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Phylogenetic Relationships of Barcheek Darters (Percidae: *Etheostoma*, Subgenus *Catonotus*) with Descriptions of Two New Species

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Etheostoma virgatum **has been treated as a species occupying three widely separated regions of the Cumberland River drainage in Kentucky and Tennessee. To test the hypothesis that the three widely disjunct populations of *E. virgatum* are monophyletic, DNA sequence data from mitochondrial and nuclear loci were gathered on *E. virgatum* and other species of *Catonotus* including all species of barcheek darters. Morphological data were analyzed from populations throughout the range of *E. virgatum*. The three widely separated populations of *E. virgatum*, although morphologically similar, do not form a monophyletic group in phylogenetic analyses of molecular data. Consistent with this result, two of the populations are described as new species. These three species had been identified as *E. virgatum* because of the shared presence of bold dark stripes along the side of the body, a feature not found in the other four species of barcheeks. It is unclear whether the presence of bold stripes represents retention of a pleisiomorphic trait (lost in other barcheeks) or whether the condition arose independently in these three species.**

ETHEOSTOMA VIRGATUM has been treated as a species occupying three widely separated regions of the Cumberland River drainage (Fig. 1) with populations in the Rockcastle River and nearby creeks in eastern Kentucky, in the upper Caney Fork system in central Tennessee, and in tributaries of the lower Cumberland River in west-central Kentucky and Tennessee from the Red River system to Stones River (Page and Braasch, 1977; Braasch and Mayden, 1985; Etnier and Starnes, 1993). This tripartite distribution has intrigued students of North American freshwater fishes for decades because the distributional data (Page and Braasch, 1977; Burr and Warren, 1986; Etnier and Starnes, 1993) are incompatible with phylogenetic hypotheses (Page, 1975; Braasch and Mayden, 1985; Porterfield et al., 1999).

The distribution of *E. virgatum* might be assumed to be the result of extirpations of populations throughout large areas of the Cumberland River drainage. However, two closely related species, *Etheostoma obeyense* and *Etheostoma smithi*, have the same habitat requirements as *E. virgatum* (Page, 1983; Braasch and Mayden, 1985; Etnier and Starnes, 1993) and occupy portions of the Cumberland drainage between areas occupied by *E. virgatum*. The geographically intermediate distributions of these species suggest that competitive displacement must be invoked to explain the modern distribution of *E. virgatum* (Page and Schemske, 1978). This explanation requires that *E. obeyense* and *E. smithi* originated in drainages other than the Cumberland River, then invaded the Cumberland River, displaced *E. virgatum*, and subsequently were extirpated in the other drainages. Although this sequence of events is possible, there is no evidence that *E. obeyense* or *E. smithi* ever occurred outside the Cumberland drainage or that environmental changes have occurred that would have caused their extirpation in adjacent drainages.

Barcheek darters differ from the other 13 species in the subgenus *Catonotus* (Page et al., 1992; Porterfield et al., 1999) by having distinctive pigment patterns. Particularly diagnostic is a unique pigment pattern referred to as a ''bar'' on the cheek (Fig. 2). The bar is detectable on juveniles and females but is most evident on breeding males. On nonbreeding individuals, the upper and lower halves of the bar are dusky white; on breeding males, the spot on the upper half is red, the spot on the lower half is bright white, and each spot is surrounded by an iridescent yellow circle. The spots in the bar on the cheek of a breeding male are hypothesized to function as egg mimics (Page, 2000). In contrast to other species of *Catonotus*, barcheek breeding males develop diagnostic color patterns in the fins. In all barcheeks the first dorsal fin is red with an anterior basal black blotch, second dorsal and caudal fins are red, pelvic fins are black, and pectoral and anal fins are red with a dark blue lower margin. Males of other species of *Catonotus* lack red and blue.

Species of barcheeks differ from one another in color pattern, configuration of the infraor-

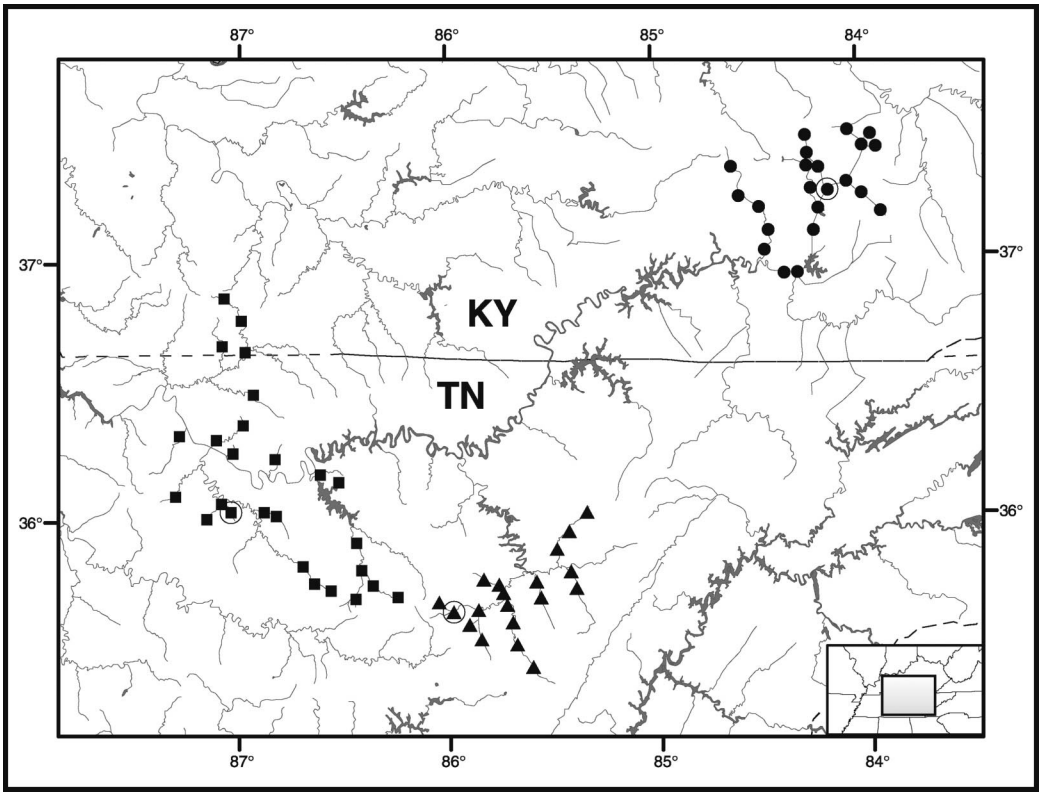

Fig. 1. Known localities for *Etheostoma virgatum* (circles), *Etheostoma basilare* (triangles), and *Etheostoma derivativum* (squares). Type locality for each species is circled. All three species have been referred to as *E. virgatum*.

bital canal, maximum body size, and modal meristic counts (Kuehne and Small, 1971; Page and Braasch, 1976, 1977). As historically recognized, *E. virgatum* is the only species with bold dark brown stripes on the side of the body (Page and Braasch, 1977; Burr and Warren 1986; Etnier and Starnes, 1993). *Etheostoma barbouri* and *Etheostoma striatulum* lack bold stripes but have rows of small brown spots that form streaks along the side. Compared to *E. barbouri*, which has a large black suborbital bar, *E. striatulum* has a much smaller teardrop but darker streaks on the side. *Etheostoma obeyense* and *E. smithi* have blotches along the side but lack obvious stripes or streaks. *Etheostoma smithi*, at a maximum total length of about 62 mm, is substantially smaller than *E. obeyense* at 84 mm and has more darkly outlined scales. *Etheostoma virgatum* (78 mm maximum total length) and *E. obeyense* (85 mm) are about 1.3–1.5 times as large as *E. smithi* (64 mm), *E. barbouri* (60 mm), and *E. striatulum* (60 mm).

To test the hypothesis that the three widely disjunct populations of *E. virgatum* are monophyletic, DNA sequence data from mitochondrial and nuclear loci were gathered on *E. virgatum* and several other species of *Catonotus* including all species of barcheek darters. Morphological data were analyzed from populations throughout the range of *E. virgatum*.

