AN ELECTROPHORETIC ANALYSIS OF HOLARCTIC HYLID FROG EVOLUTION

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Abstract.—The phylogenetic relationships of 30 taxa of Holarctic hylid frogs were examined using starch gel electrophoresis. Allelic variation was scored at 33 presumed genetic loci and genetic distance data were used to construct trees representing the phylogenetic relationships. There are two major groups: (A) *Hyla regilla, H. cadaverina, H. crucifer, Limnaoedus ocularis,* and all presently recognized species of *Pseudacris;* and (B) the remaining species of Holarctic *Hyla.* The positions of *Acris* and *H. meridionalis* are unclear. In general, the relationships based on electrophoretic data agree well with immunological, hybridization, and chromosome studies, but conflict with some morphological studies. A phylogenetic classification requires that *H. regilla, H. cadaverina, H. crucifer,* and *L. ocularis* be transferred to the genus *Pseudacris.* This genus is characterized by terrestrial species that breed during cold weather and possess small digital pads and spherical (or ovoid) dark testes. Holarctic *Hyla* are arboreal species that breed during warm weather and possess large digital pads and elongate white (or yellowish) testes. The correlation between albumin immunological distance (*AID*) and Nei's electrophoretic distance (*D*) is significant, with $1 D = 70 AID$. Calibration of the molecular clock for Holarctic hylid frogs suggests that *Pseudacris* diverged from a *Hyla*-like ancestor in the early Tertiary. The physiological traits associated with cold-weather breeding and the morphological trait (small digital pads) associated with terrestrial habits appear to have been key innovations that allowed *Pseudacris* to undergo an adaptive radiation in North America coinciding with the radiation of Holarctic *Hyla.* [Electrophoresis; phylogeny; genetic distance; molecular clock; Hylidae; *Acris; Hyla; Limnaoedus; Pseudacris.*]

Molecular techniques such as electrophoresis, microcomplement fixation, and DNA-DNA hybridization have become important tools in systematic research. However, techniques other than sequencing are indirect in their assessment of genetic differences. Redundancy of the genetic code, differences between reciprocal comparisons, and convergence in electrophoretic mobility are a few of the sources of error that may influence the results of these methods. One approach to revealing the strengths and weaknesses of molecular techniques is by comparing the results of two or more techniques applied to the same taxonomic group. Concordant results between two independent molecular data sets suggest that both may be providing a good estimate of relationships. For example, the correlation between electrophoretic distance and albumin immunological distance is usually highly significant, although slopes vary among taxonomic groups (Wyles and Gorman, 1980). In one group of salamanders (*Plethodon*), there are strong correlations among data obtained by electrophoresis, DNA-DNA hybridization, and microcomplement fixation (Macgregor et al., 1973; Highton and Larson, 1979; Maxson and Maxson, 1979). Primates have been extensively studied using several molecular techniques and the results have been generally congruent (Sibley and Ahlquist, 1984; and references therein).

The purpose of this study is to examine the relationships of Holarctic hylid frogs using electrophoresis. This group comprises about 35 described species of arboreal, terrestrial, and semiaquatic frogs that have been placed in four genera: *Acris* (2 species), *Hyla* (25), *Limnaoedus* (1), and *Pseudacris* (7). Several independent lines of evidence suggest that these species form a monophyletic (or paraphyletic) group within the family Hylidae (ca. 640 species; Frost, 1985). First, all have 24 chromosomes except the two species of *Acris* (22) and the tetraploid *H. versicolor* (48). Other *Hyla* have 18, 20, 22, 24, and 30 chromo-

somes (Bogart, 1973). Second, the ability to produce hybrids in the laboratory is relatively high among species of this group as compared with a cross between a Holarctic and a 24-chromosome Neotropical species (Ralin, 1970). Third, immunological distances are lower and, thus, show closer relationship among Holarctic species than when those species are compared with Neotropical or Australian hylids, although some Middle American species appear to be closely related to the Holarctic assemblage (Maxson and Wilson, 1975; Maxson, 1977). Savage (1973) suggested that the Nearctic species arose from a Neotropical ancestor and in turn colonized the Palearctic region via Beringia. In addition to being morphologically well known, this group has been previously examined by microcomplement fixation (Maxson and Wilson, 1975), thus providing an opportunity to compare the results of two independent molecular techniques.

MATERIALS AND METHODS

Products of 33 presumed genetic loci were examined in 30 taxa of Holarctic hylid frogs. Frogs were collected in the field and obtained from colleagues (localities and sample sizes are given in Appendix 1; the average sample size per species is 4.3). The use of small samples can be justified as long as the number of loci is large, genetic distances among taxa are large, and average heterozygosity within species is low (i.e., <10%) (Sarich, 1977; Nei, 1978; Gorman and Renzi, 1979). Nei et al. (1983) found that 30 or more loci were necessary for estimating the phylogeny of a diverse group of species. Thus, the first criterion was satisfied. I will show below that the other two criteria, large distances and low heterozygosity, also were satisfied.

Heart, liver, kidney, skeletal muscle, and in some cases, intestines were removed from each individual and homogenized in distilled water at a ratio (water:tissue) of 3:1 (justification for combining tissue is given below under *protein homology*). Dilutions of up to 6:1 were used with small individuals (e.g., *Limnaoedus ocularis*). Any food present in the intestinal tract was discarded before homogenization. After centrifugation at 2°C for 20 min and 10,000 rpm, the aqueous protein extracts were stored at −75°C. Starch gel electrophoresis was performed in the laboratory of Richard Highton, University of Maryland, using Sigma starch (S4501) at a concentration of 12.5%. Buffers were prepared following the methods of Selander et al. (1971). The loci examined, electrophoretic conditions, and stain recipes used are listed in Table 1. Assays for the following proteins were modified: ACP, 30 mg 4-methylumbelliferyl phosphate was dissolved in 20 ml 0.1 M acetic acid (pH 5.5); AK, this locus was scored on the gel assayed for pyruvate kinase, ADH, 0.8 ml absolute ethanol was substituted for 0.3 ml; CD, 10 mg 4-methylumbelliferyl acetate was dissolved in 0.75 ml acetone and combined with 50 ml 0.5 M acetate (pH 5.2); ES, 25 ml 0.2 M monobasic Na phosphate (pH 4.4), 5 ml 0.2 M dibasic Na phosphate (pH 8.7), 20 ml water, 20 mg Fast Garnet GBC, and 0.75 ml alpha-naphthyl beta-naphthyl acetate (in acetone); ICD, MnCl was not used; LDH, 20 ml water, 20 ml 0.2 M tris HCl (pH 8.0), 1 ml 0.5 M lithium lactate, 13 mg NAD, 5 mg NBT, and 5 mg PMS; MDH, 30 ml 0.2 M tris HCl (pH 8.0), 5 ml 2.0 M D-L malate, 20 mg NAD, 10 mg NBT, 10 mg MTT, and 5 mg PMS; DPEP, 25 ml 0.2 M tris HCl (pH 8.0), 40 mg D-leucylalanine, 10 mg *Crotalus atrox* venom (Sigma V7000), 20 mg peroxidase, 10 mg o-dianisidine applied as a 2% agar overlay (25 ml); PT (general protein), 112.5 mg coomassie blue R250 dissolved in 2,475 ml acetic acid/methanol/water (1:5:16) for stock solution; SOD, seen on many gels but scored on gels assayed for malic enzyme; XDH, 50 mg substrate was used.

All differences in electrophoretic mobility were assumed to be of genetic origin and inherited in a Mendelian fashion. Allelomorphs were given alphabetic designations starting with the one most anodal. When more than one locus was visible on a gel, they were numbered in order from cathode to anode. All differences in mobility were confirmed in side-by-side comparisons with electromorphs of similar

TABLE 1. Protein loci and electrophoretic conditions.

