The Auk 109(1):173–185, 1992

DNA-DNA HYBRIDIZATION EVIDENCE OF PHYLOGENETIC RELATIONSHIPS AMONG MAJOR LINEAGES OF *PARUS*

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ABSTRACT.—DNA-DNA hybrids were formed among 2 outgroups and 12 taxa of titmice and chickadees to estimate the genealogical relationships of the main *Parus* lineages. A complete matrix of reciprocal comparisons among seven parids and the Verdin (*Auriparus flaviceps*) indicated that the Blue Tit (*P. caeruleus*) and Great Tit (*P. major*) together form the sister group of the rest of the genus, and that the Bridled Titmouse (*P. wollweberi*) is more closely related to North American titmice than to the Old World crested tits. The DNA-hybridization data complement information from allozyme and mtDNA studies of closely related parids and provide historical insight into patterns of tit behavior. *Received 8 April 1991, accepted 15 July 1991.*

IN RECENT years, the titmice and chickadees (tits) have become the focus of molecular systematic studies (e.g. Braun et al. 1984, Braun and Robbins 1986, Mack et al. 1986, Gill et al. 1989, Gill and Slikas 1992). Their popularity among systematists is growing because, in terms of life history and behavior, *Parus* includes the most extensively studied species of wild birds: Black-capped Chickadee (*Parus atricapillus*), Great Tit (*P. major*), and Blue Tit (*P. caeruleus*; e.g. see *Wilson Bulletin* 101[2], 1989 for a series of papers). The genus also includes several taxa that exemplify classic vicariant distributions and hybridization zones. The Carolina Chickadee (*P. carolinensis*) and Black-capped Chickadee hybrid zone is a particularly interesting and controversial example (e.g. Braun and Robbins 1986, Mack et al. 1986).

Given the large body of information on the biology of this group, an understanding of their phylogeny would shed more light on the historical components of bird ecology and distribution than similar studies on other taxa. Efforts to interpret tit ecology and distribution in terms of phylogeny have been limited, however, because parid phylogeny remains largely unresolved. The allozyme and mitochondrial-DNA (mtDNA) restriction-fragment analyses so far employed (e.g. Gill et al. 1989, Gill and Slikas 1992) are limited in their range of effectiveness. They have worked quite well at the population and close-species levels, but less so when applied to more distantly related taxa (unpubl. data). We decided, therefore, to estimate the branching pattern among the more diverged tit lineages by DNA hybridization, which operates most effectively at and above the generic level (e.g. Sheldon 1987b, Bledsoe 1988, Madsen et al. 1988, Krajewski 1989, Sibley and Ahlquist 1990).

METHODS

Selection of taxa.—Twelve species of *Parus* and two outgroups, the Verdin (*Auriparus flaviceps*) and the White-breasted Nuthatch (*Sitta carolinensis*), were compared (Table 1). A complete matrix of pairwise measurements was made among seven of the *Parus* species and *Auriparus* (Table 2). The taxa included in the matrix were selected based on preliminary mtDNA restriction-fragment comparisons, which indicated that these seven species were members of the most diverged lineages within the genus and presented particularly interesting taxonomic problems (Gill and Slikas 1992; F. B. Gill, A. Mostrom, and A. L. Mack, unpubl. manuscript).

Biochemistry.—The DNA-hybridization procedure we used is based on that of Sibley and Ahlquist (1990), with the following modifications. DNA was extracted from frozen tissues (mainly liver and heart), as opposed to alcohol-preserved tissues or erythrocytes. These were placed in liquid nitrogen or on dry ice in the field and stored at −80°C in the laboratory. In most cases, the nuclei were separated from the mitochondria before nuclear DNA was extracted. This separation was achieved by grinding the tissues in cold STE buffer (0.25 M sucrose, 0.03 M Tris, 0.1 M EDTA) and spinning the homogenate at 3,000 rpm (700 g) for 5 min to pellet the nuclei and large tissue fragments. The pellets were then resuspended in STE, treated with pronase, and extracted as usual with

chloroform-isoamyl alcohol and phenol. The samples prepared in this way are marked with "n" for nuclear DNA in Table 1. The samples marked "w" for whole DNA were simply ground in STE, pronased and extracted. DNA was chopped to an average of about 500 base pairs by sonification with a microprobe.

Tracer DNA was prepared from single-copy DNA (C_0t 1000), which was oligo-labeled with tritium and sized (Caccone et al. 1987, Feinberg and Vogelstein 1983, Cunningham et al. 1991). Hybrids were formed with 20,000–50,000 DPM of tracer (ca. 0.002 μg) and 20–30 μg of driver DNA (tracer : driver ca. 1:10,000), and incubated at 60°C to C_0t greater than 22,000. The presence of mtDNA in some of the driver samples was not expected to affect measurements because in single-copy DNA the mtDNA will be in molar ratio with the nuclear DNA at much less than 0.1% (Powell et al. 1986).

The hybrids were fractionated on a thermal elution device similar to that of Sibley and Ahlquist (1981) and Kirsch et al. (1990), except that 35 instead of 25 hybrids were compared in a single experiment, and temperature was controlled manually. Fractions of most samples were taken at 60°C and 68° to 94°C in 2° increments by pumping 4 ml of 0.12 M sodium phosphate buffer through columns consisting of 1 ml of hydroxylapatite (HAP) in 5-ml syringe barrels and collecting the eluate in 20-ml scintillation vials. In one out of four labeled *P. bicolor* experiments, fractions were taken at 60°C and 66° to 94°C in 2° increments, and in two others fractions were taken at 60° to 95°C in 2.5° increments. The effect of differences in fractionation-temperature regimes is shown in Figures 1 and 2. We added 15 ml of biodegradable scintillation cocktail to each fraction, and the vials were shaken and counted in a scintillation counter programmed for quenching. Data in the form of disintegrations per minute (DPM) were collected directly from the counter to a computer. These are available on floppy disk.

Experimental design and data analysis.—Each experiment comprised replicate measurements of a homoduplex control (i.e. hybrids formed from labeled and unlabeled DNA of single individual), an intraspecific heteroduplex (hybrids of labeled DNA and DNA of another individual of the same species), and interspecific heteroduplexes (hybrids of labeled DNA and DNAs from other species). This approach permitted the measurement of distance and individual variation in the control, as well as other species (e.g. Caccone et al. 1987, Sheldon 1987b). To control for bias among heteroduplexes in the matrix, we sampled a variety of DNA preparations from different individual birds (see Table 1). For *P. caeruleus*, we had DNA from only two individuals; hence, the lower number of replicates for that species.