Materials and Methods

DNA sequencing.—Specimens were collected using seines and electrofishing equipment and fresh frozen in liquid nitrogen. See Appendix 1 for catalog numbers of vouchers and GenBank accession numbers for specimens sequenced. Nucleic acids were isolated from tissue using a standard protein digest and phenol-chloroform procedure followed by ethanol precipitation. Approximately 300 ng of the nucleic acid extract were used as template in the polymerase chain reaction (PCR) amplification of the target region. The complete coding regions of the mitochondrial encoded cytochrome b (*cytb*) and NADH dehydrogenase subunit 2 (ND2) genes were PCR amplified using primers and conditions given in Kocher et al. (1995) and Near et al. (2000). PCR amplification of the NADH dehydrognease subunit 4 (ND4) used primers ARG-F and Leu-R (primer sequences available

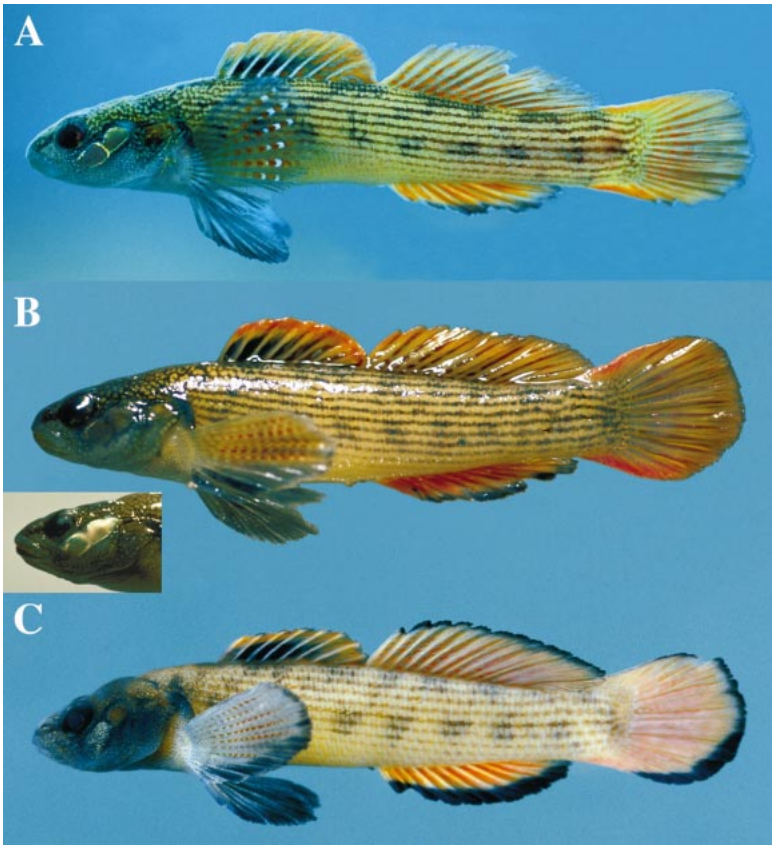

Fig. 2. (A) *Etheostoma virgatum*, 63-mm SL breeding male, Clear Creek, Rockcastle County, Kentucky, 18 April 2000. Note white spots on pectoral fin that are hypothesized to be egg mimics (Porter et al., 2002). (B) *Etheostoma basilare*, 63-mm SL breeding male, Duke Creek (Caney Fork system), Cannon County, Tennessee, 18 April 2000. Inset: *Etheostoma basilare*, 56-mm SL breeding male, Duke Creek, Cannon County, Tennessee, 3 April 1992. Note egglike spots on cheek that are hypothesized to be egg mimics (Page, 2000). (C) *Etheostoma derivativum*, 49-mm SL breeding male, West Fork Stones River, Rutherford County, Tennessee, 22 April 2002. Photo A by M. H. Sabaj, inset by K. S. Cummings, B and C by L. M. Page.

upon request) and 30 cycles with 50 C annealing temperature. PCR products were purified using the Qiagen QIAquick PCR Purification Kit and sequenced with the Perkin-Elmer Big Dye DNA Sequencing Kit according to the manufacturers' protocol with primers used in PCR and those designed to anneal at various downstream locations within the amplified regions to provide complete, double-stranded sequences. Sequenced products were purified by passing the reaction through 700μl Sephadex columns (2.0g Sephadex: 32.0mL water) and dried prior to visualization with an ABI Prism 377 automated DNA sequencer (PE Applied Biosystems).

DNA sequences were obtained for the complete mtDNA regions comprising *cytb* (1140 b.p.) and ND2 (1047 b.p.) and for the nDNA encoded first intron of the S7 ribosomal protein (527 b.p.) for all recognized barcheek darter species and specimens from the three populations of *E. virgatum* (Fig. 1). In addition, sequence data were collected from five other species of *Catonotus*: *Etheostoma flabellare*, *Etheostoma percnurum*, *Etheostoma kennicotti*, *Etheostoma oophylax*, and *Etheostoma squamiceps*. Complete DNA sequences were obtained for the mtDNA encoded ND4 (1395 b.p.) for a subset of the barcheek darter individuals sampled for the three other genes, and for three additional species of *Catonotus*: *E. percnurum*, *E. kennicotti*, and *E. squamiceps*. Based on previous studies (Page, 1975; Braasch and Mayden, 1985; Porterfield et al., 1999), *E. squamiceps* and *E. oophylax* were designated as outgroup species for phylogenetic analysis. *Etheostoma flabellare*, *E. kennicotti*, and *E. percnurum* were included to test the monophyly of the barcheeks.

The alignment of composite files for all three

mtDNA protein-coding loci was trivial and the character matrix analyzed by PAUP* (vers. 4.0b8) was generated by Sequencher 4.1. All composite files were deposited with GenBank (see Appendix 1). Clustal X was used to align the nDNA S7 intron, and adjusted by eye to minimize inferred insertions and deletions (indels). Phylogenetic tree estimation, calculation of pairwise sequence divergences, and base frequencies were obtained with PAUP* v.4.0b10. All maximum-parsimony (MP) analyses employed a heuristic search algorithm with tree-bisection-reconnection (TBR), branch swapping, and the MULPARS (save all optimal trees) option, and with 100 addition sequence replicates. Indels and heterozygous sites in the S7 intron were treated as missing data. Nodal support was assessed with 1000 nonparametric bootstrap pseudoreplicates. Where more than one most-parsimonious tree was recovered, rival topologies were summarized by their strict consensus. Initially, gene regions (*cytb,* ND2, ND4, and S7 intron) were partitioned and analyzed separately using MP. To test for significant phylogenetic conflict among data partitions prior to the combination and simultaneous analysis, the incongruence-length difference method (Farris et al., 1994) as implemented by the partition-homogeneity test in PAUP* was replicated 1000 times. A *P*-value of less than 0.05 was interpreted as evidence of significant phylogenetic conflict among partitions that should not be analyzed simultaneously (Bull et al., 1993)

Fifty-six progressively complex models of sequence evolution were assessed using hierarchical likelihood ratio tests (LRTs), with a chi-square distribution (Huelsenbeck and Crandall, 1997) to select the model at which the addition of further parameters did not significantly improve the likelihood of observing the data. Modeltest v3.06 (Posada and Crandall, 1998) was used to calculated maximum-likelihood (ML) scores, estimate model parameters, and execute LRTs on a neighbor-joining inferred tree topology. Identified optimal models and estimated model parameters were used in subsequent ML analyses of each gene partition. The TBR heuristic search algorithm was used to find the tree topologies with maximum ML scores, with 10 addition sequence replicates. Nodal support was assessed with 100 nonparametric bootstrap pseudoreplicates. A Bayesian statistical procedure based on Markov Chain Monte Carlo (MCMC) sampling methods was used in ML analysis of the concatenated data for three gene partitions (ND4 was omitted because of missing data for some species). To account for different rate parameters present in the gene partitions, a site-specific general time-reversible model of gene-sequence evolution combined with gamma rate heterogeneity was used to estimate the likelihood of each tree. In each analysis, 500,000 generations of trees were developed using the MCMC procedure, and every 10th tree was sampled. The distribution of ML score versus generation permitted identification of the optimal ML score, and all less-optimal tree generations were discarded as ''burn-in.'' From the collection of trees resulting after the burn-in, a 50% majority rule consensus tree provided values at nodes, which were interpreted as posterior probabilities.

Alternative tree topologies were compared to the Bayesian inferred tree using the Shimodaira-Hasegawa (SH) test, as executed in PAUP*. Optimal trees that represented alternative hypotheses of phylogenetic relationships were generated using MP constraint tree searches. ML scores of the Bayesian tree and each alternative hypothesis were compared. Alternative hypotheses examined were *E. virgatum* as a monophyletic clade, and a tree that represented barcheek and fantail darter groups as reciprocally monophyletic clades.

