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Morphometric variation as an indicator of genetic interactions between Black-...

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MORPHOMETRIC VARIATION AS AN INDICATOR OF GENETIC INTERACTIONS BETWEEN BLACK-CAPPED AND CAROLINA CHICKADEES AT A CONTACT ZONE IN THE APPALACHIAN MOUNTAINS

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ABSTRACT.—We studied hybridization and introgression between Black-capped (Poecile atricapillus) and Carolina (P. carolinensis) chickadees along two transects in the Appalachians using four genetic markers and multivariate analysis of morphology. Genetic data revealed that at least 58% of the birds in the center of each transect were of mixed ancestry and that recombinant genotypes predominated among hybrids, demonstrating that hybridization is frequent and that many hybrids are fertile. Genetic clines generally were steep and coincident in position, but introgression was evident well beyond the range interface. Introgression was higher at the one autosomal locus surveyed than in mitochondrial DNA or in two sexlinked markers, suggesting that the hybrid zone is a conduit for gene flow between the two forms at some loci. On a broad scale, morphometric variation was concordant with genetic variation. Clines in morphological variation based on principal components (PC) scores were steep and coincident with genetic clines. Also, a strong correlation within a population between PC scores and an individual's genetic makeup suggested that a large amount of morphological variation was genetically determined. However, morphological analysis indicated that hybrids were uncommon on one transect, whereas genetic data clearly showed that they were common on both. In addition, patterns of morphological variation were equivocal regarding introgression across the hybrid zone. Thus, genetic data provided a complementary and more detailed assessment of hybridization, largely due to the discrete nature of genetic variation. Genetic markers are useful in understanding hybridization and introgression, but diagnostic markers may underestimate average gene flow if selection against hybrids maintains steep clines at diagnostic loci. To gain a clearer picture of the genome-wide effects of hybridization, a much larger number of loci must be assayed, including non-diagnostic ones. Received 23 December 1998, accepted 1 October 1999.

HYBRID ZONES are places where differentiated forms meet, mate, and produce hybrids (Barton and Hewitt 1985). Because the boundaries between otherwise discrete taxa blur in such areas, hybrid zones are useful for investigating the process of speciation and the development of reproductive isolation between taxa (Moore and Price 1993, Hodges and Arnold 1994). Hybrid zones also may be viewed as natural laboratories where the interaction of populations differentiated at many genetic loci can be used to study microevolutionary processes, and where evolutionary events of significance in their own right can occur (Harrison 1990, Arnold 1992).

Morphological differences are often used to gauge the extent of genetic interactions between hybridizing taxa. Some of the earliest studies of hybrid zones were done on birds, partly because plumage differences facilitated the identification of hybrids (e.g. Sibley 1950, Short 1963). When populations that meet along a common boundary are morphologically similar, however, inferences about genetic interactions can be elusive. Morphological intermediacy may not be readily apparent, and hybrids may be difficult to diagnose. In such cases, it is important to use multiple independent characters to assess evidence for hybridization. Classic avian examples are the Eastern (Sturnella magna) and Western (S. neglecta) meadowlarks, and the Eastern (Contopus virens) and Western (C. sordidulus) wood-pewees (Lanyon 1966, Rohwer 1972, Rising and Schueler 1980), for which careful analyses of vocalizations and

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morphology were necessary to demonstrate that hybridization between each of the species pairs was relatively infrequent.

Black-capped (Poecile atricapillus) and Carolina (P. carolinensis) chickadees are another such pair of hybridizing taxa for which it has been difficult to ascertain the extent of genetic admixture. The two taxa meet in eastern North America along an extensive parapatric range boundary that stretches from New Jersey to Kansas, dipping southward in a peninsular fashion in the Appalachian Mountains (Brewer 1963). Poecile atricapillus and P. carolinensis are quite similar in morphology, with only moderate mensural and plumage differentiation (Rising 1968, James and Rising 1985, Pyle et al. 1987). Moreover, clinal geographic variation minimizes these phenotypic differences where the two meet (Duval 1945, Lunk 1952, James 1970). Thus, although several studies of morphology have detected intermediacy and increased variability in the contact zone suggestive of hybridization (Rising 1968, Johnston 1971, Robbins et al. 1986), others have found little evidence of intermediacy (Tanner 1952; Merritt 1978, 1981). Clearly, the morphological similarity of these birds renders it difficult or impossible to make inferences about introgression beyond the range interface based on morphology alone (Robbins et al. 1986).

These chickadees also differ in song, which provides the most reliable means of field identification. The difference in song is a principal reason why the two have traditionally been treated as separate species. Intermediate songs and/or bilingual birds occur at the range interface, suggesting that hybridization takes place (Johnston 1971, Ward and Ward 1974, Robbins et al. 1986), but such mixed singing is limited to a narrow region relative to the range of each bird. However, heterospecific song learning between these chickadees has been demonstrated in the laboratory (Kroodsma et al. 1995). Song learning probably also occurs in nature and has the potential to either mask or exaggerate apparent levels of hybridization and introgression. These and other studies (Grubb et al. 1994, Kershner and Bollinger 1999) call into question the behavioral and ecological mechanisms that have been proposed to maintain the distinctiveness of the species.

Molecular genetic analyses with diagnostic marker loci can provide direct estimates of the extent of hybridization and introgression, potentially revealing the structure of a hybrid zone in greater detail than either morphology or behavior (Dowling et al. 1989, Arnold et al. 1990, Szymura and Barton 1991). In the case of atricapillus and carolinensis, one isozyme difference (Braun and Robbins 1986, Gill et al. 1989) and three restriction fragment length differences (Mack et al. 1986; Gill et al. 1989, 1993; Sawaya 1990) are known. These four diagnostic molecular markers were used in southwestern Missouri to provide the first detailed assessment of genetic interactions between these chickadees (Sawaya 1990). Here, we use these markers to estimate levels of hybridization and introgression at the contact zone in the Appalachian Mountains and compare that with an assessment based on morphology. We evaluate the correlation of morphometric and genetic variation in these chickadees and assess the reliability of morphometric variation in reflecting genetic interactions. These comparisons enhance our ability to understand the evolutionary significance of this and other hybrid zones.

