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Allozyme Evidence for the Separation of *Rana areolata* and *Rana capito* and for the Resurrection of *Rana sevosa*

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The taxonomic status of *Rana capito* is unclear. Allozymes were surveyed for *Rana areolata* and populations throughout the range of *R. capito*, including representatives of the three putative subspecies. Fixed differences were exhibited at two loci, supporting the separation of *R. capito* and *R. areolata*. The westernmost population of *R. capito*, in the range of *Rana capito sevosa*, could be diagnosed from the remaining populations of *R. capito*. These remaining populations exhibited no substantial genetic divisions. We recommend the resurrection of *R. sevosa* as a distinct taxon.

CRAYFISH frogs (*Rana areolata*) and gopher frogs (*Rana capito*), in broad terms, are distributed in the eastern half of North America. More specifically, the distribution of *R. areolata* broadly describes a rectangle from the Gulf coast of eastern Texas north to eastern Kansas, east to western Indiana and south through northern Mississippi and northwestern Louisiana. The distribution of *R. capito* is disjunct from *R. areolata*, historically ranging from extreme southeastern Louisiana along the Gulf coast to throughout the upper three-fourths of the Florida peninsula and north along the Atlantic coast to southeastern North Carolina.

The taxonomic status of the gopher frog (*R. capito*, sensu Collins, 1990) has been unclear. It was first described as a distinct species by LeConte (1855) but was placed in the synonymy of the crayfish frog, *R. areolata*, by Cope (1875). Subsequent work either followed LeConte (e.g., Harper, 1935; Wright and Wright, 1942) or Cope (Dickerson, 1906; Goin and Netting, 1940; Neill, 1957). Recently, Collins (1990) resurrected the name *R. capito* for all populations within the historical range of the gopher frog. This classification was based on the disjunct distribution of *R. areolata* (mostly the Mississippi River drainage and west of it) and *R. capito* (mostly east of the Mississippi River drainage along the Gulf and Atlantic coasts). Currently, authors either consider the gopher frog as a distinct species with three subspecies (*Rana capito aesopus, Rana capito sevosa, Rana capito capito*; Altig and Lohoefener, 1983; Dundee and Rossman, 1989), or the contents are included as subspecies of *R. areolata* (*R. a. aesopus, R. a. areolata, R. a. capito, R. a. circulosa, R. a. sevosa*; Conant, 1975; Hillis et al., 1983).

The objective of this study was to elucidate the taxonomic status of *R. capito* by assessment of allelic variation using starch gel electrophoresis. Allozyme data have been found to be useful in the elucidation of relationships among taxa of the *Rana pipiens* complex (Hillis, 1988). Specifically, we wanted to (1) describe and quantify any allelic differences among individuals currently considered gopher frogs (*R. capito*) and individuals considered to be crawfish frogs (*R. areolata*), (2) examine allelic differences among populations of *R. capito*, regardless of current taxonomic status, (3) estimate the average heterozygosity over all the protein loci examined, and (4) reevaluate the taxonomic status of *R. capito* in light of the allozyme data.

MATERIALS AND METHODS

Liver and muscle tissues were collected from 10 individuals of *R. areolata* and 72 individuals of *R. capito* from 20 localities (Table 1, Fig. 1; see Material Examined for localities and deposition of vouchers), stored at −80 C and later homogenized separately on ice using a teflon tip attached to a power drill. The grinding buffer consisted of 0.01 M Tris and 0.001 M EDTA dissolved in deionized water. Tissues were ground as a 1:4 (tissue:buffer) ratio. Unused homogenates were stored at −80 C.

Standard horizontal starch gel electrophoresis was employed (Murphy et al., 1996). The tissue type, enzyme commission number, the buffer system, and the electrophoretic conditions for each resolved locus are listed in the Appendix. Gels were sliced into four 1-mm thick slices. Histochemical staining procedures followed Murphy et al. (1996). Alleles were assigned letters based on relative mobility of their products from cathode to anode.

Genetic distances were computed using PHYLIP (Phylogeny Inference Package, J. Felsenstein, 1993, unpubl.). Chi-square analysis was performed for each population at each locus sampled to ascertain whether the frequencies were within those expected under Hardy-Weinberg equilibrium. Average heterozygosity [(average number of heterozygous loci/individu-

TABLE 1. ABBREVIATIONS OF SAMPLED POPULATIONS AND PREVIOUSLY SUGGESTED SUBSPECIFIC DESIGNATIONS (CONANT AND COLLINS, 1991).

