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An electrophoretic comparison of gene loci in etheostoma nigrum and etheostoma olmstedi from the James River drainage, Virginia

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AN ELECTROPHORETIC COMPARISON OF GENE LOCI IN *ETHEOSTOMA NIGRUM* AND *ETHEOSTOMA OLMSTEDI* FROM THE JAMES RIVER DRAINAGE, VIRGINIA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

As many as five different populations of *Etheostoma nigrum* and *Etheostoma olmstedi* have been identified in the James River drainage, Virginia. Genetic variation of these populations was analyzed with starch gel electrophoresis and revealed only three distinct populations. Nine loci encoding five enzyme systems and two loci encoding muscle proteins were examined in tissue homogenates. All populations were monomorphic with the exception of the esterase-3 (Est-3), phosphoglucomutase (Pgm-1) and phosphoglucose isomerase (Pgi) loci. Two alleles of Est-3 exhibited activity in the homozygous and heterozygous conditions. Pgm-1 occurred as two alleles with a high degree of heterozygosity (46% for *E. nigrum* and 54% for *E. olmstedi*). The Pgi-1 allele in both the homozygous and heterozygous condition was species specific for *E. nigrum* whereas the presence of Pgi-1 and Pgi-2 in the homozygous state was diagnostic of *E. olmstedi*. *Etheostoma nigrum* inhabited the Piedmont section of the James above the Fall Line; *E. olmstedi* occupied the Coastal Plain segment of the river and the Appomattox River drainage. The two species occurred sympatrically in Falling Creek. No electrophoretic evidence was found for hybridization between the two forms. Banding patterns for all loci examined were the same for the subspecies *E. o. olmstedi* and *E. o. atromaculatum*. Overall heterozygosity was higher for *E. nigrum* (10.3%) than *E. olmstedi* (5.1%) but both were within the range reported for teleosts.

Etheostoma nigrum Rafinesque 1820, the Johnny darter, and *Etheostoma olmstedi* Storer 1842, the tesselated darter, are sibling species (subgenus *Boleosoma*) distinguishable from each other by the number of rays in the dorsal and pectoral fins and the number of infraorbital canal pores (Cole, 1965). Bruner (1980) showed that *E. nigrum* occupies an extensive range from Quebec in the northeast to Colorado in the west, and from central Canada south to Alabama. It is also present in southeastern Atlantic drainages in Virginia and North Carolina. *Etheostoma olmstedi* is an inhabitant of Atlantic Coastal streams and rivers from Quebec south to Florida (Lee and McAllister, 1980).

The two species occur sympatrically in the Lake Ontario drainage. Stone (1947) in a study of the Ottawa River section of the drainage found no evidence of hybridization. Cole (1958, 1965), however, reported a hybrid swarm of *E. nigrum* and *E. olmstedi* in western New York and introgression of an *E. nigrum* character into *E. olmstedi* from Lake Champlain. In a later study of the Ottawa River population, McAllister, et al. (1972) concluded that hybridization had occurred. Their analysis entailed a comparison of meristic characters and confirmation of the results with electrophoretic separation of muscle proteins.

Etheostoma nigrum and *E. olmstedi* occur in the James River drainage of Virginia. With the use of meristic character analysis, Cole (1965, 1967) and later Clark (1978) determined that *E. olmstedi* is represented by two subspecies, *E. o. olmstedi* and *E. o. atromaculatum*. *Etheostoma o. olmstedi* is restricted to tributaries of the upper Coastal Plain whereas *E. o.*

atromaculatum occupies the tidal James and its lower tributaries. Cole (1967) stated that the species were allopatric in the James with *E. n. nigrum* in the upper drainage and *E. olmstedi* subspp. below the Fall Line. In addition, Clark (1978) identified two populations with characters intermediate between *E. nigrum* and *E. o. olmstedi*. One was in the Appomattox River and the other in Falling Creek, tributaries of the Coastal Plain James. He concluded introgressive hybridization was occurring between established populations of *E. o. olmstedi* and introduced *E. nigrum* that entered the drainages via stream piracy on Piedmont tributaries of the James.

In view of the apparent success of McAllister, et al. (1972) with electrophoresis in confirming suspected hybridization between populations of *E. nigrum* and *E. olmstedi*, starch gel electrophoresis was used to assess Clark's (1978) conclusions on the relationship between the populations of *E. nigrum* and *E. olmstedi* in the James River and its tributaries.

MATERIALS AND METHODS

A total of 132 specimens of *E. nigrum* and *E. olmstedi* from 19 collection sites was analyzed. Specimens were collected with a 10 ft (3 m) wide one-quarter in (7 mm) mesh seine and kept alive in a styrofoam container. Upon return to the laboratory the specimens were frozen and kept at -15 C until homogenized.

Homogenates were prepared in two ways. Thirty eight specimens used for general protein (myogen) analysis were skinned, filletted, minced and homogenized in an equal volume of 2.0% 2-phenoxyethanol following Merkle, et al. (1977).

Ninety four specimens were prepared in a similar manner except that a ventral incision was made from the branchial septum to the anus after which the digestive tract was removed and discarded. Homogenates from these plus the 38 muscle homogenates were analyzed for specific enzyme activity within two weeks of preparation. Homogenates were stored at -15 C.

Starch gel electrophoresis was performed using a modification of the methods of Smithies (1955, 1959). Starch gels (Electrostarch Lot #392: Otto Hiller, Madison, Wisconsin) were heated with constant stirring in the appropriate buffer to 80 to 85 C, degassed and poured into a plastic tray 23 cm x 18 cm x 1.5 cm. A razor blade was used to slice the cooled gel along its width. Filter paper wicks soaked in the homogenate were placed in the slot formed by the slice using fine forceps to prevent contamination.

The specific loci selected for analysis of the darter populations were among those suggested by Donald A. Merkle (pers. commun.) as useful for vertebrates. Electrophoretic techniques based on studies of mouse (*Peromyscus polionotus*) serum by Selander, et al. (1971) were employed with the following modifications: All gels were run at 5 C; phosphoglucomutase (Pgm-1), phosphoglucose isomerases (Pgi-1, Pgi-2), and esterases (Est-1, Est-2, Est-3) were best demonstrated with a boric acid buffer (0.01 M Tris, 0.001 M boric acid, 0.001 M EDTA, pH = 9.0), which was used in both the gel and electrode compartment; lactate dehydrogenases (Ldh-1, Ldh-2) and malate dehydrogenases (Mdh-1, Mdh-2) were best demonstrated with a Tris-citrate buffer (0.7 M Tris, 0.15 M citric acid, pH = 8.0) diluted 1:18 for the gel buffer

and undiluted in the electrode chambers. The gel concentration of these two systems was 13.0% and 12.5%, respectively. Boric acid gels were run at 400 V for 36 to 48 hrs whereas Tris-citrate gels were run at 150 V for 18 hrs.

Muscle homogenates were electrophoresed following the procedure originally described by Tsuyuki, et al. (1962) and used by McAllister, et al. (1972). This system employs a borate buffer (gel: 0.03 M boric acid, 0.012 M NaOH; electrode: 0.3 M boric acid, 0.03 M NaOH; pH = 8.5) run at 200 V for 18 hrs. In the present study 13% gels were run at 5 C. The method of Smithies (1955) as used by McAllister, et al. (1972) and that of Buth (1982) was used for wide spectrum staining of muscle proteins (Pt-1, Pt-2). General muscle proteins were also separated with cellulose polyacetate strips (Gelman Instruments, Inc.) and the bands qualitatively compared with those obtained on starch gel. Buffer and stains were prepared as those used with the gels.

Upon completion of a run the gels were horizontally sliced with a 26 gauge steel wire. Histochemical staining was carried out at 37 C according to Selander, et al. (1971). After 30 min to an hour the slices were set with a wash solution (5 parts acetone, 5 parts methanol, 1 part glacial acetic acid), wrapped in cellophane and stored at 5 C.

As this study was intended to be complementary to the meristic one of Clark (1978), most samples were from sites listed in his study. Additional collections were made in the upper Appomattox River where Abbott, et al. (1978) reported the occurrence of several upper James species including *E. nigrum*.

Estimates of mean heterozygosity ($\bar{H}$) were calculated for populations of *E. nigrum* and *E. olmstedi* in the James River drainage according to the method of Selander, et al. (1971). Chi-square analysis was used to test the significance of the proportion of homozygosity between populations.

Results were organized similarly to those in Merkle and Guttman (1977). Isozymes of enzyme systems are reported in order of decreasing anodal mobility. Alleles present at polymorphic loci are designated alphabetically in order of decreasing anodal mobility by a superscript following the locus designation.

COLLECTION SITES: The collections are listed in an upstream-downstream sequence of the James River and its tributaries following Clark (1978) and were made in those locations where specimens used by him were collected, unless otherwise noted by an asterisk (*). The number of specimens analyzed from each site are in parentheses.

UPPER JAMES RIVER: Buffalo River at St. Rt. 778* (9), Amherst Co., 21 Oct., 1981; Rivanna River at St. Rt. 6 (8), Fluvanna Co., 12 Apr., 1982; Beaverdam and Buffalo Creek (12), Goochland Co., 2 Mar., 1982; Tuckahoe Creek at U. S. Rt. 250 (3), 20 Apr., 1982.

APPOMATTOX RIVER: Holiday Creek at St. Rt. 614* (8), Appomattox Co., 23 Dec., 1981; Appomattox River at St. Rt. 612* (6), Appomattox Co., 23 Dec., 1981; Bush River at U. S. Rt. 460 (7), Prince Edward Co., 23 Dec., 1981; Appomattox River approximately 1 km upstream of its confluence with Swift Creek (4), Chesterfield Co., 23 May, 1982.

LOWER JAMES RIVER: Swift Creek at St. Rt. 677 (4), Chesterfield Co., 24 Feb., 1982; Swift Creek at St. Rt. 653 (3), Chesterfield Co., 9 Dec., 1981; Swift Creek at U. S. Rt. 1 (8), Chesterfield Co., 23 May,

1982; Falling Creek at St. Rt. 677 (8), Chesterfield Co., 24 Feb., 1982; Falling Creek at St. Rt. 653 (16), Chesterfield Co., 9 Dec., 1981; Falling Creek at St. Rt. 638 (4), Chesterfield Co., 17 Dec., 1981; Falling Creek at U. S. Rt. 1 (3), Chesterfield Co., 17 Dec., 1981; Herring Creek approximately 2 km below Harrison Lake (8), Charles City Co., 9 Sept., 1981; Beaverdam Creek at U. S. 360 (8), Hanover Co., 10 Feb., 1982; Collins River at St. Rt. 614 (8); Charles City Co., 10 Feb., 1982; Diascund Creek at U. S. Rt. 60 (5), New Kent Co., 7 May, 1982.

RESULTS

The twelve loci examined were found to be scorable in greater than 98% of the specimens of *E. nigrum* and *E. olmstedi*. Ldh-1, Ldh-2, Mdh-1, Mdh-2, Est-1, Est-2, Pt-1 and Pt-2 were monomorphic for a common allele and occurred with a frequency of 1.00. Est-3 and Pgm-1 were polymorphic for all populations; Pgi was polymorphic for *E. nigrum* (Table 1).

Frequencies of gene loci (Table 1) and calculations of mean heterozygosity (Table 2) for populations designated as *E. o. olmstedi* and *E. o. atromaculatum* by Clark (1978) were no different and were combined for subsequent analysis.