We included eight species in the fundamental comparison matrix (Table 2) so that eight experiments, each comprising 35 hybrids, would yield four heteroduplex replicates per matrix cell and three strict (intra-individual) homoduplex measurements. Although this was our original intention, the loss of replicate measurements through equipment failure forced us to run more than eight experiments. In addition, we have added distances from some preliminary experiments to the matrix (hence, the different fractionation regimes described in previous section). These preliminary experiments also included hybrids among *Parus* species that are not part of the matrix. Data from those hybrids are summarized in Tables 3 to 5.

For each hybrid, we calculated Tm, modified Fermi-Dirac mode, ΔTm, Δmode, and percent reassociation values as described in Sheldon and Bledsoe (1989). Delta values are genetic distances calculated by subtracting heteroduplex values from the average homoduplex value. The indexes and distances are unmod-

TABLE 1. Summary of species and DNA preparations. Species name followed by information on preparation number, source locality, and preparation type. Preparation type "n" indicates that mitochondria were removed before DNA extraction and, thus, preparation comprised of only nuclear DNA; "w" signifies whole-DNA preparations.

Willow Tit (*Parus montanus*): TAI (preparation number), Sweden (locality), n (preparation type).
Black-capped Chickadee (*P. atricapillus*): 938AK, Alaska, n.
Carolina Chickadee (*P. carolinensis*): 2.1, Pennsylvania, w; 2.6a, Pennsylvania, w; 2.6bA, Pennsylvania, w; 2.60, Pennsylvania, w; 2.11a, Texas, w; 185, Pennsylvania, n; CM7, New Jersey, n; and CM10, New Jersey, n.
Boreal Chickadee (*P. hudsonicus*): JP1156, Nova Scotia, n.
Bridled Titmouse (*P. wollweberi*): 1589, Arizona, n; 1595, Arizona, n; 2257, Arizona, n; 2258, Arizona, n; and CP1, Mexico, n.
Coal Tit (*P. ater*): 2214, Szechwan, n; 2190, Szechwan, n; 2216, Szechwan, n; and 139, Sweden, n.
Grey-crested Tit (*P. dichrous*): 2131, Szechwan, n; 2142, Szechwan, n; 2143, Szechwan, n; and 2147, Szechwan, n.
Great Tit (*P. major*): 2200, Szechwan, n; 2185, Szechwan, n; 2166, Szechwan, n; and 2169, Szechwan, n.
Blue Tit (*P. caeruleus*): 138, Sweden, n; and BM2, Sweden, n.
Plain Titmouse (*P. inornatus*): 4, New Mexico, n.
Tufted Titmouse (*P. bicolor*): 204, Pennsylvania, n; 880, Louisiana, n; 882, Louisiana, n; 1992, Pennsylvania, n; 1993b, Pennsylvania, n; and 2045, Pennsylvania, n.
Black-crested Titmouse (*P. atricristatus*): 2.9, Texas, n.
Verdin (*Auriparus flaviceps*): VHT1, Arizona, n; VHT2, Arizona, n; VHT3, Arizona, n; and VHT 4, Arizona, n.
White-breasted Nuthatch (*Sitta carolinensis*): 2.1aA, Pennsylvania, w; and 2.8B, Pennsylvania, w.

TABLE 2. Matrix of *Parus* hybrid dissociation, reassociation, and dissimilarity values. Hybrids between the same species are heteroduplexes. Homoduplex values summarized in Table 6. "% R" is nonnormalized percent reassociation.

		Label													
		P. carolinensis					*P. wollweberi*					*P. ater*			
Driver	Statistic	Tm	ΔTm	Mode	ΔMode	% R	Tm	ΔTm	Mode	ΔMode	% R	Tm	ΔTm	Mode	ΔMode	% R
Parus	*n*	4	4	4	4	4	5	5	5	5	5	4	4	4	4	4
carolinensis	$\bar{x}$	83.4	0.7	85.6	0.4	86.3	81.2	2.7	83.4	2.5	89.8	81.7	2.4	83.7	2.3	91.6
	SD	0.30	0.23	0.38	0.28	12.5	0.24	0.23	0.19	0.19	4.38	0.12	0.12	0.13	0.13	3.94
	SE	0.15	0.12	0.19	0.14	6.27	0.11	0.10	0.09	0.08	1.96	0.06	0.06	0.06	0.06	1.97
P. wollweberi	*n*	4	4	4	4	4	3	3	3	3	3	4	4	4	4	4
	$\bar{x}$	80.7	3.3	83.1	2.8	79.5	83.5	0.4	85.6	0.3	84.2	81.2	2.9	83.4	2.7	80.5
	SD	0.35	0.35	0.22	0.22	11.89	0.35	0.35	0.30	0.30	2.80	0.21	0.21	0.16	0.16	14.14
	SE	0.18	0.18	0.11	0.11	5.94	0.20	0.20	0.17	0.17	1.61	0.11	0.11	0.08	0.08	7.07
P. ater	*n*	4	4	4	4	4	3	3	3	3	3	3	3	3	3	3
	$\bar{x}$	80.9	3.1	83.3	2.6	83.6	81.4	2.9	83.4	2.6	87.3	83.3	0.8	85.3	0.7	89.3
	SD	0.21	0.21	0.15	0.15	4.81	0.16	0.03	0.10	0.13	1.45	0.25	0.25	0.26	0.26	2.72
	SE	0.10	0.10	0.07	0.07	2.40	0.09	0.02	0.06	0.08	0.84	0.15	0.15	0.15	0.15	1.57
P. dichrous	*n*	4	4	4	4	4	5	5	5	5	5	4	4	4	4	4
	$\bar{x}$	81.0	3.0	83.4	2.6	84.0	81.1	2.8	83.3	2.6	80.3	81.2	2.9	83.3	2.7	86.0
	SD	0.21	0.21	0.15	0.15	5.59	0.05	0.03	0.09	0.08	10.90	0.16	0.16	0.19	0.19	3.81
	SE	0.10	0.10	0.08	0.08	2.80	0.02	0.02	0.04	0.04	4.87	0.08	0.08	0.09	0.09	1.90
P. major	*n*	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
	$\bar{x}$	80.2	3.8	82.6	3.4	82.8	80.3	3.7	82.4	3.5	80.4	80.6	3.5	82.6	3.4	85.1
	SD	0.16	0.16	0.13	0.13	6.15	0.19	0.14	0.15	0.14	4.70	0.12	0.12	0.13	0.13	3.92
	SE	0.09	0.09	0.07	0.07	3.55	0.10	0.07	0.07	0.07	2.35	0.06	0.06	0.07	0.07	1.96
P. caeruleus	*n*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	$\bar{x}$	80.5	3.5	82.9	3.1	88.5	80.1	3.8	82.4	3.5	81.9	80.8	3.3	82.9	3.1	90.3
	SD	0.07	0.07	0.07	0.07	1.21	0.28	0.28	0.21	0.21	6.25	0.27	0.27	0.25	0.25	2.12
	SE	0.05	0.05	0.05	0.05	0.85	0.20	0.20	0.15	0.15	4.42	0.19	0.19	0.18	0.18	1.50
P. bicolor	*n*	8	8	8	8	8	9	9	9	9	9	4	4	4	4	4
	$\bar{x}$	81.3	2.8	83.6	2.5	86.4	81.9	2.3	83.9	2.1	88.5	81.3	2.8	83.4	2.6	88.5
	SD	0.28	0.24	0.19	0.12	3.73	0.30	0.17	0.28	0.18	2.84	0.20	0.20	0.22	0.22	2.91
	SE	0.10	0.08	0.07	0.04	1.32	0.10	0.06	0.09	0.06	0.95	0.10	0.10	0.11	0.11	1.45
Auriparus	*n*	4	4	4	4	4	6	6	6	6	6	4	4	4	4	4
flaviceps	$\bar{x}$	75.2	8.8	77.2	8.7	77.0	75.5	8.6	77.1	8.8	76.0	75.7	8.4	77.5	8.5	76.5
	SD	0.25	0.25	0.31	0.31	10.33	0.30	0.15	0.55	0.42	7.39	0.17	0.17	0.17	0.17	10.45
	SE	0.12	0.12	0.16	0.16	5.16	0.12	0.06	0.22	0.17	3.02	0.09	0.09	0.08	0.08	5.23

