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An electrophoretic analysis of the genetic relationship between Etheostoma nigrum and Etheostoma Olmstedi (percidae: etheostomatini) in the James River drainage of Virginia

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AN ELECTROPHORETIC ANALYSIS OF THE GENETIC RELATIONSHIP BETWEEN *ETHEOSTOMA NIGRUM* AND *ETHEOSTOMA OLMSTEDI* (PERCIDAE: ETHEOSTOMATINI) IN THE JAMES RIVER DRAINAGE OF VIRGINIA

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Abstract

The genetic relationship between Etheostoma nigrum and Etheostoma olmstedi in the James River drainage of Virginia was analyzed with horizontal starch gel electrophoresis. Seventeen loci (six polymorphic) were resolved from 13 enzyme-protein systems in 189 specimens from 10 collection sites. Distribution of individuals within genotypic classes was in agreement with Hardy-Weinberg in two-thirds of all populations at each locus. Disagreement with Hardy-Weinberg was due to a shortage of heterozygotes in every instance. Species occurred sympatrically in Falling Cr. and the Appomattox R. and were so similar genetically that fusion into a single population seems to have occurred because of widespread hybridization. Low distance coefficients (<0.134) revealed a close genetic relationship among all James River populations regardless of species; however, some geographical clustering was evident. Results suggest the subspecies designation E. n. olmstedi for the coastal plain form.

Introduction

Etheostoma nigrum Rafinesque 1820, the Johnny darter, ranges from Alabama to the Hudson Bay and from Colorado east to the Atlantic slope in the James, Roanoke, Tar and Neuse Rivers (Page, 1983). Etheostoma olmstedi Storer 1842, the tesselated darter, is restricted to the east coast in the Atlantic and Lake Ontario drainages (Page, 1983). In the James and Roanoke River systems in Virginia E. nigrum occupies the piedmont and montane regions, whereas E. olmstedi primarily inhabits the coastal plain (Cole, 1967).

The relationship between the morphologically similar species of darters (subgenus Boleosoma) is controversial and has been investigated by several authors. Stone (1947), using multiple character analysis of the two forms in the zone of overlap in the Lake Ontario drainage, concluded that they were separate species. Hubbs and Lagler (1958) treated E. olmstedi as a subspecies of E. nigrum. Based on meristic data in a study of the two forms, Cole (1958, 1965 and 1967) supported Stone's conclusions that separate species status is justified in drainages in New York, Virginia, and North Carolina. Scott and Crossman (1973) referred all Canadian Johnny darters to E. nigrum until it could be determined whether variation in the widespread form was somatic or genetic.

Electrophoretic protein pattern analysis of the relationship between the two darters was initiated by McAllister et al. (1972) who included morphological data in their investigation of populations in the Ottawa River, Canada. Even though they recognized interspecific hybrids (7.6%) in the zone of sympatry, they concurred with the interpretation of separate species.

Stillman (1984) compared morphological characters of E. olmstedi and E. nigrum from allopatric and sympatric populations in New York State and found the two species were more alike in sympatric areas. She estimated a 2% overall hybridization rate in sympatric areas and observed E. nigrum character index scores skewed toward those of E. olmstedi in one sympatric area in Lake Ontario. Brett (1985) used starch gel electrophoresis to investigate gene-flow in E. nigrum and found substantial evidence of interbreeding between E. n. nigrum and E. n. olmstedi in areas of sympatry. Brett (pers. comm.) believes E. olmstedi should be reduced to subspecific status based on her and Stillman's (1984) studies.

Clark (1978) identified five populations of E. olmstedi and E. nigrum in the James River drainage using proportional measurements and meristic character analyses. He proposed that introgressive hybrid populations, intermediate to E. nigrum and E. o. olmstedi, were in the Appomattox R. and Falling Cr., a small tributary of the coastal plain James

R. with headwaters near the fall line in the Piedmont. Falls (1982) used horizontal starch gel electrophoresis to examine the relationship of the two species and found no supportive evidence for hybridization.

Johnson (1973) and Buth (1984) said electrophoretic methods are valuable in providing additional data for testing systematic hypotheses based on morphological criteria, but should not be the sole determinant. Isozymes, more specifically allozymes, have been used often to estimate genetic relationships and structure within and between Etheostoma species (McAllister et al., 1972; Martin and Richmond, 1973; Page and Whitt, 1973; Echelle et al., 1975; Echelle et al., 1976; Wiseman et al., 1978; Wolf et al., 1979; Buth et al., 1980; McLeod et al., 1980; Buth, 1982; and Falls, 1982).

The present study, using electrophoretic techniques,was undertaken in an effort to resolve the genetic relationship between E. olmstedi and E. nigrum in the James River drainage. Widespread hybridization was found in areas of sympatry, in Falling Cr. and the Appomattox R. Low distance coefficients revealed a close relationship among all James R. populations regardless of species; however, some geographical clustering was evident. Results suggest the subspecies designation, E. n. olmstedi, for the coastal plain form.

Materials and Methods

A total of 189 specimens of E. nigrum and E. olmstedi was collected with seines at 10 collection sites in the James River drainage system (Fig. 1). Specimens were frozen on dry ice at capture and stored at -20 C prior to extract preparation. Electrophoretic examination of each sample was completed within four months of its collection date.

Before preparation for electrophoresis each specimen was identified to species by the morphological character, interrupted (E. nigrum) or uninterrupted (E. olmstedi) infraorbital canal (Cole, 1967). The opercle bones and fins were removed to facilitate homogenization. Next a ventral incision was made from anus to branchial septum and the viscera were removed. The remainder of each specimen was macerated, diluted (1:1, weight:volume) with 0.1 M Tris-HCl buffer at pH 7.5, and homogenized with a motorized teflon and glass homogenizer. The homogenate was centrifuged at approximately 12,000 g for 30 min at 4 C. The supernatant was placed in 2 ml cryogenic tubes, frozen at -50 C in a methanol bath, and stored at -20 C for one to four days until electrophoretic analysis was completed. Horizontal starch gel electrophoresis was run using 13% starch (Lot#392; Electrostarch Co., Madison, Wisc. 53701) following the methods of Selander et al. (1971), Falls (1982), and Werth (1985) (Appendix I). Twenty-three enzyme systems were initially examined, but ten were discontinued because of

faint or poor resolution of banding patterns. The remaining 13 enzyme-protein systems were analyzed (Table 1; Appendix II). Enzyme and locus terminology follow guidelines proposed by Buth (1983). Allelic terminology was based on relative electrophoretic mobilities and expressed by numerical superscripts as percents of the most common allele at each locus in E. olmstedi from Herring Creek.

Allelic frequencies were calculated from the observed genotypes and used to calculate mean heterozygosity, similarity coefficients (Rogers, 1972), and distance coefficients (Nei, 1978) for each population. Rogers' (1972) genetic similarity coefficient (S), which ranges from 0 (no allele shared at any locus) to 1 (100% similarity between populations), estimates the normalized identity (I) of genes between populations. Nei's (1978) genetic distance coefficient (D), which is an estimate of accumulated allele differences per locus between populations ($D = -\log_e I$), ranges from 0 to infinity. The higher the value of D the greater the difference (on the average).

The Chi-square goodness of fit test was used for testing conformance of observed genotypic frequencies with Hardy-Weinberg equilibrium expectations for each population at each variable locus. The expected frequencies were calculated using Levene's (1949) formula for small samples and X^2 was calculated using Yates (1934) correction for continuity when the degrees of freedom = 1. In some cases to compensate

for suspect X^2 values where some expected frequencies were too low, genotypes were pooled to form three classes. They were: homozygotes for the most common allele; heterozygotes for the most common allele and one other allele; and all other genotypes.

F-statistics, specifically the inbreeding Coefficient (F_{IS}) and the Fixation Index (F_{ST}), were computed for each polymorphic locus. F_{IS} measures the reduction in heterozygosity of an individual due to nonrandom mating within its subpopulation (Hartl, 1981). Values near 0 indicate random mating within subpopulations. Negative values indicate an excess of heterozygotes and those approaching 1 indicate an excess of homozygotes. F_{ST} measures the reduction of heterozygosity of a subpopulation due to random genetic drift and also may be influenced by mutation, migration, and natural selection (Hartl, 1981). F_{ST} has a minimum value of 0 (indicating no genetic divergence) and a maximum of 1 (indicating a fixation for alternative alleles in subpopulations), "but generally values >0.25 indicate very great genetic differentiation" (Hartl, 1981).

A cluster phenogram based on Nei's (1978) D (distance) was obtained using the unweighted pair-group method with arithmetic averaging (UPGMAA; Sneath and Sokal 1973). The BIOSYS-1 program of Swofford and Selander (1981) was used to compute all statistical tests in this paper.

COLLECTION SITES: Numbers in parentheses are specimens examined.

Virginia:

Lower James River drainage: Herring Cr., approximately 2 km below Harrison Lake, St. Rt. 5, Charles City Co., 4 Sept. 1984 (7), 31 Oct. 1984 (8), 20 Dec. 1984 (13), 28 Dec. 1984 (14); Falling Cr. (upper), Co. Rt. 653, Chesterfield Co., 30 Oct. 1984 (8), 23 Feb. 1985 (4); Falling Cr. (middle), Co. Rt. 649, Chesterfield Co., 2 Oct. 1984 (4); Falling Cr. (lower), Co. Rt. 651, Chesterfield Co., 2 Oct. 1984 (5), 23 Feb. 1985 (5).

