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Lactate Dehydrogenase Isozymes of Darters and the Inclusiveness of the Genus *Percina*

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THE DARTERS (Etheostomati), with over 110 recognized species, comprise the second largest group of North American freshwater fishes. At present, the most widely accepted classification of darters is a proposal of Bailey progressively formulated in Bailey (1951), Bailey et al. (1954), and Bailey & Gosline (1955).

The latter paper lists three darter genera (*Percina*, *Ammocrypta*, *Etheostoma*) and 22 subgenera and arranges them according to evolutionary advancement. This classification is based on years of study by Bailey and reflects to a high degree the true relationships among the darters. However, little documentation was presented in the 1955 paper, and little has been presented subsequently, for the classification. Thus some of the groupings and arrangements deserve closer scrutiny.

Collette (1965) compared breeding tubercle patterns among all darters and used the same genera and subgenera as had Bailey & Gosline except for the recognition of *Villora* as an additional subgenus of *Etheostoma*. More recently *Doration* and *Vaillantia* have also been recognized as valid subgenera of *Etheostoma* (Cole 1967, Howell 1968).

Systematic studies of darters have been mainly descriptions of species. Few attempts have been made to elucidate relationships among species. Although recent descriptions of new species include remarks on similarities between the *species novum* and the assumed closest relatives, they seldom contain analyses involving more than two or three species.

A controversial aspect of Bailey's classification has been the acceptance of the genus *Percina*. Some ichthyologists believe *Percina caprodes* and its closest relatives *P. rex*, *P. burtoni* and *P. macrolepidia* deserve separate generic status and assign the remaining species to *Hadropterus* (Hubbs & Lagler 1958, Hubbs 1967, Minckley 1963, Stevenson 1971, Winn 1958) or to *Hadropterus* and *Cottogaster* (Curd 1967).

The decision to combine the species of *Percina* and *Hadropterus* in the same genus was based primarily on the ubiquitous presence of modified (enlarged and strongly toothed) scales on the breasts of males of all species in both groups (Bailey et al. 1954). Darters in other genera do not have the modified scales.

Data are herein presented on another characteristic that is seemingly ubiquitous among, and nearly unique to, the species of *Percina*. The characteristic is the electrophoretic mobility of a readily distinguishable homopolymeric lactate dehydrogenase (LDH) isozyme. The pres-

ence of a unique morphological characteristic and a nearly unique enzymatic characteristic among species of *Percina* is considered strong evidence of a monophyletic origin, attests to the genetic continuity of the group, and strongly supports relegation of *Hadropterus* to the synonymy of *Percina*.

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METHODS AND MATERIALS

The head of each darter was homogenized by hand in two volumes of tris-HCl buffer (0.1 M, pH 7.0) at 4°C. The homogenates were centrifuged for 30 minutes in a Sorvall RC2-B centrifuge at 4°C at 48,200 g. The supernatant was recentrifuged under the same conditions. The final supernatant fraction was immediately subjected to starch gel electrophoresis. The immunochemical procedure was described in Whitt (1970).

Vertical starch gel electrophoresis (Büchler instruments) was accomplished in a 14 percent gel at 8V/cm for 16-20 hours at 4°C. Two different buffer systems were employed. The tris-citrate pH 6.8 stock buffer contained 0.75 M tris and 0.25 M citric acid (monohydrate). Dilution of the stock buffer for both electrode chambers was 1:7 and for the gel it was 1:20. The EBT stock buffer contained EDTA (tetrasodium, 1×10^{-4} M), boric acid (2.5×10^{-2} M) and tris (4.5×10^{-2} M) and had a pH of 8.7. Dilutions of the stock buffer for gel, cathode chamber, and anode chamber were 1:20, 1:5, and 1:7, respectively. The staining procedure for LDH isozymes has been described in Shaw & Prasad (1970). The control for nonspecific reductants or "nothing dehydrogenases" was accomplished by incubating one-half of the gel in all the staining components except for the substrate, L-lactate.