Morphology.—Meristic counts, including data from Page and Braasch (1977), were compiled on 446 specimens. Body and fin measurements were made on 55 specimens over 35 mm SL (CU 51551 (2), KU 16222 (2), INHS 37908 (6), 55587 (1), 55610 (7), 64776 (8), 79060 (4), 79353 (2), 84179 (4), 91975 (11), UAIC 2415 (4), USNM 204349 (2), UT 91.552 (2)). Measurements taken were standard length, head length, head width, interorbital width, snout length, gape width, body depth, caudal peduncle depth, first and second dorsal-fin base lengths, and length of the anal, first dorsal, second dorsal, and pectoral fins. Counts and measurements were made as described by Hubbs and Lagler (1958) except body depth was measured at the origin of the first dorsal fin. Head canal pore counts were made as described by Hubbs and Cannon (1935). Counts of bilateral features were made on the left side. Data are presented on only those counts and measurements that showed significant variation. Color observations were on live and freshly preserved individuals. Standard length is used throughout unless total length is stated. Institutional abbreviations are as given in Leviton et al. (1985); MEB = private collection of M. E. Braasch.

RESULTS

DNA sequence data.—The nucleotide compositions of *cytb,* ND2 and ND4 gene sequences

TABLE 1. MEAN NUCLEOTIDE COMPOSITION AND PHYLOGENETIC CHARACTER INFORMATION FOR THE GENE REGION DATA PARTITIONS.

Data partition	A	C	G	T	χ^2	No. characters	No. constant	No. parsimony informative	No. parsimony informative (ingroup)
cyt*b*	0.228	0.295	0.170	0.306	$P = 0.999$ df = 48	1140	708	340	312
ND2	0.242	0.326	0.154	0.278	$P = 0.999$ df = 48	1047	588	379	350
ND4	0.243	0.281	0.174	0.302	$P = 0.999$ df = 33	1395	856	375	349
S7 intron	0.253	0.181	0.237	0.326	$P = 1.0$ df = 48	527	433	55	29

were typical for protein-coding mitochondrial genes among vertebrates (Lydeard and Roe, 1997). Mean uncorrected genetic distances for all interspecific comparisons among sampled taxa were: *cytb* 14.45 ± 0.40%; ND2 16.85 ± 0.41%; ND4 15.30 ± 0.49%; and S7 intron 1 4.71 ± 0.33%. Restricting interspecific comparisons to barcheek darter species, uncorrected genetic distances were: *cytb* 11.9 ± 0.85%; ND2 14.6 ± 0.90%; ND4 13.84 ± 0.86%; and S7 intron 1 1.7 ± 0.16%. As expected, the rate of nucleotide substitution was substantially higher among the protein-coding mtDNA genes than observed in the nuclear encoded intron.

Results of the partition homogeneity test (Table 1) suggested incongruence for all comparisons between the mtDNA genes and the nuclear S7 intron (*cytb* vs S7 $P = 0.035$; ND2 vs S7 $P = 0.028$, and *cytb*-ND2 vs S7 $P = 0.025$); however, no incongruence was detected between the two mtDNA genes genes in the most taxon-inclusive analysis (*cytb* vs ND2 $P = 0.933$). When the partition homogeneity test was restricted to phylogenetically informative characters (Table 1), *P*-values from mtDNA and nuclear gene partition comparisons decreased by an order of magnitude.

Table 2 summarizes the results from hierarchical LRTs and describes the optimal model of DNA sequence evolution for each gene region data partition. These models were used in subsequent ML analyses of each data partition. Modeltest identified the generalized-time-reversible (GTR) model (Lanave et al., 1984) with corrections for the proportion of invariant sites and rate heterogeneity (GTR+I+Γ) for ND2 and ND4 partitions and the same model without the correction for invariant sites (GTR+Γ) for the *cytb* partition. For the S7 intron 1 partition, the less parameter-rich Hasegawa-Kishino-Yano (HKY; Hasegawa et al., 1985) model with correction for rate heterogeneity (HKY+Γ) was identified as the optimal model.

Molecular phylogenetic analyses.—Monophyly of the barcheek darters was supported in the MP and ML analyses of the nuclear S7 data and the ML and Bayesian analyses of combined sequence data. In contrast, all analyses of mitochondrial data and the MP analysis of all sequence data suggest that the barcheeks are not monophyletic. In these latter analyses, members of the fantail group (*E. flabellare*, *E. kennicotti*, and *E. percnurum*) were positioned within the clade of all barcheeks. None of the phylogenetic analyses (Figs. 3–6) supported the monophyly of *E. virgatum* sensu vetere (in the old sense) or a sister-species relationship between any two of the three populations previously named *E. virgatum* (Fig. 1).

Among barcheeks, the most consistently and strongly supported clade was one containing the lower Cumberland River population of *E. virgatum*, described below as *E. derivativum*, as sister to *E. smithi* plus *E. striatulum*. The only trees in which the relationships among these species were not supported were those based solely on S7 data.

In all trees except those based only on S7 data, *E. obeyense* was sister to *E. derivativum* + (*E. smithi* + *E. striatulum*), and *E. virgatum* (Rockcastle River) was sister to *E. obeyense* + (*E. derivativum* + [*E. smithi* + *E. striatulum*]). Support for these nodes varied but was high in most analyses.

The remaining barcheek species, *E. barbouri* and the Caney Fork population of *E. virgatum*, described below as *E. basilare*, were hypothesized to be sister species in the MP (with moderate support) and ML analyses of S7 data and in the Bayesian analysis of all data (with high support).

TABLE 2. OPTIMAL MODELS OF SEQUENCE EVOLUTION USING MAXIMUM-LIKELIHOOD RATIO TESTS FOR GENE REGION DATA PARTITIONS.

Data partition or combination	Model	Frequencies				*P*-Invar.	Γ-shape	Substitution rates				
		A	C	G	T			A ↔ C	A ↔ G	A ↔ T	C ↔ G	C ↔ T	G ↔ T
cyt*b*	GTR+I+Γ	0.234	0.307	0.159	0.300	0.479	1.006	2.257	23.483	0.883	1.103	11.092	1.0
ND2	GTR+I+Γ	0.258	0.330	0.142	0.270	0.473	2.083	1.041	17.327	0.643	0.909	5.455	1.0
ND4	GTR+I+Γ	0.252	0.284	0.166	0.299	0.476	1.497	1.806	20.142	0.964	1.178	10.396	1.0
S7 intron	HKY+Γ	0.244	0.189	0.249	0.318	—	0.371	Transition:Transversion expected ratio: 0.795 κ = 1.643					
mtDNA	GTR+I+Γ	0.247	0.303	0.158	0.292	0.471	1.406	1.705	19.250	0.794	0.989	8.355	1.0
S7 + mtDNA	GTR+I+Γ	0.248	0.287	0.168	0.297	0.483	1.254	1.535	14.355	0.707	0.933	7.211	1.0

In other analyses, the hypothesized relationships of these two species varied.

Monophyly of the fantail darter group was supported by MP analyses of *cytb* and ND2 (but without bootstrap support) and ML analysis of *cytb*. The sister-taxon relationship between *E. flabellare* and *E. percnurum* hypothesized by Jenkins (Jenkins and Burkhead, 1993) was supported by MP (with bootstrap support) and ML analysis of *cytb* and ND2, and the Bayesian analysis of all data (with high bootstrap support). In contrast, a sister-species relationship between *E. kennicotti* and *E. percnurum* was supported in the MP (with bootstrap support) and ML analysis of S7.

SH tests of alternative topologies to the Bayesian inferred tree rejected the monophyly of *E. virgatum* sensu vetere (difference in lnL = 180.79, $P < 0.001$). Although the fantail darter group is not shown as monophyletic in the Bayesian tree, the hypothesis that the fantail darter group and barcheek darter group are each monophyletic could not be rejected in the SH test (difference in lnL = 2.13, $P = 0.650$).

Morphology.—Although similar phenotypically, a close examination of morphology reveals several characteristics that distinguish *E. virgatum*, *E. basilare*, and *E. derivativum*. These traits are described below in taxonomic accounts and summarized in Table 3.