Protein[a]	Locus	Enzyme Commission number[a]	Electrophoretic conditions[b]	Stain reference[c]
Acid phosphatase	Acp	3.1.3.2	5	*
Adenylate kinase	Ak	2.7.4.3	1	1
Alcohol dehydrogenase	Adh	1.1.1.1	6	2*
Aminopeptidase	Apep	3.4.11.1	4	3
Aspartate aminotransferase	Aat-1	2.6.1.1	1, 2	3
Aspartate aminotransferase	Aat-2	2.6.1.1	1, 2	3
Carbonate dehydratase	Cd	4.2.1.1	6	*
Creatine kinase	Ck	2.7.3.2	6	2
Cytochrome b₅ reductase	Cr	1.6.2.2	1	2
Dipeptidase	Dpep	3.4.13.11	4	*
Esterase	Es-1	3.1.1.1	6	*
Esterase	Es-2	3.1.1.1	6	· *
Glucosephosphate isomerase	Gpi	5.3.1.9	5	3
Glutamate dehydrogenase	Glud	1.4.1.3	1	4
Glutathione reductase	Gsr-2	1.6.4.2	6	2
Glycerol-3-phosphate dehydrogenase	Gpd	1.1.1.8	5	3
Isocitrate dehydrogenase	Icd-1	1.1.1.42	1	3*
Isocitrate dehydrogenase	Icd-2	1.1.1.42	1	3*
Lactate dehydrogenase	Ldh-1	1.1.1.27	3	*
Lactoyl-glutathione lyase	Lgl	4.4.1.5	6	2
Malate dehydrogenase	Mdh-1	1.1.1.37	2	3*
Malate dehydrogenase	Mdh-2	1.1.1.37	2, 3	3*
Malate dehydrogenase (NADP-dependent)	Me	1.1.1.40	3, 5	5
Mannosephosphate isomerase	Mpi	5.3.1.8	5	5
Phosphoglucomutase	Pgm	2.7.5.1	2	3
Phosphogluconate dehydrogenase	Pgd	1.1.1.44	5	3
Protein 1	Pt-1	—	3, 4	*
Protein 2	Pt-2	—	3, 4	*
Protein 3	Pt-3	—	3, 4	*
Pyruvate kinase	Pk	2.7.1.40	1	1
Superoxide dismutase	Sod	1.15.1.1	3	*
Xanthine dehydrogenase	Xdh-1	1.2.1.37	6	3*
Xanthine dehydrogenase	Xdh-2	1.2.1.37	6	3*

[a] Nomenclature Committee of the International Union of Biochemistry (1979).

[b] (1) Tris-citrate pH 8.0, 130 V, 5 h; (2) Tris-citrate pH 6.7, 140 V, 5 h; (3) Poulik, 240 V, 4 h; (4) Lithium hydroxide, 325 V, 5 h; (5) Tris-versene-borate, 200 V, 5 h; (6) Tris-HCl, 200 V, 3 h.

[c] (1) Buth and Murphy (1980); (2) Harris and Hopkinson (1976); (3) Selander et al. (1971); (4) Shaw and Prasad (1970); (5) Siciliano and Shaw (1976). An asterisk indicates that a technique is included in the text.

mobility, this procedure accounting for a majority of the electrophoretic runs.

Protein homology.—A common misconception concerning multilocus enzyme systems is that homologies cannot be determined unless specific tissues (heart, liver, kidney, and skeletal muscle) are run separately. In most cases, this is not true. Although it is known that some enzymes are restricted to certain tissues, there are many ways of determining protein homology other than by tissue specificity. For example, Holarctic hylids possess two isozymes of lactate dehydrogenase: one is found mostly in skeletal muscle (*Ldh-1*); the other is found mostly in the heart

(*Ldh-2*). When skeletal muscle and heart homogenates are run separately and stained for LDH, the differential in enzyme concentration of the respective tissues results in only one of the two isozymes being predominantly expressed. When heart, liver, kidney, and skeletal muscle are mixed (as in this study), both isozymes of LDH appear on the gel but the homologies are still clear: *Ldh-2* bands are considerably lighter because heart makes up a smaller proportion of the total homogenate than skeletal muscle (Fig. 1). In other cases, cell fractionation methods (Henderson, 1965) can be used to isolate isozymes that occur at higher concentra-
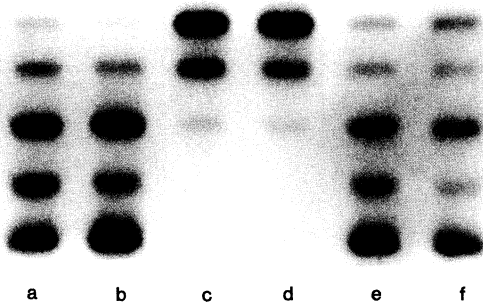
FIG. 1. Electrophoretic banding patterns of lactate dehydrogenase in two individuals (a, c, e; and b, d, f) of *Pseudacris regilla* illustrating protein homology. (a and b) Skeletal muscle homogenate: dark bands at bottom are gene products of *Ldh*-1 and faint bands at top are gene products of *Ldh*-2; the three intermediate bands represent heteropolymers of the gene products of the two presumed loci. (c and d) Heart homogenate: *Ldh*-2 predominates and *Ldh*-1 is barely visible. (e and f) Mixture of heart, liver, kidney, and muscle homogenates: *Ldh*-1 and *Ldh*-2 are both present but are easily distinguished due to differential band intensity.

tions in mitochondria than in the cytoplasm. Color differences also help in distinguishing protein homologues (Webster, 1973). Probably the most common means of assessing protein homology is by their relative separation on the gel. For example, when two loci are present and the allelomorphs of each locus vary within a zone about 10 mm wide, yet the two "zones" are separated by 100 mm of gel, homology can usually be assumed.

Despite these methods for determining homology, there will still be some multilocus systems that pose problems. In this study, malate dehydrogenase (MDH) and NADP-dependent malate dehydrogenase (ME) each possessed two loci and fell into this category. I determined that if assays for each enzyme system were performed on a Poulik gel, only one locus of each enzyme system would appear. Using two buffer systems, both MDH loci were accurately scored. Only one locus of ME was scored (on Poulik) due to difficulties with resolving the other locus in some species when the two occurred together (on TVB). In this study, *Ldh*-2 was not resolvable in some taxa so that locus was omitted.

Genetic distance analysis.—Farris (1981)

has criticized the use of genetic distance measures, arguing that some data are non-metric and sometimes result in inferred ancestors that could not have existed. Although these are valid criticisms and represent a weakness of genetic distance data, they are not major problems unless genetic distances are interpreted as absolute path lengths. If they are interpreted as "expected" distances subject to stochastic error (Nei et al., 1983; Felsenstein, 1984), they can be useful in estimating the phylogenetic relationships of organisms. Under the expected distance interpretation, even nonmetric measures that sometimes result in negative branch lengths are explainable in terms of stochastic error. However, the assumptions of additivity and independence should still be met by these measures under this interpretation (Felsenstein, 1984).

There are currently at least 10 different genetic distance measures available for electrophoretic data (Swofford and Selander, 1981). In addition, there are several tree-building methods commonly used with genetic distances: Fitch and Margoliash (1967); UPGMA (Sneath and Sokal, 1973); distance-Wagner (Farris, 1972); and modified versions of the distance-Wagner (Swofford, 1981; Tateno et al., 1982). This necessitates a judgement as to which combination of distance measure and tree-building method is best.

Recently, Nei et al. (1983) assessed the abilities of several electrophoretic genetic distance measures and tree-building methods at estimating a "known" phylogeny using computer simulations and assuming a constant rate of molecular evolution. For obtaining an accurate topology, a modified Cavalli-Sforza distance combined with the UPGMA method or a modified version of the distance-Wagner method (Tateno et al., 1982) performed best. For estimating branch lengths, Nei's standard distance (Nei, 1972) and the UPGMA method was the best combination. Unlike most other distance measures, Nei's standard distance (D) has a nearly linear relationship with the number of amino-acid substitutions (Nei, 1972).

Since neither a constant nor variable rate of protein evolution could be assumed, both the UPGMA and distance-Wagner methods were used to construct phylogenetic trees from the genetic distance data. The modified Cavalli-Sforza distance was used with the UPGMA method because of its superior performance in computer simulations (Nei et al., 1983). The original Cavalli-Sforza and Edwards (1967) chord distance was used with the distance-Wagner method because it is a metric that agrees with Wright's (1978:102) criteria for an optimal distance measure. The distance-Wagner tree was constructed using the multiple-addition criterion (maxtree = 30) and rooted at the midpoint of the longest path (Swofford, 1981). An additional tree was produced using Nei's unbiased distance (a modified version of D for small sample sizes; Nei, 1978) and the UPGMA method to obtain rough estimates of divergence times. All trees were produced using the BIOSYS-1 program (Swofford and Selander, 1981). This program was modified to incorporate the Cavalli-Sforza distance used by Nei et al. (1983).

Goodness-of-fit.—One way to compare trees generated by genetic distance data is by examining their goodness-of-fit measures. When trees to be compared are generated using the same clustering method, this is an objective way of determining the tree that best represents the data. However, when different methods are involved, each operating under a different set of assumptions, comparison of goodness-of-fit statistics may not be appropriate. This point was made by Swofford (1981), who realized that methods which allow negative homoplasies (e.g., UPGMA) have an unfair advantage in terms of goodness-of-fit over those that do not (distance-Wagner). For this reason, he described an optimization procedure that allows negative homoplasies in the generation of distance-Wagner trees so that they could be objectively compared with other trees. Unfortunately, there are other assumptions associated with these methods, particularly the constant-rate assumption of the UPGMA method, that still reduce

the effectiveness of goodness-of-fit measures in comparing different trees (Nei et al., 1983). They will be used here simply as a means of determining which tree best represents the data but not necessarily which is the best tree, the latter decision depending on the assumption one wishes to invoke. Several different goodness-of-fit statistics are available to compare trees (Swofford, 1981), but all are highly correlated so only one is used here—Prager and Wilson's (1976) F statistic.