TABLE 2. Continued.

		Label														
		P. dichrous					*P. major*					*P. caeruleus*				
Driver	Statistic	Tm	ΔTm	Mode	ΔMode	% R	Tm	ΔTm	Mode	ΔMode	% R	Tm	ΔTm	Mode	ΔMode	% R
Parus	*n*	5	5	5	5	5	5	5	5	5	5	3	3	3	3	3
carolinensis	$\bar{x}$	81.7	2.7	83.8	2.4	91.6	80.7	3.3	82.9	2.9	90.6	80.7	3.6	82.9	3.2	89.9
	SD	0.24	0.30	0.22	0.25	2.81	0.58	0.32	0.48	0.24	3.74	0.06	0.06	0.07	0.07	2.94
	SE	0.11	0.13	0.10	0.11	1.26	0.26	0.14	0.22	0.11	1.67	0.03	0.03	0.04	0.04	1.70
P. wollweberi	*n*	5	5	5	5	5	5	5	5	5	5	4	4	4	4	4
	$\bar{x}$	81.6	2.7	83.7	2.5	84.8	80.6	3.4	82.7	3.0	82.9	80.5	3.7	82.7	3.4	83.6
	SD	0.32	0.07	0.27	0.06	8.33	0.30	0.07	0.24	0.10	8.46	0.14	0.14	0.10	0.10	6.30
	SE	0.14	0.03	0.12	0.03	3.72	0.13	0.03	0.11	0.04	3.78	0.07	0.07	0.05	0.05	3.15
P. ater	*n*	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4
	$\bar{x}$	81.5	2.8	83.6	2.6	87.3	80.6	3.4	82.8	3.0	86.0	80.7	3.6	82.8	3.3	86.4
	SD	0.54	0.08	0.45	0.14	2.60	0.53	0.23	0.46	0.17	2.96	0.04	0.04	0.10	0.10	1.91
	SE	0.24	0.04	0.20	0.06	1.16	0.27	0.12	0.23	0.08	1.48	0.02	0.02	0.05	0.05	0.95
P. dichrous	*n*	3	3	3	3	3	5	5	5	5	5	4	4	4	4	4
	$\bar{x}$	83.9	0.3	85.8	0.3	89.5	80.7	3.3	82.8	3.0	86.2	80.7	3.5	82.9	3.2	86.8
	SD	0.18	0.18	0.14	0.14	2.98	0.40	0.12	0.33	0.05	1.84	0.17	0.17	0.09	0.09	2.55
	SE	0.11	0.11	0.08	0.08	1.72	0.18	0.06	0.15	0.02	0.82	0.08	0.08	0.05	0.05	1.28
P. major	*n*	5	5	5	5	5	3	3	3	3	3	4	4	4	4	4
	$\bar{x}$	81.0	3.4	83.0	3.2	84.9	83.4	0.4	85.4	0.3	87.5	81.4	2.9	83.5	2.7	87.9
	SD	0.31	0.05	0.32	0.12	3.52	0.13	0.13	0.13	0.13	5.77	0.20	0.20	0.17	0.17	3.92
	SE	0.14	0.02	0.14	0.06	1.57	0.08	0.08	0.08	0.08	3.33	0.10	0.10	0.09	0.09	1.96
P. caeruleus	*n*	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1
	$\bar{x}$	80.8	3.5	82.9	3.2	89.0	81.2	2.7	83.4	2.3	88.9	83.5	0.7	85.5	0.6	95.1
	SD	0.19	0.19	0.11	0.11	1.98	0.36	0.36	0.26	0.26	2.25					
	SE	0.13	0.13	0.08	0.08	1.40	0.25	0.25	0.19	0.19	1.59					
P. bicolor	*n*	4	4	4	4	4	5	5	5	5	5	4	4	4	4	4
	$\bar{x}$	81.8	2.6	83.8	2.5	88.1	80.7	3.3	82.8	3.0	88.0	80.7	3.6	82.9	3.3	85.2
	SD	0.30	0.05	0.26	0.05	2.08	0.39	0.12	0.33	0.06	1.70	0.17	0.17	0.15	0.15	2.10
	SE	0.15	0.03	0.13	0.03	1.04	0.18	0.05	0.15	0.03	0.76	0.08	0.08	0.08	0.08	1.05
Auriparus	*n*	4	4	5	5	4	4	4	4	4	4	4	4	4	4	4
flaviceps	$\bar{x}$	76.2	8.2	78.0	8.2	77.2	75.8	8.2	77.5	8.3	76.3	75.7	8.6	77.6	8.5	73.8
	SD	0.10	0.30	0.29	0.26	9.47	0.43	0.31	0.50	0.33	8.01	0.16	0.16	0.20	0.20	9.92
	SE	0.05	0.15	0.13	0.12	4.73	0.22	0.16	0.25	0.16	4.01	0.08	0.08	0.10	0.10	4.96

TABLE 2. Continued.

