Copeia, 2001(3), pp. 656–667

Phylogenetic Relationships of Cyprinid Fishes in Subgenus *Notropis* Inferred from Nucleotide Sequences of the Mitochondrially Encoded Cytochrome *b* Gene

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Cytochrome *b* sequences (1140 bp) from 16 species considered to be members of subgenus *Notropis* and several outgroup taxa were analyzed phylogenetically. Phylogenetic analysis of cytochrome *b* was sensitive to sampling of outgroup taxa, probably resulting from substitutional rate heterogeneity among outgroups. Maximum-likelihood analysis was more robust to these effects as compared to maximum parsimony. Both maximum parsimony and maximum-likelihood analysis supported removal of *Notropis candidus* and *Notropis shumardi* from subgenus *Notropis*. Monophyly of the 14 remaining species was supported by the maximum likelihood analysis. Regardless of the method of analysis or use of outgroup taxa, phylogenetic analysis of cytochrome *b* indicated strong support for the following hypothesis of relationships: ((*Notropis rubellus*, *Notropis suttkusi*), (*Notropis amoenus*, *Notropis stilbius*, ((*Notropis atherinoides*, *Notropis oxyrhynchus*), (*Notropis amabilis*, *Notropis jemezanus*)))). This phylogenetic hypothesis implies that morphological characters associated with increased olfaction were derived independently among members of subgenus *Notropis*, perhaps in response to life in more turbid habitats.

THE subgenus *Notropis* is an ecologically diverse yet morphologically conservative group of North American cyprinids. Distributions of the 17 nominal species range from highly restricted (*Notropis oxyrhynchus,* found only in the Brazos River, TX) to nearly continental (*Notropis atherinoides,* found throughout the central United States from the Gulf of Mexico to Canada). Habitats are primarily riverine and range from broad channels of turbid rivers (*Notropis girardi*) to clear creeks and streams (*Notropis ariommus*). Subgenus *Notropis* are broadcast spawners, scattering eggs and sperm without preparation of substrate, although at least two species, *Notropis amoenus* and *Notropis rubellus,* are known to spawn as nest associates with other species (Johnson and Page, 1992). Members of subgenus *Notropis* are relatively small and round bodied and lack striking coloration. However, two species, *N. rubellus* and *Notropis suttkusi,* display red and orange coloration on the head and base of fins during the breeding season.

Coburn (1982) conducted a detailed morphological study of *N. atherinoides,* the type species for genus *Notropis,* and employed a cladistic analysis to define a monophyletic subgenus *Notropis* based upon synapomorphies shared between *N. atherinoides* and other species of genus *Notropis*. Coburn (1982) described four distinct species-groups within subgenus *Notropis*: (1) the *atherinoides* group (*N. atherinoides, N. amabilis, N. rubellus, Notropis perpallidus*); (2) the *shumardi* group (*Notropis shumardi, Notropis candidus, N. oxyrhynchus, Notropis. jemezanus*); (3) the *photogenis* group *(Notropis photogenis, N. amoenus, Notropis stilbius*); and (4) the *ariommus* group (*N. ariommus, Notropis scepticus, Notropis semperasper, Notropis telescopus*). Coburn (1982) proposed a hypothesis of relationships for these species groups, placing the *shumardi* group sister to the *atherinoides* group and the *photogenis* group sister to the *ariommus* group.

Aside from placement of *N. rubellus* (formerly of subgenus *Hydrophlox*) in subgenus *Notropis* (Dowling and Brown, 1989; Mayden, 1991), modification of subgenus *Notropis* since it was defined by Coburn (1982) has centered primarily on members of the *N. shumardi* species group. Based upon a cladistic analysis of variation in chromosomal NORs, Amemiya and Gold (1990) hypothesized that *N. oxyrhynchus* (formerly of *N. shumardi* species group) was more closely related to *N. atherinoides* (*N. atherinoides* species group). Based upon reinterpretation of characters associated with the olfactory capsule, Coburn and Cavender (1992) placed *N. girardi* (formerly of subgenus *Alburnops*) in the *N. shumardi* species group and moved *N. oxyrhynchus* (formerly of *N. shumardi* species group) into the *N. atherinoides* species group. Coburn and Cavender (1992) also raised the question of affinities of species placed in the *N. shumardi* species group by noting that characters of the olfactory capsule used to diagnose the *N. shumardi* species group were distributed widely outside of sub-

genus *Notropis*. Prior to the analysis of Coburn (1982), *N. shumardi* was placed outside of subgenus *Notropis* in subgenus *Alburnops* (Snelson, 1968). Two recently described species, *N. candidus* (Suttkus, 1980) and *N. suttkusi* (Humphries and Cashner, 1994), represent geographically distinct populations formerly considered to be part of *N. shumardi* and *N. rubellus,* respectively.