DISCUSSION

The three populations previously identified as *E. virgatum*, although morphologically similar, do not form a monophyletic group. Consistent with this result, two of the populations are described below as new species. These three species are sufficiently similar morphologically to have gone unrecognized until the phylogenetic analysis of DNA sequence data showed them to be distinct. All were identified as *E. virgatum* because of the bold stripes along the side. This character state (bold stripes) has remained unchanged in these three species (whereas lost in other barcheeks) as a result of selection or evolutionary constraint (Williamson, 1987; Colborn et al., 2001; Burt, 2001), or it arose independently in *E. virgatum*, *E. basilare*, and *E. derivativum* through selection or drift.

Some phylogenetic analyses of molecular data, that is, MP and ML analyses of the nuclear S7 data and the ML and Bayesian analysis of all sequence data, indicate that barcheek darters form a monophyletic group. Alternatively, all analyses of mitochondrial data and the MP analysis of all sequence data indicate that the barcheeks are not monophyletic. These alternative

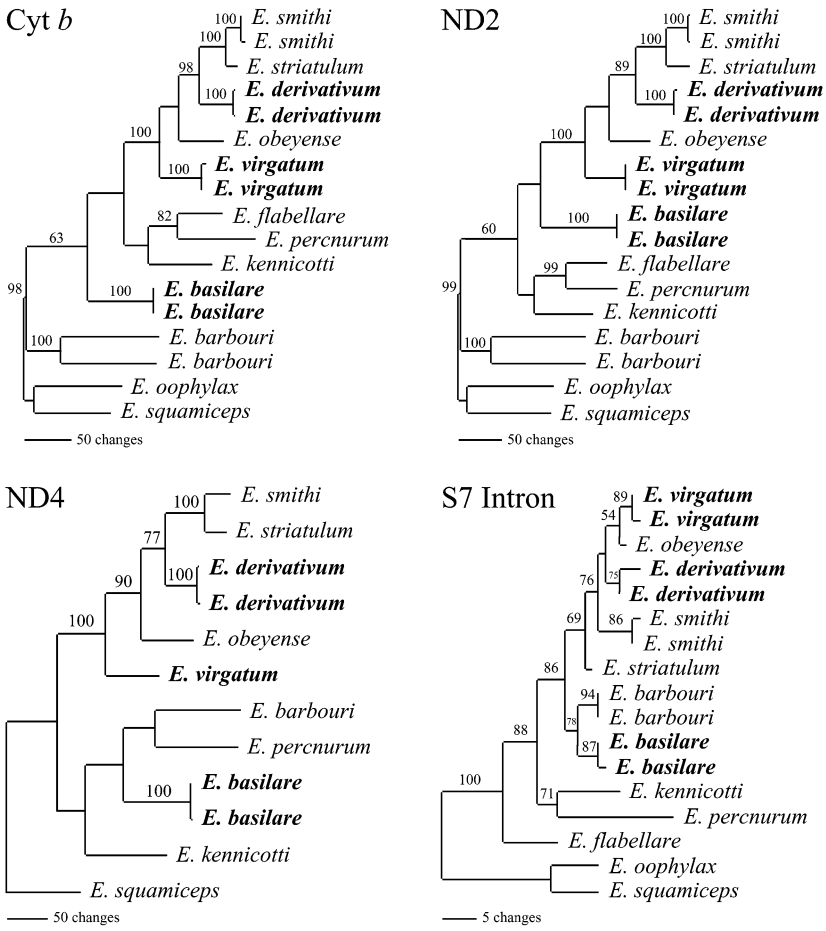

Fig. 3. Maximum-parsimony phylograms for each gene partition. Values above nodes are nonparametric bootstrap percentages (1000 pseudoreplicates). *Cytb* tree length = 985, CI = 0.58, RI = 0.66; ND2 tree length = 1061, CI = 0.59, RI = 0.66; ND4 tree length = 1079, CI = 0.64, RI = 0.59; S7 intron tree length = 118, CI = 0.86, RI = 0.86.

hypotheses have *E. basilare* and/or *E. barbouri* as more distantly related to other barcheeks than are *E. flabellare*, *E. percnurum*, or *E. kennicotti*. However, these alternative hypotheses are highly variable in the arrangement of species and rarely receive bootstrap support (Figs. 3, 5). It seems likely that the more basal positions of *E. basilare* and *E. barbouri* have led to increased homoplasy and that this increase obscures phylogenetic relationships. Given the morphological synapomorphies (described below) for barcheeks, the support for monophyly in several analyses of molecular data, and the variation in species relationships in trees in which monophyly is rejected, monophyly of the barcheeks remains the most strongly supported hypothesis.

Barcheek Darters

Included species.—*Etheostoma virgatum*, *E. obeyense*, *E. barbouri*, *E. smithi*, *E. striatulum*, *E. basilare* n. sp., and *E. derivativum* n. sp.

Diagnosis.—Barcheek darters are members of the subgenus *Catonotus* of *Etheostoma* (Percidae), as diagnosed most recently by Page (1981) and Braasch and Mayden (1985). Barcheeks differ from other species of *Catonotus* by possessing the bar pattern on the cheek (illustrated in color by Kuehne and Barbour [1983], Page [1983], and Etnier and Starnes [1993]), red and blue in the fins of breeding males, a crenulate preopercular margin, modally nine or 10 anal rays, and the absence of distinct black bands in the caudal fin. Other species of *Catonotus* lack the

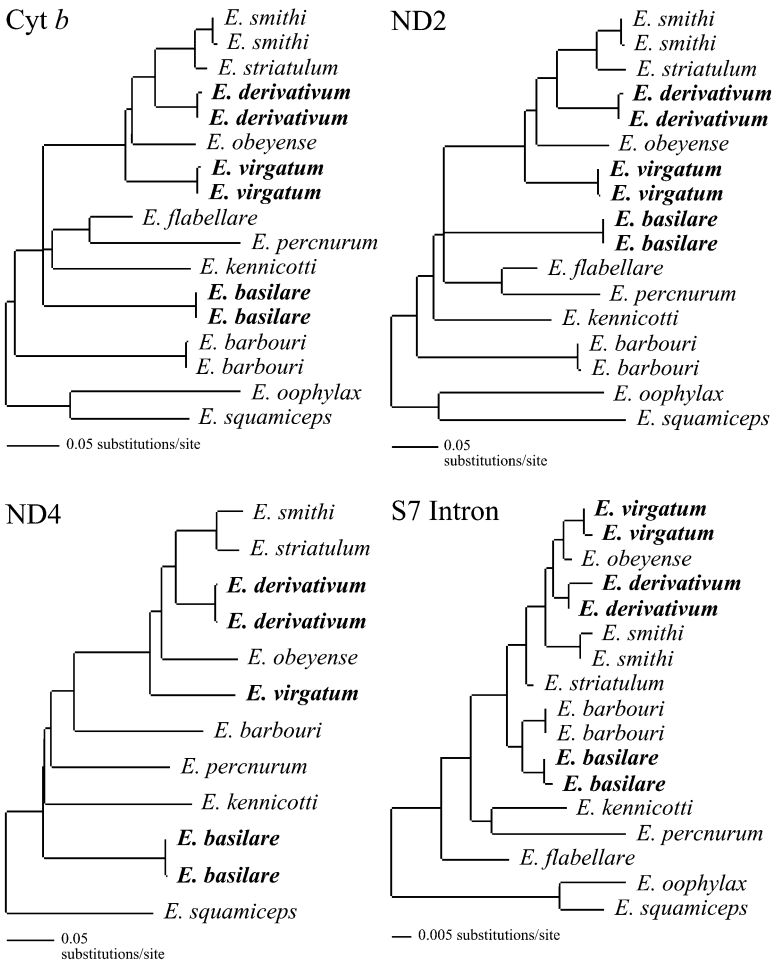

Fig. 4. Maximum-likelihood phylograms for each gene partition. *Cytb* model = GTR+I+Γ, *ln* L = 5644.30; ND2 model = GTR+I+Γ, *ln* L = 5780.03; ND4 model = GTR+I+Γ, *ln* L = 6351.31; S7 intron model = HKY+Γ, *ln* L = 1450.63. See Table 2 for model parameters of each gene partition.

bar on the cheek and red and blue colors, and have a smooth edge on the preopercle, black bands in the caudal fin, and modally seven or eight anal rays.

In common with the *E. flabellare* species group as defined by Page (1975) and Braasch and Mayden (1985), but in contrast to the 10 species in the *E. squamiceps* species group (Page et al., 1992), barcheek darters have a wide interruption in the infraorbital canal with three or four pores anteriorly and one or two pores posteriorly, develop swollen ridges on lower body scales of breeding males (Mayden, 1985), lack scales on the nape and prepectoral area, and lack the vertical row of three black spots at the origin of the caudal fin.