Driver	Statistic	Label										
		P. bicolor					*Auriparus flaviceps*					
		Tm	ΔTm	Mode	ΔMode	% R	Tm	ΔTm	Mode	ΔMode	% R	
Parus carolinensis	*n*	10	10	10	10	10	4	4	5	5	4	
	$\bar{x}$	81.1	2.4	83.6	2.3	82.6	75.2	8.8	77.2	8.6	77.8	
	SD	0.36	0.19	0.55	0.16	8.20	0.18	0.12	0.35	0.19	4.98	
	SE	0.11	0.06	0.17	0.05	2.59	0.09	0.06	0.16	0.09	2.49	
P. wollweberi	*n*	9	9	9	9	9	5	5	5	5	5	
	$\bar{x}$	81.3	2.2	83.8	2.0	60.4	75.0	8.9	77.1	8.8	69.8	
	SD	0.40	0.17	0.57	0.19	34.37	0.08	0.18	0.08	0.16	10.99	
	SE	0.13	0.06	0.19	0.06	11.46	0.03	0.08	0.04	0.07	4.92	
P. ater	*n*	4	4	4	4	4	5	5	5	5	5	
	$\bar{x}$	80.4	2.8	82.7	2.6	82.0	75.1	8.8	77.1	8.7	69.2	
	SD	0.28	0.28	0.26	0.26	4.87	0.29	0.31	0.32	0.35	4.64	
	SE	0.14	0.14	0.13	0.13	2.44	0.13	0.14	0.14	0.16	2.08	
P. dichrous	*n*	4	4	4	4	4	5	5	5	5	5	
	$\bar{x}$	80.4	2.8	82.7	2.6	80.8	75.1	8.9	77.1	8.8	74.2	
	SD	0.17	0.17	0.14	0.14	3.42	0.19	0.13	0.19	0.10	4.74	
	SE	0.09	0.09	0.07	0.07	1.71	0.08	0.06	0.08	0.04	2.12	
P. major	*n*	2	2	3	3	2	5	5	5	5	5	
	$\bar{x}$	79.6	3.6	81.9	3.4	79.8	75.1	8.8	77.1	8.7	67.7	
	SD	0.05	0.05	0.05	0.05	5.84	0.13	0.27	0.18	0.25	6.16	
	SE	0.04	0.04	0.03	0.03	4.13	0.06	0.12	0.08	0.11	2.75	
P. caeruleus	*n*	3	3	4	4	3	3	3	3	3	3	
	$\bar{x}$	79.9	3.3	82.2	3.2	84.2	75.4	8.6	77.4	8.6	81.0	
	SD	0.22	0.22	0.22	0.22	2.27	0.86	0.53	0.79	0.50	4.16	
	SE	0.12	0.12	0.11	0.11	1.31	0.49	0.31	0.45	0.29	2.40	
P. bicolor	*n*	1	1	1	1	1	5	5	5	5	5	
	$\bar{x}$	83.0	0.2	85.1	0.3	91.0	75.2	8.7	77.1	8.7	78.4	
	SD						0.15	0.18	0.27	0.23	3.02	
	SE						0.07	0.08	0.12	0.10	1.35	
Auriparus flaviceps	*n*	3	3	3	3	3	4	4	4	4	4	
	$\bar{x}$	74.9	8.3	76.9	8.5	70.5	83.5	0.4	85.7	0.2	81.2	
	SD	0.31	0.31	0.27	0.27	12.16	0.35	0.27	0.27	0.17	9.38	
	SE	0.18	0.18	0.16	0.16	7.02	0.17	0.14	0.13	0.09	4.69	

ified and presented in Tables 2 to 7. Data from hybrids that failed because of mechanical problems or were aberrant, under the criteria outlined in Sheldon (1987a), were not included in the analyses. (Two out of 314 were considered aberrant.)

To produce more additive dissimilarity values, ΔTm's were transformed to ΔT50H's using an empirically derived equation:

$$\Delta T50H = 1.08(\Delta Tm) + 0.007(\Delta Tm)^2 \qquad (1)$$

(Sheldon and Bledsoe 1989, unpubl. data). The ΔT50H values were further transformed by the Jukes and Cantor (1969) equation to adjust for multiple mutations at single base sites. These transformations and their logic and assumptions are discussed by Springer and Kirsch (1989), Springer and Krajewski (1989a, b), and Werman et al. (1990:243). The final transformed values were adjusted for asymmetry with A. Dickerman's program ("Symboot"), which performs the corrections outlined in Springer and Kirsch (1989:333–334) based on the average "percent nonreciprocity" of Sarich and Cronin (1976). Percent nonreciprocity is equal to 100 times the reciprocal differences divided by the reciprocal sums.

Phylograms of Tm-values and their various transformations were derived using the programs "Fitch" and "Kitsch" in J. Felsenstein's phylogenetic computer package, PHYLIP 2.8. The options available in PHYLIP were set so that, in searching through various tree topologies having positive branches, unweighted least-squares regression (Cavalli-Sforza and Edwards 1967) was employed to find the tree with the minimum residual sum of squares. Unweighted least squares was used in lieu of weighted (Fitch and

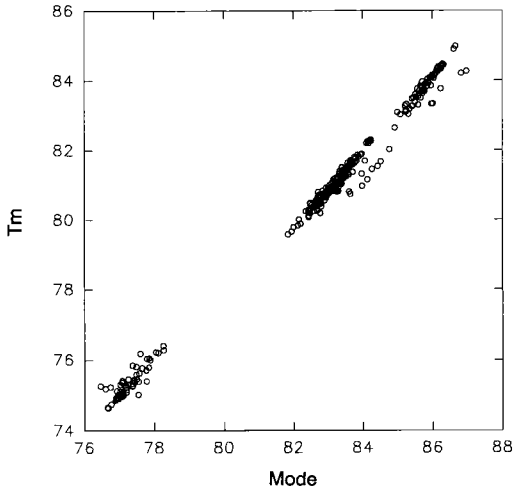

Fig. 1. Mode versus Tm from data summarized in Table 2.

Margoliash 1967), because variance does not seem to increase with genetic distance in HAP-based DNA-hybridization data (Sheldon 1987a, Sibley and Ahlquist 1990). The "Fitch" program does not depend on the assumption of a molecular clock and is likely to provide good estimates of phylogeny even if evolutionary rates vary from lineage to lineage (Bledsoe 1987, Sheldon 1987a, Springer and Krajewski 1989b, Bledsoe and Sheldon 1990).

