MOLECULAR PHYLOGENETICS AND EVOLUTION
Vol. 10, No. 3, December, pp. 343–353, 1998
ARTICLE NO. FY980542

Phylogenetic Relations among Percid Fishes as Inferred from Mitochondrial Cytochrome *b* DNA Sequence Data

Choon Bok Song,[1] Thomas J. Near,[2] and Lawrence M. Page

Center for Biodiversity, Illinois Natural History Survey, 607 East Peabody Drive, Champaign, Illinois 61820

Received September 19, 1997; revised February 18, 1998

Hypotheses of relationship among genera of Percidae have been conflicting. Based on different phylogenetic premises, the evolution of small benthic forms in Percidae has been interpreted as resulting from either convergence or common ancestry. In order to assess various phylogenetic hypotheses of Percidae we collected complete sequences (1140 bp) of mitochondrially encoded cytochrome *b* for 21 species of percids. Seven species representing four additional families of Perciformes were used as outgroups. Maximum parsimony and minimum evolution analyses both recovered single shortest trees, and the results of these analyses were generally congruent with one another. All analyses consistently recovered three monophyletic groups in Percidae: Etheostomatinae (*Ammocrypta, Crystallaria, Etheostoma,* and *Percina*), Percinae (*Perca* and *Gymnocephalus*), and Luciopercinae (*Stizostedion, Zingel,* and *Romanichthys*). As a result of this analysis we present a revised classification of Percidae and discuss the phylogenetic evidence for the independent evolution of small benthic species within Etheostomatinae and Luciopercinae.

INTRODUCTION

Percidae is a family of 10 genera and about 195 species of freshwater fishes confined to the northern hemisphere. In North America the family is represented by more than 180 species. In contrast, only 14 species of percids are found in Eurasia. Despite the intensive study of percid taxonomy, hypotheses of relationship among the Percidae based on morphology and behavior have led to conflicting classifications (Collette, 1963; Hubbs, 1971; Collette and Banarescu, 1977; Page, 1985; Wiley, 1992).

Collette (1963) and Collette and Banarescu (1977) recognized two subfamilies, Percinae and Luciopercinae, each of which was composed of two tribes; Percinae contained Percini (*Perca, Gymnocephalus,* and *Percarina*) and Etheostomatini (*Ammocrypta, Etheostoma,* and *Percina*), and Luciopercinae contained Lucioperсini (*Stizostedion*) and Romanichthyini (*Zingel* and *Romanichthys*). The hypothesis of relationships presented by Collette (1963) and Collette and Banarescu (1977) was based on the condition of the first anal pterygiophore and the size of the anal spines; however, these analyses did not include a character matrix and did not assess whether the preferred topology represented the most-parsimonious arrangement of character states. This hypothesis suggests that in both the Etheostomatini and Romanichthyini small benthic forms have evolved in response to independent shifts from slow-moving bodies of water to faster flowing stream habitats. Characters shared by Etheostomatini and Romanichthyini (small size, absence of a predorsal bone, absence of a swimbladder, and presence of breeding tubercles) were considered to be the result of convergent evolution. Collette (1965) presented evidence for the convergent nature of breeding tubercles by noting differences in the distributions of tubercles in small European lucioperсines (*Zingel* and *Romanichthys*) and in the darters (*Ammocrypta, Etheostoma,* and *Percina*). The tubercles in *Zingel* and *Romanichthys* are concentrated on the dorsal and dorsolateral surfaces, while in the darters the tubercles are distributed on the ventral and ventrolateral surfaces. Collette (1963) argued that the characters that differentiate Percinae from Lucioperсinae (size of interhaemal bone and anal spines) are more "conservative" than characters hypothesized to be convergent.

Hubbs (1971) conducted hybridization experiments on *Perca, Stizostedion, Etheostoma,* and *Percina* to investigate genetic relationships among percids. No taxa representing the Romanichthyini (*Romanichthys* and *Zingel*) were included. Hubbs concluded that Percidae should contain three subfamilies, Percinae, Lucioperсinae, and Etheostomatinae. However, if two of the subfamilies were to be grouped together, Hubbs (1971) recommended that *Stizostedion, Zingel,* and *Romanich-*

[1] Present address: Department of Marine Biotechnology, Cheju National University, Cheju City 690-756, Korea.

[2] To whom correspondence should be addressed. Fax: (217) 333-4949. E-mail: near@mail.inhs.uiuc.edu.

1055-7903/98 $25.00

thys be placed in Etheostomatinae with the darters rather than in a separate subfamily, Luciopercinae.

Page (1985) presented a hypothesis of relationships among percid taxa based on reproductive behaviors and recognized two subfamilies, Percinae and Etheostomatinae. Percinae contained only the tribe Percini (*Perca, Percarina,* and *Gymnocephalus*), which is characterized by encasing eggs in long gelatinous strands, a behavior referred to as egg-stranding. Etheostomatinae included the tribes Luciopercini (*Stizostedion*), Etheostomatini (*Ammocrypta, Etheostoma,* and *Percina*), and Romanichthyini (*Zingel* and *Romanichthys*). Luciopercini exhibits broadcasting behavior, a primitive mode of reproductive behavior among fishes which involves discharging eggs and sperm over the substrate. Etheostomatini displays reproductive behaviors (egg-burying, egg-attaching, egg-clumping, and egg-clustering) that are derivatives of broadcasting. The grouping of Luciopercini with Etheostomatini was based on the hypothesis that the most primitive mode of reproduction in darters, egg-burying, had evolved from the broadcasting behavior exhibited by *Stizostedion* (Page, 1985). The reproductive behavior of Romanichthyini is not known, and it was grouped with Luciopercini following Collette (1963).

Wiley (1992) studied the condition of the swim bladder, breeding tubercles, general body shape, and 27 osteological characters in a phylogenetic analysis of percid relationships. The resultant hypothesis grouped *Gymnocephalus, Stizostedion, Zingel,* and *Romanichthys* with the darters (*Crystallaria, Etheostoma,* and *Percina*) in the subfamily Etheostomatinae. The genus *Perca* was basal to all other percid genera and placed in a monotypic subfamily Percinae. The presence of a reduced swim bladder, breeding tubercles, and a frenum were 3 of 5 character states that diagnosed the clade containing *Zingel, Romanichthys,* and the darter genera. Therefore, a hypothesis of a single origin of adaptations to benthic environments in all small percids is supported by Wiley's (1992) analysis. This contrasts with the hypothesis of convergent evolution of these characteristics in North American and European percids (Collette, 1963; Collette and Banarescu, 1977).

Scale morphology in Percidae was studied by Coburn and Gaglione (1992), and 8 characters were scored for phylogenetic analysis. After removing 3 characters which were autapomorphous in darter genera, the 5 remaining characters supported two equally parsimonious trees. One tree united *Perca, Gymnocephalus,* and *Stizostedion* to a clade consisting of *Zingel* and *Romanichthys.* This group was the sister taxon of the darters (*Ammocrypta, Crystallaria, Etheostoma,* and *Percina*). The other tree united *Zingel, Romanichthys,* and the darters. This second arrangement of percid genera was favored by Coburn and Gaglione (1992) because it was in general agreement with the conclusions of Wiley's (1992) cladistic analysis.

