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## AN ASSESSMENT OF SONG ADMIXTURE AS AN INDICATOR OF HYBRIDIZATION IN BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*) AND CAROLINA CHICKADEES (*P. CAROLINENSIS*)

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**ABSTRACT.**—Vocal admixture often occurs where differentiated populations or species of birds meet. This may entail song sympatry, bilingually singing birds, and songs with intermediate or atypical characteristics. Different levels of vocal admixture at the range interface between Black-capped Chickadees (*Poecile atricapillus*) and Carolina Chickadees (*P. carolinensis*) have been interpreted as indicating that hybridization is frequent at some locations but not others. However, song ontogeny in these birds has a strong nongenetic component, so that inferences regarding hybridization based on vocal admixture require confirmation. We used diagnostic genetic markers and quantitative analyses of song to characterize population samples along two transects of the chickadee contact zone in the Appalachian Mountains. More than 50% of individuals at the range interface were of hybrid ancestry, yet only 20% were observed to be bilingual or to sing atypical songs. Principal component analysis revealed minimal song intermediacy. This result contrasts with an earlier analysis of the hybrid zone in Missouri that found considerable song intermediacy. Re-analysis of the Missouri data confirmed this difference. Correlation between an individual's genetic composition and its song type was weak in Appalachian hybrid populations, and genetic introgression in both forms extended far beyond the limits of vocal admixture. Therefore, song is not a reliable indicator of levels of hybridization or genetic introgression at this contact zone. Varying ecological factors may play a role in producing variable levels of song admixture in different regions of the range interface. Received 18 October 2004, accepted 6 August 2006.

**Key words:** Black-capped Chickadee, Carolina Chickadee, hybrid zone, intermediacy, introgression, *Poecile atricapillus*, *P. carolinensis*, song admixture.

### Una Evaluación de la Mixtura de Cantos como Indicador de Hibridación en *Poecile atricapillus* y *P. carolinensis*

**RESUMEN.**—La mixtura vocal ocurre usualmente donde poblaciones diferenciadas o especies de aves se encuentran. Esto puede implicar simpatria en los cantos, aves bilingües en sus cantos y cantos con características intermedias o atípicas. Los diferentes niveles de mixtura vocal en la interfase de los rangos de *Poecile atricapillus* y *P. carolinensis* han sido interpretados como indicadores de que la hibridación es frecuente en algunas localidades, pero no en otras. Sin

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embargo, la ontogenia del canto en estas aves presenta un fuerte componente no genético, por lo que las inferencias sobre la hibridación basadas en la mixtura de vocalizaciones requieren ser confirmadas. Usamos marcadores genéticos diagnósticos y análisis cuantitativos de los cantos para caracterizar las muestras poblacionales a lo largo de dos transectas de la zona de contacto entre las especies de estudio en las Montañas Apalaches. Más del 50% de los individuos de la interfase de los rangos fueron de origen híbrido, aunque se observó que sólo el 20% de los individuos fueron bilingües o cantaron canciones atípicas. Análisis de componentes principales revelaron que los cantos intermedios fueron mínimos. Estos resultados contrastan con un análisis anterior de la zona híbrida de Missouri que encontró niveles considerables de cantos intermedios. El re-análisis de los datos de Missouri confirma esta diferencia. La correlación entre la composición genética de los individuos y su tipo de canto fue débil en las poblaciones híbridas de los Apalaches, y la introgresión genética en ambas formas se extendió considerablemente más allá de los límites de la mixtura de voces. Por lo tanto, los cantos no son un indicador confiable de los niveles de hibridación o de introgresión genética en esta zona de contacto. Los factores ecológicos cambiantes podrían jugar un papel en producir niveles variables de mixtura de cantos en diferentes regiones de la interfase de los rangos.

HYBRIDIZATION IN BIRDS is relatively widespread (Grant and Grant 1992) and provides the potential for genetic exchange between taxa. Yet species-specific visual and vocal communication systems usually function to ensure assortative mating and reproductive isolation (Paterson 1985, Gill 1998). Because such avian communication systems rely heavily on imprinting and other forms of learning, however, they are susceptible to breakdown. Contact zones between related taxa with similar recognition systems are a case in point. The physical proximity of differentiated taxa at the contact zone provides opportunities for misdirected imprinting and learning that can lead to interbreeding, with the subsequent potential for genetic exchange between them. In birds, plumage intermediacy usually provides clear evidence of such events (Rising 1983).

Black-capped Chickadees (*Poecile atricapillus*) and Carolina Chickadees (*P. carolinensis*) share an extensive contact zone across the eastern half of North America where the potential for genetic exchange exists (Brewer 1963, Mostrom et al. 2002, Curry 2005). They also share a similar vocal repertoire (Smith 1972, Ficken et al. 1978, Hailman 1989; but see Hailman and Ficken 1996). Because the plumage and mensural differences that distinguish the two are subtle, the first evidence usually noted that suggests possible hybridization between them

is not morphological intermediacy, but vocal admixture, including sympatry of song types, presence of birds singing atypical or intermediate songs, and bilingual birds that sing both species' songs (Brewer 1963, Johnston 1971, Ward and Ward 1974, Robbins et al. 1986). When these behavioral observations are followed by detailed morphological analyses, subtle intermediacy is typically revealed that supports the conclusion that hybridization is present (Brewer 1963, Rising 1968, Johnston 1971, Robbins et al. 1986, Sattler and Braun 2000).

On the other hand, at other portions of the contact zone between *atricapillus* and *carolinensis*, a minimal level of atypical singing and morphological intermediacy has been found, leading to the conclusion that hybridization between them is rare or absent at these locations (Tanner 1952; Brewer 1963; Merritt 1978, 1981; but see Grubb et al. 1994). However, because of the central role of learning in song ontogeny, song can be an unreliable marker of hybridization and introgression in some songbird hybrid zones (Ficken and Ficken 1967, Emlen et al. 1975), given that social interactions among individuals shape song development (Payne 1981, Payne and Payne 1997). Likewise, a number of factors can make levels of morphological intermediacy an imperfect and even misleading measure of genetic interactions between taxa, especially when two forms are similar (Sattler



and Braun 2000 and references therein). The ecological setting of a hybrid zone can also vary geographically and influence the interactions between taxa at different locations (Cook 1975, Grubb et al. 1994). This may lead to geographically varying levels of vocal intermediacy that are unrelated to levels of hybridization.

Molecular genetic analyses offer a means of more accurately assessing genetic interactions between hybridizing taxa. Species-specific marker loci are now available that are diagnostic for *atricapillus* and *carolinensis* (Mack et al. 1986; Gill et al. 1989, 1993; Sawaya 1990; Sattler 1996). Use of these markers to probe the genetic structure of their contact zone has revealed a high proportion of hybrids in the center and extensive introgression across the contact zone at each location studied, including Missouri, Virginia, West Virginia, and Ohio (Sawaya 1990, Sattler 1996, Sattler and Braun 2000, Bronson et al. 2005). Comparison of morphometric and genetic variation in Virginia and West Virginia showed that, on a broad scale, there is concordance between morphology and genes. However, the genetic markers detected more hybridization and introgression than was indicated by morphological analysis alone (Sattler and Braun 2000). Accurate assessment of genetic ancestry helped demonstrate that endogenous selection because of genetic incompatibility is largely responsible for maintaining the hybrid zone (Bronson et al. 2003a, 2005) and that social dominance of males is more important than genetic ancestry in female mate preference (Bronson et al. 2003b).

Here, we examine vocal and genetic variation across Virginia, West Virginia, and Missouri transects of the chickadee hybrid zone to assess the reliability of song as a marker of hybridization. Such an assessment is especially important for a contact zone in which even multivariate analyses of morphology are inadequate to assess hybridization, because vocal intermediacy is then relied on to make such estimates. Our analysis reveals that song type is not a good indicator of genetic ancestry of individual chickadees in and near the hybrid zone. Although hybridization is extensive on each transect, the degree of vocal admixture varies. We suggest that this variation may be related to differences in the abruptness of ecological transitions at the range interface on the three transects.