Upper James River drainage: Buffalo R., Co. Rt. 778, Amherst Co., 13 Nov. 1984 (32), 29 Dec. 1984 (26); Wreck Island Cr., Co. Rt. 666, Appomattox Co., 23 Oct. 1984 (19).

Upper Appomattox River drainage: North Branch Appomattox R. St. Rt. 24, Appomattox Co., 2 Mar. 1985 (20); South Fork Appomattox R., Co. Rt. 634, Appomattox Co., 2 Mar. 1985 (11); Rocky Run Cr., Co. Rt. 616, Appomattox Co., 2 Mar. 1985 (5); Appomattox R., Co. Rt. 618, Appomattox Co., 2 Mar. 1985 (8).

Results

Seventeen anodally migrating loci from 13 enzyme systems were scored in 189 specimens (Table 1). Six loci were polymorphic (Table 2) and eleven were monomorphic (Cbp-A and B, Cat-A and B, Me-A, Mdh-A, Mpi-B, Ldh-A, Icd-A, Lap-A, and Pgm-A). A locus was considered polymorphic if the

frequency of the most common allele did not exceed 95%. The number of polymorphic loci in each population ranged from 0 in E. olmstedi from upper Falling Cr., South Fork Appomattox R., Appomattox R., and E. nigrum from Rocky Run Cr. to 6 (35.3%) in E. olmstedi from Herring Cr. (Table 2). Average polymorphism for all 16 populations was 2.6 out of 17 loci, or 15.3%. Mean heterozygosity ($\bar{H}$) ranged from 0.000 in three small samples of E. olmstedi and one of E. nigrum to 0.096 in E. nigrum from lower Falling Cr. (Table 2). Average $\bar{H}$ for all 16 populations was 0.04. The direct count $\bar{H}$ did not differ significantly from the Hardy-Weinberg expected $\bar{H}$ in any population; however, the direct count $\bar{H}$ was lower than the expected $\bar{H}$ in all populations except E. nigrum from middle and lower Falling Cr. (Table 2).

Fifteen alleles were resolved from six loci of five enzyme systems. Results of electrophoretic staining patterns and analyses of allele frequencies from each population are reported for each enzyme system (Table 2).

SOD - Four alleles were resolved at one locus which exhibited a dimeric allozymal banding pattern (Fig. 2). Sod-A^{100} was observed only in the populations of E. olmstedi from Herring Cr. and E. nigrum from middle Falling Cr. Sod-A^{40} was present only in two populations of E. nigrum from Buffalo R. and Wreck Island Cr. The rarest allele, Sod-A^{72}, was in four populations of E. nigrum from Buffalo R., Wreck Island Cr., upper and lower Falling Cr., and

was also in the E. olmstedi population from Herring Cr. Sod-A^{26} was present in all populations ranging from a low frequency of 0.03 in E. nigrum from Wreck Island Cr. to 1.00 in nine different populations of both species (Table 2).

GPI - Two loci were resolved for the GPI enzyme system, but the less anodally migrating locus, Gpi-B, was not clearly interpretable. Three alleles were resolved at the Gpi-A locus which had a dimeric banding pattern (Fig. 3). Gpi-A^{100} was found throughout the populations and had a high allele frequency (>0.80) in all except the E. nigrum from Buffalo R., upper Falling Cr. and Wreck Island Cr. Gpi-A^{141} was the rarest allele and restricted to E. nigrum from the Buffalo R.

ES - Two alleles at each of two loci, Es-B and D, which exhibited monomeric banding patterns, were clearly resolved (Fig. 4). The other two loci, Es-A and C, were not interpretable. Es-B^{100} was observed in all populations except E. olmstedi from upper Falling Cr. and always at a higher frequency than Es-B^{95}. The latter was not present in seven populations of both species (four of E. olmstedi and three of E. nigrum) (Table 2). Es-D^{90} was found in all populations except E. olmstedi from the South Fork Appomattox R., and present at a higher frequency than Es-D^{100} in ten populations of both species (four of E. olmstedi and six of E. nigrum) (Table 2).

AAT - Two loci were observed, but only Aat-B was scored because of the diffuse nature of the Aat-A locus. Aat-B exhibited monomeric enzyme banding with two alleles resolved (Fig. 5). Aat-B^{100}, the most common allele, had a higher frequency (>0.70) than did Aat-B^{70} in all but two populations, E. nigrum from Wreck Island Cr. and E. olmstedi from lower Falling Cr.

CBP - Two monomorphic loci (Cbp-A and B) and one monomeric banding polymorphic locus (Cbp-C) were resolved (Fig. 6). Cbp-B possibly is equivalent to Ck-A (creatine kinase) of Buth (1982). Two alleles (Cbp-C^{100} and Cbp-C^{80}) were present only in E. olmstedi from Herring Cr. with the rarer allele, Cbp-C^{80}, having a frequency of 0.28.

At the Sod-A, Gpi-A, and Cbp-C loci, X^2 values were low and the associated probabilities (P) were greater than 0.05. Therefore, there was no justification to reject the hypothesis that the genotypic frequencies were in Hardy-Weinberg proportions for these loci (Table 3). Chi-square values for Es-B also were low and P>0.05 for E. nigrum from lower Falling Cr., Wreck Island Cr., North Branch Appomattox R. and South Fork Appomattox R., and E. olmstedi from North Branch Appomattox R. The Hardy-Weinberg model was not rejected for these populations. Chi-square was large and P<0.05 at Es-B for E. nigrum from upper Falling Cr. and Buffalo R., and E. olmstedi from Herring Cr. The goodness of fit was sufficiently poor to reject the Hardy-

Weinberg model because chance alone is not likely to produce deviation as large as was obtained. At the Es-D locus there was no reason to reject Hardy-Weinberg in the populations of E. olmstedi from lower Falling Cr. and Rocky Run Cr., or in E. nigrum from middle and lower Falling Cr., Buffalo R. and North Branch Appomattox R. Hardy-Weinberg was rejected in populations of E. olmstedi from Herring Cr. and North Branch Appomattox R., and E. nigrum from Wreck Island Cr., South Fork Appomattox R. and Appomattox R. The Hardy-Weinberg model was not in agreement with any polymorphic populations at Aat-B except E. olmstedi from lower Falling Cr.

Sod-A and Cbp-C possessed negative F_{IS} values, whereas Aat-B had the largest F_{IS} value, 0.92 (Table 4). The average F_{IS} over the six polymorphic loci was 0.27. All six polymorphic loci had F_{ST} values of >0.25 (Table 4). Sod-A exhibited the highest value (0.65) and Cbp-C the lowest (0.26). The mean F_{ST} across the six polymorphic loci was 0.47.

Genetic distance was small between all populations regardless of whether they were paired intraspecifically or interspecifically (Table 5). Distance ranged from 0.000 in three pairings of populations from the Appomattox R. drainage to 0.133 in the pairings of E. nigrum from Buffalo R. with E. nigrum from Rocky Run Cr. and the former with E. olmstedi from the Appomattox R. All D coefficients were <0.062

between the eight populations from the Appomattox R. drainage and <0.086 between the five Falling Cr. populations. Average genetic similarity was 96.3% between all populations from the Appomattox R. drainage and 91.6% between all Falling Cr. populations. The greatest divergence occurred between populations of E. nigrum from Wreck Island Cr. and E. olmstedi from upper Falling Cr., which shared a relatively high similarity of 81.9%.

Phenograms generated by a cluster analysis showed that all populations were clustered at a divergence level of 0.104 (Fig. 7). The populations formed three subgroups. The largest one was comprised of E. olmstedi and E. nigrum populations from the four Appomattox R. drainage locations and from the three Falling Cr. locations. Etheostoma nigrum from Buffalo R. and Wreck Island Cr. formed the second group and the third was E. olmstedi from Herring Cr.

Discussion

Genetic variation within James River drainage populations of E. nigrum and E. olmstedi (avg polymorphic loci = 15.3%; avg $\bar{H}$ = 4.0%) agreed with that given by Nevo (1978) for Perciformes (avg polymorphic loci = 15.2 $\pm$ 9.8%; avg $\bar{H}$ = 5.13 $\pm$ 3.38%). The Appomattox R. populations (avg polymorphic loci = 8.8%; avg $\bar{H}$ = 1%) heavily influenced the results.

Low distance coefficients revealed a close relationship among all James R. populations regardless of species or geographical location; however, some geographical clustering

was evident. Nei (1976) has estimated that genetic distance for species is >0.200 and for subspecies ranges from 0.020 to 0.200. All distance coefficients for the present study were <0.134, regardless of morphological identification. Genetic distance estimates are little affected by sample size and even a single individual may be used to represent a species for interspecific comparisons when the average heterozygosity is low (Nei, 1978; Gorman and Renzi, 1979). The resulting estimated D shows systematic error that usually overestimates the full population D estimate (Gorman and Renzi, 1979). Therefore, the James R. populations may be even more closely related than the results indicate due to the two single specimen samples included in the investigation (Table 2).