Least-squares regression lines, product-moment correlation coefficients, and matrix correlation coefficients (Sneath and Sokal, 1973) were computed for the comparison between D and AID. Levels of significance for the matrix correlations were calculated using the Mantel test (Schnell et al., 1985:appendix). All goodness-of-fit statistics and measures of variability for the electrophoretic data were obtained using BIOSYS-1.

RESULTS

There were 306 alleles identified at 33 presumed genetic loci. Only four loci were monomorphic (*Adh, Cr, Gsr-2,* and *Pt-3*). Estimates of average heterozygosity across all 33 loci ranged from zero in *H. arborea, H. cadaverina,* and *P. s. illinoensis* to 13% (SE = 5.0) in *P. nigrita* with a mean of 5.3% (SE = 3.55) among all taxa. Of the taxa represented by one or two individuals, only one, *P. s. streckeri* (12.1%, SE = 5.8) exceeded 10% average heterozygosity. The percentage of polymorphic loci ranged from 0 to 33 with a mean of 11.5. Allelic variation in all taxa for the 29 polymorphic loci is given in Appendix 2. Cavalli-Sforza and Edwards' chord distances (D_C) and modified Cavalli-Sforza distances (D_A) are presented in Table 2.

Phenograms constructed by UPGMA clustering of D_A and D are identical in topology and, therefore, only the former is presented (Fig. 2). Two large groups are defined: one group (A) contains *Hyla regilla, H. cadaverina, H. crucifer, Limnaoedus ocularis,* and all *Pseudacris* taxa, whereas the other group (B) contains *A. crepitans, A. gryllus,* and all remaining Holarctic *Hyla*.

TABLE 2. Cavalli-Sforza and Edwards (1967) chord distances above diagonal and modified Cavalli-Sforza distances (Nei et al., 1983) below diagonal for all pairs of taxa.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13
1 feriarum		.33	.34	.52	.53	.59	.61	.63	.67	.65	.68	.68	.72
2 kalmi	.14		.36	.51	.52	.57	.63	.58	.63	.60	.67	.67	.71
3 brachyphona	.14	.16		.50	.53	.58	.60	.64	.66	.63	.67	.67	.72
4 triseriata	.33	.32	.31		.59	.63	.58	.67	.70	.69	.71	.71	.75
5 brimleyi	.34	.34	.35	.43		.63	.63	.64	.65	.63	.65	.65	.70
6 nigrita	.44	.40	.42	.49	.49		.59	.65	.63	.67	.69	.68	.72
7 clarki	.46	.48	.44	.42	.50	.43		.63	.67	.70	.72	.70	.70
8 crucifer	.48	.42	.51	.55	.51	.53	.49		.64	.63	.66	.64	.73
9 ocularis	.55	.48	.54	.61	.53	.48	.55	.51		.62	.68	.67	.72
10 ornata	.53	.45	.49	.59	.50	.56	.60	.49	.48		.45	.46	.68
11 streckeri	.58	.55	.56	.62	.51	.60	.64	.53	.58	.24		.32	.71
12 illinoensis	.58	.55	.55	.62	.51	.57	.61	.51	.56	.26	.13		.69
13 regilla	.63	.63	.63	.70	.60	.64	.61	.65	.63	.58	.62	.59	
14 cadaverina	.64	.64	.64	.70	.64	.67	.64	.71	.65	.60	.61	.61	.37
15 arborea	.79	.79	.79	.82	.76	.82	.82	.72	.79	.72	.70	.70	.76
16 japonica	.76	.72	.76	.79	.73	.77	.79	.75	.68	.68	.70	.70	.70
17 andersoni	.73	.74	.76	.79	.73	.76	.79	.77	.76	.70	.70	.70	.79
18 ogechiensis	.76	.74	.76	.79	.72	.79	.79	.76	.72	.67	.67	.67	.79
19 avivoca	.76	.74	.76	.79	.72	.79	.79	.76	.72	.67	.67	.67	.79
20 versicolor	.76	.74	.75	.79	.68	.79	.77	.76	.69	.67	.66	.66	.77
21 chrysoscelis	.76	.72	.76	.79	.69	.79	.79	.76	.68	.66	.67	.67	.77
22 meridionalis	.67	.63	.67	.70	.64	.73	.73	.67	.64	.58	.60	.63	.67
23 arenicolor	.70	.65	.70	.71	.67	.71	.73	.70	.68	.64	.67	.64	.67
24 eximia	.70	.65	.70	.76	.67	.72	.76	.69	.65	.55	.59	.59	.59
25 femoralis	.70	.70	.70	.70	.66	.73	.67	.70	.66	.65	.64	.64	.73
26 gryllus	.61	.60	.60	.65	.58	.60	.64	.60	.62	.52	.52	.52	.58
27 crepitans	.61	.61	.61	.64	.58	.63	.67	.61	.60	.57	.55	.55	.64
28 squirella	.66	.67	.67	.68	.64	.70	.70	.69	.61	.64	.64	.64	.69
29 gratiosa	.69	.69	.70	.73	.67	.73	.73	.72	.64	.65	.65	.65	.76
30 cinerea	.70	.67	.70	.73	.67	.68	.70	.73	.61	.67	.67	.67	.76

In the former, the most distant division separates a cluster containing H. regilla and H. cadaverina and one containing the remaining taxa in group A. Of those latter taxa, P. ornata, P. s. streckeri, and P. s. illinoensis form one cluster; H. crucifer, L. ocularis, and the remaining Pseudacris taxa form another cluster. Within group B, the most distant taxon is H. arborea. The other taxa form two large subgroups: one contains H. andersoni, H. a. avivoca, H. a. ogechiensis, H. chrysoscelis, H. versicolor, and H. japonica; the other contains the remaining taxa in group B. Within the former subgroup, H. japonica is the most distant taxon; H. andersoni clusters with H. a. avivoca and H. a. ogechiensis; that cluster, in turn, joins one containing H. versicolor and H. chrysoscelis. In the other subgroup of group B, H. meridionalis is the most distant taxon; H. eximia and H. arenicolor form a cluster and join with another cluster containing H. femoralis, Acris (crepitans and gryllus), and a unit containing H. squirella, H. gratiosa, and H. cinerea.

The phylogenetic tree produced by the distance-Wagner method (Fig. 3) is similar overall to the UPGMA tree (Fig. 2), yet it differs in a few details. Group A defined in the UPGMA tree now includes a subgroup containing H. meridionalis and the two species of Acris. In the optimized version of this tree (not shown), the topology is identical except that these three species are in Group B. Another difference between Figures 2 and 3 is that the branching order of the cluster containing H. cadaverina and H. regilla and the one containing P. s. streckeri, P. s. illinoensis, and P. ornata is reversed. Also, H. crucifer clusters with P. nigrita and P. clarki in Figure 3; P. t. feriarum clusters with P. brachy-

TABLE 2. Continued.