The purpose of the present investigation was to use complete sequences of the mitochondrially encoded cytochrome *b* (cyt *b*) gene to infer a hypothesis of phylogenetic relationships among species of subgenus *Notropis* and, in particular, to investigate phylogenetic affinities of species in the *N. shumardi* species group. Mitochondrial genes have been useful in studies of the phylogenetic relationships of several groups of North American cyprinids. Schmidt and Gold (1995) used partial cyt *b* sequences to infer phylogenetic affinities of *Notropis topeka,* and complete cyt *b* sequences provided the basis for a hypothesis of relationships among species of the cyprinid genera *Luxilus* (Dowling and Naylor, 1997) and *Lythrurus* (Schmidt et al., 1998). Cyt *b* even has been used to investigate cyprinid relationships at the subfamilial level (Briolay et al., 1998). Other mitochondrial genes also have been used effectively in studies of North American cyprinids (e.g., Simons and Mayden, 1997; Broughton and Gold 2000).

Five species of subgenus *Notropis* (*N. candidus, N. girardi, N. jemezanus, N. oxyrhynchus,* and *N. shumardi*) exhibit pronounced enlargement of brain structures (olfactory bulb and facial lobe) associated with olfaction (Coburn, 1982, pers. comm.). Previously, four of these were considered a monophyletic group (*shumardi* species group; Coburn 1982). However, with the exception of *N. shumardi* and *N. candidus,* our analysis of cyt *b* indicated that species with pronounced enlargement of brain structures associated with olfaction were not closely related. Because these species also tend to prefer more turbid waters (Huber and Rylander, 1992), our findings support the hypothesis that enlarged olfactory capabilities might have evolved within subgenus *Notropis* to compensate for reduced visibility in turbid waters of large rivers.

Materials and Methods

Taxa examined.—Cyt *b* sequences were acquired from 16 of 17 nominal species of subgenus *Notropis* and from eight species belonging to five other assemblages within genus *Notropis* (see Materials Examined). Whole fish were captured with minnow seines, transported in either liquid nitrogen or 95% ethanol, and stored at −80 C until processed. Collection localities, drainage, and catalog number of voucher specimens are presented in the Materials Examined section. A single specimen of *Notropis semperasper* was collected from the James River, Virginia, but reliable sequences of its cyt *b* gene could not be obtained. The cyt *b* gene (1140 bp) was sequenced directly for representatives of 24 species of *Notropis*; cyt *b* sequences of *N. rubellus* were kindly provided by T. E. Dowling of Arizona State University.

DNA extraction, amplification, and sequencing.—Whole fish were ground in liquid nitrogen, and DNA was extracted by phenol-chloroform treatment and ethanol precipitation (Sambrook et al., 1989). DNA preparations were used as template for primer-directed amplification of target DNA sequences via polymerase chain reaction (PCR). PCR amplification used the primers LA, LC, HA, HB, and HD described in Schmidt et al. (1998). PCR thermal profile consisted of 35–45 cycles of 95 C denaturation for 1 min, 48–50 C annealing for 1 min, and 72 C extension for 45 sec. Excess primers, nucleotides, and polymerase were removed from DNA amplification products by using Prep-A-Gene® DNA purification system (BioRad). Double-stranded DNA amplification products were sequenced directly with ABI PRISM™ (Perkin Elmer) dye-terminator cycle-sequencing kits and an Applied Biosystems (Perkin Elmer) 377 automated DNA sequencer. Sequencing used primers LA, LC, HA, HB, and HD described in Schmidt et al. (1998). The cyt *b* sequence of an individual was determined from a minimum of two independent sequencing-reactions for each primer.

Patterns of sequence variation.—Measures of nucleotide composition were obtained by using PAUP* version 4.0d63 (D. L. Swofford, Sinauer, Sunderland, MA, 1998, unpubl.). Homogeneity of nucleotide composition was tested among taxa by using chi-square tests of contingency tables of nucleotide counts, as implemented in PAUP*. Maximum likelihood provided the statistical basis for testing expectations of the molecular clock (Yang et al., 1995). Likelihood ratio tests of the molecular clock were conducted by using the optimal topology obtained from maximum-likelihood analysis of all ingroup and outgroup taxa.