There was no observable difference between the general protein bands on starch gel and cellulose polyacetate media. Two myogen bands were always present regardless of the separation media or the staining method (Fig. 1).

Est-3 was present in two forms, *a* and *b* (Table 1; Figs. 2A and 2B). The *a* allele was present at a much lower frequency (approximately 1:4) in all populations (Table 1). There was no difference between *E.*

nigrum and *E. olmstedi* for heterozygosity at this locus (Table 3).

Pgm-1 was expressed as two alleles, *a* and *b* (Table 1; Figs. 3A and 3B). There was no significant difference between genotypes of *E. nigrum* and *E. olmstedi* for the Pgm locus (Table 3). A high degree of heterozygosity was observed (46% for *E. nigrum*, 54% for *E. olmstedi*).

The most notable difference between the enzyme bands of the two species was observed at the Pgi loci. *Etheostoma nigrum* was identified by the Pgi-1*a* and Pgi-2*a* alleles in either the homozygous or heterozygous state whereas the Pgi-1*c* and Pgi-2*b* alleles in the homozygous condition was diagnostic of *E. olmstedi* (Table 1; Figs. 4A and 4B).

The banding patterns of the Pgi alleles identified *E. nigrum* and *E. olmstedi*. The Piedmont section of the James River above the Fall Line was determined to contain *E. nigrum* exclusively. *Etheostoma olmstedi* occupied the tidal James, the Appomattox River and its tributary Swift Creek. The two species were sympatric in Falling Creek where samples were comprised of 45% *E. nigrum* and 55% *E. olmstedi* (Fig. 5).

There was significantly greater ($p < .001$) heterozygosity at the Pgi loci in the combined populations of *E. nigrum* than in populations of *E. olmstedi* (Table 3). This contributed to a considerably higher estimate of overall heterozygosity for *E. nigrum*. When the Pgi loci are included in the calculation for this species the value obtained is 10.3%, twice that for *E. olmstedi* (5.1%). If the Pgi loci are deleted from the calculation for *E. nigrum* the value obtained is 5.0%, virtually the same as *E. olmstedi* (Table 2).

An exception to the high degree of heterozygosity for *E. nigrum*

at the Pgi loci was in the Falling Creek population (Table 4). *Etheostoma nigrum* in this stream was significantly more ($p < .05$) homozygous (43%) than *E. nigrum* from all other locations (16%).

DISCUSSION

The taxonomic relationship of *E. nigrum* and *E. olmstedi* has been a controversial one. Stone (1947), using discriminant function analysis, first compared sympatric populations of the two species in the Ottawa River, then compared allopatric populations of *E. nigrum* from Michigan and western New York with those of *E. olmstedi* from eastern New York. After comparing results of the studies, he suggested that the two darters represented distinct species and reported greatest character divergence within the zone of overlap. Reproductive character displacement is a well-documented phenomenon (Grant, 1975).

Hubbs and Lagler (1964) did not agree with Stone's (1947) findings and treated *E. olmstedi* as a subspecies of *E. nigrum*. Cole (1965, 1967) made a detailed study of the two forms throughout their range and concluded that they were valid species. He recognized three subspecies of *E. nigrum* and four of *E. olmstedi*. Zorach (1971) studied *E. o. olmstedi* and *E. o. atromaculatum* in southeastern Virginia and encountered problems identifying subspecies with the meristic characters used by Cole (1967). Clark (1978) studied *E. nigrum* and *E. olmstedi* in the James River, Virginia and recognized five populations; *E. nigrum* in the Piedmont section of the river, *E. o. olmstedi* in the creeks of the upper Coastal Plain, and *E. o. atromaculatum* throughout the tidal section of the James. Two distinct and different populations of morphological intermediates,

which he concluded to be introgressive hybrids between *E. nigrum* and *E. o. olmstedi*, occurred in the Appomattox River and Falling Creek.

Cole (1958) previously made reference to a hybrid swarm between *E. nigrum* and *E. olmstedi* in a zone of overlap in a stream in western New York, part of the Lake Ontario drainage. McAllister, et al. (1972) later detailed apparent hybridization between *E. nigrum* and *E. olmstedi* in the Ottawa River, another area of sympatry in the Lake Ontario drainage. Ten morphological characters of 132 specimens were analyzed with a simple character index. Morphological intermediates constituted 7.6% of the population. Hybridization was confirmed with an analysis of general muscle proteins after electrophoretic separation. Muscle homogenates of 21 specimens yielded species specific protein bands of unassigned loci (designated "A" for *E. nigrum*, "B" for *E. olmstedi* according to order of anodal migration). Seven of eight morphological intermediates had both bands and only one had "A". Of five specimens identified as *E. olmstedi*, three had the "B" band, one had "A" and one had both.

In the present study electrophoretic analysis was applied to the populations reported by Clark (1978). The general protein bands of 38 specimens showed all of Clark's populations to be monomorphic for two bands, Pt-1 and Pt-2, in contrast to the findings of McAllister, et al. (1972). At least three different factors acting independently or collectively could account for this. One is the geographical separation of the two studies. Merkle, et al. (1977) implied that extensive isozyme variation over a large geographic area is the rule rather than the exception in vertebrates. A positive correlation between differing environments and observed genetic variability in organisms has been

provided by both experimental and theoretical data (McDonald and Ayala, 1974). Second, the bands observed by McAllister, et al. (1972) may represent separate alleles, not separate loci. Avise and Selander (1972) reported this to be the case for Pgm-1 in *Astyanax mexicanum*, the Mexican cavefish. The double band of the intermediate could indicate a heterozygous subpopulation rather than a true hybrid. This would explain why McAllister, et al. (1972) found all three banding patterns in five specimens that had been identified with meristic characters as *E. olmstedi*. Third, the monomorphic condition for Pt-1 and Pt-2 as found for *E. nigrum* in the present study also was reported by Buth (1982) for this species. He isolated two bands and assigned loci to them by combining general and specific staining techniques. Pt-1 was identified as calcium binding protein and Pt-2 as an allele of creatine kinase A. In the current study the general protein stain used by McAllister, et al. (1972) and the one used by Buth (1982) were compared by staining different slices of the same gel. There was no difference in the banding pattern obtained for the two although Buth's was cleaner and simpler to use. Considering the impact that these three factors could have on results, the conclusion of McAllister, et al. (1972) that hybridization was occurring should be viewed with skepticism. Even they cautioned that the discriminating power of the "A" band for *E. nigrum* was only 65% and the "B" band for *E. olmstedi* was only 67%.

Comparison of the zymograms of muscle and total body homogenates revealed that 11 of 12 loci exhibited strong activity in pure muscle tissue. With the exception of the Pgi-2 locus, muscle homogenates alone would suffice for analysis of loci in these two species. This is important because of the small size (3 to 5 cm) of most specimens.

Obtaining sufficient and consistent quantities of specific organs (e.g., brain and liver) is virtually impossible without combining the organs of many specimens. Similar findings from comparison of visceral and muscle homogenates in minnows (*Rhinichthys cataractae*) for Mdh, Ldh, Pgm and Est (Merritt, et al., 1978) permits a generalization that muscle homogenates would be sufficient for studies on most enzyme systems of small teleosts.

Ldh and Mdh isozymes have been found to exist in as many as three and two distinct forms, respectively, in A. *mexicanum* by Avise and Selander (1972). The monomorphic state for these two loci in *E. nigrum* and *E. olmstedi* in the James drainage is supported by data on *Etheostoma* from other locales. Page and Whitt (1973a) used morphology, Ldh-1 and Ldh-2 to establish the tribal relationship of Etheostomatini (including *E. nigrum* and *E. olmstedi*) collected from Virginia to Illinois. The same authors (1973b) studied Mdh-1 and Mdh-2 isozymes and reported that the same pattern obtained in the present study was shared by 18 species and 7 subgenera of *Etheostoma*, including *E. nigrum*. They concluded a probable common origin for a diverse number of species assigned to this genus and believed the trend in Mdh evolution was the same as that in Ldh: "differentiated patterns tend to be unique to individual species and are therefore presumably rather recent developments" (Page and Whitt, 1973b).

Etheostoma nigrum and *E. olmstedi* were monomorphic for Est-1 and Est-2, supporting the findings of Martin and Richmond (1973) for *E. nigrum*. Two alleles for Est-3 were found with consistent frequency for all James River populations with Est-3*a* the rarer. Intraspecific

allelic variability has been found previously for Est loci in other species of *Etheostoma* by Martin and Richmond (1973) and for *R. cataractae* by Merritt, et al. (1978). The variability in *R. cataractae* was attributed to genetic drift rather than selection.

Pgm exists as a single locus in fishes (Pgm-1) in contrast to three typically found in mammals (Avise and Selander, 1972). *Etheostoma nigrum* and *E. olmstedi* showed activity at this locus. The high degree of heterozygosity (46% of *E. nigrum* and 54% of *E. olmstedi*) for the Pgm-1 locus is common in fishes. Avise and Selander (1972) found 48% of specimens of a population of *A. mexicanum* to be heterozygous. Merritt, et al. (1978) stated that results for Pgm in *R. cataractae* "were as described by Avise and Selander (1972) for *Astyanax mexicanum*" but reported only overall heterozygosity, none for individual loci. Ferris, et al. (1982) reported average heterozygosity of a population of *Catastomus plebeius*, the Rio Grande Mountain sucker, to be .017, primarily attributable to heterozygosity at the Pgm-1 locus.

Pgi has been found only as a single locus in arthropods and tetrapods (Selander, et al., 1971). In *A. mexicanum* and other teleosts, however, two independent loci are present. This is attributed to gene duplication (Avise and Selander, 1972). Duplication of genes ranges from small duplication involving only a single locus or few loci to polyploidy, i.e., multiplication of the entire genome. Such changes are significant factors in evolution (Manwell and Baker, 1970). Each Pgi loci encodes a subunit, two of which combine to form the functional dimeric enzyme. Individuals homozygous at both Pgi loci exhibit three widely spaced bands. Specimens heterozygous at

either locus show six bands resulting from all possible combinations of subunits. Specimens presumed to be heterozygous at both loci have phenotypic patterns that are fully compatible with this model (Avise and Selander, 1972). Genotype could be assigned only to the combined loci of *E. nigrum* since heterozygosity at either locus results in the same banding pattern.

This pattern has been documented in *A. mexicanum* by Avise and Selander (1972) and *R. cataractae* by Merritt, et al. (1978). The patterns found in the current study supported these findings. The Pgi-1*a* and Pgi-2*a* alleles in either the homozygous or heterozygous condition identified *E. nigrum*. The homozygous condition for Pgi-1*c* and Pgi-2*b* for all *E. olmstedi* was diagnostic for the species.

There have been controversial reports of the occurrence of *E. nigrum* and *E. olmstedi* in the Appomattox drainage. Abbott, et al. (1978) identified *E. nigrum* in the upper Appomattox River and its tributary, Holiday Creek. Clark (1978), with samples from sites throughout the Appomattox drainage, concluded that introgressive hybrids of *E. nigrum* and *E. olmstedi* formed most of the population of this drainage. An exception was the pure *E. olmstedi* population in Swift Creek, a Coastal Plain confluent of the Appomattox River. Locations sampled in both of the above studies were included in the sampling regimen of the present study. Pgi isozymes showed that *E. olmstedi*, and not *E. nigrum* nor introgressive hybrids, is in the entire drainage. This was not unexpected since the Appomattox River empties into the James River in the Coastal Plain at Hopewell, Virginia, approximately 33 river km below the Fall Line. Therefore, it is not a drainage easily accessible to *E. nigrum*, which is restricted to the

river above the Fall Line.