14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
.72	.80	.78	.77	.78	.78	.78	.78	.74	.75	.75	.75	.70	.70	.73	.75	.75
.72	.80	.76	.77	.77	.77	.78	.77	.71	.72	.72	.75	.70	.70	.74	.75	.74
.72	.80	.78	.78	.78	.78	.78	.78	.74	.75	.75	.75	.70	.70	.74	.75	.75
.75	.81	.80	.80	.80	.80	.80	.80	.75	.76	.78	.75	.72	.72	.74	.77	.77
.72	.78	.77	.77	.76	.76	.74	.75	.72	.74	.74	.73	.68	.68	.72	.74	.74
.74	.81	.79	.79	.80	.80	.80	.80	.77	.76	.76	.77	.70	.71	.75	.77	.74
.72	.81	.80	.80	.80	.80	.79	.80	.77	.77	.78	.74	.72	.74	.75	.77	.75
.76	.76	.78	.79	.79	.79	.78	.78	.74	.75	.75	.75	.70	.70	.75	.77	.77
.73	.80	.74	.78	.76	.76	.75	.74	.72	.74	.73	.73	.71	.70	.70	.72	.70
.70	.76	.74	.75	.74	.74	.74	.73	.69	.72	.67	.73	.65	.68	.72	.73	.74
.70	.75	.75	.75	.74	.74	.73	.74	.70	.74	.69	.72	.65	.67	.72	.73	.74
.70	.75	.75	.75	.74	.74	.73	.74	.71	.72	.69	.72	.65	.67	.72	.73	.74
.55	.78	.75	.80	.80	.80	.79	.79	.74	.74	.69	.77	.69	.72	.75	.78	.78
	.80	.77	.78	.78	.78	.78	.78	.75	.75	.74	.75	.75	.75	.77	.78	.77
.79		.68	.72	.72	.72	.71	.72	.68	.72	.70	.74	.71	.72	.72	.74	.74
.73	.58		.69	.67	.67	.65	.63	.71	.67	.71	.75	.69	.68	.70	.67	.70
.76	.64	.58		.46	.45	.50	.49	.74	.71	.74	.68	.72	.73	.72	.66	.68
.76	.65	.55	.26		.11	.49	.47	.72	.69	.72	.70	.74	.74	.72	.67	.68
.76	.64	.55	.25	.02		.49	.47	.73	.70	.72	.70	.74	.74	.72	.67	.68
.76	.63	.52	.31	.30	.30		.28	.72	.69	.69	.69	.69	.69	.68	.64	.66
.76	.64	.49	.30	.27	.27	.10		.72	.68	.68	.68	.67	.67	.67	.62	.65
.70	.58	.63	.67	.65	.65	.64	.64		.72	.67	.70	.67	.65	.67	.67	.68
.70	.64	.55	.62	.59	.60	.59	.57	.64		.62	.61	.64	.65	.65	.66	.67
.67	.61	.62	.67	.64	.64	.59	.58	.55	.47		.70	.65	.69	.70	.69	.67
.70	.67	.70	.58	.61	.61	.59	.58	.61	.46	.61		.63	.63	.57	.59	.63
.69	.61	.58	.64	.67	.67	.58	.55	.55	.50	.52	.49		.32	.56	.59	.67
.70	.64	.58	.66	.67	.67	.58	.55	.52	.53	.58	.49	.13		.55	.57	.61
.73	.64	.61	.64	.64	.64	.58	.55	.55	.52	.61	.40	.39	.37		.45	.54
.76	.67	.56	.54	.55	.55	.51	.47	.55	.54	.59	.43	.43	.40	.25		.45
.73	.67	.61	.58	.58	.58	.53	.52	.58	.55	.56	.49	.55	.46	.37	.25	

phona, instead of *P. t. kalmi*. In group B, the only difference is that *H. arborea* now clusters, although distantly, with *H. japonica*.

Prager and Wilson's (1976) F values for each of the three trees are: UPGMA of D_A (Fig. 2), 6.70; UPGMA of D (not shown), 12.7; unoptimized distance-Wagner (Fig. 3), 6.62; optimized distance-Wagner (not shown), 2.03. Thus, the UPGMA tree (Fig. 2) and the unoptimized distance-Wagner tree (Fig. 3) have virtually identical fit measures but the best-fit tree is the optimized distance-Wagner. Of the two UPGMA trees, the one using D_A values (Fig. 2) has a considerably better fit than the one using D values, yet they are identical in topology. Although the distance-Wagner tree has the best fit, it may not be the best estimate of the evolution of this group using electrophoretic data. If the rate of protein evolution in hylid frogs was known to be relatively constant, the UPGMA tree (Fig. 2) might be a better "species tree" even though it is not the best "gene tree" (Nei et al., 1983). In fact, the evenness of branch lengths in Figure 3 suggests that the rate of protein evolution, among lineages of this group, is not highly variable.

DISCUSSION

Two major groups of Holarctic hylid frogs are indicated by the electrophoretic data. Although the positions of *A. crepitans*, *A. gryllus*, and *H. meridionalis* are unclear, all other Holarctic species can be placed in one of the two groups by genetic distance data analyzed both phenetically (Fig. 2) and cladistically (Fig. 3). In addition to electrophoretic data, these two groups (A and B) are distinctive in other ways. Albumin immunological data iden-
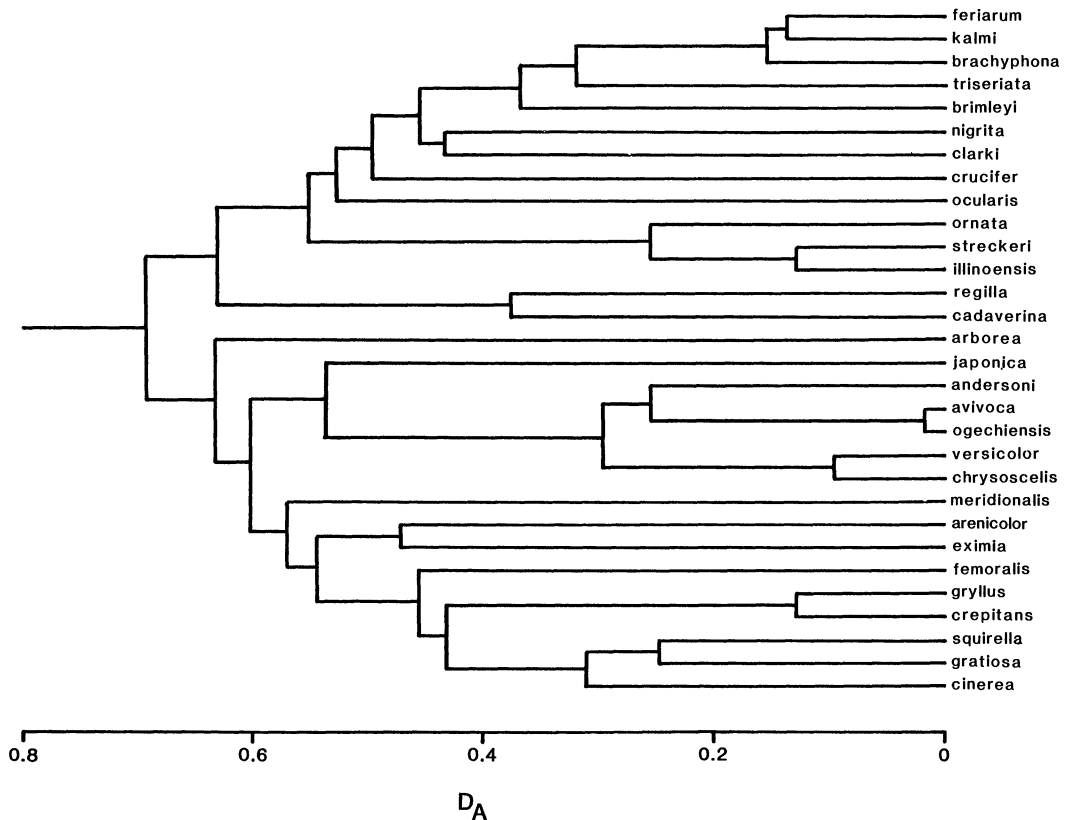
FIG. 2. Phylogenetic tree constructed by UPGMA clustering of modified Cavalli-Sforza distances. Prager and Wilson's (1976) F value = 6.70.

tify *H. crucifer, H. regilla,* and *H. cadaverina* as being closer to *Pseudacris* species than to other *Hyla* (Maxson and Wilson, 1975). Also, Mecham (1965) and Ralin (1970) both found that *H. crucifer* hybridizes more readily with *Pseudacris* than with other Holarctic *Hyla*. Karyotypically, *H. crucifer* has secondary constrictions and arm ratios unique to *Pseudacris* and not found in southeastern *Hyla* (Wiley, 1982). In addition, the mating calls of *H. crucifer* and *Limnaoedus ocularis* are similar to the calls of some species of *Pseudacris* (Blair, 1958).

Most group A species have relatively small digital pads and possess spherical (or ovoid) testes surrounded by darkly pigmented peritoneum (Ralin, 1970), whereas nearly all group B species have large digital pads and elongate, white or yellowish testes. The exceptions in group A are *H.*

cadaverina, H. crucifer, and *H. regilla,* all of which have digital pads that are intermediate in size. The only exceptions in group B are *H. eximia* (intermediate digital pads), *H. meridionalis* (ovoid dark testes), and *Acris* (small digital pads and spherical, dark testes). Although some individuals of *H. avivoca* have dark testes (K. Anderson, pers. comm.), they are elongate like other group B species and are white in other individuals. Testes of some other hylid species related to the Holarctic assemblage but not included in this study (*Anotheca spinosa, H. euphorbiacea, H. plicata,* and *H. savignyi*) are elongate and white.

