MITOCHONDRIAL DNA: A SOURCE OF GENETIC MARKERS FOR STUDIES OF SIMILAR PASSERINE BIRD SPECIES

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ABSTRACT.—Restriction enzyme analyses of mitochondrial DNA (mtDNA) of the Black-capped Chickadee (*Parus atricapillus*), Carolina Chickadee (*P. carolinensis*), and Tufted Titmouse (*P. bicolor*) indicate substantial genetic divergence (p = 0.04–0.09). Eleven of 14 enzymes produced fragment patterns that distinguish the two chickadees, revealing a bounty of markers for studies of these sibling species. *Received 23 December 1985, accepted 18 April 1986.*

DIFFERENCES in mitochondrial DNA (mtDNA) are valuable for biochemical comparisons of closely related taxa. MtDNA is a moderately sized molecule (15,700–19,500 base pairs) amenable to detailed comparative study. Size, content, and gene organization of mtDNA vary little among vertebrates. These molecules contain few repeated or nontranscribed sequences. Large sequence rearrangements and deletions are less common than in nuclear DNA. Apparently subject to high mutation rates and rapid rates of evolution, mtDNA often yields better resolution of closely related taxa than can nuclear DNA (Brown 1983).

Divergence in mtDNA base pair composition can be revealed through application of restriction endonucleases, which cut the mtDNA molecule into fragments, the number and size of which are specific for each organism. The sizes of the fragments sum to that of the original, circular molecule. Mutations that alter any of the base pairs in a sequence recognized by an enzyme will produce corresponding differences in the number and size of the fragments produced by that enzyme. Thus, differences in fragment patterns can be used as a measure of the amount of change between any pair of mtDNAs. Restriction enzyme analyses of mtDNA have been useful in the study of evolutionary relationships among higher primates (Johnson et al. 1983), rodents (Avise et al. 1979; Brown et al. 1981; Ferris et al. 1981, 1983b), reptiles (Brown and Wright 1979, Wright et al. 1983), and ducks (Kessler and Avise 1984), as well as in the resolution of patterns of gene exchange between hybridizing vertebrate species (Ferris et al. 1983a; Avise et al. 1984; Avise and Saunders 1984; Spolsky and Uzzell 1984, 1986; Gyllensten et al. 1985; Szymura et al. 1985).

We performed a restriction enzyme analysis of mtDNA nucleotide sequences in the three common species of *Parus* of the eastern United States, the morphologically similar Black-capped Chickadee (*Parus atricapillus*) and Carolina Chickadee (*P. carolinensis*), and the dissimilar Tufted Titmouse (*P. bicolor*). Whether *P. atricapillus* and *P. carolinensis* comprise two distinct species remains unresolved (Braun and Robbins 1986). The more northern *atricapillus* replaces *carolinensis* abruptly along a long, narrow contact zone stretching from New Jersey to Kansas and also at high elevations in the Appalachians (Tanner 1952; Brewer 1963; Merritt 1978, 1981). They hybridize where their breeding ranges overlap in Missouri (Robbins et al. 1986), Kansas (Rising 1968), Illinois (Brewer 1963), and Indiana (Merritt 1981) and probably most other regions of contact. The reproductive relations of *P. atricapillus* and *P. carolinensis* remain uncertain. Clear morphological or genetic markers that would permit analysis of the extent of hybridization and the limits of

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introgression beyond the contact zone are unknown. These two forms are extremely difficult to identify; vocalizations provide some of the best clues to identity, but they too may not be reliable (Robbins et al. 1986). A recent survey of allozymic variation at 35 presumed genetic loci revealed no fixed allelic differences (Braun and Robbins 1986). Indeed, the protein genetic distance (*D*; Nei 1978) between *P. atricapillus* and *P. carolinensis* is only 0.001, less than that usually distinguishing avian subspecies (Barrowclough 1983).

The preliminary survey of mtDNA we report here revealed a bounty of genetic markers for future studies of *P. atricapillus* and *P. carolinensis*. Eleven of 14 restriction enzymes tested yielded diagnostic fragment patterns. The level of divergence we found in *Parus* mtDNA is comparable to that found among morphologically distinct species of ducks (Kessler and Avise 1984).