Description (all species of barcheeks).—Head unscaled; body scaled except for nape, breast, and prepectoral area; belly fully scaled; mouth terminal, frenum moderately broad. Supratemporal canal interrupted medially with two pores on either side; supraorbital canal with four pores; preoperculomandibular pores usually 10 (usually nine in *E. barbouri*; Kuehne and Small 1971); branchiostegal rays 6, membranes slightly joined.

Juveniles, females, and nonbreeding males are yellow-brown dorsally with 6–8 dark brown rectangular saddles on the middorsum; first saddle beneath anterior half of first dorsal fin and last at origin of caudal fin; nape sometimes with a weakly developed saddle just anterior to the first dorsal fin but more often with dark brown vermiculations. Nine to 11 rectangular dark brown blotches along side; ventral extensions of the posterior 2–4 blotches often encircle lower half of the caudal peduncle. Variously devel-

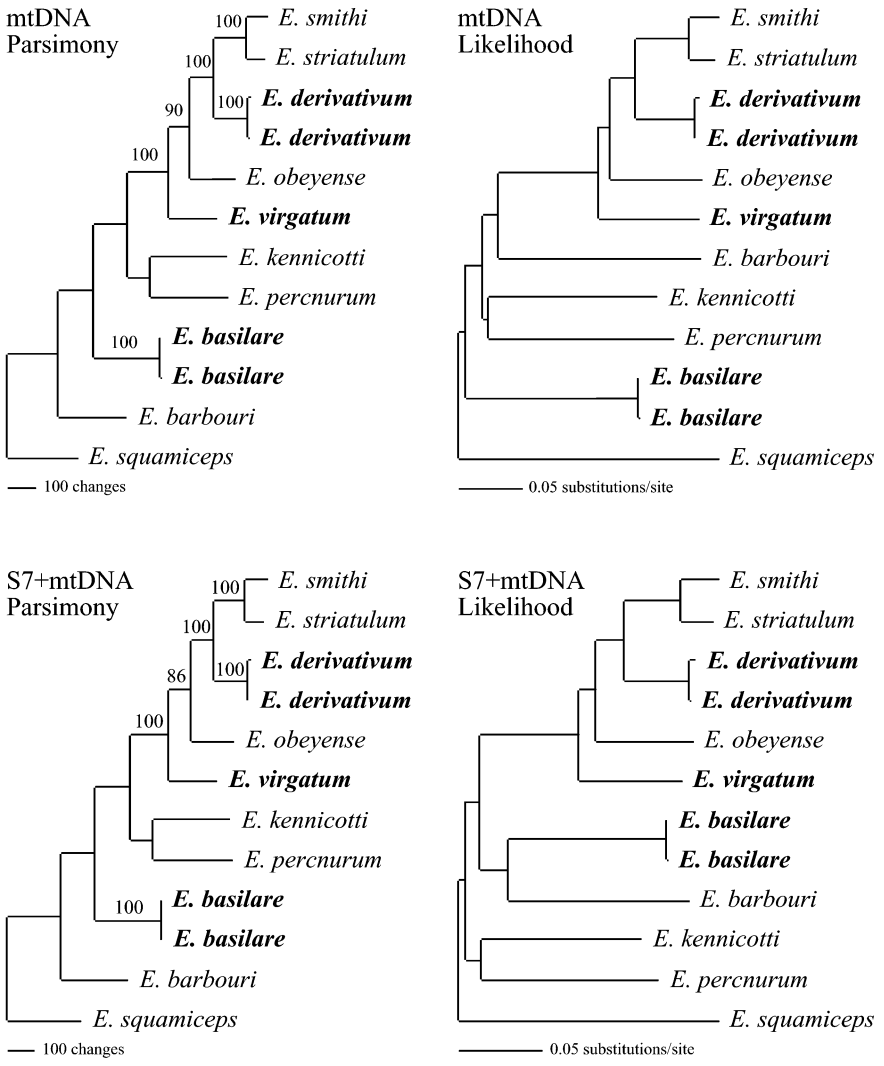

Fig. 5. Maximum-parsimony and likelihood phylograms for the mtDNA and all data combined. Values above nodes are nonparametric bootstrap percentages (1000 pseudoreplicates). MtDNA model = GTR+I+Γ, *ln* L = 16360.15; S7 intron + mtDNA model = GTR+I+Γ, *ln* L = 17890.43. See Table 2 for model parameters of each dataset.

oped dark brown vermiculations connect dorsal and midlateral blotches. Some species with stripes along side (see descriptions and key to species below). Humeral spot is large and black. Breast and belly are greenish white, often spotted with melanophores.

Head is light brown dorsally with darker brown vermiculations; black preorbital bars converge on upper lip but do not meet. Black suborbital bar variously developed, sometimes reduced to a spot below eye; side of head heavily spotted with melanophores. Cheek bar has black outline; on nonbreeding individuals the upper and lower halves of the bar on the cheek are dusky white. Undersides of head and branchiostegal membranes are heavily spotted with melanophores.

First dorsal fin is heavily spotted with melanophores, has a large black basal blotch and a thin red-brown margin. Second dorsal, caudal, and pectoral fins have bands formed by concentrations of yellow, brown, and black pigment on fin membranes. Pelvic fins are clear or have melanophores on the membranes anteriorly. Anal fin is often heavily spotted with melanophores.

Breeding male has red first dorsal fin with an anterior basal black blotch, red second dorsal and caudal fins, black pelvic fins, and red pec-

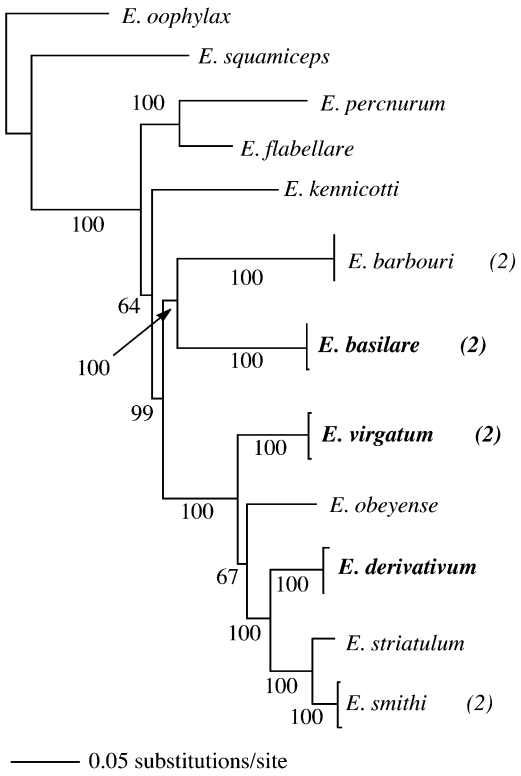

Fig. 6. Maximum-likelihood (ML) tree obtained using Bayesian Markov chain Monte Carlo sampling. ML score = 12,558.16. Rate parameters used in site-specific rates model were, *cytb* first codon 0.270559, *cytb* second codon 0.078838, *cytb* third codon 2.942669, ND2 first codon 0.535517, ND2 second codon 0.154986, ND2 third codon 3.164224, and S7 intron 1 0.226306. Numbers at nodes represent posterior probabilities of the clade among sampled tree topologies. (2) = two populations sampled.

toral and anal fins with dark blue margins. Head is dark and swollen. Cheek bar is vivid; upper half is red, lower half is bright white (interspecific variation described below).

Etheostoma virgatum (Jordan 1880)
Striped Darter
Figure 2

Poecilichthys virgatus Jordan 1880:236 (original description).

Etheostoma virgatum.—Jordan, 1887:868 (catalog of fishes of North America).

Types.—*Poecilichthys virgatus* was described by Jordan in 1880 from specimens collected in the Rockcastle River at Livingston, Rockcastle County, Kentucky. Jordan and Evermann (1896) subsequently selected a lectotype (USNM 23456). Collette and Knapp (1966) located and examined the lectotype and one paralectotype (UMMZ 187511).

Material examined.—Rockcastle River: AUM 12041 (5), 27273 (4), EKU 1 (2), INHS 37939 (3), 55582 (6), 55610 (10), 76019 (3), 79060 (10), KU 11461 (30), 16224 (4), UAIC 12458.05 (1), UMMZ 168047 (1), 171488 (9), USNM 204349 (12); Buck Creek: INHS 76019 (3), NLU 6053 (12), 10837 (10).