## METHODS

*Population samples.*—We studied singing behavior of chickadees during April–July, 1989–1992, at 12 sites that comprise two transects (Fig. 1 and Table 1). The transects crossed the contact zone in the Appalachian Mountains, one on the eastern slope (Virginia transect) and one on the western slope (West Virginia transect). Morphological and genetic variation of chickadee populations at these sites was described by Sattler and Braun (2000), and detailed localities can be found therein. Nine sites represent closely spaced samples of the hybrid zone on the two transects (VA1–VA5 and WV1–WV5). Allopatric population samples of *carolinensis* (VA and OH) served as terminal parental populations of *carolinensis* for the Virginia and West Virginia transects, respectively (Fig. 1). Initially, a single site (VA1/WV1) from the central Appalachians served as a common terminal population of *atricapillus* for both transects. However, because birds at this site showed evidence of genetic introgression from *carolinensis* (Sattler and Braun 2000), we added a more distant allopatric sample (PA) in northern Pennsylvania to represent pure parental *atricapillus*. We then treated PA as a terminal population sample of both transects.

At each site, we located chickadees visually, by their spontaneous calls or song, or by response to a playback tape. Birds were usually encountered in pairs. After locating birds, we evaluated the vocal response of males to playback of both *atricapillus* and *carolinensis* song. In populations where *atricapillus* song predominated, 2 min of *carolinensis* song was broadcast, followed by 2 min of silence, then 2 min of *atricapillus* song. In populations where *carolinensis* song predominated, the order of song presentation was reversed. In WV3, where both species' songs were common, we alternated the order of song presentation.

During and following responsiveness trials, we recorded samples of whistled song using a Sony TCM-5000EV cassette recorder with a Sennheiser ME-80 shotgun microphone. If a bird sang more than one type of song, an effort was made to record each song type sung. Collecting birds for genetic and morphometric analyses was crucial, however, so recording sessions were not lengthy (usually 5–20 min), and song types recorded from a given bird do not necessarily represent its full repertoire.

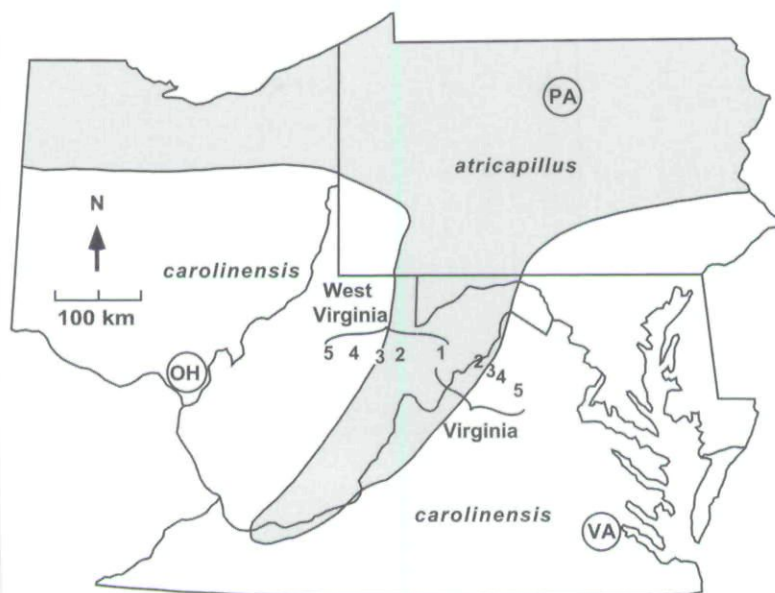


FIG. 1. Range of *Poecile atricapillus* and *P. carolinensis* in the Appalachian region and locations of study sites that comprise the Virginia and West Virginia transects, including parental samples OH, PA, and VA. Exact localities are given in Sattler and Braun (2000). Range boundaries are approximate (Peterjohn 1989, Virginia Department of Game and Inland Fisheries–Virginia Society of Ornithology 1989, Brauning 1992, Buckelew and Hall 1994).

An additional transect crossing the contact zone in Missouri (Table 1) has previously been analyzed vocally (Robbins et al. 1986) and genetically (Sawaya 1990). We re-analyzed vocal data from that transect for comparison with our Appalachian samples. The Missouri transect comprised six samples: an *atricapillus* reference sample from northwestern Missouri (MO), a *carolinensis* reference sample from Louisiana (LA), and four samples from near the hybrid zone in southwestern Missouri (MO1–MO4; see also Robbins et al. 1986, their figure 1). Robbins et al. (1986) restricted vocal analyses to MO1–MO4, combining MO1 and MO2 as a reference *atricapillus* sample, and we treated them similarly.

**Song types.**—We classified songs into song types based on discontinuous variation in number, structure, and pattern of notes (Kroodsma 1982, Nowicki et al. 1994). Within a song type, there was more limited variation, mainly in frequency and duration characteristics of homologous notes. We found this classification preferable to that of Robbins et al. (1986), who designated song types solely by the number of notes in a song, because that method sometimes

lumps songs that differ appreciably in frequency or patterning into a single song type. When an individual sang more than one song type, songs of each type were treated separately as song bouts. Song was analyzed from 133 individuals from the Appalachian transects, all of which were sexed as males by examination of gonads, with the exception of one male sexed genetically by the method of Griffiths et al. (1998) and three singing individuals that were not collected. Nineteen individuals sang more than one song type, and so were represented more than once in song analyses. We analyzed 157 bouts of song from these Appalachian birds (Table 1; see also table 6 in Sattler 1996). A total of 88 song bouts from 56 individuals from Missouri populations MO1–MO4 was re-analyzed here (Table 1; see also table 8 in Sattler 1996). Thirteen of these individuals sang more than one song type, and so were represented more than once in song analyses.

The song of *atricapillus* consists of two whistled notes (Fig. 2), the first slightly higher in pitch than the second (Dixon and Stefanski 1970), and is highly stereotypic throughout most of the bird's range (Hailman 1989, Kroodsma et



TABLE 1. Sample sizes for genetic and vocal analyses along with proportion of hybrids estimated genetically for three transects of the chickadee hybrid zone.

Virginia transect						West Virginia transect					
Popu- lation	Distance <sup>a</sup> (km)	Genetic ( <i>n</i> )	Hybrids (%)	Number of males <sup>b</sup>		Popu- lation	Distance <sup>a</sup> (km)	Genetic ( <i>n</i> )	Hybrids (%)	Number of males <sup>b</sup>	
				Indiv <sup>c</sup>	Bouts					Indiv <sup>c</sup>	Bouts
PA <sup>d</sup>	0.0	20	0.0	14 (0)	14	PA <sup>d</sup>	0.0	20	0.0	14 (0)	14
VA1/WV1 <sup>e</sup>	100.0	20	15.0	6 (0)	6	VA1/WV1 <sup>e</sup>	100.0	20	15.0	6 (0)	6
VA2	153.6	33	45.0	15 (3)	18	WV2	155.4	20	15.0	7 (0)	7
VA3	164.5	24	62.5	9 (2)	12	WV3	172.7	31	58.1	19 (8)	30
VA4	171.4	21	28.6	6 (0)	6	WV4	188.7	19	57.9	10 (5)	16
VA5	205.5	20	10.0	8 (0)	8	WV5	212.5	19	47.4	7 (1)	8
VA	387.0	21	0.0	16 (0)	16	OH	344.9	20	40.0	16 (0)	16