Relationships among the darters (*Ammocrypta, Crystallaria, Etheostoma,* and *Percina*), which include about 177 species are ambiguous. Relationships have been investigated using morphology (Bailey and Gosline, 1955; Page, 1974, 1981; Williams, 1975; Bailey and Etnier, 1988; Simons, 1991, 1992), allozymes (Page and Whitt, 1973a,b; Wood, 1996; Wood and Mayden, 1997), karyotypes (Ross, 1973), survival of interspecific hybrids (Hubbs and Strawn, 1957; Hubbs, 1971), reproductive behaviors (Page, 1985), and mitochondrial DNA sequence data (Turner, 1997). Although most darter species are easily grouped into genera and subgenera that are thought to be monophyletic, relationships among genera, subgenera, and species in subgenera are poorly understood (Page, 1981; Bailey and Etnier, 1988).

This investigation was initiated to provide a new molecular dataset to assess previous hypotheses of percid relationships. Cytochrome *b* was selected because it contains discrete character classes, (i.e., the three codon positions) which exhibit rates of mutation ranging from rapid to conservative (Irwin *et al.,* 1991). Due to the variable rate of evolution among the codon positions, cytochrome *b* appears to be useful for testing phylogenetic hypotheses for a wide range of divergences within actinopterygians (Lydeard and Roe, 1997). Complete double-stranded sequences were collected for 21 percids and one moronid (temperate bass) in order to investigate the evolution of the cytochrome *b* gene and its phylogenetic implications relative to percid fishes.

MATERIALS AND METHODS

Nucleic Acid Isolation, Polymerase Chain Reaction, and Sequencing

Specimens were collected and stored at ultracold temperatures (−70 to −80°C) until nucleic acids were extracted. The species used in this analysis and their classification (*sensu* Nelson, 1994; Page, 1983), with subgenera listed for Etheostomatini, and GenBank Accession Numbers are given in Table 1. Tissues from individual specimens were ground to a powder in liquid nitrogen. Approximately 0.1 g of ground tissue was added to 700 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA, and 3% SDS) and digested by adding proteinase K (final concentration 100 µg/ml) and incubated at 50°C. The supernatant was extracted once with chloroform:phenol (1:1). The nucleic acids were precipitated in a solution containing 10 µl of 3 M sodium acetate (pH 5.2) and 1 ml of absolute isopropanol. The resulting pellet was washed with cold 70% ethanol, dried, and resuspended in 50 µl of distilled water.

The polymerase chain reaction (PCR) was used to amplify the complete cytochrome *b* gene in 22 perciform species. Primers that anneal to tRNA genes flanking the cytochrome *b* region were designed from published

TABLE 1

Species Examined

Species (abbreviation)	Family	GenBank Accession No.	Locality/source
Etheostoma (Catonotus) kennicotti (Eken)	Percidae	AF045341	Poor Fork, KY, U.S.A.
Etheostoma (Catonotus) flabellare (Efla)	Percidae	AF045342	Blackwater R., VA, U.S.A.
Etheostma (Litocara) sagitta (Esag)	Percidae	AF045343	Red Bird Cr., KY, U.S.A.
Etheostoma (Oligocephalus) spectabile (Espe)	Percidae	AF045344	Linker Cr., AR, U.S.A.
Etheostoma (Boleichthys) gracile (Egra)	Percidae	AF045345	Dismal Cr., IL, U.S.A.
Etheostoma (Boleosoma) podostemone (Epod)	Percidae	AF045346	Blackwater R., VA, U.S.A.
Etheostoma (Ioa) vitreum (Evit)	Percidae	AF045347	Blackwater R., VA, U.S.A.
Etheostoma (Nothonotus) camurum (Ecam)	Percidae	AF045348	Rockcastle R., KY, U.S.A.
Etheostoma (Allohistium) cinereum (Ecin)	Percidae	AF045349	Rockcastle R., KY, U.S.A.
Ammocrypta clara (Acla)	Percidae	AF045350	Zumbro R., MN, U.S.A.
Ammocrypta beani (Abea)	Percidae	AF045351	Big Black R., MS, U.S.A.
Crystallaria asprella (Casp)	Percidae	AF045352	Cahaba R., AL, U.S.A.
Percina (Alvordius) maculata (Pmac)	Percidae	AF045353	Red Bird Cr., KY, U.S.A.
Percina (Percina) caprodes (Pcap)	Percidae	AF045354	Embarras R., IL, U.S.A.
Percina (Odontopholis) stictogaster (Psti)	Percidae	AF045355	Red Bird Cr., KY, U.S.A.
Gymnocephalus cernuus (Gcer)	Percidae	AF045356	St. Louis R., WI, U.S.A.
Perca flavescens (Pfla)	Percidae	AF045357	Silver Lake, WI, U.S.A.
Perca fluviatilis (Pflu)	Percidae	AF045358	Windmere, U.K.
Stizostedion vitreum (Svit)	Percidae	AF045359	Mississippi R., IL, U.S.A.
Zingel streber (Zstr)	Percidae	AF045360	Turiec R., Slovakia
Romanichthys valsanicola (Rval)	Percidae	AF045361	Vilsan R., Romania
Morone mississippiensis (Mmis)	Moronidae	AF045362	Illinois R., IL, U.S.A.
Dicentrarchus labrax (Dlab)	Moronidae	X81566	Cantatore *et al.*, 1994
Micropterus salmoides (Msal)	Centrarchidae	L14074	Whitmore *et al.*, 1994
Boops boops (Bboo)	Sparidae	X81567	Cantatore *et al.*, 1994
Sarda sarda (Ssar)	Scombridae	X81562	Cantatore *et al.*, 1994
Scomber scombrus (Ssco)	Scombridae	X81564	Cantatore *et al.*, 1994
Thunnus thynnus (Tthy)	Scombridae	X81563	Cantatore *et al.*, 1994

sequences available on GenBank. PCR was performed in 25-µl volumes containing 2.0 mM $MgCl_2$, 0.4 mM each of the deoxynucleotides, 1.0 µM of each primer (forward primer 5′-GTGACTTGAAAAACCACCGTTG-3′, reverse primer 5′-CTCCATCTCCGGTTTACAAGAC-3′), 1.0 unit of *Thermus aquaticus* DNA polymerase in a reaction buffer of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.1% Triton X-100. Of template DNA 100 ng was used in PCR. Temperature conditions for amplification were 90°C (1 min), 42°C (1 min), and 72°C (2 min). A final incubation of 7 min (72°C) was performed to completely extend the amplified product. The size of resulting PCR product was verified by electrophoresis in a 0.8% agarose gel using DNA size standards.

The amplified cytochrome *b* gene was isolated from PCR reactants by agarose gel isolation using the GeneClean II protocol (Bio 101, Inc.). Isolated PCR product was treated with Klenow Fragment (Promega), ligated to pBluescript (SK^-) vector, and used to transform *E. coli* XL1-Blue competent cells. Colonies that were positive by blue/white selection were screened using *Eco*RI and *Xho*I to verify the size of the insert. Plasmid DNA was isolated from individual clones and used as template for the sequencing reactions. At least two clones from each individual were sequenced.

Sequencing reactions were performed for most taxa using the Sequenase 2.0 kit (Amersham United States Biochemical) with ^{32}P-dATP as the radionuclide. Cytochrome *b* sequence of *Romanichthys valsanicola* was generated using Delta Taq cycle sequencing kit (Amersham United States Biochemical). Each species was sequenced for both strands using a total of 10 primers (2 PCR primers, 6 internal primers, and 2 vector primers). Sequences of internal forward primers with their 5′ annealing positions on the cytochrome *b* gene were ATYCGHAAYMTDCATKCYAA (225F), GGCTTYTCMGTVGAYAABSC (498F), and CAYATYMARCCHGARTGATA (798F). Reverse internal primers were TARTTHACRTCHCGRCARAT (205R), GSVTTRTCBACKGARAAGCC (498R), and TATCAYTCDGGYTKRATRTG (798R). Sequencing products were separated by electrophoresis in 6% polyacrylamide/8.3 M urea gels and visualized by autoradiography.