Population	Abbreviation	County	Subspecific status
Alabama	AL	Covington	*R. c. sevosa*
Florida 1	FL1	Okaloosa	*R. c. sevosa*
Florida 2	FL2	Duval	*R. c. aesopus*
Florida 3	FL3	Levy	*R. c. aesopus*
Florida 4	FL4	Alachua	*R. c. aesopus*
Florida 5	FL5	Putnam	*R. c. aesopus*
Florida 6	FL6	Marion	*R. c. aesopus*
Florida 7	FL7	Lake	*R. c. aesopus*
Georgia 1	GA1	Chattahoochee	*R. c. sevosa/ capito*
Georgia 2	GA2	Baker	*R. c. aesopus*
Georgia 3	GA3	Charlton	*R. c. aesopus*
Georgia 4	GA4	McIntosh	*R. c. capito*
Georgia 5	GA5	Liberty	*R. c. capito*
Mississippi	MS	Harrison	*R. c. sevosa*
North Carolina 1	NC1	Scotland	*R. c. capito*
North Carolina 2	NC2	Pender	*R. c. capito*
North Carolina 3	NC3	Carteret	*R. c. capito*
Arkansas	AR	White	*R. areolata*
Kansas	KS	Anderson	*R. areolata*
Missouri	MO	Cass	*R. areolata*

al)/total number of loci] was calculated for each population as an estimate of genetic variation.

A hierarchical clustering analysis was performed using the unweighted pair-group method with arithmetic averaging (UPGMA), with the recognition that intraspecific relationships may not exhibit nested hierarchical structure (de Queiroz and Good, 1997). Genetic distances for populations represented by a single individual were not included in this analysis. Only a single genetic distance (Nei's D; Nei, 1972) was used because most genetic distance measures are highly correlated (Gorman and Renzi, 1979).

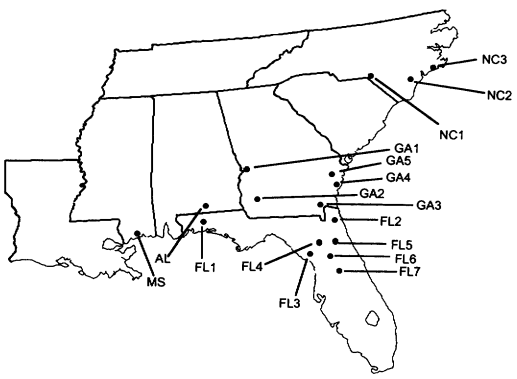

Fig. 1. Collection localities for all populations of *Rana capito* sampled in this study. Refer to Table 1 explanation of the abbreviations.

Populations were used as Operational Taxonomic Units (OTUs) for a cladistic analysis. Populations represented by a single individual were not included in this analysis. The data were coded following the mutation model coding method (Murphy, 1993). This model equates apomorphies with the origin of the novel state, that is, a mutation event. This model is also unique in that character state definitions are based on the polarization and ordering of the allelic arrays. The mutation model avoids the pitfalls of independent allele models and other locus-as-character coding approaches. Frequency data were not used because of the problems associated with recognizing frequencies as synapomorphies and because of problems inherent to the methods that employ them (e.g., Crother, 1990; Kornet and Turner, 1999; Murphy and Doyle, 1998).

PAUP* (vers. 4.01b1,D. L. Swofford, Sinauer Assoc., Inc., Sunderland, MA, 1998, unpubl.) was employed to infer the phylogeny. The outgroups consisted of two populations (from Missouri and Kansas) of *R. areolata.* The trees were inferred with the branch-and-bound algorithm. As noted above, 13 characters were analyzed as ordered and one as unordered. However, additional runs were conducted to see whether treating all the characters as unordered effected the outcome.