Finally, there are ecological similarities which link the species in each of these two groups. Nearly all group A species are terrestrial and breed during cold weather (winter and early spring). In his account
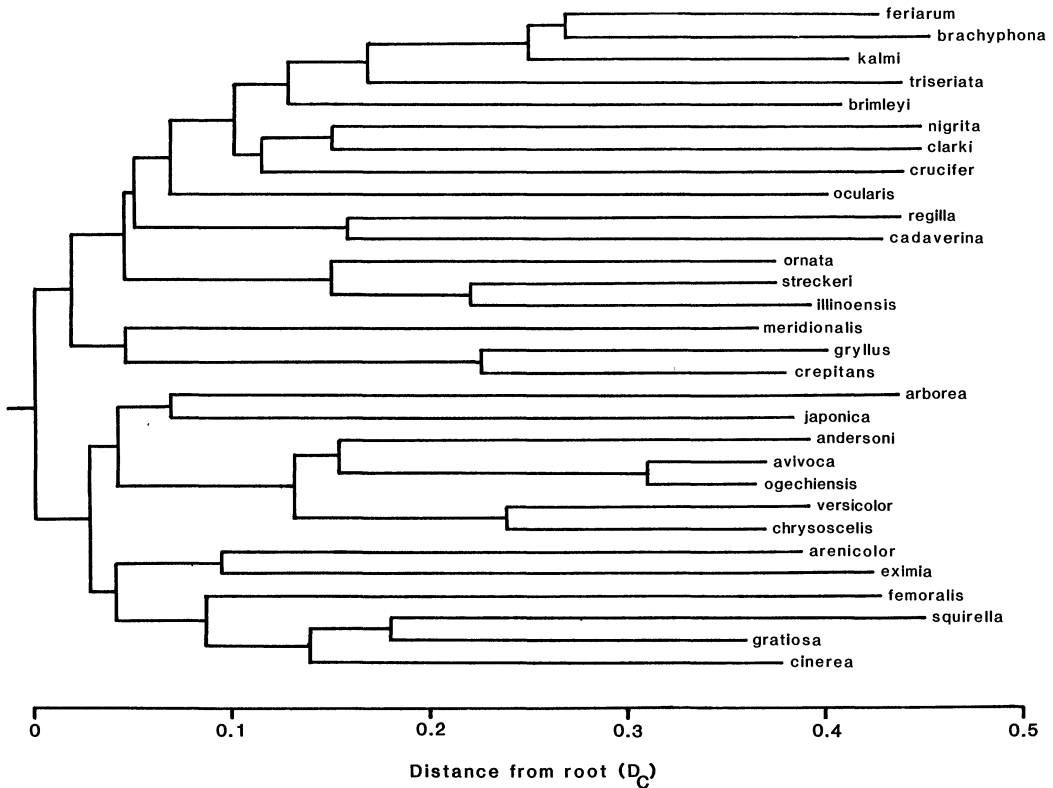
FIG. 3. Phylogenetic tree constructed by distance-Wagner method (Farris, 1972) using the multiple-addition criterion (Swofford, 1981) and rooted at the midpoint of the longest path. The distance measure used is the Cavalli-Sforza and Edwards (1967) chord distance. Prager and Wilson's (1976) F value = 6.62.

of *H. regilla*, A. H. Wright (Wright and Wright, 1949) noted similarities between that species and *Pseudacris* and remarked (p. 335) "the early season and the chorus each make me think of *Pseudacris* choruses in early Spring." In the southwestern United States and adjacent Mexico, *H. cadaverina* breeds later than *H. regilla* (late winter to late spring), although at "temperatures much cooler than for *arenicolor*," a species of group B (Gorman, 1960). *Limnaoedus ocularis* breeds during cold or warm weather (Conant, 1975). In contrast, all group B species (where their ecologies are known) are warm-weather breeders (late spring and summer).

Despite the considerable evidence supporting electrophoretic groups A and B, some data are not in complete agreement with this arrangement. Ralin (1970) found

that *Acris*, *H. regilla*, and *H. cadaverina* hybridize poorly with other species of Holarctic hylids. Also, Duellman's (1970) *H. eximia* group contained two group A species (*H. regilla* and *H. cadaverina*) and one group B species (*H. eximia*). His grouping was based on similarities in external morphology, osteology, tadpoles, and mating call. Other evidence not supporting the phylogenetic arrangement proposed here comes from osteological studies. All four genera of Holarctic hylids are osteologically distinct (Chantell, 1968a, b). Gaudin (1974) performed a phenetic study of 20 osteological characters in 32 Holarctic hylid species, concluding that: (1) *L. ocularis* was the most distinct of the 32 species; (2) *Limnaoedus* is more similar to *Acris* and least similar to *Hyla*; (3) *Pseudacris* and *Acris* are more closely related than
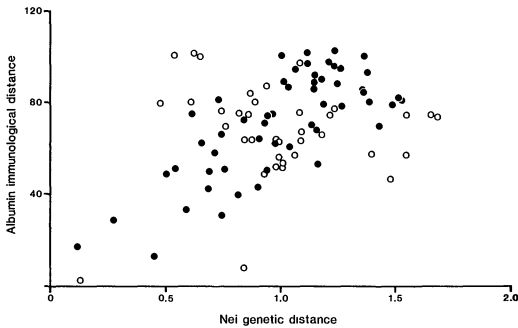
FIG. 4. The relationship between albumin immunological distance (from Maxson and Wilson [1975] and Maxson [1978]) and Nei's genetic distance (this study) in Holarctic hylid frogs. Open circles are distances involving *Acris crepitans, A. gryllus,* and *Hyla arborea* (see text); closed circles are distances involving other species ($r_p = 0.46$, $n = 91$ for all data; $r_p = 0.75$, $n = 54$ for data represented by closed circles).

either is to *Limnaoedus* or *Hyla;* and (4) *H. crucifer* occupies "a position linking *Pseudacris* to the other Holarctic *Hyla.*" These conclusions, except for (1), are contradicted by both his data and phenogram (Gaudin, 1974:fig. 11). According to his phenogram, *H. crucifer* is more similar to other *Hyla* than to *Pseudacris,* and not a "link" between the two genera. Also, *Pseudacris* is more similar to *Hyla* than to *Acris* (not the reverse) and *Acris* is more similar to all other species than to *Limnaoedus.* In six of the seven osteological characters distinguishing *Limnaoedus* from all other Holarctic hylids, the character state possessed by *Limnaoedus* was a reduction or loss of a structure. This condition might be expected in one of the smallest frogs in the world and is phylogenetically uninformative.

The phylogenetic relationships proposed here indicate that two seemingly minor morphological characters, testis shape and color, are taxonomically useful. This was suggested by Ralin (1970, 1977) but has not been recognized by other authors. The extent of variation in these characters among other hylid species is largely unknown, thus making it difficult to establish polarity. West Indian hylids (*Calyptahyla, Hyla,* and *Osteopilus*) have white or yellowish (green in *H. heilprini*) elongate testes and none of those species

are closely related to the Holarctic assemblage (Trueb and Tyler, 1974; Maxson and Wilson, 1975; Hedges, unpubl.). This suggests that the spherical or ovoid dark testes of the group A species represent synapomorphic character states and that *Acris* and *H. meridionalis* may belong in group A as suggested by Figure 3. Another morphological character that is in agreement with the relationships proposed here is digital pad size. Since all hylid frogs possess disc-shaped intercalary cartilages between the terminal and penultimate phalanges and most possess clawlike terminal phalanges (Duellman, 1970), presumably adaptations for arboreality (Greene, 1979), it is likely that the ancestral hylid was arboreal. This suggests that the large digital pads of arboreal species are plesiomorphic, whereas the small digital pads possessed by terrestrial hylids are apomorphic. If true, it would provide additional support for including *Acris* with group A because of its small digital pads.

Convergence.—Convergence and parallel evolution are not uncommon phenomena in amphibian evolution as evidenced by most molecular studies on this group (e.g., Maxson and Wilson, 1974; Maxson, 1977). The remarkable convergence between *H. regilla* and *H. eximia* detected by immunology (Maxson and Wilson, 1974) was confirmed by electrophoresis (Case et al., 1975; this study). Another equally striking case of convergence involves *H. andersoni.* This green treefrog resembles another species found in eastern North America, *H. cinerea,* in both morphology and in mating call (Blair, 1958). In a paper describing parallel evolution and convergence in Holarctic hylids, Jameson and Richmond (1971) concluded that these striking resemblances indicated relatedness and *not* convergence: "The measurements alone are not indicative of the relationship apparent from five minutes of observation of how they sit, stand, jump, and look when alive." Chromosome data first indicated that these two species are not closely related but convergent (Wiley, 1982). My results (Figs. 2, 3) support this conclusion.