Analysis of Sequence Data

Sequences were manually aligned and PAUP* (4.0d54) was used to assess levels of pairwise nucleotide variation and to determine nucleotide composition for each taxon. Nucleotide saturation was assessed at each codon position by plotting numbers of transitions and transversions against *p* distance values (Moritz *et al.*, 1992). All phylogenetic analyses were executed using PAUP* (4.0d54). Complete cytochrome *b* se-

quences from 6 non-percid perciform species (Table 1) were downloaded from GenBank. These 6 species and *Morone mississippiensis* were designated as outgroup taxa in all analyses. In maximum parsimony analysis only minimal-length trees were retained and zero length branches were collapsed. The heuristic search algorithm was used and employed 10 random orders of taxa and tree bisection-reconstruction (TBR) branch swapping. Bootstrap analysis (1000 replications) was used to assess the relative robustness of inferred monophyletic groups. The robustness of the clades recovered in the phylogenetic hypothesis also was evaluated in a decay analysis (Bremer, 1988). The amount of phylogenetic information in the aligned cytochrome *b* dataset was assessed by examination of the g^1 value of the tree length distribution for 10^5 randomly generated trees (Hillis and Huelsenbeck, 1992). Minimum evolution analysis was executed using LogDet/paralinear distances (Lake, 1994; Lockhart *et al.*, 1994), and trees were recovered using a heuristic search with 10 random orders of taxa and tree bisection-reconstruction branch swapping. Minimum evolution bootstrap analysis involved 1000 replications.

Alternative phylogenetic hypotheses (Fig. 1) were

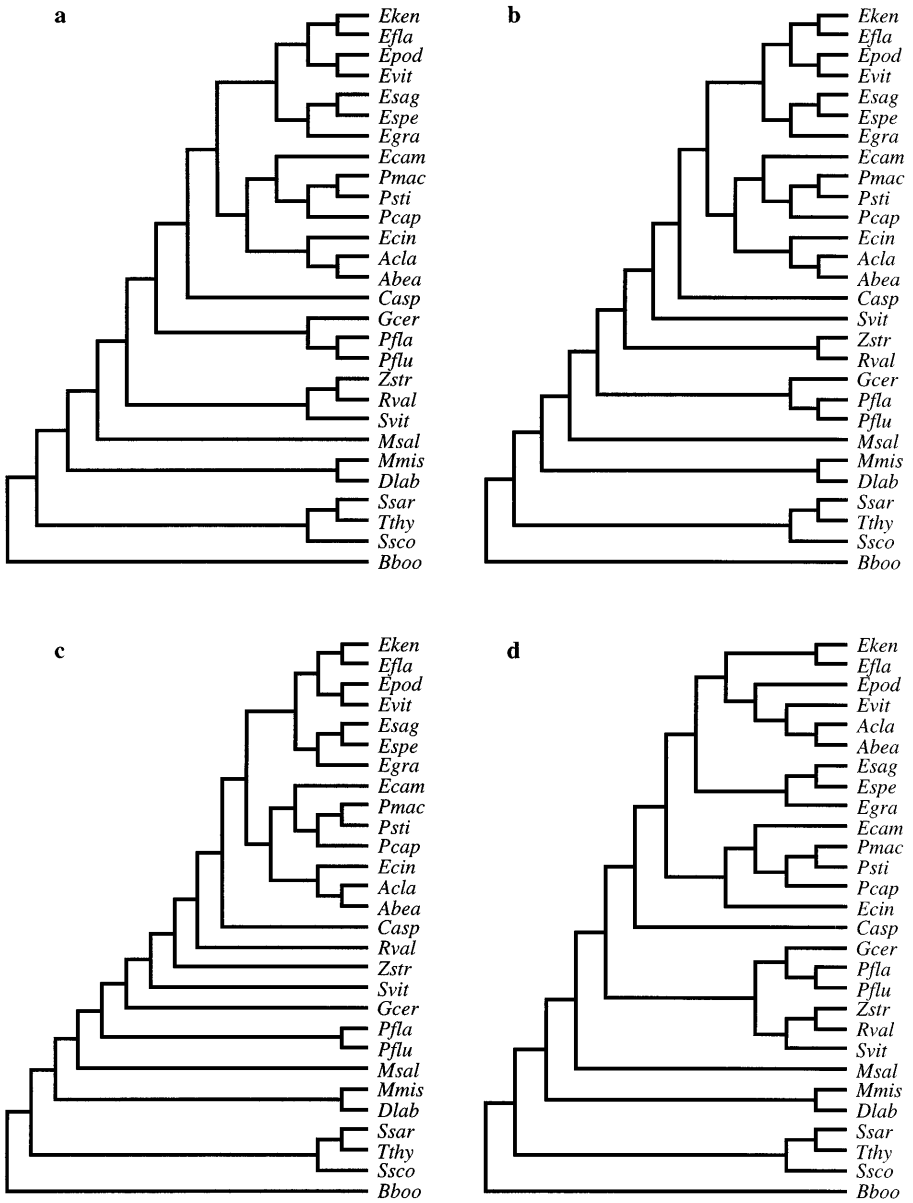

FIG. 1. Alternative phylogenetic hypotheses of Percidae shown with species included in cytochrome *b* analyses: (a) Collette (1963) and Collette and Banarescu (1977); (b) Page (1985); (c) Wiley (1992); (d) Simons (1992). See Table 1 for species abbreviations.

tested statistically by two methods using the tree scores option in PAUP* (4.0d54). Topologies were assessed using a pairwise parsimony method proposed by Templeton (1983) and modified by Felsenstein (1993), and by maximum likelihood criteria using the method of Kishino and Hasegawa (1989). The topologies examined by these criteria were representative of previous systematic or phylogenetic hypotheses of percid relationships and included the hypothesis of Collette and Banarescu (1977), which depicts a sister taxon relationship between the Percinae and Etheostomatinae (Fig. 1a); a classification of Percidae proposed by Page (1985), which grouped Etheostomatini, Luciopercini, and Romanichthyini in the subfamily Etheostomatinae and, within this group, hypothesized *Stizostedion* to be the sister taxon to Etheostomatini (Fig. 1b); the phylogenetic hypothesis of Wiley (1992), where *Romanichthys* is presented as the sister taxon of the Etheostomatini (Fig. 1c); and the phylogenetic hypothesis of Simons (1992), which grouped *Ammocrypta* as the sister taxon of *Etheostoma vitreum* (Fig. 1d).