METHODS

POPULATION SAMPLES

We sampled 268 individuals from 12 populations in the study (Tables 1, 2). The sites that we sampled formed two transects that cross the range interface at the base of the Appalachian Mountains (Fig. 1), one on the eastern slope (Virginia transect) and one on the western slope (West Virginia transect). Where population samples were closely spaced (VA2 to VA4), population boundaries were determined by elevation. For example, VA3 included all birds collected on the floor of the Shenandoah Valley, whereas VA2 and VA4 were collected on the adjoining ridges above the valley floor to the west and east, respectively. Initially, we sampled a central "atricapilluslike" population (VA1/WV1) to serve as a common terminal population for both transects (Fig. 1). However, because this sample showed genetic evidence of introgression from carolinensis (see Results), we collected a distant allopatric sample in northern Pennsylvania to represent pure parental atricapillus. This parental sample (PA) was then treated as one of the terminal population samples of both transects (Tables 1, 2). Parental samples OH and VA were collected to represent the terminal carolinensis populations of the West Virginia and Virginia transects, respectively.

All birds were collected with shotguns, frozen within one to four hours on dry ice or in liquid nitrogen, and transferred to a -80° C freezer. Collect-

Popula- tionª	n ^b	Location ^c	Distance ^d	USNM ^e	Date
		West Virginia transect			
PA	20	PA: Potter Co., 2.5 km S, 4.5 km E of Ole Bull State Park; 41°31'N, 77°39'W	0.0	600060 to 600077	Jul 91
WV1	20	WV: Pendleton and Tucker Cos., 9 km S, 11 km W of Petersburg; 38°54'N, 79°15'W	100.0	600078 to 600094	May 90
WV2	20	WV: Randolph Co., 2 km S, 3.5 km E of Belington; 38°59'N, 79°54'W	155.4	600114 to 600131	May 90
WV3	31	WV: Upshur Co., 3 km S, 9 km E of Buckhannon; 38°57'N, 80°08'W	172.7	600132 to 600162	Apr 90, 92
WV4	19	WV: Upshur Co., 3 km S, 7.5 km W of Buckhannon; 38°57'N, 80°20'W	188.7	600212 to 600229	Apr 90
WV5	19	WV: Lewis Co., 10 km S, 13 km W of Weston; 38°56'N, 80°37'W	212.5	600230 to 600247	Apr, May 90
OH	20	OH: Lawrence Co., 9 km S, 5 km E of Lawrence; 38°43'N, 82°34'W	344.9	597882 to 597900	Jul 91
		Virginia transect			
PA	20	PA: Potter Co., 2.5 km S, 4.5 km E of Ole Bull State Park; 41°31'N, 77°39'W	0.0	600060 to 600077	Jul 91
VA1	20	WV: Pendleton and Tucker Cos., 9 km S, 11 km W of Petersburg; 38°54'N, 79°15'W	100.0	600078 to 600094	May 90
VA2	33	VA: Shenandoah Co., 2.5 km N, 2 km E of Liberty Furnace; 38°54'N, 78°41'W	153.6	600288 to 600319	Jul 89, Apr 91
VA3	24	VA: Shenandoah Co., 1 km S, 3 km W of Woodstock; 38°52'N, 78°33'W	164.5	600320 to 600342	Jul 89
VA4	21	VA: Shenandoah Co., 6 km E of Edinburg; 38°50'N, 78°30'W	171.4	600267 to 600287	Jun, Jul 89
VA5	20	VA: Rappahannock Co., 2 km S, 3.5 km E of Flint Hill; 38°45'N, 78°03'W	205.5	600095 to 600113	Apr 90
VA	21	VA: Charles City Co., 5.5 km N, 17.5 km W of Williamsburg; 37°20'N, 77°51'W	387.0	600039 to 600059	Jun 91

TABLE 1. Data for chickadees collected on the West Virginia and Virginia transects, 1989 to 1992.

* PA is treated as the atricapillus parental population sample for both transects. WV1 and VA1 are the same samples from the central Appalachians and serve as the second population at the atricapillus end of both transects.

^b Includes all individuals used in genetic analyses. Morphological analyses performed on males only.

^c Approximate center of area in which collection was made. Population diameters spanned from a few km to a few tens of km.

^d Measured relative to the *atricapillus* terminus of each transect and perpendicular to the range interface as determined from Peterjohn (1989), BDGIF (1989), Brauning (1992), and Buckelew and Hall (1994). The linear distance between PA and VA1/WV1 is corrected because PA is displaced from the east/west-oriented transects. This distance was estimated by measuring the distance from PA to the closest point of the range interface.

* Catalog numbers for specimens at USNM. Skins of 18 birds included in genetic analyses were not salvageable and are not represented here. Tissue samples from these 18 birds are archived at the Laboratory of Molecular Systematics.

Popula-	п	Minimum hybrids (%)	Potential F ₁ _ (%) ^b	Percent atricapillus alleles			
tion ^a				mtDNA	GDA	C7	ski
			West V	irginia transe	ct		
PA	20	0.0	0.0	100.0	100.0	100.0	100.0
WV1	20	15.0	0.0	100.0	100.0	100.0	92.5
WV2	20	15.0	0.0	95.0	100.0	100.0	95.0
WV3	31	58.1	16.1	64.5	59.6	59.6	62.9
WV4	19	57.9	5.3	5.3	6.7	6.7	31.6
WV5	19	47.4	0.0	0.0	0.0	0.0	23.7
OH	20	40.0	0.0	0.0	0.0	0.0	20.0
			Virg	inia transect			
PA	20	0.0	0.0	100.0	100.0	100.0	100.0
VA1	20	15.0	0.0	100.0	100.0	100.0	92.5
VA2	33	45.5	12.1	87.9	94.0	94.0	77.3
VA3	24	62.5	0.0	4.2	4.9	4.9	37.5
VA4	21	28.6	9.5	4.8	11.4	11.4	19.0
VA5	20	10.0	0.0	0.0	0.0	0.0	5.0
VA	21	0.0	0.0	0.0	0.0	0.0	0.0

TABLE 2. Sample size and percentage of hybrids, potential F₁ hybrids, and *atricapillus* alleles at four diagnostic genetic markers.

*PA is treated as the *atricapillus* parental population sample for both transects. WV1 and VA1 are the same samples from the central Appalachians and serve as the second population at the *atricapillus* end of both transects.

^b Potential male F_1 hybrids must be heterozygous at the two sex-linked loci (GDA, C7) as well as at the one autosomal locus (*ski*) because they are the homogametic sex. Potential female F_1 hybrids must be heterozygous at *ski*, and their mtDNA haplotype must be the opposite of that of the two sex-linked loci, because their mitochondrial genome is maternally inherited, whereas their single Z chromosome is paternally inherited.