Diagnosis.—Member of *E. virgatum* (barcheek darter) complex. Brown stripes on side. Maximum body size = 65.0 mm SL; 78.0 mm TL (breeding male; EKU 1). Modally 9 anal fin rays (Table 4), 13 dorsal fin rays (Table 5), 48–53 lateral scales (Table 6), and 13 or more pored lateral-line scales (Table 7). Breeding male has bright white spots on pectoral fin (Fig. 2; also see Porter et al., 2002), lacks bold dark blue margin on second dorsal and caudal fins, has black spot in dorsal fin of breeding male beginning on first membrane (first four membranes are black). Spots on cheek bar do not change during the spawning season from red to yellow-gold.

Comparisons.—Only *E. basilare* and *E. derivativum* have brown stripes on side of body similar to those of *E. virgatum*. *Etheostoma barbouri* and *E. striatulum* have streaks but not well-defined stripes; *E. obeyense* and *E. smithi* lack dark stripes or streaks on side. Compared to *E. virgatum*, *E. basilare* has modally 10 anal-fin rays (Table 4), 14 dorsal-fin rays (Table 5) and 41–48 lateral scales (Table 6), and reaches only 59 mm SL. *Etheostoma derivativum* has modally 15 or fewer pored lateral-line scales (Table 7), the black spot in the first dorsal fin restricted to the second to fourth membranes, bold dark blue margin on the second dorsal and caudal fins on the breeding male, and reaches only 57 mm SL. Breeding males of *E. basilare* and *E. derivativum* lack bright white spots on pectoral fin; in *E. basilare* the spots on the cheek bar change during the spawning season from red and white to yellow-gold.

Variation.—Specimens from Buck Creek tend to have fewer lateral scales than specimens from Rockcastle River (Table 6). Specimens from Buck Creek modally have 13 pectoral-fin rays; those from Rockcastle River modally have 12 rays (Table 8).

Distribution.—Cumberland River drainage, eastern Kentucky. Species is known from the Rock-

TABLE 3. MORPHOLOGICAL CHARACTERISTICS DISTINGUISHING *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	Etheostoma virgatum	*Etheostoma basilare*	*Etheostoma derivativum*
Margin of second dorsal and caudal fins on breeding male	Clear to dusky	Clear to dusky	Dark blue
Black spot in first dorsal fin of breeding male	Begins on 1st membrane; first four membranes black	Begins on 1st membrane; first four membranes black	Begins in 2nd membrane; 2nd–4th membranes black (1st membrane may be dusky)
Spots on cheek bar of breeding male	Remain red and white	Change from red to yellow-gold during spawning	Remain red and white
Pectoral fin of breeding male	Develops bright white spots	Lacks white spots	Lacks white spots
Maximum body size, mm	65.0 SL, 78.0 TL	59.4 SL, 71.3 TL	57.0 SL, 69.0 TL
Modal no. anal-fin rays	9 (93% have 9 or fewer)	10 (54% have 10 or more)	9 (79% have 9 or fewer)
Modal no. dorsal-fin rays	13 (83% have 13 or fewer)	14 (66% have 14 or more)	13 (61% have 13 or fewer)
Modal no. lateral scales	48–53 (76% have 48 or more)	41–48 (92% have 48 or fewer)	44–50 (82%)
Modal no. pored lateral-line scales	13–18 (86% have 13 or more)	12–18 (78% have 12 or more)	8–15 (89% have 15 or fewer)

castle River, Buck Creek and Beaver Creek systems (Fig. 1). Locally common.

Etheostoma basilare, sp. nov.
Corrugated Darter
Figure 2

Holotype.—INHS 90759; a breeding male 50.6 mm (63.1 mm TL), Duke Creek, 9.6 km south of Sheybogan, Rt. 53 bridge, Collins River–Caney Fork drainage, Cannon County, Tennessee, 18 April 2000, M. H. Sabaj and L. M. Page.

Paratypes.—INHS 55616 (4 specimens; 43.1–54.6 mm), same collection data as holotype. UAIC 10140.03 (4 specimens; 35.8–44.0 mm), same locality data as holotype, 28 September 1991. All other paratypes ex. INHS 64776: trib., Duke Creek, 4.8 km east of Hollow Springs, Cannon County, Tennessee, 2 May 1989: AUM 34592 (2 specimens, 39.5–46.8 mm); SIUC 43056 (2 specimens, 42.9–45.5 mm); TU 193623 (2 specimens, 40.3–43.8 mm); UF 119607 (2 specimens, 40.2–48.8 mm); USNM 367644 (2 specimens, 34.2–52.2 mm).

Other material examined.—Caney Fork: AUM 3238 (2), 11049 (4), CU 51551 (2), KU 11598 (2), 16215 (12), MEB (Calfkiller River) (5), TU 30324 (2), UAIC 8654.01 (5), 9816.10 (7), 10119.11 (6), UT 91.279 (4), 91.552 (12); Collins River: INHS 27838 (2), 29484 (2), 37908 (6), 58366 (5), 64679 (8), 75030 (2), 75033 (3), KU 12061 (10), TU 33488 (6), 33494 (15), UAIC 2415 (10), UT 91.49 (1), 91.7 (1).

Diagnosis.—Member of *E. virgatum* (barcheek darter) complex. Brown stripes on side. Maximum body size = 59.4 mm SL; 71.3 mm TL (breeding male; INHS 29484). Modally 10 anal-fin rays (Table 4), 14 dorsal-fin rays (Table 5), 41–48 lateral scales (Table 6), 12 or more pored lateral-line scales (Table 7). Breeding male lacks bold dark blue margin on second dorsal and caudal fins and lacks bright white spots on pectoral fin. Black spot in dorsal fin of breeding male begins on first membrane (first four membranes are black). Spots in cheek bar change during the spawning season from red and white to yellow-gold and are surrounded by a dark ring of melanophores that gives the spots a spherical egglike appearance (Fig. 2; also see photo in Etnier and Starnes, 1993:547).

Comparisons.—Only *E. virgatum* and *E. derivativum* have brown stripes on side of body similar to those of *E. basilare*. *Etheostoma barbouri* and *E. striatulum* have streaks but not well-defined stripes; *E. obeyense* and *E. smithi* lack dark stripes

TABLE 4. COUNTS OF ANAL-FIN RAYS IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	7	8	9	10	11	*n*	χ	SD
Species/drainage								
Etheostoma virgatum								
Rockcastle River	1	35	44	8		88	8.7	0.57
Buck Creek		9	16			25	8.6	0.46
Totals	1	44	60	8		113	8.7	0.55
Etheostoma basilare								
Caney Fork		10	26	20		56	9.2	0.59
Collins River		1	28	46	11	86	9.8	0.55
Totals		11	54	66	11	142	9.5	0.65
Etheostoma derivativum								
Stones River		11	18	8		37	8.9	0.55
White Creek		1	2			3	8.7	0.44
Marrowbone Creek	1	7	11	3		22	8.7	0.62
Sycamore Creek		2	6			8	8.8	0.38
Harpeth River		26	44	20	2	92	8.9	0.55
Louise Creek		2	5	2		9	9.0	0.44
Red River		1	14	5		20	9.2	0.40
Totals	1	50	100	38	2	191	8.9	0.52

or streaks on side. Compared to *E. basilare*, *E. virgatum* and *E. derivativum* have modally nine anal-fin rays (Table 4), and 13 dorsal-fin rays (Table 5). *Etheostoma virgatum* also has modally 48–53 lateral scales (Table 6), bright white spots on the pectoral fin of the breeding male, and reaches 65 mm SL. *Etheostoma derivativum* also has modally 15 or fewer pored lateral-line scales (Table 7), the black spot in the first dorsal fin restricted to the second to fourth membranes, and dark blue margin on the second dorsal and caudal fins on the breeding male. Unlike in *E. basilare*, in *E. virgatum* or *E. derivativum*, the spots on the cheek bar do not change during the spawning season from red and white to yellow-gold.