Clark's (1978) interpretation of the introgressive structure of the Appomattox River population was based on meristic values skewed toward *E. nigrum* and the presence of a complete infraorbital canal, a character of *E. olmstedi*. There was no electrophoretic evidence for hybridization in the Appomattox drainage populations. Rather, the condition appears to be like that reported by Cole (1967) for the various subspecies of *E. olmstedi* that occur in the Atlantic drainages of Virginia and North Carolina. He found that *E. o. vexillare*, the Piedmont subspecies of *E. olmstedi* in the Rappahannock River, Virginia, responded to a steeper-gradient upstream habitat with meristic values similar to those of *E. nigrum*. Lateral line scales and rays of the dorsal, anal and pectoral fins were fewer in number than those for *E. olmstedi*. He further suggested that character convergence could account for the high prevalence of incomplete infraorbital canals, a character of *E. nigrum*, in *E. o. maculaticeps* in the upper Cape Fear River, North Carolina. Lack of any known stream exchange between the Cape Fear and the Neuse River to the north, which contains *E. nigrum*, precluded character introgression. Cole (1967) further reported the trend from greater meristic values for *E. o. atromaculatum*, the form in the Coastal Plain James River, to lower values (characteristic of *E. nigrum*) for *E. o. olmstedi*, an inhabitant of the upper Coastal Plain tributaries. Further evidence for ecologically correlated variation is given by Kott and Humphreys (1978). They found that Lake Superior populations of *E. nigrum* had subconical, rounded snouts characteristic of *E. olmstedi* whereas the Nith River (Lake Erie drainage) form, in flowing water, possessed the bluntly rounded snout, a typical character of *E. nigrum*.

These demonstrations of the pliability of characters of the two species in response to environmental pressures probably explain the difference in results of the current study and that of Clark (1978) (Table 5). The population in the Appomattox shows a morphological trend toward *E. nigrum* (Clark, 1978), but has the homozygous Pgi-1c allele characteristic of *E. olmstedi*. Even though the mouth of the Appomattox River is well below the Fall Line and readily accessible only to *E. olmstedi*, much of the river lies within the Piedmont Province. The population of *E. olmstedi* in this drainage has taken on the characteristics of *E. nigrum*, the species characteristic of the James River in the Piedmont section, via character convergence as postulated by Cole (1967) for the various subspecies of *E. olmstedi*.

The Swift Creek population possesses the morphological and electrophoretic characters diagnostic of *E. olmstedi*. Swift Creek (gradient: 1.9 m/km) joins the Appomattox River on the Coastal Plain and even though it extends onto the lower Piedmont it retains characteristics of upper Coastal Plain tributaries, typical *E. olmstedi* habitat.

A different relationship exists between *E. nigrum* and *E. olmstedi* in Falling Creek. This stream empties into the James River in the Coastal Plain, approximately 6 km below the Fall Line. Its headwaters are in the lower Piedmont, less than one km from tributaries of the James in the Piedmont. According to Clark (1978), meristic values characteristic of *E. nigrum* in the headwaters of Falling Creek decreased downstream, approaching those of *E. olmstedi*. He concluded that *E. nigrum* occupied the headwaters and introgressive hybrids between *E. nigrum* and *E. olmstedi* composed the remaining part of the population. Clark attributed the presence of *E. nigrum* in Falling Creek to headwater

stream piracy of James River tributaries. Although this is feasible it would seem that the severe flooding that has occurred frequently in the James could cause the introduction of this form into the creek either through its headwaters or its confluence with the James. Access of fishes from the Coastal Plain region of the James to the Piedmont section of Falling Creek has been restricted since 1928 by a dam approximately 5 m high. There was no evidence for hybridization between the two species, although Pgi analysis showed that the two were in the creek and existed sympatrically. Interestingly, Pgi bands from specimens at the headwater were characteristic of *E. olmstedi*, whereas Clark identified *E. nigrum* in this section. Small sample size in the present study might have precluded identification of *E. nigrum* from this location.

Any *E. nigrum* x *E. olmstedi* hybrid would have to show activity at the Pgi-1*a* locus because it is species specific for *E. nigrum*. Of the 14 specimens from Falling Creek with this locus, 43% were homozygous, a condition in which the Pgi-1c locus characteristic of *E. olmstedi* is absent. This is significantly greater ($p < .05$) than the 16% homozygosity at this locus in *E. nigrum* from other locales. Martin and Richmond (1973) used similar electrophoretic evidence to discount hybridization between *Etheostoma spectabile* and *Etheostoma caeruleum*. They ruled out hybridization when suspected hybrids were homozygous at four different loci (tetrazolium oxidase, Ldh, Mdh and Est-1); the heterozygous condition should have existed based on parental genotypes. Whitt (1981) reported that gene expression in hybrids is often perturbed and alleles may actually be repressed, which might account for high degrees of homozygosity. However, this is most evident during

embryogenesis and persists into the adult stage only in intergeneric hybrids. All fishes analyzed in the present study were mature adults. The difference in homozygosity is more likely due to the environment. The niche-width variation hypothesis states that populations occupying more varied niches should be relatively more polymorphic since a greater number of complexes of environmental factors will be likely to select for different phenotypes (Avise and Selander, 1972). Falling Creek is a small, relatively fast flowing stream (gradient: 3.5 m/km) with little variety of niches for fishes when compared with the James in the Piedmont Province. Reduced variation in response to fewer niches is reflected in a homozygous genotype.

Winn (1958) reported the mating habits of *E. nigrum* and *E. olmstedi* (then considered as subspecies) to be the same. The male excavates under rocks and awaits acceptance by the female. Females select the largest males, enter the nest and attach eggs to the underside of the rock as the male sheds milt. The female then leaves and the male guards the nest. The similar breeding habit of the two makes hybridization possible but is contingent upon interspecific acceptance of the male by the female.

Hubbs (1955) stated that it is almost a universal rule that natural interspecific hybrids are intermediate between their parental species for all characters in which these species differ including shape, color, form, structures and number of parts. Clark (1978) pointed out that the characters of the Falling Creek population lie toward *E. nigrum* and are not exactly intermediate. He concluded that introgressive hybridization between established *E. olmstedi* and *E. nigrum* introduced by stream capture produced this distribution of

characters. Introgression requires maintenance of some degree of hybrid fertility. Hubbs (1967) stated that based on laboratory breeding experiments, most darter combinations are sterile and those that are fertile typically are much less so than comparable controls. He summarized by declaring that "introgression does not seem to apply to darters."

Hybridization is probably not a factor in the character distribution of the Falling Creek population of *E. nigrum* and *E. olmstedi*. The more plausible conclusion is that *E. olmstedi* is undergoing character convergence toward *E. nigrum* in response to environmental conditions. The high degree of homozygosity for Pgi in *E. nigrum* from Falling Creek indicates it is maintaining its genetic integrity. The question of hybridization between *E. nigrum* and *E. olmstedi* in this stream could probably be resolved by an electrophoretic analysis of the isozymes of laboratory produced hybrids between the two. Hubbs (1959), after successfully crossing a large number of *Etheostoma* species, concluded that virtually any interspecific hybrid of this genus could be produced in the laboratory.

There was no useful electrophoretic character for separating the subspecies *E. o. olmstedi* and *E. o. atromaculatum*. Mean heterozygosity was virtually identical (.050 and .051, respectively). Subspecific status implies a genetic difference. Even though none was found in this study, only a few of many possible loci were examined, prohibiting a definitive statement.

Values for mean heterozygosity ranging from 4.1% to 11.2% were compiled by Merritt, et al. (1978) for six species of freshwater fishes. Similar figures for mean heterozygosity were obtained for *E. nigrum* and

E. olmstedi from the James River. The 10.3% determined for *E. nigrum* is in the high end of the range and is considerably higher than that for *E. olmstedi* (5.1%). The high value for *E. nigrum* can be attributed to the Pgi loci and the small number of total possible loci examined. If Pgi is eliminated from the calculation, the estimate for *E. nigrum* becomes 5.0%. These figures are in good agreement with the 5.8% estimate given for all vertebrate species (Selander and Johnson, 1973).

In summary, *E. nigrum* can be distinguished from *E. olmstedi* as they occur in the James River drainage by the presence of the Pgi-1*a* allele. *E. nigrum* occupies the Piedmont part of the James whereas *E. olmstedi* inhabits the river in the Coastal Plain, the Appomattox River and Swift Creek. In Falling Creek the two species occur sympatrically. Solid evidence for hybridization was missing. Rather, morphological character convergence of *E. olmstedi* into *E. nigrum* was indicated in the Appomattox River and Falling Creek. Subspecific separation of *E. o. olmstedi* and *E. o. atromaculatum* could not be verified based on electrophoretic data. Mean heterozygosity for both species is in agreement with those values published previously for fishes and other vertebrates.

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Table 1. Allele frequency of polymorphic loci found in James River, Virginia populations of *Etheostoma n. nigrum*, *Etheostoma o. olmstedi* and *Etheostoma o. atromaculatum*. Designation of collection sites for *E. o. atromaculatum* follow Clark (1978).

		Alleles*									
		Est-3		Pgm-1		Pgi-1			Pgi-2		
Species:	location (N)	*a*	*b*	*a*	*b*	*a*	*b*	c	*a*	*b*	c
E. n. n.:	Buffalo R. (9)	.22	.78	.61	.39	.40	.30	.30	.40	.30	.30
	Rivanna R. (8)	.25	.75	.44	.56	.50	.25	.25	.50	.25	.25
	Beaverdam Cr. (12)	.17	.83	.42	.58	.44	.28	.28	.44	.28	.28
	Tuckahoe Cr. (3)	.00	1.00	.50	.50	.34	.33	.33	.34	.33	.33
	Middle Falling Cr. (12)	.21	.79	.46	.54	.66	.17	.17	.66	.17	.17
	Lower Falling Cr. (2)	.00	1.00	.50	.50	.34	.33	.33	.34	.33	.33
E. o. o.:	Holiday Cr. (8)	.12	.88	.44	.56	.00	.00	1.00	.00	1.00	.00
	Upper Appomattox R. (6)	.17	.83	.34	.66	.00	.00	1.00	.00	1.00	.00
	Bush R. (7)	.21	.79	.64	.36	.00	.00	1.00	.00	1.00	.00
	Lower Appomattox R. (4)	.25	.75	.50	.50	.00	.00	1.00	.00	1.00	.00
	Upper Swift Cr. (7)	.25	.75	.50	.50	.00	.00	1.00	.00	1.00	.00
	Beaverdam Cr. (8)	.12	.88	.44	.56	.00	.00	1.00	.00	1.00	.00
	Collins R. (8)	.31	.69	.56	.44	.00	.00	1.00	.00	1.00	.00
	Upper Falling Cr. (8)	.19	.81	.50	.50	.00	.00	1.00	.00	1.00	.00
	Middle Falling Cr. (4)	.25	.75	.50	.50	.00	.00	1.00	.00	1.00	.00
	Lower Falling Cr. (5)	.30	.70	.75	.25	.00	.00	1.00	.00	1.00	.00
E. o. a.:	Lower Swift Cr. (8)	.25	.75	.50	.50	.00	.00	1.00	.00	1.00	.00
	Herring Cr. (8)	.44	.56	.56	.44	.00	.00	1.00	.00	1.00	.00
	Diascund Cr. (5)	.20	.80	.30	.70	.00	.00	1.00	.00	1.00	.00

* esterase-1, phosphoglucomutase-1, phosphoglucose isomerase 1 and 2

Table 2. Estimates of mean heterozygosity ($\overline{H}$) for James River, Virginia populations of *Etheostoma n. nigrum*, *Etheostoma o. olmstedi* and *Etheostoma o. atromaculatum* calculated with Pgi* loci and without Pgi loci.

