Copeia, 2000(3), pp. 701–711

Evolution of Cytochrome *b* and the Molecular Systematics of *Ammocrypta* (Percidae: Etheostomatinae)

THOMAS J. NEAR, JEAN C. PORTERFIELD, AND LAWRENCE M. PAGE

Investigations of phylogenetic relationships using comparative morphological characters have led to the hypothesis that *Ammocrypta* is the sister taxon of the *Etheostoma* subgenus *Ioa.* Subsequent molecular analyses have not recovered this proposed relationship. In this investigation, variation in complete cytochrome *b* sequences sampled from 27 percid species, representing all darter genera and all species of *Ammocrypta,* is examined in a phylogenetic context. Third codon purine transitions are saturated when all darter species examined are compared; however, saturation is not detected in any character class among species of *Ammocrypta.* Maximum-parsimony analysis, which excludes potentially homoplasious character classes, and maximum-likelihood analysis of cytochrome *b* sequences statistically reject the hypothesis that *Ammocrypta* is the sister taxon of the *Etheostoma* subgenus *Ioa.* *Etheostoma* is not recovered as monophyletic; however, the hypothesis of monophyly cannot be rejected using maximum-parsimony and maximum-likelihood methods. Morphological characters are combined with cytochrome *b* sequences in a total evidence analysis of relationships within *Ammocrypta.* A novel hypothesis of relationships is proposed, which includes a sister-taxon relationship between *A. clara* and *A. pellucida.* With regard to relationships among species of *Ammocrypta,* the morphological data do not significantly conflict with the cytochrome *b* data. Morphological characters provide additional synapomorphies supporting the hypothesis that *A. beani* and *A. bifascia* are sister taxa. The biogeographic implications of the total evidence analysis are discussed, and the continued taxonomic placement of *Ammocrypta* in *Etheostoma* is discouraged.

CURRENT recognition of darter genera is based primarily on Bailey et al. (1954) and Bailey and Gosline (1955), who proposed that the darters (Etheostomatinae) constituted a natural group within Percidae and recognized three genera, *Percina, Ammocrypta,* and *Etheostoma.* This was a reduction from the 31 genera recognized by Jordan et al. (1930) and was prompted by the lack of diagnostic characteristics and the propensity of convergent evolution in many morphological features used to historically describe and diagnose darter genera (Bailey et al., 1954:141).

Although generally accepted (Page, 1981; Bailey and Etnier, 1988), this hypothesis of darter genera has been challenged. Moore (1968) and Miller and Robison (1973) recognized the monotypic genus *Crystallaria* as distinct from *Ammocrypta,* and Simons (1992) recognized *Ammocrypta* as a subgenus of *Etheostoma.* The recognition of *Crystallaria* was supported in a cladistic examination of eight morphological characters (Simons, 1991) and by descriptions of protolarval morphology in *Crystallaria* and *Ammocrypta* (Simon et al., 1992). Additional cladistic analysis (Simons, 1992) of 38 morphological characters led to the hypothesis that *Ammocrypta* s.s. is the sister taxon of *Etheostoma vitreum,* the only species in the subgenus *Ioa.* The *Ioa-Ammocrypta* clade was found to be the sister taxon of the subgenus *Boleosoma,* and *Ammocrypta* s.s. was recognized as a subgenus of *Etheostoma* (Simons, 1992). The placement of *Ammocrypta* in *Etheostoma* and the elevation of *Crystallaria* are both controversial and have not been uniformly recognized (Etnier and Starnes, 1993; Jenkins and Burkhead, 1994; Mettee et al., 1996).

Two molecular analyses have not supported the hypothesis that *Ammocrypta* is the sister taxon of *Ioa.* Phylogenetic examination of allozyme variation recovered a monophyletic *Etheostoma* (excluding *Ammocrypta*) and a monophyletic clade containing *Ammocrypta* and *Crystallaria* (Wood and Mayden, 1997). Additionally, phylogenetic analysis of complete mitochondrially encoded cytochrome *b* sequences in percid fishes did not recover a sister-taxon relationship between *Ammocrypta* and *Ioa* (Song et al., 1998). This analysis of cytochrome *b* sequences also did not recover *Etheostoma* as monophyletic; however, nodes depicting a paraphyletic *Etheostoma* were not supported in bootstrap resampling and decay analyses (Song et al., 1998).

Interspecific relationships and intraspecific population structure within *Ammocrypta* s.s. were examined by Wiley and Hagen (1997) using a

© 2000 by the American Society of Ichthyologists and Herpetologists

total evidence analysis of selected morphological characters from Simons (1992) and 422 bp of mtDNA sequence data, which included the 3′ end of tRNA glutamate and a 5′ portion of cytochrome *b*. Using Simons' (1992) placement of *Ammocrypta* in *Etheostoma* as justification, only *E.* (*Ioa*) *vitreum* and *E.* (*Boleosoma*) *nigrum* were used as outgroup taxa. This small sample did not adequately represent the diversity of darters and therefore did not test the hypothesis that *Ammocrypta* is a subgenus of *Etheostoma*. Cladistic analysis of partial cytochrome *b*, morphology, and total evidence datasets (Wiley and Hagen, 1997) recovered phylogenetic topologies among the six *Ammocrypta* species that were incongruent with relationships proposed by Simons (1992).

In this study, hypotheses of darter generic relationships, the validity of recognizing *Ammocrypta* s.s. as a genus, and the phylogenetic relationships of the six species of *Ammocrypta* are reexamined in a phylogenetic analysis of complete cytochrome *b* sequences sampled from 21 darter species, including those examined by Song et al. (1998). In addition, morphological characters presented by Simons (1992) that are variable within *Ammocrypta* are combined with the cytochrome *b* dataset for total evidence analysis (Kluge, 1989). Patterns of nucleotide sequence divergence in darters are documented, and resulting phylogenetic hypotheses are used to investigate the historical biogeography and speciation processes in *Ammocrypta*.