Maximum-likelihood analysis of DNA substitution models.—Prior to estimating a topology, an optimal DNA substitution model for analysis of the cyt *b* was selected by using maximum likelihood.

A hierarchy of candidate DNA substitution models was constructed based upon three substitution matrices and four models of among-sites rate variation. Substitution matrices included the F81 matrix (Felsenstein, 1981), the HKY85 matrix (Hasegawa et al., 1985), and the GTR matrix (Yang, 1994a). Models of among-sites rate variation were (1) equal rates among sites (Poisson model); (2) Poisson model plus a correction for invariant sites (Poisson + INV model; Gu et al., 1995); (3) variable rates among sites, modeled using a discrete approximation to the Gamma distribution (Gamma model; Yang, 1994b); and (4) variable rates among sites, plus a correction for invariant sites (Gamma + INV model).

Likelihood ratio tests (Goldman, 1993) were used to identify a candidate model that provided maximum explanatory power using the fewest parameters. Models were tested in a pairwise fashion and in a two-stage process. The first stage was to test substitution matrices in order of increasing complexity (F81 vs HKY85 and HKY85 vs GTR). The second stage was to test the relative fit of each models of among-sites rate variation by using the optimal substitution matrix. The chi-square distribution was used to evaluate the gain in likelihood associated with using a more complex model (Yang et al., 1995).

Maximum-likelihood scores were computed for each model by using PAUP*. With the exception of nucleotide frequencies, all parameter values for a given model, including the topology, were estimated using maximum likelihood. Computational limitations prevented estimation of substitution parameters concurrently with estimation of a tree for all 24 taxa; hence, models were evaluated using subsets of taxa. Ingroup taxa were placed randomly into two subsets of taxa, whereas outgroups were placed into a third subset. Subsets of taxa were composed as follows: Set 1—*N. ariommus, N. atherinoides, N. candidus, N. girardi, N. jemezanus, N. photogenis, N. rubellus,* and *N. stilbius*; Set 2—*N. amabilis, N. amoenus, N. oxyrhynchus, N, perpallidus, N. shumardi, N. scepticus, N. suttkusi,* and *N. telescopus*; and Set 3—*N. boops, N. chrosomus, N. edwardraneyi N. longirostris, N. nubilus, N. potteri, N. texanus,* and *N. volucellus.*

Phylogenetic analysis.—Phylogenetic analyses were carried out by using a test version of PAUP*. Heuristic searches under maximum parsimony (unweighted) were performed with the tree bisection and reconnection branch swapping (TBR) algorithm. TBR branch swapping was performed on 100 starting trees obtained using random addition of sequences. Heuristic maximum-likelihood analysis was carried out using the DNA substitution model that provided the best fit to these cyt *b* sequences. With the complete set of taxa, computational constraints prevented estimation of substitution parameters during tree search; consequently, approximate parameter values were used (Yang, 1996; Yang et al., 1994, 1995). Approximate parameter values were obtained by maximum-likelihood estimation, using the best-fit model and an approximate topology. The latter was generated via neighbor-joining analysis (Saitou and Nei, 1987) of a GTR distance matrix.

Because of controversy over the sister group to subgenus *Notropis* (Coburn, 1982; Mayden, 1989; Coburn and Cavender, 1992), eight species from other lineages of genus *Notropis* were sampled for use as outgroups. These included representatives from subgenera *Alburnops* and *Hydrophlox,* and the *N. dorsalis, N. texanus,* and *N. volucellus* species groups (set 3). Based upon characters associated with osteology of the skull, M. M. Coburn (pers. comm.) hypothesized that subgenus *Alburnops* is the most basal of these lineages within genus *Notropis.* Consequently, *N. edwardraneyi* and *N. potteri* (subgenus *Alburnops*) were employed as outgroups to the remaining *Notropis.*

Relative support for individual nodes of a topology was assessed by nonparametric bootstrapping (Felsenstein, 1985; Penny and Hendy, 1985). Bootstrap proportions (P_B), obtained using the maximum-parsimony criterion, were based on 1000 pseudoreplications. When using the maximum-likelihood criterion, bootstrap analysis is necessarily constrained by computational limitations; hence, P_B was estimated (100 pseudoreplications) by fixing substitution parameters to their maximum likelihood values. Statistical tests of alternative hypotheses were conducted using the method of Kishino and Hasegawa (1989).