RESULTS AND DISCUSSION

Nucleotide Variation, Base Compositional Bias, and Saturation

The aligned sequences of the complete cytochrome *b* gene (1140 bp) from 21 percid and 7 perciform outgroup species exhibited 553 variable sites, 485 of which were phylogenetically informative for maximum parsimony analysis. The distribution of phylogenetically informative sites by codon position in all 28 species is similar to patterns observed in other vertebrates, with 94 in the first, 27 in the second, and 364 in the third codon position. The base composition and nucleotide bias indices of compositional differences in cytochrome *b* of

TABLE 2

Empirical Base Composition and Calculated Base Compositional Bias (Irwin *et al.*, 1991) of the Cytochrome *b* Gene in Percidae and Seven Additional Perciform Species (See Table 1 for Species Abbreviations)

					Codon position											
	All				First				Second				Third			
Species	A	T	C	G	A1	T1	C1	G1	A2	T2	C2	G2	A3	T3	C3	G3
Eken	23.2	30.0	30.4	16.5	23.4	23.9	25.8	26.8	19.2	40.8	25.5	14.5	26.8	25.3	39.7	8.2
Efla	23.9	30.1	30.1	16.0	23.9	23.2	26.8	26.1	20.0	40.8	25.5	13.7	27.6	26.3	37.9	8.2
Esag	20.7	29.4	31.1	18.8	22.1	23.7	26.6	27.6	20.0	40.8	25.5	13.7	20.0	23.7	41.3	15.0
Espe	20.6	30.7	29.9	18.8	22.9	23.2	27.1	26.8	19.7	40.3	26.1	13.9	19.2	28.7	36.6	15.5
Egra	22.4	30.3	30.5	16.8	21.8	23.4	27.1	27.6	19.7	40.8	25.5	13.9	25.5	26.6	38.9	8.9
Epod	22.9	29.8	30.6	16.7	22.1	22.6	27.6	27.6	20.0	40.8	25.5	13.7	26.6	26.1	38.7	8.7
Evit	22.8	30.1	29.8	17.3	22.4	23.2	26.6	27.9	20.0	40.8	25.5	13.7	26.1	26.3	37.4	10.3
Ecam	22.6	29.1	31.1	17.2	22.6	24.5	25.3	27.6	20.0	40.5	25.5	13.9	25.3	22.4	42.4	10.0
Ecin	22.3	28.1	32.5	17.1	22.9	23.2	26.8	27.1	19.7	40.8	25.8	13.7	24.2	20.3	45.0	10.5
Acla	23.7	30.8	30.2	15.4	22.6	24.2	26.3	26.8	19.7	40.8	25.5	13.9	28.7	27.4	38.7	5.3
Abea	21.9	28.3	32.9	16.8	22.9	22.6	27.6	26.8	19.7	40.8	25.5	13.9	23.2	21.6	45.5	9.7
Casp	23.4	29.5	30.4	16.7	22.4	22.4	27.9	27.4	20.5	40.8	25.0	13.7	27.4	25.3	38.4	8.9
Pmac	22.5	28.2	32.4	16.9	22.6	23.7	26.3	27.4	19.5	40.6	25.9	14.0	25.3	20.3	45.0	9.5
Pcap	22.4	28.6	32.2	16.8	22.6	23.4	26.6	27.4	19.7	40.5	25.8	13.9	24.7	21.8	44.2	9.2
Psti	22.4	29.8	30.8	17.0	22.4	24.5	25.5	27.6	19.7	40.5	25.8	13.9	25.0	24.5	41.1	9.5
Gcer	25.4	29.5	30.4	14.6	23.7	24.2	26.1	26.1	19.7	41.3	25.0	13.9	32.9	22.9	40.3	3.9
Pfla	24.0	30.6	29.7	15.6	22.6	25.0	25.3	27.1	19.7	40.8	25.3	14.2	29.7	26.1	38.7	5.5
Pflu	24.1	30.5	29.9	15.4	23.2	25.5	24.2	27.1	19.7	40.5	25.8	13.9	29.5	25.5	39.7	5.3
Zstr	24.7	31.8	28.4	15.1	22.6	23.7	26.6	27.1	19.5	41.1	25.3	14.2	32.1	30.5	33.4	3.9
Rval	23.1	32.9	27.4	16.7	22.6	24.2	25.8	27.4	19.5	40.8	25.3	14.5	27.1	33.7	31.1	8.2
Svit	24.5	30.7	30.0	14.8	23.4	24.5	25.5	26.6	19.7	40.5	25.8	13.9	30.3	27.1	38.7	3.9
Mmis	24.2	31.3	29.6	14.9	24.2	26.1	25.8	23.9	20.3	41.1	25.3	13.4	28.2	26.8	37.6	7.4
Dlab	24.8	32.8	26.0	16.4	23.2	26.1	24.5	26.3	20.5	41.8	23.9	13.7	30.8	30.5	29.5	9.2
Msal	23.2	28.6	32.7	15.5	22.1	24.5	26.8	26.6	20.0	40.5	26.1	13.4	27.4	20.8	45.3	6.6
Bboo	24.5	28.6	31.4	15.5	23.7	22.9	27.1	26.3	20.0	40.5	25.3	14.2	29.7	22.4	41.8	6.1
Ssar	25.2	28.9	31.1	14.8	22.6	24.2	26.6	26.6	19.5	41.1	26.1	13.4	33.4	21.6	40.5	4.5
Ssco	23.4	28.1	31.3	17.2	21.3	23.2	26.8	28.7	19.2	41.3	25.5	13.9	29.7	19.7	41.6	8.9
Tthy	24.7	28.9	31.2	15.1	22.6	23.2	27.4	26.8	19.5	41.1	26.1	13.4	32.1	22.6	40.3	5.0
Mean	23.3	29.9	30.5	16.3	22.8	23.9	26.4	27.0	19.8	40.8	25.5	13.9	27.4	24.9	39.6	8.1
Bias[a]		0.139				0.045				0.217				0.227		

[a] $B = (2/3)\,\Sigma_{i=1}^{4}|c_i - 0.25|$

the 28 species are shown in Table 2. Examination of the entire gene reveals a moderate lack of guanine (G) and a slight abundance of cytosine (C) in the H-strand. The nucleotide composition of the first codon position is relatively unbiased; however, the second and third positions exhibit moderate biases (Table 2). The bias in the second position involves a slight lack of adenine (A), a moderate lack of guanine (G), and an abundance of thymine (T) in the H-strand. The third position exhibits the highest bias of the codon positions, with an abundance of cytosine (C) and a pronounced lack of guanine (G) in the H-strand (Table 2). The calculated compositional bias indices for the second (0.217) and third (0.227) codon positions are similar to one another. In birds (Edwards *et al.*, 1991; Helm-Bychowski and Cracraft, 1993; Nunn and Cracraft, 1996) and mammals (Irwin *et al.*, 1991) the third position exhibits a much higher compositional bias than the second codon position. The absence of extreme bias in the third codon position of the 28 species examined can be attributed to the higher percentage of guanine (G) in perciform fishes (8.1%) than that observed in sharks (2.4%), mammals (3.6%), and passerine birds (3.1%) (Irwin *et al.*, 1991; Martin, 1995; Nunn and Cracraft, 1996).

Plots of the numbers of transitions and transversions against *p* distances for each codon position revealed that third position transitions and transversions are saturated in comparisons between the percids and the outgroups (Fig. 2). Transitions and transversions at both first and second codon positions were found to increase linearly with increasing *p* distances, indicating that substitutions at first and second codon positions are not saturated in comparisons between percids and outgroups (plots not shown). Transitions and transversion substitutions at the third codon position do not appear to be saturated for pairwise comparisons within Percidae (Fig. 2).

Phylogenetic Analyses

Strong phylogenetic signal was detected in the aligned cytochrome *b* data set, as evidenced by the skewed distribution of 10^5 random trees ($g^1 = -0.633858$, $P < 0.01$). Maximum parsimony analysis recovered a single most-parsimonious tree, with a length of 3242 steps and a consistency index (excluding uninformative characters) of 0.286 (Fig. 3). Percidae is monophyletic with respect to the other perciform species in the analysis, and this clade is supported with high bootstrap (91%) and decay (10) values. Etheostomatinae is recovered as the sister taxon of a monophyletic Luciopercinae-Percinae clade. The monophyly of the Luciopercinae-Percinae clade is supported with moderate bootstrap (63%) and low decay (2) values (Fig. 3).

