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Geographic Protein Variation in *Pseudacris brimleyi* (Anura: Hylidae): Analysis by Sequential Electrophoresis

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ABSTRACT.—**Geographic protein variation in 26 genetic loci from six samples of *Pseudacris brimleyi* was analyzed by standard and sequential starch-gel electrophoresis. A single population of *P. feriarum* was included as an outgroup. In these seven populations, standard methods detected 59 alleles while sequential analysis revealed 31 additional alleles at 14 loci, a 53% increase over the number of alleles using standard methodology. Mean heterozygosity in *P. brimleyi* increased from 8.5% to 12.0% and the percentage of polymorphic loci increased from 26% to 34%. The four northern populations of *P. brimleyi* from North Carolina and Virginia are all quite similar to each other genetically, while the two from South Carolina are more differentiated. These results are consistent with the hypothesis that *P. brimleyi* may have recently expanded its range northward.**

The purpose of this study is to analyze geographic protein variation in Brimley's chorus frog, *P. brimleyi* Brandt and Walker, based on samples of frogs from six sites scattered throughout the range of the species. Most previous studies of geographic variation using electrophoretic methods have analyzed variation at each locus on a single buffer system, although a few loci are sometimes compared on additional buffer systems. It has been estimated that only about 40% of the real protein variation is revealed by this procedure (Ramshaw et al., 1979; McLellan, 1984). On the other hand, by using different electrophoretic conditions successively (sequential electrophoresis) over 90%

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TABLE 1. Number of alleles per locus in successive buffer systems in six samples of *P. brimleyi* and one sample of *P. feriarum*. If a number is not indicated, then additional buffer systems failed to resolve that locus satisfactorily.

	Buffer system			
Locus	1	2	3	4
ACP	1[a]			
ACON-1	4[b]	5[c]		
AK	1[b]	1[c]		
ADH	1[d]	1[a]	1[b]	
AAT-1	1[a]	1[b]	1[e]	
AAT-2	4[b]	4[a]	8[e]	
ESD	1[d]			
GPI	4[a]	7[e]		
GLUD	1[b]	3[a]		
GPD	3[a]			
ICD-1	1[b]	1[c]	2[a]	
ICD-2	5[b]			
LDH-1	3[b]	6[a]		
LDH-2	2[b]	3[a]		
MDH-1	4[b]	6[c]	7[a]	
MDH-2	3[b]	3[c]		
ME	3[f]	3[d]	6[a]	
PEP-1	4[e]	5[b]		
PEP-2	2[e]	3[b]		
PGM	3[b]	4[c]	6[a]	6[f]
PT-1	1[f]			
PT-2	1[e]	1[f]	2[d]	2[a]
PT-3	2[f]	2[d]		
PT-4	1[f]	1[e]		
PK	2[b]	3[c]	6[a]	
XDH	1[d]			
Total no. alleles	59	75	90	90

[a] Tris-versene-borate, 250 v, 6 h.
[b] Tris-citrate pH 8.0, 130 v, 6 h.
[c] Tris-citrate pH 6.7, 150 v, 6 h.
[d] Tris-Hcl, 250 v, 4 h.
[e] Lithium hydroxide, 300 v, 8 h.
[f] Poulik, 300 v, 6 h.

of protein variants may be detected, including those with amino acid replacements that do not affect charge (Coyne, 1982; Lewontin, 1985).

In sequential electrophoresis, proteins are first compared on a single buffer system. All electromorphs, hereafter referred to as alleles, that appear to have the same mobility are then systematically compared on a second buffer system. Alleles that still appear to have the same mobility are then compared successively on additional buffer systems. Only those alleles that still have the same mobility after the use of multiple buffer systems are regarded as genetically the same. Hedges (1989a, b), Hass (1991), Burnell and Hedges (1990) and Hedges and Burnell (1990) utilized sequential electrophoresis to study relationships of species within genera of West Indian frogs and lizards, but it has not been used previously to analyze geographic variation within a species. In order to compare the difference between the results of standard electrophoresis and sequential electrophoresis, the data on *P. brimleyi* obtained for up to four buffer systems were analyzed separately and compared.