Materials and Methods

Collection of nucleotide sequence data.—Nucleic acids were extracted from frozen tissues using standard phenol-chloroform extraction and ethanol precipitation procedures. Localities and GenBank accession numbers for all specimens sequenced in this study are in Material Examined. Nucleic acid extractions were used as template for PCR amplification using the primers GLU (GAC TTG AAG AAC CAC CGT TG) and THR (TCC GAC ATT CGG TTT ACA AG). PCR was performed in 50 μl reactions containing 0.8 mM of dNTP, 2.5 mM MgCl2, 0.5 μM of each primer, and 2.5 U of taq DNA polymerase in a reaction buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100. The amount of template DNA used in PCR ranged from 100–300 ng. Thermal cycling conditions consisted of an initial denaturation step of 94 C for 3 min followed by 30 cycles of 94 C (30 sec), 55 C (30 sec), and 72 C (1.5 min). A final incubation of 72 C for 5 min was added to ensure complete extension of amplified products. Amplified DNA was purified by centrifugal filtration using Millipore Ultrafree-MC (30,000 NMWL) filter units. Filtered PCR product was ligated into pGem-T vector plasmid using T_4 ligase (Promega) and used to transform DH5α-*E. coli*. Isolated cloned plasmids were used as template for chain-terminating cycle sequencing, which was performed using the Delta-Taq Cycle Sequencing kit (Amersham-USB, Cleveland, OH). Each specimen was sequenced with six primers for both strands using two separate clones. Products from sequencing reactions were separated by electrophoresis in 6% polyacrylamide/8.3 M urea gels and visualized by autoradiography. Complete cytochrome *b* sequences were assembled by overlapping individual sequence files using the program Sequencher (Gene Codes, Ann Arbor, MI). Any ambiguities among individual sequence files were rechecked and resolved by sequencing additional clones.

Data analysis.—Sequences were aligned by hand to the percid cytochrome *b* dataset of Song et al. (1998). Pairwise genetic distances, pairwise transition:transversion ratios, and base composition values were calculated using PAUP* 4.0b1. Saturation of nucleotide substitutions was assessed by plotting observed numbers of transitions versus observed transversions. This method is preferred over the common practice of plotting numbers of transitions and transversions versus pairwise genetic distance, where the genetic distance serves as a substitute for time (Moritz et al., 1992). The problem with plotting observed substitutions versus genetic distance is that the estimate of genetic distance depends on the numbers of transitions and transversions, and this introduces additional correlations between the two axes (Edwards, 1997). The substitution dynamics of transition substitutions were determined by separately plotting the observed numbers of the two types of transition substitutions (C ↔ T and A ↔ G) for third codon positions versus observed number of third codon position transversions (Kocher and Carleton, 1997). A linear relationship between transitions and transversions with the slope equal to the transition:transversion ratio is expected of data that are not saturated with multiple substitutions.

Assessment of rate heterogeneity.—Both maximum-likelihood and parsimony-based strategies were employed to determine whether cytochrome *b* exhibits a clocklike pattern of substitution within *Ammocrypta*. First, a maximum-likelihood approach was used via likelihood ratio tests (Huel-

senbeck and Crandall, 1997), which were executed by determining the likelihood scores of the maximum-likelihood-inferred topology using a substitution model that assumes a molecular clock (Ho: HKY85INV+Γc) and the maximum-likelihood-inferred topology using a substitution model without the molecular clock (H1: HKY85INV+Γ). The HKY85INV+Γ model was chosen because it takes into account the proportion of invariable sites and different rates of substitution between transitions and transversions; it allows for the occurrence of unequal base frequencies, and the Γ (alpha shape parameter) takes into consideration observed among-site rate variation (Yang, 1996; Huelsenbeck and Crandall, 1997). The likelihood ratio test statistic $[-2 \log L = -2(\log L_o - \log L_1]$ was then compared to a chi-square distribution with $s - 2$ degrees of freedom, where s is equal to the number of sequences in the analysis (Huelsenbeck and Crandall, 1997). Second, relative rates of substitution within *Ammocrypta* were studied using a parsimony-based approach (Mindell et al., 1997). MacClade (vers. 3.0, W. P. Maddison and D. R. Maddison, Sinauer, Sunderland MA, 1992, unpubl.) was used to determine numbers of unambiguous autapomorphic substitutions at third codon positions for all possible two-taxon combinations within *Ammocrypta* using *Crystallaria asprella* as the outgroup. Two sets of tests were done: one including all third position substitutions; and one including only third position transversions. Binomial tests were used to determine whether there was significant departure from an expectation of equal amounts of character change in the two taxa relative to the outgroup (Mindell et al., 1996).

Phylogenetic analysis of sequence data.—Maximum-parsimony and maximum-likelihood analyses were executed using PAUP* 4.0b1. For the 28 OTU dataset (seven specimens of *Ammocrypta* and 21 other percid species), heuristic searches were used with tree bisection-reconnection (TBR) branch swapping with steepest descent option and 100 random addition sequences. Exhaustive searches for the most-parsimonious tree(s) were used for the 8 OTU dataset, which was restricted to the six species of *Ammocrypta* and *Crystallaria asprella* as the outgroup. Bootstrap (2000 replications) and decay analyses were used to examine levels of relative support for inferred monophyletic groupings. Maximum-likelihood analysis invoked the following options: assumed nucleotide frequencies estimated from the data, number of substitution types set at 2, and rates assumed to follow a gamma distribution with the alpha shape parameter and proportion of invariable sites estimated via maximum likelihood. The gamma approximation was set to four rate categories, and the average for each category was represented by the mean. The HKY85 invariant model (Hasegawa et al., 1985) with rate heterogeneity was used, with the transition:transversion ratio estimated via maximum likelihood and starting branch lengths obtained using the Rogers-Swofford approximation. Heuristic searches were used to find the topology with the best likelihood score using tree bisection-reconnection (TBR) with steepest descent option and 20 random addition sequences. Bootstrap analysis within the maximum-likelihood optimality criteria used 100 pseudoreplications.

Analysis of morphological characters and total evidence.—Of the 38 characters presented in Simons (1992), 12 are variable within *Ammocrypta* (Table 1); these were analyzed with a maximum-parsimony exhaustive search using PAUP* 4.0b1. Unfortunately, character state information for the morphological characters in Simons (1992) is lacking for most darters for which cytochrome *b* sequences are available; therefore, total evidence analysis cannot yet be used to assess the higher level relationships of darter genera and, more specifically, the phylogenetic affinities of *Ammocrypta*. However, the morphological characters from Simons (1992) combined with cytochrome *b* sequence data presented in this study permit a total evidence analysis of relationships among *Ammocrypta* species.