Effects of individual outgroup taxa on estimation of a topology for ingroup taxa were investigated by jackknifing (Lanyon, 1985), that is, each outgroup taxon was excluded singly and maximum-parsimony and maximum-likelihood analysis was carried out on the reduced dataset. The Robinson and Foulds (1981) tree metric was used to summarize differences between topologies. The value of this tree metric indicates the number of collapsed and reassembled branches required to convert one topology into another. Impact of outgroup taxa also was investigated by comparing topologies generated using only ingroup taxa to those obtained using all ingroup and outgroup taxa. Topologies ob-

TABLE 1. LOG-LIKELIHOOD SCORES FOR DIFFERENT MODELS OF DNA SUBSTITUTION

Models of among-sites rate variation	Substitution matrix		
	F81	HKY85	GTR
Set 1 (l_{max} = −3355.9)			
1—Poisson	−4475.7	−4161.2	−4129.5
2—Poisson + INV	−4279.5	−3913.8	−3884.7
3—Gamma	−4281.3	−3906.3	−3881.9
4—Gamma + INV	−4279.5	−3905.3	−3884.9
Set 2 (l_{max} = −3329.4)			
1—Poisson	−4248.0	−3940.3	−3913.1
2—Poisson + INV	−4104.5	−3761.3	−3732.4
3—Gamma	−4104.6	−3752.6	−3729.8
4—Gamma + INV	−4102.7	−3748.7	−3730.2
Set 3 (l_{max} = −3488.14)			
1—Poisson	−4694.0	−4382.7	−4354.2
2—Poisson + INV	−4457.1	−4098.8	−4072.7
3—Gamma	−4459.5	−4091.1	−4070.6
4—Gamma + INV	−4457.1	−4089.3	−4070.4

Note: F81 is the substitution matrix of Felsenstein (1981); HKY85 is the substitution matrix of Hasegawa et al. (1985); and GTR is the general-time-reversible substitution matrix of Yang (1994a). l_{max} indicates the log likelihood score of a dataset under the unconstrained multinomial model. Species composition in Sets 1–3 is given in text.

tained from analyses that excluded outgroup taxa were rooted a posteriori to tree selection. The unrooted topology for ingroup taxa was specified as a "backbone" constraint upon which location of outgroups was estimated by using either parsimony or likelihood criteria.

RESULTS

Sequence variation.—There were 368 variable positions among the nominal species of subgenus *Notropis* and 400 variable positions among all species. Average percent nucleotide sequence divergence, excluding conspecific comparisons, between nominal members of subgenus *Notropis* was 11 ± 2% (average ± SD). Average percent sequence divergence among outgroups was 13 ± 2% (average ± SD). Nucleotide sequence variation within subgenus *Notropis* was predominantly at synonymous sites (96%), most of which (88%) were at third codon positions. Patterns of variation among codon positions followed those expected for protein-coding sequences sampled from closely related taxa (Kocher and Carleton, 1997), that is, sequence divergence within subgenus *Notropis* was 2.6 ± 1.1%, 0.34 ± 0.2%, and 31.1 ± 6.3% (average ± SD) at first, second, and third codon positions, respectively. Average amino acid sequence difference among all species from genus *Notropis* was 1.1 ± 0.6% (average ± SD).

With one exception, overall patterns of nucleotide composition were similar among all sampled species. Nucleotide composition among nominal species of subgenus *Notropis* was homogenous at all three codon positions: first positions, χ^2 = 0.88, 45 df, P = 1.0; second positions, χ^2 = 0.21, 45 df, P = 1.0; and third positions, χ^2 = 45.4, 45 df, P = 0.46. Nucleotide composition among outgroup taxa, however, exhibited significant heterogeneity at third codon positions: first positions, χ^2 = 2.19, 21 df, P = 1.0; second positions, χ^2 = 0.07, 21 df, P = 1.0; and third positions, χ^2 =40.2, 21 df, P = 0.007.

Maximum-likelihood analysis of DNA substitution models.—Likelihood ratio tests were based on log-likelihood scores for each candidate model (Table 1). Regardless of the set of taxa or model of among-sites rate variation, the optimal substitution matrix was the GTR matrix (Table 2). The fit of the GTR matrix was improved by modeling variation in substitution rates among sites (Table 3). However, the Gamma model did not provide significantly better fit to cyt *b* than did correcting for the presence of conserved sites (Poisson + INV; Table 3). Based on the foregoing, we employed the GTR substitution matrix (Yang, 1994a) combined with a correction for invariant sites.