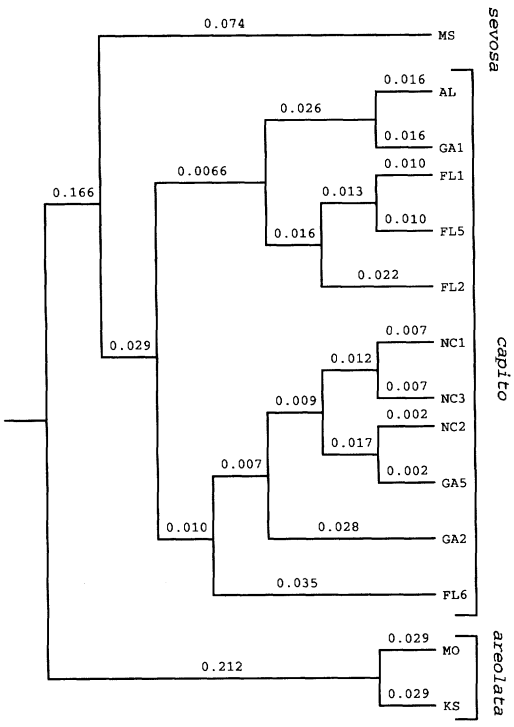

Fig. 2. UPGMA phenogram depicting the overall similarity among the frog populations sampled in this study. Numbers represent branch lengths. See Table 1 for key to OTU abbreviations and text for further discussion.

A priori, we note our recognition of species is based on the employment of the Phylogenetic Species Concept (Cracraft, 1983, 1987) as the operational form of the Evolutionary Species Concept (Wiley, 1978; Frost and Hillis, 1990). Because we adhere to these concepts, species recognition at this level must be considered in the context of a phylogenetic hypothesis.

Results

Fifty-one distinct alleles were detected at 26 presumptive genetic loci. Ten loci were monoallelic, and 16 were variable within and among populations of the two taxa (data available from BIC upon request). Of the variable loci, two (*CK, SOD*) showed a fixed allelic difference (mutually exclusive and homozygous) between *R. capito* and *R. areolata.* The locus *MPI,* although not fixed, exhibited mutually exclusive alleles between *R. capito* and *R. areolata,* and the outgroup showed a unique allele for ADH. One locus (*SOD*) showed a fixed difference for the individuals of *R. capito* collected from Mississippi. Estimates of average heterozygosity calculated for all populations across all loci ranged from 0 to 0.15 with an average of 0.072 (0.0439 SD). Nei's genetic distances for all populations ranged from 0.0304 to 0.6863. Chi-square analysis showed that all populations conformed to Hardy-Weinberg expectations at all loci.

Table 2. Data Matrix Derived from the Mutation Model Coding of the Allozyme Data and Employed in the Phylogenetic Analysis.

	Character													
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MS	1	1	1	1	1	0	0	1	0	1	1	0	0	1
AL	1	1	1	1	1	0	0	0	0	1	1	1	0	2
NC1	1	1	0	1	1	0	0	1	0	0	1	0	0	2
NC2	1	1	0	1	1	0	0	1	0	0	1	0	0	2
NC3	1	1	1	1	1	0	0	1	0	0	1	1	0	2
GA1	1	1	1	1	1	1	0	1	0	0	1	1	0	2
GA2	0	1	1	1	1	0	0	0	0	0	1	1	0	2
GA5	1	1	0	1	1	0	1	1	0	0	1	0	0	2
FL1	1	1	0	1	0	0	0	1	1	0	1	0	0	2
FL2	1	1	1	1	1	1	1	1	0	1	1	1	0	2
FL5	1	1	0	1	0	1	0	1	0	0	1	1	1	2
FL6	1	1	0	1	0	0	0	1	0	0	1	1	0	2
MO	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KS	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The UPGMA analysis recovered two large groupings corresponding to the populations of *R. areolata* (Missouri, Kansas) and *R. capito* (Fig. 2). The Mississippi population is clearly separated genetically from the other gopher frog populations. Within non-Mississippi gopher frogs, the Florida, Alabama, and Georgia1 populations cluster, whereas the more eastern Georgia5 and Georgia2 populations group with the North Carolina populations.

The mutation model coding yielded 14 characters (Table 2). Character state definitions for 13 of the characters were unambiguous and were analyzed as ordered. The 14th (*SOD*) was multistate and could not unambiguously be ordered and was analyzed as unordered. A single most-parsimonious tree (Fig. 3) was recovered. Six characters (four unambiguous) diagnosed the *R. capito* populations relative to *R. areolata.* Three populations (Mississippi, Florida 1, Florida 5) have uniquely derived alleles and substructure exists within *R. capito.*

Discussion

The average heterozygosity calculated for the populations fell into three categories for vertebrates: low (< 4%), typical (4–8%), and high (> 8%; Gorman and Renzi, 1979). Most popu-

