

EXTENSIVE PROTEIN SIMILARITY OF THE HYBRIDIZING CHICKADEES *PARUS* *ATRICAPILLUS* AND *P. CAROLINENSIS*

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ABSTRACT.—Starch gel electrophoresis of proteins was used to assess genetic differentiation and introgression across a contact zone between *Parus atricapillus* and *P. carolinensis*. Little or no differentiation was found at 35 presumed genetic loci, even between distantly allopatric population samples. Nei's (1978) genetic distance (D) was ≤ 0.001 for all comparisons. In contrast, *Parus gambeli*, another chickadee known to hybridize with *atricapillus*, was well differentiated at 3 loci ($D \approx 0.065$). While the data suggest that *atricapillus* and *carolinensis* are closely related, they do not allow conclusions on the extent of introgression across the contact zone. The implications of these data are discussed in the light of the emerging pattern of isozyme variation in birds. Received 26 August 1985, accepted 28 March 1986.

THE evolutionary implications of hybridization in a contact zone depend on the extent to which gene flow is promoted by that hybridization. If the rate of gene flow is substantial, the hybridizing organisms form an evolutionary unit in the sense that most of the genetic variation available to one form is also available to the other. If gene flow is seriously impaired in the contact zone, then the hybridizing forms may be more or less genetically independent of one another. For most contact zones, the rate of gene flow can be inferred from the extent of introgression of parental characteristics across the zone. In the case of the Black-capped and Carolina chickadees (*Parus atricapillus* and *P. carolinensis*), however, achieving an accurate assessment of gene flow across their contact zone has been, and continues to be, difficult.

Previous studies of hybridization between these chickadees have established that interbreeding is common in many areas of contact (Brewer 1963, Rising 1968, Johnston 1971, Robbins et al. 1986). However, the amount of genetic introgression resulting from this interbreeding is unknown for two reasons. First, the two parental forms are extremely similar in all

morphological and ecological characteristics. Thus, an *atricapillus* population introgressed with *carolinensis* characters would be hardly distinguishable from a pure *atricapillus* population, and vice versa. Furthermore, because the contact zone parallels an obvious ecotone in some areas, the possibility remains that selection gradients have produced the observed morphological differences (Robbins et al. 1986). Second, although the songs of the two forms are strikingly different, the value of vocal characters as markers of gene flow is questionable because learning may be involved in song ontogeny (Ficken 1981, Ficken pers. comm.), or dispersing individuals may adopt the song type of the local population in which they breed (Payne 1981).

These difficulties with traditional characters led to interest in other markers that could be used to trace gene flow across the contact zone. Isozymic traits determined by protein electrophoresis are attractive prospects as genetic markers because they provide discontinuous characters that generally are inherited in a simple Mendelian fashion. If a large portion of protein variation is selectively neutral, as has been suggested repeatedly (e.g. King and Jukes 1969, Kimura 1983, Barrowclough et al. 1985), then loci that are differentiated across contact zones may provide silent markers through which demographic processes can be observed. A number of surveys of avian contact zones have employed this technique (Corbin et al.

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1979, Barrowclough 1980, Corbin 1981, Johnson and Zink 1983, Braun et al. 1984). Such studies yield valuable information on the genetics of wild bird populations in general, as well as on the evolutionary implications of contact zones (Barrowclough 1983).

MATERIALS AND METHODS

Sixty-nine specimens were analyzed electrophoretically, including 20 *atricapillus* from northern Missouri (Nodaway Co.), 20 *carolinensis* from Louisiana (St. Charles Parish), 14 birds from the contact zone in west-central Missouri (Bates and St. Clair cos.), and 15 *Parus gambeli* from California (San Bernardino Co.). Of the 14 contact zone birds, 13 were used previously in a multivariate analysis of morphological intermediacy in the contact zone (Robbins et al. 1986). Although these 13 birds cannot be identified safely as parentals, hybrids, etc. in terms of their morphological discriminant scores, they were representative of the entire contact zone sample, because the distribution of their discriminant scores did not differ significantly from the distribution of the entire sample (Kolmogorov-Smirnov test). All birds were collected with shotguns and frozen immediately on dry ice in the field. Later, the birds were thawed and heart, liver, kidney, and pectoral muscle collected. Tissue samples were stored at -60°C until use. Study skins and frozen tissue samples are preserved in the collections of the Louisiana State University Museum of Zoology.