Missouri transect					
Popu- lation	Distance <sup>a</sup> (km)	Genetic ( <i>n</i> )	Hybrids (%)	Number of males <sup>b</sup>	
				Indiv <sup>c</sup>	Bouts
MO	0.0	20	0.0	–	–
MO1	218.0	17	0.0	14 <sup>f</sup> (0)	14 <sup>f</sup>
MO2	251.0	14	28.6		
MO3	259.5	36	44.4	30 (9)	56
MO4	296.5	21	4.8	12 (4)	18
MO5	1,209.5	21	0.0	–	–

<sup>a</sup>Distances are measured in relation to the *atricapillus* terminus of each transect and perpendicular to the range interface as determined from Peterjohn (1989), Virginia Department of Game and Inland Fisheries-Virginia Society of Ornithology (1989), Brauning (1992), and Buckelew and Hall (1994). The linear distance between PA and WV1-VA1 is corrected, because PA is displaced from the east-west oriented Virginia and West Virginia transects. This distance was estimated by measuring the distance from PA to the closest point of the range interface (in southeastern Pennsylvania) and subtracting from it the distance between VA1-WV1 and the closest point of the range interface (in Virginia). Distances are from population centroids estimated by eye. Population sample diameters ranged from a few kilometers to a few tens of kilometers, owing to the density of birds.

<sup>b</sup>Number of singing males recorded. Some males sang more than one song type, each of which was analyzed as a separate song bout.

<sup>c</sup>Number of singing males recorded that sang multiple song types are in parentheses.

<sup>d</sup>Serves as the *atricapillus* parental population sample for both the Virginia and West Virginia transects.

<sup>e</sup>VA1 and WV1 are the same sample from the central Appalachian Mountains, which serves as the second population at the *atricapillus* end of both the Virginia and West Virginia transects.

<sup>f</sup>Song data from MO1 and MO2 were combined as one population in song analysis.

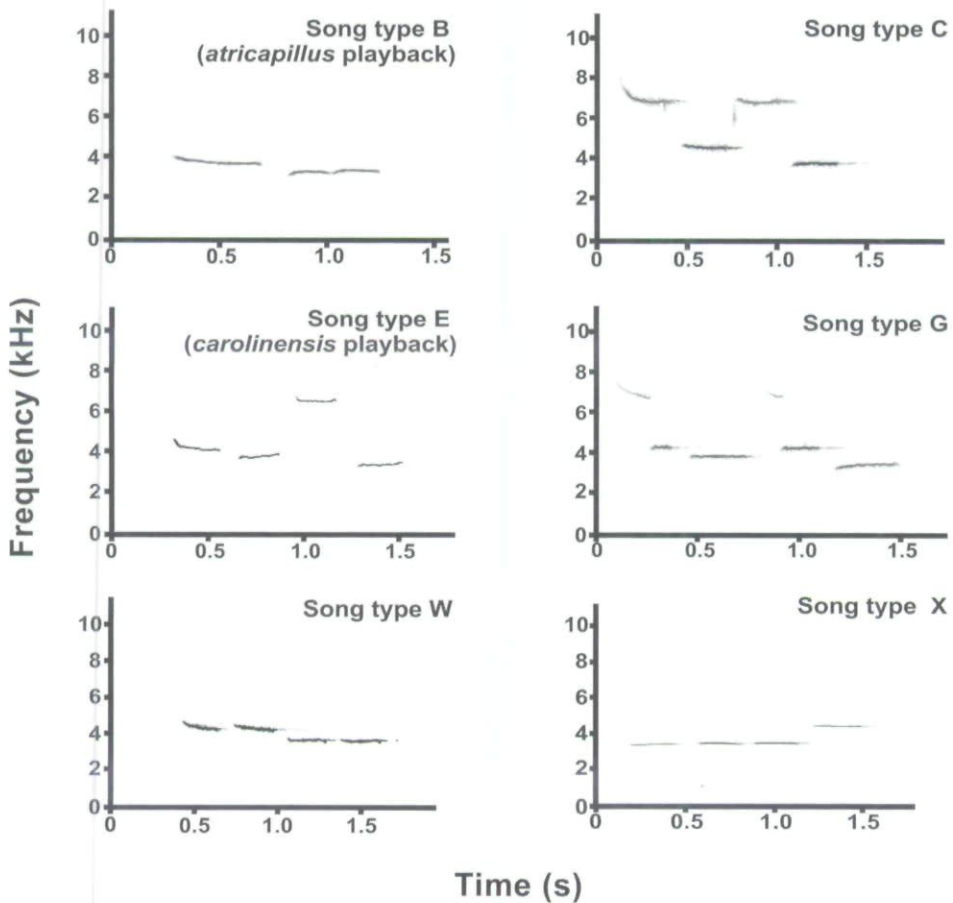


FIG. 2. Representative spectrograms of song types of *atricapillus* (B) and *carolinensis* (E) that were used in playback, two additional *carolinensis* song types (C, G), and two atypical song types (W, X). Populations and locations (or source) of songs depicted are as follows: B = New York (Peterson 1983), C = VA (Charles City County), E = Adams County, Ohio (Peterson 1983), G = WV4 (Upshur County), W = VA5 (Rappahannock County), X = MO4 (St. Clair County). Representations of other songs not depicted are found as follows: D (Ward 1966, fig. 4 DN1-1), F (Ward 1966, fig. 5 VK1-1), H (Ward 1966, fig. 4 VN4-1), I (Ward 1966, fig. 5 FV5-3), Y (Sattler 1996, fig. 7I; Ballard 1988, fig. 13 A3), Z (Ward and Ward 1974, fig. 2 B).

al. 1999). Well-known amplitude modulation (Kroodsma et al. 1995, 1999) and frequency shifts (Ratcliffe and Weisman 1985, Horn et al. 1992) are commonly heard in most populations, but these are relatively minor variations on the basic song, so we recognized only one *atricapillus* song type, B (Fig. 2).

*Poecile carolinensis* displays extensive individual and geographic variation in its song (Ward 1966). We recognized a total of seven *carolinensis* song types (C–I) in our samples (Fig. 2). All but song type G have been reported in other *carolinensis* populations distant from the range

interface with *atricapillus* (Ward 1966, Lohr 1995). Each possesses at least one descending interval between a high note in the frequency range of 5.9–7.4 kHz and a low note in the frequency range of 3.0–4.8 kHz. Lohr (1995) showed that such a descending two-note interval in the frequency range appropriate for *carolinensis* is the minimal song characteristic necessary to elicit a full species-typical response in this species.

Occasional song variants deviating from one of the common *carolinensis* song types occurred interspersed in bouts of typical song. These variants appeared to be formed by the addition



or deletion of one or more notes from the end of otherwise typical songs (Ward 1966, G. D. Sattler pers. obs.). We ignored these variant songs unless they formed the predominant song of an individual. On the other hand, Robbins et al. (1986) included these variants as separate song types (eight individuals), so in our re-analysis of their data we did likewise.

Four additional song types were encountered that were not easily attributable to either species. Two of these, Y and Z, were found only in populations at the range interface and have been reported previously from other portions of the range interface (Ward and Ward 1974, Tove 1980, Ballard 1988, Sattler 1996). Two other song types, W and X (Fig. 2), have not been reported previously for either species. Both were encountered in *carolinensis*-like populations near the range interface. We refer to these four song types as atypical song types.

*Quantitative analyses of whistled song.*—Spectral analysis of songs was performed using CANARY, version 1.1 (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, New York). A 176-Hz filter bandwidth setting was used in most cases to measure song parameters, unless greater resolution was needed in measuring note duration, in which case a 1,400-Hz bandwidth setting was used. We measured eight variables in each song: the duration and onset, midpoint, and offset frequencies of both high and low notes. Mindful of the characteristics of *carolinensis* song demonstrated by Lohr (1995), we chose for analysis the highest note of a song and the note following it, unless the highest note of the song was the last note (e.g., song type X; Fig. 2). In that case, we analyzed the highest note and the one preceding it. This procedure was followed throughout, including our re-analysis of the Missouri transect data of Robbins et al. (1986). Those authors measured the same eight variables on the first two notes of every song, regardless of pitch. We avoided that procedure because it tends to produce an artifactual appearance of intermediacy in the small percentage of *carolinensis* songs that do not begin with a high note followed by a low note (e.g., song type E; Fig. 2).