The consistency and reproducibility of "Fitch" trees was examined by two methods: Krajewski and Dickerman's (1990) bootstrapping and Lanyon's (1985) jackknife strict-consensus. The bootstrapping method assigns quantitative levels of stability to branching

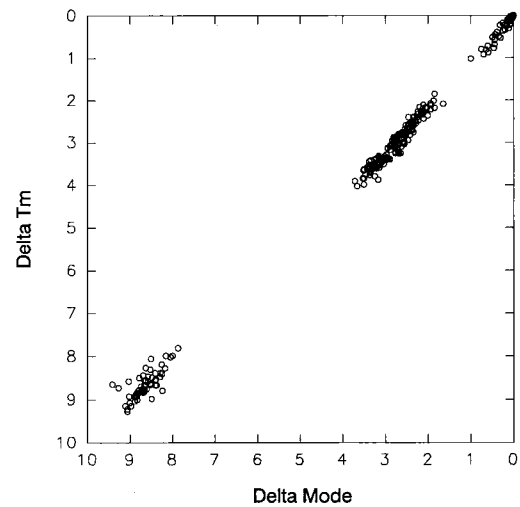

Fig. 2. Delta mode versus ΔTm from data summarized in Table 2.

TABLE 3. Dissociation, reassociation, and dissimilarity values for labeled *Parus carolinensis* comparisons not included in Table 2.[a]

Statistic	Tm	ΔTm	Mode	ΔMode	% R
			Parus carolinensis (homoduplex)		
n	4	4	4	4	4
$\bar{x}$	84.18	−0.00	86.18	0.00	90.55
SD	0.04	0.03	0.10	0.09	1.51
SE	0.02	0.02	0.05	0.05	0.76
			P. carolinensis (heteroduplex)		
n	4	4	4	4	4
$\bar{x}$	83.92	0.26	86.05	0.14	66.03
SD	0.11	0.14	0.01	0.07	2.59
SE	0.08	0.10	0.01	0.05	1.83
			P. hudsonicus		
n	4	4	4	4	4
$\bar{x}$	83.20	0.99	85.46	0.72	68.66
SD	0.10	0.09	0.12	0.17	1.50
SE	0.05	0.04	0.06	0.08	0.75
			P. atricapillus		
n	3	3	3	3	3
$\bar{x}$	83.76	0.42	86.20	−0.01	80.20
SD	0.05	0.05	0.57	0.62	1.93
SE	0.03	0.03	0.33	0.36	1.11
			P. montanus		
n	4	4	4	4	4
$\bar{x}$	82.89	1.29	85.13	1.05	83.27
SD	0.21	0.22	0.34	0.33	1.13
SE	0.11	0.11	0.17	0.17	0.56
			Sitta carolinensis		
n	4	4	4	4	4
$\bar{x}$	75.39	8.79	77.42	8.76	54.95
SD	0.45	0.47	0.75	0.71	2.24
SE	0.22	0.23	0.37	0.36	1.12

[a] Homoduplex DNA derived from Pennsylvania population. Driver DNA in *P. carolinensis* heteroduplexes came from Texas population.

points by sampling sets of replicate measurements. Lanyon's jackknifing assays the effects that additional taxa have on tree topology. As noted by Krajewski and Dickerman (1990), the two methods complement one another; jackknifing operates at the level of matrix columns and rows, and bootstrapping at the level of matrix cells.

Bootstrapping was performed on uncorrected Tm's and on T50H's corrected for multiple mutations at single base sites. We used A. Dickerman's program "Bootstrap," which resamples replicate homoduplex and heteroduplex values with replacement, recalculates the average distance for each cell to produce a pseudoreplicate matrix, smooths for reciprocal measurement discrepancies, estimates the best-fit tree with "Fitch," and writes the tree to a file summarizing all pseudoreplicate trees (Krajewski and Dickerman 1990). One thousand such trees were estimated for Tm and for corrected T50H, and a majority-rule consensus tree

TABLE 4. Dissociation, reassociation, and dissimilarity values for labeled *Parus bicolor* comparisons not included in Table 2.

Statistic	Tm	ΔTm	Mode	ΔMode	% R
	Parus bicolor (homoduplex)				
n	4	4	4	4	4
$\bar{x}$	83.44	0.00	85.96	0.00	0.81
SD	0.22	0.19	0.26	0.26	0.02
SE	0.11	0.09	0.13	0.13	0.01
	P. atricristatus				
n	4	4	4	4	4
$\bar{x}$	82.87	0.56	85.69	0.26	0.48
SD	0.28	0.17	0.19	0.20	0.01
SE	0.14	0.08	0.10	0.10	0.00
	P. inornatus				
n	4	4	4	4	4
$\bar{x}$	82.09	1.35	84.77	1.19	0.69
SD	0.20	0.09	0.17	0.18	0.01
SE	0.10	0.05	0.08	0.09	0.01
	Sitta carolinensis				
n	3	3	3	3	3
$\bar{x}$	75.71	7.82	77.54	8.38	0.62
SD	0.09	0.09	0.33	0.33	0.01
SE	0.05	0.05	0.19	0.19	0.01

TABLE 5. Dissociation, reassociation, and dissimilarity values for labeled *Auriparus flaviceps* comparisons not included in Table 2.

Statistic	Tm	ΔTm	Mode	ΔMode	% R
	Auriparus flaviceps (homoduplex)				
n	3	3	3	3	3
$\bar{x}$	84.20	0.00	86.16	0.00	89.91
SD	0.31	0.31	0.17	0.17	7.21
SE	0.18	0.18	0.10	0.10	4.16
	Parus atricristatus				
n	3	3	3	3	3
$\bar{x}$	74.88	9.32	76.69	9.47	66.10
SD	0.03	0.03	0.08	0.08	1.14
SE	0.02	0.02	0.05	0.05	0.66
	P. inornatus				
n	3	3	3	3	3
$\bar{x}$	75.45	8.75	77.41	8.75	71.44
SD	0.09	0.09	0.11	0.11	4.67
SE	0.05	0.05	0.06	0.06	2.69
	P. hudsonicus				
n	3	3	3	3	3
$\bar{x}$	75.40	8.80	77.16	9.00	69.21
SD	0.18	0.18	0.35	0.35	3.09
SE	0.11	0.11	0.20	0.20	1.78
	P. atricapillus				
n	3	3	3	3	3
$\bar{x}$	75.51	8.70	77.47	8.69	75.17
SD	0.03	0.03	0.13	0.13	1.97
SE	0.02	0.02	0.08	0.08	1.14
	P. montanus				
n	2	2	2	2	2
$\bar{x}$	75.63	8.58	77.59	8.57	78.69
SD	0.10	0.10	0.01	0.01	5.59
SE	0.07	0.07	0.01	0.01	3.95

was constructed with PHYLIP's (version 3.2) "Consense" program (Felsenstein 1985).