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The LDH isozymes of 494 specimens of 68 species were examined. Patterns of 67 species are illustrated in Fig. 1: *Ammocrypta (Ammocrypta) beani*: Leaf River, Jones Co., Miss.; *A. (A.) pellucida*: Middle Fork, Vermilion Co., Ill.; *Etheostoma (Allohistium) cinereum*: Big South Fork, Scott Co., Tenn.; *E. (Boleosoma) longimanum*: trib., James River, Botetourt Co., Va.; *E. (B.) nigrum*: trib., Gasconade River, Maries Co., Mo.; *E. (B.) olmstedii*: Tar River, Franklin Co., N. C.; *E. (Catonotus) barburi*: Brushy Fork, Casey Co., Ky.; *E. (C.) flabellare*: Maries River, Osage Co., Mo.; *E. (C.) kennicotti*: Big Creek, Hardin Co., Ill.; *E. (C.) obeyense*: Ferguson Creek, Livingston Co., Ky.; *E. (C.) squamiiceps*: Sugar Creek, Livingston Co., Ky.; *E. (C.) virgatum*: Harpeth River, Williamson Co., Tenn.; *E. (C.)* species: Cypress Creek, Wayne Co., Tenn.; *E. (Doration) stigmaeum*: Little River, Blount Co., Tenn.; Clarks Fork, Marshall Co., Ky.; *E. (Etheostoma) blennioides*: Embarras River, Cumberland Co., Ill.; *E. (E.) histrio*: Leaf River, Jones Co., Miss.; *E. (E.) rupestris*: Cahaba River, Bibb Co., Ala.; *E. (E.) tetrazonum*: Gasconade River, Maries Co., Mo.; *E. (E.) thalassinum*: trib., Saluda River, Pickens Co., S. C.; *E. (E.) zonale*: Gasconade River, Maries Co., Mo.; *E. (Holelepis) gracile*: Clear Creek, Union Co., Ill.; *E. (H.) serriferum*: Collie Swamp, Martin Co., N. C.; *E. (Ioa) vitreum*: Tar River, Franklin Co., N. C.; *E. (Litocara) nianguae*: Big Tavern Creek, Miller Co., Mo.; *E. (Microperca) microperca*: Coon Creek, Kane Co., Ill.; *E. (M.) proeliare*: Alcorn Creek, Pope Co., Ill.; *E. (Nothonotus) bellum*: Brushy Fork, Casey Co., Ky.; *E. (N.) camurum*: Middle Fork, Vermilion Co., Ill.; *E. (N.) jordani*: Conasauga River, Bradley Co., Tenn.; *E. (N.) maculatum*: Big South Fork, Scott Co., Tenn.; *E. (N.) rufilineatum*: Little River, Blount Co., Tenn.; *E. (N.) tippecanoe*: Big South Fork, Scott Co., Tenn.; *E. (Oligocephalus) asprigene*: Clear Creek, Union Co., Ill.; *E. (O.) caeruleum*: Embarras River, Cumberland Co., Ill.; James River, Webster Co., Mo.; *E. (O.) ditrema*: Sugar Creek, Bradley Co., Tenn.; *E. (O.) exile*: Otter Creek, Winnebago Co., Ill.; *E. (O.) juliae*: James River, Webster Co., Mo.; *E. (O.) spectabile*: Stony Creek, Vermilion Co., Ill.; *E. (O.) swaini*: Leaf River, Jones Co., Miss.; *E. (Psychromaster) trisella*: Conasauga River, Bradley Co., Tenn.; *E. (Ulocentra) coosae*: Sugar Creek, Bradley Co., Tenn.; *E. (U.) duryi*: Cypress Creek, Wayne Co., Tenn.; *E. (U.) simoterum*: Little River, Blount Co., Tenn.; *E. (U.)* species A: Flat Creek, Maury Co., Tenn.; *E. (U.)* species B: trib., Perdido River, Escambia Co., Ala.; *E. (U.)* species C: Clarks Fork, Marshall Co., Ky.; *E. (Vaillantia) chlorosomum*: Clear Creek, Union Co., Ill.; *E. (Villora) edwini*: trib., Perdido River, Escambia Co., Ala.; *Percina (Alvordius)¹ crassa*: Tar River, Franklin Co., N. C.; *P. (A.) maculata*: Clarks Fork, Marshall Co., Ky.; *P. (A.) peltata*: Tar River, Franklin Co., N. C.; *P. (A.) notogramma*: trib., James River, Botetourt Co., Va.; *P. (A.)¹ roanoka*: Roanoke River, Montgomery Co., Va.; *P. (A.)* species: Conasauga River, Bradley Co., Tenn.; *P. (Cottogaster) copelandi*: Big South Fork, Scott Co., Tenn.; Shoal Creek, Cherokee Co., Kan.; *P. (Ericosma) evides*: Gas-