Methods

This study is based on specimens of *P. atricapillus*, *P. bicolor*, and *P. carolinensis* obtained during the breeding season. The *P. atricapillus* were from the vicinity of Eckville, Pennsylvania in Berks Co., 24 km north of the contact zone. The *P. carolinensis* were from the pine barrens (Wharton State Forest) in the vicinity of Dunbarton, Gloucester Co., New Jersey about 90 km south of the contact zone. The *P. bicolor* were from extreme northeastern Lancaster Co. and from Eckville, Berks Co., Pennsylvania. Study skins of the specimens are deposited in the collection of The Academy of Natural Sciences in Philadelphia.

We found that fresh tissues were not required, because frozen tissues yield substantial amounts of purified mtDNA. This greatly simplifies sampling wild birds, which can be frozen in the field to await laboratory preparation. We transported freshly shot specimens on dry ice and transferred them to laboratory storage at −70°C until we isolated and purified mtDNA from heart and liver tissues using established procedures (Spolsky and Uzzell 1984, 1986). The addition of a sucrose step gradient allowed us to obtain highly purified mtDNA from frozen tissues with little nuclear contamination. Lysis and purification by organic gradients yielded up to 1.5 μg of purified mtDNA per individual.

We digested 5–10 ng of the purified mtDNA of each species with 18 commercially available (Bethesda Research Labs and New England Biolabs) type II restriction enzymes that recognize and cleave at 6-base pair nucleotide sequences (Table 1). We followed standard digestion procedures using the supplier's recommended assay conditions. We labeled the fragments with alpha-^{32}P-deoxynucleoside triphosphates and separated the fragments electrophoretically by length on horizontal 1% agarose gels. Fragments longer than about 400 base pairs could be scored on such gels. Each gel included markers of known fragment sizes: PM2 and lambda phage DNA each cut with Hind III. We dried the gels onto 3MM chromatography paper and made autoradiograms for determination of fragment sizes (Brown 1980).

We detected no differences among conspecific individuals (2–4 *P. atricapillus*: Eco RV, Hind III, Hinc II, Hae II; 5 *P. bicolor*: Ava I, Eco RI), even between the two Pennsylvania localities. Although we did not survey many conspecific individuals, variation in mtDNA at this level is typically minor relative to interspecific variation. Most estimates of individual variation have been made in mammals and range from less than 0.5% in *Peromyscus* from a single locality (Avise et al. 1979) to 4% for *Rattus* worldwide (Brown et al. 1981). Kessler and Avise (1984) found intraspecific variation to be less than 0.5% in several species of ducks. We therefore used one individual of each species for our interspecific comparisons.

The total proportion of shared fragments (F) was calculated from Nei and Li's (1979) Eq. 21. We translated this value to the estimated number of base pair substitutions per nucleotide (p) using Upholt's (1977) formula and assumptions.

Results

Four of the restriction enzymes (Kpn I, Nru I, Xho I, Xor II) failed to cut any of the *Parus* mtDNAs; the 14 remaining enzymes each yielded 0–6 fragments per species and a total of 1–10 fragments for the three species together. A total of 84 distinct fragments (revealing 84 cleavage sites) resulted from restriction enzyme digestions (Table 1). By summing fragment sizes we estimated genome sizes for these species of *Parus* to be 16,700 ± 800 base pairs. This genome size is typical for birds and mammals (Brown 1983, Kessler and Avise 1984). The sites recognized totaled 40 in *P. carolinensis*, 39 in *P. atricapillus*, and 36 in *P. bicolor*.

The two chickadee mtDNAs shared 51% of the 80 fragments produced by the 18 restriction enzymes. Eleven of the 14 enzymes produced mtDNA fragment profiles that could serve as genetic markers for the two species of chickadees (Fig. 1). Only about a fifth (21% and 24%) of the mtDNA fragments from either chickadee matched the *bicolor* mtDNA fragments (Table 2). Conversion of the proportions of shared fragments to estimates of nucleotide sequence

TABLE 1. Results of restriction enzyme digests of *Parus* mtDNA. Pc = *Parus carolinensis*, Pa = *Parus atricapillus*, Pb = *Parus bicolor*. nc = no cuts.