Allopatric designations of E. olmstedi from Herring Cr. and E. nigrum from the Buffalo R. and Wreck Island Cr. agreed with those of Cole (1967), Clark (1978) and Falls (1982). Both species were morphologically identified in the Appomattox R. drainage, and in upper and lower Falling Creek in the present investigation. Clark (1978) also found E. olmstedi and E. nigrum throughout the Appomattox R., but Falls (1982) reported only the occurrence of E. olmstedi. Abbott et al. (1978), in a limited survey, found only E. nigrum. Clark (1978) reported E. nigrum in Falling Cr. and proposed introgressive hybridization was occurring between E. nigrum and E. olmstedi, whereas Falls (1982)

said both species were in Falling Cr. and found no evidence of hybridization. Distance coefficients between the populations from Falling Cr. (both species) and E. olmstedi from Herring Cr. decreased downstream. This finding agreed with that of Clark (1978), where meristic values characteristic of E. nigrum progressively approached those of E. olmstedi in the downstream Falling Cr. collecting sites. Etheostoma olmstedi and E. nigrum were so genetically similar in the overwhelming majority of Falling Cr. and Appomattox R. populations that fusion of the two species into a single population seems to have occurred as a result of widespread hybridization. However, the sympatric populations from upper Falling Cr. at the Gpi-A and Es-B loci, and lower Falling Cr. at the Aat-B locus exhibited some restricted gene flow which resulted in unexplained allelic distributions. Allelic frequencies of both species from Rocky Run Cr. at the Es-D also did not conform to the expected frequencies, possibly due to the E. nigrum sample size of only one fish.

The distribution of individuals within genotypic classes was in agreement with the Hardy-Weinberg model in two-thirds of all populations at each locus including all those at the Sod-A, Gpi-A, and Cbp-C loci. Significant ($P \leq 0.05$) disagreement (X^2) with Hardy-Weinberg in four populations of E. olmstedi and ten of E. nigrum across three loci, Es-B, Es-D, and Aat-B, was caused by a shortage of heterozygotes in every instance. F_{IS} values confirmed a shortage of

heterozygotes for those three loci, especially that of Aat-B. Several possibilities have been used to explain these deviations, e.g. nonrandom mating (inbreeding and assortive mating), selection, migration, mutation or sampling error (Hartl, 1980; Hartl, 1981; Buth and Crabtree, 1982; Tamarin, 1982). The large F_{ST} values indicate a high tendency for fixation of alternative alleles in populations at all six polymorphic loci, particularly at Sod-A. This reduced gene flow occurred among the upper James R. (Buffalo R. and Wreck Island Cr.), lower James R. (Herring Cr.), and combined Falling Cr. and Appomattox R. populations. Several authors (Hartl, 1981; Buth and Crabtree, 1982; Tamarin, 1982) have proposed that reduced gene flow between populations may reflect founder effect, random genetic drift or selective pressure. Probably the latter would more accurately portray the relationship of the James R. E. nigrum (Piedmont) and E. olmstedi (Coastal Plain) populations. Clark (1978) in explaining the distribution of the two species in the James R. drainage, supported Cole's (1971) postulation that E. nigrum and E. olmstedi evolved in isolation from common stock in geological times predating Pleistocene glaciation. Etheostoma olmstedi then moved into the James R. basin within the early Pleistocene and utilized expanded coastal plain drainages during periods of sea level decline. The Fall Line probably limited E. olmstedi to the lower Piedmont and coastal streams. The E. nigrum population represents

an intrusion from the west as a result of stream capture between the James R. headwaters and the New R. Clark (1978) further postulated that the E. nigrum of the Appomattox R. and Falling Cr. entered by stream piracy from Piedmont tributaries of the James R.

There were several conflicts between the results of the present study and those of Falls (1982). Cbp-A and B, and Ldh-A were monomorphic in both studies, but in the present one Ldh-B was not observed. Unlike Falls (1982), no polymorphisms were observed at Pgm-A. In contrast to Falls' results, Gpi-B was not monomorphic, but the locus was uninterpretable because resolution of bands was poor. It was difficult to compare the ES system in the two studies because four loci (none monomorphic) were observed in this study and three (two monomorphic) in that of Falls (1982). The Gpi-A locus was polymorphic in each study and the allele frequencies were similar at common collecting sites except the Buffalo R., where one additional allele was resolved in the present study. Falls described Gpi-A^{2} (analagous to Gpi-A^{100}) as diagnostic of E. olmstedi when in the homozygous condition, i.e. the homozygous condition did not occur in E. nigrum. In contrast Gpi-A^{100} was observed in the heterozygous state in 14.6% of the E. olmstedi from Herring Cr. and in the homozygous condition in E. nigrum (genotypic frequency = 3.4 - 100%) from every location in the present

study. Homozygotes of Gpi-A^{100} occurred in E. olmstedi at a genotypic frequency that ranged from 85 - 100%.

No electrophoretic species- specific characters (alleles) were detected consistently at any collection site in areas of sympatry. Sod-A^{40} was present only in allopatric E. nigrum populations in the upper James R. (Buffalo R. and Wreck Island Cr.) and was the most common allele (frequencies >0.82) in those populations. Data presented here do not support a difference at the species level between E. nigrum and E. olmstedi in the James R. drainage. Genetic distance coefficients suggest a subspecies relationship following Nei's (1978) guidelines. This finding disagrees with Clark (1978) and Falls (1982), who supported Cole's (1965) two-species interpretation. However, Clark did state that hybrid populations occurred in the Appomattox R. and Falling Cr. Stillman (1984) and Brett (1985) found interbreeding in sympatric areas of the Genesee R. in New York State. Extremely small genetic divergence (<0.061) between sympatric populations suggests the character used in this study to identify the fish prior to homogenization, infraorbital canal completeness, may not be reliable for differentiating between sympatric E. nigrum and E. olmstedi. Stillman (1984) found that, while 99% of all allopatric specimens of both species in New York state could be correctly identified by this character, its reliability declined considerably when the two species occurred sympatrically (28-33% of E. olmstedi specimens

had incomplete infraorbital canals). This reduction concurs with Cole (1965), who reported a value of 30% for this character when the two forms occurred together in New York State.

In summary, overall genetic variability among populations was low; however, if genetic distance and allelic frequencies are considered, it is possible to cluster three geographical groups, upper James R., Herring Cr. and the combined Falling Cr. and Appomattox R. populations. The geographical grouping may be due to an original colonization of the lower James R. during early Pleistocene by E. olmstedi and a later intrusion of the upper James R. from the New R. drainage by E. nigrum. The hybrid populations of Appomattox R. and Falling Cr. emphasizes the close relationship of the two forms, strengthening the support for subspecific designation of E. olmstedi.

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Table 1. Electrophoretically Examined Enzyme-Protein Systems of E. nigrum and E. olmstedi.

Enzyme-Protein	Abbr.	E. C. No.	Loci scored	Buffer system	Stain Recipe
Oxidoreductases					
Lactate dehydrogenase	LDH	1.1.1.27	1	TC[1]	Shaw and Prasad 1970
Malate dehydrogenase	MDH	1.1.1.37	1	TC	Brewer 1970
Malic enzyme	ME	1.1.1.40	1	TC	Ayala et al. 1972
Isocitrate dehydrogenase	ICD	1.1.1.42	1	TC	Shaw and Prasad 1970
Catalase	CAT	1.11.1.6	2	LiOH[2]	Shaw and Prasad 1970
Superoxide dismutase	SOD	1.15.1.1	1	LiOH	Johnson et al. 1970
Tranferases					
Aspartate aminotransferase	AAT	2.6.1.1	1	TC	Schwartz et al. 1963
Phosphoglucomutase	PGM	2.7.5.1	1	TC	Spencer et al. 1964
Hydrolases					
Esterase	ES	3.1.1.1	2	LiOH	Brewer 1970
Leucine aminopeptidase	LAP	3.4.1.1	1	TC	Shaw and Prasad 1970
Isomerases					
Mannose phosphate isomerase	MPI	5.3.1.8	1	TC	Nichols et al. 1973
Glucosephosphate isomerase	GPI	5.3.1.9	1	LiOH	DeLorenzo and Ruddle 1969
Nonenzymatic					
Calcium-binding protein	CBP	-------	3	LiOH	Buth 1982

[1]Tris-citrate, pH 8: Electrode - 0.687M Tris, 0.143M Citric acid; Gel - 1:29 dilution of electrode (Selander et al. 1971).

[2]Lithium hydroxide: Solution A (Electrode), pH 8.1 - 0.03M Lithium hydroxide, 0.19M Boric acid; Solution B, pH 8.4 - 0.05M Tris, 0.008 Citric acid; Gel - 1 part A + 9 parts B (Selander et al. 1971).

Table 2. Allelic Frequencies, % Polymorphism, and Mean Heterozygosity ($\bar{H}$)

E. nigrum from the James R. Drainage.

	Locus	Sod-A				Gpi-A			Es-B	
Population	Allele	100	72	40	26	141	130	100	100	95
HC	*E.o.*	0.82	0.02	——	0.17	——	0.07	0.93	0.56	0.44
FCU	*E.o.*	——	——	——	1.00	——	——	1.00	——	1.00
	E.n.	——	0.10	——	0.90	——	0.55	0.45	0.80	0.20
FCM	*E.n.*	0.25	——	——	0.75	——	0.13	0.88	1.00	——
FCL	*E.o.*	——	0.50	——	0.50	——	——	1.00	1.00	——
	E.n.	——	0.31	——	0.69	——	0.13	0.88	0.75	0.25
BR	*E.n.*	——	0.02	0.83	0.15	0.13	0.73	0.14	0.42	0.58
WIC	*E.n.*	——	0.08	0.90	0.03	——	0.47	0.53	0.55	0.45
NAR	*E.o.*	——	——	——	1.00	——	——	1.00	0.77	0.23
	E.n.	——	——	——	1.00	——	0.06	0.94	0.94	0.06
SAR	*E.o.*	——	——	——	1.00	——	——	1.00	1.00	——
	E.n.	——	——	——	1.00	——	——	1.00	0.90	0.10
RRC	*E.o.*	——	——	——	1.00	——	——	1.00	1.00	——
	E.n.	——	——	——	1.00	——	——	1.00	1.00	——
AR	*E.o.*	——	——	——	1.00	——	——	1.00	1.00	——
	E.n.	——	——	——	1.00	——	0.17	0.83	1.00	——

[1]Direct count mean heterozygosity.