To jackknife, we removed one taxon at a time from the 8 × 8 Tm matrix and calculated seven majority-rule consensus trees by bootstrapping each pseudoreplicate matrix 100 times. A strict-consensus tree was constructed from the majority-rule trees by examining the latter for inconsistent branching patterns using Lanyon's (1985) program. This process was repeated for the corrected T50H matrix.

RESULTS

DATA CHARACTERISTICS

Reproducibility.—Hybrid replicate distribution statistics are provided in Table 8. Variance is typical of similar studies in which DNA hybrids are fractionated on HAP and the data are uncorrected. For example, in sets of heron and nine-primaried oscine hybrids, Sheldon and Bledsoe (1989) found the average sample standard deviation (SD) of ΔTm's to be 0.28 and of Δmodes to be 0.27. In his crane study, Krajewski (1989) derived an average ΔTm SD of 0.48. The homoduplex delta-value SD's in our study are less than those of heteroduplexes, presumably because homoduplexes are formed from a single preparation of a single individual's DNA, whereas heteroduplexes comprise different preparations of DNA from different individuals.

Measurement symmetry.—Symmetry describes the extent of similarity between reciprocal comparisons. Its relevance to DNA hybridization is discussed in detail by Bledsoe and Sheldon (1989) and Springer and Krajewski (1989a). The ΔTm asymmetry in this study ranges from 0.0–0.7, with a mean of 0.26 (SD = 0.22). Matrix-wide asymmetry, expressed as average percent nonreciprocity, is 3.3%. Other published examples of percent nonreciprocity are 3.12 and 11.37 for phalangerid marsupials (Springer and Kirsch 1989, Springer et al. 1990). In itself, percent nonreciprocity is not a particularly useful value, because the average is inversely proportional to distance, but its calculation is required when asymmetry is corrected (Springer and Kirsch 1989).

TABLE 6. Homoduplex dissociation and reassociation values of species compared in Table 2.

Experiment	Tm	Mode	% R
	Parus carolinensis		
P57-1	84.2	86.0	95.8
P57-2	83.9	85.9	95.7
P57-3	84.1	86.0	95.8
P26-2	84.2	86.2	91.8
P26-17	84.2	86.1	91.9
	P. wollweberi		
P49-9	84.1	85.9	90.7
P49-34	84.0	85.9	88.8
P50-1	84.0	85.9	90.5
P50-3	83.9	85.9	90.3
P50-4	83.9	85.9	90.5
P57-35	82.6	84.9	86.6
P61-4	84.4	86.2	91.6
P61-2	84.4	86.2	91.7
P61-3	84.3	86.1	93.4
	P. ater		
P58-1	83.9	85.8	90.3
P58-2	84.3	86.2	88.4
P58-3	84.1	86.0	87.5
P58-4	84.2	86.1	88.3
	P. dichrous		
P49-35	85.0	86.7	91.1
P49-17	84.9	86.6	90.9
P51-1	84.3	86.1	88.9
P51-2	84.3	86.1	88.7
P51-3	84.2	86.1	90.4
P51-4	84.2	86.1	88.9
P55-35	83.6	85.7	84.9
	P. major		
P49-25	84.5	86.3	85.8
P52-1	83.8	85.7	85.2
P52-2	83.8	85.7	85.6
P52-3	84.0	85.7	85.1
P52-4	83.8	85.6	84.3
	P. caeruleus		
P60-1	83.8	85.7	94.4
P60-2	84.4	86.3	96.6
P60-3	84.3	86.2	95.6
P60-4	84.4	86.3	96.4
	P. bicolor		
P59-1	83.2	85.2	84.6
P59-2	83.3	85.4	86.4
P59-3	83.3	85.4	86.3
P59-4	83.1	85.3	84.9
P024-a	83.3	86.0	81.0
P024-b	83.3	86.0	81.0
P025-a	83.8	86.2	77.6
P025-b	83.3	85.6	82.4
P034-a	84.3	87.0	69.0
P034-21	84.2	86.8	73.1
	Auriparus flaviceps		
P55-32	84.4	86.3	94.1
P55-33	84.4	86.3	94.0
P56-1	83.7	85.6	93.8
P56-2	83.6	85.6	93.1
P56-3	83.9	85.8	93.9
P56-4	84.1	86.0	93.3

Indexes of duplex stability.—Plots of mode versus Tm and Δmode versus ΔTm are shown in Figures 1 and 2. The correlation coefficient for mode to Tm is 0.98, and for Δmode to ΔTm is 0.99. The high degree of correspondence between these two indexes dispels the contention of Sarich et al. (1989) and Schmid and Marks (1990) that mode is the better guide to DNA hybridization melting distributions, at least when closely related birds are under study. Given the high correlation, we conducted the tree-building analyses (described below) with Tm, which is more easily and accurately calculated than mode.

In Figure 1, a series of points between modal values 83–87 lie lower than the bulk of the data and appear to form a parallel line. These "aberrant" points derive from the *P. bicolor* experiments in which extra low-temperature fractions were sampled, as described in the Methods section. They show a larger mode-to-Tm differential than most of the data because the addition of low-temperature fractions in a melting distribution reduces its median (Tm), but has no effect on its mode. Changes in fractionation-temperature regime have little effect on delta values (Fig. 2), because shifts in heteroduplex Tm-values are compensated by shifts in homoduplex Tm-values.

RATES OF EVOLUTION

Rates of evolution were examined by folding the matrix in Table 2 and comparing the distances from *Auriparus* to the *Parus* species by ANOVA and the Neuman-Keuls procedure. Following the suggestion of Swofford and Olsen (1990) that individual matrix-cell SD's may be misleading, we used the average heteroduplex SD in the tests. There was no evidence of

TABLE 7. DNA-hybrid summary data taken from two experiments performed by C. Sibley and J. Ahlquist (pers. comm.) in February 1982 at Yale University. *Parus atricapillus* was tracer species. Methods used are described in Sibley and Ahlquist (1990).