Etheostomatinae (*Ammocrypta*, *Crystallaria*, *Etheostoma*, and *Percina*) is recovered as monophyletic; however, this clade receives only moderate support in bootstrap (52%), no support in decay analysis, and has a branch length of 31 steps (Fig. 3). Within the Etheostomatinae, both *Percina* and *Ammocrypta* are monophyletic and supported with high bootstrap and decay values. The placement of *E. (Nothonotus) camurum* and *E. (Allohistium) cinereum* confounds the recovery of a monophyletic *Etheostoma*. The phylogenetic placement of these two species within Etheostomatinae

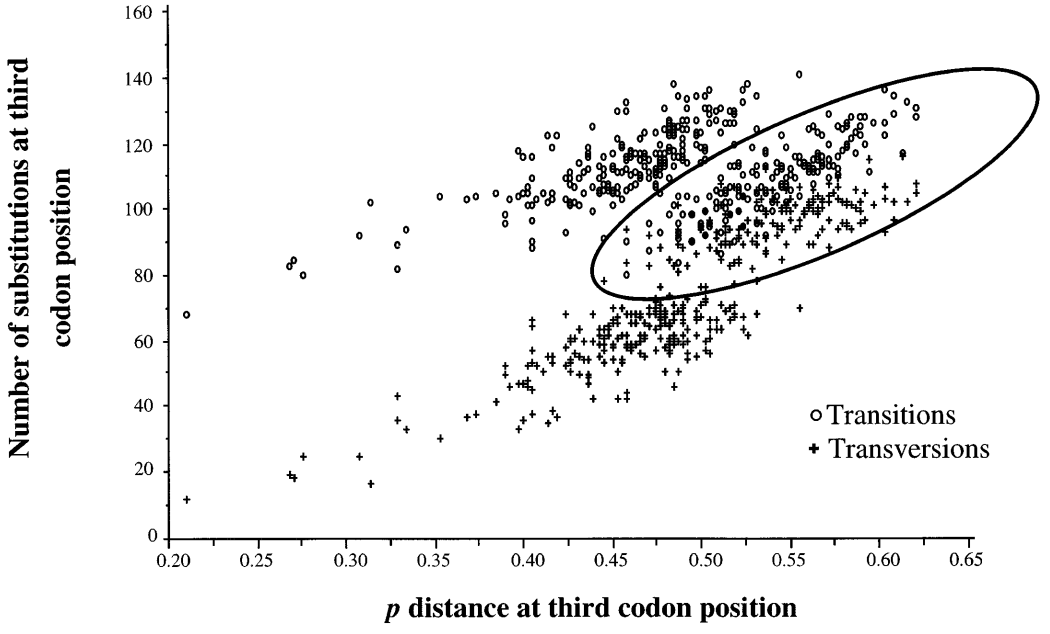

FIG. 2. Plots of the numbers of transitions (open circles) and transversions (crosses) at the third codon position against *p* distance values at third codon position. Points enclosed in oval are comparisons between percid species and nonpercid perciform outgroups.

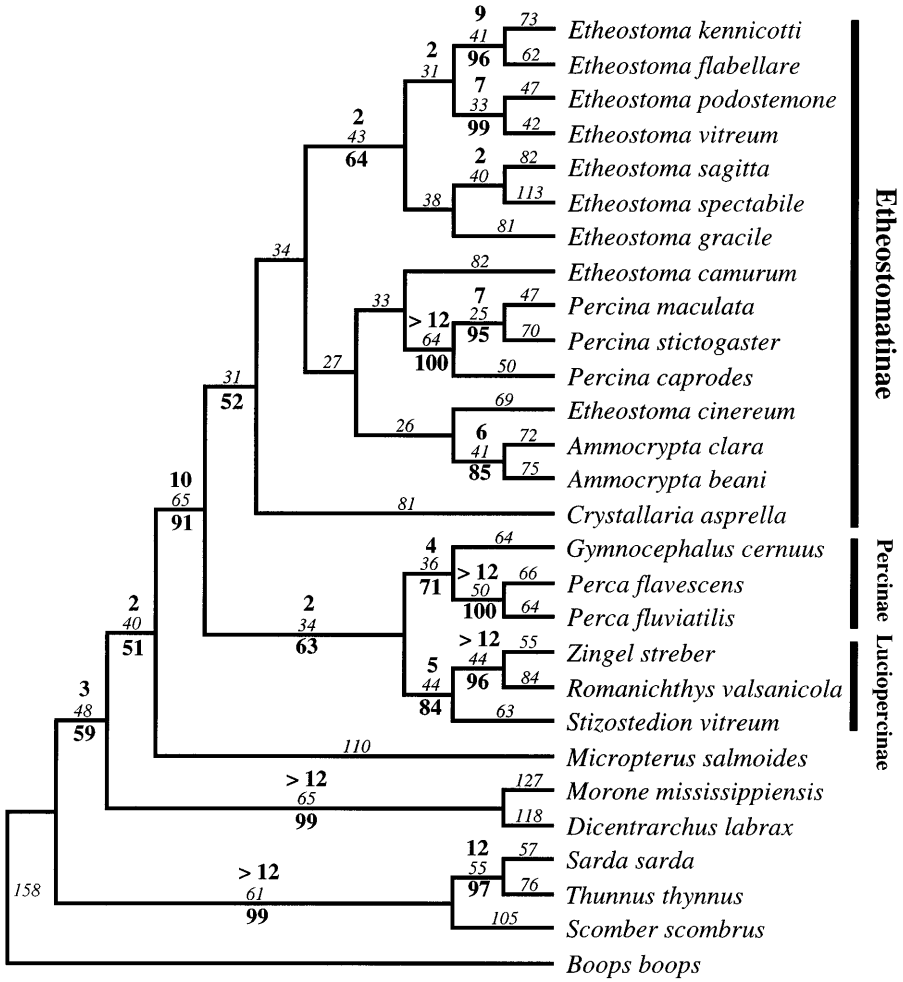

FIG. 3. Single most-parsimonious tree (TL = 3242; CI = 0.286) recovered in maximum parsimony analysis of cytochrome *b* (1140 bp). Decay values (in bold) and branch lengths (in italics) are given above branches. Bootstrap values (1000 replications) are indicated below branches in bold. Subfamilies of Percidae are listed to the right.

cannot be resolved with the cytochrome *b* dataset, as evidenced by the lack of bootstrap and decay support. The aberrant placement of *E. (Nothonotus) camurum* was critically examined by including a complete cytochrome *b* sequence of *E. (Nothonotus) aquali* (unpublished data). The two *Nothonotus* species were strongly monophyletic and continued to be placed outside the rest of *Etheostoma* (not shown). A similar experiment could not be done for *E. (Allohistium) cinereum* since this species occurs in a monotypic subgenus. Within the monophyletic *Etheostoma* clade, the subgenus *Catonotus* (*E. kennicotti* and *E. flabellare*) and the *Boleosoma* species group (*E. podostemone* and *E. vitreum*) are each monophyletic with high bootstrap and decay support (Fig. 3).

Luciopercinae (*Stizostedion, Romanichthys,* and *Zingel*) is recovered as monophyletic; moderate bootstrap (84%) and decay (5) values support this clade. In addition, the branch leading to Luciopercinae has a length of 44 steps, which is moderate to high for an internal node in Percidae (Fig. 3). Within Luciopercinae, *Stizostedion vitreum* is recovered as the sister taxon of a monophyletic clade consisting of *Zingel streber* and *Romanichthys valsanicola.* In the parsimony analysis the *Zingel-Romanichthys* clade is supported with high bootstrap (96%) and decay (>12) values, and the length of the branch leading to this clade is 44 steps.