[2]Hardy-Weinberg expected mean heterozygosity - an unbiased estimate based

[3]P = % polymorphic loci; A locus is considered polymorphic if the frequency

[4]Mean Sample Size Per Locus

HC, Herring Cr.; FCU, upper Falling Cr.; FCM, middle Falling Cr.; FCL, North Branch Appomattox R.; SAR, South Fork Appomattox R.; RRC, Rocky

at Six Polymorphic Loci in 16 Populations of E. olmstedi and

Es-D		Aat-B		Cbp-C		$\bar{H}$			
100	90	100	70	100	80	DC[1]	HW[2]	P[3]	N[4]
0.83	0.17	0.71	0.29	0.72	0.28	0.087	0.123	35.3	30.4
----	1.00	1.00	----	1.00	----	0.000	0.000	0.0	2.0
----	1.00	0.80	0.20	1.00	----	0.029	0.081	23.5	10.0
0.13	0.88	1.00	----	1.00	----	0.059	0.055	17.7	4.0
0.25	0.75	0.50	0.50	1.00	----	0.088	0.108	17.7	2.0
0.38	0.63	1.00	----	1.00	----	0.096	0.094	23.5	8.0
0.53	0.47	0.76	0.24	1.00	----	0.085	0.124	29.4	53.9
0.53	0.47	0.41	0.59	1.00	----	0.090	0.131	29.4	18.6
0.32	0.68	1.00	----	1.00	----	0.032	0.048	11.8	11.0
0.23	0.72	0.89	0.11	1.00	----	0.020	0.050	23.5	9.0
1.00	----	1.00	----	1.00	----	0.000	0.000	0.0	1.0
0.90	0.10	0.90	0.10	1.00	----	0.012	0.033	17.7	10.0
0.63	0.38	1.00	----	1.00	----	0.015	0.032	5.9	4.0
----	1.00	1.00	----	1.00	----	0.000	0.000	0.0	1.0
----	1.00	1.00	----	1.00	----	0.000	0.000	0.0	2.0
0.33	0.67	1.00	----	1.00	----	0.020	0.046	11.8	6.0

on conditional expectations (Levene,1949; Nei,1978).

of the most common allele is ≤ 0.95.

lower Falling Cr.; BR, Buffalo R.; WIC, Wreck Island Cr.; NAR, Run Cr.; AR, Appomattox R.

Table 3. Chi-square Test[1] for Deviation from Hardy-Weinberg Equilibrium at Each Polymorphic Locus of 16 Populations of *E. olmstedi* and *E. nigrum* from the James R. Drainage.

Population		Sod-A		Gpi-A		Es-B		Es-D		Aat-B		Cbp-C	
		X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
HC	E.o.	0.323[1]	0.570[1]	0.000	1.000	18.203	0.000	3.866	0.049	10.469	0.001	2.562	0.109
FCU	E.o.	NO POLYMORPHIC LOCI											
	E.n.	0.000	1.000	0.881	0.348	7.105	0.008	-----	-----	7.105	0.008	-----	-----
FCM	E.n.	0.000	1.000	0.000	1.000	-----	-----	0.000	1.000	-----	-----	-----	-----
FCL	E.o.	0.021	0.885	-----	-----	-----	-----	0.000	1.000	0.688	0.407	-----	-----
	E.n.	0.239	0.625	0.000	1.000	0.028	0.867	0.896	0.344	-----	-----	-----	-----
BR	E.n.	0.889[1]	0.346[1]	0.999[1]	0.317[1]	20.435	0.000	0.816	0.366	18.778	0.000	-----	-----
WIC	E.n.	0.000[1]	1.000[1]	0.000	1.000	1.462	0.227	10.315	0.001	15.026	0.000	-----	-----
NAR	E.o.	-----	-----	-----	-----	0.051	0.822	5.150	0.023	-----	-----	-----	-----
	E.n.	0.000	1.000	-----	-----	0.000	1.000	3.006	0.083	4.352	0.037	-----	-----
SAR	E.o.	NO POLYMORPHIC LOCI											
	E.n.	-----	-----	-----	-----	0.000	1.000	4.854	0.028	4.854	0.028	-----	-----
RRC	E.o.	-----	-----	-----	-----	-----	-----	0.208	0.648	-----	-----	-----	-----
	E.n.	NO POLYMORPHIC LOCI											
AR	E.o.	NO POLYMORPHIC LOCI											
	E.n.	-----	-----	0.000	1.000	-----	-----	4.023	0.045	-----	-----	-----	-----

[1]Pooled data.

Table 4. Summary of F-Statistics of All Polymorphic Loci in 16 Populations of *E. olmstedi* and *E. nigrum* from the James R. Drainage.

Locus	F_{IS}	F_{ST}
Sod-A	-0.43	0.65
Cbp-C	-0.38	0.26
Gpi-A	0.04	0.43
Es-B	0.20	0.46
Es-D	0.49	0.43
Aat-B	0.92	0.31
Mean	0.27	0.47

Table 5. Matrix of Genetic Similarity and Distance Coefficients of

Above Diagonal: Rogers (1972) Genetic Similarity

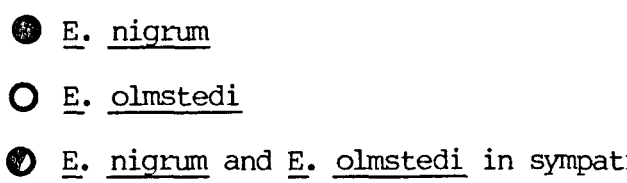
Below Diagonal: Nei (1978) Unbiased Genetic Distance

Population		HC	FCU		FCM	FCL		BR
		E.o.	E.o.	E.n.	E.n.	E.o.	E.n.	E.n.
HC	E.o.	———	0.832	0.841	0.862	0.864	0.883	0.862
FCU	E.o.	0.119	———	0.903	0.912	0.868	0.908	0.833
	E.n.	0.111	0.060	———	0.931	0.900	0.926	0.881
FCM	E.n.	0.073	0.064	0.015	———	0.930	0.954	0.844
FCL	E.o.	0.063	0.085	0.026	0.011	———	0.930	0.844
	E.n.	0.057	0.047	0.021	0.006	0.007	———	0.874
BR	E.n.	0.097	0.122	0.073	0.101	0.098	0.077	———
WIC	E.n.	0.078	0.132	0.083	0.097	0.058	0.075	0.015
NAR	E.o.	0.073	0.042	0.025	0.006	0.019	0.003	0.104
	E.n.	0.079	0.059	0.019	0.002	0.009	0.005	0.107
SAR	E.o.	0.066	0.125	0.086	0.048	0.052	0.031	0.129
	E.n.	0.057	0.102	0.070	0.039	0.038	0.022	0.115
RRC	E.o.	0.066	0.084	0.045	0.015	0.023	0.009	0.114
	E.n.	0.111	0.061	0.021	0.002	0.019	0.015	0.133
AR	E.o.	0.111	0.061	0.021	0.002	0.019	0.015	0.133
	E.n.	0.082	0.069	0.018	0.001	0.017	0.005	0.101

E. olmstedi and E. nigrum Populations from the James R. Drainage.

WIC	NAR		SAR		RRC		AR	
E.n.	E.o.	E.n.	E.o.	E.n.	E.o.	E.n.	E.o.	E.n.
0.873	0.871	0.868	0.877	0.895	0.875	0.839	0.839	0.857
0.819	0.936	0.918	0.882	0.888	0.904	0.941	0.941	0.912
0.875	0.930	0.935	0.879	0.897	0.901	0.938	0.938	0.928
0.846	0.953	0.962	0.926	0.921	0.949	0.971	0.971	0.971
0.879	0.924	0.940	0.897	0.903	0.919	0.926	0.926	0.926
0.877	0.970	0.954	0.923	0.929	0.945	0.938	0.938	0.962
0.945	0.856	0.853	0.827	0.844	0.849	0.824	0.824	0.853
-----	0.857	0.855	0.828	0.846	0.850	0.825	0.825	0.855
0.100	-----	0.978	0.947	0.952	0.969	0.968	0.968	0.976
0.097	0.000	-----	0.944	0.957	0.967	0.971	0.971	0.980
0.121	0.030	0.031	-----	0.982	0.978	0.941	0.941	0.951
0.104	0.021	0.022	0.001	-----	0.972	0.935	0.935	0.945
0.106	0.006	0.005	0.006	0.003	-----	0.963	0.963	0.973
0.125	0.008	0.004	0.061	0.050	0.021	-----	1.000	0.971
0.125	0.008	0.004	0.061	0.050	0.021	0.000	-----	0.971
0.100	0.002	0.000	0.027	0.020	0.003	0.006	0.006	-----

Fig. 1. Map of James River drainage of Virginia showing the distribution of Etheostoma olmstedi and Etheostoma nigrum by collection site.