Pseudacris brimleyi ranges in the Atlantic Coastal Plain of the United States from southeastern Virginia to southeastern Georgia. Hoffman (1983) has reviewed the literature on the species. Hedges (1986) analyzed the relationships of 30 taxa of Holarctic hylid frogs by an electrophoretic analysis of genetic variation in 33 protein loci and his data have been further evaluated by Highton (1991, 1993). The results indicate that *P. brimleyi* is probably the sister species to a group of three closely related species: *P. brachyphona*, *P. feriarum*, and *P. triseriata*. No natural hybrids between *P. brimleyi* and sympatric species of *Pseudacris* (*P. feriarum*, *P. nigrita*, *P. ornata*, *P. crucifer*, and *P. ocularis*) have been reported, but Mecham (1965) reared, beyond metamorphosis, laboratory induced hybrids of *P. brimleyi* with the first three of the above mentioned sympatric species as well as hybrids with *P. brachyphona*.

MATERIALS AND METHODS

Protein variation in six series of *Pseudacris brimleyi* and one sample of *P. feriarum* (outgroup) from populations listed in Appendix I were analyzed electrophoretically by sequential electrophoresis. The methods used were the same as in Hedges (1986, 1989a, b). The loci and buffer systems used are listed in Table 1. Data on *P. brimleyi* population 1 and the outgroup *P. feriarum* population have been reported previously in Hedges (1986), although the sample sizes have been increased for this study.

Nei (1972) genetic distances, UPGMA trees (Sokal and Rohlf, 1962) and their bootstrap frequencies were calculated by the BIOSYS-1 program (Swofford and Selander, 1981), modified for bootstrapping by Rob Billerbeck and SBH. Cavalli-Sforza and Edwards (1967) chord distances as modified by Nei et al. (1983), neighbor-joining trees (Saitou and Nei, 1987), and their bootstrap frequencies were calculated using the DISPAN program by Tatsuya Ota. Bootstrapping is a procedure that resamples the loci with replacement in order to obtain confidence limits for the topology at each node of the tree (Felsenstein, 1985).

RESULTS

The total number of alleles detected for 26 loci in the first buffer systems within the six populations of *P. brimleyi* was 48, after the second buffer system (20 loci) the total number of alleles increased to 58 (an increase of 21%), and

TABLE 2. The effect of sequential electrophoresis on measures of variability in six samples of *Pseudacris brimleyi*. The mean heterozygosity is an unbiased estimate.

Number of buffer systems	Mean H	Mean no. of alleles per locus	Total no. of alleles	Mean percent loci polymorphic per population (0.99 criterion)
1	0.085	1.30	48	26.3
2	0.107	1.39	58	30.1
3	0.120	1.45	64	34.0

after the third buffer system (9 loci) the total number of alleles revealed was 63, an increase of 31% over the first buffer system (Table 1). The equivalent numbers of alleles for the outgroup population, *P. feriarum*, were 32, 34, and 41, an increase of 6% and 28% for the second and third buffer systems respectively (Appendix II). The equivalent numbers of alleles for all populations (ingroup and outgroup) were 59, 75, and 90, an increase of 27% and 53% for the second and third buffer systems respectively. Two loci were examined on a fourth buffer system but no additional alleles were resolved on that system. The increases in the mean heterozygosity, mean number of alleles per locus, total alleles, and the percentage of polymorphic loci are shown in Table 2.

Table 3 compares the Nei (1972) genetic distances between samples using the results from one and three buffer systems. The four northern samples (1–4) of *P. brimleyi* are all extremely similar with Nei (1972) genetic distances below 0.02 (0.02–0.05 after sequential electrophoresis). These four populations are all more distant from the remaining populations 5 and 6 (0.07–0.08 before, and 0.09–0.13 after sequential electrophoresis). Samples 5 and 6 also have a large genetic distance (0.07 before, and 0.10 after sequential electrophoresis) to each other even though their geographic distance is only 117 km (Fig. 2; Table 3).

Fig. 2 shows UPGMA and the neighbor-joining trees based on data from one, two and three buffer systems. Also included are the bootstrap confidence percentages for each tree based on 2000 UPGMA trees and 10,000 neighbor-joining trees. The confidence in the topology of the trees as indicated by the bootstrap values does not improve noticeably as a result of sequential electrophoresis.