FIG. 1. Distribution of *Poecile atricapillus* and *P. carolinensis* in the Appalachian region, with locations of study sites comprising the West Virginia and Virginia transects, including parental samples. Exact localities are given in Table 1. Range boundaries are approximate (Peterjohn 1989, VDGIF 1989, Brauning 1992, Buckelew and Hall 1994).

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Popu- lationª	п	Mass (g)	Wing chord (mm)	Tail length (mm)	Tail:wing	PC1	PC2	PC3
19.00			h feither b	Wes	t Virginia trans	ect	266 BBS - 6	
PA	13	11.5 ± 0.2	66.0 ± 0.6	61.1 ± 0.6	0.928 ± 0.008	1.39 ± 0.31	0.30 ± 0.18	-0.20 ± 0.16
WV1	13	11.2 ± 0.1	66.9 ± 0.4	62.7 ± 0.6	0.936 ± 0.007	1.62 ± 0.20	-0.44 ± 0.14	-0.18 ± 0.11
WV2	13	11.7 ± 0.2	66.6 ± 0.6	62.1 ± 0.7	0.934 ± 0.006	1.83 ± 0.31	0.27 ± 0.19	-0.18 ± 0.12
WV3	21	11.1 ± 0.1	65.8 ± 0.4	60.1 ± 0.7	0.913 ± 0.007	0.91 ± 0.22	-0.01 ± 0.13	-0.08 ± 0.09
WV4	11	10.6 ± 0.2	64.5 ± 0.6	56.9 ± 0.5	0.883 ± 0.008	-0.25 ± 0.28	-0.02 ± 0.27	0.09 ± 0.16
WV5	11	11.1 ± 0.1	65.0 ± 0.4	56.9 ± 0.5	0.876 ± 0.005	0.17 ± 0.21	0.37 ± 0.15	0.25 ± 0.07
OH	16	$10.2~\pm~0.1$	64.4 ± 0.4	54.8 ± 0.4	0.852 ± 0.004	-0.90 ± 0.21	-0.27 ± 0.08	0.39 ± 0.09
				v	irginia transect	t		
PA	13	11.5 ± 0.2	66.0 ± 0.6	61.1 ± 0.6	0.928 ± 0.008	1.39 ± 0.31	0.30 ± 0.18	-0.20 ± 0.16
VA1	13	11.2 ± 0.1	66.9 ± 0.4	62.7 ± 0.6	0.936 ± 0.007	1.62 ± 0.20	-0.44 ± 0.14	-0.18 ± 0.11
VA2	15	11.1 ± 0.1	66.1 ± 0.5	60.7 ± 0.7	0.919 ± 0.009	1.06 ± 0.22	-0.18 ± 0.22	-0.10 ± 0.13
VA3	12	10.3 ± 0.1	63.0 ± 0.2	54.6 ± 0.5	0.867 ± 0.007	-1.25 ± 0.11	0.08 ± 0.17	0.00 ± 0.07
VA4	14	10.2 ± 0.2	63.1 ± 0.4	54.6 ± 0.7	0.866 ± 0.009	-1.30 ± 0.31	-0.04 ± 0.12	0.02 ± 0.11
VA4 ^b	13	10.0 ± 0.1	62.8 ± 0.3	54.0 ± 0.5	0.861 ± 0.008	-1.58 ± 0.17	-0.10 ± 0.11	0.02 ± 0.12
VA5	14	10.4 ± 0.1	62.9 ± 0.3	54.1 ± 0.4	0.860 ± 0.005	-1.31 ± 0.17	0.26 ± 0.11	0.05 ± 0.09
VA	15	9.7 ± 0.2	62.1 ± 0.3	53.1 ± 0.3	0.857 ± 0.003	-2.08 ± 0.16	-0.17 ± 0.18	-0.04 ± 0.06

TABLE 3. Sample size (males only), morphological measures, and principal components scores of chickadee populations comprising the West Virginia and Virginia transects. Values are $\bar{x} \pm SE$.

* PA is treated as the *atricapillus* parental population sample for both transects. WV1 and VA1 are the same samples from the central Appalachians and serve as the second population at the *atricapillus* end of both transects.

^b Omits one pure atricapillus individual from predominantly carolinensis-like VA4.

ing was done during the breeding seasons (April to July) of 1989 to 1992 (Table 1), prior to or following the rearing of young. Study skins and tissue specimens were deposited at the United States National Museum of Natural History (USNM). All 268 individuals, represented by 178 males, 82 females, and 8 unsexed individuals (see below), were used in genetic analyses. Because these birds are sexually dimorphic in size, only males were included in morphometric analyses. Owing to excessive plumage wear or damage, we omitted 10 males from morphometric analyses, resulting in a total morphometric sample size of 168 males (Table 3).

In using the generic names *Poecile* and *Baeolophus*, we follow AOU (1998), while remaining unconvinced by available data of the advisability of this revision (i.e. Slikas et al. 1996).

MORPHOMETRIC ANALYSIS

Specimens were thawed in the laboratory, where the sex and age of each bird was determined by examination of gonads and skull ossification, respectively. Birds were weighed to the nearest 0.1 g, and wing chord and tail length were measured by GDS to the nearest 0.5 mm by ruler before tissue collection and specimen preparation. Samples VA2, VA3, and VA4 each contained four to eight immature birds (i.e. hatched in that breeding season). We found no significant differences between adults and immatures for the three morphometric variables (Mann-Whitney *U*-tests, all P > 0.10), so the two age classes were combined in each sample.

Other mensural and plumage characters have previously been used to distinguish these chickadees (Rising 1968, James and Rising 1985). However, all mensural differences are highly correlated with overall size. Also, the subtle differences in plumage coloration between these birds are difficult to score on worn specimens (Rising 1968), and many of our birds were worn because they were collected during and immediately after the breeding season (when wear is greatest) to insure that we were working with locally breeding individuals. Robbins et al. (1986) found that mass, wing length, and tail length were sufficient to discriminate among parental populations of the two species and to illuminate patterns of intermediacy in the hybrid zone; we follow that strategy here.