Phylogenetic analysis of ingroup and outgroup taxa.—Maximum-parsimony analysis yielded a single most-parsimonious tree with a length of 1676 steps (Fig. 1). Bootstrap analysis indicated very strong support (P_B = 99%) for monophyly of *N. rubellus, N. suttkusi, N. amoenus, N. stilbius, N. atherinoides, N. oxyrhynchus, N. amabilis,* and

TABLE 2. LIKELIHOOD RATIO TESTS OF DIFFERENT SUBSTITUTION MATRICES.

Model of among-sites rate variation	F81 vs HKY85	HKY85 vs GTR
	df = 1, $\chi^2_{0.05}$ = 7.48[a]	df = 4, $\chi^2_{0.05}$ = 12.76[a]
Set 1		
1—Poisson	2δ = 629.0*	2δ = 63.4*
2—Poisson + INV	2δ = 731.4*	2δ = 58.2*
3—Gamma	2δ = 750.0*	2δ = 48.8*
4—Gamma + INV	2δ = 748.4*	2δ = 40.8*
Set 2		
1—Poisson	2δ = 615.4*	2δ = 45.4*
2—Poisson + INV	2δ = 686.4*	2δ = 57.8*
3—Gamma	2δ = 704.0*	2δ = 45.6*
4—Gamma + INV	2δ = 708.0*	2δ = 37.0*
Set 3		
1—Poisson	2δ = 622.6*	2δ = 57.0*
2—Poisson + INV	2δ = 716.6*	2δ = 52.2*
3—Gamma	2δ = 736.8*	2δ = 41.0*
4—Gamma + INV	2δ = 735.6*	2δ = 37.0*

Note: F81 is the substitution matrix of Felsenstein (1981); HKY85 is the substitution matrix of Hasegawa et al. (1985); and GTR is the general-time-reversible substitution matrix of Yang (1994a). Species composition of Sets 1–3 is given in text. Asterisks (*) indicate a significant gain in likelihood.

[a] Critical value of chi-square is Bonferroni corrected for eight contrasts.

N. jemezanus (Fig. 1). Within this clade there was bootstrap support for monophyly of *N. amoenus, N. stilbius, N. atherinoides, N. oxyrhynchus, N. amabilis,* and *N. jemezanus* (P_B = 69%); monophyly of *N. atherinoides, N. amabilis, N. jemezanus,* and *N. oxyrhynchus* (P_B = 52%); a sister-group relationship between *N. amabilis* and *N. jemezanus* (P_B = 70%); a sister-group relationship between *N. atherinoides* and *N. oxyrhynchus* (P_B = 98%); and a sister-group relationship between *N. suttkusi* and *N. rubellus* (P_B = 100%; Fig. 1). There also was strong support (P_B = 100%) for a sister group relationship between *N. candidus* and *N. shumardi*; however this clade was placed sister to *N. texanus* (*N. texanus* species-group). Although not strongly supported by the bootstrap analysis, placement of the remaining species traditionally considered members of subgenus *Notropis* (*N. perpallidus, N. photogenis, N. telescopus, N. scepticus,* and *N. ariommus*) also suggested closer relationship to species previously not considered members of subgenus *Notropis* (Fig. 1).

Maximum-likelihood analysis yielded three trees of equal likelihood ($-ln$ L = 8016.3); the strict consensus of these three trees is presented in Figure 2. In contrast to maximum-parsimony analysis, maximum-likelihood analysis indicated monophyly (P_B = 51%) of all species traditionally placed in subgenus *Notropis* examined except *N. shumardi* and *N. candidus* (Fig. 2). Bootstrap analysis indicated strong support (P_B = 100%) for the monophyly of *N. rubellus, N. suttkusi, N. amoenus, N. stilbius, N. atherinoides, N. oxyrhynchus, N. amabilis,* and *N. jemezanus* (Fig. 2). Within this clade, there was support for the same relationships supported by bootstrap analysis of the maximum-parsimony topology (Fig. 2). Placement of *N. amoenus* and *N. stilbius* also

TABLE 3. LIKELIHOOD RATIO TESTS OF DIFFERENT MODELS OF AMONG-SITES RATE VARIATION (ASRV).