HC, Herring, Cr.; FCU, upper Falling Cr.; FCM, middle Falling Cr.; FCL, lower Falling Cr.; BR, Buffalo R.; WIC, Wreck Island Cr.; NAR, North Branch Appomattox R.; SAR, South Fork Appomattox R.; RRC, Rocky Run Cr.; AR, Appomattox R.

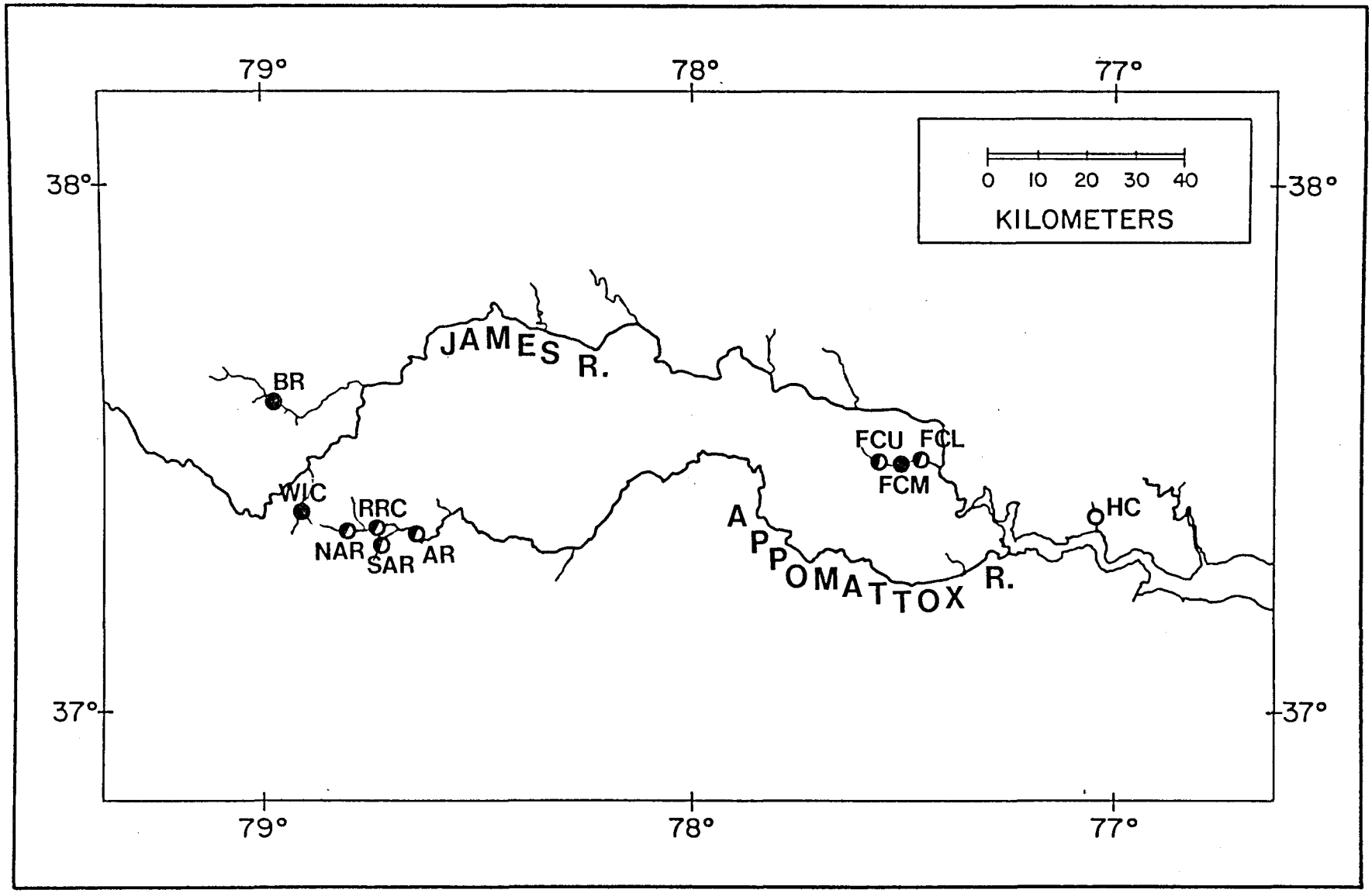

79°
78°
77°
38°
37°
0 10 20 30 40
KILOMETERS
JAMES R.
BR
WIC
RRC
NAR
SAR
AR
FCU
FCL
FCM
HC
APPOMATTOX R.

Fig. 2. Superoxide dismutase (SOD) starch gel zymogram showing dimeric banding pattern at the Sod-A locus in *Etheostoma olmstedi* and *Etheostoma nigrum* from the James R. drainage in Virginia.