Percinae (*Perca* and *Gymnocephalus; Percarina* is absent from our analysis) is recovered as monophyletic and is supported by moderate bootstrap (71%) and decay (4) values (Fig. 3). The length of the branch leading to Percinae is 36 steps. The two species of *Perca* (*P. flavescens* and *P. fluviatilis*) are monophyletic with high bootstrap (100%) and decay (>12) values, and a branch length of 50 steps.

Minimum evolution analysis using LogDet/paralinear distances recovered a single shortest tree with a minimum evolution score of 2.588 (Fig. 4). This tree is generally congruent with the maximum parsimony tree

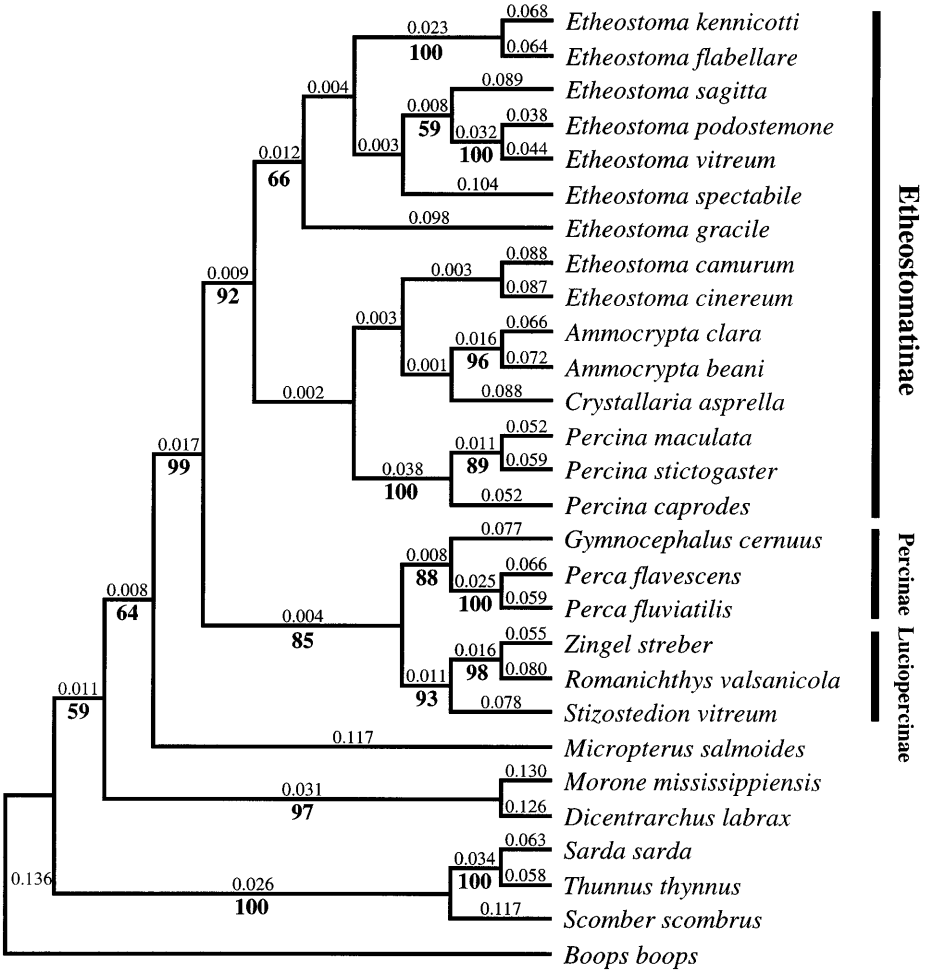

FIG. 4. Single shortest tree (ME score = 2.588) recovered in minimum evolution analysis using LogDet/paralinear distances. Numbers above branches are branch lengths and numbers below branches indicate bootstrap values (1000 replications). Subfamilies of Percidae are listed to the right.

(Fig. 3); the only differences involve relationships of species and genera in Etheostomatinae. As in the maximum parsimony analysis, the genus *Etheostoma* is not monophyletic. The minimum evolution bootstrap analysis consistently recovered monophyletic groups at a higher frequency than the maximum parsimony analysis (Fig. 3); for instance, minimum evolution bootstrap values for the genus *Ammocrypta* (96% vs 85%), Etheostomatinae (92% vs 52%), Percinae (88% vs 71%), Luciopercinae (93% vs 84%), and the monophyletic Percinae-Luciopercinae clade (85% vs 63%) were higher in comparison to values in the maximum parsimony bootstrap analysis.

A potential confounding factor in the phylogenetic analyses of cytochrome *b* in the Percidae is the sensitivity of ingroup taxa resolution to the choice of outgroup taxa (Smith, 1994). Relationships of basal perciforms are unresolved and the sister taxon to Percidae has not been identified (Johnson, 1984; Wiley, 1992). The potential problem of appropriate perciform outgroups to polarize character-state transformations within Percidae is exacerbated by the short internodal branch lengths in both the maximum parsimony and minimum evolution analyses (Figs. 3 and 4) and the determination that third codon substitutions are saturated when comparing percids to all outgroup taxa in the data set (Fig. 2). In order to assess the effect of distant outgroups on the inferred topology of species in the Percidae, we reanalyzed the cytochrome *b* data set with all nonpercid taxa removed. The resultant maximum parsimony tree was rooted with both species of *Perca*. This outgroup choice was based on the phylogenetic analysis of Wiley (1992), which found that *Perca* was morphologically most similar to other perciform families examined. Two most-parsimonious trees resulted with a length of 2155 steps and a consistency index (excluding uninformative characters) equal to 0.332. The strict consensus of the two trees (Fig. 5) reveals that the only area of conflict between the two most-parsimonious topologies is within *Etheostoma*. One surprising result