DISCUSSION

The results of studies on genetic differentiation among populations using the method of sequential electrophoresis are not comparable

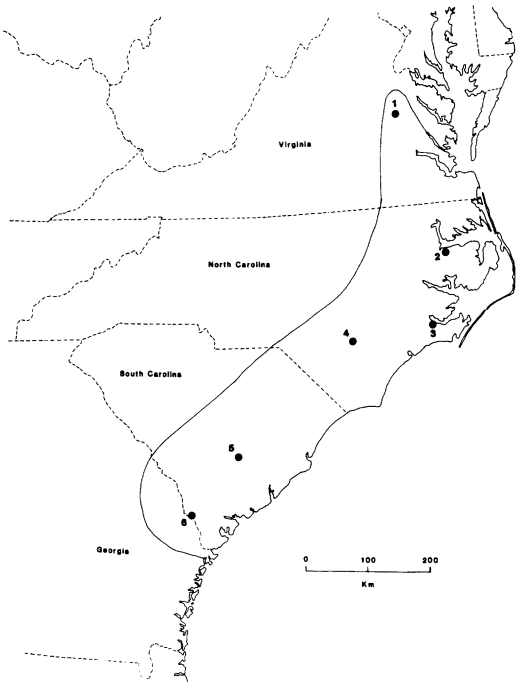

FIG. 1. Distribution of *Pseudacris brimleyi* showing the collection sites for the six samples.

to those for studies using a single buffer system. This is because much more genetic variation is revealed by sequential electrophoresis and therefore genetic distances between populations would be expected to be higher than for comparable studies utilizing standard electrophoretic methods.

One goal of this study was to compare the trees obtained by using the successive data sets obtained by sequential electrophoresis in order to evaluate the amount of improvement in the estimates of relationships by comparison with the geographic relationships of the populations studied. This attempt was not successful because of the genetic patterns of relationships among the sampled populations. As noted above, samples 1–4 are very similar; at most loci they are almost as similar as might be expected if four different samples had been taken from a single population. Because the genetic relationships of four of the six populations of *P. brimleyi* are so close, this is not a sufficient data set to use in comparing the results of sequential electrophoresis to those of standard methods in a geographic genetic variation study. Comparisons of our data with geography are of limited use in this regard, but sequential analysis might be used in other species in order to find out whether the extra time and expense required for sequential electrophoresis yields superior results over standard methods. Better results

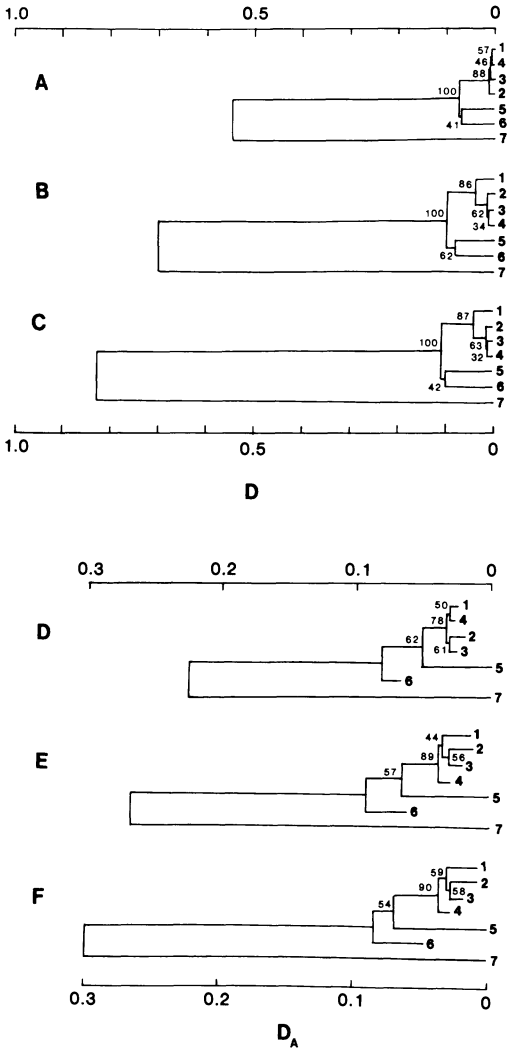

FIG. 2. UPGMA phenograms for Nei (1972) genetic distances (D) based on electrophoretic analysis based on one (A), two (B) and three (C) buffer systems. Neighbor-joining trees for Cavalli-Sforza and Edwards' (1967) chord distances (D_A) based on analysis of one (D), two (E) and three (F) buffer systems. Percentages at each node of the UPGMA trees are the proportion of 2000 bootstrap trees that support the topology at that node while those of the neighbor-joining trees are based on 10,000 bootstrap trees.

might be inferred if there is a higher correlation of genetic distance to geography (nearby samples more similar than more geographically distant samples), or if there is a better fit of sequential electrophoretic results to other independent molecular or morphological data. Sequential electrophoresis is obviously desirable because it provides more accurate genetic data, but the question remains as to whether or not the improvement in quality of the data is sufficient to justify so much additional work.