The cytochrome *b* and the edited morphology datasets were combined into a single character matrix for all six species of *Ammocrypta* and *Crystallaria asprella*. The choice of *C. asprella* as the outgroup is based on its placement as the sister taxon of *Ammocrypta* in phylogenetic analysis of allozymes (Wood and Mayden, 1997). Morphological characters for *C. asprella* were coded as missing data. Prior to total evidence analysis, the relative level of heterogeneity between the two datasets was assessed by calculating the incongruence length difference (ILD; Farris et al., 1994). The statistical significance of the ILD (Farris et al., 1995) was assessed using a randomization procedure (2000 replicates, branch-and-bound search) as implemented in the partition homogeneity test in PAUP* 4.0b1. Total evidence analysis of the combined dataset was executed using an exhaustive tree search in PAUP* 4.0b1.

Examination of alternative phylogenetic hypotheses.—Six hypotheses of *Ammocrypta* relationships were examined with the cytochrome *b* sequence data

TABLE 1. DATA MATRIX OF MORPHOLOGICAL CHARACTERS: NUMBERS FOLLOW THOSE PRESENTED IN SIMONS (1992).

	Transformation Series											
	4	5	8	16	18	19	20	28	30	31	34	37
A. beani	0	1	1	0	1	1	1	1	1	1	1	2
A. bifascia	0	1	1	0	1	1	1	1	1	0	1	2
A. meridiana	0	0	0	1	1	0	1	0	0	0	0	3
A. vivax	0	0	0	1	1	0	1	0	0	0	0	2
A. clara	0	0	0	0	0	0	0	0	0	0	1	2
A. pellucida	1	0	0	1	1	0	1	0	0	0	0	3

[4] Premaxillary frenum (0) present, (1) absent
[5] Premaxillary socket (0) V-shaped, (1) U-shaped
[8] Notch posterior to the articular process of the quadrate (0) shallow or absent, (1) deep
[16] Preopercle (0) nonserrate, (1) serrate
[18] Opercle spine (0) present, (1) absent
[19] Opercle strut (0) elongate, (1) reduced
[20] Extension of subopercle (0) elongate, (1) not elongate
[28] Postcleithrum 2 (0) present, (1) absent
[30] Struts on anal pterygiphores (0) present, (1) absent
[31] Anterior process on first anal pterygiphore (0) process for insertion present, (1) process for insertion absent
[34] Body scalation (0) complete, (1) reduced
[37] Breeding tubercles (2) present on anal and pelvic fins, not on belly, (3) present only on pelvic fin

using both maximum-parsimony and maximum-likelihood methods. The edited morphological dataset and the combined dataset were used to test alternative hypotheses using maximum parsimony methods. The first two hypotheses tested address the generic placement of *Ammocrypta* and the monophyly of *Etheostoma*: the hypothesis of Simons (1992), which depicted *Ammocrypta* s.s. as a subgenus within *Etheostoma* and the sister taxon of *E.* (*Ioa*) *vitreum*, and a tree topology that represents *Etheostoma* as a monophyletic group. The remaining four hypotheses specifically address the relationships among species of *Ammocrypta* s.s. (Fig. 1A–D) and were examined using the cytochrome *b*, morphology, and combined datasets. Statistical significance of the differences between alternative hypotheses and optimal topologies was assessed using the test proposed by J. Felsenstein (PHYLIP, vers. 3.5c, Univ. of Washington, Seattle, 1993, unpubl.), which is a modification (MT) of the Templeton (1983) test. The maximum-likelihood method (K-H) of Kishino and Hasegawa (1989) was also used to test the alternative topologies with the cytochrome *b* dataset.

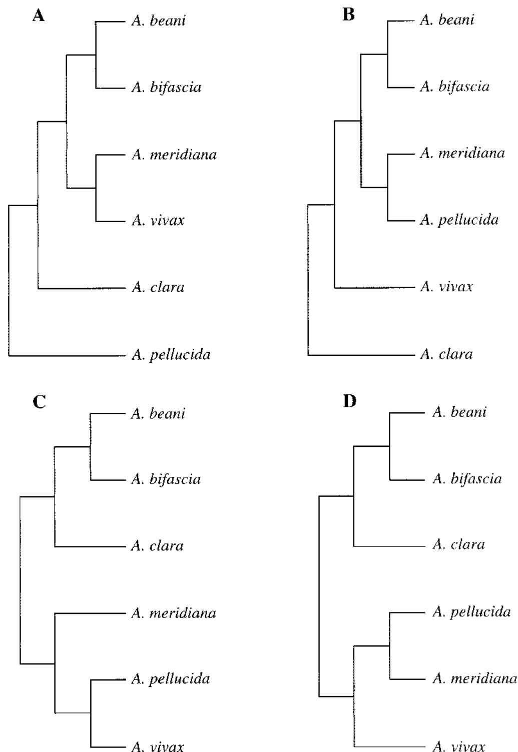

Fig. 1. Alternative hypotheses of relationships among *Ammocrypta* species. (A) Wiley and Hagen (1997); (B) Simons (1992); C. Page (1981); and D. Williams (1975).

RESULTS AND DISCUSSION

Cytochrome b variation.—Within the 28 percid OTUs sampled, 474 of 1140 (41.6%) sites are variable, and 413 of these sites are potentially informative for parsimony analysis. The amount of sequence variation (*p*-distance) between OTUs ranges from 2.5% to 21.1% with a mean of 16.8%. Transition:transversion ratios range between 1.33 and 6.83 with a mean of 2.29. The distribution of substitutions by codon position exhibits the typical pattern that has been reported for cytochrome *b* in percid fishes (Song et al., 1998; Porterfield et al., 1999), with the majority of substitutions (including "informative sites") occurring at the third codon posi-

tion (364/474; 76.8%). Among the seven sequences sampled for *Ammocrypta*, 266 (23.3%) sites are variable and 184 of these sites are potentially parsimony-informative. The majority of the cytochrome *b* variation within *Ammocrypta* involves third codon substitutions (237/266; 89.1%), and first codon substitutions are dominated (53.6%) by nonsynonymous changes at sixfold degenerate leucine codons (YTN). Pairwise *p*-distances within *Ammocrypta* range from 4.1–13.5% with a mean of 11.1%. Transition: transversion ratios are high and range between 2.86 and 6.83 with a mean of 4.13.