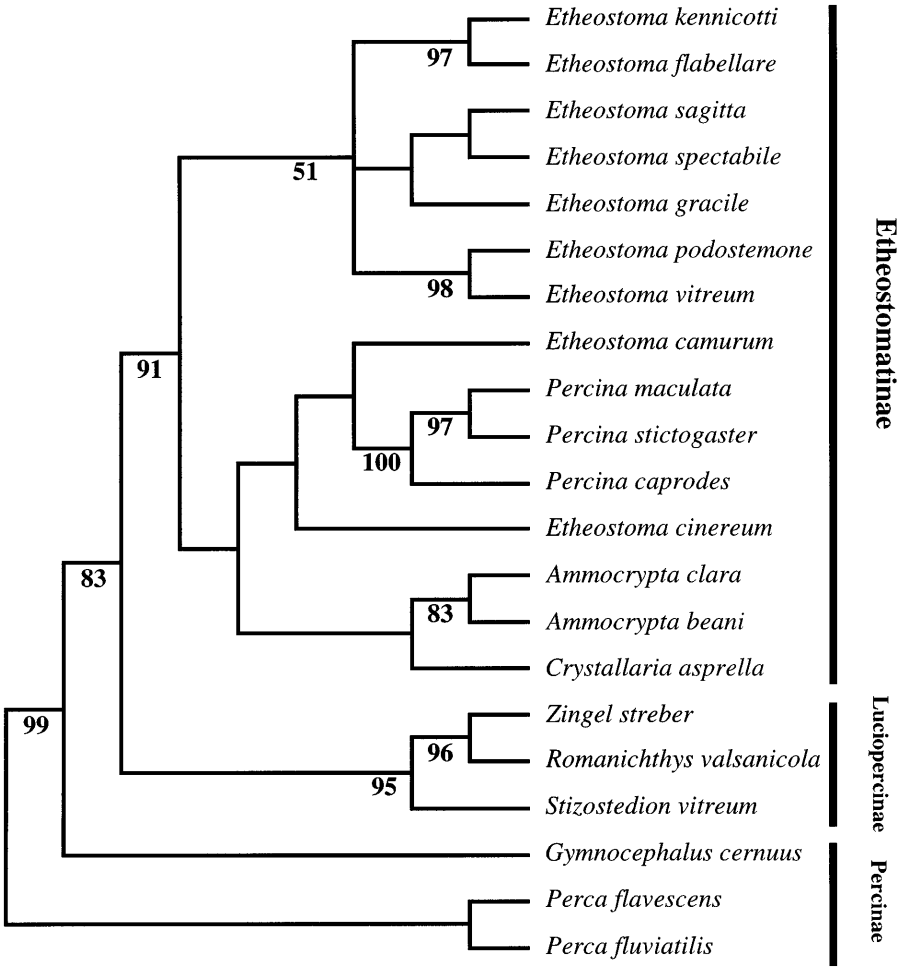

FIG. 5. Strict consensus of two trees (TL = 2155; CI = 0.332) recovered in maximum parsimony analysis of cytochrome *b* (1140 bp) with all nonpercid perciforms removed. The tree is rooted with *Perca*. Subfamilies of Percidae are listed to the right. Numbers below branches are bootstrap values (1000 replications).

from this analysis is that a monophyletic Etheostomatinae is strongly supported in bootstrap analysis (91%), in contrast to the weak support for this clade (bootstrap = 52%) in the maximum parsimony analysis including the nonpercid perciform outgroups (Fig. 3).

Comparison of Alternative Topologies

The results of the Hasegawa-Kishino test of alternative topologies, using both maximum parsimony and maximum likelihood criteria, indicate that cytochrome *b* is able to discriminate and reject most of the alternative hypotheses of percid phylogeny (Table 3). The minimum evolution tree (Fig. 4) is not significantly different from the maximum parsimony tree (Fig. 3). This is not surprising since the two trees are congruent with respect to the monophyly of the three major groups in Percidae. The hypothesis of Collette (1963) and Collette and Banarescu (1977) (Fig. 1a) is not significantly different from the most-parsimonious topology using both parsimony and maximum likelihood criteria. All other topologies examined (Figs. 1b, 1c, and 1d) are significantly different from the most parsimonious tree (Fig. 3) using both maximum parsimony and maximum likelihood criteria (Table 3). We also examined Wiley's (1992) hypothesis (Fig. 1c) by comparing it to the topology resulting from a percid-only dataset rooted with *Perca* (Fig. 5); the topology proposed by Wiley (1992) (Fig. 1c) was 29 steps longer and significantly different ($P < 0.003$) from the most parsimonious trees (Fig. 5). This last comparison indicates that the inclusion of potentially distant outgroups in the dataset is not altering the ability of cytochrome *b* to discriminate hypotheses that are significantly less parsimonious.

Conclusions: Taxonomic Suggestions and Hypotheses of Percid Evolution

The cytochrome *b* dataset for 21 species of Percidae strongly supports the monophyly of three major groups, Etheostomatinae, Luciopercinae, and Percinae. In all

TABLE 3

Statistical Comparison of Alternative Topologies

	Maximum parsimony			Maximum likelihood		
Topology	Parsimony length (SD)	Worse by parsimony?[a]	Consistency index[b]	*ln* L	SD of *ln* L difference	Worse by likelihood?[a]
Figure 2	3242	Best	0.286	−15,879.27	—	Best
Figure 4	3252 (12.17)	No	0.285	−15,882.41	26.20	No
Figure 1a	3249 (4.58)	No	0.285	−15,889.77	9.80	No
Figure 1b	3267 (6.20)	Yes[c]	0.284	−15,923.32	13.45	Yes[c]
Figure 1c	3285 (9.56)	Yes[c]	0.282	−15,979.78	22.58	Yes[c]
Figure 1d	3299 (9.06)	Yes[c]	0.281	−16,052.17	25.80	Yes[c]

[a] Probability of getting a more extreme T value under the null hypothesis of no difference between the two trees (Swofford, 1997).
[b] Excluding uninformative characters.
[c] Significant at $P < 0.05$.

analyses employing available perciform outgroups, Percinae and Luciopercinae are sister taxa. However, the hypothesis that Etheostomatinae and Percinae are sister taxa (Collette, 1963; Collette and Banarescu, 1977) (Fig. 1a) cannot be rejected using criteria of parsimony and maximum likelihood (Table 3). Due to low internodal branch lengths, the topology of the three major groups in Percidae is unresolved. In spite of the inability to discriminate hypotheses addressing the topology of major percid groups (Table 3), we present a revised classification for the family (Table 4) recognizing three monophyletic subfamilies. In addition, we strongly advocate the recognition of the genus *Ammocrypta* based on the statistical rejection of Simons' (1992) hypothesis (Table 3) and the fact that *Ammocrypta* never clustered with the *Boleosoma* species group in the phylogenetic analyses (Figs. 3, 4, and 5).

The phylogenetic analysis of cytochrome *b* in percids contradicts the hypothesis of Wiley (1992) that the Etheostomatinae and the small luciopercines (*Zingel* and *Romanichthys*) are monophyletic. The monophyletic clade consisting of *Stizostedion*, *Zingel*, and *Romanichthys* is recovered in all analyses (Figs. 3, 4, and 5), and a topology that depicts *Romanichthys* as the sister taxon of Etheostomatinae (Fig. 1c) is strongly rejected by maximum parsimony and maximum likelihood criteria (Table 3). Based on these results, we interpret character states used by Wiley (1992) to identify a *Zingel-Romanichthys*-Etheostomatinae clade to have evolved as a result of convergent evolution. These characters include (with transformation series from Wiley, 1992, indicated) occurrence of a frenum (TS 26), presence of breeding tubercles (TS 10), reduction of the supraoccipital crest (TS 7), absence of a supraneural bone (TS 6), and reduction of the swimbladder (TS 5). We concur with the hypothesis of Collette (1963) and Collette and Banarescu (1977) that convergence has occurred among percids as both Etheostomatinae and members of the Luciopercinae have independently invaded benthic rheophilic habitats. This hypothesis is further supported by the fact that Etheostomatinae is found only in North America and the small luciopercines (*Zingel* and *Romanichthys*) occur only in Europe.

TABLE 4

Revised Classification of the Percidae

Family Percidae Bonaparte
- Subfamily Percinae Bonaparte
  - Genus *Perca* Linnaeus
  - Genus *Percarina* Nordmann
  - Genus *Gymnocephalus* Bloch
- Subfamily Luciopercinae Jordan and Evermann
  - Genus *Stizostedion* Rafinesque
  - Genus *Zingel* Cloquet
  - Genus *Romanichthys* Dumitrescu, Banarescu, and Stoica
- Subfamily Etheostomatinae Agassiz
  - Genus *Ammocrypta* Jordan
  - Genus *Crystallaria* Jordan and Gilbert
  - Genus *Etheostoma* Rafinesque
  - Genus *Percina* Haldeman

ACKNOWLEDGMENTS

Several colleagues provided specimens or DNA aliquots; we thank H. L. Bart for *Crystallaria asprella*, N. Billington for *Perca fluviatilis* and *Zingel streber*, E. Marsden for *Perca flavescens* and *P. fluviatilis*, and E. O. Wiley for *Romanichthys valsanicola*. Most of the DNA sequences for this study was collected by the first author (C.B.S.) for a Ph.D. thesis in Ecology, Ethology, and Evolution at the University of Illinois at Urbana-Champaign. We thank J. C. Porterfield, C. H. Dietrich, and three anonymous reviewers for helpful and constructive comments. Partial funding for this study was provided by the University of Illinois Research Board.