The genetic similarity among the four northernmost *P. brimleyi* samples is similar to that found in northern populations of several species of woodland salamanders of the genus *Plethodon* (Highton and Webster, 1976; Highton, 1989; unpubl. data). This is interpreted as an indication that each of these species has recently expanded its range northward as a result of warming climates since the retreat of the Wisconsin glaciation during the last 10,000 years. During glacial maxima, the northernmost populations of several *Plethodon* species may have occupied a rather small area, as might also be expected in a frog such as *P. brimleyi*, whose present east-west range is now only about 150-200 km in width and is limited to the Coastal Plain physiographic province. At the maximum advance of the Wisconsin glaciation, its northernmost populations may have occupied a limited area and might have been genetically very similar to each other. As the continental glacier retreated, if the northernmost populations were the sole source of northward migrants, then the present genetic uniformity of *P. brimleyi* in the northern half of its range would be expected because these populations have not yet had time to diverge genetically in such a short period of time. The source populations(s) of the northern migrants may have been in southern North Carolina and/or northeastern South Carolina.

Although southern populations are more differentiated from each other and from northern populations, the amount of genetic differentiation observed between them is similar to that found within numerous amphibian and other vertebrate species that have been studied (Thorpe, 1982). Thus there is no indication that there are any cryptic species within *P. brimleyi*.

Several workers, including Brandt and Walker (1933), Brandt (1936), and Hoffman (1983), have noted that the calls of *P. brimleyi* and *P. brachyphona* are quite similar and have suggested that the two species might be closely related. However, Hedges' (1986) genetic data indicates that *P. brachyphona* is closer to *P. feriarum* than it is to *P. brimleyi* and Highton (1993) found this relationship was supported statistically in 99.9% of 1000 bootstrap UPGMA trees. Therefore it is likely that the call similarities between *P. brachyphona* and *P. brimleyi* are due to convergence. The calls of both are similar to those of several other species of chorus frogs of the genus *Pseudacris* including *nigrita, feriarum, maculata,* and *triseriata,* except that the calls of *brachyphona* and *brimleyi* are much more rapid. Because parts of the ranges of the latter two species overlap those of *nigrita* and *feriarum,* by the simple expedient of speeding up their calls, both may have

TABLE 3. Nei (1972) genetic distances among samples based on a single buffer system (above diagonal) and based on sequential electrophoresis using all buffer systems (below diagonal).

Population	*P. brimleyi*						*P. feriarum*
	1	2	3	4	5	6	7
1		0.017	0.010	0.006	0.067	0.076	0.549
2	0.049		0.009	0.009	0.080	0.082	0.555
3	0.040	0.016		0.006	0.076	0.079	0.541
4	0.035	0.018	0.016		0.072	0.070	0.543
5	0.120	0.120	0.116	0.094		0.070	0.612
6	0.128	0.102	0.094	0.090	0.101		0.483
7	0.841	0.832	0.841	0.853	0.862	0.739	

evolved a similar premating reproductive isolating mechanism to prevent interbreeding with their closely related congeners. The alternate hypothesis, that *brimleyi* and *brachyphona* share the ancestral call type and that the other species have all shortened their calls, would seem to be much less likely since the probable outgroup species (*nigrita*) also has a shorter call. Patterns of evolution in calls of *Pseudacris* have been discussed recently by Cocroft and Ryan (1995).

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APPENDIX I. Localities of samples.

Population no.	State	County	N Latitude °	N Latitude ′	N Latitude ″	W Longitude °	W Longitude ′	W Longitude ″
P. brimleyi								
1	VA	Caroline	37	49	27	77	19	58
2	NC	Washington	35	55	24	76	35	37
3	NC	Craven	34	57	09	77	02	00
4	NC	Sampson	34	48	56	78	21	54
5	SC	Orangeburg	33	19	55	80	25	36
6	SC	Hampton	32	33	44	81	16	44
P. feriarum								
7	TN	Blount	35	41	28	83	47	56

APPENDIX II. Genetic data for 18 variable loci in six samples of *P. brimleyi* and one sample of *P. feriarum*. Eight loci (ACP, AK, ADH, AAT-1, ESD, Pt-1, PT-4, and XDH) were monomorphic in all samples on all buffer systems. Alleles distinguished on the first buffer system are indicated by small letters. Additional alleles identified on the second buffer system are given numbered superscripts and those distinguished on the third buffer system are indicated by capital letter superscripts.