The morphological similarity among the Palearctic species (*arborea, japonica,* and *meridionalis*), although not as striking, is indicated by the fact that all three were considered conspecific until relatively recently (Ralin, 1970, 1977). The results presented here show that these three species are not closely related to each other or to other Holarctic hylids (albumin immunological data [Maxson, 1978] indicate a closer relationship between *H. arborea* and *H. japonica*). In general, the independence of morphological and molecular evolution (Wilson et al., 1977) suggests that many more cases of convergence and parallel evolution will be found as additional groups are studied using molecular techniques.

Taxonomic implications.—Several taxonomic changes are necessary to produce a classification of Holarctic hylid frogs that is in closer agreement with their phylogenetic relationships proposed here. *Hyla cadaverina, H. crucifer, H. regilla,* and *L. ocularis* are all transferred to the genus *Pseudacris,* which now corresponds to group A. The type species of *Hyla* is *arborea* (Duellman, 1970:173) and, thus, group B species retain that generic name. Since the exact relationship of *Acris* and *H. meridionalis* to other Holarctic hylids is unclear, no taxonomic change is proposed here for those taxa.

These taxonomic changes do not solve all of the problems of paraphyly (or polyphyly) in the genus *Hyla*. There are many additional species of *Hyla* in Middle and South America that are not closely related to the Holarctic assemblage (Duellman, 1970; Maxson and Wilson, 1975). Also, Maxson and Wilson (1975) and Maxson (1977) found that several genera of Middle American hylids are closely related to the Holarctic hylids. These include *Anotheca, Nyctimantis, Plectrohyla,* and several species of *Hyla* (*elaeochroa, hazalae, pseudopuma, siopela,* and *staufferi*). Most of these species appear to form a group on the basis of immunological data but their exact relationship to the Holarctic hylids must await further studies.

Within *Pseudacris,* the two currently recognized species groups (Mecham, 1965) are supported by this study: the *ornata* group includes *ornata* and *streckeri;* and the *nigrita* group includes *brachyphona, brimleyi, clarki, feriarum, nigrita,* and *triseriata*. In addition, I propose that the following three groups be added: the *crucifer* group, containing *Pseudacris crucifer* (new comb.); the *ocularis* group, containing *Pseudacris ocularis;* and the *regilla* group, containing *Pseudacris cadaverina* (new comb.) and *Pseudacris regilla* (new comb.). Ralin's (1970) recognition of *feriarum* as a full species is supported by my electrophoretic data. My findings also suggest that *illinoensis* and *kalmi* warrant recognition as full species, but that decision must await detailed studies on geographic variation within *streckeri* and *feriarum*. However, since *kalmi* is geographically and genetically more similar to *feriarum* than to *triseriata,* it should be tentatively recognized as a subspecies of the former species: *Pseudacris feriarum kalmi* (new comb.).

Within Holarctic *Hyla,* there is disagreement as to the number and composition of the species groups (Blair, 1958; Duellman, 1970; Ralin, 1970, 1977; Jameson and Richmond, 1971; Maxson, 1978; Wiley, 1982). Nonetheless, there is general agreement that: the *cinerea* group contains at least *cinerea, gratiosa,* and *squirella;* and the *versicolor* group contains at least *avivoca, chrysoscelis,* and *versicolor*. The electrophoretic data are in agreement with these "core" groups but suggest some changes. *Hyla femoralis* is here considered a distant member of the *cinerea* group, and the inclusion of *andersoni* in the *versicolor* group by Wiley (1982) is supported. A Palearctic species, *japonica,* is here considered a distant member of the *versicolor* group. No evidence was found for including *arenicolor* with the *versicolor* group (Blair, 1958) but, instead, I consider it a member of the *eximia* group which now includes *arenicolor, eximia, euphorbiacea, plicata,* and *walkeri*. The last three species were not examined in this study but *euphorbiacea* was shown to be closely related to *eximia* by Maxson
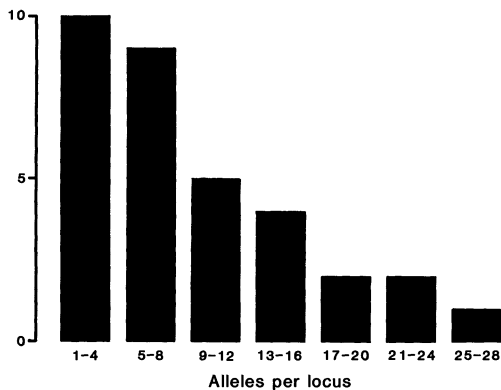
FIG. 5. Histogram of the number of alleles per locus at 33 loci in Holarctic hylid frogs.

and Wilson (1974) and Case et al. (1975). The remaining species, *arborea*, is not close to any of the other taxa examined and is here considered as a separate group.

Immunological distance versus genetic distance.—The product-moment correlation coefficient (r_p) for *AID* (Maxson and Wilson, 1975; Maxson, 1978) versus D (my study) is 0.46 ($n = 91$; Fig. 4). However, only a subset of those distance values form a square matrix and, thus, are statistically testable (Sokal, 1979; Schnell et al., 1985). The matrix correlation coefficient (r_m) for those data is 0.48 and is significant ($n = 45$; Mantel test statistic $[t] = 3.62$; $P < 0.01$). A major lack of congruence between the results of Maxson and Wilson's study and this study is in the placement of *Acris* and *H. arborea* (Swofford's [1981] reanalysis of their data is in better agreement with my results). If all data involving *Acris* and *Hyla arborea* are not included, a higher correlation coefficient is obtained ($r_p = 0.74$, $n = 54$; $r_m = 0.81$, $n = 28$, $t = 3.97$, $P < 0.001$).

Wyles and Gorman (1980) reviewed the comparison of *AID* and *D* in nine studies of vertebrates. Slope values, useful for calibrating the molecular clock between these two techniques, ranged from 22 to 55. The slope value for Holarctic hylid frogs using my electrophoretic data, adjusted to pass through the origin (and $\bar{x}$, $\bar{y}$) is 70 (71 with *Acris* and *Hyla arborea* removed). This is the highest slope value yet reported and is twice as high as the slope (35) obtained

by Case et al. (1975) with several species of *Hyla*. In their study, only 14 (or fewer) loci were used (some loci were omitted in some taxa due to technical difficulties), and the slope was calculated with several distances above $D = 2$. As Maxson and Maxson (1979) pointed out, D values above 2 are not very meaningful due to chance convergence of electromorph mobilities ("saturation effect"), making it difficult to compare the slope of Case et al. (1975) and the slope reported here.

Another factor that influences the slope of *AID*-versus-*D* comparisons is the proportion of "fast" and "slow" evolving loci used (Sarich, 1977). Fast loci are those that accumulate electrophoretically detectable substitutions at a greater rate than other, more slowly evolving loci. A greater proportion of fast-versus-slow loci will produce relatively greater electrophoretic distances and hence a lower slope in the *AID*-versus-*D* comparison (Sarich, 1977). Usually there are both invariant loci and variable loci in electrophoretic studies of a group of species. It has not been shown whether certain loci are highly variable in all groups and therefore can be designated as "fast-evolving." In fact, the suggestion by Sarich (1977) of a tenfold difference in the rate of electrophoretically detectable substitutions among loci can be tested. As long as both fast- and slow-evolving loci are included, a histogram of the number of alleles per locus should exhibit a bimodal distribution. This is not the case for Holarctic hylid frogs (Fig. 5). Also, if some loci are typically fast- or slow-evolving, there should be a significant correlation between the number of alleles per locus in any two studies.

Highton and Larson (1979) scored 317 alleles at 29 loci in 26 species of salamanders, thus providing a similar-sized group for comparison. The correlation between the number of alleles at 18 common (and presumably homologous) loci in Highton and Larson's study and my study is not significant ($r_p = 0.01$; $P > 0.05$). Thus, the concept of fast- and slow-evolving loci, as defined by Sarich (1977) does not appear to hold true for Holarctic hylid frogs. As

a result, the high slope value (70) for *AID*-versus-*D* in this study is not "adjustable" by computing the proportion of these loci used since fast- and slow-evolving loci cannot be identified. Instead, it is an indication that substitution rates, as measured by electrophoresis or microcomplement fixation (or both) differ among groups studied. As long as these differences occur among groups and not within groups, methods of analysis that assume rate constancy (UPGMA) may still prove useful.