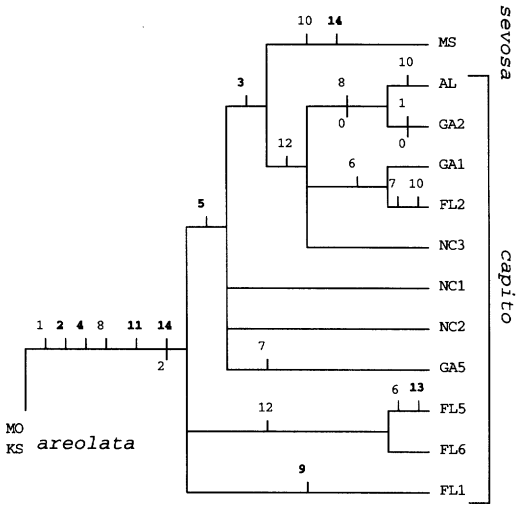

Fig. 3. Inferred phylogenetic relationships of the *Rana capito* and *Rana areolata* populations. This is the single most parsimonious tree: length = 22, CI = 0.68 (0.65 minus uninformative characters), RI = 0.76. Numbers along the branches refer to the character. Character states for each character are 1, except where noted on the tree. Bold numbers are unambiguous synapomorphies; regular fonts designate homoplasies. See Table 1 for key to OTU abbreviations and text for further discussion. Key: 1-*ADA*, 2-*ADH*, 3-*FBA*, 4-*CK*, 5-*FUM*, 6-*G3PDH-A*, 7-*G3PDH-B*, 8-*GCDH*, 9-*ICDH*, 10-*IDDH*, 11-*MPI*, 12-*PEP A,C,S*, 13-*PGM*, 14-*SOD*.

lations fell into the typical and high categories. Gorman and Renzi (1979) have shown that average heterozygosity estimates are more affected by number of loci sampled than number of individuals sampled, and demonstrated (for lizards) that error in estimation of heterozygosity varied among species from 0 to 6.6% when considering only two individuals at 25–27 loci sampled. Thus, average heterozygosity for several populations with small sample size in the present study (n = 1–3) may be subject to similar levels of error.

The allozyme data collected in this study support a separation of *R. capito* and *R. areolata*. At three loci (CK, MPI, and SOD) *R. capito* and *R. areolata* were characterized by some or all alleles that did not overlap; that is, they exhibited mutually exclusive differences. These three were all unambiguous synapomorphies. The locus ADH also separated *R. areolata* from *R. capito* unambiguously (CI = 1.0) but not because of fixed differences or mutually exclusive differences. In addition, two other loci (ADA, GCDH), although homoplastic (CI = 0.5 for each), supported the separation of *R. areolata* and *R. capito*. These allozyme differences corroborate past information indicating a distinction between the two taxa, including geographical separation (Collins, 1990), cranial morphological differences (Harper, 1935), mating call differences (S. Doody, pers. obs.), and color pattern (Conant and Collins, 1991). Although Hillis et al. (1983) were able to diagnose *R. areolata* relative to other *Rana*, they did not include any putative specimens of *R. capito*. Based on the character differences between *capito* and *areolata*, it seems clear that *capito* is distinct from *areolata*.

Three populations of *R. capito* were diagnosed by synapomorphy: Florida1, Florida5, and Mississippi. Florida1 possessed a unique allele (but not fixed, i.e., all individuals were not homozygous for that allele) relative to all the other ingoup and outgroup OTUs but was represented by only two individuals. Florida5 also possessed a unique allele, but like Florida1, it was not fixed. In both Florida1 and Florida5, the other allele was present in all the ingoup and outgroup OTUs. In our opinion, temporally variable evidence like frequencies are improper characters for diagnosing lineages.

The population of *R. capito* from Mississippi (Mississippi) displayed a single fixed allelic difference (at the SOD locus) in relation to other *R. capito* populations. Like many other populations of *R. capito*, the Mississippi population is geographically isolated from other breeding populations, and it is currently the only known population in the state (G. Johnson, pers. comm.). It might be expected that this population would show divergence resulting from isolation; however, its geographic separation from the nearest known breeding population (Alabama, Fig. 1) is no greater than separations between other isolated breeding sites that were not found to exhibit any fixed differences. Genetic divergence even in small isolated populations may take several hundred generations to occur (Avise and Ball, 1990), although given the estimated effective population size of the Mississippi population (75–150; R. A. Seigel, pers. comm.) several hundred generations is surely an extreme in this case. Perhaps the explanation for the fixed allozyme difference found in the Mississippi population is that the length of the separation of this population may be longer than previously thought (less than 60 years based on Allen, 1932). Regardless of the explanation for the divergence at the *SOD* locus, the divergence indicates that the population is on a unique evolutionary trajectory and may represent a species of gopher frog that was previously recognized (*R. sevosa*; Goin and Netting, 1940).