Variation in song characteristics across the Appalachian contact zone was examined by performing a principal component analysis (PCA) in SAS, version 8.2 (PROC PRINCOMP; SAS Institute, Cary, North Carolina), on the

matrix of correlations among averages for the eight song variables. All 12 populations of the two Appalachian transects were included in the analysis. Note duration was normally distributed in most cases, whereas note-frequency variables were non-normally distributed in a number of cases because of the presence of multiple song types in a population. Transformations failed to normalize the frequency variables in these populations, so untransformed values were retained in the PCA. We also performed a PCA on the remeasured data of Robbins et al. (1986) from Missouri, using the procedures described above.

*Genetic analysis.*—After recording sessions, birds were collected with a shotgun and frozen on dry ice for transport to the lab for specimen preparation and tissue extraction. Four diagnostic genetic markers were used to detect hybridization and introgression (Fig. 3). One was the isozyme guanine deaminase (GDA) (Gill et al. 1989, Sawaya 1990), for which electrophoresis was carried out on liver tissue according to Sattler and Braun (2000). The other three markers were restriction-fragment-length polymorphism (RFLP) differences analyzed on Southern blots. DNA extraction and Southern analysis followed Sattler and Braun (2000). The three probes used to detect RFLPs were (1) a cloned fragment of the chicken oncogene *ski* (Li et al. 1986), (2) a randomly cloned fragment of Tufted Titmouse (*Baeolophus bicolor*) DNA designated C7 (Sawaya 1990), and (3) CsCl gradient-purified mitochondrial DNA (mtDNA) of *P. carolinensis*.

The four genetic markers have previously been shown to be diagnostic for *atricapillus* and *carolinensis* (Mack et al. 1986, Gill et al. 1989, Sawaya 1990, Sattler and Braun 2000). For example, population samples MO (20 *atricapillus* from northern Missouri) and LA (21 *carolinensis* from southern Louisiana) are fixed for alternative alleles at each of the four marker loci (Sawaya 1990), as are samples PA (20 *atricapillus* from northern Pennsylvania) and VA (21 *carolinensis* from southeastern Virginia; Sattler and Braun 2000). Scoring of alternative alleles is straightforward and unambiguous; banding patterns for each locus are shown in Figure 3.

Because the parental populations do not share alleles, hybrid ancestry can be inferred if an individual is found to have any admixture of *atricapillus* and *carolinensis* alleles among these four diagnostic markers. In other words, pure *atricapillus* must have only *atricapillus* alleles,



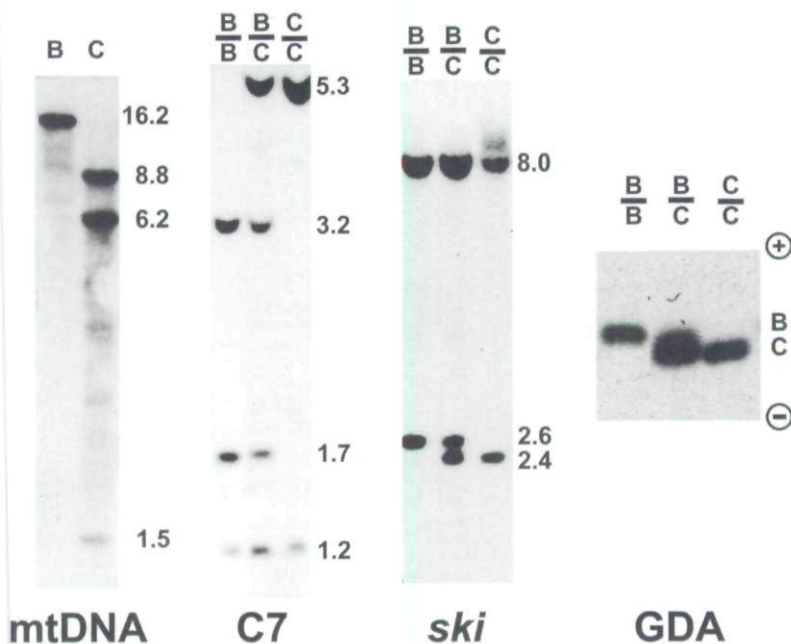


FIG. 3. Banding patterns of alternative genotypes for the four genetic marker loci used. GDA is an isozyme; mtDNA, C7, and *ski* are RFLPs (see text). B = *atricapillus*, C = *carolinensis*, B/C = heterozygote. Numbers beside DNA bands are molecular size estimates in kilobases. Cleavage patterns shown are produced by the restriction enzyme *Pst* 1 for mtDNA and C7, and by *Eco* R1 for *ski*. In both C7 and GDA panels, one lane has been removed to show the three genotypes juxtaposed.

and pure *carolinensis* must have only *carolinensis* alleles. Estimates of hybrid frequency are conservative because some later generation and backcross hybrids may have parental genotypes by chance from reassortment among the four markers. The frequency of female hybrids is especially likely to be underestimated, because two of the marker loci, GDA and C7, are sex-linked (Sattler and Braun 2000), and females are the heterogametic sex. These two markers may also be physically linked on the Z chromosome, resulting in nonindependence and further increasing the chance of misclassifying hybrids as parentals. Sattler and Braun (2000) gave allele frequencies in each of the 12 Appalachian populations for all four loci. Here, we classify birds as hybrids or potential parentals on the basis of their genotypes (Table 1).

#### RESULTS

*Levels of song admixture in Appalachian transects.*—We encountered chickadees that sang *atricapillus* and *carolinensis* songs syntopically

in population samples nearest the range interface on both the Virginia (VA2–VA4) and West Virginia (WV3–WV4) transects (Fig. 4). These same populations had the highest proportion of hybrids determined genetically (Table 1 and Fig. 4). Atypical song types Y and Z were found at the Appalachian range interface (VA2 and WV3) but were uncommon in our samples (Table 2). Species-typical song types predominated at the center of each transect, and these songs did not sound abnormal or intermediate to our ears. Frequency and duration characteristics of species-typical songs were maintained, even in sympatry (Table 3). Variability in note duration and frequency within populations was greatest for *carolinensis* (Table 3), where multiple song types were always present.

In a PCA of song bouts based on duration and frequency variables, there was little evidence of intermediacy in either Appalachian transect. The first principal component (PC1) explained 68.2% of total variation (Table 4) and was correlated positively with note frequency and negatively with note duration. It exhibited

