombigbee River. Those data teased us with the possibility of cryptic, allopatric taxa diagnosable solely by mtDNA restriction sites. The new samples reported here reveal that chickadees with eastern and western haplotypes coexist and probably interbreed freely at localities that vary clinally in the proportions of alternative haplotypes. Hence, in sympatry, individuals carrying eastern versus western haplotypes "behave" as a single biological species. An option remains for formal taxonomic distinction as subspecies to recognize both long-standing evolutionary independence and the current geographic structuring of haplotypes. To

do so would be novel. We know of no other case of an avian taxon being defined solely by a single biochemical character, and we do not advocate such a taxonomic distinction at this time.

Our definition of the geographic structure of Carolina Chickadees based on divergent mtDNA haplotypes adds to the evidence for significant fragmentation and genetic divergence of vertebrate populations in the Southeast (Avise 1994). The lowlands of the southeastern United States provided refugia for taxa that experienced range compression during glacial maxima (Highton and Webster 1976, Pielou 1991, Avise 1992). Not only was *P. carolinensis* restricted to the Southeast during glacial maxima (Brewer 1963), but its populations apparently fragmented into eastern and western fractions, allowing allopatric fixation and 2.7% divergence of alternative mtDNA sequences. No evidence supports the alternative hypothesis of local divergence as a haplotype polymorphism (Avise 1989). The primary split of *P. carolinensis* into eastern and western components predates recent Pleistocene glaciations. We project that split back 1 to 1.5 mya, based on a standard rate of 2% per million years corrected for divergence among variants of eastern haplotypes and among western haplotypes, respectively (Nei 1987, Gill et al. 1993).

Certain major river systems, such as the Appalachicola and Tombigbee (Mobile Basin), acted as barriers, particularly when these water barriers were at full dimensions during maximum sea levels (Avise 1992). Longitudinal disjunctions at the Tombigbee River system currently include such diverse taxa as water snakes (Lawson 1987), minnows (Wiley and Mayden 1985), and perhaps swamp rabbits (Chapman and Feldhamer 1981). Among birds, divergent song populations of the Northern Parula (*Parula americana*) also make contact at the Tombigbee River, but they exhibit no mtDNA divergence based on a survey with 22 restriction enzymes (Moldenhauer 1992, Moldenhauer and Regelski 1996).

We hypothesize that the Mobile Basin served as the primary historical barrier between eastern and western populations of *P. carolinensis*, allowing divergence of their mtDNA genomes. We do not know, however, to what degree the step cline described in this paper is due to recent secondary contact, or whether it is a static or dynamic one, because we lack data on dispersal or recent population dynamics of *P. carolinensis* in the southeastern states. *Poecile carolinensis* is expanding its range northward (Brewer 1963, Peterjohn 1989, Sibley 1994), but the haplotype correlates of that expansion are not known. Indeed, the range expansion of *P. carolinensis* has been poorly documented in some regions because of the difficulties of distinguishing this species from *P. atricapillus* in the field (Parkes 1966, 1987).

To date, the analysis of ornithological biogeography in southeastern North America, and the detection of major range expansions and replacements, generally have been limited to morphologically different taxa. Yet, species such as *Poecile carolinensis* that occupied distributions at low latitudes exhibit greater genetic population structure than do species such as *P. atricapillus* that responded opportunistically to climatic changes at higher latitudes (Slatkin 1987; Capparella 1988, 1991; Gill et al. 1993; Highton 1995; Zink 1996). The final message from this study is that we face a growing need for molecular sampling of avian populations, first, to determine whether other species of birds in the Southeast comprise cryptic biochemical populations, and second, to detect the geographic spread and replacement of alternative avian genotypes at major geographic scales.

Acknowledgments.—We gratefully acknowledge the field assistance of G. Armistead, A. Ingersoll, C. Witt, and F. H. Sheldon. Especially helpful to this work was access to key collecting localities granted by Mrs. E. Johnson, D. Stringer, M. Vanardo, and the Scott Paper Company (T. Morgan, R. Taylor). Our research was supported by a National Science Foundation grant (BSR 88-06647).

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Received 19 February 1998, accepted 13 July 1998.
Associate Editor: R. M. Zink