Etymology.—The name *basilare*, a Latin adjective meaning "at the base," refers to the relatively basal phylogenetic position of this species

TABLE 5. COUNTS OF DORSAL-FIN RAYS IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	11	12	13	14	15	16	17	*n*	χ	SD
Species & drainage										
Etheostoma virgatum										
Rockcastle River		27	48	12	1			88	12.9	0.52
Buck Creek		1	18	6				25	13.2	0.38
Totals		28	66	18	1			113	12.9	0.46
Etheostoma basilare										
Caney Fork		1	23	27	5			56	13.6	0.59
Collins River		2	22	40	18	3	1	86	14.0	0.62
Totals		3	45	67	23	3	1	142	13.9	0.63
Etheostoma derivativum										
Stones River		4	21	11	1			37	13.2	0.54
White Creek			3					3	13.0	0.00
Marrowbone Creek		4	11	7				22	13.1	0.55
Sycamore Creek			6	2				8	13.3	0.38
Harpeth River	1	9	43	34	5			92	13.4	0.65
Louise Creek			3	4	2			9	13.9	0.59
Red River			12	8				20	13.4	0.48
Totals	1	17	99	66	8			191	13.3	0.60

TABLE 6. COUNTS OF LATERAL SCALES IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	56	58	61	*n*	χ	SD
Species & drainage																							
Etheostoma virgatum																							
Rockcastle River						2	2	3	4	1	6	9	17	13	13	9	5	1	2	1	88	50.4	2.27
Buck Creek				4		1	2	2	2	4	3	2	3	2							25	46.4	2.64
Totals				4		3	4	5	6	5	9	11	20	15	13	9	5	1	2	1	113	49.5	2.69
Etheostoma basilare																							
Caney Fork				5	6	3	4	6	5	9	9	4	4		1						56	45.8	2.40
Collins River	1	4	7	10	16	13	10	7	7	3	5	1	2								86	43.3	2.14
Totals	1	4	7	15	22	16	14	13	12	12	14	5	6		1						142	44.3	2.55
Etheostoma derivativum																							
Stones River			1		1	2	3	5	7	3	7	2	4	1	1						37	46.6	2.10
White Creek											2	1									3	48.3	0.44
Marrowbone Creek										1	2	2	6	5	4	2					22	50.5	1.27
Sycamore Creek								2	2	2	1		1								8	46.8	1.25
Harpeth River				1	4	5	12	13	11	14	9	6	11	2	1	2	1				92	46.6	2.23
Louise Creek							2	3	1			1		1	1						9	46.8	2.59
Red River							2	1	4	3	5	4	1								20	47.2	1.40
Totals			1	1	5	7	19	24	25	23	26	16	23	9	7	4	1				191	47.2	2.24

TABLE 7. COUNTS OF PORED LATERAL-LINE SCALES IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	0	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	23	24	25	N	χ	SD
Species & drainage																											
E. virgatum																											
Rockcastle River				2		1					5	5	13	19	12	5	8	5	3	3	3	1		1	86	14.9	2.47
Buck Creek					1				1		1		1	2	2	3	1	4	3	1	1	1	2	1	25	17.1	3.56
Totals				2	1	1			1		6	5	14	21	14	8	9	9	6	4	4	2	2	2	111	15.4	2.90
E. basilare																											
Caney Fork	1					1	2		3	3	4	3	3	6	6	6	5	7	2	4					56	14.2	3.18
Collins River			1		1	2	2			6	5	12	7	15	8	6	3	5	4	3	5	1			86	14.2	3.01
Totals	1		1		1	3	4		3	9	9	15	10	21	14	12	8	12	6	7	5	1			142	14.2	3.08
E. derivativum																											
Stones River		1				1	2	5	2	6	2	5	1	3	3		2								33	10.8	2.63
White Creek										1			1				1								3	13.3	2.44
Marrowbone Creek				1				2				4	2	3	3	3	4								22	13.5	2.50
Sycamore Creek						1			1			4			1	1									8	11.8	2.13
Harpeth River				3	1	4	3	8	5	10	12	9	14	9	4	6	2	1							91	11.3	2.55
Louise Creek				1				1	1		1		1	2	1				1						9	11.9	3.46
Red River			1	1	1	2	1	2	2	2	3	1	3		1										20	9.2	2.70
Totals		1	1	6	2	8	6	18	11	19	18	23	22	17	13	10	9	1	1						186	11.3	2.78

TABLE 8. COUNTS OF PECTORAL-FIN RAYS IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	9	10	11	12	13	14	*n*	χ	SD
Species & drainage									
Etheostoma virgatum									
Rockcastle River			9	71	8		88	12.0	0.20
Buck Creek				2	22	1	25	12.9	0.15
Totals			9	73	30	1	113	12.2	0.45
Etheostoma basilare									
Caney Fork	1	1	9	45			56	11.8	0.40
Collins River		1	4	61	20		86	12.2	0.39
Totals	1	2	13	106	20		142	12.0	0.28
Etheostoma derivativum									
Stones River			12	23	2		37	11.7	0.47
White Creek				3			3	12.0	0.00
Marrowbone Creek				11	11		22	12.5	0.50
Sycamore Creek			3	2	3		8	12.0	0.75
Harpeth River			7	75	10		92	12.0	0.21
Louise Creek				8	1		9	12.1	0.20
Red River			3	16	1		20	11.9	0.27
Totals			25	138	28		191	12.0	0.29

among barcheek darters. The common name, corrugated darter, refers to the appearance of alternating folds and ridges caused by the bold stripes along the side that usually appear larger and more distinct in this species than in other species of barcheeks (e.g., Fig. 2).

Variation.—Specimens of *E. basilare* from the two major tributaries of Caney Fork, Collins River and Caney Fork proper, show substantial variation in meristic counts. Specimens from Caney Fork modally have nine dorsal-fin spines, a higher mean number of lateral scales, and a lower mean number of anal-fin rays; those from Collins River modally have eight dorsal-fin spines, fewer lateral scales, and tend to have 10 rather than nine anal-fin rays (Tables 4, 6, 9). Both populations modally have 12 pectoral-fin rays, but specimens from Collins River are also likely to have 13 rays; those from Caney Fork are more likely to have 11 rays (Table 8).

Distribution.—Upper Caney Fork system (Collins River and Caney Fork), Cumberland River drainage, central Tennessee (Fig. 1). Locally common.

Etheostoma derivativum, sp. nov.
Stone Darter
Figure 2

Holotype.—INHS 90760; a breeding male 54.9 mm SL (65.5mm TL), McCory Creek, 1.6 km southeast of Rudderville on EuDailey-Covington Road, Harpeth River drainage, Williamson County, Tennessee, 21 April 2001, C. E. Johnston and L. M. Page.

Paratypes.—INHS 91975 (3 specimens, 32.3–57.0 mm), same collection data as holotype. All other paratypes ex. INHS 91975: AUM 29022 (2 specimens, 45.2–46.7 mm); SIUC 43055 (2 specimens, 40.1–50.7 mm); TU 193622 (2 specimens, 37.0–47.4 mm); UF 119606 (2 specimens, 39.3–50.3 mm); USNM 367643 (3 specimens, 36.1–50.3 mm). UAIC 3775.16 (9 specimens, 30.4–42.2 mm), Harpeth River, 16 km south of Nashville, Williamson County, Tennessee, 10 April 1970.

Other material examined.—Stones River: CU 37264 (5), INHS 55587 (5), 75034 (10), NLU 15810 (12), TU 33163 (1), UAIC 11597.14 (4); White Creek: INHS 91965 (3); Marrowbone Creek: INHS 75031 (2), 79353 (9), UAIC 3758.09 (6), 3776.11 (5); Sycamore Creek: INHS 91977 (8); Harpeth River: INHS 75032 (8), 84179 (10), 91973 (8), KU 14197 (2), NLU 15730 (10), TU 14662 (6), UMMZ 120172 and 120173 (19), 177609 (7), UT 91.76 (7); Louise Creek: NLU 9795 (1), 12252 (7), 25635 (1); Red River: INHS 68316 (1), 91983 (17), KU 16222 (2).