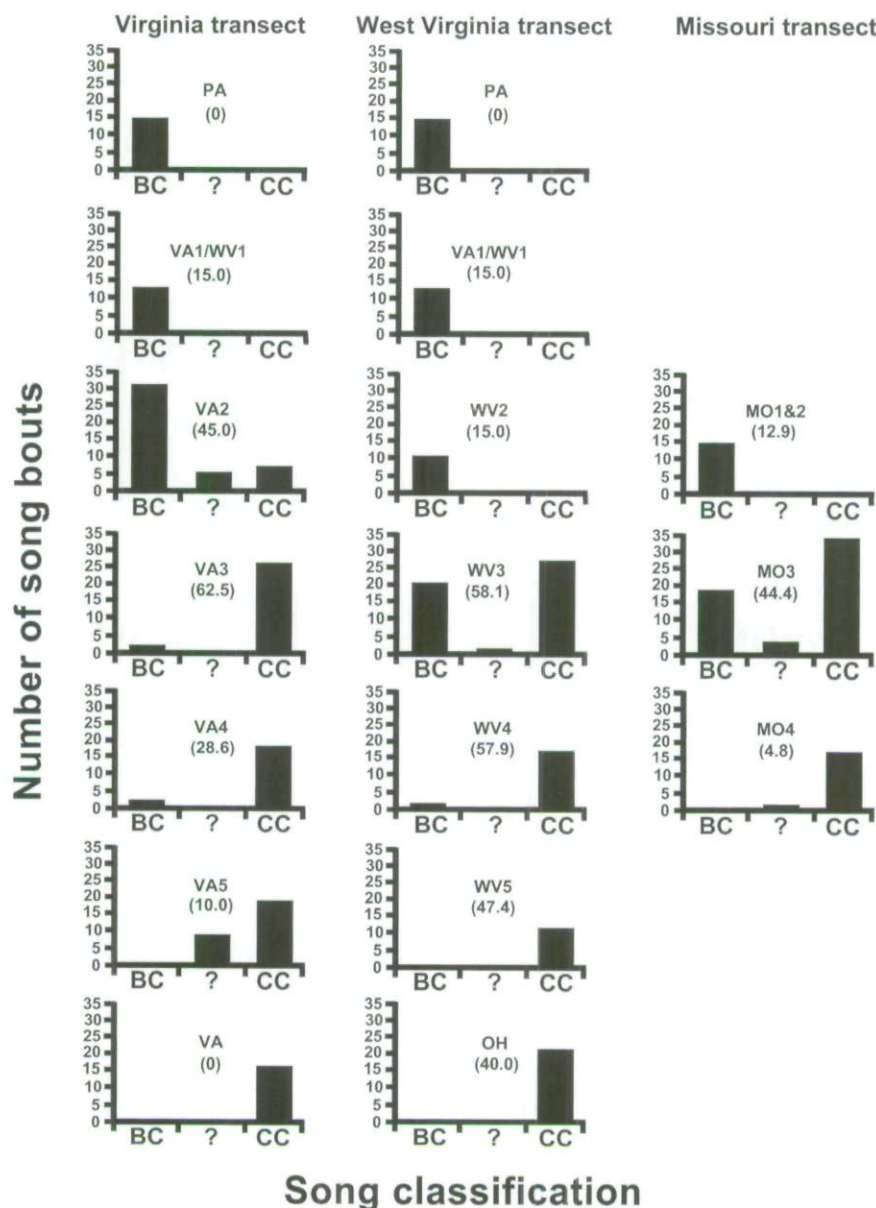


FIG. 4. Number of *atricapillus* (BC), *carolinensis* (CC), and atypical (?) song bouts heard or recorded in each population of the three transects. Numbers in parentheses indicate the percentage of hybrids estimated genetically for each population. Ninety-three bouts heard in the field but not taped are included.

a bimodal distribution in range interface populations of each Appalachian transect (Fig. 5), separating *atricapillus* from *carolinensis* song bouts. In scatterplots of PC1 versus PC2, only a few song bouts from the Appalachian hybrid populations fell between the ranges of the

parental samples from the same transect (Fig. 6). The distinctions maintained between chickadee songs at both sites on the Appalachian range interface contrast with the extensive hybridization and intermediacy present in the same populations, demonstrated by genetic



TABLE 2. Number of singing birds in central populations of the three transects according to several broad repertoire categories. Only a subset of these songs was recorded and included in analyses for intermediacy, and no individual is represented more than once.

Song type	Population(s)			
	VA2 and VA3	WV3	VA + WV total	MO3
<i>atricapillus</i> only (B)	26	9	35	10
<i>carolinensis</i> only (C-I)	24	7	31	12
<i>atricapillus</i> and atypical	2	0	2	2
<i>carolinensis</i> and atypical	0	1	1	0
Bilingual	3	9	12	5
Bilingual and atypical	2	0	2	1
Bilingual-atypical subtotal	7 (12%)	10 (38%)	17 (20%)	8 (27%)
Total	57	26	83	30

(Table 1 and Fig. 5) and morphological analyses (Sattler and Braun 2000).

Some vocal admixture between Appalachian *atricapillus* and *carolinensis* was evident in the presence of birds with bilingual or atypical song repertoires near the range interface of each transect. Such birds were especially common in the West Virginia transect, where they made up 38% of the birds heard singing in WV3, compared with just 12% of the birds in VA2 and VA3 combined (Table 2). The different frequency of bilingual singing in the two Appalachian transects was mirrored in the geographic extent of song sympatry as well. Both *atricapillus* and *carolinensis* songs were common in an area  $\geq 9$  km wide in WV3. By contrast, the area in which both *atricapillus* and *carolinensis* songs were common at the range interface between VA2 and VA3 spanned only 4 km.

*Levels of song admixture in the Missouri transect.*—The lack of song intermediacy in the Appalachian transects contrasts with the extensive intermediacy reported by Robbins et al. (1986) at the range interface in Missouri. In their contact-zone population (MO3), discriminant analysis classified 37% of song bouts as intermediate in duration and frequency characteristics. They also encountered atypical song types Y and Z and a moderate level of bilingual singing (27% of individuals; Table 2) spanning an area  $\sim 9$  km wide. Yet the Missouri transect had lower levels of hybridization and introgression determined genetically (Table 1).

Re-analysis of the Missouri song data of Robbins et al. (1986) by PCA according to our song-type definitions confirmed the apparent difference between Missouri and Appalachian

transects. Eigenvalues and factor loadings from PCA were comparable to the analysis of Appalachian populations (Table 4). In contrast to the bimodal distribution of PC1 scores in the Appalachian transect interface populations, MO3 exhibited a unimodal distribution in PC1 scores (Fig. 5). This contrast can be quantified by estimating the proportion of intermediate songs in each transect in scatterplots of the first two PCA scores (Fig. 6). If songs falling between the limits of reference parental populations of each transect are considered intermediate, 39.3% of songs in the central population of the Missouri transect (MO3) exhibit intermediacy. This contrasts with only 13.3% and 16.7% intermediate songs in the central populations of the Virginia (VA2 and 3) and West Virginia (WV3) transects, respectively. Song type E had a greater tendency toward intermediacy in the PCAs than other song types, but deleting it made little difference in the proportion of intermediate songs in any transect (data not shown).

*Correlation of song and genetics.*—To assess the relationship of song to genetic ancestry, we looked at the association between song, as measured by PC1 scores, and genetic composition, as measured by the number of *atricapillus* alleles per individual. Combining data from all three transects at the population level, there was a very strong correlation between a population's average PC1 song score and its average genetic composition (Spearman's  $r_s = -0.91$ , one-tailed  $P < 0.01$ ,  $n = 15$ ). This correlation is not surprising, however, because both variables are related to geographic position of populations along the transects. We can factor out the effect of geography by looking at the association

TABLE 3. Averages for univariate measurements of species-typical song for population samples comprising the West Virginia and Virginia transects. Values are mean  $\pm$  SD.