Molecular clock estimates of divergence times.—If the molecular-clock hypothesis (Wilson et al., 1977; Thorpe, 1982) is in large part correct, it can be used to date divergence events in the evolutionary history of Holarctic hylid frogs. However, such a clock needs calibration. Nei (1972) originally suggested a calibration of 1 D = 5 Myr. In plethodontid salamanders, 1 D = 14 Myr has generally been used based on a calibration with the albumin clock (Maxson and Maxson, 1979). Smith and Coss (1984) calibrated the electrophoretic clock in ground squirrels based on fossil and geologic information at 1 D = 6.8 Myr. Thorpe (1982) reviewed studies pertaining to the molecular clock hypothesis and arrived at an average calibration of 1 D = 18.9 Myr. Considering the diversity of calibrations and the evidence that individual protein loci evolve at different rates in different groups, this last electrophoretic calibration should not be used unless other data are unavailable. Although it is rare that the fossil record is complete enough to establish an exact date of divergence of two lineages, the first occurrence of those two lineages in an incomplete fossil record provides a minimum estimate of the time of their separation. The fossil record for most amphibians is poor, but some fossil hylids exist that allow a rough calibration to be made. Chantell (1964) described a diverse hylid fauna from the Miocene–Pliocene boundary (approximately 5 million years ago) in Nebraska that includes fossils assigned to *H. cinerea*, *H. gratiosa*, and *H. squirella*. The lineages leading to these three species are distinct at a D of

0.25 (the divergence of *H. squirella* and *H. gratiosa*). If this D value corresponds to 5 Myr based on the hylid fossil record, then the electrophoretic clock is calibrated at 1 D = 20 Myr. This calibration is obviously a conservative estimate since the actual divergence occurred prior to the Miocene–Pliocene boundary.

Another independent calibration can be made using the albumin clock (Sarich and Wilson, 1967). Albumin immunological distance has been shown to be roughly correlated with divergence time in several groups of animals (Carlson et al., 1978). One *AID* corresponds to approximately 0.6 Myr. If albumin evolution is relatively constant among groups (Maxson et al., 1975), then the albumin clock may be used to calibrate the electrophoretic clock. Using the slope value (70) of the *AID*-versus-*D* relationship in hylid frogs (Fig. 4), 1 D corresponds to about 42 Myr. This is more than twice the value obtained by calibration with the fossil record. The difference in these two independent estimates is not unreasonable considering that the fossil *Hyla* used in the first calibration probably diverged a considerable amount of time before they were preserved as recognizable species in the fossil record. Using the albumin calibration (1 D = 42 Myr) as a rough approximation, and the hylid fossil calibration (1 D = 20 Myr) as a minimum estimate, it appears that the divergence of the two major groups of Holarctic hylids probably occurred by the early Eocene (50 Myr ago) but no later than the mid-Oligocene (27 Myr ago). There are no other hylid fossils that have a direct bearing on the time of this divergence. Fossil *Acris*, *Hyla*, and an "intermediate" genus, *Proacris*, are known from the lower Miocene (22 Myr ago) of Florida (Holman, 1967). If the fossil *Hyla* from that site represents a Holarctic lineage, then the divergence of *Acris* and Holarctic *Hyla* must have occurred prior to that time. These findings are in agreement with Hecht (1963), who postulated (based on limited fossil evidence) that most genera of living frogs were probably present in the early Tertiary.

Two adaptive radiations.—Adaptive radiations are believed to occur when a large number of ecological niches suddenly become available and are then rapidly filled by the descendents of a single lineage through adaptation (Romer, 1966). Relative to other hylid groups, the Holarctic assemblage lacks diversity (Duellman, 1970) but nonetheless can be viewed as representing two adaptive radiations.

Larson et al. (1981) used molecular data to show that two "key innovations" appeared during a relatively short period of time preceding the radiation of the salamander genus *Aneides*. Likewise, the two major features that distinguish *Pseudacris* from Holarctic *Hyla*, small digital pad size and cold-weather breeding, probably evolved in a short period of time relative to the duration of the group. The early divergence of the *regilla* and *ornata* groups (Figs. 2, 3) and the fact that both groups have species with reduced digital pads and cold-weather breeding provides support for that view.

Hylid frogs are believed to have originated in the New World tropics (Duellman, 1970) and, thus, it is likely that warm-weather breeding was the ancestral condition. Cold-weather breeding does not appear to be linked to any major morphological trait although it has probably been accompanied by a number of physiological adaptations. Several species of the anuran genera *Bufo* and *Rana* have also adapted to cold-weather breeding. This dichotomy in breeding time (winter/early spring versus late spring/summer) has resulted in two distinct anuran larval communities (Wilbur, 1980). Species belonging to the cold-weather community are adapted for growth and development at lower temperatures than species belonging to the warm-weather community. Density independent mortality due to freezing of the eggs can be important in the cold-weather community, whereas drought may be a problem in the warm-weather community (Wilbur, 1980). Studies have shown that competition and predation can have significant effects on the structure of anuran larval communities

(Wilbur, 1980, 1982; Morin, 1981, 1983; Wilbur et al., 1983). *Pseudacris* are smaller, on the average, than Holarctic *Hyla* and have relatively small larvae. Also, at least one species, *P. crucifer*, is a poor competitor with other sympatric anuran species (Morin, 1983). Thus, cold-weather breeding may have allowed *Pseudacris* to escape from interspecific competition during the larval stage. Also, the preference for shallow ponds or ditches for breeding instead of deeper and more permanent bodies of water may have been a way of reducing interspecific competition with other cold-weather breeding anurans, as well as a way of avoiding predation by invertebrates (Smith, 1983). Ambystomatid salamander larvae are the major predators of the cold-weather community, whereas invertebrate predation is strongest in the warm-weather community (Wilbur, 1980). Natricine snakes are also predators on anurans and their larvae (Whitaker, 1971; Wassersug and Sperry, 1977; Wilbur et al., 1983). The early emergence and explosive breeding of *Pseudacris* may be an adaptation to reduce snake predation on adult frogs.

The semiaquatic habits of *Acris* represent a third lifestyle found in Holarctic hylids. The major morphological innovation associated with this lifestyle is the presence of webbing between the toes. Unlike Holarctic *Hyla* and *Pseudacris*, *Acris* apparently did not undergo an adaptive radiation as it is represented by only two closely related extant species.

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APPENDIX 1

Localities and Sample Sizes

Acris crepitans ($n = 5$), Maryland, Prince Georges Co.; *A. gryllus* ($n = 5$), Georgia, Effingham Co., 7.2 km SE Pineora; *Hyla andersoni* ($n = 2$), New Jersey, Burlington Co., Chatsworth; *H. arborea* ($n = 1$), Italy; *H. arenicolor* ($n = 1$), Arizona, Pima Co., Sabin Canyon; *H. a. avivoca* ($n = 6$), Alabama, Greene Co.; *H. a. ogechiensis* ($n = 1$), South Carolina, Jasper Co., 2.6 km WNW Tillman; *H. cadaverina* ($n = 2$), California, San Bernardino Co., San Gorgonio Pass; *H. chrysoscelis* ($n = 3$), Texas, Travis Co., 1.6 km W Bee Cave; *H. cinerea* ($n = 5$), Maryland, Dorchester Co., Smithville; *H. crucifer* ($n = 4$), Virginia, Caroline Co., 19.4 km S Bowling Green; *H. eximia* ($n = 2$), Mexico, Mexico, 0.4 km E Rio Frio; *H. femoralis* ($n = 5$), Georgia, Effingham Co., 7.2 km SE Pineora; *H. gratiosa* ($n = 6$), Georgia, Effingham Co., 7.2 km SE Pineora; *H. japonica* ($n = 1$),

Japan, Shizuoka Prefecture, Shizuoka; *H. meridionalis* ($n = 5$), Morocco, Tetouan Province, 13.4 km S Asilah; *H. regilla* ($n = 4$), California, Riverside Co., Vail Reservoir; *H. squirella* ($n = 1$), Georgia, Effingham Co., Meldrim; *H. versicolor* ($n = 5$), Texas, Montgomery Co., Woodlands; *Limnaoedus ocularis* ($n = 5$), Florida, Alachua Co., Gainesville; *Pseudacris brachyphona* ($n = 5$), Georgia, Pickens Co., 6.2 km W, 2.4 km N Talking Rock; *P. brimleyi* ($n = 4$), Virginia, Caroline Co., 4.0 km E Dawn; *P. clarki* ($n = 6$), Texas, Travis Co., Manor; *P. nigrita* ($n = 4$), South Carolina, Berkeley Co., 1.0 km W Jamestown; *P. ornata* ($n = 6$), South Carolina, Aiken Co., Flamingo Bay; *P. s. illinoensis* ($n = 6$), Illinois, Mason Co., 8 km S Bath; *P. s. streckeri* ($n = 1$), Texas, Parker Co., near Fort Worth; *P. t. feriarum* ($n = 4$), Tennessee, Blount Co., 3.8 km W, 1.6 km S Townsend; *P. t. kalmi* ($n = 4$), Maryland, Kent Co., 4.2 km E, 1.6 km N Massey; *P. t. triseriata* ($n = 2$), Illinois, Cass Co., 3.2 km E Chandlerville.