Nucleotide compositional biases in percid cytochrome *b* are discussed in Song et al. (1998). Although nucleotide counts within Percidae are homogenous for first and second positions (first positions, $\chi^2 = 8.044$, 81 df, $P > 0.05$; second positions, $\chi^2 = 0.969$, 81 df, $P > 0.05$), significant heterogeneity was detected at third codon positions ($\chi^2 = 195.302$, 81 df, $P < 0.001$). Among *Ammocrypta* and *Crystallaria*, cytochrome *b* sequences (8 OTU dataset), variation of heterogeneity in nucleotide counts between species was not detected among the three codon positions (first positions, $\chi^2 = 1.282$, 21 df, $P > 0.05$; second positions, $\chi^2 = 0.226$, 21 df, $P > 0.05$; third positions, $\chi^2 = 22.388$, 21 df, $P > 0.05$).

Plotting the absolute number of transitions versus the absolute number of transversions reveals that nucleotide substitutions are saturated among the most divergent taxa in the 28 OTU percid dataset (not shown). In contrast, nucleotide substitutions among *Ammocrypta* species are not saturated; however, moderate saturation of nucleotide substitutions is detected between *C. asprella* and *Ammocrypta* species (not shown). Plotting the absolute numbers of transitions and transversions for each codon position versus *p*-distances (plots not shown) indicate that third position transitions represent the only character class in the 28 OTU percid dataset that exhibits saturation of nucleotide substitutions. The majority of cytochrome *b* nucleotide substitutions in both the 28 and eight OTU datasets are dominated by third codon position transitions; this class of substitutions in a mitochondrial protein coding gene (NADH 2) has been shown to saturate rapidly in cichlids (Kocher and Carleton, 1997). Discrimination of the two possible types of transitions (purines, A ↔ G; pyrimidines, C ↔ T) reveals that the initial rate of divergence for the two transition classes are equal (Fig. 2A); however, as divergence increases, purines (A ↔ G) appear to saturate more rapidly than pyrimidines (C ↔ T). This same pattern is observed when restricting the plots of pairwise nucleotide substitutions to

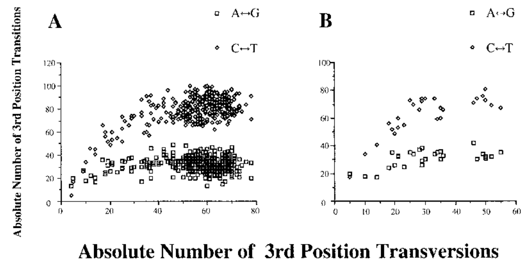

Fig. 2. The accumulation of transition differences at the third codon position in cytochrome *b*, differences involving purines (squares) and pyrimidines (diamonds) are illustrated. (A) Comparisons in the 28 OTU percid dataset. (B) Comparisons in the 8 OTU dataset (*C. asprella* and *Ammocrypta* species).

comparisons involving *Ammocrypta* species and *C. asprella* (Fig. 2B). The rapid saturation of purine relative to pyrimidine nucleotide substitutions has been explained as resulting from the greater inequality between A and G than observed between C and T and the low frequency of G at the third codon position (Kocher and Carleton, 1997).

Rate heterogeneity of cytochrome b in Ammocrypta.—Neither maximum-likelihood nor parsimony approaches detected any significant rate heterogeneity in cytochrome *b* sequences among species of *Ammocrypta*. The log-likelihood scores for the HKY85+Γc (−3868.01) and HKY85+Γ (−3865.05) models were used in a likelihood ratio test, which did not reject the HKY85+Gc model ($\chi^2 = 5.92$, 6 df, $P > 0.05$). Similarly, in the parsimony relative-rate analysis no two-taxon comparisons within *Ammocrypta*, using *C. asprella* as an outgroup, exhibited significant departures from uniform rates of sequence evolution (all $P > 0.05$). No significant differences in rate heterogeneity among *Ammocrypta* species were detected when considering all third position substitutions or when the analysis only considered third position transversions.

Phylogenetic analysis of sequence data.—Maximum-parsimony analysis ignored third position purine substitutions, because this character class appears to be severely saturated with multiple substitutions (Fig. 2A). A single most-parsimonious tree is recovered in which *C. asprella* is the sister taxon of a monophyletic *Ammocrypta*; however, the node is not supported in bootstrap analysis and has a relatively low decay score (Fig. 3). *Etheostoma* is paraphyletic because the two species of the subgenus *Nothonotus* are sister to a monophyletic *Percina*, and this clade is the sister taxon of *E.* (*Allohistium*) *cinereum*. These

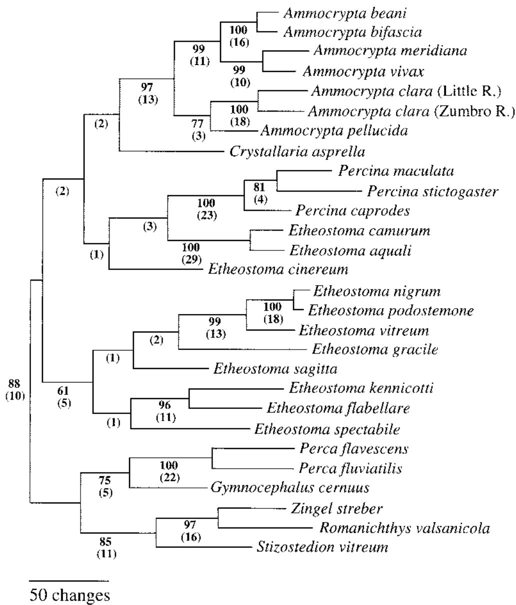

Fig. 3. Phylogram depicting the single most-parsimonious tree resulting from maximum parsimony analysis ignoring third codon purine (A ↔ G) substitutions. The tree length is 1955 steps, CI (excluding uninformative characters) = 0.283. Bootstrap pseudoreplicate scores (2000 iterations) are listed in bold below branches and decay indices are presented in parentheses. Scale of optimized character changes at bottom of figure.