Note 1						Note 2				
Sample	Birds (n)	Bouts (n)	Duration (ms)	Onset frequency (kHz)	Midpoint frequency (kHz)	Offset frequency (kHz)	Duration (ms)	Onset frequency (kHz)	Midpoint frequency (kHz)	Offset frequency (kHz)
West Virginia transect										
PA	14	14	4.46 ± 0.44	4.04 ± 0.29	3.84 ± 0.25	3.78 ± 0.23	4.34 ± 0.19	3.20 ± 0.21	3.30 ± 0.19	3.31 ± 0.18
WV1	6	6	3.91 ± 0.33	4.34 ± 0.15	4.08 ± 0.18	3.98 ± 0.14	4.20 ± 0.16	3.46 ± 0.11	3.54 ± 0.10	3.54 ± 0.11
WV2	7	7	3.88 ± 0.33	3.93 ± 0.25	3.77 ± 0.22	3.74 ± 0.19	4.06 ± 0.33	3.41 ± 0.29	3.31 ± 0.17	3.31 ± 0.17
WV3 <sup>a</sup>	16 <sup>b</sup>	16 <sup>c</sup>	3.92 ± 0.38	4.11 ± 0.23	3.92 ± 0.23	3.91 ± 0.22	3.96 ± 0.40	3.34 ± 0.22	3.44 ± 0.19	3.44 ± 0.19
WV3 <sup>d</sup>	10 <sup>b</sup>	13	2.18 ± 0.79	6.98 ± 0.65	6.66 ± 0.24	6.59 ± 0.22	2.98 ± 0.30	3.43 ± 0.27	3.50 ± 0.21	3.50 ± 0.21
WV4	10	15 <sup>e</sup>	2.32 ± 0.48	7.70 ± 1.00	6.92 ± 0.31	6.69 ± 0.22	2.74 ± 0.35	3.71 ± 0.26	3.78 ± 0.23	3.78 ± 0.23
WV5	7	8	2.69 ± 0.43	7.34 ± 0.87	6.51 ± 0.37	6.49 ± 0.36	2.92 ± 0.30	3.69 ± 0.41	3.76 ± 0.37	3.76 ± 0.36
OH	16	16	3.26 ± 0.43	6.98 ± 0.66	6.32 ± 0.33	6.29 ± 0.33	3.19 ± 0.20	3.59 ± 0.64	3.65 ± 0.51	3.65 ± 0.51
Virginia transect										
PA	14	14	4.46 ± 0.44	4.04 ± 0.29	3.84 ± 0.25	3.78 ± 0.23	4.34 ± 0.19	3.20 ± 0.21	3.30 ± 0.19	3.31 ± 0.18
VA1	6	6	3.91 ± 0.33	4.34 ± 0.15	4.08 ± 0.18	3.98 ± 0.14	4.20 ± 0.16	3.46 ± 0.11	3.54 ± 0.10	3.54 ± 0.11
VA2	15	16 <sup>f</sup>	3.81 ± 0.43	4.04 ± 0.23	3.84 ± 0.23	3.79 ± 0.23	4.05 ± 0.45	3.21 ± 0.18	3.30 ± 0.19	3.32 ± 0.18
VA3	9	12	2.99 ± 0.51	6.55 ± 0.65	6.25 ± 0.56	6.22 ± 0.56	2.98 ± 0.25	3.60 ± 0.36	3.68 ± 0.27	3.67 ± 0.27
VA4	5 <sup>g</sup>	5	3.07 ± 0.40	7.59 ± 0.87	6.58 ± 0.28	6.54 ± 0.27	2.94 ± 0.15	3.83 ± 0.37	3.87 ± 0.32	3.87 ± 0.32
VA5	8	8	2.75 ± 0.33	6.25 ± 1.06	5.93 ± 0.92	5.72 ± 0.87	3.37 ± 0.73	3.98 ± 0.13	3.91 ± 0.14	3.91 ± 0.13
VA	16	16	3.29 ± 0.32	7.23 ± 0.56	6.52 ± 0.30	6.35 ± 0.59	3.70 ± 0.58	4.26 ± 0.54	4.19 ± 0.50	4.17 ± 0.50

<sup>a</sup> *Atricapillus* song type (B).  
<sup>b</sup> Of the 19 birds analyzed vocally from WV3, 8 were bilingual and are included in both WV3 averages.  
<sup>c</sup> A bout of song type X from one individual was omitted.  
<sup>d</sup> *Carolinensis* song types (C and D).  
<sup>e</sup> A bout of *atricapillus* song from one bilingual individual was omitted.  
<sup>f</sup> *Carolinensis* song from two bilingual individuals was omitted.  
<sup>g</sup> One individual singing only *atricapillus* song was omitted.



TABLE 4. Correlations between song variables and principal component scores.

Character	Virginia and West Virginia		Missouri	
	PC1	PC2	PC1	PC2
<b>Note 1</b>				
Duration	-0.65	0.61	-0.65	0.70
Onset frequency	0.95	-0.13	0.94	-0.11
Midpoint frequency	0.94	-0.25	0.94	-0.20
Offset frequency	0.92	-0.28	0.93	-0.20
<b>Note 2</b>				
Duration	-0.70	0.54	-0.50	0.70
Onset frequency	0.77	0.60	0.80	0.55
Midpoint frequency	0.81	0.57	0.84	0.51
Offset frequency	0.81	0.57	0.84	0.50
Eigenvalue	5.45	1.84	5.35	1.88
Variation explained	0.682	0.230	0.669	0.235

between PC1 scores and genetic makeup of individuals from the only two range interface populations (WV3 and MO3) that had an appreciable mix of the songs and genes of both forms. There was a weakly significant correlation in WV3, and none in MO3 (Fig. 7). Many hybrid individuals in these populations (and others) sang accurate renditions of one or both species' song types, and a number of individuals genetically pure for the four marker loci sang accurate renditions of the other species' song (Fig. 7). Thus, the duration and frequency characteristics of song bouts were poor predictors of the genetic ancestry of individual singers.

As an additional test, we examined the association between bilingual singing and hybridity in contact-zone populations of all three transects (VA2, VA3, WV3, and MO3). To minimize potential bias, only individuals collected within 5 km of the estimated range interface were included in the analysis. A higher proportion of bilingual birds were hybrids (10 of 12, 83.3%) than nonbilingual birds (19 of 35, 54.3%), but the association was not significant (Fisher's exact test, one-tailed,  $P = 0.072$ ), again indicating that song type was a poor predictor of genetic ancestry.

DISCUSSION

*Vocal admixture as an indicator of hybridization.*—Song is an unreliable criterion for assessing levels of hybridization and introgression in these chickadees. Although >50% of contact-zone

chickadees on both Appalachian transects were determined genetically to be hybrids, song intermediacy at these sites was minimal. Other forms of vocal admixture, such as bilingual or atypical singing, were also uncommon at the Virginia range interface. Although bilingual and atypical singing was more common and occurred over a broader area in the West Virginia transect, the geographic extent of any form of vocal admixture on either transect was very narrow in relation to genetic mixing. Moreover, there was little or no correlation between an individual's PC1 score for song and its genetic ancestry. Numerous individuals with *carolinensis*-like genotypes sang "normal" *atricapillus* songs, and vice versa.

Song has been found to be an equally unreliable marker of hybridization in other songbird hybrid zones (Ficken and Ficken 1967, Emlen et al. 1975, Sorjonen 1986). Also, song does not reflect paternal family lineages in many birds (Payne 1996, Payne and Payne 1997). In each of these cases, the discordance between genes and song has been attributed to a strong component of learning in song ontogeny. Learning is important in the development of vocalizations in all oscine songbirds studied, including several members of the genera *Parus* and *Poecile* (Kroodsma and Baylis 1982, Ficken and Popp 1995, Hughes et al. 1998). Most pertinently, *atricapillus* nestlings tutored with a tape of *carolinensis* song learned most elements of the heterospecific song, whereas *carolinensis* nestlings developed songs nearly identical to an *atricapillus* tutor tape (Kroodsma et al. 1995). Therefore, caution