GTR matrix + ASRV model	2δ	df	$\chi^2_{0.05}$[a]
Taxa Set 1 (ingroup)			
Poisson vs. Poisson + INV	489.6*	1	6.24
Poisson vs. Gamma	495.6*	1	6.24
Gamma vs. Gamma + INV	[b]	1	6.24
Poisson + INV vs. Gamma + INV	[b]	1	6.24
Taxa Set 2 (ingroup)			
Poisson vs. Poisson + INV	361.4*	1	6.24
Poisson vs. Gamma	366.6*	1	6.24
Gamma vs. Gamma + INV	[b]	1	6.24
Poisson + INV vs. Gamma + INV	4.4	1	6.24
Taxa Set 3 (outgroup)			
Poisson vs. Poisson + INV	563.0*	1	6.24
Poisson vs. Gamma	567.2*	1	6.24
Gamma vs. Gamma + INV	4.6	1	6.24
Poisson + INV vs. Gamma + INV	0.4	1	6.24

Note: GTR is the general-time-reversible substitution matrix (Yang, 1994a). Species composition of Sets 1–3 is given in text. Asterisks (*) indicate a significant increase in likelihood.

[a] Critical value of chi-square is Bonferroni corrected for four contrasts.

[b] Increasing the complexity of the model did not yield an increase in likelihood score.

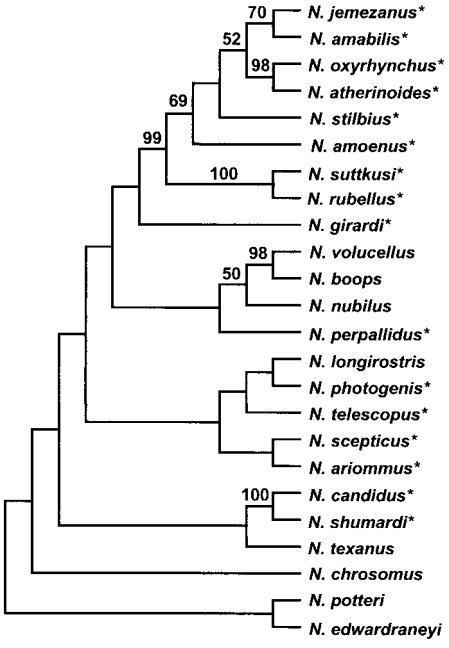

Fig. 1. Single most-parsimonious tree (1676 steps) derived from maximum-parsimony analysis of complete cyt *b* sequences (CI = 0.34; RI = 0.37). Numbers above nodes represent nonparametric bootstrap proportions (1000 replications). Bootstrap proportions less than 50% are not shown. Asterisks (*) indicate hypothesized members of subgenus *Notropis*.

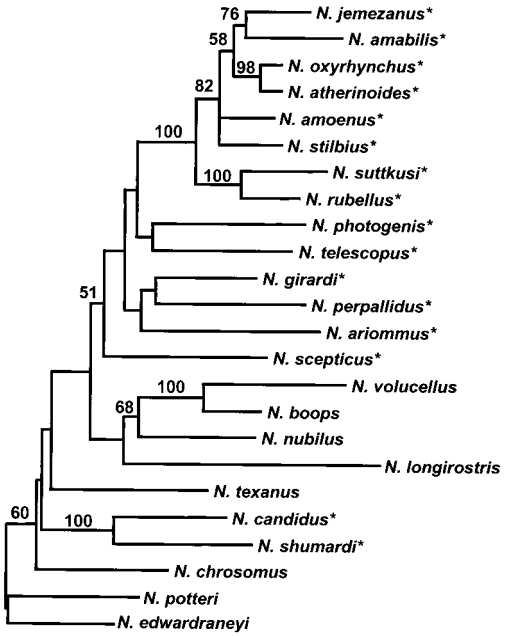

Fig. 2. Optimal topology ($-lnL = 8016.3$) recovered from maximum-likelihood analysis of cyt *b* sequences, using the GTR substitution matrix combined with a correction for invariant sites. Numbers above nodes are bootstrap proportions (100 replications). Bootstrap proportions less than 50% are not shown. Asterisks (*) indicate hypothesized members of subgenus *Notropis*.

could not be resolved using maximum likelihood (Fig. 2). There was strong support for a sister-group relationship between *N. candidus* and *N. shumardi,* although in the maximum-likelihood topology this clade was not sister to *N. texanus* (Fig. 2).