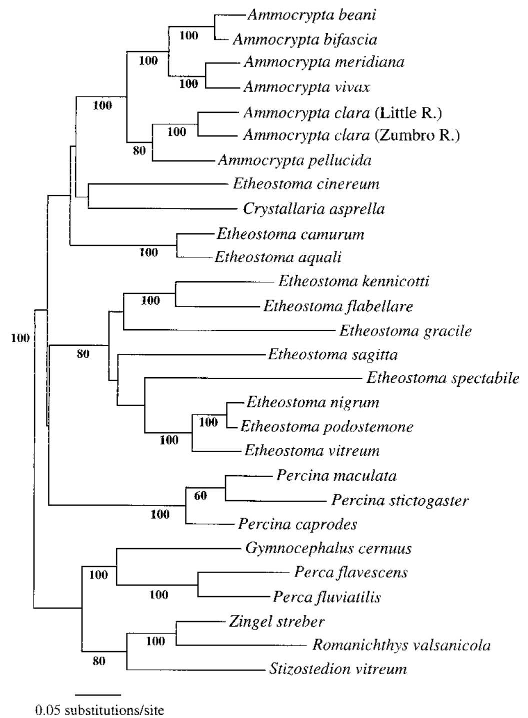

Fig. 4. Phylogram depicting the single tree recovered in maximum-likelihood analysis of all substitutions ($Ln = -11{,}066.23$). The HKY85inv+Γ model parameters were transition:transversion ratio (ti:tv) = 5.01, proportion of invariable sites = 0.56, and alpha shape parameter of the gamma distribution (α) = 1.27. Bootstrap pseudoreplicate scores (100 iterations) are listed in bold below branches. Scale of inferred substitutions per site is at bottom of figure.

nodes representing *Etheostoma* as a paraphyletic group are not supported in bootstrap analysis and exhibit low decay values (Fig. 3). Despite the recovery of many nodes that are not well supported, *Ammocrypta* is monophyletic with high bootstrap and decay scores and did not group with *E.* (*Ioa*) *vitreum* or any other species of the *Boleosoma* group represented in this analysis (Fig. 3). Maximum-likelihood analysis of the 28 OTU dataset was congruent to the maximum-parsimony analysis (Fig. 3) in terms of the failure to recover *Ammocrypta* as the sister of *E.* (*Ioa*) *vitreum*, the recovered relationships among *Ammocrypta* species, and the recovery of a paraphyletic *Etheostoma* with short nodes, which are not supported in bootstrap analysis (Fig. 4)

Analysis of morphology and total evidence.—Exhaustive searches of the morphological dataset (Table 1) found a single most-parsimonious tree (12 steps, CI = 1.0), which shows identical relationships to those proposed by Simons (1992; Fig. 1B). In the combined dataset of cytochrome *b* sequences and coded morphological transformation series (TS; Table 1), the two datasets were not found to be significantly heterogeneous in partition homogeneity tests (ILD = 3, $P > 0.05$).

A single most-parsimonious tree was recovered from the combined dataset using the exhaustive search algorithm (Fig. 5). *Ammocrypta beani* and *A. bifascia* are recovered as sister taxa with 10 unique (CI = 1.0) nucleotide and five unique (CI = 1.0) morphological changes. No other morphological character is optimized as a synapomorphy for a clade of two or more *Ammocrypta* species (Fig. 5). The only morphological characters that are optimized as homoplasious on the total evidence topology are serration of the preopercle (TS 16), body scalation (TS 34), and breeding tubercles (TS 37).

The total evidence tree (Fig. 5) is identical at the species level to one of two sets of consensus trees resulting from analysis of partial cytochrome *b* sequences (Wiley and Hagen, 1997); it differs only in the placement of the root, which results in a monophyletic *A. clara–A. pel-*

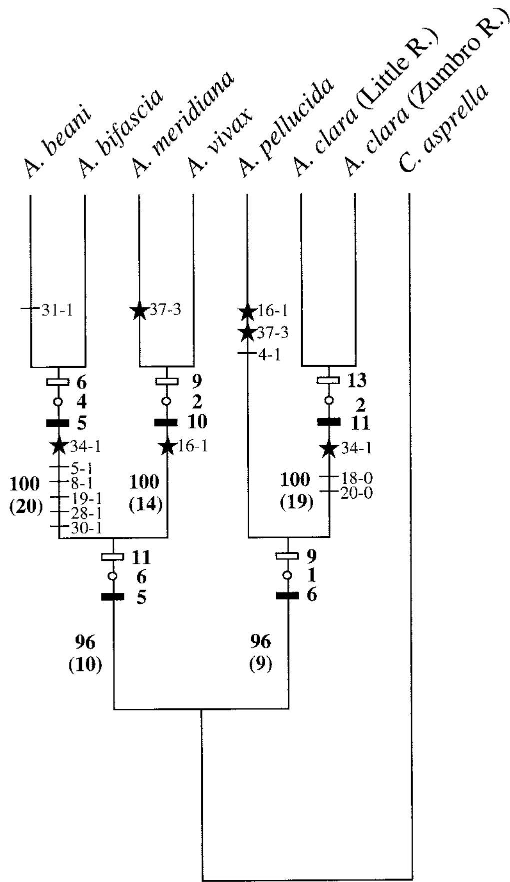

Fig. 5. Single most-parsimonious tree resulting from maximum-parsimony analysis of the combined cytochrome *b* and morphology dataset. The tree length is 547 steps, CI (excluding uninformative characters) = 0.624. Open bars denote number of unique transitions (CI = 1.0), and circles denote unique transversions (CI = 1.0). Dark bars tally the number of nucleotide substitutions that are homoplasious along a particular internode. Unique morphological changes (CI = 1.0) are marked with a dash, and homoplasious morphological changes are indicated with a star. Bootstrap replicate scores and decay indices (in parentheses) are listed to left of the appropriate internode.

lucida clade in datasets using complete cytochrome *b* sequences (Figs. 4–5). This node is supported with 10 unique (CI = 1.0) nucleotide substitutions, nine decay steps, and high bootstrap recovery (Fig. 5). The remaining clades were previously recovered in analyses of partial cytochrome *b* sequences (Wiley and Hagen, 1997). Comparison with the partial cytochrome *b* dataset indicates that complete cytochrome *b* sequences have the effect of providing stronger character support for nodes, as measured by the number of unique substitutions. Slightly more than 70% (144 of 204) of the phylogenetically informative characters from cytochrome *b* mapped on the total evidence tree (Fig. 5) are located in portions of the gene that are not sampled with "universal" primers.