Species (N)	$\overline{H}$ with Pgi loci	$\overline{H}$ without Pgi loci
E. n. n. (46)	.103	.050
E. o. o. (65)	.051	.061
E. o. a. (21)	.052	.062

* phosphoglucose isomerase

Table 3. Genotype distribution of polymorphic loci for combined populations of *Etheostoma nigrum* and *Etheostoma olmstedi* in the James River, Virginia.

Species (N)	Alleles* Est-3 Homo-zygous	Est-3 Hetero-zygous	Pgm-1 Homo-zygous	Pgm-1 Hetero-zygous	Pgi Homo-zygous	Pgi Hetero-zygous
E. n. (46)	.96	.04	.54	.46	.76	.24
E. o. (86)	.94	.06	.46	.54	.00	1.00
Significance	N.S.		N.S.		$p < .001$	

* esterase-3, phosphoglucomutase-1, phosphoglucose isomerase

Table 4. Comparison of homozygosity at the Pgi* loci in *Etheostoma nigrum* from Falling Creek and *E. nigrum* from other locales in the James River.

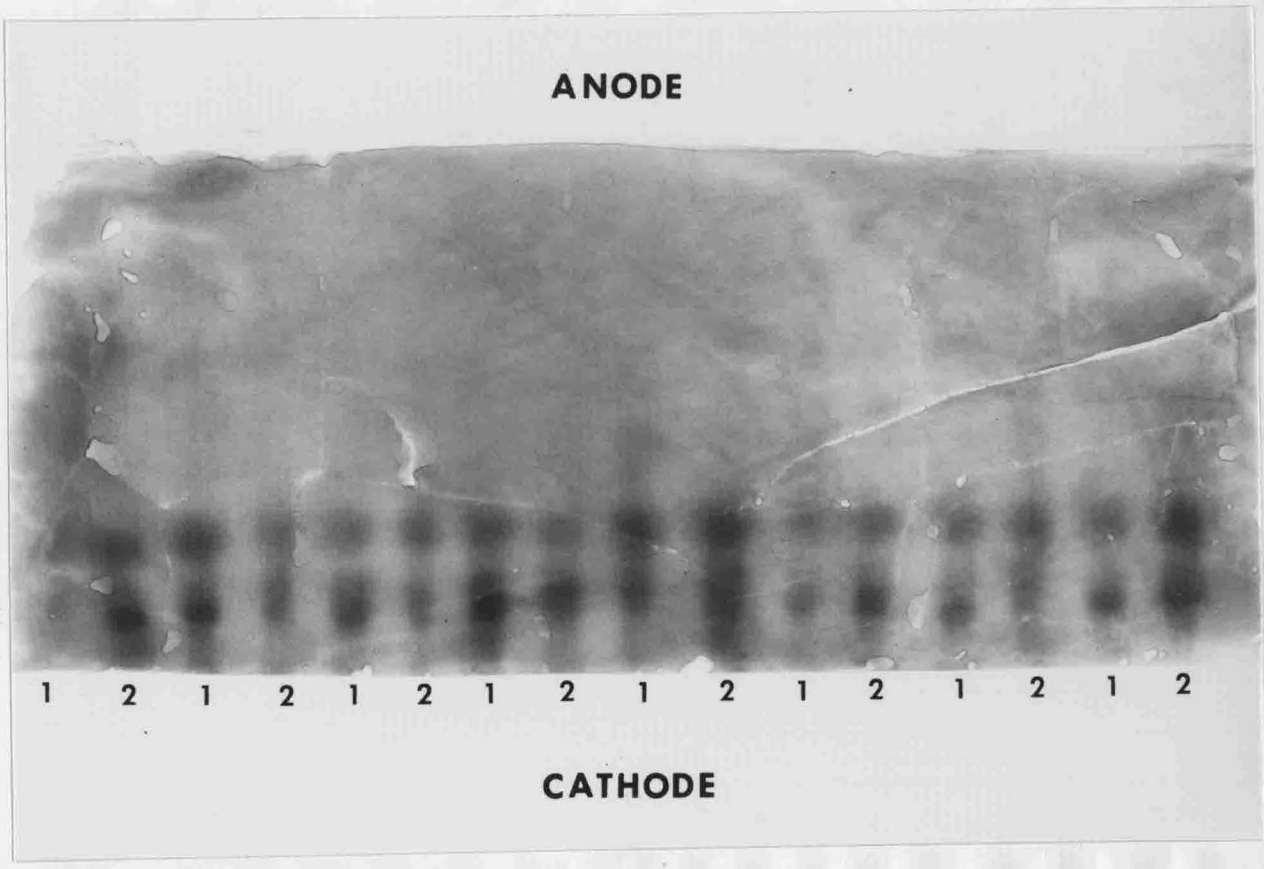
Species: location (N)	Proportion of specimens homozygous for Pgi	Significance
E. n.: Falling Cr. (14)	.43	$p < .05$
E. n.: Other locales (32)	.16	

* phosphoglucose isomerase

Table 5. A comparison of the distribution of populations of *Etheostoma nigrum* and *Etheostoma olmstedi* in the James River drainage, Virginia as reported in Clark (1978) and the current study.

Populations	Population Designation	
	Clark	Current Study
Upper James R.	*E. nigrum*	*E. nigrum*
Lower main James R.	*E. o. atromaculatum*	*E. olmstedi*
Lower James R. tribs.	*E. o. olmstedi*	*E. olmstedi*
Appomattox R.	*E. nigrum* x *E. o. olmstedi*	*E. olmstedi*
Upper Swift Cr.	*E. o. olmstedi*	*E. olmstedi*
Lower Swift Cr.	*E. o. atromaculatum*	*E. olmstedi*
Upper Falling Cr.	*E. nigrum*	*E. olmstedi*
Mid and lower Falling Cr.	*E. nigrum* x *E. o. olmstedi*	*E. olmstedi* and *E. nigrum*

Fig. 1. Photographs of starch gel (top) and cellulose polyacetate (bottom) electrophoretograms of general muscle proteins Pt-1 and Pt-2. (1) *Etheostoma nigrum*, (2) *Etheostoma olmstedi*.


ANODE
PT1
PT2
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
CATHODE

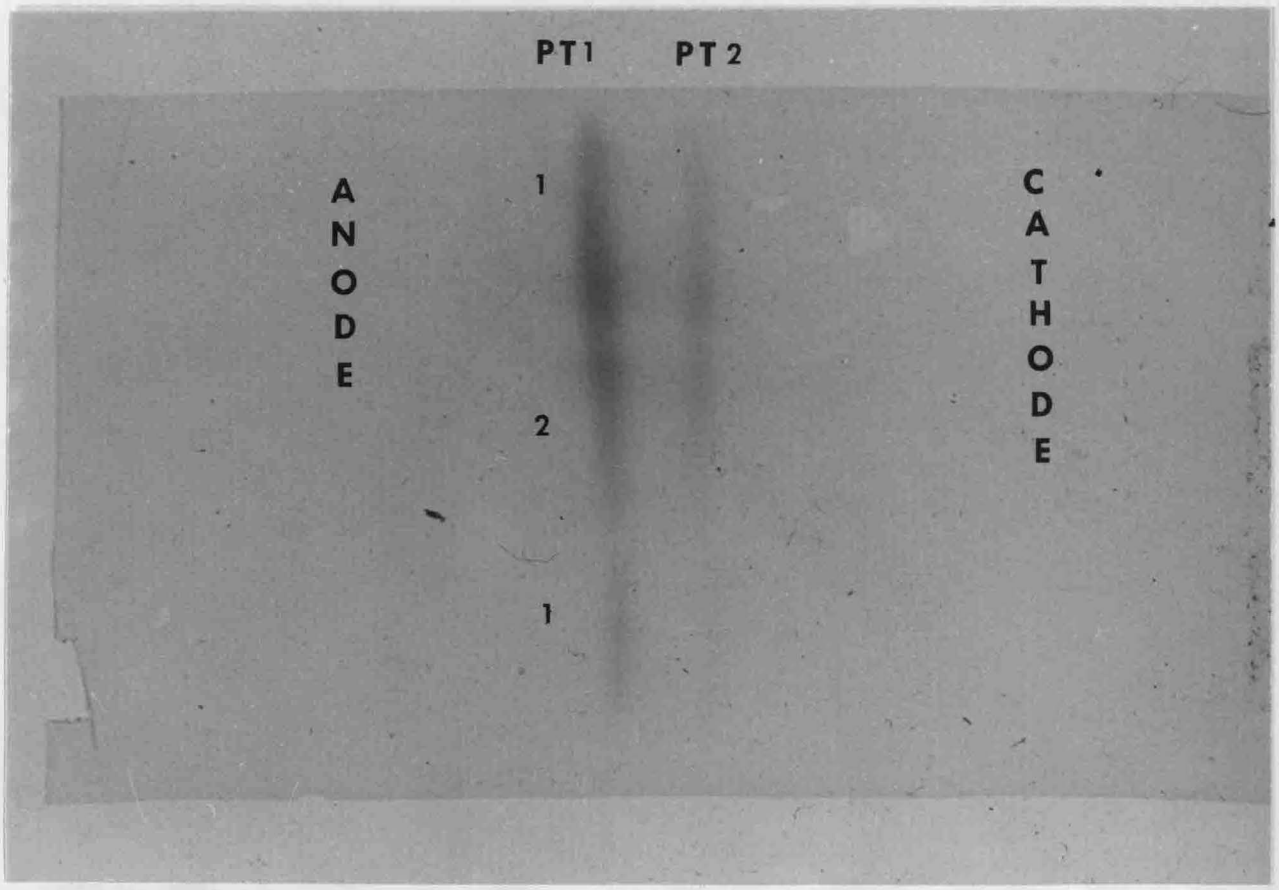
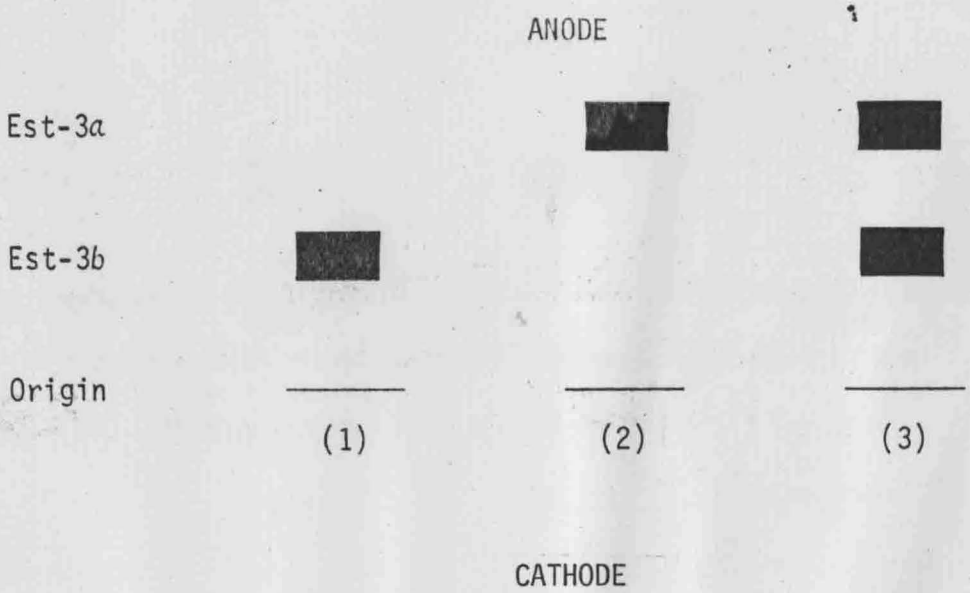

PT1 PT2
ANODE
1
2
1
CATHODE

Fig. 2A. Banding patterns for esterase-3 in *Etheostoma nigrum* and *Etheostoma olmstedi* from the James River, Virginia drainage. (1) homozygous 3*b* , (2) homozygous 3*a*, (3) heterozygous.