A. O = origin

(1) $A^{40}\ A^{26}$

(2) $A^{40}\ A^{40}$

(3) $A^{100}\ A^{100}$

(4) $A^{100}\ A^{26}$

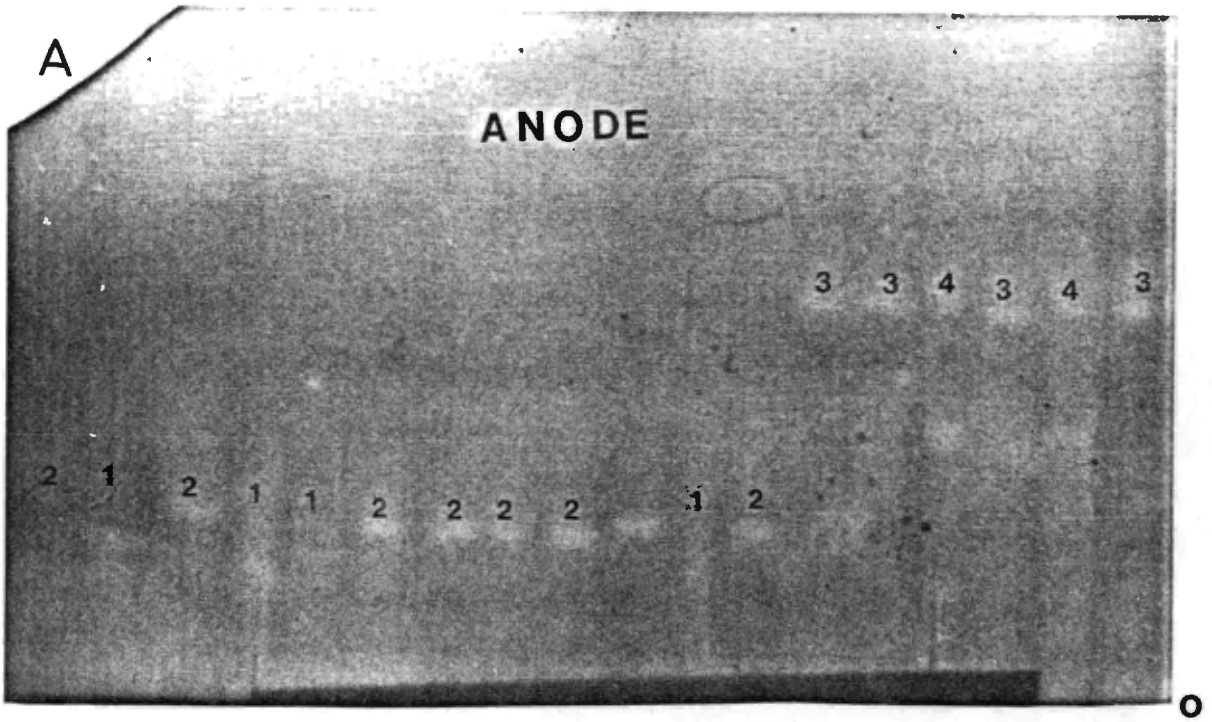
B. O = origin

(1) $A^{40}\ A^{26}$

(4) $A^{100}\ A^{26}$

(5) $A^{26}\ A^{26}$

(6) $A^{72}\ A^{40}$


A
ANODE
3 3 4 3 4 3
2 1 2 1 1 2 2 2 2 1 2
O

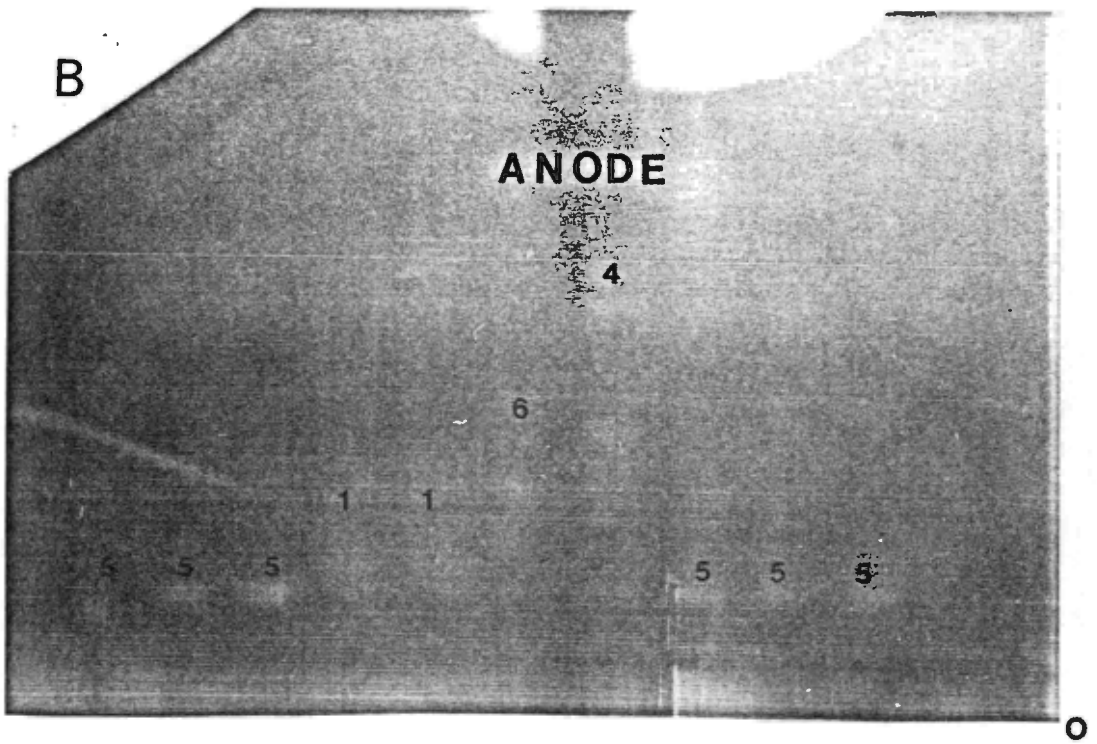

B
ANODE
4
6
1 1
5 5 5 5 5 5
O

Fig. 3. Glucosephosphate isomerase (GPI) starch gel zymogram showing dimeric banding pattern at the Gpi-A locus in Etheostoma olmstedi and Etheostoma nigrum from the James R. drainage in Virginia.

O = origin

(1) $A^{130}\ A^{130}$

(2) $A^{141}\ A^{141}$

(3) $A^{141}\ A^{130}$

(4) $A^{100}\ A^{100}$

(5) $A^{130}\ A^{100}$

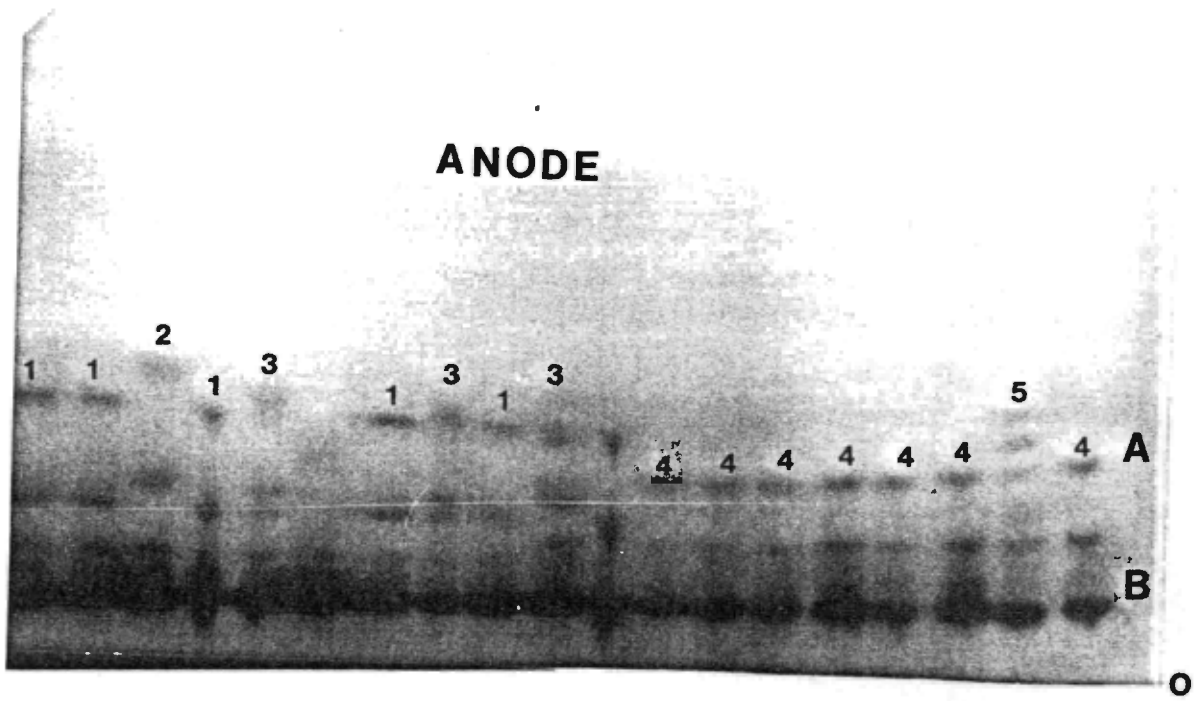

ANODE
1
1
2
1
3
1
3
1
3
4
4
4
4
4
4
5
4
A
B
O

Fig. 4. Esterase (ES) starch gel zymogram showing monomeric banding pattern at the Es-D locus in Etheostoma olmstedi and Etheostoma nigrum from the James R. drainage in Virginia.

O = origin

(1) D^{100} D^{100}

(2) D^{100} D^{90}

(3) D^{90} D^{90}

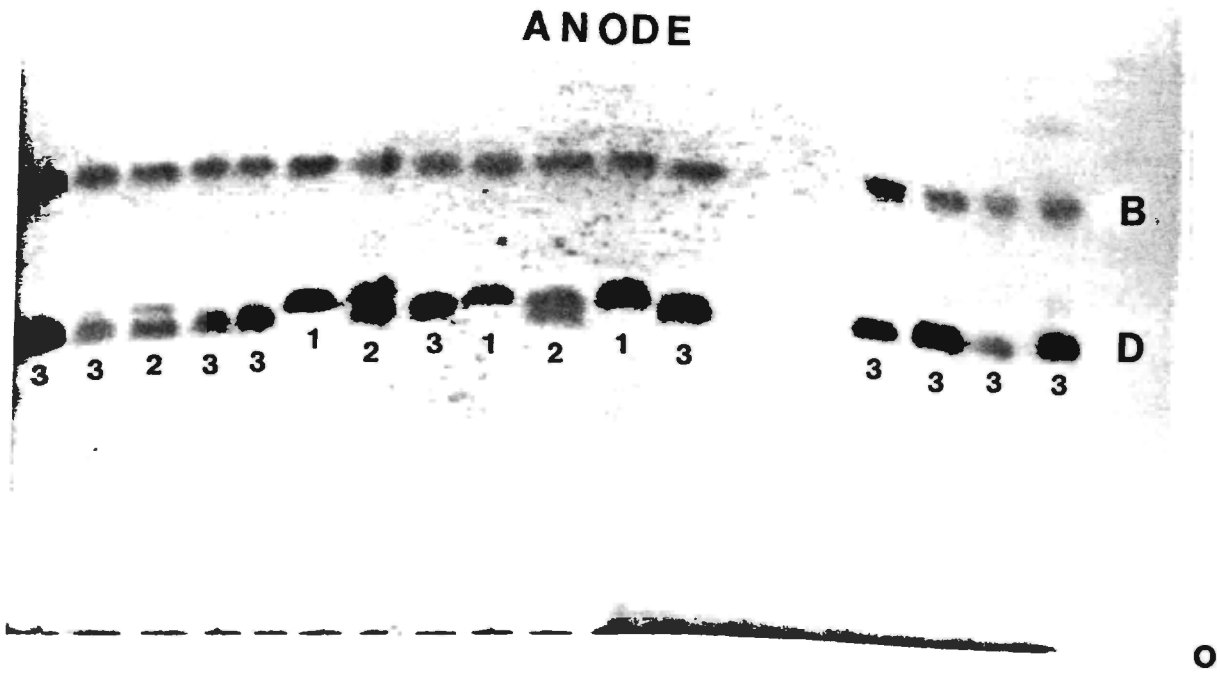

ANODE
B
D
3 3 2 3 3 1 2 3 1 2 1 3
3 3 3 3
O

Fig. 5. Aspartate aminotransferase (AAT) starch gel zymogram showing monomeric banding pattern at the Aat-B locus in Etheostoma olmstedi and Etheostoma nigrum from the James R. drainage in Virginia.