Examination of alternative hypotheses.—The topology that depicts *Ammocrypta* as the sister of *E.* (*Ioa*) *vitreum* (Simons, 1992) is significantly longer (59 steps) than the most-parsimonious tree (Fig. 3) and has a significant *ln* L (121.13) difference from the best maximum-likelihood tree (Table 2). The shortest topology, recovered in constraint tree searches, which depicts *Etheostoma* as monophyletic is only seven steps longer and is not significantly different than the most-parsimonious tree (Table 2). Similarly, a monophyletic *Etheostoma* is different from the best maximum-likelihood tree by *ln* L of only 9.41 and is not significantly less likely (Table 2). The topology inferred from maximum-likelihood analysis (Fig. 4) was only 15 steps longer than the most-parsimonious tree (Fig. 3), and this difference was determined not to be significantly different ($0.01 < P < 0.05$) in the MT test (Table 2). The two trees resulting from maximum-parsimony (Fig. 3) and maximum-likelihood (Fig. 4) analyses are congruent with regard to relationships within *Ammocrypta*, and the failure to recover *Ammocrypta* as the sister taxon of *E.* (*Ioa*) *vitreum* or any other species of the *Boleosoma* group. The maximum-parsimony topology is not significantly different from the best likelihood tree in the K-H test (Table 2).

Three of the four alternative hypotheses of relationships within *Ammocrypta* are significantly different in MT and K-H tests (Table 2). The hypothesis presented in Wiley and Hagen (1997; Fig. 1A) is only nine steps longer than the most-parsimonious tree and differs from the best maximum-likelihood tree by an *ln* L of 0.33 (Table 2). The relationships within *Ammocrypta* proposed by Simons (1992; Fig. 1B), Page (1981; Fig. 1C), and Williams (1975; Fig. 1D) are significantly longer than the optimal tree in MT and K-H tests (Table 2). Tests of the alternative hypotheses of relationships within *Ammocrypta* using the total evidence dataset produced results that did not differ from the results obtained using the 8 OTU cytochrome *b* dataset (Table 2).

When the alternative topologies of relationships within *Ammocrypta* were assessed using the morphological dataset (Table 1), no hypotheses were found to be significantly different. The unrooted most-parsimonious tree resulting from analysis of the edited morphological dataset

TABLE 2. STATISTICAL COMPARISON OF ALTERNATIVE TOPOLOGIES USING MODIFIED TEMPLETON TEST (MT) AND KISHINO-HASEGAWA (K-H) TEST.

Topology	Maximum Parsimony				Maximum Likelihood		
	Tree length	Length Diff.	CI	*P*	*ln* L	*ln* L Diff.	*P*
Genus level hypotheses							
MP, Fig. 3	1948	best	0.283	—	−11,083.59	17.35	0.1180
ML (HKY85 + Γ)	1963	15	0.280	0.1583	−11,066.23	best	—
Etheostoma monophyletic[a]	1955	7	0.218	0.5500	−11,086.31	20.08	0.2054
Etheostoma monophyletic[b]	1966	18	0.280	0.0947	−11,079.29	13.05	0.1655
Simons (1992)	2007	59	0.290	<0.0001	−11,187.37	121.14	<0.0001[c]
Species level hypotheses							
MP, Fig. 5	532	best	0.619		−3,865.05	best	
Wiley and Hagen (1997), Fig. 1A	541	9	0.606	<0.0605	−3,865.38	0. 33	0.7098
Simons (1992), Fig. 1B	584	52	0.550	<0.0001[c]	−3,924.15	59.10	<0.0001[c]
Page (1981), Fig. 1C	587	55	0.547	<0.0001[c]	−3,923.30	58.25	<0.0001[c]
Williams (1975), Fig. 1D	589	57	0.545	<0.0001[c]	−3,923.30	58.25	<0.0001[c]

[a] Most-parsimonious tree from constraint search.
[b] Maximum-likelihood tree from constraint search.
[c] Probability of getting a more extreme T-value under the null hypothesis of no difference between the two trees (two-tailed test).

(Table 1) and the hypotheses of Simons (1992) and Williams (1975) are all identical in tree length (12 steps, CI = 1.0). The tree proposed in this study (Fig. 5) and the hypothesis of Wiley and Hagen (1997) differ only in the placement of the outgroup root, and exhibit the same tree length when the morphological data are optimized onto these hypotheses (15 steps, CI = 0.727; $P > 0.05$). It appears that the morphological characters are less able than the cytochrome *b* nucleotide characters to discriminate among alternative hypotheses. This is probably attributable to the disparity in the number of characters in the two datasets (12 vs 1140).

Biogeography of Ammocrypta.—The phylogenetic relationships recovered in this investigation allow development and reevaluation of historical biogeographic hypotheses for *Ammocrypta*. The grouping of *Ammocrypta* species into two major clades is generally congruent with the distribution of these species. The clade consisting of *A. beani, A. bifascia, A. vivax,* and *A. meridiana* (Fig. 5) is distributed almost exclusively in Gulf of Mexico and lower Mississippi River drainages. Sister species pairs within this clade provide evidence for vicariant speciation in the Mobile Basin and Gulf of Mexico drainages to the east and west. The sister-taxon relationship of *A. beani* and *A. bifascia* has been cited as an example of vicariant speciation following separation of the Escambia, Yellow, and Blackwater Rivers from the Mobile Basin (Simons, 1992; Wiley and Mayden, 1985; Williams, 1975). The sister-taxon relationship of *A. vivax* and *A. meridiana* suggests a vicariant event separating the Mobile Basin and Gulf of Mexico drainages to the west. Two species of the *Lythrurus roseipinnis* group exhibit this same vicariant pattern of speciation (Wiley and Siegel-Causey, 1994). Potentially complicating the interpretation of a Mobile-West Gulf Coast vicariance in *Ammocrypta* is the fact that *A. vivax* is not restricted to Gulf of Mexico drainages, because the species is found in various tributaries of the Lower Mississippi River (Williams, 1975). The hypothesis of a Mobile vicariance would be supported if an intraspecific analysis of *A. vivax* determined that populations in the Gulf of Mexico contained ancestral haplotypes with regard to populations in the Mississippi Drainage.