Fig. 2B Photograph of esterase starch gel zymogram showing all three loci and the three banding patterns of Est-3. (1) *Etheostoma olmstedi*, homozygous 3*b*, (2) *Etheostoma nigrum*, homozygous 3*a*, (3) *E. nigrum*, heterozygous.

2A


ANODE
Est-3a
Est-3b
Origin
(1)
(2)
(3)
CATHODE

2B

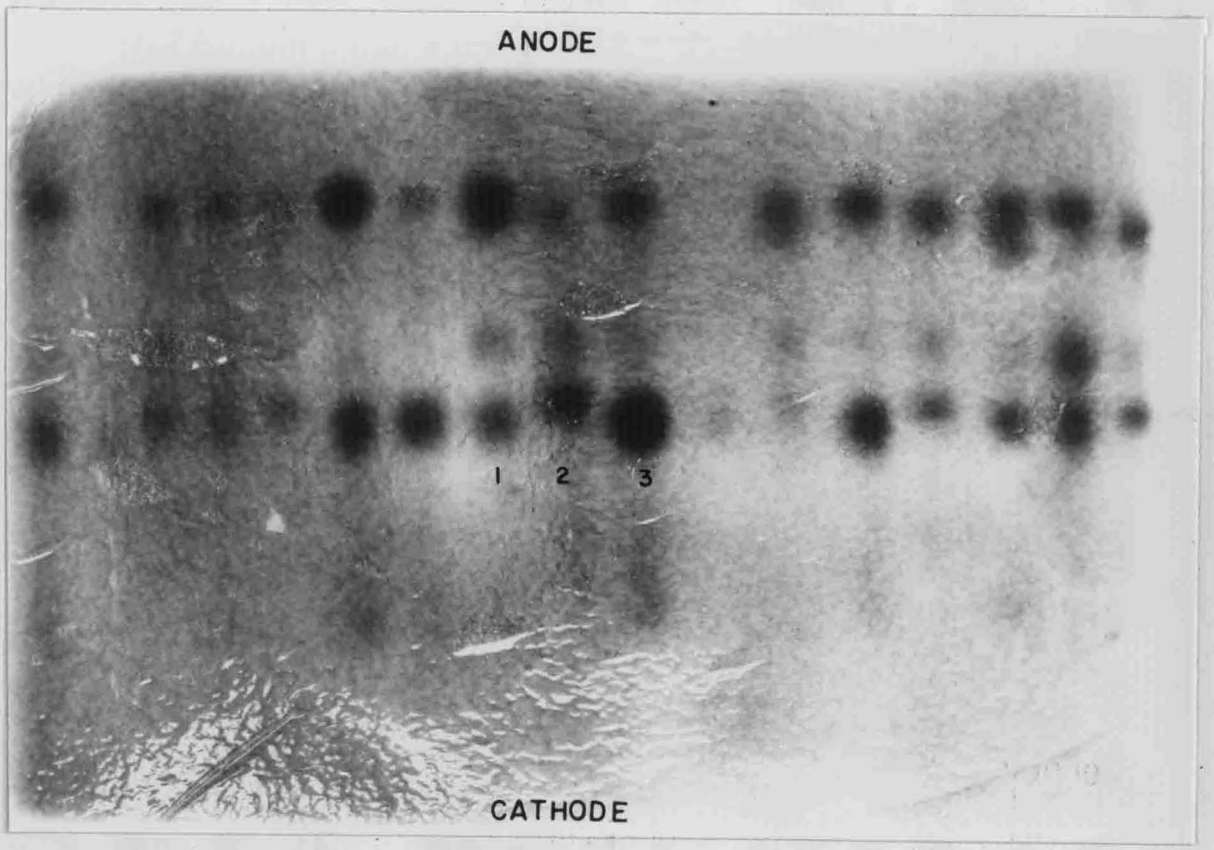
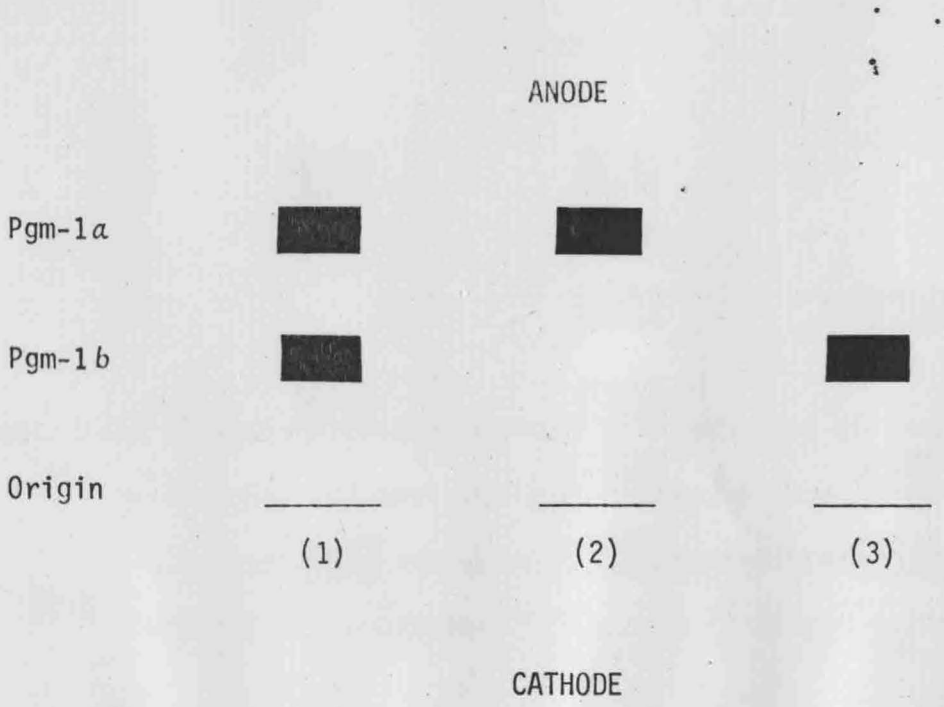

ANODE
EST1
EST2
EST3
1
2
3
CATHODE

Fig. 3A. Banding patterns for phosphoglucomutase in *Etheostoma nigrum* and *Etheostoma olmstedi* from the James River, Virginia drainage. (1) heterozygous, (2) homozygous 1*a*, (3) homozygous 1*b*.

Fig. 3B. Photograph of phosphoglucomutase starch gel zymogram showing the three banding patterns. (1) *Etheostoma olmstedi*, heterozygous, (2) *E. olmstedi*, homozygous 1*a*, (3) *Etheostoma nigrum*, homozygous 1*b*.

3A



3B

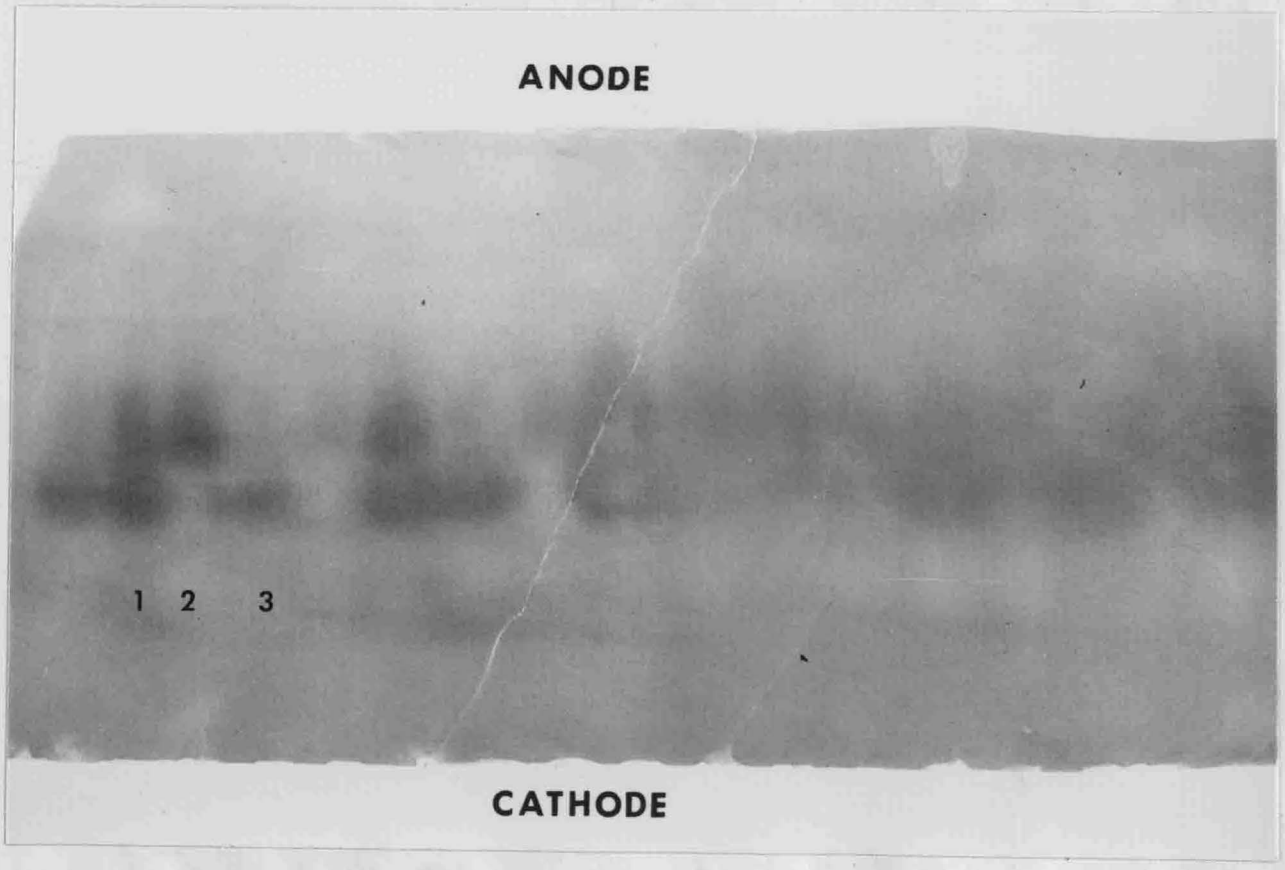
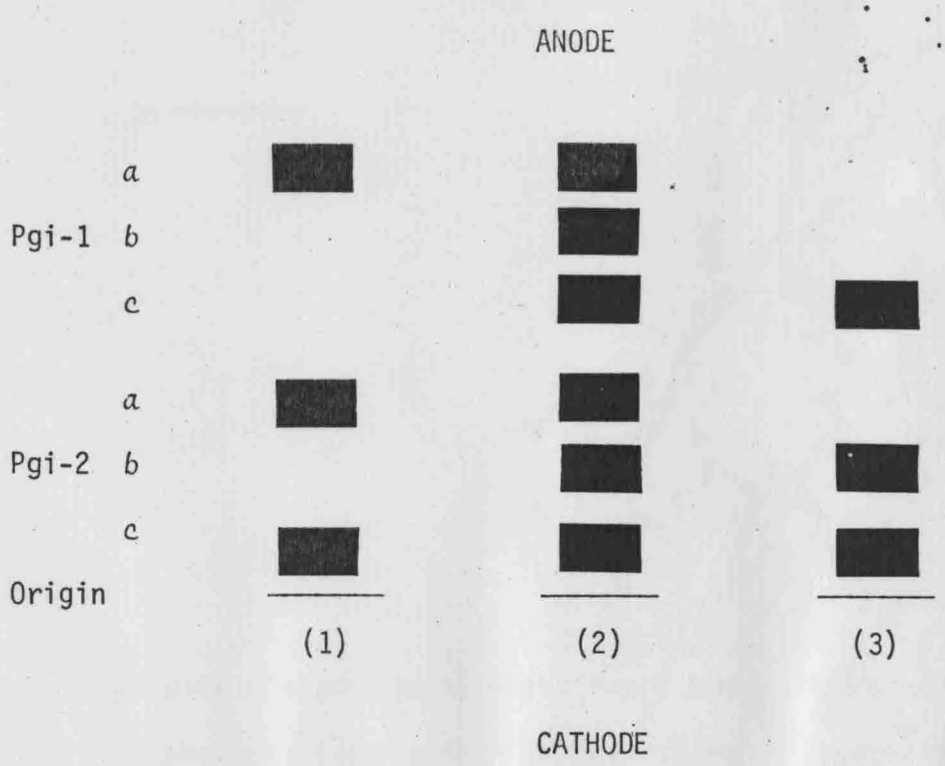