We performed principal components analysis (PCA) on the untransformed data using the correlation matrix, thus weighting all variables equally (SAS 1987). All 12 population samples were included in the analysis. The three morphometric variables were distributed normally in each sample with the following exceptions. Mass, wing chord, and tail length were not normally distributed in VA4 due to the presence of a single individual characterized by our genetic markers as a pure atricapillus in this predominantly carolinensis sample. PCA was performed both with and without this individual, with little effect on the overall analysis. Mass also was not normally distributed in VA, VA5, and WV4, and wing chord was not normally distributed in VA3. Transformations failed to normalize the variables in these populations, so untransformed values were retained

in the PC analyses. Extracted components were distributed normally in each population sample. Oneway analyses of variance (ANOVA) tests of the components were done for the West Virginia and Virginia transects separately followed by *a posteriori* Tukey tests to assess the significance of morphometric differences among populations. All tests were performed with SAS (1987).

GENETIC ANALYSIS

Isozyme analysis.-Liver tissue was thawed and 0.05 to 0.2 g homogenized in 150 µL of deionized water with a pestle. Samples were centrifuged for 2 min and the supernatant aliquoted and stored at -80°C until use. Isozymes were separated on Titan III thinlayer cellulose acetate plates using Zip Zone electrophoresis chambers (Helena Laboratories, Beaumont, Texas). Gels were run at 200 V for 50 to 120 min using a 50 mM Tris/20 mM maleate buffer (pH 7.8), and stained by agar overlay using the guanine deaminase (GDA) staining recipe of Richardson et al. (1986). GDA is diagnostic for atricapillus and carolinensis (Gill et al. 1989, Sawaya 1990). It is also believed to be sex linked in these chickadees; only male hybrids display a heterozygous pattern for the marker (Sawaya 1990, Sattler 1996).

Genomic DNA extraction .- Pectoral muscle was thawed and 0.7 g from each bird mechanically homogenized in 7.0 mL of extraction buffer (0.1 M NaCl, 0.1 M EDTA, 0.01 M Tris, pH = 8.0). The homogenate was digested overnight at 55°C with proteinase K (200 μ g/mL) in the presence of 0.5% SDS and then digested with RNase (100 μ g/mL) for 1 h at room temperature. NaCl was added to 0.2 M concentration and samples were extracted once in an equal volume of a phenol-chloroform-isoamyl alcohol solution (25:24:1) and twice in an equal volume of a chloroform-isoamyl alcohol solution (24:1), incubating each extraction at 55°C for 20 min. Total DNA was recovered by overlaying the aqueous solution with two volumes of cold 95% ethanol and spooling the high molecular weight DNA onto a glass rod, rinsing in 70% ethanol, and resuspending in 800 μ L of TE (10 mM Tris, 1 mM EDTA, pH = 8.0).

Restriction analysis.—Restriction enzyme digestions were carried out overnight according to manufacturer's recommendations. Four micrograms of total genomic DNA were digested with 20 units of restriction enzyme and electrophoresed in 0.6% agarose gels using TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.4) overnight at 30 to 50 V. Gels were soaked for 30 to 45 min in 1 L of 0.4 M NaOH and 0.8 M NaCl under gentle agitation to denature the DNA, then soaked in 1 L of 1.5 M NaCl and 0.5 M Tris HCl for 30 to 60 min prior to blotting onto MSI Magnagraph nylon membranes (Southern 1975). Transfer was accomplished over 6 to 20 h using 10× SSC (1.5 M NaCl, 0.15 M sodium citrate). DNA was crosslinked to membranes using a Stratagene UV Stratalinker 1800. Membranes were then rinsed in $2 \times$ SSC (0.3 M NaCl, 0.03 M sodium citrate), air dried, and stored at -20° C.

Probes were labeled to high specific activity (10⁸ to 10° dpm/µg) with alpha 32P dATP by random priming (Feinberg and Vogelstein 1983). Transfer membranes were prehybridized in a solution of 1 M NaCl, 1.0% SDS, and 10.0% dextran sulfate for 1 to 3 h at 65°C. Labeled probe was then added to a concentration of 2×10^6 to 2×10^7 dpm/mL (1 to 2×10^5 dpm/ mL for mitochondrial probe) and hybridization carried out for 18 to 24 h at 65°C. One low-stringency wash ($1.0 \times$ SSC, 0.5% SDS, 1 mM EDTA) and two high-stringency washes (0.2× SSC, 0.1% SDS, 1 mM EDTA) were done at 48°C. Membranes were then wrapped in cellophane without drying and exposed to Kodak XRP film for 20 to 200 h using two Dupont Cronex intensifying screens. After autoradiography, some membranes were stripped of radioactivity in two changes of boiling 0.1% SDS (1 L each) and reprobed with mtDNA. Fragment lengths were estimated by comparison with a size marker consisting of Hind III-digested bacteriophage lambda DNA and Hae III-digested bacteriophage φ X174 DNA. We did not attempt to score fragments smaller than 400 base pairs (bp).

We used three probes to detect restriction fragment length variants that were diagnostic for *atricapillus* and *carolinensis*. The first was a 1,200-bp fragment of the chicken oncogene *ski* (Li et al. 1986) that was used to probe *Eco* RI digests (Sawaya 1990). The second was a randomly cloned 4,000-bp fragment of Tufted Titmouse (*Baeolophus bicolor*) DNA designated C7 that was used to probe *Pst* I digests (Sawaya 1990). The third was mitochondrial DNA (mtDNA) from *carolinensis* purified by subcellular fractionation and CsCl equilibrium-gradient centrifugation following Dowling et al. (1990). Three restriction enzymes (*Pst* I, *Pvu* II, and *Ava* II) were used to identify species-specific mtDNA haplotypes (Mack et al. 1986, Sawaya 1990).

Restriction fragment sizes for *ski*, C7, and mtDNA agreed with those reported earlier (Sawaya 1990). Some intraspecific polymorphisms in restriction fragment pattern occurred in both *atricapillus* and *carolinensis* for C7 and mtDNA haplotypes, but all fragment patterns could be unambiguously assigned to one or the other species based on their distribution in samples from parental populations and/or their relationship to parental haplotypes (Sawaya 1990, Sattler 1996). *Ski* is autosomal in these chickadees, whereas C7 is sex linked (Sawaya 1990, Sattler 1996).