Tissue samples were homogenized with 1–2 volumes of distilled water and clarified by centrifugation. Fifteen to 20 μl of the supernatant were loaded onto 10% starch gels and vertical electrophoresis carried out at 6 V/cm for 12–24 h. Specific staining techniques similar to those described by Harris and Hopkinson (1976) and Brewer (1970) were used to identify 35 presumed genetic loci. The loci assayed and the electrophoretic conditions used are summarized in the Appendix. When more than one locus with a particular enzymatic activity was scored, the loci were numbered beginning with the most anodal. Multiple alleles at a locus were designated alphabetically, beginning with the most anodal. Each bird was assigned a presumed genotype based on the pattern of bands in its electrophoretic phenotype.

The BIOSYS-1 computer program (Swofford and Selander 1981) was used to calculate indices of genetic variability and the genetic distances of Nei (1978) and Rogers (1972). The conformance of each population to Hardy-Weinberg expectations was assessed using a Chi-square test and an exact probability test (Elston and Forthofer 1977). To preserve sample sizes for tests of loci with more than two alleles, genotypes were pooled into three classes: homozygotes for the most common allele, heterozygotes for

the most common allele and another allele, and all other genotypes.

F-statistics were calculated by the method of Wright (1978) using BIOSYS-1. These statistics provide a means for analyzing population breeding structure in terms of correlations between uniting gametes. Barrowclough (1983) discussed the use of these statistics in analyzing avian populations. F_{is} and F_{it} are fixation indices of the individual with respect to its subpopulation and the total sample, respectively. F_{st} provides a measure of the differentiation of the subpopulations in terms of their progress toward complete fixation for alternate alleles. F_{st} has advantages over genetic distance in that it can be used to compare more than two populations simultaneously. It should be borne in mind, however, that F_{st} measures progress toward fixation, not absolute differentiation, and generally has smaller values when heterozygosity is high (Wright 1978).

RESULTS

Variation within populations.—Of 35 loci surveyed, 15 were polymorphic in one or more populations (Appendix). Eleven loci were only slightly polymorphic, however, having a single allele at a frequency greater than 0.05. Mean heterozygosities and percentages of polymorphic loci (Table 1) indicated that the variability of each population was within the range of values previously observed in passerine birds (Barrowclough 1980) and vertebrates in general (Nevo 1978). Values of F_{is} for polymorphic loci (Table 2) were generally small, indicating little deviation from panmixia within local populations. A possible exception involved the dipeptidase-1 locus (see below). The mean of F_{is} over all polymorphic loci was not significantly different from zero (t -test).

Tests for conformance to Hardy-Weinberg equilibrium revealed only one case of heterozygote deficiency that approached statistical significance: the dipeptidase-1 locus in the contact zone population ($P = 0.031$, Chi-square test; $P = 0.064$, exact probability test). The biological significance of this deviation is questionable, however, because it results from an observation of two heterozygous individuals when only 4.7 heterozygotes were expected. Furthermore, because even allopatric *atricapillus* and *carolinensis* population samples were virtually identical in allelic frequency at this locus (Appendix), the relationship of any possible heterozygote deficiency to interbreeding between the two forms is unclear.

TABLE 1. Measures of genetic variability at 35 loci in 4 chickadee populations.