Fig. 4A. Banding patterns for phosphoglucose isomerase in *Etheostoma nigrum* and *Etheostoma olmstedi* from the James River, Virginia drainage. (1) homozygous *E. nigrum*, (2) heterozygous *E. nigrum*, (3) homozygous *E. olmstedi*.

Fig 4B. Photograph of phosphoglucose isomerase starch gel zymogram showing the three banding patterns (legend same as in Fig. 4A).

4A


ANODE
a
Pgi-1 b
c
a
Pgi-2 b
c
Origin
(1)
(2)
(3)
CATHODE

4B

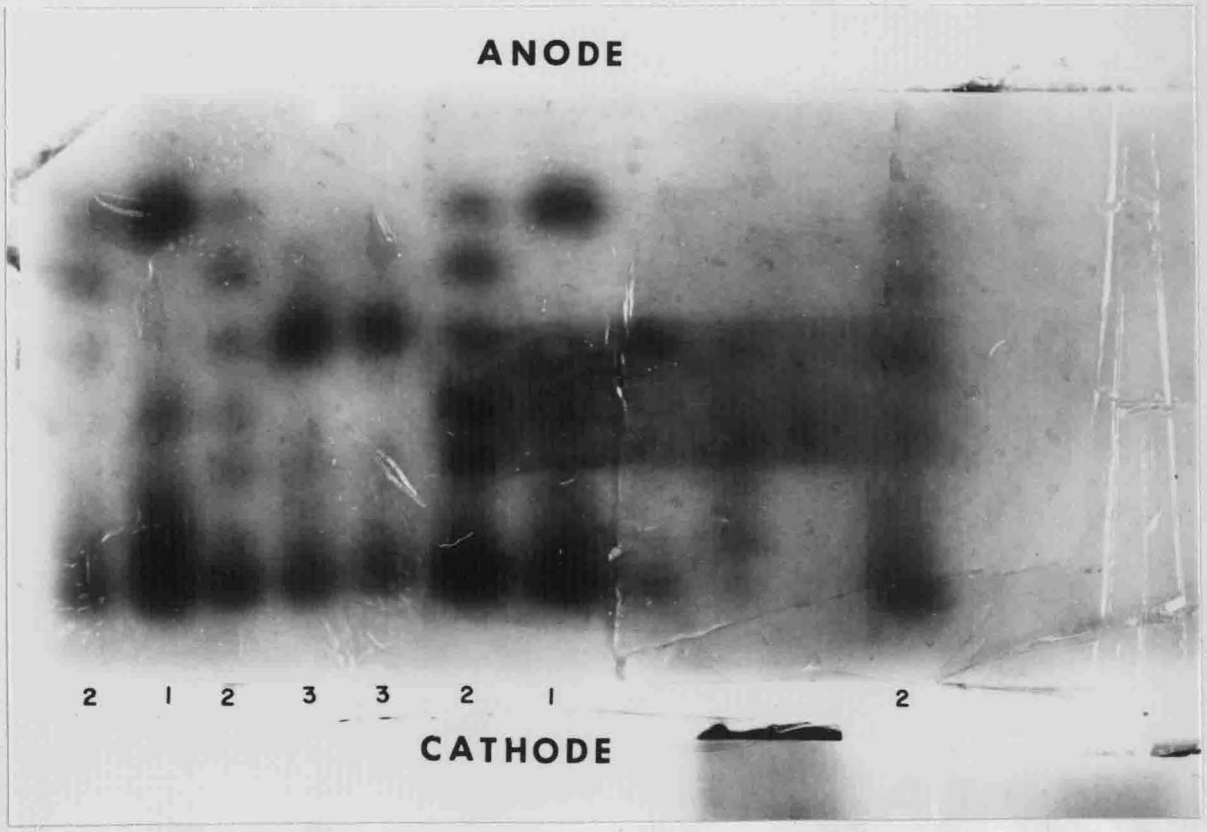
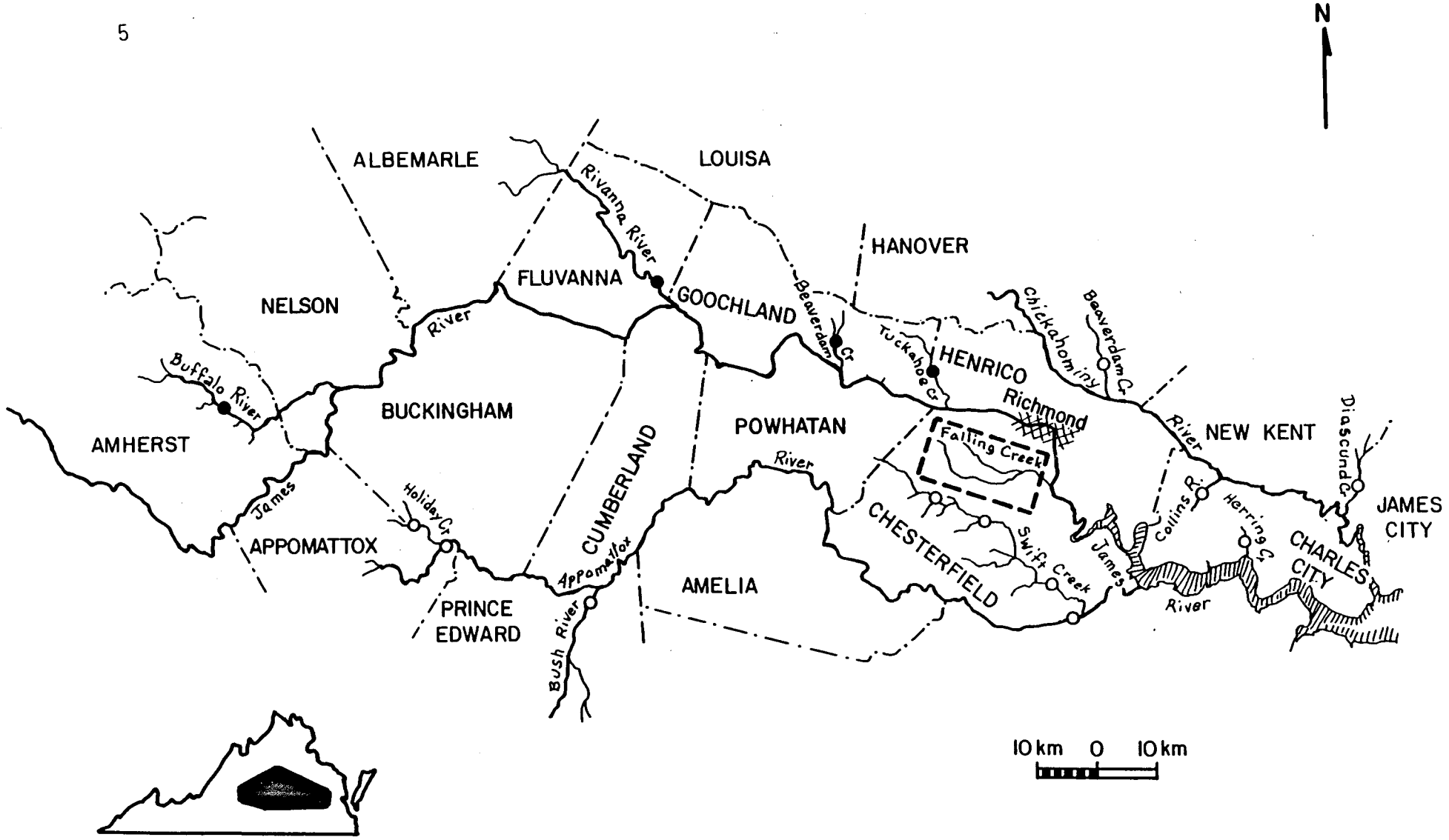

ANODE
2 1 2 3 3 2 1 2
CATHODE

Fig. 5. Map of the James River drainage of Virginia showing distribution of *Etheostoma nigrum* and *Etheostoma olmstedi* by sampling site.

● *Etheostoma nigrum*

o *Etheostoma olmstedi*

- - - - -
' - - - - ' *E. nigrum and E. olmstedi* in sympatry


N
ALBEMARLE
LOUISA
Rivanna River
HANOVER
FLUVANNA
NELSON
GOOCHLAND
Beaverdam Cr.
Chickahominy
Beaverdam Cr.
Tuckahoe Cr.
HENRICO
Richmond
Buffalo River
James River
BUCKINGHAM
POWHATAN
Falling Creek
NEW KENT
Diascund Cr.
AMHERST
James River
Holiday Cr.
CUMBERLAND
CHESTERFIELD
Swift Creek
Collins R.
Herring Cr.
JAMES CITY
APPOMATTOX
Appomattox River
AMELIA
James River
CHARLES CITY
PRINCE EDWARD
Bush River
10 km 0 10 km

APPENDIX I

Fig. 1. Photograph of lactate dehydrogenase zymogram showing the monomorphic condition for *Etheostoma nigrum* and *Etheostoma olmstedi*. (1) *Etheostoma nigrum*, (2) *Etheostoma olmstedi*, (A) is Ldh-1 dimer, (B) is Ldh-1 and Ldh-2 dimer, (C) is Ldh-2 dimer.