REFERENCES

Bailey, R. M., and Etnier, D. A. (1988). Comments on the subgenera of darters (Percidae) with descriptions of two new species of *Etheostoma* (*Ulocentra*) from southeastern United States. *Misc. Publ. Mus. Zool. Univ. Michigan* **175:** 1–48.

Bailey, R. M., and Gosline, W. A. (1955). Variation and systematic

significance of vertebral counts in the American fishes of the family Percidae. *Misc. Publ. Mus. Zool. Univ. Michigan* **93:** 1–44.

Bremer, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42:** 795–803.

Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaleta, M. N., and Saccone, C. (1994). Evolutionary analysis of cytochrome *b* sequences in some Perciformes: Evidence for a slower rate of evolution than in mammals. *J. Mol. Evol.* **39:** 589–597.

Coburn, M. M., and Gaglione, J. I. (1992). A comparative study of percid scales (Teleostei: Perciformes). *Copeia* **1992:** 986–1001.

Collette, B. B. (1963). The subfamilies, tribes, and genera of the Percidae (Teleostei). *Copeia* **1963:** 615–623.

Collette, B. B. (1965). Systematic significance of breeding tubercles in fishes of the family Percidae. *Proc. U.S. Natl. Mus.* **117:** 567–614.

Collette, B. B., and Banarescu, P. (1977). Systematics and zoogeography of the fishes of the family Percidae. *J. Fish. Res. Board Can.* **34:** 1450–1463.

Edwards, S. V., Arctander, P., and Wilson, A. C. (1991). Mitochondrial resolution of a deep branch in the genealogical tree for perching birds. *Proc. R. Soc. London B* **243:** 99–107.

Felsenstein, J. (1993). "PHYLIP: Phylogenetic Inference Package," Ver. 3.5c, University of Washington, Seattle.

Helm-Bychowski, K., and Cracraft, J. (1993). Recovering phylogenetic signal from DNA sequences: Relationships within the corvine assemblage (Class Aves) as inferred from complete sequences of the mitochondrial DNA cytochrome *b* gene. *Mol. Biol. Evol.* **10:** 1196–1214.

Hillis, D. A., and Huelsenbeck, J. P. (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *J. Hered.* **83:** 189–195.

Hubbs, C. (1971). Survival of intergroup percid hybrids. *Japan. J. Ichthyol.* **18:** 65–75.

Hubbs, C., and Strawn, K. (1957). Survival of F_1 hybrids between fishes of the subfamily Etheostomatinae. *J. Exp. Zool.* **134:** 33–62.

Irwin, D. M., Kocher, T. D., and Wilson, A. C. (1991). Evolution of the cytochrome *b* gene of mammals. *J. Mol. Evol.* **32:** 128–144.

Johnson, G. D. (1984). Percoidei: Development and relationships. *In* "Ontogeny and Systematics of Fishes" (H. G. Moser *et al.*, Eds.), pp. 464–498, Spec. Publ. No. 1, Amer. Soc. Ichthyol. and Herp.

Kishino, H., and Hasegawa, M. (1989). Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* **29:** 170–179.

Lake, J. A. (1994). Reconstructing evolutionary trees from DNA and protein sequences: Paralinear distances. *Proc. Natl. Acad. Sci. USA* **91:** 1455–1459.

Lockhart, P. J., Steel, M. A., Pendy, M. D., and Penny, D. (1994). Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* **11:** 605–612.

Lydeard, C., and Roe, K. J. (1997). The phylogenetic utility of the mitochondrial cytochrome *b* gene for inferring relationships among actinopterygian fishes. *In* "Molecular Systematics of Fishes" (T. D. Kocher and C. A. Stepien, Eds.), pp. 285–303, Academic Press, New York.

Martin, A. P. (1995). Mitochondrial DNA sequence evolution in sharks: Rates, patterns, and phylogenetic inferences. *Mol. Biol. Evol.* **12:** 1114–1123.

Moritz, C., Schneider, C. J., and Wake, D. B. (1992). Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Syst. Biol.* **41:** 273–291.

Nelson, J. S. (1994). "Fishes of the World," Wiley, New York.

Nunn, G. B., and Cracraft, J. (1996). Phylogenetic relationships among major lineages of birds-of-paradise (Paradisaeidae) using mitochondrial DNA gene sequences. *Mol. Phylogenet. Evol.* **5:** 445–459.

Page, L. M. (1974). The subgenera of *Percina* (Percidae: Etheostomatini). *Copeia* **1974:** 66–86.

Page, L. M. (1981). The genera and subgenera of darters (Percidae, Etheostomatini). *Occ. Pap. Mus. Nat. Hist. Univ. Kansas* **90:** 1–69.

Page, L. M. (1983). "Handbook of Darters," TFH, Neptune City.

Page, L. M. (1985). Evolution of reproductive behaviors in percid fishes. *Illinois Nat. Hist. Surv. Bull.* **33:** 275–295.

Page, L. M., and Whitt, G. S. (1973a). Lactate dehydrogenase isozymes, malate dehydrogenase isozymes and tetrazolium oxidase mobilities of darters (Etheostomatini). *Comp. Biochem. Physiol.* **44B:** 611–623.

Page, L. M., and Whitt, G. S. (1973b). Lactate dehydrogenase isozymes of darters and the inclusiveness of the genus *Percina*. *Illinois Nat. Hist. Surv. Biol. Notes* **82:** 1–7.

Ross, M. R. (1973). A chromosome study of five species of etheostomine fishes (Percidae). *Copeia* **1973:** 163–165.

Simons, A. M. (1991). The phylogenetic relationships of the crystal darter, *Crystallaria asprella*. *Copeia* **1991:** 927–936.

Simons, A. M. (1992). Phylogenetic relationships of the *Boleosoma* species group (Percidae: *Etheostoma*). *In* "Systematics, Historical Ecology, and North American Freshwater Fishes" (R. L. Mayden, Ed.), pp. 268–292, Stanford Univ. Press, Stanford.

Smith, A. B. (1994). Rooting molecular trees: Problems and strategies. *Biol. J. Linn. Soc.* **51:** 279–292.

Swofford, D. (1997). "PAUP*" Ver. 4.0 d56, Sinauer, Sunderland, MA.

Templeton, A. R. (1983). Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution* **37:** 221–244.

Turner, T. F. (1997). Mitochondrial control region sequences and phylogenetic systematics of darters (Teleostei: Percidae). *Copeia* **1997:** 319–338.

Whitmore, D. H., Thai, T. H., and Craft, C. M. (1994). The largemouth bass cytochrome *b* gene. *J. Fish Biol.* **44:** 637–645.

Wiley, E. O. (1992). Phylogenetic relationships of the Percidae (Teleostei: Perciformes): A preliminary hypothesis. *In* "Systematics, Historical Ecology, and North American Freshwater Fishes" (R. L. Mayden, Ed.), pp. 247–267, Stanford Univ. Press, Stanford.

Williams, J. D. (1975). Systematics of the percid fishes of the subgenus *Ammocrypta*, genus *Ammocrypta*, with descriptions of two new species. *Bull. Alabama Mus. Nat. Hist.* **1:** 1–56.

Wood, R. M. (1996). Phylogenetic systematics of the darter subgenus *Nothonotus* (Teleostei: Percidae). *Copeia* **1996:** 300–318.

Wood, R. M., and Mayden, R. L. (1997). Phylogenetic relationships among selected darter subgenera (Teleostei: Percidae) as inferred from analysis of allozymes. *Copeia* **1997:** 265–274.