Screening with *Pst* I, *Pvu* II, and *Ava* II produced a size estimate for the mtDNA genome of these chick-adees of 16,200 bp. MtDNA fragment profiles produced by each enzyme were concordant in establishing *atricapillus* or *carolinensis* haplotypes for all individuals. Although two distinct mitochondrial hap-

lotypes occur in *carolinensis* along an east / west cline (Sawaya 1990; Gill et al. 1993, 1999), we encountered only the eastern haplotype.

Identification of hybrids .- We defined a hybrid as any individual that possessed a mixture of atricapillus and carolinensis alleles among the four diagnostic loci. Estimates of hybrid frequency are conservative because of the limited number of genetic markers available to characterize genetic ancestry. For this reason also, birds are classified for convenience into a small number of genetic classes (parental, potential F_{ν} and backcross or later-generation hybrid). The actual genetic composition of the hybrid zone populations is surely much more complex than this. Estimates of hybrid frequency are more conservative for females than for males because two of the marker loci are sex linked (GDA and C7), and females, being the heterogametic sex, have only one allele at such loci. The possibility of physical linkage of GDA and C7 on the Z chromosome could result in nonindependence of these markers, further increasing the chances of misclassifying later-generation hybrids as parentals. On the other hand, the number of potential F1 individuals will exceed the actual number in the population, because some later-generation hybrids will have genotypes consistent with F1 status.

RESULTS

Genetic analysis.-The marker loci yielded unambiguous evidence of extensive hybridization in both Appalachian transects. At least 58% of the birds in the center of each transect were of hybrid ancestry, based on admixture of the four marker loci (Table 2). The frequency of hybrids declined rapidly away from the range interface, except for the carolinensis side of the West Virginia transect. Here, hybrids remained at frequencies of 40% or higher as far as sample OH. Also, the central Appalachian populations VA1/WV1 and WV2 still included 15% hybrids. However, all hybrids found more than 20 km from the center of either transect were classified as such on the basis of a single foreign allele at ski.

Allele frequency clines for all four diagnostic markers were coincident in position, with the center of the hybrid zone lying between WV3 and WV4 in West Virginia and between VA2 and VA3 in Virginia (Table 2). Allele frequencies at three of the marker loci, GDA, C7, and mtDNA, were similar in all populations, and evidence for introgression at these loci was limited to the central three samples of each transect, WV2-4 and VA2-4 (Table 2). Introgression at *ski* was greater in both directions across the hybrid zone, affecting five populations in the Virginia transect and six in the West Virginia transect. In fact, the terminal *carolinensis* sample of the West Virginia transect, OH, which lies about 160 km west of the center of the hybrid zone, still had a frequency of 20% *atricapillus* alleles at *ski*.

With the exception of WV3, genes of one species or the other predominated in each sample (Table 2). In all population samples, backcross or other recombinant genotypes predominated among hybrids (Table 2). In the West Virginia transect, 75.0% of all hybrids were identified as such on the basis of a single foreign allele among the loci surveyed, whereas in the Virginia transect the figure was 63.4%. In the West Virginia transect, potential F_1 hybrids made up less than 20% of any sample, whereas in the Virginia transect, potential F_1 hybrids constituted less than 15% of any sample.

Morphometric analysis.-Both parental population samples of carolinensis (OH, VA) averaged smaller than the parental sample of atricapillus (PA) in all univariate measurements and in the ratio of tail length to wing chord (Table 3), a character commonly used to distinguish these species (Tanner 1952; Johnston 1971; Merritt 1978, 1981). Population samples from the center of each transect were intermediate between the appropriate parental samples in these measures. Poecile atricapillus-like samples from higher elevations in the central Appalachians (VA1/WV1, WV2) often averaged larger than the parental atricapillus sample (PA), although these differences were rarely significant.

Principal components analysis and discriminant function analysis are two multivariate approaches often used in the phenetic analysis of taxonomic groups engaged in hybridization (Rising 1968, Rohwer 1972). In addition to more stringent assumptions, discriminant analysis requires correct classification of individuals in reference samples (Neff and Smith 1978). As noted above, although reference samples of atricapillus and carolinensis were collected in areas of allopatry, genetic analysis revealed long-distance introgression into two of these samples (VA1/WV1 and OH). PCA is a more suitable method of analysis in this situation, as long as a large proportion of the variation present in the data discriminates among

TABLE 4. Eigenvectors generated by a principal components analysis of three morphometric variables for all chickadees comprising the West Virginia and Virginia transects.

Character	PC1	PC2	PC3
Mass	0.54	0.84	0.02
Wing chord	0.59	-0.40	0.70
Tail length	0.59	-0.37	-0.72
Eigenvalue	2.41	0.41	0.18
Variance explained	0.805	0.135	0.060

the taxa being analyzed, as is clearly the case with the variables analyzed here.

PCA provided good separation of chickadee populations in morphometric space. The first principal component (PC1) accounted for 80.5% of the total variance, and the second and third components (PC2 and PC3) explained 13.5% and 6.0%, respectively (Table 4). PC1 had positive factor loadings for all three variables and thus was closely related to overall body size. The PC2 axis primarily contrasted mass with wing chord and tail length, whereas the PC3 axis primarily contrasted wing chord with tail length. PC1 and PC3 scores closely tracked the proportion of *atricapillus* alleles in all populations, but PC2 showed no consistent differences between the two forms (Fig. 2, Table 3).

All of the genetically *atricapillus*-like population samples (PA, VA1/WV1, WV2, VA2) were relatively uniform with respect to both PC1 and PC3 and showed no significant differences in either measure by ANOVA (Table 3, Fig. 2). However, all genetically *atricapillus*-like samples had higher PC1 scores and lower PC3 scores than did all genetically *carolinensis*-like samples (OH, WV4, WV5, VA3–VA5, VA). For PC1, all of these differences were significant (P < 0.05, Tukey tests). For PC3, the only significant differences were between OH and all samples of predominantly *atricapillus* genetics.

Consistent trends also occurred in these two parameters among genetically *carolinensis*-like samples on either side of the Appalachians. For PC1, all such samples of the West Virginia transect had larger scores than equivalent samples of the Virginia transect (Table 3, Fig. 2), making them more like *atricapillus*. All of the differences were significant (P < 0.05, Tukey tests), except in the case of the parental sample OH. In contrast, all *carolinensis*-like samples of the Virginia transect had smaller PC3 scores than did those in West Virginia, making them more *atri*- *capillus*-like on this axis. Only the difference between OH and VA was significant (P < 0.05, Tukey test).