conade River, Maries Co., Mo.; *P. (E.) palmaris*: Conasauga River, Bradley Co., Tenn.; *P. (Hadropterus) lentacula*: Leaf River, Jones Co., Miss.; *P. (H.) nigrofasciata*: Leaf River, Jones Co., Miss.; *P. (H.) sciera* (eight specimens): Embarras River, Cumberland Co., Ill.; *P. (Hypohomus) aurantiaca*: Little Pigeon River, Sevier Co., Tenn.; *P. (Imostoma) shumardi*: Main Ditch, New Madrid Co., Mo.; *P. (I.) uranidea*: Main Ditch, Mississippi Co., Mo.; *P. (I.)* species: Conasauga River, Bradley Co., Tenn.; *P. (Percina) caprodes carolinaria*: Big Tavern Creek, Miller Co., Mo.; *P. (P.) c. caprodes x semifasciata*: Embarras River, Cumberland Co., Ill.; *P. (Swainia) phoxocephala*: Embarras River, Cumberland Co., Ill.; *P. (?)¹ cymatotaenia*: Gasconade River, Pulaski Co., Mo.

Fig. 2: *E. caeruleum*: Embarras River, Coles Co., Ill.; *E. flabellare*: Gasconade River, Pulaski Co., Mo.; *E. gracile*: Clear Creek, Union Co., Ill.; *E. nigrum*: Embarras River, Cumberland Co., Ill.; *P. caprodes*: Embarras River, Cumberland Co., Ill.; *P. cymatotaenia*: Gasconade River, Pulaski Co., Mo.; *P. maculata*: Gibbons Creek, Pope Co., Ill.; *P. phoxocephala*: Embarras River, Cumberland Co., Ill.; *P. sciera*: Embarras River, Cumberland Co., Ill.; *P. uranidea*: Main Ditch, Mississippi Co., Mo.

Fig. 4: *E. cinereum*: Little River, Blount Co., Tenn.; *E. duryi*: Cypress Creek, Wayne Co., Tenn.; *E. edwini*: trib., Perdido River, Escambia Co., Ala.; *E. flabellare*: Cypress Creek, Wayne Co., Tenn.; *E. microperca*: Coon Creek, Kane Co., Ill.; *E. proeliare*: Alcorn Creek, Pope Co., Ill.; *E. trisella*: Conasauga River, Bradley Co., Tenn.; *P. caprodes*: Maries River, Osage Co., Mo.; *P. sciera*: Leaf River, Jones Co., Miss.

RESULTS AND DISCUSSION

The suitability of electrophoretic data for assessment of evolutionary relationships among fishes has been demonstrated by Tsuyuki et al. (1965), Ohno et al. (1968), Markert & Holmes (1969), Lush et al. (1969), Koehn (1969), and Whitt & Horowitz (1970). As specific gene products, isozymes (multiple molecular forms of an enzyme, Markert & Møller 1959) are useful as taxonomic characters. The LDH isozymes of fishes have proven to be excellent gene markers (Markert & Faulhaber 1965, Goldberg 1966, Morrison & Wright 1966, Odense et al. 1969, and Whitt 1970). The following analysis includes only those isozymes formed by the assembly of LDH subunits encoded in the LDH A and B loci (Markert & Faulhaber 1965). The activity of the LDH E locus was too low to employ the retinal-specific E₁ isozyme (Whitt et al. 1971, Horowitz & Whitt 1972) as a genetic marker.