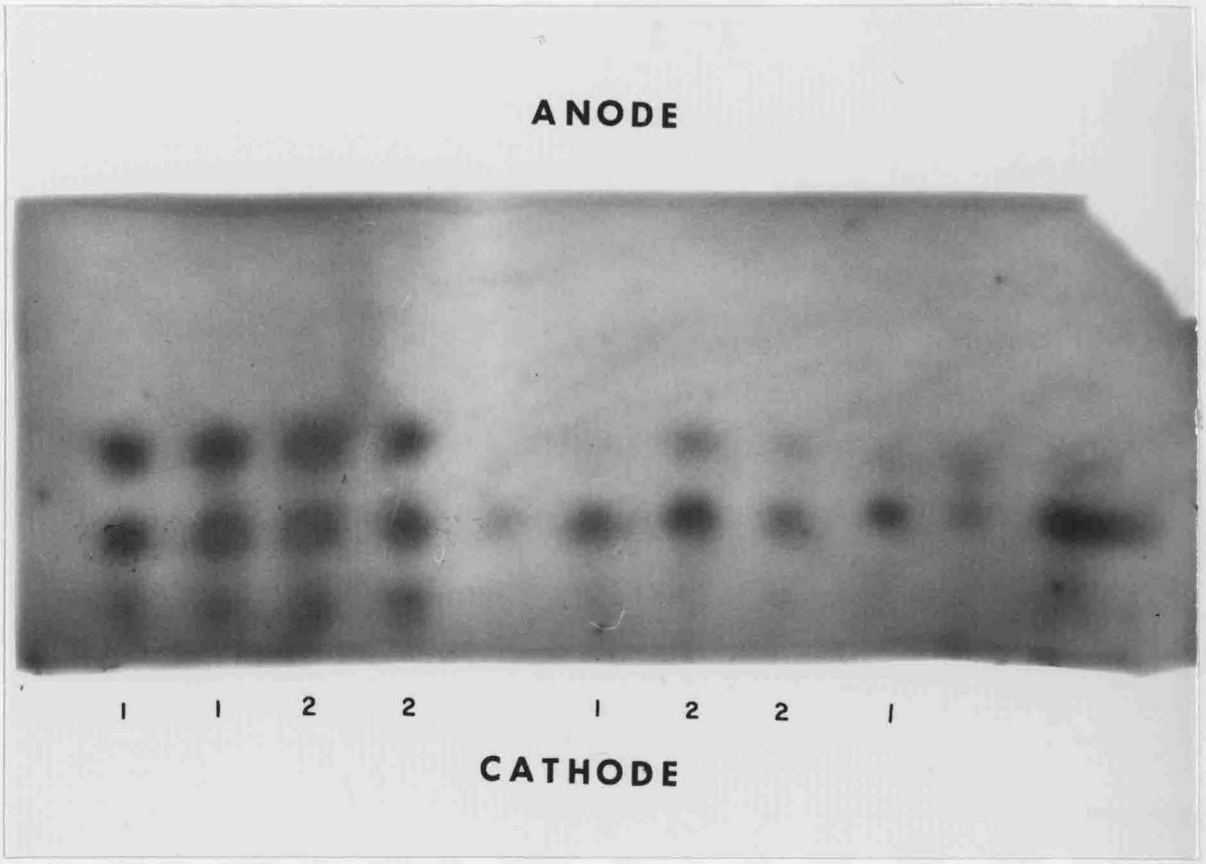
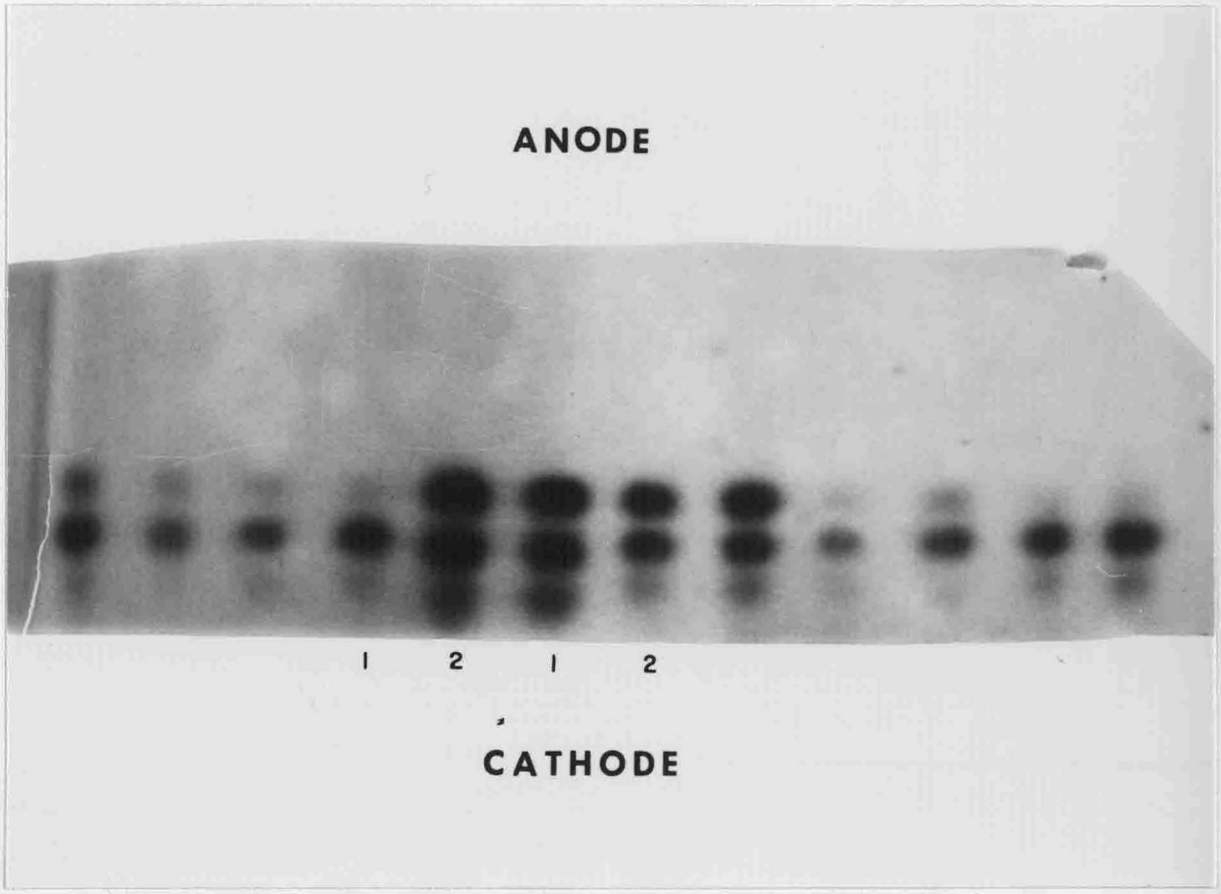

ANODE
A
B
C
1 1 2 2 1 2 2 1
CATHODE

Fig. 2. Photograph of malate dehydrogenase zymogram showing the monomorphic condition for *Etheostoma nigrum* and *Etheostoma olmstedi*. (1) *Etheostoma nigrum*, (2) *Etheostoma olmstedi*, (A) is Mdh-1 dimer, (B) is Mdh-1 and Mdh-2 dimer, (C) is Mdh-2 dimer.


ANODE
A
B
C
1
2
1
2
CATHODE

APPENDIX II

The following is a description of the detailed methodology used in the present study in the preparation of tissue homogenates and starch gel zymograms. It is intended as a guide for those interested in studying evolutionary relationships and genetic variation in smaller fishes. The guide can be applied to any organism with appropriate modification of tissue storage and homogenation.

PREPARING HOMOGENATES: Store the whole specimens at -15 to -20 C in freezer bags until ready to homogenize. To homogenize, place specimens in 2.0% 2-phenoxyethanol (Sigma # P-1126) on ice to thaw. All homogenizing procedures are carried out with vessels on ice. Wear gloves during the procedure to prevent contamination of the homogenate with your enzymes and to protect your skin from the homogenizing solution.

The following procedure is best carried out in a glass petrie dish. If muscle homogenates are desired, skin the specimen and remove the skeletal muscle, blot the tissue on a kimwipe and weigh to the nearest 0.1 g. For total body homogenates, open the whole specimen from the branchial septum to the anus and remove and discard the digestive tract to prevent contamination by the food item's enzymes. Blot the tissue dry and weigh to the nearest 0.1 g. For muscle or total body homogenates, mince the tissue into fine pieces with a razor blade, place in a glass homogenizing tube with an equal volume of 2.0% 2-phenoxyethanol (e.g., for 1.0 g of tissue, add 1.0 ml solution) and homogenize at 1,000 to 1,500 rpm for 16 complete strokes. Place the homogenate in a vial marked to indicate species, individual, collection site and date of homogenation. Store at -15 to -20 C. Maximum enzyme

activity will last for 2-3 wks, diminishing completely after 6 mos.

BUFFERS: The following recipes (from Merkle, pers. commun.) are useful. Many others can be found in the literature. Some may be useful for a given enzyme in some species and not others so some experimentation to determine the best system is necessary. Measure all reagents to the nearest 0.01 g and adjust all pH's with the pH meter. Accuracy and consistency is imperative.

Recipes (Sigma Chemical order no. is in parentheses):

Salamander B	EDTA		0.58 g	
	Boric Acid		1.08 g	Use for gel
400 V	Tris (T-1378)		21.00 g	and electrode
	Distilled water to 2 l			
Lithium Hydroxide	Solution A:	LiOH	2.40 g	
	pH=8.1	Boric Acid	23.80 g	
350 V		Dist. water to 2 l		
	Solution B:	Citric Acid (monohydrate)	3.20 g	
	pH=8.4	Tris	12.40 g	
		Dist. water to 2 l		
	Electrode buffer: use solution A			
	Gel buffer: use 60 ml of A, 540 ml of B			
Poulik	Electrode buffer:	Boric Acid	37.10 g	
	pH=8.2	NaOH	4.80 g	
250 V		Dist. water to 2 l		
	Gel buffer:	Tris	18.42 g	
	pH=8.7	Citric Acid (mono)	2.10 g	
		Dist. water to 2 l		
Citrate (pH=8.0)	Electrode buffer:	Tris	166.40 g	
		Citric Acid (mono)	66.00 g	
150 V		Dist. water to 2 l		
	Gel buffer: 19.5 ml of electrode buffer			
	580.5 ml dist. water			
Versene	Electrode buffer:	Tris	121.20 g	
		Boric Acid	80.00 g	
200 V		Disodium EDTA	12.00 g	
		Dist. water to 2 l		

Gel buffer: 40 ml electrode buffer
360 ml dist. water

PREPARING GEL: Dry the powder at 37 C for 48 hrs prior to use. Use a 12.5% or 13.0% gel (e.g., for 13.0%, 65 g of gel diluted to 500 ml with the appropriate buffer in a volumetric flask). Concentration varies with the type of buffer and must be experimented with to determine the best combination. Place the gel/buffer mixture into a 1 l filter flask. Wearing heavy gloves, swirl the flask over an open flame. In 4-6 min the gel mixture will go from a white slurry to an amber color solution with a thick consistency. Keep swirling and the solution gradually thins. When the large bubbles (30 to 50 mm in diameter) rising in the solution become smaller (5 to 10 mm in diameter) due to the thinner consistency, the gel is ready to degas. Place a stopper in the flask and hook the flask to a faucet aspirator. Turn on the water and swirl the flask to keep the gel from bubbling out. Degas until only large (30 to 50 mm) bubbles are being pulled from the bottom of the vessel. Remove the stopper, turn off the water (do not turn off water first as the pressure equalization pulls tap water into the flask), and gently pour the gel into the plexiglass form. After pouring, remove any bubbles remaining in the gel with a spatula. If it is properly degassed only a few (less than 20) bubbles will remain but these must be removed. Let the gel set for 30 min at room temperature, then at 5 C for 1-2 hrs before introducing the samples.