The best morphometric separation of parental populations was achieved with a scatterplot of PC1 and PC3 scores (Fig. 3). In the Virginia transect, parental samples of atricapillus (PA) and carolinensis (VA) were well resolved morphometrically from one another (Figs. 3A, B). Nonparental population samples of this transect (VA1-VA5) fell into two distinct clusters (Figs. 3C, D) despite the presence of a high proportion of hybrids in some populations (Table 2). Most of the hybrids were backcrosses or other recombinant progeny (see above), and each sorted morphologically with the appropriate parental species based on its predominant marker alleles. The lone genetically atricapillus individual from the predominantly carolinensis population VA4 was clearly atricapillus-like in morphology (Figs. 3C, D).

If morphological intermediacy is defined on the basis of an intermediate position between parental polygons in the scatterplots, 22 individuals in the Virginia transect were intermediate. These birds represented 32.4% of the 68 individuals in VA1–VA5 for which morphological data were available, a proportion similar to the proportion of hybrids determined genetically (25 of 75 individuals, or 33.3%). However, more than half of these morphologically "intermediate" individuals were classified as genetically "pure" *atricapillus* or *carolinensis* based on our four marker loci (Fig. 3D).

For the West Virginia transect, parental samples of *atricapillus* (PA) and *carolinensis* (OH) separated on the scatterplot of PC1 and PC3 (Figs. 3A, B), but the degree of separation was less than that of parental samples of the Virginia transect. Because of this higher morphometric similarity between PA and OH, the region between them that defines morphometric intermediacy is narrow, and only 6 of 69 birds (8.7%) in WV1–WV5 fell within this morphometric space compared with 30 birds (43.5%) that were genetically defined as hybrids in the same samples (Figs. 3E, F).

Allele frequencies at four nonparental West Virginia sites were strongly skewed toward either *atricapillus* (WV1, WV2) or *carolinensis* (WV4, WV5) alleles, as in Virginia nonparental populations. In WV3, representation of *atricapillus* and *carolinensis* alleles was more evenly balanced (Table 2). Instead of falling into two distinct clusters in a PC plot as in Virginia, the West Virginia nonparental populations were distributed in one more or less continuous cluster (Figs. 3E, F). Again, hybrids in this transect were predominantly backcrosses or other recombinant genotypes and showed a strong tendency to fall morphometrically with the appropriate parental species on the basis of their genetic makeup (Fig. 3F).

Because of the more balanced representation of both species' genes in WV3, we could directly assess the relationship between morphometric and genetic variation in this sample. Significant correlations existed between two PC scores (PC1 and PC3) and the number of *atricapillus* alleles for individuals of WV3 (Fig. 4). Thus, the correlation between morphology and genetics evident at the regional or population scale (Fig. 2) was maintained on a local level among individuals.

DISCUSSION

Levels of hybridization.-Data from four diagnostic genetic markers clearly demonstrate the presence of a high proportion of hybrids at the range interface of atricapillus and carolinensis in Virginia and West Virginia. The estimated proportion of hybrids in the Virginia and West Virginia hybrid zones (>58%) is comparable to that in southwestern Missouri (Sawaya 1990), where at least 44% of 36 individuals sampled at the range interface were of mixed ancestry based on the same marker loci. The presence of a majority of non-F₁ hybrids among progeny of mixed ancestry at these three locations demonstrates that some hybrids are fertile and that backcrossing occurs frequently. The genetic data confirm several previous morphological analyses of this hybrid zone that found patterns of variation suggestive of substantial backcrossing (Rising 1968, Johnston 1971, Robbins et al. 1986). The significance of other morphological studies that found little evidence of intermediacy (Tanner 1952; Merritt 1978, 1981) is less clear (see below).

Correlation of morphometric variation with genetic ancestry.—Many morphological traits in birds are under polygenic control (Buckley 1987), making them potentially useful for assessing genetic interactions within a hybrid zone. However, the specific mode of inheritance of morphological traits generally is unknown, and in some cases, geographic variation in morphology is environmentally induced (James 1983, 1991). Thus, it is crucial to separate environmental from genetic effects to fully understand the meaning of morphological data in studies of hybridization.

In our study, PC1 and PC3 exhibited abrupt transitions across the contact zone that were coincident in position with changes in allele frequency at the four marker loci (Fig. 2). This suggests (but does not prove) a genetic basis for the morphological variation. The morphological clines also could relate to environmental gradients associated with elevation. On the other hand, it is unlikely that the strong correlation of PC1 and PC3 with an individual's genotype in WV3 (Fig. 4) was induced environmentally because all individuals were collected in a 16km² area within which environmental variation surely was minimal. Thus, the morphological measures of size and shape that we used probably were strongly influenced by genetics. This inference is possible because of the great range of genotypic variation present in hybrid zones (Hewitt 1988).

Hybridization assessed from morphology versus direct molecular analysis.—Although general agreement occurred between morphology and genetics in assessing hybridization in this zone, careful comparison revealed that these assessments differed in important details. Individuals from the Virginia transect fell into two relatively discrete clusters in morphometric space (Figs. 3C, D), whereas those from the West Virginia transect were distributed in essentially one cluster (Figs. 3E, F). This difference might be taken to indicate that less admixture has occurred in Virginia, yet the genetic analysis revealed comparably high levels of hybridization in both transects (Table 2).

What is responsible for this apparent discordance? First, a preponderance of advanced generation hybrids can phenotypically mask extensive hybridization and introgression (Arnold 1993, Arnold et al. 1993). Although the proportion of F_1 hybrids was comparable among the two transects, no Virginia F_1 hybrids were males, compared with five males out of six total F_1 hybrids in West Virginia. Thus, no F_1 hybrids from the Virginia transect were included in the PCA. This sampling artifact might accentuate



FIG. 2. Population averages (\pm SE) of principal components scores and proportion of *Poecile atricapillus* alleles (Table 2) at diagnostic genetic loci in population samples from the West Virginia and Virginia transects. PA and VA1/WV1 each constitute part of both transects. PA appears once as the central *P. atricapillus* origin of each transect, whereas VA1/WV1 appears twice as the second population sample of both transects. PA is

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the appearance of bimodality in the Virginia transect.