The criteria on which to base the recognition of species are controversial, in part because of

the rejection by many workers (explicit or otherwise) of the need for a sound philosophical basis for choosing among species concepts. We accept (and follow herein) the arguments of Frost and Kluge (1994) that the Phylogenetic Species Concept (Cracraft, 1983, 1987) is the operational form of the Evolutionary Species Concept (Wiley, 1978; Frost and Hillis, 1990). Under this concept, any type of evidence (intrinsic or extrinsic) that indicates a population or cluster of populations is a unique evolutionary lineage capable of maintaining its lineage separate from other lineages can be used to diagnose a species.

The frequency differences in Florida1 and Florida5 do not represent evidence that indicates lineages maintaining a separate identity. In the case of the Mississippi population, the fixed allelic difference coupled with distinctive external morphological characters (as noted in Goin and Netting, 1940) and geographic isolation from other gopher frog populations is sufficient evidence for species recognition. Because the locality of the population falls within the range of Goin and Netting's (1940) species *R. sevosa*, it is recommended that this name be elevated from its current standing as a subspecific epithet for *capito* to a specific epithet. The standard English name for *R. sevosa* is the dusky gopher frog. The elevation of the Mississippi population to species renders the remainder of *R. capito* paraphyletic.

Intraspecific relationships are complex and may not be representable as mutually exclusive hierarchical nested groups (de Queiroz and Good, 1997). Because similarities need not exhibit a hierarchical pattern as forced by UPGMA, the interpretation of the phenogram warrants caution. As such, direct inspection of the distance matrix itself may be more appropriate (de Queiroz and Good, 1997). The data indicate no fixed genetic divisions among the remaining populations, and among the presumptive subspecies. In some cases, there is a trend for geographically close populations to be genetically similar. In the case of Florida3 and Florida4 populations, the relatively high distance (0.4053, not shown in table) is likely because of sample size ($n = 1$ for both). Both individuals were found to have different alleles at several loci, but in each case, the two alleles overlapped when all the populations were considered. Florida7 demonstrated similar trends. The absence of fixed or mutually exclusive differences among the putative subspecies in both the phenetic and cladistic results leads us to recommend that the subspecific designations be dropped for those populations still considered *R. capito*.

To conclude, we address two possible points of contention, sample size and species concepts. It is well known that broad geographic sampling is more critical to the discovery of allelic variation than dense sampling within a single population. If intraspecific variation exists, it is more likely to be pronounced between populations than within them (e.g., see Buth, 1984). We collected individuals from 20 localities, perhaps the most geographically diverse sample of any allozyme study undertaken on *Rana*. It is also well known that number of loci is more critical for assessment of genetic variation and relationships than number of individuals from a single population (e.g., see Gorman and Renzi, 1979; Hillis, 1987). As a rule of thumb for determination of phylogenetic relationships based on allozyme data, Murphy et al. (1996) recommended a minimum of three times as many loci as the number of species involved: we resolved products of 26 loci for three species.

Debates about species concepts are many, and criticism has been leveled at all notions. Probably no single species concept can apply to all forms of life. However, for diploid, sexually reproducing organisms, a philosophically justified concept of species is the evolutionary species concept (ESC) of Simpson as modified by Wiley and expanded upon by Frost and Hillis (1990). The drawback to that concept is its lack of empirical utility. It has been shown by Frost and Kluge (1994) and has been accepted for a long time prior, that the phylogenetic species concept (PSC) is the appropriate operational version of the ESC.

The view might be held that we have taken the PSC to the extreme. A single fixed diagnosable character state is all that is required for recognition of species under this concept. It might be suggested that we have comfortably separated *R. areolata* from *R. capito* with three mutually exclusive differences but have not demonstrated the same for *R. capito* and *R. sevosa* with one fixed difference. There is no sliding scale. The concept is what it is.

Material Examined

Institutional abbreviations follow Leviton et al. (1985), except as noted. Abbreviations used: ONF, Ocala National Forest; SLU, Southeastern Louisiana University; USC, University of Southern California (at SLU); WMA, Wildlife Management area.