The method of Kishino and Hasegawa (1989) was used to test the hypothesis that subgenus *Notropis,* excluding *N. shumardi* and *N. candidus,* provided a significantly better fit to cyt *b* sequences than did a subgenus *Notropis* that included *N. candidus* and *N. shumardi.* Under maximum parsimony, the best tree consistent with a monophyletic subgenus *Notropis* (including *N. candidus* and *N. shumardi*) resulted in a significant decrease in fit to cyt *b* sequences relative to the topology shown in Figure 1 (length difference = 26, $t = 2.02$, $P < 0.05$). Retesting the best tree consistent with a monophyletic subgenus *Notropis,* excluding *N. candidus* and *N. shumardi,* did not indicate a significant difference relative to the most-parsimonious tree (length difference = 17, $t = 1.25$, $P > 0.05$). This result indicates significant support under the maximum-parsimony criterion for exclusion of only *N. candidus* and *N. shumardi* from subgenus *Notropis.* Under maximum likelihood, the best tree consistent with a monophyletic subgenus *Notropis,* including *N. candidus* and *N. shumardi,* provided a significant decrease in fit to cyt *b* relative to a monophyletic subgenus *Notropis* where *N. candidus* and *N. shumardi* were excluded ($\Delta l = 21.8$, $t = 2.14$, $P < 0.05$). Our finding that subgenus *Notropis* does not include *N. shumardi,* and *N. candidus* does not depend on the assumed location of the root, as no alternative location for the root could yield a monophyletic subgenus *Notropis.*

Phylogenetic analyses also were carried out using multiple representatives of the following taxa: *N. atherinoides, N. photogenis, N. rubellus, N. scepticus, N. stilbius, N. suttkusi,* and *N. telescopus* (see Materials Examined for locality information). Results of analyses where multiple representatives of these species were used were similar to those obtained by using a single representative of each species (data not shown).

Relative rates of evolution among taxa.—Homogeneity in rate of nucleotide substitution was investigated by using the maximum-likelihood consensus topology for all ingroup and outgroup taxa (Fig. 2). Rate homogeneity was re-

TABLE 4. ROBINSON AND FOULDS (1981) TREE DISTANCE BETWEEN TOPOLOGIES OBTAINED USING ALL OUTGROUP TAXA AND TOPOLOGIES OBTAINED BY JACKKNIFING OUTGROUP TAXA.

Outgroup taxon excluded in jackknife analysis	Maximum parsimony	Maximum likelihood
N. boops	0	0
N. chrosomus	14	8
N. edwardraneyi	12	0
N. longirostris	10	8
N. nubilus	7	8
N. potteri	10	0
N. texanus	7	0
N. volucellus	0	0

jected either when using members of subgenus *Alburnops* to place the root ($2\delta = 86.4$, $P < 0.05$) or the more conservative maximum-likelihood estimate of the root under the assumption of a molecular clock ($2\delta = 53.32$, $P < 0.05$). When using the more conservative location for the root, four of the six largest deviations from clocklike expectations were associated with the outgroup taxa *N. boops, N. longirostris, N. nubilus,* and *N. volucellus*. When using members of subgenus *Alburnops* to place the root, the six largest deviations from clocklike expectations were associated with the outgroup taxa *N. boops, N. chrosomus, N. edwardraneyi, N. longirostris, N. texanus,* and *N. volucellus*.

Effects of outgroups on ingroup topology.—Jackknifing outgroup taxa, followed by maximum-parsimony and maximum-likelihood analysis, revealed different levels of sensitivity to outgroup sampling. Maximum parsimony was the more sensitive, as greater differences in topology were observed among jackknifed datasets (Table 4). Maximum-likelihood topologies, however, also exhibited dependence upon outgroup sampling (Table 4). Despite sensitivity of both phylogenetic analyses to outgroup taxa, *N. candidus* and *N. shumardi* consistently were placed outside of a clade comprised of the remaining species of subgenus *Notropis,* that is, placement of *N. candidus* and *N. shumardi* outside of subgenus *Notropis* was not dependent on any one outgroup taxon. Furthermore, under maximum likelihood, monophyly of a subgenus *Notropis* that excluded *N. candidus* and *N. shumardi* was not contradicted by jackknifing outgroup taxa.

Topologies obtained from phylogenetic analysis after exclusion of outgroup taxa, *N. candidus,* and *N. shumardi* are shown in Figures 3 and 4. Under maximum parsimony, exclusion of these taxa resulted in a general decrease in resolution: Relationships of *N. ariommus, N. girardi, N. perpallidus,* and *N. scepticus* were not resolved, and there was a loss of resolution relative to placement of *N. amoenus* and *N. stilbius* (Fig. 3). The maximum-likelihood topology generated after exclusion of outgroup taxa, *N. candidus* and *N. shumardi,* exhibited no loss of resolution but differed from that previously obtained (using all taxa) in placement of *N. photogenis* and *N. scepticus* (Fig. 4). Relationships previously supported by bootstrap analysis were unaffected by exclusion of taxa.