Driver	Preparation	Tm	ΔTm	Mode	ΔMode	% R
Parus atricapillus	87	84.8	0.0	87.8	0.0	0.55
P. atricapillus	87A	83.1	0.0	86.2	0.0	0.40
P. bicolor	447	82.6	2.2	84.9	2.9	0.59
P. bicolor[a]	447	81.2	1.9	83.6	2.6	0.53
P. major	910	80.5	4.2	83.1	4.7	0.57
P. major[a]	910	78.9	4.2	82.1	4.1	0.40
Auriparus flaviceps	395	76.6	8.2	79.4	8.3	0.37
Sitta carolinensis	661	72.1	11.0	74.3	11.9	0.41
Sitta carolinensis	661	75.0	9.8	77.1	10.7	0.49

[a] Driver DNAs of these two hybrids apparently interchanged. We have switched them back in this table.

different rates among the *Parus* species in the matrix. Rates were similarly checked in Table 5, which summarizes comparisons between *Auriparus* and various *Parus* species not included in the Table 2. The distance from *Auriparus* to *P. atricristatus* in Table 5 (ΔTm 9.3) is unusually large. It does not, however, necessarily imply a rate increase. The *atricristatus* distance is the average of three measurements to a single DNA preparation, which may have been shorter-stranded and, thus, less stable as duplex than other parid samples. To determine whether DNA anomaly or evolutionary rates caused the unusually large distance would require knowledge of the hybrid-duplex base-pair length or additional measurements with other preparations of *atricristatus* DNA. Without such data, it is impossible to say whether *atricristatus* has evolved faster than other parids. A rate difference seems unlikely, however, given the apparent rate constancy of all the other *Parus* species and the genetic similarity of *atricristatus* to one of those species, *P. bicolor* (e.g. Avise and Zink 1988).

PHYLOGENY

All phylograms built from the matrix in Table 2 with the "Fitch" and "Kitsch" programs, whether or not the data were transformed to increase additivity, smoothed for reciprocal measurement discrepancy, jackknifed with different numbers of taxa, and bootstrapped with different sets of replicates, are consistent with the strict-consensus tree depicted in Figure 3. *Parus wollweberi* groups with *bicolor*, and *major* groups with *caeruleus* as the sister taxon to all other *Parus* species. The positions of *carolinensis*, *ater*, and *dichrous* relative to one another and to *bicolor* and *wollweberi* remain uncertain.

When the 8 × 8 Tm and corrected-T50H matrices were bootstrapped 1,000 times each, the *caeruleus-major* node was supported more than 97% of the time, and all the other resolved nodes in Figure 3 were supported 100% of the time. In the Tm majority-rule consensus tree, *carolinensis* appeared as the sister taxon to *dichrous*, *ater*, and *wollweberi-bicolor*. In the T50H tree, *dichrous* appeared as the sister taxon to *carolinensis*,

TABLE 8. Summary of distribution statistics for data presented in Tables 2 and 6.

Statistic	Tm	ΔTm	Mode	ΔMode	% R[a]
		Average homoduplex melting temperature (n = 50)			
$\bar{x}$	83.98	−0.00	85.97	−0.00	88.82
SD	0.46	0.12	0.39	0.12	5.85
SE	0.06	0.02	0.06	0.02	0.82
		Average heteroduplex standard deviation (n = 264)			
$\bar{x}$	0.24	0.19	0.24	0.18	5.66
SD	0.06	0.03	0.06	0.03	2.87

[a] Nonnormalized.

ater, and *wollweberi-bicolor*. This suggests that *ater* is the sister taxon to *wollweberi-bicolor*. However, in the jackknifed trees, all four of these taxa moved around in a rather unpredictable way and, although it is tempting to provide the tree with more structure, it is prudent not to do so.

The bootstrapping method used in this study is unusually stringent, because it relies on melting values rather than distances. Homoduplex as well as heteroduplex values were sampled before distances were calculated and made into trees. Thus, both homoduplex and heteroduplex variation have been taken into account. The method is stringent because homoduplexes from more than one experiment are sampled, and the distances computed after sampling are not normalized for interexperimental effects (e.g. Felsenstein 1987, Sheldon and Bledsoe 1989, Sibley et al. 1990).

Figure 4 presents a summary tree, which includes additional taxa taken from Tables 3 to 5. We simply inserted these taxa by hand into the tree in Figure 3, according to the relative distances of the taxa. As a result, this summary tree is more speculative than the one built from a complete matrix with a fitting algorithm, and we have been careful not to overstate its resolution. Nevertheless, the structure in the titmouse clade (*wollweberi, inornatus, atricristatus,* and *bicolor*) is well-supported by the data in Table 4, given the relatively constant rate of parid evolution (Tables 2 and 5). *Parus atricristatus* presents the only potential problem in the clade. Its distance from *bicolor* may be exaggerated because of short-stranded DNA or increased evolutionary rate (see Table 5 and the section on rates of evolution). If anything, *atricristatus* is closer to *bicolor* than depicted. More structure could be postulated for the chickadee clade (*carolinensis, atricapillus, hudsonicus,* and *montanus*) based on the distances in Table 3, but the span of ΔTm's among these species (0.4–1.2) is probably too short to be resolved into branches by DNA hybridization, even with a complete matrix of comparisons.

The positions of *Sitta* and *Auriparus* relative to *Parus* also are unclear. The distance from *Parus* to *Auriparus* ranges from 8.2–8.9, with one odd value of 9.3 discussed in the Rates-of-Evolution section above (Tables 2, 5, and 7). Distances from *Parus* to *Sitta* are more variable. We measured average values of 7.8 (Table 4) and 8.8 (Table 3) and, from sample raw data provided by C. G. Sibley (pers. comm.), we computed distances of 9.8 and 11 (Table 7). The 7.8 value is a clear example of long-distance ΔTm compression (described in detail by Sheldon and Bledsoe 1989). Why the Sibley and Ahlquist distances are so different is more perplexing, especially as their *Parus* to *Auriparus* distance of 8.2 fits with our data. It is possible that the discrepancy was caused by an unrecorded technical problem. The other data of Sibley and Ahlquist (1990, pers. comm.), which compare *P. atricapillus* to *P. bicolor* and *P. major* (Table 7), produce distances and branching consistent with ours, despite differences in their labeling method, amount of DNA used, and distance computation (ΔT50H).

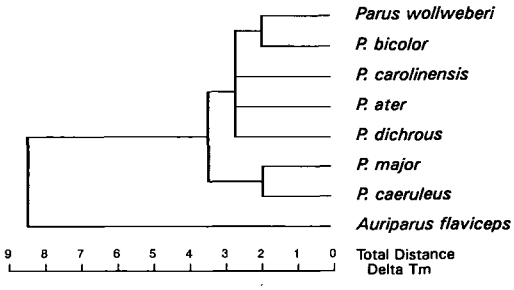

Fig. 3. Consensus tree derived from Tm values in Table 2.

Discussion

Comparison to Previous Phylogenetic Analyses and Classifications

Gill et al. (1989) reviewed the problems and issues of tit systematics. Of these, we address several below.