Second, morphological separation of parental populations in the Virginia transect was greater than for the West Virginia transect (Figs. 3A, B). Ironically, although this increases the ability to detect individual hybrids on the basis of morphological intermediacy, it also increases the appearance of bimodality at the population level (Figs. 3C, D). It is unclear whether the lower separation of parentals in the West Virginia transect relative to those in the Virginia transect was due to long-distance introgression, geographic variation, or both (see below).

A third factor that probably contributed to the seeming disparity between genetics and morphology was a difference in the hybrid zone's structure between the Virginia and West Virginia transects. Both genetic and morphological clines along the Virginia transect were steeper than along the West Virginia transect. The range interface in the Virginia transect is located at a very sharp ecological transition where the Shenandoah Valley meets the first steep ridge of the Appalachians. Samples VA2 and VA3 were taken on the ridges and valley floor, respectively, but essentially were contiguous in that there was no room for another sample between them (Sattler 1996). Because of the sharpness of the transition, VA2 is predominantly atricapillus-like and VA3 is predominantly carolinensis-like, both morphometrically and genetically (Table 2, Fig. 2). Yet, the discrete nature of molecular genetic variation allows the identification of many of these birds as hybrids, which the morphological variation alone would not. In contrast, the West Virginia transect crossed the range interface in a region where the ecological transition was more gradual. Consequently, it was possible to collect a population sample (WV 3) in a region where intermediate birds predominated.

Morphological and genetic assessments of hybridization differed in other important details. Introgression beyond the range interface was evident in the genetic data but was not clearly discernable in the morphological data (see below). Also, many morphologically "intermediate" individuals were classified genetically as "pure" *atricapillus* or *carolinensis* based on our four marker loci, and vice versa. This last result probably relates to the quantitative nature of morphological variation, the preponderance of later-generation hybrids, and the limited number of loci actually surveyed in either the morphological or genetic data sets. Non-F₁ hybrids can easily be missed by either type of data.

These examples illustrate that caution is necessary when inferring hybridization processes on the basis of phenetic evidence alone. Character intermediacy and increased character variability in a population can often be reliable means of phenetically identifying the occurrence of hybridization (Schueler and Rising 1975). However, the converse may not always be true. The conclusion that limited hybridization occurs at some portions of the atricapillus/ carolinensis contact zone based on morphological analyses (Tanner 1952; Merritt 1978, 1981) requires verification with genetic data. The local structure of the contact zone in the areas examined by those studies may resemble the Virginia range interface, making detection of hybridization difficult on the basis of morphology alone (Braun and Robbins 1986, Robbins et al. 1986, Grubb et al. 1994).

Extent of introgression.—Genetic introgression across the chickadee range interface was higher than has previously been appreciated. We found *P. carolinensis* alleles in all Appalachian populations of *atricapillus* that we examined and *atricapillus* alleles in many *carolinensis* populations. In fact, introgression was so extensive that two of the three samples originally collected to represent "pure" parentals, OH and VA1/WV1, contained significant numbers of foreign alleles. Introgression certainly has affected parental populations of both forms in a broad swath, extending a considerable distance from the range interface.

The extent of genetic introgression varies with respect to genetic locus as evidenced in the more extensive penetration of *ski* alleles relative to those at the other loci (Table 2). Of the

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displaced from the east/west transects, and its equivalent position relative to the other population samples is calculated as described in Table 1.

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FIG. 3. Scatterplots of individual PC1 and PC3 scores from a PCA of three morphometric variables (mass, wing chord, and tail length). All 168 males from 12 population samples comprising the Virginia and West Virginia transects were included in the PCA. Parental populations (PA, OH, VA) identified by population (A) and genetic ancestry (B). (C–D) Virginia transect populations (VA1–VA5). (E–F) West Virginia transect populations (WV1–WV5). Dashed polygons in C to F denote parental populations for each transect from A and B.

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four marker loci, *ski* is the only one that is autosomal, and hence is the only diploid locus. Perhaps the other three loci are exposed to stronger selection against hybrid gene combinations because they are haploid or sex linked. This effect is believed to be the reason that a disproportionate number of sex-linked loci are involved in reproductive isolation (Coyne and Orr 1989).

Introgression of atricapillus ski alleles into the range of carolinensis also varies with respect to geographic location. Introgression along the West Virginia transect is greater than along the Virginia transect, extending as far as the parental carolinensis population sample OH (Table 2). Alternatively, these foreign atricapillus alleles could be interpreted as an ancestral polymorphism, because we do not have a terminal population fixed for the carolinensis allele in OH. However, their absence in allopatric *carolinensis* populations on the Virginia transect, as well as in Louisiana (Sawaya 1990) and at an additional site in western Virginia (G. Sattler and M. Braun unpubl. data), makes introgression a more plausible explanation.

Consistent with the idea of gene flow as the source of these alleles is the fact that the Ohio range interface was in a more southerly position in historic times (Wheaton 1882), thereby favoring introgression in southern Ohio and West Virginia. In addition, winter incursions of atricapillus south of its normal range occasionally reach southern Ohio (Peterjohn 1989). If some individuals undertaking these movements remain to breed successfully, they would provide another source of atricapillus alleles in the range of carolinensis. Such southerly occurrences of atricapillus are virtually unknown along the coastal plain of Virginia (VSO 1987), where carolinensis populations show no evidence of introgression.

Long-distance introgression across the *atricapillus* / *carolinensis* range boundary is evident in the genetic data but is not so clear in the morphological data (Table 2, Fig. 2). Within the

FIG. 4. Scatterplots of principal components scores vs. number of *Poecile atricapillus* alleles at the four diagnostic loci for 21 individuals in WV3. Correlations were significant for PC1 ($r_s = 0.62$, P = 0.0027) and PC3 ($r_s = -0.50$, P = 0.022) but not for PC2 ($r_s = -0.03$, P = 0.889).

range of carolinensis, PC1 scores are more atricapillus-like in the West Virginia transect, but PC3 scores are more atricapillus-like in the Virginia transect. Introgression could affect one morphological component but not the other. Alternatively, the differences could reflect large-scale ecogeographic variation (James 1991). For instance, both atricapillus and carolinensis increase in size from south to north across their ranges in accordance with Bergmann's rule (Duvall 1945, Lunk 1952, James 1970). Likewise, Lunk (1952) observed an increase in size and in tail/wing ratio in carolinensis from east to west across the southern portion of its range, where a genetic influence from atricapillus is unlikely. Because the West Virginia and Virginia transects run essentially east/west but traverse steep elevational changes, both of these trends could be relevant. Distinguishing ecogeographic variation from that caused by genetic introgression is difficult in this case because they yield similar expectations. In fact, both might be operating. In any case, the observed morphological variation in carolinensis does not correspond to recognized subspecies, because all samples fall within the range of P. carolinensis extimus (AOU 1957).