LDH patterns tended to be highly conservative. For the 494 specimens (68 species) examined, intraspecific variation was limited to infrequent polymorphism, occasional low activities of individual bands, slight geographic variation in a few species, and marked geographic variation in *Percina copelandi* (Page & Whitt 1973).

¹ Subgeneric reassignments in *Percina* are based on conclusion by the senior author (Page 1974).

Although data herein presented are expressions of only one character (the LDH isozyme pattern), they are especially significant at the generic level. The LDH B₄ isozyme of 19 species of *Percina* possessed an identical electrophoretic mobility; that mobility was absent in 45 of 46 species of *Etheostoma* and in the 2 species of *Ammocrypta* examined (Fig. 1). Although not resolved for the specimen of *P. uranidea* in Fig. 1, in a separate run using another specimen the band was as prominent in this species as in other *Percina* (Fig. 2). In addition to the 67 species presented in Fig. 1, *E. punctulatum* was examined and was without the B₄ mobility of *Percina*.

Isozymes may be selectively precipitated by the addition of antisera to the enzyme extract prior to electrophoresis. The anodal band of *Percina* (designated B₄)

was precipitated by the anti-B serum but not the anti-A serum (Fig. 3) indicating that it contained only B subunits and was the B₄ homopolymer.

Electrophoresis of LDH isozymes in EBT buffer (Fig. 4) showed more clearly the unique mobilities of LDH isozymes of certain species of *Etheostoma* (*E. cinereum*, *E. microperca*, *E. edwini*, *E. trisella*, and *E. duryi*) which appeared in tris-citrate buffer (Fig. 1) to possess a band with a mobility possibly identical to that of the B₄ of *Percina* species. As demonstrated in EBT buffer, *E. cinereum* does possess a band with the same mobility as the *Percina* B₄; the other species do not. The most anodal band of *E. squamiceps* also appears in Fig. 1 to have the same mobility as the B₄ band of *Percina*; however, electrophoresis in other gels demonstrated it to be different.