Popula- tion	Percent- age of poly- morphic loci ^a	Mean heterozygosity \pm SE	
		Direct count	Hardy-Weinberg expected (Nei 1978)
<i>carolin- ensis</i>	31.4	0.042 \pm 0.015	0.043 \pm 0.015
<i>atricapil- lus</i>	22.9	0.046 \pm 0.021	0.044 \pm 0.020
Contact zone	20.0	0.035 \pm 0.018	0.041 \pm 0.019
<i>gambeli</i> ^b	15.2	0.020 \pm 0.009	0.020 \pm 0.009

^a A locus was considered polymorphic if no allele exceeded 0.99 in frequency.

^b Values for *gambeli* are over 33 loci.

Variation among populations.—The northern Missouri *atricapillus* sample and the Louisiana *carolinensis* sample were extremely similar in allelic frequencies at the 35 loci studied (Appendix). Only one allele occurred in one sample at an appreciable frequency and not in the other. This was the proline dipeptidase-2a allele, which occurred in *atricapillus* at a frequency of 0.125 but was not found in the *carolinensis* sample. An allele with such a low frequency is not promising for use as a genetic marker, however, because of the potential sampling error involved. For example, if *carolinensis* populations actually had the allele at the same frequency as the *atricapillus* sample, it would require a sample of about 50 individuals to ensure with 95% probability that the allele would be found (Gregorius 1980). With a sample size of 20 for each population, it is far from certain that the two populations actually differ in the frequency of proline dipeptidase-2a.

For the multilocus comparisons, the genetic distances between *atricapillus*, *carolinensis*, and contact zone samples were very small (Table 3). These values indicate a level of divergence more typical of local avian populations rather than levels generally achieved by separate avian species (Barrowclough 1980). The same conclusion may be drawn from the summary of F -statistics (Table 2). All F_{st} values were small, and the mean of F_{st} over all polymorphic loci (\bar{F}_{st}) is practically identical to the mean of \bar{F}_{st} (0.022) from a number of previous studies of conspecific bird populations (Barrowclough 1983).

Given the high degree of molecular similarity of the *atricapillus* and *carolinensis* samples, it

TABLE 2. F -statistics (Wright 1978) for 15 variable loci in 3 chickadee populations (*P. gambeli* not included). Values shown are the weighted averages across alleles at a locus. See the Appendix for locus abbreviations.

Locus	F_{is}	F_{it}	F_{st}
TRI-1	-0.037	-0.012	0.024
PRO-1	-0.076	-0.028	0.045
DIP-1	0.165	0.167	0.002
PRO-2	0.051	0.083	0.034
TRI-2	-0.026	-0.008	0.017
DIP-2	-0.053	-0.017	0.034
EST-1	-0.043	-0.020	0.023
ICD-2	-0.056	-0.018	0.036
GPD-2	-0.037	-0.012	0.024
PGM	-0.026	-0.013	0.013
AAT-1	-0.032	-0.016	0.016
AAT-2	-0.026	-0.008	0.017
MPI	-0.076	-0.051	0.024
CK-M	-0.026	-0.008	0.017
IDH	-0.034	-0.011	0.022
$\bar{x} \pm$ SD	-0.022 \pm 0.059	0.002 \pm 0.054	0.023 \pm 0.011

was of interest to determine if other species of chickadees could be distinguished on the basis of protein patterns. We compared a sample of 15 *Parus gambeli* with the eastern chickadee samples. The *P. gambeli* were fixed for an alternate allele at the alanine aminotransferase-2 locus, and showed large frequency differences at both the proline dipeptidase-1 and the dipeptidase-1 loci (Appendix). These differences were reflected in the overall genetic distance measures (Table 3). Clearly, molecular differentiation was detectable in *gambeli*, a species closely related to the *atricapillus-carolinensis* complex (see below).