Significance of introgression.—We have taken the *ski* data as evidence that substantial genetic introgression occurs across this hybrid zone, but these data are from a single genetic locus. Focusing instead on the sharp clines for the other three markers and the equivocal morphological evidence, one might adopt the alternative view that introgression on the whole is minimal. Which of these perspectives is more realistic?

In evaluating the available data, it is important to note that we used only diagnostic marker loci to infer hybridization and introgression. This is the norm in studies of hybrid zones because such markers allow unambiguous determination of hybrids. Yet, diagnostic markers in a narrow, quasi-stable hybrid zone such as this one are likely to be under selection, either directly or indirectly (Barton and Gale 1993). Relevant modes of selection will oppose the movement of alleles across the zone, resulting in estimates of introgression that are lower than might be obtained with neutral loci.

The greater the strength of selection, the greater this bias will be. As mentioned above, there is an *a priori* expectation for selection to

be stronger on mtDNA, GDA, and C7 because these markers are haploid and/or sex linked. For these reasons, it seems likely that all of these loci will yield underestimates of introgression genome-wide. The *ski* data probably represent an underestimate as well, but it can be taken as a minimum estimate of introgression at neutral loci.

To obtain a quantitative sense of gene flow across the entire genome, evidence from many more marker loci would be desirable. Such studies are demanding but now are feasible (Reisberg et al. 1999). However, a dilemma exists here. Marker loci should be a representative sample of the genome as a whole, but the more diagnostic a marker is, the more likely it will experience selection against hybrids and the more limited introgression will be. Regions of the genome that experience neutral diffusion (or positive selection) will have few diagnostic marker loci because gene flow will tend to erase differences. Thus, it may be difficult to achieve a representative sampling of the genome. This problem is exacerbated in groups like birds where sample sizes per population are limited by practical or ethical constraints; statistical significance can be achieved only with marker loci that are strongly differentiated.

The problem then becomes one of determining what portions of the genome are experiencing selection against hybridization and introgression, and what the strength of that selection is. This problem is no more tractable than that noted above, but some inferences can be made. For example, we can safely assume that regions of the genome that are identical cannot be experiencing selection against hybrids. In fact, the overall genomic divergence between these birds is small. DNA-DNA hybridization studies yield an estimate of nucleotide sequence divergence between atricapillus and carolinensis of roughly 0.5% over the entire singlecopy genome (Slikas et al. 1996, Werman et al. 1996). When interspecific divergences are so small, intraspecific variation becomes significant. Available estimates of intraspecific variation in vertebrates average around 0.3% sequence mismatch (Werman et al. 1996). Thus, the actual proportion of fixed sequence divergence between atricapillus and carolinensis is likely to be in the neighborhood of 0.2%, or one base in 500. It should be noted that these rough estimates do not account for several sources of

One in 500 bases seems like a small fraction of the genome. However, because the complexity of avian genomes typically is on the order of 10° bp, this proportion implies that atricapillus and carolinensis differ at about 2 million nucleotide positions. If these differences were distributed randomly throughout the genome (they probably are not), they could involve a large fraction of all genetic loci. Again, a rough estimate of the proportion of differentiated loci can be made from existing surveys. Only one of 40 isozyme loci that have been studied was found to be differentiated (Braun and Robbins 1986, Gill et al. 1989). In searching for restriction fragment length differences, Sawaya (1990) effectively examined more than 2,000 bp of sequence from nine nuclear loci in order to detect the two diagnostic nuclear markers used here. Obviously, these surveys are biased in disparate ways with respect to coding vs. non-coding sequences and sensitivity for detecting divergence. Nevertheless, one gains the impression that perhaps only a relatively small fraction of the genome is differentiated and therefore could be potentially involved in the maintenance of the hybrid zone.

What, then, of the rest of the genome? Loci from undifferentiated regions of the genome may be subject to a certain amount of neutral diffusion, and alleles under positive selection surely will be able to cross a hybrid zone (Barton 1979). Recombinant genotypes must be present to facilitate this process, but we now know that many viable and fertile hybrids and a preponderance of recombinant genotypes occur in the chickadee hybrid zone. The analogy emerges that some hybrid zones act as semipermeable membranes that provide a conduit for gene flow at some loci and restrict it at others. Such an interface would increase the range of genetic variation available at some loci, while allowing local adaptation (to the environment) and coadaptation (among loci) at others. A structure such as this has been proposed for other hybrid zones (e.g. Tegelström and Gelter 1990, Dod et al. 1993, Parsons et al. 1993). To determine how well the analogy applies to these chickadees will require more data on introgression at diagnostic and non-diagnostic loci, as well as more information on reproductive success of hybrids (Brewer 1963, Rising 1968).

One species or two?—The demonstration that gene flow at some autosomal loci reaches populations far from the hybrid zone refutes previous contentions that hybridization is irrelevant to the species status of these birds (Gill et al. 1993). The same authors appear to believe that, because atricapillus and carolinensis may not be sister on a mtDNA phylogeny, they cannot belong to the same species. This ignores the fact that single-gene phylogenies may differ from organismal phylogenies (Nei 1987) and the possibility that mtDNA (and other sexlinked genes) in birds may be particularly prone to divergence early in the differentiation process, while gene flow at other loci continues (Tegelström and Gelter 1990). To apply phylogenetic reasoning to questions about species status requires broadening of simple principles when microevolutionary processes may result in true differences among gene phylogenies (Maddison 1997).

Ultimately, any useful debate about species status must be preceded by a clear definition of a species concept. Unfortunately, rigorous and operationally relevant definitions have been elusive for most species concepts. Also, because speciation processes extend over significant periods of time, many assemblages of differentiated populations defy categorization. We lack the data to predict whether these chickadees eventually will satisfy the criteria of one or another currently popular species concept. Still, we are heartened by the thoughts of Hennig (1966:30): "Groups of individuals that are interconnected by tokogenetic relationships are called species. The fact that the species concept as used in systematics is much more complicated need not concern us at the moment." Research programs that will deepen our understanding of the diversification of these birds are worth pursuing regardless of nomenclatural considerations.

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