APPENDIX 2

Protein Variation for 30 Taxa of Holarctic Hylid Frogs at 29 Polymorphic Loci

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Acp	h	h	h	h	h	h	h	h (.88) i (.12)	c (.9) h (.1)	h	h	h	h	h	b
Ak	d	d	d	d	d	d	d	d	d	d (.67) h (.33)	d	d	d	d	g
Apep	d	d	d	d	d	d	d	d	c	a	a	a	b	b	a
Aat-1	a	a	a	a	a	a	a	a (.88) d (.12)	a	a	a	a	d	b	e
Aat-2	b	b	b	b	b	b	a (.92) e (.08)	a (.13) d (.87)		e	h (.5) j (.5)	h	a	f	k
Cd	b	b	b	b	b	b	b	c	b	b	b	b	b	b	a
Ck	b	b	b	b	b	b	b	b	b	b	b	b	b	b	c
Dpep	g	g	g	g	a	f	f	j	h	h	h	h	e	i	k
Es-1	d	d	d	d	d	d	d	d	d (.4) e (.6)	d	b	d	c	b	h
Es-2	c	c	c	c	c	c	c	c	c	c	c	c	b	a	c
Gpi	j	b (.13) h (.62) q (.25)	o	m	e (.13) l (.87)	h (.25) q (.75)	m	c (.88) r (.12)	d (.1) k (.9)	o (.17) r (.83)	p (.5) r (.5)	p	b	a	c
Glud	b	b	b	b	b	a	b	b	b	b	b	b	b	b	n
Gpd	l	c (.13) l (.87)	l	c (.75) l (.25)	g	f	d (.08) f (.92)	i (.88) p (.12)	f (.4) o (.6)	m	m	m	g (.5) m (.5)	m	n
Icd-1	e	e	e	e	e	b	e	c (.13) h (.87)	g	h	h	h	c	e	c
Icd-2	c	c	c	c	c (.88) d (.12)	c (.75) d (.25)	c	c	b (.3) e (.7)	d (.75) e (.25)	d	d	e	e	f
Ldh-1	a	a	a (.9) o (.1)	a	m	a (.63) o (.37)	e	e (.25) f (.5) i (.25)	e	d	o (.5) p (.5)	o	c	c	l
Lgl	d (.75) f (.25)	d (.5) f (.5)	d (.2) f (.8)	f	d	e	e (.08) h (.92)	b	e	b (.08) e (.92)	b	b	e	e	b
Mdh-1	f	g	l	j	j (.38) p (.62)	m	k	g	g	g	o	i	k	k	a
Mdh-2	b (.5) j (.5)	j	j (.7) p (.3)	c (.25) h (.75)	k	o (.63) s (.37)	a (.17) e (.67) h (.08) q (.08)	h	i	e (.17) p (.75) s (.08)	p (.5) s (.5)	p	f	f	f

APPENDIX 2

Continued

	Taxon[a]														
Locus	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Acp	c	d	f	f	c	c	e	a	e	g	h	h	c	c	c
Ak	g	b	e	e	b	b	h	d	a	d	d	d	d	c (.67) f (.33)	c (.5) f (.5)
Apep	b	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Aat-1	c	b	b	b	b	b	b	c	c	b	c	c	c	c	c
Aat-2	i	k	k (.5) l (.5)	k	c	c	g (.9) h (.1)	i	f	i	i	i	i	i	i
Cd	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
Ck	c	a	a	a	a	a	b	b	b	b	b	b	b	b	b
Dpep	e	b	b	b	b	b	e	c	c	c	e	e	d	b	b
Es-1	h	g	g	g	h	h	h	h	h	a (.3) f (.7)	c (.1) h (.9)	h	f	a (.25) f (.75)	f
Es-2	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Gpi	h	i	f	f	g	g	b	h	b (.75) h (.25)	s	g (.9) n (.1)	g	n	n	h
Glud	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
Gpd	b	q	h	h	g	g	n	a	g	e (.2) j (.8)	k	k	n	n	n
Icd-1	f	f	f	f	f	f	d	c	c	c	c	a	c	a	a
Icd-2	e	f	f	f	f	f	a	f	f	f	f	f (.8) g (.2)	f	f	f
Ldh-1	l	h	b (.5) k (.5)	b (.67) k (.33)	g (.6) n (.4)	j	k	k	l	j	j	j	j	j	j
Lgl	b	c	c	c	c	c	b	c	c	c	a (.1) b (.8) e (.1)	b	g	c	c
Mdh-1	h	b	e	b (.75) n (.25)	c	c	g	i	g	b	c	c	c	c	d
Mdh-2	r	r (.75) v (.25)	r	r (.83) u (.17)	m	g (.33) 1 (.5) r (.17)	f	n	f	n	e	d	c	r	m (.2) t (.8)

APPENDIX 2

Continued

Locus	Taxon[a]														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Me	c	c	c (.6) f (.4)	a	g	c	b (.5) c (.5)	b (.63) c (.37)	h	g	g	g	c	c	g
Mpi	o (.13) t (.87)	q (.5) t (.37) v (.13)	i (.1) r (.4) u (.5)	m (.25) s (.75)	b (.13) n (.75) q (.12)	r	r	q (.63) t (.37)	i (.2) n (.3) q (.5)	q (.75) u (.25)	q	q	d (.25) g (.5) o (.25)	c	k
Pgm	g	g	c (.1) g (.9)	c	c (.13) g (.87)	f	c	j	i	e	m	m	c	d	n
Pgd	v (.4) aa (.4) bb (.2)	k (.13) n (.75) t (.12)	q (.1) u (.6) z (.2) aa (.1)	j (.75) o (.25)	w (.13) y (.87)	j (.13) t (.5) x (.37)	s	k (.75) n (.25)	d (.3) g (.1) k (.3) n (.3)	n (.92) t (.08)	o	o	e	g	a
Pt-1	e	e	e	e	e	d	d	g	d	e	e	f	f	f	b
Pt-2	b	b	b	b	b	b	b	b	b	c	c	c	c	c	e
Pk	b	b (.13) h (.87)	b	a	d	a	b	i	c	g	g	g	d	d	e
Sod	m	m	m	m	i (.13) k (.87)	g (.38) j (.62)	i	g (.25) i (.5) 1 (.25)	e (.3) g (.7)	i (.42) m (.58)	i	i	b	a	h
Xdh-1	c	c	c	a	c	c	a	c	c	c	c	c	c	c	c
Xdh-2	d	d	d	b	d	d	a (.08) f (.92)	d	d	d	d	d	d	c	e

APPENDIX 2

Continued

Locus	Taxon[a]														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Me	g	g	g	g	g	g	g	i	d	g	f (.1) g (.9)	g	g	g	e (.1) g (.9)
Mpi	a (.5) l (.5)	t	q	q	i (.6)	j (.17) q (.83)	a (.2) f (.8)	l (.5) s (.5)	e	k	g (.1) s (.6) v (.3)	s	h (.5) o (.5)	l (.08) p (.84) t (.08)	p
Pgm	o	o	o	o	n (.28) o (.72)	k (.16) o (.84)	a	l (.5) o (.5)	e (.75) m (.25)	l	b	b	a	a	h
Pgd	n (.5) p (.5)	m	m	m	m (.7) p (.1) r (.2)	m (.67) n (.33)	b	n	h	g	l	l	i	c (.58) p (.25) r (.17)	f
Pt-1	b	h	h	h	h	h	e	a	a	c	c	c	c	c	d
Pt-2	a	a	a	a	a	a	b	d	c	b	b	b	b	b	b
Pk	f	e	e	e	e	e	c	d	g	c	g	c	c	c (.83) g (.17)	c
Sod	e	j	e	e	e (.4) i (.4) o (.2)	e	c	h	e	i	j	f (.7) j (.1) n (.2)	f	d	f
Xdh-1	c	c	c	c	c	c	c	c	c	b	c	c	c	c	c
Xdh-2	g	g	g	g	g	g	e	g	d	g	d	d	g	g	g

[a] Numbers correspond to following taxa: (1) *feriarum*; (2) *kalmi*; (3) *brachyphona*; (4) *triseriata*; (5) *brimleyi*; (6) *nigrita*; (7) *clarki*; (8) *crucifer*; (9) *ocularis*; (10) *ornata*; (11) *streckeri*; (12) *illinoensis*; (13) *regilla*; (14) *cadaverina*; (15) *arborea*; (16) *japonica*; (17) *andersoni*; (18) *ogechiensis*; (19) *avivoca*; (20) *versicolor*; (21) *chrysoscelis*; (22) *meridionalis*; (23) *arenicolor*; (24) *eximia*; (25) *femoralis*; (26) *gryllus*; (27) *crepitans*; (28) *squirella*; (29) *gratiosa*; and (30) *cinerea*.