DISCUSSION

The extensive molecular similarity of *P. atricapillus* and *P. carolinensis* samples in this study was striking. The samples represent populations that are differentiated morphologically and vocally and are currently considered to belong to distinct species. The populations sampled are separated by 1,200 km. Also, the narrow contact zone presumably could restrict gene flow. Yet, these samples are essentially indistinguishable at the protein loci examined. We considered several possible explanations for this observation.

First, it seemed possible that the lack of pro-

TABLE 3. Genetic distances over 35 loci between 4 chickadee populations. Below diagonal: Nei's (1978) distance; above diagonal: Rogers's (1972) distance.

Population	1	2	3	4
1 <i>carolinensis</i>	—	0.021	0.023	0.089
2 <i>atricapillus</i>	0.001	—	0.011	0.080
3 Contact zone	0.001	0.000	—	0.085
4 <i>gambeli</i> *	0.072	0.063	0.066	—

* Values for *gambeli* are over 33 loci.

tein differences reflected something unusual about the cellular biology or genetics of chickadees that precluded detectable molecular differentiation. This possibility was considered by Barrowclough and Corbin (1978) and Aquadro and Avise (1982) in exploring small genetic distances between other bird species. Although it is unclear what mechanism could bring about such an effect, it probably would be related to an alleged slowdown in avian molecular evolution (Prager et al. 1974). We reasoned that if electrophoretically detectable molecular differentiation was, in fact, forbidden in chickadees, other chickadee species also would be indistinguishable by electrophoresis. Therefore, we compared eastern chickadees with *P. gambeli*, a close relative of the *atricapillus-carolinensis* complex. Although widely sympatric in the western United States, *gambeli* and *atricapillus* hybridize along the Rio Grande in New Mexico (W. Howe pers. comm.). Compared with population samples from the eastern complex, *gambeli* was strongly differentiated at several loci. Thus, there appears to be no consistent bias against electrophoretically detectable protein differentiation between chickadee species. We note also that at least some putatively conspecific *Parus* populations are known to have genetic divergence detectable by electrophoresis. Braun et al. (1984) found significant molecular differentiation between the black-crested and gray-crested forms of the Tufted Titmouse (*Parus bicolor*). These forms hybridize locally in a narrow contact zone in Texas (Dixon 1955). These studies of other *Parus* populations imply that there is nothing unusual about the genus that would preclude electrophoretic detection of differentiation between *atricapillus* and *carolinensis*.

Next, it was pertinent to determine whether our techniques uncovered all the differentiation actually present at the loci surveyed. In-

creased electrophoretic variation can be detected by systematically varying the experimental conditions (e.g. Coyne et al. 1979). The number of hidden alleles, however, may amount to only 10–20% of the final total of alleles (Aquadro and Avise 1982). A number of factors tended to ameliorate this shortcoming in our data; for example, a large number of buffer vs. locus combinations were examined in optimizing the clarity of banding patterns. Also, it is evident from the data of Aquadro and Avise (1982) that a significant portion of "hidden" variation may be detected by simply running gels longer. We routinely performed overnight runs. Finally, no electrophoretic survey can be truly exhaustive. There are certain to be many alleles that are undetectable by electrophoresis (Coyne et al. 1979), and staining techniques exist for only a small fraction of all structural genes. Considering these limitations, it appeared more reasonable to survey many loci for differentiation rather than to investigate intensively all the variation at a few.

The simplest conclusion is that these two forms have undergone very little divergence, at least at the loci analyzed. The Nei genetic distance ($D = 0.001$) between *atricapillus* and *carolinensis* is among the lowest ever reported for putative biological species. Few nonavian species comparisons approach this level of similarity. For example, in a survey of 616 pairwise comparisons of nonavian congeners, Avise et al. (1975) found only a few with $D < 0.05$. However, the emerging pattern of isozyme variation in birds entails much lower genetic distances than are generally found in nonavian groups (reviewed by Avise and Aquadro 1982). For instance, Barrowclough (1980) reported a mean D of 0.044 for 72 comparisons among bird congeners. Among parulid warblers, Avise et al. (1980) found a mean D of 0.056 among congeneric species (74 comparisons). In 21 of these comparisons (28%), D was of the same order of magnitude as that between *atricapillus* and *carolinensis* in the present case ($D < 0.01$). All comparisons were among forms for which there was no doubt about their species status. As Avise et al. (1980) correctly intimated, the confidence interval of such small genetic distances is relatively large, given the sample sizes used. Nevertheless, these examples and others (Yang and Patton 1981, Johnson and Zink 1983) suggest that Nei distances less than 0.01 between