Diagnosis.—Member of *E. virgatum* (barcheek darter) complex. Brown stripes on side. Maximum body size = 57.0 mm SL; 69.0 mm TL (breeding male; INHS 91975). Modally 9 anal-

TABLE 9. COUNTS OF DORSAL-FIN SPINES IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	7	8	9	10	*n*	χ	SD
Species & drainage							
Etheostoma virgatum							
Rockcastle River	1	17	69	1	88	8.8	0.35
Buck Creek		3	18	4	25	9.0	0.31
Totals	1	20	87	5	113	8.9	0.33
Etheostoma basilare							
Caney Fork	2	10	44		56	8.8	0.39
Collins River	3	67	16		86	8.2	0.32
Totals	5	77	60		142	8.4	0.52
Etheostoma derivativum							
Stones River	1	5	28	3	37	8.9	0.34
White Creek		1	2		3	8.7	0.44
Marrowbone Creek	1	13	8		22	8.3	0.50
Sycamore Creek		3	5		8	8.6	0.47
Harpeth River	4	27	56	5	92	8.7	0.54
Louise Creek		6	3		9	8.3	0.44
Red River	1	11	8		20	8.4	0.52
Totals	7	66	110	8	191	8.6	0.55

fin rays (Table 4), 13 dorsal-fin rays (Table 5), 44–50 lateral scales (Table 6), and 15 or fewer pored lateral-line scales (Table 7). Breeding male has bold dark blue margin on second dorsal and caudal fins; black spot in dorsal fin of breeding male begins on second membrane (second to fourth membranes are black). In the breeding male, white spots are absent on the pectoral fin and the spots on the cheek bar remain red and white (i.e., do not change during the spawning season to yellow-gold).

Comparisons.—Only *E. virgatum* and *E. basilare* have brown stripes on side of body similar to those of *E. derivativum*. *Etheostoma barbouri* and *E. striatulum* have streaks but not well-defined stripes; *E. obeyense* and *E. smithi* lack dark stripes or streaks on side. Unlike *E. derivativum*, breeding males of *E. basilare* and *E. virgatum* lack dark blue margin on the second dorsal and caudal fins, and the black spot in the first dorsal fin begins on the first, rather than the second, membrane. In contrast to *E. derivativum*, *E. basilare* has modally 10 anal rays (Table 4), 14 dorsal-fin rays (Table 5), and 12 or more pored lateral-line scales (Table 7); in the breeding male, the spots on the cheek bar change during the spawning season from red and white to yellow-gold. *Etheostoma virgatum* has modally 13 or more pored lateral-line scales (Table 7), bright white spots on the pectoral fin of the breeding male, and reaches 65 mm SL.

Etymology.—The name *derivativum*, a Latin adjective meaning "proceeding from," refers to the relatively derived phylogenetic position of this species among barcheek darters. The common name, stone darter, refers both to Stones River, where this species is common, and to the stony slab-rock pools inhabited by this species.

Variation.—*Etheostoma derivativum* occupies several tributaries of the Cumberland River and shows more intraspecific variation than do *E. virgatum* or *E. basilare*. *Etheostoma derivativum* from Marrowbone Creek is the most unusual in that it tends to have more lateral scales and pored lateral-line scales than other populations (Tables 6–7) and has 13 pectoral-fin rays as often as it has 12; other populations (except possibly Sycamore Creek where only eight specimens were examined) modally have 12 pectoral rays (Table 8). The modal number of dorsal-fin spines varies from eight in Marrowbone Creek, Louise Creek, and Red River, to nine in Stones River and Harpeth River (Table 9). *Etheostoma derivativum* from Red River almost always has only one pore in the posterior segment of the infraorbital canal; all other populations (and all populations of *E. virgatum* and *E. basilare*) have two pores (Table 10).

Distribution.—Cumberland River drainage, southern Kentucky and north-central Tennessee from the Red River system in Todd and Logan

TABLE 10. COUNTS OF INFRAORBITAL PORE COUNTS IN *Etheostoma virgatum*, *Etheostoma basilare*, AND *Etheostoma derivativum*.

	Anterior segment of canal					Posterior segment of canal				
	No. pores					No. pores				
Species/drainage	3	4	*n*	χ	SD	1	2	*n*	χ	SD
Etheostoma virgatum										
Rockcastle River	2	81	83	4.0	0.05	10	73	83	1.9	0.21
Buck Creek	1	24	25	4.0	0.08	9	16	25	1.6	0.46
Totals	3	105	108	4.0	0.05	19	89	108	1.8	0.29
Etheostoma basilare										
Caney Fork	1	48	49	4.0	0.04	7	42	49	1.9	0.24
Collins River	2	77	79	4.0	0.05	6	73	79	1.9	0.14
Totals	3	125	128	4.0	0.05	13	115	128	1.9	0.18
Etheostoma derivativum										
Stones River	6	30	36	3.8	0.28	10	26	36	1.7	0.40
White Creek	2	1	3	3.3	0.44	3		3	1.0	0.00
Marrowbone Creek	2	18	20	3.9	0.18	2	18	20	1.9	0.18
Sycamore Creek	2	6	8	3.8	0.38	3	5	8	1.6	0.47
Harpeth River	8	84	92	3.9	0.16	17	75	92	1.8	0.30
Louise Creek	1	9	10	3.9	0.18		10	10	2.0	0.00
Red River	1	17	18	3.9	0.10	17	1	18	1.1	0.10
Totals	22	165	187	3.9	0.21	52	135	187	1.7	0.40

counties, KY, to West Fork Stones River, TN (Fig. 1). Locally common.

KEY TO BARCHEEK DARTERS

1. Distinct brown stripes on side of body 2
1. No distinct brown stripes on side of body (thin dark streaks may be present) 4
2. Black spot in first dorsal fin begins on second membrane (first membrane may be slightly dusky); 15 or fewer pored lateral-line scales (89% of individuals); breeding male has bold dark blue margin on 2nd dorsal and caudal fins; tributaries of lower Cumberland River in west-central Kentucky and Tennessee from the Red River system to Stones River ..... *E. derivativum*
2. Black spot in first dorsal fin begins on first membrane; 12 or more pored lateral-line scales (80% of individuals); breeding male lacks dark blue margin on second dorsal and caudal fins 3
3. Modally nine anal rays and 48–53 lateral scales; bright white spots on pectoral fin of breeding male; no yellow-gold spots on cheek bar of breeding male; Rockcastle River and adjacent tributaries of Cumberland River, eastern Kentucky ..... *E. virgatum*
3. Modally 10 anal rays and 41–48 lateral scales; no bright white spots on pectoral fin; yellow-gold spots on cheek bar of breeding male; upper Caney Fork system, central Tennessee ..... *E. basilare*
4. Rows of dark spots form thin dark streaks on side of body 5
4. No dark streaks on side of body 6
5. White areas in front of and behind large black teardrop; modally nine dorsal spines, nine preoperculomandibular pores; wide bar on cheek (covers most of cheek); confined to upper and middle Green River system, Kentucky and Tennessee ..... *E. barbouri*
5. Dusky areas in front of and behind small teardrop; modally eight dorsal spines, 10 preoperculomandibular pores; narrow bar on cheek; confined to upper and middle Duck River system, central Tennessee ..... *E. striatulum*
6. Scales darkly outlined; infraorbital canal has three pores anteriorly and one pore posteriorly; fewer than 14 pored lateral-line scales; lower Cumberland River drainage (below Caney Fork) and lower Tennessee River drainage (lower Duck River and downstream), Kentucky and Tennessee ..... *E. smithi*
6. Scales not darkly outlined; infraorbital canal has four pores anteriorly and two pores posteriorly; 10–26 pored lateral-line scales; middle Cumberland River drainage from Big South Fork to Obey River, Kentucky and Tennessee ..... *E. obeyense*

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APPENDIX 1

Voucher catalog and GenBank accession numbers for specimens sequenced. *Etheostoma barbouri* (2 specimens): INHS 27864, AF412542–3, AY251541, AF412528–9, AF412559–60; *Etheostoma basilare* (2): INHS 27838, AF412548, AF412551, AY251548, AY251551, AF123043, AF412534, AF412565, AF412568; *Etheostoma derivativum* (2): INHS 91975, AF412549–50, AY251549–50, AF412532–3, AF412566–7; *Etheostoma flabellare*: INHS 45883, AF412540, AF412526, AF412557; *Etheostoma kennicotti*: no voucher, AF412541, AY251542, AF412527, AF412558; *Etheostoma obeyense*: INHS 48194, AF412544, AY251543, AF123035, AF412561; *Etheostoma oophylax*: INHS 44563, AF412538, AF412524, AF412555; *Etheostoma percnurum*: INHS 48196, AF412539, AY251544, AF412525, AF412556; *Etheostoma smithi* (2): INHS 28316, 51622, AF412545–6, AY251545, AF412530–1, AF412562–3; *Etheostoma squamiceps*: INHS 48199, AF412537, AY251546, AF412523, AF412554; *Etheostoma striatulum*: INHS 48193, AF412547, AY251547, AF123042, AF412564; *Etheostoma virgatum* (2): INHS 27832, AF412552–3, AY251552, AF412535–6, AF412569–70.