O = origin

(1) B^{100} B^{100}

(2) B^{100} B^{70}

(3) B^{70} B^{70}

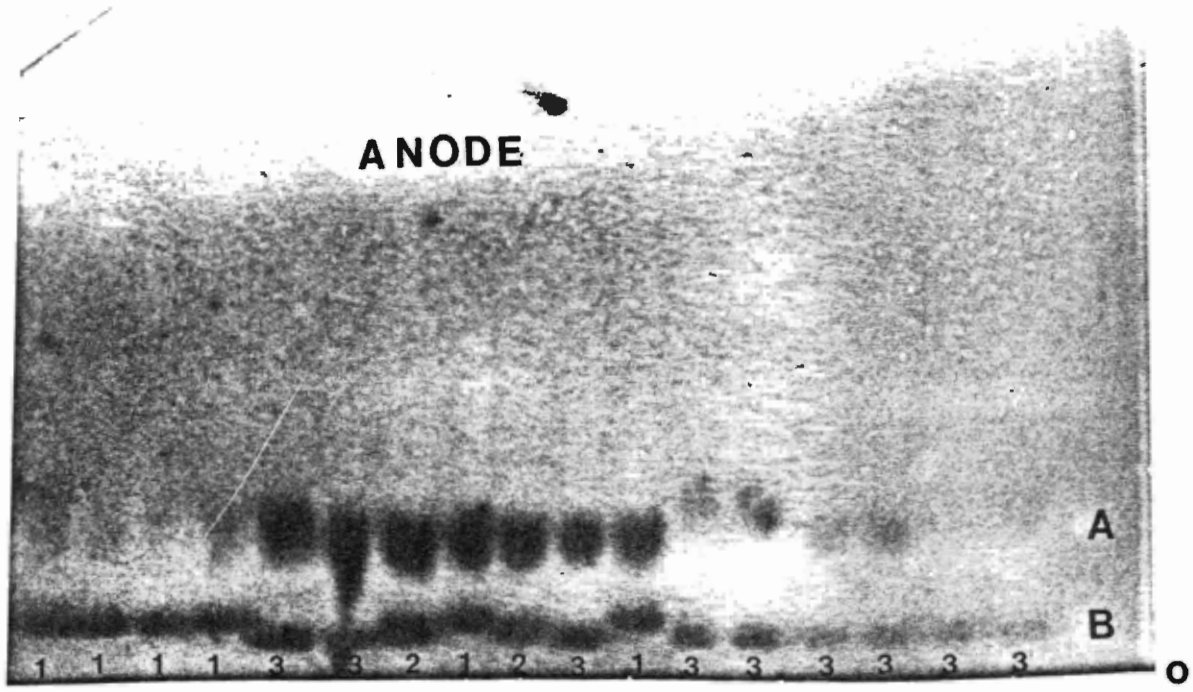

ANODE
A
B
1 1 1 1 3 3 2 1 2 3 1 3 3 3 3 3 3
O

Fig. 6. Calcium binding protein (CBP) starch gel zymogram showing monomeric banding pattern at the Cbp-C locus in Etheostoma olmstedi and Etheostoma nigrum from the James R. drainage in Virinia.

O = origin

(1) C^{100} C^{100} (all unlabeled fish)

(2) C^{100} C^{80}

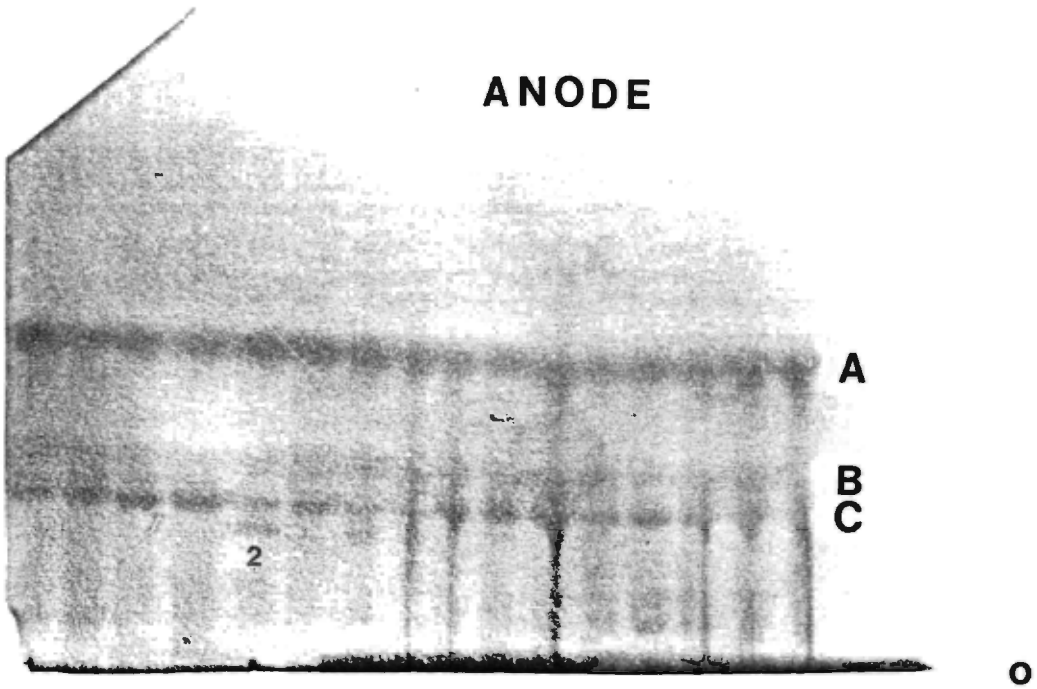
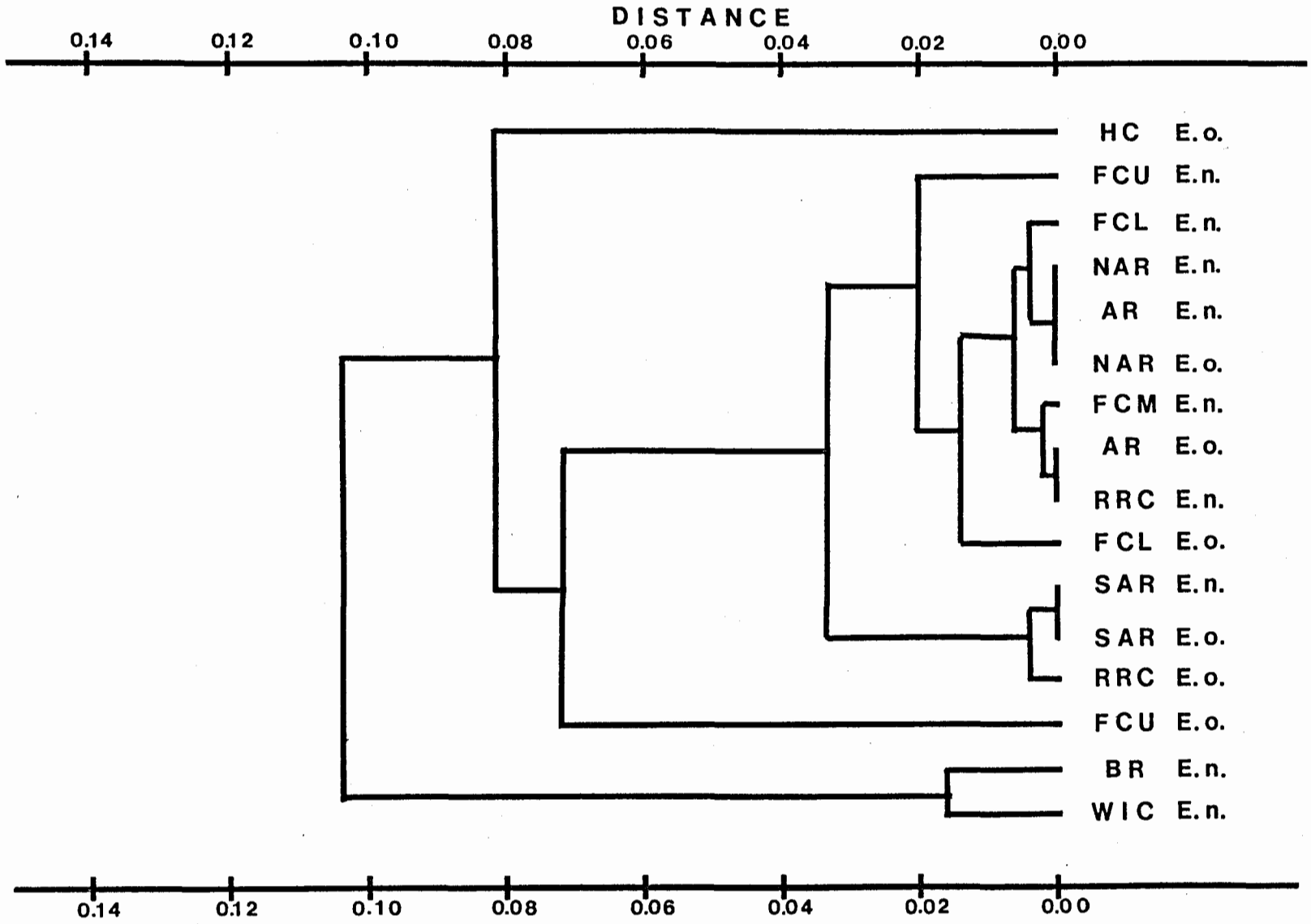

ANODE
A
B
C
2
O

Fig. 7. Phenogram of cluster analysis (UPGMAA) using Nei's (1978) unbiased genetic distance of Etheostoma olmstedi and Etheostoma nigrum from the James River drainage in Virginia.


DISTANCE
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
HC E.o.
FCU E.n.
FCL E.n.
NAR E.n.
AR E.n.
NAR E.o.
FCM E.n.
AR E.o.
RRC E.n.
FCL E.o.
SAR E.n.
SAR E.o.
RRC E.o.
FCU E.o.
BR E.n.
WIC E.n.
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

APPENDIX I

Methodology for Electrophoresis Adapted from Selander et al. (1971), Falls (1982), and Werth (1985).

Preparation of Gel: Corn starch (Lot #392: Electrostarch Co.) was heated at 37 C for at least three days prior to gel preparation to remove unknown volatiles. Appropriate gel buffer (lithium hydroxide or tris-citrate) was mixed with 97.5 g of starch to a final volume of 750 ml making a 13% starch gel. Mixture was heated over a bunsen burner with constant vigorous swirling in a 2 liter flask for approximately five minutes until the starch grains burst and a colloid was formed. At that time the mixture was thick and noticeably clearer. After additional heating and swirling for about one minute, the mixture thinned and boiled. The mixture was degassed for one minute until bubbles were uniform and rising from the bottom. The flask was immediately removed from the aspirator and the gel gently poured into a 20 cm X 17.5 cm X 1.5 cm plexiglass gel tray. Bubbles, lumps, or foreign matter were removed quickly with a spatula, the flask immediately filled with cold water (to facilitate gel removal), and the gel allowed to cool at room temperature for 1/2 to one hour. The gel was then covered with plastic wrap and placed in the refrigerator (4 C) until next morning.