The distributions of *A. pellucida* and *A. clara* suggest an east–west Central Highlands vicariance that is commonly associated with speciation in North American freshwater fishes (Wiley and Mayden, 1985; Mayden, 1987; Strange and Burr, 1997). The *A. pellucida–A. clara* clade is distributed in the Eastern and Interior Highlands, and there are disjunct populations of *A. clara* in the Cumberland, Green, and Upper Tennessee Rivers (Williams, 1975; Etnier and Starnes, 1993). The two species have historically been collected together in the Wabash and Green River systems (Williams, 1975). This is the only instance where two sister species of *Ammocrypta* are sympatric. Examination of morphometric and meristic characters has not revealed any instances of hybridization between *A. clara* and *A. pellucida* (Williams, 1975). If a vicariant hypothesis of speciation is adopted, then *A. pel-*

lucida may have originated in the headwaters of the preglacial Teays or the Laurentian (Pflieger, 1971) and dispersed westward to the Green and Wabash Rivers after *A. clara* was established in these drainages.

The relict populations of *A. clara* in the eastern highlands may reflect Quaternary-induced vicariance and extinction of populations in a previously more widespread species. Evidence for divergence within *A. clara* is found in meristic differences between Upper Tennessee River and western populations (Starnes et al., 1977). The two *A. clara* cytochrome *b* sequences collected for this investigation exhibit a moderate divergence (6.8%), exceeding the divergence observed between *A. beani* and *A. bifascia* (4.1%) and between *A. vivax* and *A. meridiana* (6.0%).

Taxonomic and evolutionary conclusions.—Phylogenetic analysis of allozymes (Wood and Mayden, 1997) and cytochrome *b* sequences (Fig. 4) clearly indicate that *Ammocrypta* should be considered a distinct genus. Continued placement of *Ammocrypta* species in *Etheostoma* should be abandoned unless substantial new data are presented to support the placement as the sister taxon of *E.* (*Ioa*) *vitreum*. Such data should represent at least the diversity of darters sampled here with cytochrome *b* sequences (Figs. 3–4) and by Wood and Mayden (1997) with allozyme variation. Recognition of *Etheostoma* as a paraphyletic group is premature, because both maximum-parsimony and maximum-likelihood analyses are not able to reject the hypothesis that *Etheostoma* is monophyletic (Table 2). Additionally, the paraphyly of *Etheostoma* depicted in both the maximum-parsimony and maximum-likelihood trees (Figs. 3–4) would require either the recognition of *Allohistium* and *Nothonotus* as genera or the inclusion of *Ammocrypta, Crystallaria*, and *Percina* as subgenera of *Etheostoma*. Clearly, such taxonomic rearrangements are not adequately supported in these analyses. It appears that cytochrome *b* sequences are able to discriminate certain hypotheses of darter generic relationships [e.g., *Ammocrypta* as the sister taxon of *E.* (*Ioa*) *vitreum*], whereas it is unable to test others (e.g., relationships among *Percina, Crystallaria, Ammocrypta*, and *Etheostoma*). Perhaps relationships among darter genera can be resolved through examination of other mitochondrial and nuclear gene sequences.

The placement of *Ammocrypta* in *Etheostoma* leads to an evolutionary hypothesis that is not restricted to taxonomic issues. The identification of *Ammocrypta* and *E.* (*Ioa*) *vitreum* as sister taxa supports a hypothesis that various characters associated with dwelling in sand runs (semitranslucent and elongated bodies, protrusible premaxillaries, and behavior of burying in sand) are shared becasue of common ancestry (Simons, 1992). Based on the phylogenetic relationships recovered in this study, characteristics shared by *Ammocrypta* and *E.* (*Ioa*) *vitreum* are interpreted as resulting from independent evolution of specializations in sand run habitats (Page and Swofford, 1984).

Material Examined

Localities and GenBank accession numbers for species sequenced during this study follow. Data for other species are given in Song et al. (1998). *Ammocrypta bifascia*—INHS 38107 Escambia County, Alabama, 10 March 1996 (AF183940). *Ammocrypta clara*—New Madrid County, Missouri, 17 February 1996 (AF183941). *Ammocrypta meridiana*—INHS 38679 Wilcox County, Alabama, 12 March 1996 (AF183942). *Ammocrypta pellucida*—Cumberland County, Illinois, 24 February 1996 (AF183943). *Ammocrypta vivax*—INHS 40535 New Madrid County, Missouri, 21 March 1997 (AF183944). *Etheostoma nigrum*—INHS 39507 Beltrami County, Minnesota, 27 July 1996 (AF183945).

Acknowledgments

J. Armbruster, P. Ceas, T. M. Near, and R. Steinberg assisted in collecting specimens used in this study. Financial support was provided by the Illinois Natural History Survey H. H. Ross Memorial Fund and the Illinois Department of Transportation. We thank C. Phillips, A. Simons, and three anonymous reviewers for helpful and constructive comments on the manuscript.

Literature Cited

Bailey, R. M., and D. A. Etnier. 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. Mich. 175:1–48.

———, and W. A. Gosline. 1955. Variation and systematic significance of vertebral counts in the American fishes of the family Percidae. *Ibid.* 93:1–44.

———, H. E. Winn, and C. L. Smith. 1954. Fishes from the Escambia River, Alabama and Florida, with ecological and taxonomic notes. Proc. Acad. Nat. Sci. Phila. 106:109–164.

Edwards, S. V. 1997. Relevance of microevolutionary processes to higher level molecular systematics, p. 251–278. *In:* Avian molecular evolution and systematics. D. P. Mindell (ed.). Academic Press, San Diego, CA.

ETNIER, D. A., AND W. C. STARNES. 1993. The fishes of Tennessee. Univ. of Tennessee Press, Knoxville.

FARRIS, J. S., M. KALLERSJO, A. G. KLUGE, AND C. BULT. 1994. Testing significance of incongruence. Cladistics 10:315–319.

———, M. KALLERSJO, A. G. KLUGE, AND C. BULT. 1995. Constructing a significance test for incongruence. Syst. Biol. 44:570–572.

HASEGAWA, M., H. KISHINO, AND T. YANO. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22:160–174.

HUELSENBECK, J. P., AND K. A. CRANDALL. 1997. Phylogeny estimation and hypothesis testing using maximum likelihood. Annu. Rev. Ecol. Syst. 28: 437–466.

JENKINS, R. E., AND N. M. BURKHEAD. 1994. Fishes of Virginia. American Fisheries Society, Bethesda, MD.