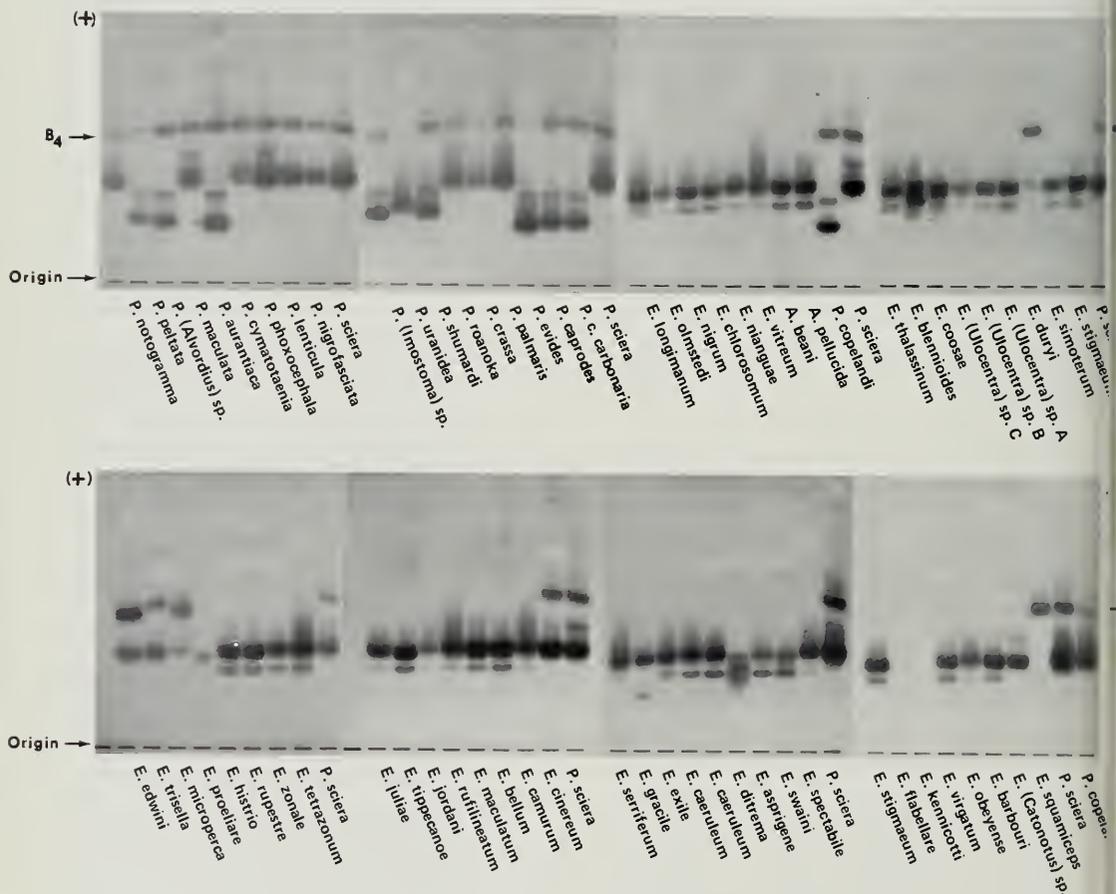


Fig. 1.—Lactate dehydrogenase isozymes from head extracts of 67 species of darters. *Percina sciera* is present on each gel as a marker species to facilitate comparisons between gels. Electrophoresis was in pH 6.8 tris-citrate buffer.

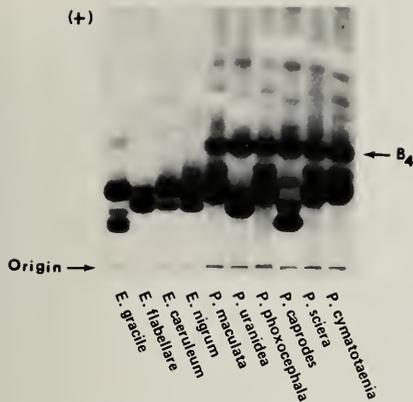


Fig. 2.—Lactate dehydrogenase isozymes of six species of *Percina* and four of *Etheostoma*. Electrophoresis was in pH 6.8 tris-citrate buffer.

Fig. 4 also shows more detailed patterns of species poorly resolved in Fig. 1 (*E. flabellare* and *E. proeliare*).

Percina characteristics—such as less elaborate spawning behavior than in *Etheostoma*, the presence of a gas bladder in most species, generally larger size, and a complete lateral line—leave no doubt that this genus includes the primitive darters, and the distinctive B_4 isozyme mobility is probably an expression of genetic conservatism absent in species of the more advanced genera (except *E. cinereum*).

The genetic continuity demonstrated by the identical mobility of the B_4 isozyme band in all 19 species of *Percina* examined is strong evidence of relationships among the species currently assigned to the genus. The 19 species analyzed included representatives of all 8 recognized subgenera (Bailey & Gosline 1955, Collette 1965).

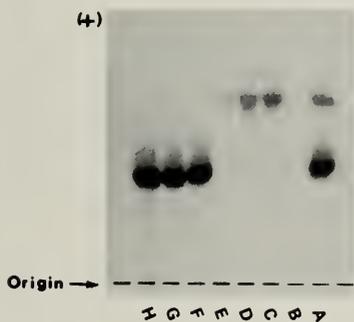


Fig. 3.—Effects of antisera on the lactate dehydrogenase isozymes of *Percina sciera*. (A) untreated LDH isozymes, (B) antiserum alone, (C,D,E) effect of anti-LDH A serum on the *P. sciera* isozymes, (F,G,H) effect of anti-LDH B serum on the *P. sciera* isozymes. The anodal LDH band is the B_4 isozyme.