Relationships of the crested tits.—On the basis of morphology and distribution, Thielcke (1968) placed *wollweberi* and *dichrous* in the subgenus *Lophophanes* with the Crested Tit (*cristatus*). Eck (1988) concurred, placing the three species in a "species group," *cristatus*. We found, however, that *wollweberi* clusters with the North American titmice, *inornatus, bicolor,* and *atricristatus* (subgenus *Baeolophus*) and is not particularly closely related to *dichrous* (Fig. 4). In allozyme studies, Gill et al. (1989) also noted the relationship between *wollweberi* and titmice. As for *dichrous,* we were unable to establish its closest affinities. This enigmatic species of the coniferous forests of western China and Tibet may be closely related to *cristatus,* but our data do not address this possibility. Within *Baeolophus,*

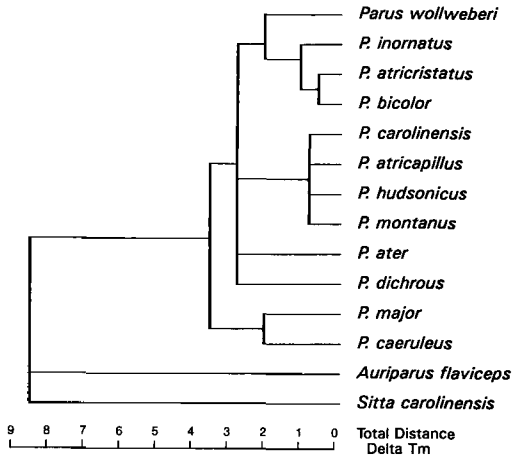

Fig. 4. Consensus tree of Figure 3 with additional taxa from Tables 3 to 5.

TABLE 9. Samples of divergence values between *Parus bicolor* and selected congeners derived by different biochemical methods. DNA-hybridization estimates based on 1:1 relationship between genetic distance and percent nucleotide divergence (Bonner et al. 1973). Two different allozyme distances (Rogers's [1972] and Cavalli-Sforza and Edwards's [1964] chord) are from Gill et al. (1989). The mtDNA divergence values are from Gill and Slikas (1991) and have been calculated with Upholt's (1977) formula for shared restriction sites. All values expressed as percentages.

Parus species	DNA hybridization	Allozyme		mtDNA
		Roger's	C-S&E	
atricristatus	0.6	—	—	0.4
inornatus	1.4	1.1	2.7	6.3
wollweberi	2.3	2.1	3.9	9.7
carolinensis	2.6	3.2	5.1	—
major	3.5	3.1	4.9	—

all molecular evidence including ours supports the established taxonomic hierarchy, which groups *bicolor* and *atricristatus* as conspecies (e.g. AOU 1983) or semispecies (e.g. Sibley and Monroe 1990), and places *inornatus* as their sister taxon.

Relationships of chickadees.—The chickadees were placed in the subgenus *Poecile* by Thielcke (1968). Of particular interest are the branching patterns of the North American brown- and black-capped varieties, the extent of divergence between *atricapillus* and *carolinensis*, and the position of the Old World *montanus*. The latter looks superficially like *atricapillus* and, at one time, the two were considered conspecific (discussion and references in Mayr and Short 1970). The chickadees we examined cluster on a single branch relative to other parids. We did not attempt to distinguish their precise branching hierarchy and, thus, cannot speak to issues of their interrelationship other than to indicate that our data suggest *montanus* is no closer to *atricapillus* than are *hudsonicus* and *carolinensis*. However, even if we had attempted a complete matrix of comparisons, it is unlikely that DNA hybridization could resolve the branching pattern of such closely related taxa.

Relationships of Coal, Great, and Blue tits.—Thielcke (1968) placed these three species in separate subgenera (*Periparus, Parus,* and *Cyanistes,* respectively), and Eck (1988) put them in separate species groups (*ater, major,* and *caeruleus*). No one has seriously attempted to define their positions relative to other parid species groups. Gill et al. (1989) suggested that *ater* and *caeruleus* may lie in sister lineages. Our data do not distinguish the precise relationship of *ater*. However, our results link *caeruleus* and *major* unequivocally in a clade that is the sister group to the rest of *Parus*.

Other Considerations

The data and results of this study open several lines of investigation in systematics and provide an historical perspective to an active area of ecological research.

Comparison of distances.—It will soon be possible to develop extensive lists of divergence values (such as in Table 9), which will reveal relative patterns and idiosyncrasies of distances derived by different methods. Even the few values listed in Table 9 are suggestive. The allozyme measurements of the more-diverged tit taxa appear to have reached a threshold, after which they are compressed and uninformative. Relative to nuclear distances, mtDNA distances depict slow divergence between the hybridizing taxa *bicolor* and *atricristatus* (suggesting an interchange of mtDNA) and fast change between nonhybridizing forms. Such a pattern is expected (e.g. Ferris et al. 1983a, b, Shields and Wilson 1987, Gill and Slikas 1992).

Disparity of avian genera.—The genetic divergence among tit species is much greater than expected for such similar-looking birds. The most divergent members of *Parus* are as different from one another as species divided into 4 different genera of cranes (Krajewski 1989), 5

genera of ducks (Madsen et al. 1988), 8 genera of herons (Sheldon 1987b), 10 genera of nine-primaried oscines (Bledsoe 1988), and 12 genera of swallows (Sheldon and D. Winkler, unpubl. data). Only a few genera, such as *Harpactes* trogons, comprise species that are more highly diverged than those in *Parus* (Sibley and Ahlquist 1990). Because it does not appear to be a particularly old or fast-evolving group (Sibley and Ahlquist 1990), *Parus* (like trogons) must be unusually conserved morphologically. In general, the wide range of differentiation among species within genera of birds raises the issue of subjectivity in avian classification. Perhaps genetic, rather than morphological, criteria should be employed to distinguish taxonomic hierarchy, as suggested by Sibley and Ahlquist (1990).

Phylogeny and ecology.—In light of recent research in tit behavior (e.g. Ekman 1989, Sherry 1989, Krebs et al. 1990), our discovery that *caeruleus* and *major* form the sister clade to the rest of the parids is particularly interesting. Most tits hoard food, and this habit is correlated with coherent flocking behavior. *Parus caeruleus* and *major*, however, differ from all other tits in that they do not cache food. Apparently, the neurophysiological changes necessary for caching and associated flocking behavior developed after *caeruleus* and *major* diverged from the rest of *Parus*.

ACKNOWLEDGMENTS

We are grateful to the following colleagues who helped us obtain tissues for this study: K. Bryant, P. Escalente, D. Gibson, W. Howe, T. Huels, J. D. Ligon, R. Moldenhauer, A. Navarro, J. Pitocchelli, J. V. Remsen, M. B. Robbins, and S. Russell. C. G. Sibley kindly provided *Parus* DNA-hybridization data, and A. Dickerman and S. Lanyon sent copies of their programs for bootstrapping and jackknifing. J. Cracraft, J. Love, and A. Mostrom commented on the manuscript. This research was supported by the National Science Foundation (BSR 88-06647 and 88-06890).

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