Rana areolata, (White County, Searcy, AR): SLU 0006; (Cass County, MO): USC 8167–8168,

8176; (Anderson County, KS): KU 222818–823. *Rana capito*, (Conecuh National Forest, Covington County, AL): LSUMZ 57951, SLU 0046, 0050, 0056, 0065, 0068; (Locholoosa WMA, Alachua County, FL): USC 7981; (Palm Valley, Duval County, FL): USC 7979–7980, 8170–8175; (ONF, Lake County, F.): SLU 0069; (Levy County, Florida): USC 8205; (ONF, Marion County, FL): SLU 0003, 0041; (Eglin Air Force Base, Okaloosa County, FL): USC 7984–7985; (Ordway Preserve, Putnam County, FL): SLU 0004–0005, 0045, 0058, 0066–0067; (Baker County, GA): SLU 0042, 0049, 0052, 0059, 0064; (Okefenokee, Charlton County, GA): SLU 0063; (Fort Benning, Chattahoochee County, GA): SLU 0043–0044, 0047–0048, 0053–0054, 0061–0062, 0070; (Fort Stewart, Liberty County, GA) SLU 0060; (Liberty County, GA): SLU 0014–0018; (Desoto National Forest, Harrison County, MS): USC 8206–8210, 8236, SLU 0040, 0051, 0055, 0057; (Peltier, Carteret County, NC): LSUMZ 57949–52, USC 8211–8212; (Pender County, NC): SLU 0013, 0019–0020; (Scotland County, NC): SLU 0021–0025).

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APPENDIX. LIST OF LOCI RESOLVED.

Enzyme system	Enzyme commission number	Locus	Tissue	*Assay conditions
Adenosine deaminase	3.5.4.4	*Ada-A*	Muscle	A
Alcohol dehydrogenase	1.1.1.1	*Adh-A*	Liver	B
Fructose-biphosphate aldolase	4.1.2.13	*Fba-B*	Liver	A
Calcium Binding protein	Nonspecific	*CBP*	Muscle	B
Creatine kinase	2.7.3.2	*Ck-A*	Muscle	B
Fumarate hydratase	4.2.1.2	*Fum-A*	Liver	C
Glycerol-3-phosphate dehydrogenase	1.1.99.5	*G3pdh-A*	Muscle	A
Glycerol-3-phosphate dehydrogenase	1.1.99.5	*G3pdh-B*	Liver	A
Glucose 1-dehydrogenase	1.1.1.47	*Gcdh-A*	Liver	D
Glutamate dehydrogenase	1.4.1.2	*Gtdh-A*	Liver	A
L-Iditol 2-dehydrogenase (IDDH)	1.1.1.14	*Iddh-A*	Liver	C
Isocitrate dehydrogenase (NADP+)	1.1.1.42	*mIcdh-A*	Muscle	A
Isocitrate dehydrogenase (NADP+)	1.1.1.42	*sIcdh-A*	Liver	A
L-Lactate dehydrogenase	1.1.1.27	*Ldh-A*	Muscle	A
L-Lactate dehydrogenase	1.1.1.27	*Ldh-B*	Muscle	A
Malate dehydrogenase (NAD+)	1.1.1.37	*mMdh-A*	Muscle	E
Malate dehydrogenase (NAD+)	1.1.1.37	*sMdh-A*	Muscle	E
Malate dehydrogenase (NADP+)	1.1.1.40	*mMdhp-A*	Liver	E
Malate dehydrogenase (NADP+)	1.1.1.40	*sMdhp-A*	Liver	E
Mannose-6-phosphate isomerase	5.3.1.8	*Mpi-A*	Muscle	A
Tripeptide aminopeptidase-B (L-leucylglyclgycine)	3.4.13.4	*Pep-B*	Liver	D
Peptidase-cytosol nonspecific (glycyl-L-leucine)	3.4.13.18	*Pep-A,C,S*	Liver	D
XAA-PRO dipeptidase (L-phenylalanyl-L-proline)	3.4.13.9	*Pep-D*	Liver	D
6-Phosphogluconate dehydrogenase	1.1.1.44	*Pgdh-1*	Liver	E
Phosphoglucomutase	5.4.2.2	*Pgm-A*	Muscle	B
Superoxide dismutase	1.15.1	*sSod-A*	Liver	B

* A: Tris-citrate pH 8.0, 6V/cm 6h; B: "Poulik" system, 11V/cm 6–10h; C: Lithium hydroxide, 14.3V/cm 5h; D: Tris-HCL pH 8.5, 14.3V/cm 6h; E: Tris-citrate pH 7.0, 5 V/cm 24h. Slight modifications in voltage or time were employed in some instances to obtain better resolution. Histochemical stains followed Murphy et al. (1996).