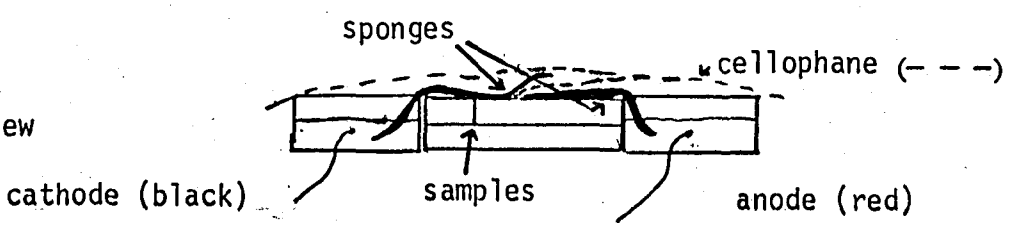
SAMPLE APPLICATION: Prior to slicing the gel, cut 10 mm x 5 mm wicks from Whatmann #3 filter paper. Make a slice along the width of the gel 4.5 cm from the end. Use a single edge razor blade with the guard

removed; double edge blades are too flexible. Wear gloves at all times. Let the homogenate thaw just enough to soak the wick. Spread the gel with your fingers and, using fine forceps, insert the first wick into the slice 1.5 cm from the left edge of the tray. Insert the wick with its long axis perpendicular to the bottom of the tray. Place subsequent samples 1.0 cm apart. Keep track of the samples applied by using a reproduction of the flow sheet appearing at the end of the appendix. Mix the samples up on the gel if you are comparing different populations or species so that a skewed run will not give a false indication of different banding patterns.

Separate the gel from the sides of the tray with a spatula behind the slice. Cut strips of Whatmann #3 to fit snugly between the gel and the back of the tray. Place 5 to 6 thicknesses into the tray to push the slab of gel behind the samples forward snugly against the wicks. The gel is now ready to run.

ELECTROPHORESIS: Place an empty gel frame upside down in the refrigerator and place the gel containing the samples on top of it. The apparatus is set up as indicated in Fig. 1. Electrode compartments

Fig. 1:
Side View

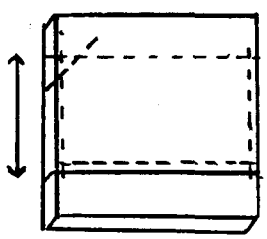


are fashioned from staining boxes (VLchek plastics #401). Remove the tops and burn pinholes in the sides so a wire can be run through the inside of the box parallel to its width. Seal the wires with clear silicon. Use platinum wire for the cathode and nickel:chromium

(80%:20%) for the anode. Handi-wipes work quite well for sponges; cut them to exactly fit the width of the gel. Place the appropriate buffer in the electrode compartments so it comes above the wires. Soak the sponges in the buffer before placing them on the gel. All air bubbles between the gel and the sponges and cellophane must be removed prior to running the gel. After this is accomplished, place a pan of ice on top of the gel. Hook the electrodes to a DC power supply capable of generating 500 V. Run the gels at the proper voltage for 1,000 mAmp·hrs. The resistance varies with each gel. Read the mAmps being generated after allowing the gel to cool for two hours. Divide 1,000 by the number of mAmps being generated to obtain the time in hours the gel should run (e.g., if 20 mAmps are being generated, 50 hours are needed). This is a rule of thumb for darters and use may vary for other organisms.

GEL SLICING: At the completion of a run, shut off the power and remove the gel. Slice while the gel is still cool. Trim the edges and cut the front of the gel to fit the staining box (Fig. 2). Slice the left corner off to mark the gel. CAREFULLY remove the gel slab

Fig. 2: Top View



Slice gel as depicted by the dotted lines

from the frame and place on moistened glass. Do not invert. Cover the slab with a flat, smooth surfaced weight (approximately 2 kg). Clean the wire on the slicer, lubricate the glass with water and pull the slicer across the gel. This will remove a 2 mm slice. Remove the weight, turn over the slab and peel off the slice. Place the slice

in a clean staining box. The first slice is good for general myogen stain. Repeat the slicing procedure for the rest of the slab to obtain 4-5 slices. Place individual boxes in the refrigerator while stains are prepared.

GEL STAINING: All enzymes will not be encoded in all organisms, therefore it is recommended that a number of different stains be tried. Always run one sample of mouse serum to test whether the stain is working or if the locus is absent in the species you are testing. Mouse serum does contain all of the following (compiled by Donald A. Merkle from Selander, et al., 1971) (Sigma Chemical order no. is in parentheses):

Esterases (Est)

25 ml phosphate A
5 ml phosphate B
20 ml dist. water
1 ml α-napthyl acetate solution
20 mg fast blue RR (F-0500)

Lactate dehydrogenase (Ldh)

30 ml Tris HCl
6 ml LiLactate
1.3 ml NAD
.3 ml NBT
.5 ml PMS

Malate dehydrogenase (Mdh)

30 ml Tris HCl
5 ml DL-malate
2 ml NAD
2 ml NBT
.5 ml PMS

Isocitrate dehydrogenase (Idh)

30 ml Tris HCl
2 ml Isocitric acid
.2 ml $MnCl_2$
.8 ml NADP
.3 ml NBT
.3 ml MTT
.5 ml PMS

α-Glycerophosphate dehydrogenase (α-Gpdh)

30 ml Tris HCl
.5 ml $MnCl_2$
25 mg DL-glycerophosphate (G-6126)
1 ml NAD
.7 ml NBT
.3 ml PMS

Alcohol dehydrogenase (Adh)

3 ml .5 M phosphate
25 ml dist. water
2 ml 95% ethanol
2.2 ml NAD
1.2 ml NBT
.3 ml PMS

6-Phosphogluconate dehydrogenase (6-Pgdh)

10 ml Tris HCl
7 ml $MgCl_2$
20 mg 6-phosphogluconic acid (P-7627)
.1 ml NADP
.4 ml MTT
.1 ml PMS

Glutamic oxaloacetic transaminase (Got)

50 ml Tris HCL
.5 mg P-5-P (P-9255)
200 mg aspartic acid (A-9256)
100 mg ketoglutaric acid (K-1750)
150 mg Fast Blue BB (F-0250)

Phosphoglucose isomerase (Pgi)

30 ml Tris HCl
10 ml $MgCl_2$
4 ml F-6-P
1 ml G-6-PDH
1 ml NADP
2 ml MTT
.5 ml PMS

Phosphoglucomutase (Pgm)

4 ml Tris HCl
20 ml dist. water
4 ml G-1-P
4 ml G-1,6-di P
4 ml $MgCl_2$
3.2 ml G-6-PDH
.4 ml NADP
.4 ml MTT
.2 ml PMS

Stain solutions:

Phosphate A (pH 4.4), 0.2 M $NaH_2PO_4 \cdot H_2O$; 27.6 g to 1 l with dist. water

Phosphate B (pH 8.7), 0.2 M $Na_2HPO_4 \cdot 7H_2O$; 53.6 g to 1 l with dist. water

Tris-HCl (pH 8.0); 24.2 g Trizma (T-1378) to 1 l with dist. water adjust pH with conc. HCl

α-Napthyl acetate solution; α-napthyl acetate (N-6750), 1.0 g in 99 ml acetone

0.5 M LiLactate; DL-Lactic acid (L-1500), 4.8 g in 100 ml dist. water

2.0 M DL-malate (pH 7.0); DL-malic acid (M-0875), 268.2 g to 1 l with dist. water, adjust pH with NaOH (about 150 g)

0.1 M Isocitric acid; DL-isocitric acid (I-1252); 300 mg in 10 ml dist. water

G-6-PDH; glucose-6-phosphate dehydrogenase (G-7877), 100 units in 10 ml dist. water

18mM Fructose-6-P; D-fructose-6-phosphate (F-3627), 547 mg in 100 ml dist. water

0.026 M G-1-P; D-glucose-1-phosphate (G-7000), 1.7 g in 100 ml dist. water

0.0005 M G-1, 6-di P; D-glucose 1, 6-di-P (G-5875), 10 mg in 45 ml dist. water

0.1 M $MgCl_2$; $MgCl_2 \cdot 6H_2O$, 2.03 g in 100 ml dist. water

0.25 M $MnCl_2$; $MnCl_2 \cdot 4H_2O$, 4.9 g in 100 ml dist. water

0.5 M Potassium phosphate for Adh; solution (A), 87.1 g K_2HPO_4 (anhydrous) to 1 l with dist. water, solution (B), 68 g KH_2PO_4 (anhydrous) to 1 l with dist. water; for the working solution, mix 500 ml (A), 490 ml (B) and 10 ml dist. water

NAD (N-7004), NADP (N-0505), PMS (P-9625), NBT (N-6876) and MTT (M-2128) are all mixed 100 mg in 10 ml dist. water

According to Dr. Merkle (pers. commun.), Idh and 6-Pgdh must be stained for in homogenates prepared less than 24 hrs prior to running. They are very labile and denature quickly. Also, Dr. Merkle has never found the Adh stain to work in organisms he has worked with and cautions that α-Gpdh is not present in skeletal muscle.

Mix the stain in a flask and pour it over the gel slice. Place the slice in an oven at 37 C and check it about every ten minutes. Do not overstain. When the bands are well defined, wash the slice in wash solution (5 parts acetone, 5 parts methanol, 1 part glacial acetic acid) twice. If possible, photograph the gel at this time. Wrap the slice in cellophane, identify it as to the enzyme stained for and specimens analyzed, and store at 5 C.

SCORING ISOZYMES: Before attempting to score enzymes, go to the literature that pertains to genetic variation in the forms with which you are working. This will save much confusion and help avoid misinterpretation. It is essential to know whether the enzyme is a monomer, dimer, tetramer, etc. as each type has distinctive banding patterns. Scoring is done on a qualitative basis by simply comparing the banding patterns of different specimens.

The basis of these procedures was provided by Dr. D. A. Merkle, Professor of Biology at Longwood College, who works with amphibians and reptiles. Modifications to fit the requirements of the current study are mine.

Electrophoresis No. _____ Date _____ Buffers

Gel _____ pH _____

Tray _____ pH _____

Material _____

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2.						
3.						
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24.						

Stained:

T _____

Comments:

VITA

Randall Keith Falls was born November 20, 1954 in Lynchburg, Virginia. After graduation from Liberty High School in Bedford, Virginia he attended the University of Richmond, graduating with a B.S. degree in biology and chemistry in May, 1977. He was married in June, 1977 to Mary Kathleen Lyle and has a son, Randall Keith, Jr., born in May, 1981. From August, 1977 to August, 1980 he worked as a chemist in a medical research laboratory. He entered the Graduate School of the University of Richmond in August, 1980 and expects to receive his M.S. degree in August, 1982. He will continue his education at the Medical College of Virginia School of Medicine beginning August, 1982.