	Sample						
	P. brimleyi						*P. feriarum*
Locus	1	2	3	4	5	6	7
N	11	6	12	5	2	19	19
First buffer system:							
ACON-1	b	b	b (.88) c (.13)	a (.10) b (.90)	a (.75) b (.25)	a (.95) b (.05)	c (.84) d (.16)
AAT-2	b (.86) d (.14)	a (.17) b (.83)	b (.92) d (.08)	b	a (.25) c (.75)	a (.11) b (.84) c (.05)	b (.97) d (.03)
GPI	a (.05) c (.64) d (.32)	c (.92) d (.08)	c (.58) d (.42)	c (.80) d (.20)	c (.75) d (.25)	a (.16) c (.76) d (.08)	b (.97) d (.03)
GLUD	a	a	a	a	a	a	a
GPD	b	a (.08) b (.92)	a (.04) b (.96)	b	b	b	b (.08) c (.92)
ICD-1	a	a	a	a	a	a	a
ICD-2	c (.82) e (.18)	c	a (.04) c (.92) e (.04)	b (.10) c (.90)	c (.50) e (.50)	c (.79) e (.21)	c (.68) d (.32)
LDH-1	c	a (.17) c (.83)	a (.08) c (.92)	c	a (.25) c (.75)	a (.34) c (.66)	b
LDH-2	b	b	b	b	b	b	a
MDH-1	c	b (.25) c (.67) d (.08)	b (.17) c (.83)	c	c	c	a (.34) c (.66)

APPENDIX II. Continued.

	Sample						
	P. brimleyi						*P. feriarum*
Locus	1	2	3	4	5	6	7
N	11	6	12	5	2	19	19
MDH-2	a (.55) c (.45)	a (.17) c (.83)	a (.17) c (.83)	a (.30) c (.70)	a (.50) c (.50)	b (.97) c (.03)	b
ME	c	b (.08) c (.92)	c	c	c	c	a
PEP-1	b (.05) c (.95)	c	c	b (.10) c (.90)	c (.75) d (.25)	c	a
PEP-2	b	b	b	b	b	b	a
PGM	b	b	a (.13) c (.88)	b	b (.75) c (.25)	b	b
PT-2	a	a	a	a	a	a	a
PT-3	a	a	a	a	a	a	b
PK	b	b	b	b	b	a (.03) b (.97)	a
Second buffer system:							
ACON-1			c^2 (.13)				c^1 (.84)
GPI	c^1 (.64) d^2 (.32)	c^1 (.92) d^2 (.08)	c^1 (.54) c^2 (.04) d^2 (.42)	c^1 (.80) d^2 (.20)	c^1 (.75) d^1 (.25)	c^1 (.74) c^2 (.03) d^1 (.03) d^2 (.05)	d^3 (.03)
GLUD	a^1	a^1 (.67) a^3 (.33)	a^1	a^1	a^1	a^1	a^2
LHD-1		a^4 (.17)	a^3 (.08)		a^1 (.25)	a^2 (.11) a^3 (.24)	
LDH-2	b^1	b^1	b^1	b^1 (.90) b^2 (.10)	b^1	b^1	
MDH-1	c^2	c^2 (.58) c^3 (.08)	c^2 (.50) c^3 (.33)	c^2	c^2	c^2 (.63) c^3 (.37)	c^1 (.21) c^2 (.45)
PEP-1	c^1 (.82) c^2 (.14)	c^1 (.08) c^2 (.92)	c^1 (.13) c^2 (.88)	c^2 (.90)	c^2 (.75)	c^2	
PEP-2							a^1 (.87) a^2 (.13)
PGM	b^1	b^1	b^1 (.88)	b^1	b^1 (.75)	b^1	b^2
PK	b^2	b^2	b^2	b^2	b^1 (.50) b^2 (.50)	b^1 (.40) b^2 (.58)	
Third buffer system:							
AAT-2	b^A (.86)	a^B (.17) b^A (.83)	b^A (.92)	b^A	a^C (.25)	a^A (.05) a^B (.03) a^C (.03) b^A (.84)	b^B (.26) b^C (.71)
ICD-1	a^B	a^B	a^A (.04) a^B (.96)	a^A (.20) a^B (.80)	a^A (.50) a^B (.50)	a^B	a^B
MDH-1							a^A (.32) a^B (.03)
ME	c^B	c^B (.83) c^C (.08)	c^B (.96) c^C (.04)	c^B (.90) c^C (.10)	c^B	c^A (.08) c^B (.71) c^C (.21)	a^A (.18) a^B (.82)
PGM							b^{2A} (.11) b^{2B} (.79) b^{2C} (.11)
PT-2	a^B	a^B	a^B	a^B	a^B	a^B	a^A
PK						a^A (.03)	a^B (.05) a^C (.32) a^D (.63)