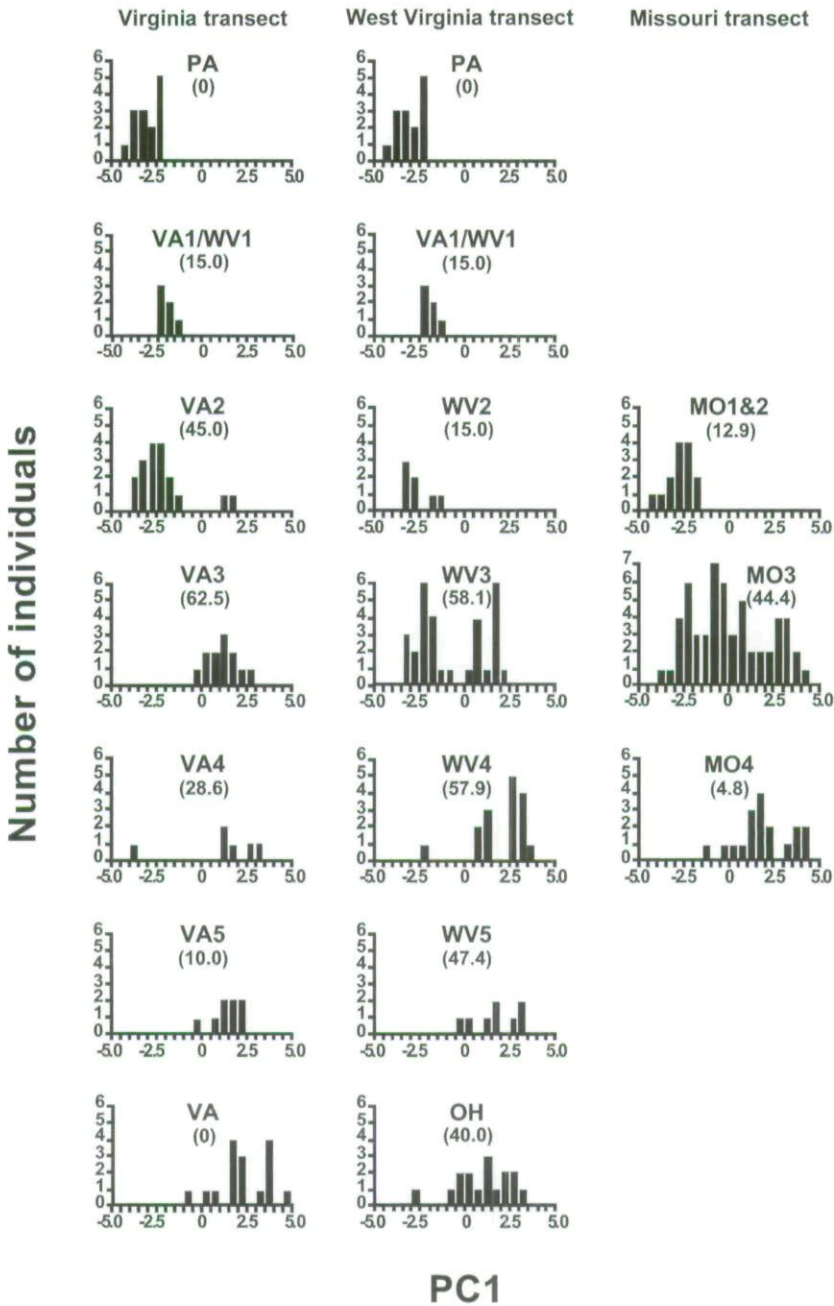


FIG. 5. Principal component 1 (PC1) scores of songs exhibited a bimodal distribution in the center of the Virginia transect (VA2 and VA3) and West Virginia transect (WV3) but a unimodal distribution in the center of the Missouri transect (MO3). Numbers in parentheses indicate the percentage of hybrids estimated genetically for each population. The outlier in OH at -2.5 was a *carolinensis* E song type that appeared *atricapillus*-like on the basis of PC1 but was well separated from all *atricapillus* by its low PC2 score (Fig. 6).



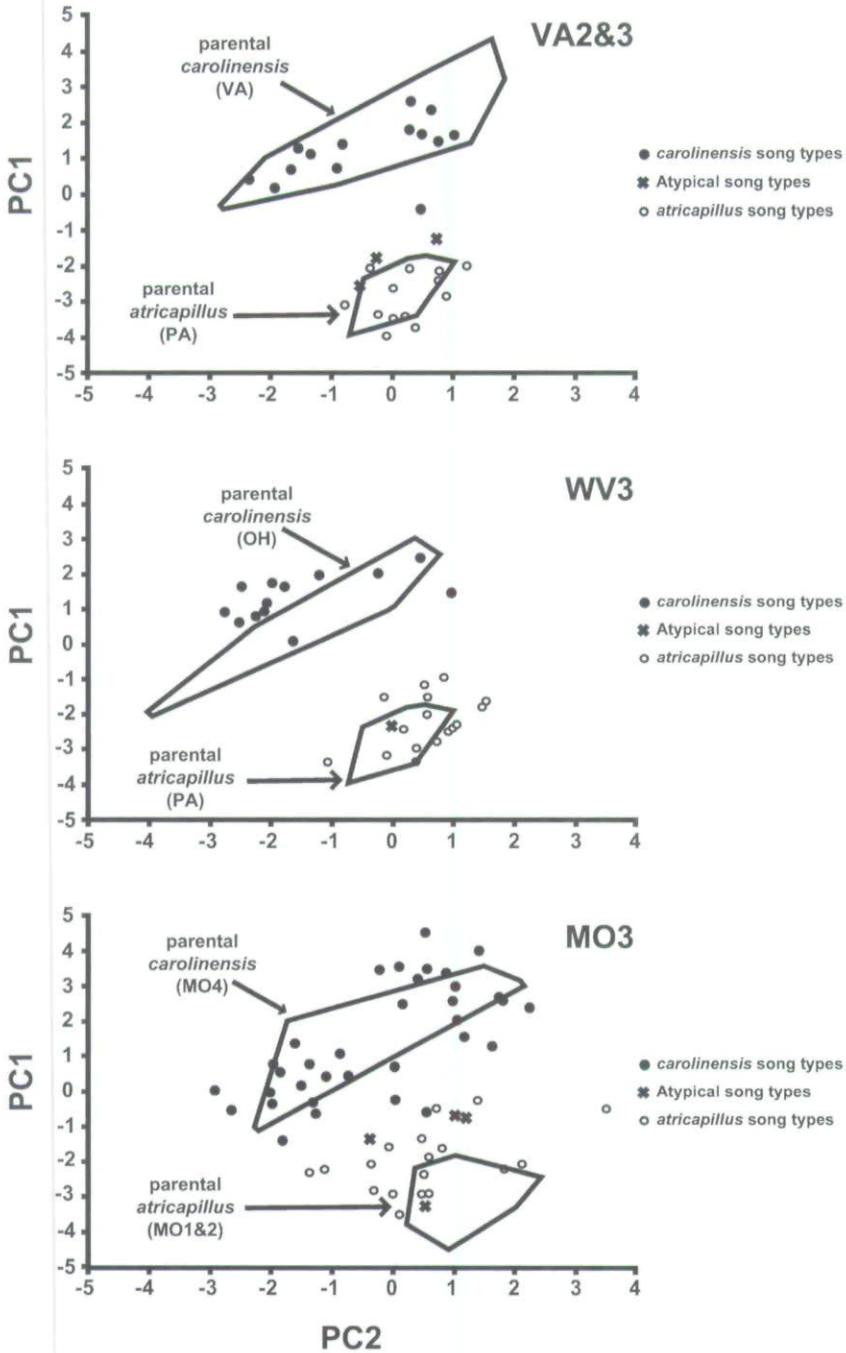


FIG. 6. There was a distinct separation between *atricapillus* and *carolinensis* song types in scatterplots of the first two PCA scores of songs for central populations in the Virginia transect (VA2 and VA3) and West Virginia transect (WV3), but not in the Missouri transect (MO3). Thirty-two Appalachian individuals that sang more than one song type are represented multiple times, as are seven individuals in MO3 that sang versions of a single song type differing in number of notes. Polygons enclose the positions of reference samples from each transect (individual data points not shown).