biological species are not unusual in birds. The fact that low genetic distances seem to characterize birds has evoked considerable discussion (Sibley and Ahlquist 1982, Avise 1983) and may be related to the large effective population size and high vagility of most avian species (Barrowclough 1983). In any case, extreme protein similarity cannot by itself be taken as strong evidence for conspecificity of bird populations.

Data compiled in this and a related study (Robbins et al. 1986) suggest that Black-capped and Carolina chickadees are extremely similar genetically and interbreed freely in at least one zone of contact (southwestern Missouri). The most likely reproductive isolating mechanism, song, breaks down completely in this zone. This evidence might be sufficient grounds for considering two forms conspecific in most cases; yet the evidence falls short of conclusively demonstrating introgression between their gene pools. Paradoxically, this occurs because no known differences between these chickadees are sufficient to be useful for measuring introgression across the contact zone. In this regard, it is ironic that Black-crested and Tufted titmice, two *Parus* forms much more differentiated at the morphological and molecular levels than are *atricapillus* and *carolinensis* (Dixon 1955, Braun et al. 1984), are now considered subspecies (A.O.U. 1983) because their differences allowed assessment of genetic interaction between them.

We probably would not hesitate to treat Black-capped and Carolina chickadees as a single species were it not for repeated reports of narrow gaps in their breeding ranges (Tanner 1952; Brewer 1963; Merritt 1978, 1981) that have been suggested to be induced competitively (Tanner 1952) or to act as a reproductive isolating mechanism (Brewer 1963). Robbins et al. (1986) discussed possible alternative explanations for the apparent allopatry observed by Brewer (1963) and Merritt (1978, 1981) in Indiana and Illinois. The elevational gap in the Great Smoky Mountains might indicate a reluctance of *atricapillus* and *carolinensis* populations in this area to interbreed despite the presence of apparently suitable habitat (Tanner 1952). The two forms hybridize in several localities in the Appalachian region (Tanner 1952, Johnston 1971), suggesting that their separation in the Great Smokies is a local phenomenon that must be studied carefully before universal significance

can be attached to it. Kendeigh and Fawver (1981) demonstrated that chickadees are segregated by habitat in this area. Perhaps chickadee populations in the Great Smokies have innate habitat preferences and remain differentiated in that area. Different habitat preferences might have developed during postulated periods of Pleistocene isolation (Brewer 1963). The Great Smokies support nearly virgin forests of exceptionally high plant species diversity (Whittaker 1956), and have a greater area at high elevation than much of the rest of Appalachia (Tanner 1952). These factors help explain how habitat segregation could be more effective in the Great Smokies than in other areas of Appalachia. The idea that innate habitat preferences result in elevational separation and maintain differentiation in these populations is at least as attractive as Tanner's (1952) hypothesis that elevational separation is competitively induced, because in most areas chickadee populations interbreed extensively at the range interface (Robbins et al. 1986). Although one can understand how one species might competitively replace another, it is hard to conceive how competition could induce a gap where a significant amount of habitat is not used by either species, as occurs in the present case.