Enzyme	Fragment sizes in base pairs		
	Pc	Pa	Pb
Ava I	5,600	5,600	5,600
			4,750
	3,450	3,450	3,450
	2,950	2,950	
	2,150	2,150	
	1,750	1,750	
			1,600
			1,300
	800	800	
Bam HI			16,700
	10,300	10,300	
	6,400		
		4,800	
		1,600	
Eco RI	nc	15,500	
			10,400
			6,300
		1,200	
Eco RV	16,700		16,700
		10,000	
		3,700	
		3,000	
Hae II		11,500	
	8,600		
			7,000
	5,200	5,200	
			4,500
			3,200
	2,900		
			2,000
Hinc II	8,500	8,500	
			6,400
			3,800
	3,000	3,000	3,000
	1,800	1,800	
	1,600	1,600	1,600
			1,300
	1,200	1,200	
	600	600	600
Hind III	8,700	8,700	
			7,100
			6,100
	3,800	3,800	
			3,500
		2,100	
	1,500	1,500	
	1,100		
	1,000		
	600	600	
Hpa I		11,300	
			10,400
	6,400		
	5,400		
		3,300	
	2,900		2,900
	2,000	2,000	2,000
			1,400
Nde I		16,700	
	14,000		
			7,000
			6,300
			3,400
	1,600		
	1,100		
Pst I	16,700	16,700	
			10,300
			6,400
Pvu II	11,400		
		10,000	10,000
		6,700	6,700
	5,300		
Sal I		16,700	
	15,500		
			8,400
			8,300
	1,200		
Sma I	16,700		16,700
		9,600	
		4,200	
		2,900	
Xba I		16,700	nc
	7,400		
	5,800		
	3,500		

divergence (p) indicated that 4% of the base pairs of *atricapillus* and *carolinensis* have undergone point mutations since they diverged from a common ancestor. The divergence between these chickadees and their common ancestor with *P. bicolor* was more than twice that, or about 9%.

DISCUSSION

Our preliminary survey of differences in mtDNA nucleotide sequences of the chickadees indicates that many genetic markers may be present in taxa with similar proteins. The protein genetic distance (*D*; Nei 1978) between *P. carolinensis* and *P. atricapillus* is only 0.001, a value typical of conspecific avian populations (Braun and Robbins 1986). Not surprisingly, our comparisons of *Parus* mtDNA reveal that *P. atricapillus* and *P. carolinensis* are more similar to each other than either is to *P. bicolor*. On the basis of external morphology, Ridgway (1904)