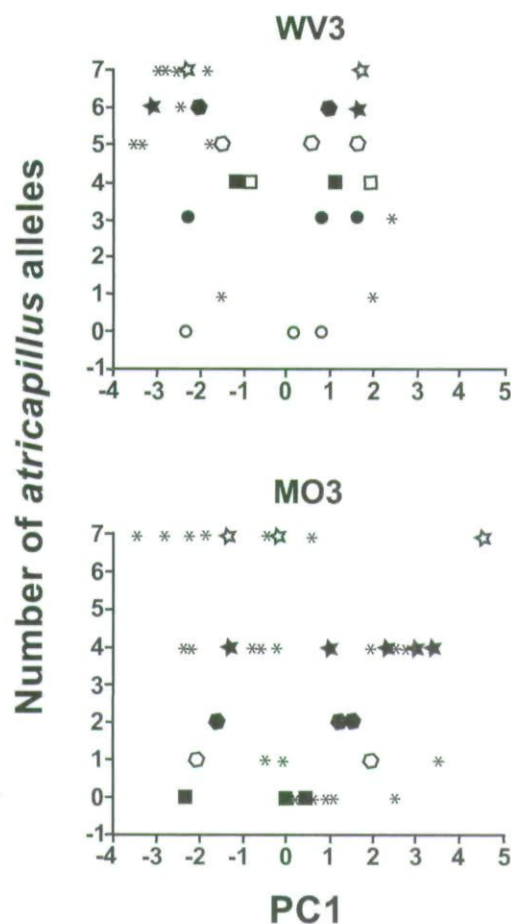


FIG. 7. Scatterplots of song PC1 scores versus number of *atricapillus* alleles of the singing individual show that, in the hybrid zone, song characteristics were poor predictors of the singer's genetic ancestry. Data are from the two range-interface populations where there was an equal representation of *atricapillus* and *carolinensis* song. When rank-transformed data were tested by Spearman's rank correlation, there was a significant correlation in WV3 ( $r_s = -0.40$ , one-tailed  $P = 0.014$ ,  $n = 30$ ) but not in MO3 (Spearman's  $r_s = -0.21$ , one-tailed  $P = 0.098$ ,  $n = 38$ ). Each asterisk represents a bird that sang a single song type. Open and closed squares, circles, stars, and hexagons identify song bouts of different song types sung by the same individual.

must be exercised in drawing conclusions about hybridization between *atricapillus* and *carolinensis* on the basis of vocalizations alone.

More broadly, finding a weak relationship between levels of vocal admixture and levels of genetic intermediacy appears to be related more directly to the function of vocal communication systems and ecological interactions between the two forms than to genetic ancestry. Social adaptation of singing behavior to the local population norms in which a bird attempts to find a mate and hold a territory may decouple the cultural transmission of song from parental lineages (Payne 1996). Also, song learning allows individuals of some species to develop repertoires with shared song types that can be matched as a means of sending warning signals to neighbors, improving competitive ability (Burt et al. 2001). Both processes may operate within this and other hybrid zones.

The distinctive vocal boundary between these chickadees can be likened to that between song-type dialects within a species. Although dialect boundaries may be linked to reductions in gene flow (MacDougall-Shackleton and MacDougall-Shackleton 2001), they often are not (Soha et al. 2004). In the chickadee case, the primary barrier to gene flow appears not to be an exogenous social factor such as vocal communication, but rather endogenous genetic ones (Bronson et al. 2003a). The barrier appears to act more stringently on some genetic loci than on others (Sattler and Braun 2000, Bronson et al. 2005).

*Geographic variation in vocal admixture.*—The clear multivariate discrimination of *atricapillus* and *carolinensis* songs at the range interface in Appalachia stands in contrast to the intermediacy found by Robbins et al. (1986) in Missouri. We substantiated this difference by a re-analysis of the earlier data in the same statistical framework used here. Vocal intermediacy at the range interface in Missouri is reminiscent of vocal interactions at a Siberian hybrid zone between two subspecies of Great Tit (*Parus major*), where both intermediate songs and bilingual singing are found (Martens 1996). It differs from the more common situation where hybrids sing the songs of one or both species and intermediate vocalizations are rare (Ficken and Ficken 1967, Payne 1980, Morrison and Hardy 1983). However, intermediacy in the Missouri contact zone is subtle; most songs sounded more or less typical of one or the other parental species to the



human ear (Robbins et al. 1986). Multivariate analysis was required to detect intermediacy, and the presence of bilingual singers was the more obvious vocal indication that hybridization was taking place. The situation is therefore similar to that found with hybridizing Pied Flycatchers (*Ficedula hypoleuca*) and Collared Flycatchers (*F. albicollis*) (Gelter 1987).

Differences in vocal admixture were also noted between our two Appalachian transects. Intermediate songs were rare in both, but the proportion of bilingual singers and the area of song sympatry were greater in West Virginia than in Virginia. Similar variation is found in the contact between Alpine and lowland forms of Willow Tit (*Poecile montanus*), where vocal intermediacy is found in some areas but not in others (Thönen 1962, Martens and Nazarenko 1993).

*Factors affecting occurrence and detection of vocal admixture.*—Three sampling issues could affect apparent levels of admixture. First, the full repertoire of some birds was probably not sampled in our relatively short recording sessions, especially for *carolinensis*. This could lead to a bias if certain song types are under-represented in our sample. Second, birds may match their songs to the playback tapes used. Horn et al. (1992) found that *atricapillus* frequency-matched their songs to playback tapes in a manner analogous to song matching using multiple song types (Krebs et al. 1981, Otter et al. 2002). Such behavior could produce a bias against detecting song intermediacy. Third, playback tapes clearly influenced recorded repertoires in that presentation of the second song type sometimes stimulated birds to switch types and exhibit bilingual singing. Thus, our estimates of the frequency of bilingual singing are undoubtedly higher than those that would be obtained by listening passively to birds singing spontaneously for the same amount of time. None of these potential biases could explain the differences in admixture found in Missouri (Robbins et al. 1986) and the Appalachians (present study). The same playback tapes and recording protocol were used in both studies. Ward and Ward (1974) also used playback tapes at the contact zone in southeastern Pennsylvania and found substantial numbers of bilingual and atypical singers.

Ecological factors are a plausible explanation for geographic variation in vocal admixture. At most points along the *atricapillus*–*carolinensis*

contact zone, there is no appreciable change in elevation or other ecological transition associated with the range interface. In Missouri, there is a moderate transition, because the contact zone parallels the boundary between the forested Ozark Plateau and the largely treeless Great Plains (Robbins et al. 1986). In Appalachia, however, ecological variation associated with elevation is much greater, and *atricapillus* is restricted to higher elevations in this region. Chickadee dispersal may be inhibited by habitat preferences and sharp habitat transitions, or the fitness of either form may be reduced in the alternative habitat. In either case, both hybridization and social interaction may be more restricted along the Appalachian transects because of ecological segregation. Lower intermediacy in species characteristics in Appalachia is the expected outcome, and this effect may be greater for culturally transmitted vocal traits than for morphological or genetic ones.

Other factors may contribute to geographic variation in vocal admixture. Suboptimal habitat or other conditions that depress population densities of chickadees at the contact zone in the Midwest (Robbins et al. 1986, Grubb et al. 1994) and in the Smoky Mountains (Tanner 1952, Tove 1980) may produce a sharp vocal interface that masks significant levels of hybridization. If population densities are low, there may be fewer opportunities for vocal interactions among the two forms, yet the proportion of hybrid pairings might actually increase because of scarcity of mates. Also, temporal differences in the age of contact may have allowed more or less vocal admixture to accumulate in different regions. Finally, genetic differences between eastern and western populations of *carolinensis* (Gill et al. 1989, 1999; Sawaya 1990; Sattler 1996) have the potential to influence interactions between *atricapillus* and *carolinensis* at many levels.

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