Elevational separation of this sort is not unknown for populations considered to represent subspecies of a single species. The Red-shafted and Gilded races of the Northern Flicker (*Colaptes auratus*) hybridize in several Arizona localities, but in other areas are separated elevationally (Phillips et al. 1964). The distribution of the Alpine and Willow races of the Willow Tit (*Parus m. montanus* and *P. m. salicarius*) in the Swiss Alps provides a striking parallel to the distribution of chickadees in Appalachia (Thönen 1962). The two European forms have vocal and morphological differences at least as great as those of *atricapillus* and *carolinensis*. There are three known zones of hybridization for the European forms, but there are also areas where no hybrids have been found even though only the Willow song type occurs on one side of a valley and only the Alpine song type on the other. The similarity extends to the behavior of individual contact zone birds, which often sing both song types. The situation in the *montanus-salicarius* complex is nearly identical to the *atricapillus-carolinensis* situation, yet the vaga-

ries of taxonomy have not yet resulted in equal categorical rank in both cases.

Although additional information may be necessary before a decision about the specific status of *carolinensis* and *atricapillus* can be made, they have passed one commonly applied test of conspecificity. Whenever the two forms breed syntopically, they hybridize readily and probably at random (Robbins et al. 1986). Thus, they do not qualify as a case where local breakdown of reproductive isolating mechanisms has caused hybridization between species that are clearly distinct in other areas, such as the towhees *Pipilo ocai* and *P. erythrophthalmus* (Sibley and Sibley 1964, Braun 1983), the paradise flycatchers *Terpsiphone rufiventer*, *T. rufocinerea*, and *T. viridis* (Chapin 1948), or the sparrows *Passer domesticus* and *P. hispaniolensis* (Meise 1936). Fortunately, in the chickadee case, further research probably will yield information useful in understanding the evolution and dynamics of their contact zone and in making an objective decision on their species status.

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APPENDIX. Allele frequencies and electrophoretic conditions for polymorphic protein loci.^{a,b}

E.C. no.	Locus (acro- nym)	<i>P. carolinensis</i>	<i>P. atricapillus</i>	Contact zone	<i>P. gambeli</i>	Tis- sue ^c	Buffer
1.1.1.8	Glycerol-3-phosphate dehydrogenase(NAD ⁺)-1 (GPD-1)					M	Tris-borate, pH 8.1
	a	—	—	0.036	—		
	b	1.000	1.000	0.964	1.000		
1.1.1.14	L-Iditol dehydrogenase (IDH)					L	Tris-citrate, pH 7.5 (NAD) ^d
	(n)	15	18	0	0		
	a	0.967	1.000	—	—		
	b	0.033	—	—	—		
1.1.1.42	Isocitrate dehydrogenase(NADP ⁺)-2 (ICD-2)					M	Tris-borate, pH 8.1 (NADP) ^d
	(n)	19	20	14	15		
	a	0.947	1.000	1.000	1.000		
	b	0.053	—	—	—		
2.6.1.1	Aspartate aminotransferase-1 (AAT-1)					M	Phosphate-citrate, pH 6.0
	a	—	0.025	—	—		
	b	1.000	0.975	0.964	1.000		
	c	—	—	0.036	—		
2.6.1.1	Aspartate aminotransferase-2 (AAT-2)					M	Phosphate-citrate, pH 6.0
	a	0.025	—	—	—		
	b	0.975	1.000	1.000	1.000		
2.6.1.2	Alanine aminotransferase-2 (ALAT-2)					M	Phosphate-citrate, pH 6.0
	(n)	16	13	14	15		
	a	—	—	—	1.000		
	b	1.000	1.000	1.000	—		
2.7.3.2	Creatine kinase-2 (CK-2)					M	Tris-citrate, pH 7.5
	(n)	20	20	13	15		
	a	1.000	0.975	1.000	1.000		
	b	—	0.025	—	—		
2.7.5.1	Phosphoglucomutase (PGM)					H	Tris-maleate, pH 7.5
	a	0.025	—	—	—		
	b	0.975	0.975	1.000	1.000		
	c	—	0.025	—	—		
3.1.1.2	Umbelliferyl acetate esterase-1 (EST-1)					M	Tris-borate, pH 8.1
	a	—	0.025	—	—		
	b	0.950	0.975	1.000	1.000		
	c	0.050	—	—	—		
3.4.11.4	Tripeptide aminopeptidase-1 (TRI-1)					M	Tris-citrate, pH 7.5
	a	—	—	0.036	—		
	b	1.000	1.000	0.964	1.000		
3.4.11.4	Tripeptide aminopeptidase-2 (TRI-2) ^e					M	Tris-citrate, pH 7.5
	a	0.025	—	—	0.033		
	b	0.975	1.000	1.000	0.967		
3.4.13.9	Proline dipeptidase-1 (PRO-1) ^e					M	Tris-citrate, pH 7.5
	a	—	0.050	—	0.033		
	b	0.175	0.375	0.357	0.933		
	c	0.825	0.575	0.643	0.033		
3.4.13.9	Proline dipeptidase-2 (PRO-2) ^e					M	Tris-citrate, pH 7.5
	a	—	0.125	0.036	0.067		
	b	0.950	0.825	0.821	0.933		
	c	0.050	0.050	0.143	—		