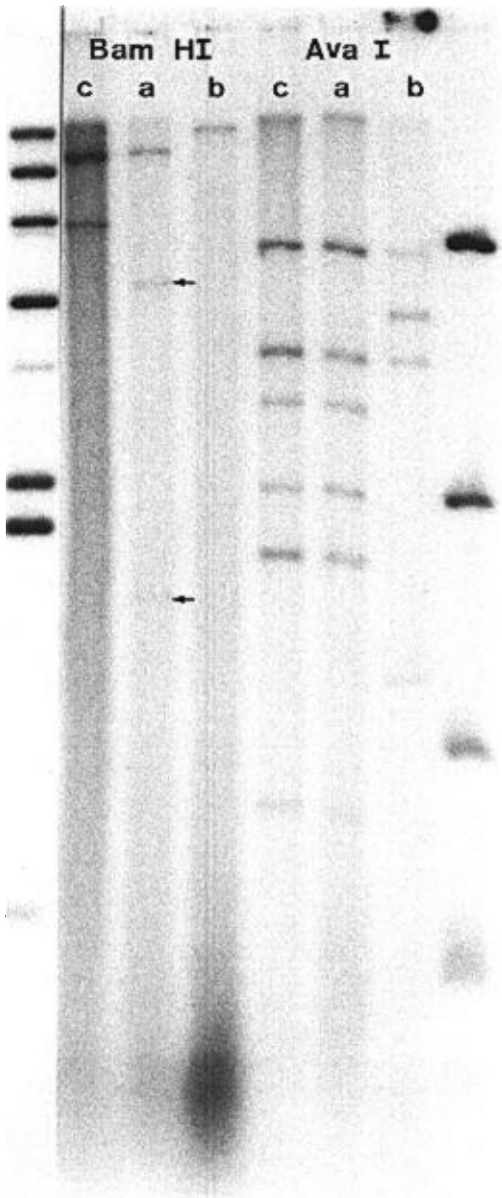

Fig. 1. Restriction fragments of mitochondrial DNA of three species of *Parus* after digestion with the enzymes Bam HI and Ava. I. Fragments were separated by electrophoresis on 1% agarose gels in standard TBE buffer; autoradiograph exposed for 7 h at room temperature. a = *P. atricapillus*, b = *P. bicolor*, c = *P. carolinensis*. Dark bars on outer gels are marker fragments of known sizes: left (lambda/Hind III) = 23,130, 9,420, 6,560, 4,370, 2,320, 2,030 base pairs; right (PM2/Hind III) = 5,450, 2,150, 1,050 base pairs.

TABLE 2. Genetic divergence (mtDNA) of chickadees and titmice. Figures above diagonal are estimates (p) of nucleotide sequence divergence. Figures below diagonal are values of F, the proportion of shared fragments.

	atricapillus	*carolinensis*	*bicolor*
atricapillus	—	0.04	0.09
carolinensis	0.51	—	0.09
bicolor	0.24	0.21	—

separated the crested American titmice, including *P. bicolor* as the genus *Baeolophus*. The protein genetic distance between *P. carolinensis* and *P. bicolor* is 0.09 (Patton and Avise unpubl. data, based on a survey of allozyme variability at 20 loci in 10 individuals of *P. carolinensis* and 4 individuals of *P. bicolor*). A *D* value of 0.09 is within the normal range of values for congeneric species of birds (Barrowclough 1980, Avise 1983, Corbin 1983).

Kessler and Avise's (1984) data on mtDNA divergence among ducks permit a comparison of *Parus* with the nonpasserine genera *Anas* and *Aythya*. Divergence between the chickadee mtDNAs is approximately the same as the average divergence between species of *Aythya* ($\bar{p} = 0.03$, range 0.02–0.04), but is less than the average divergence between species of *Anas* ($\bar{p} = 0.06$, range 0.004–0.09). MtDNA divergence between titmice and chickadees matches extreme values for the genus *Anas*, such as the divergence between the Northern Shoveler (*Anas clypeata*) and Northern Pintail (*Anas acuta*).

Preliminary attempts to estimate the rate of change in mammalian mtDNA suggest the possibility of linear, clocklike divergence during the first 8 million years, with fixations of base pair subsitutions occurring at a rate of 2–4% per

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Digestion by BAM HI yielded five distinct fragments for the three species. The *P. bicolor* mtDNA was cut only once to yield a linearized mtDNA molecule of full genome size (16,700 base pairs). *P. carolinensis* mtDNA was cut twice to yield two fragments of 10,300 and 6,400 base pairs. *P. atricapillus* mtDNA was cut at the same two sites as was *P. carolinensis* mtDNA and also at a third cleavage site within the 6,400 fragment to yield two fragments of 4,800 and 1,600 base pairs, which appear faintly (arrows) in this reproduction. The two chickadees thus shared the 10,300 base pair fragment.

million years (Brown et al. 1979, 1982). Assuming equivalent rates of base pair subsitution in birds, our results suggest that *atricapillus* and *carolinensis* mtDNAs had a common ancestor 1–2 million years ago, and further that these chickadee mtDNAs shared a common ancestor with *P. bicolor* mtDNA 2.5–4.5 million years ago. Thus, the beginning of the Pleistocene or end of the Pliocene may be the appropriate time to look for the conditions responsible for speciation of *P. carolinensis* and *P. atricapillus*, earlier than either the Illinoian or Wisconsin periods proposed by Brewer (1963) and Selander (1965).

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The following application has been received by the **International Commission on Zoological Nomenclature** and has been published in vol. 43, part 2, of the *Bulletin of Zoological Nomenclature* (9 July 1986). Comments or advice on it is welcomed and should be sent **c/o The British Museum (Natural History), Cromwell Road, London SW7 5BD, U.K.** Comments will be published in the *Bulletin*.

Case No. 1051 *Bubo* Dumeril, 1806 and *Surnia* Dumeril, 1806 (Aves): proposed confirmation on the Official List.

Visual Resources for Ornithology (VIREO) accession numbers for voucher photographs for Nichols et al. (Auk 103: 825–828) are V06-1-002 through V06-1-005.