Sample Preservation: Specimen identification and homogenate preparation were done on ice and all freezing (-50 C methanol bath) and thawing (37 C water bath) of protein extracts were

conducted as rapidly as possible to minimize loss of enzymatic activity. When thawed, extracts were immediately placed on ice until samples were applied and extracts refrozen.

Sample Application: A straight incision was made perpendicular to the gel about 5 cm from and parallel to the 17.5 cm end. Individual filter paper wicks (1.5 cm X 0.5 cm; Whatmann # 3) were soaked thoroughly with each protein extract; excess moisture was removed by blotting, and the wicks were then inserted into the origin (incision). Wicks were placed 2-3 mm apart reaching the bottom of the gel (repositioning avoided), so 20 samples and one bromphenol blue wick to monitor migration were fitted comfortably. The gel was separated with a spatula from sides of the tray and a plastic drinking straw placed between gel and tray at origin end to ensure a good sample contact.

Electrophoresis: Electrodes were devised from either hard plastic staining boxes (Ain Plastics), platinum wire (cathode) and nickel:chromium (80%:20%) wire (anode) or soft plastic desk dividers (K-Mart) and carbon welding rods (anode and cathode). There was no obvious difference in performance. The gel was positioned with origin toward the cathode electrode (negative; black) and electrode compartments were filled above electrode with appropriate buffer. A buffer-soaked sponge (Handi-wipe) was placed from the anode reservoir (positive; red) over about 4 cm of gel. The sponge and reservoir were covered with plastic wrap to about 2 cm short of the origin. Another soaked sponge was placed from the cathode reservoir over the origin to rest

on plastic wrap. This sponge and reservoir were also covered with plastic wrap so the entire apparatus was insulated. Bubbles were carefully removed, and a pan of ice was placed on top of the gel and periodically refilled. Electrodes were connected to D.C. power source. Current, adjusted to 75 mAmps, was passed through the gel by use of two electrode buffers wicked onto the gel with sponges. Amperage was checked regularly for readjustment. After 30 min the current was turned off and the wicks were removed. The apparatus was reassembled and the power switched on to continue electrophoresis at 75 mAmps until the bromphenol blue dye marker reached a distance of 10 cm from origin.

Gel Slicing: Gels were sliced into thin sheets so several different enzymes could be stained for from a single electrophoretic run. The power was cut off, the apparatus disassembled, and the gel was trimmed by cutting away the portions anodal to dye and cathodal to origin. The cathodal portions were stained for back migration of enzymes on initial gels. The upper left corner was cut for orientation purposes. The gel was removed from the tray and placed on moistened glass. A smooth weight was applied to the top of the gel and a modified Buchler gel slicer with clean and tight wire was pulled across the gel with a smooth and constant motion. The gel was turned over, a 1-2 mm slice removed and placed in 18 cm X 13 cm X 3.5 cm staining box (Ain Plastics). The procedure was repeated until all slices were made alternating the side of the gel cut to help keep an even gel thickness. The top slice was discarded and the bottom one used for experimental stains.

Staining: Enzymes were stained according to the recipes listed in Appendix II. After staining, gels were rinsed in tap water, photographed, and scored before preservation. The gels stained with MTT were preserved by soaking in 50% glycerol and then wrapped in plastic wrap. Those stained with NBT were soaked in water: methanol: acetic acid (5:5:1) and wrapped. Both types of stained gels were stored at 4 C.

APPENDIX II

RECIPES FOR BUFFER SYSTEMS AND ENZYME-PROTEIN STAINS

Electrophoretic Buffer Systems

Lithium hydroxide (LiOH) (Selander et al. 1971)

Solution A (Electrode):	Molarity	Amount per liter
Lithium hydoxide	0.03	1.20 g
Boric acid	0.19	11.89 g
pH 8.1		
Solution B:		
Tris	0.05	6.20 g
Citric acid (monohydrate)	0.008	1.60 g
pH 8.4		

Gel buffer is 1 part A + 9 parts B.

Tris-citrate pH 8.0 (Selander et al. 1971)

Electrode:	Molarity	Amount per liter
Tris	0.687	83.2 g
Citric acid (monohydrate)	0.143	33.0 g

Gel buffer is 1:29 dilution of electrode buffer.

Enzyme-Protein Stain Recipes

Lactate dehydrogenase

0.2 M Tris-HCl, pH 8.0	50.0 ml
1.0 M Lithium lactate, pH 8.0	5.0 ml
10 mg/ml NAD (Nicotinamide adenine dinucleotide)	1.0 ml
5 mg/ml NBT (Nitro blue tetrazolium)	1.0 ml
5 mg/ml PMS (Phenzine methosulfate)	1.0 ml

Incubate in dark at 37 C.

(Modified from Shaw and Prasad 1970)

Malate dehydrogenase

0.2 M Tris-HCl, pH 8.0	50.0 ml
2.0 M DL-Malic acid	5.0 ml
10 mg/ml NAD	1.0 ml
5 mg/ml NBT	1.0 ml
5 mg/ml PMS	1.0 ml

Incubate in dark at 37 C.

(Modified from Brewer 1970)

Malic enzyme (NADP-dependent MDH)

0.2 M Tris-HCl, pH 8.0	50.0 ml
0.1 M $MgCl_2 \cdot 6H_2O$	3.0 ml
2.0 M DL-Malic acid, pH 8.0	5.0 ml
NADP (solid only)	0.02 g
5 mg/ml NBT	1.0 ml
5 mg/ml PMS	1.0 ml

Incubate in dark at 37 C.

(Modified from Ayala et al. 1972)

Isocitrate dehydrogenase

0.2 M Tris-HCl, pH 8.0	50.0 ml
0.1 M $MgCl_2 \cdot 6H_2O$	3.0 ml
0.1 M DL-Isocitric acid	3.0 ml
10 mg/ml NADP	1.0 ml
5 mg/ml NBT	1.0 ml
5 mg/ml PMS	1.0 ml

Incubate in dark at 37 C.

(Modified from Shaw and Prasad 1970)

Catalase

0.5% H_2O_2	50.0 ml

Incubate for two minutes; then rinse twice with distilled H_2O; then add the following solution:

5 M KI	1.0 ml
Acetic acid (conc.)	1.0 ml
Distilled H_2O	98.0 ml

Photograph and score as soon as white bands on dark blue background are evident.

(Modified from Shaw and Prasad 1970)

Superoxide dismutase

0.2 M Tris-HCl, pH 9.0	50.0 ml
5 mg/ml NBT	2.0 ml
5 mg/ml PMS	2.0 ml

Expose to light at ambient temperature until white bands on blue background are evident.

(Modified from Johnson et al. 1970)

Aspartate aminotransferase

0.2 M Tris-HCl, pH 8.0	50.0 ml
Dissolve the following:	
α-Ketoglutaric acid	0.10 g
L-Aspartic acid	0.23 g
Readjust pH to 8.0 with 4 N NaOH.	
Then add:	
Pyridoxal 5-phosphate	0.01 g
Fast Blue BB salt	0.10 g

Incubate in dark at 37 C.

(Modified from Schwartz et al. 1963)

Phosphoglucomutase

0.2 M Tris-HCl, pH 8.0	50.0 ml
0.1 M $MgCl_2 \cdot 6H_2O$	5.0 ml
Glucose-1-phosphate	0.1 g
40 NAD-U/ml Glucose-6-phosphate dehydrogenase	1.0 ml
10 mg/ml NAD	2.0 ml
5 mg/ml NBT	1.0 ml
5 mg/ml PMS	1.0 ml

Incubate in dark at 37 C.

(Modified from Spencer et al. 1964)

Esterase

0.2 M Tris-HCl, pH 7.0	50.0 ml
α-Naphthyl acetate (1% solution in 50% acetone)	3.0 ml
Fast Blue BB salt	0.05 g

Incubate in dark at ambient temperature.

(Modified from Brewer 1970)

Leucine aminopeptidase

0.2 M Tris-maleate, pH 5.2	50.0 ml
2.5% L-leucyl-β-naphthylamide HCl	1.0 ml
Incubate 15-30 minutes in dark, then add:	
Fast Garnet GBC	50. mg

Incubate in dark at 37 C.

(Modified from Shaw and Prasad 1970)

Calcium-binding protein

Distilled H_2O	50.0 ml
Dissolve:	
Brilliant Blue G dye	0.05 g
Then add:	
Trichloroacetic acid	7.50 g
Sulfosalicylic acid	2.50 g

Incubate in dark at 37 C.

(Modified from Buth 1982)

Enzyme Systems Discontinued.

Acid phosphatase

Adenylate kinase

Alanine dehydrogenase

Alcohol dehydrogenase

Alkaline phosphatase

Creatine kinase

Glucose-6-phosphate dehydrogenase

Glutamate dehydrogenase

Hexokinase

Phosphogluconate dehydrogenase

Vita

John Stuart Prince, Jr. was born October 16, 1956 in Richmond, Va. He attended public schools in Greensville County and graduated from high school in 1975. He received a Bachelor of Science degree in Chemistry from the University of Richmond in 1979. He completed a Master of Science degree in Biology at the University of Richmond in August 1985 and will enter the School of Medicine at the Medical College of Virginia in the fall of 1985.