A genus may be defined as a “monophyletic group of species, which is separated from other taxa of the same rank [other genera] by a decided gap” (Mayr 1969). The enzymatic (LDH B_4 mobility) and morphological (modified scales) evidence for a monophyletic origin for all species in *Percina* is persuasive. Although the subgenus *Percina* is quite distinct in several ways from other subgenera, to grant the subgenus *Percina* separate generic status (as suggested by Curd 1967, Hubbs & Lagler 1958, Hubbs 1967, Minckley 1963, Stevenson 1971, and Winn 1958) because of its distinctiveness from other *Percina* negates the criterion of monophyly (Hennig 1966) for the genus containing the remaining species.

If the subgenus *Percina* were to be recognized as a separate genus, it would be a monophyletic group of four species, and the remaining species presumably would be put in *Hadropterus* as outlined by Bailey (1951). Of this composition, *Hadropterus* would not include all species descended from a common ancestor (Fig. 5), and therefore would not be a valid, monophyletic genus. The subgenus *Percina* shares with most other *Percina* subgenera (*Hadropterus*, *Swainia*, *Alvordius*, *Ericosma*, *Cottogaster*) the presence of a row of modified scales on the midbelly. The row is lacking in the subgenus *Hypohomus* (Fig. 5). Since it is extremely doubtful that a midbelly row of modified scales independently evolved twice, the separation of the subgenus *Percina* from the other subgenera with the row of modified scales almost certainly occurred after the separation of the subgenus without the row (*Hypohomus*). Therefore if *Percina* is to be a genus of four species, and genera are to be monophyletic, there also must at least be a genus *Hypohomus* and a genus *Hadropterus* (containing the subgenera *Hadropterus*, *Swainia*, *Ericosma*, *Alvordius*, *Cottogaster*, and ? *Imostoma*). The morphological gaps among the species within the latter two genera are in some instances larger than that between genera, although the genera would probably be monophyletic.

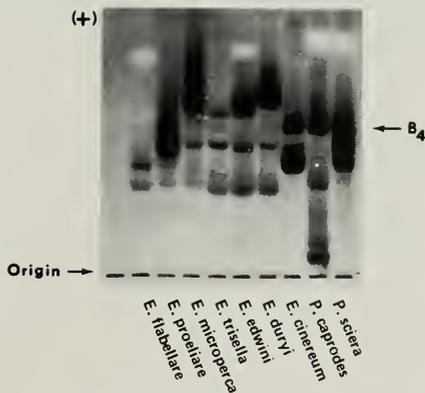


Fig. 4.—Lactate dehydrogenase isozymes of nine species of darters. Electrophoresis was in pH 8.7 EBT buffer.

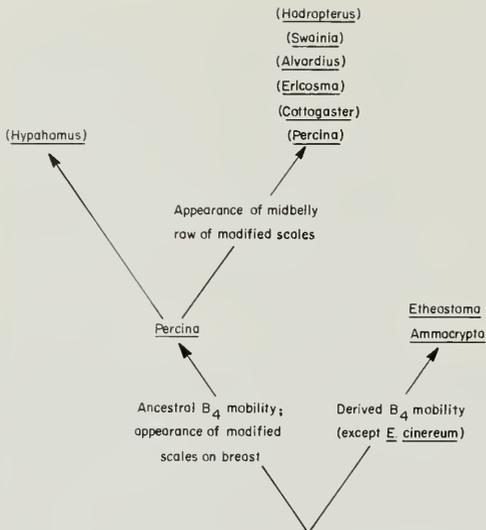


Fig. 5.—Hypothetical phylogeny of Etheostomini based on modified scale characteristics and the mobility of the lactate dehydrogenase B_4 isozyme.

If *Percina s. s.* is afforded separate generic status, the 26 described species of *Percina* should be in genera created by the elevation of each of the subgenera, and if this were to occur the other darter genera should receive similar analysis. Such splitting of genera seems unnecessary. The genus *Percina* as treated in this paper contains a monophyletic group of species (separated from other genera by a decided gap) and is therefore a valid assemblage. The genus *Percina* may be diagnosed by the presence of modified (enlarged and strongly toothed) scales on the breast of the male, the distinctive electrophoretic mobility of the LDH B_4 isozyme, a complete lateral line (often extending onto the caudal fin), and two anal spines.

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