JORDAN, D. S., B. W. EVERMANN, AND H. W. CLARK. 1930. Checklist of the fish and fishlike vertebrates of North and Middle America north of the boundary of Venezuela and Columbia. Rep. U.S. Comm. Fish., App. 10, Washington, DC.

KISHINO, H., AND M. HASEGAWA. 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. 31:151–160.

KLUGE, A. G. 1989. A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). Syst. Zool. 38:7–25.

KOCHER, T. D., AND K. L. CARLETON. 1997. Base substitution in fish mitochondrial DNA: patterns and rates, p. 13–24. *In:* Molecular systematics of fishes. T. D. Kocher and C. A. Stepien (eds.). Academic Press, San Diego, CA.

MAYDEN, R. L. 1987. Pleistocene glaciation and historical biogeography of North American central-highland fishes, p. 141–151. *In:* Quaternary environments of Kansas. W. C. Johnson (ed.). Kansas Geological Survey, Guidebook Series 5, Lawrence, KS.

METTEE, M. F., P. E. O'NEIL, AND J. M. PIERSON. 1996. Fishes of Alabama and the Mobile Basin. Oxmoor House, Birmingham, AL.

MILLER, R. J., AND H. W. ROBISON. 1973. The fishes of Oklahoma. Oklahoma State Univ. Press, Stillwater.

MINDELL, D. P., A. KNIGHT, C. BAER, AND C. J. HUDDLESTON. 1996. Slow rates of molecular evolution in birds and the metabolic rate and body temperature hypothesis. Mol. Biol. Evol. 13:422–426.

———, M. D. SORENSON, C. J. HUDDLESTON, H. C. MIRANDA JR., A. KNIGHT, S. J. SAWCHUCK, AND T. YURI. 1997. Phylogenetic relationships among and within select avian orders based on mitochondrial DNA, p. 213–247. *In:* Avian molecular evolution and systematics. D. P. Mindell (ed.). Academic Press, San Diego, CA.

MOORE, G. A. 1968. Fishes, p. 21–165. *In:* Vertebrates of the United States. W. F. Blair, A. P. Blair, F. Brodkorb, F. R. Cagle, and G. A. Moore (eds.). McGraw-Hill Co., New York.

MORITZ, C., C. J. SCHNEIDER, AND D. B. WAKE. 1992. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. Syst. Biol. 41:273–291.

PAGE, L. M. 1981. The genera and subgenera of darters. Occ. Pap. Mus. Nat. Hist. Univ. Kans. 90:1–69.

———, AND D. L. SWOFFORD. 1984. Morphological correlates of ecological specialization in darters. Environ. Biol. Fish. 11:139–159.

PFLIEGER, W. L. 1971. A distributional study of Missouri fishes. Univ. Kans. Publ. Mus. Nat. Hist. 20: 225–570.

PORTERFIELD, J. C., L. M. PAGE, AND T. J. NEAR. 1999. Phylogenetic relationships among fantail darters (Percidae: *Etheostoma*: *Catonotus*): total evidence analysis of morphological and molecular data. Copeia 1999:551–564.

SIMON, T. P., E. J. TYBERGHEIN, K. J. SCHEIDEGGER, AND C. E. JOHNSTON. 1992. Descriptions of protolarvae of the sand darters (Percidae: *Ammocrypta* and *Crystallaria*) with comments on systematic relationships. Ichthyol. Explor. Freshwaters 3:347–358.

SIMONS, A. M. 1991. Phylogenetic relationships of the crystal darter, *Crystallaria asprella* (Teleostei: Percidae). Copeia 1991:927–936.

———. 1992. Phylogenetic relationships of the *Boleosoma* species group (Percidae: *Etheostoma*), p. 268–292. *In:* Systematics, historical ecology, and North American freshwater fishes. R. L. Mayden (ed.). Stanford Univ. Press, Stanford, CA.

SONG, C. B., T. J. NEAR, AND L. M. PAGE. 1998. Phylogenetic relations among percid fishes as inferred from mitochondrial cytochrome *b* DNA sequence data. Mol. Phylogenet. Evol. 10:343–353.

STARNES, W. C., D. A. ETNIER, L. B. STARNES, AND N. H. DOUGLAS. 1977. Zoogeographic implications of the rediscovery of the percid genus *Ammocrypta* in the Tennessee River drainage. Copeia 1977:783–786.

STRANGE, R. M., AND B. M. BURR. 1997. Intraspecific phylogeography of North American highland fishes: a test of the Pleistocene vicariance hypothesis. Evolution 51:885–897.

TEMPLETON, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the humans and apes. *Ibid.* 37:221–244.

WILEY, E. O., AND R. H. HAGEN. 1997. Mitochondrial DNA sequence variation among the sand darters (Percidae: Teleostei), p. 75–96. *In:* Molecular systematics of fishes. T. D. Kocher and C. A. Stepien (eds.). Academic Press, San Diego, CA.

———, AND R. L. MAYDEN. 1985. Species and speciation in phylogenetic systematics, with examples from the North American fish fauna. Ann. Mo. Bot. Gard. 72:596–635.

———, AND D. SIEGEL-CAUSEY. 1994. A phylogenetic analysis of the *Lythrurus roseipinnis* species group (Teleostei: Cyprinidae), with comments on the relationships of other *Lythrurus*. Occ. Pap. Mus. Nat. Hist. Univ. Kans. 171:1–20.

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

WOOD, R. M., AND R. L. MAYDEN. 1997. Phylogenetic

relationships of selected darter subgenera (Teleostei: Percidae) as inferred from analysis of allozymes. Copeia 1997:265–274.

YANG, Z. 1996. Among-site rate variation and its impact on phylogenetic analyses. Trends Ecol. Evol. 11:367–372.

ILLINOIS NATURAL HISTORY SURVEY, 607 EAST PEABODY, CHAMPAIGN, ILLINOIS 61820. PRESENT ADDRESSES: (TJN) CENTER FOR POPULATION BIOLOGY, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA 95616; AND (JCP) CENTRE COLLEGE, DANVILLE, KENTUCKY 40422. E-mail: (TJN) near@mail.inhs.uiuc.edu. Send reprint requests to TJN. Submitted: 5 March 1999. Accepted: 20 Oct. 1999. Section editor: J. D. McEachran.