APPENDIX. Continued

E.C. no.	Locus (acronym)	<i>P. carolinensis</i>	<i>P. atricapillus</i>	Contact zone	<i>P. gambeli</i>	Tissue ^c	Buffer
3.4.13.11	Dipeptidase-1 (DIP-1) ^a					M	Tris-citrate, pH 7.5
	a	0.150	0.150	0.179	—		
	b	—	—	—	0.033		
	c	0.750	0.775	0.786	—		
	d	0.075	0.075	0.036	0.900		
	e	0.025	—	—	—		
	f	—	—	—	0.067		
3.4.13.11	Dipeptidase-2 (DIP-2) ^a					M	Tris-citrate, pH 7.5
	a	—	—	—	0.033		
	b	0.950	1.000	1.000	0.933		
	c	0.050	—	—	—		
	d	—	—	—	0.033		
5.3.1.8	Mannosephosphate isomerase (MPI)					M	Tris-borate, pH 8.1
	a	—	0.025	0.036	—		
	b	0.900	0.950	0.964	1.000		
	c	0.100	0.025	—	—		

^a The following loci were monomorphic in all individuals assayed: (E.C. numbers and acronyms in parentheses): lactate dehydrogenase-1 & 2 (1.1.1.27; LDH), malate dehydrogenase-1 & 2 (1.1.1.37; MDH), isocitrate dehydrogenase(NADP⁺)-1, (1.1.1.42; ICD), phosphogluconate dehydrogenase (1.1.1.43; PGD), glyceraldehyde-phosphate dehydrogenase (1.2.1.12; GAPDH), glutamate dehydrogenase (1.4.1.2; GLUD), superoxide dismutase-1 & 2 (1.15.1.1; SOD), alanine aminotransferase-1 (2.6.1.2; ALAT), creatine kinase-1 (heart locus; 2.7.3.2; CK), adenylate kinase (2.7.4.3; AK), acid phosphatase (3.1.3.2; ACP), adenosine deaminase (3.5.4.4; ADA), glucosephosphate isomerase (5.3.1.9; GPI), myoglobin (Mb), hemoglobin- α & β (Hb).

^b Unless otherwise noted, $n = 20$ for *carolinensis* and *atricapillus*, $n = 14$ for contact zone, and $n = 15$ for *gambeli*.

^c M = muscle, H = heart, L = liver.

^d Nicotine adenine dinucleotide (NAD⁺) or nicotine adenine dinucleotide phosphate (NADP⁺) added to gel buffer at 2–5 micromolar concentration.

^e Peptidase substrates were leucylglycylglycine for tripeptide aminopeptidases, leucylalanine for dipeptidases, and phenylalanylproline for proline dipeptidases.