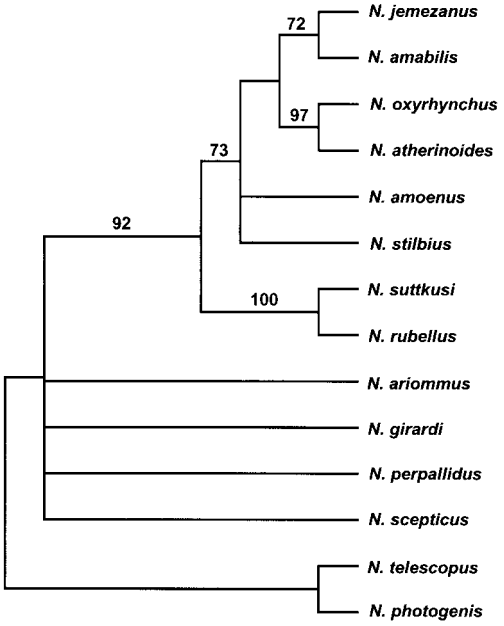

Fig. 3. Strict consensus of six equally parsimonious trees (857 steps) derived from maximum-parsimony analysis of cyt *b* sequences of 14 species of subgenus *Notropis* (CI = 0.50; RI = 0.38). Numbers above nodes represent nonparametric bootstrap proportions (1000 replications). Bootstrap proportions less than 50% are not shown. Location of the root was inferred a posteriori to tree selection.

DISCUSSION

Phylogenetic affinities of Notropis candidus and Notropis shumardi.—Both maximum parsimony and maximum-likelihood analysis of all 24 species of genus *Notropis* examined were incompatible with a monophyletic subgenus *Notropis* (sensu Coburn, 1982). Maximum-likelihood analysis, however, supported monophyly of a clade comprised of all species of subgenus *Notropis* examined except for *N. candidus* and *N. shumardi*. Jackknife analysis under maximum likelihood indicated that exclusion of *N. candidus* and *N.*

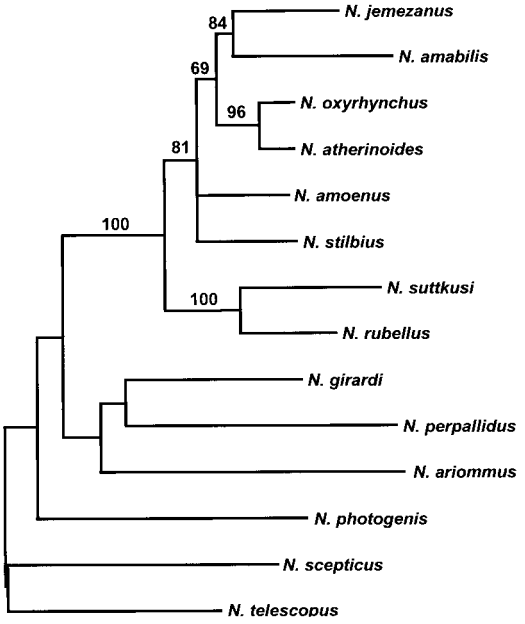

Fig. 4. Optimal topology ($-ln$L = 5037.5) recovered from maximum-likelihood analysis of cyt *b* sequences of 14 species of subgenus *Notropis*. The model used was the GTR matrix combined with a correction for invariant sites. Numbers above nodes represent bootstrap proportions (100 replications). Bootstrap proportions less than 50% are not presented. Location of the root was inferred a posteriori to tree selection.

shumardi from a monophyletic subgenus *Notropis* was not dependent on any individual outgroup taxon. Although maximum-parsimony analysis (and bootstrap analysis) supported monophyly of only eight species of subgenus *Notropis,* K-H tests indicated that only exclusion of *N. candidus* and *N. shumardi* was supported under the parsimony criterion. These results indicate that *N. candidus* and *N. shumardi* should be excluded from subgenus *Notropis*. Exclusion of *N. shumardi* from subgenus *Notropis* is not without precedent, as Snelson (1968) suggested that *N. shumardi* might be more closely related to members of subgenus *Alburnops*. The cyt *b* data, however, do not indicate a relationship between *N. candidus* and *N. shumardi* and the two members of subgenus *Alburnops* (*N. edwardraneyi* and *N. potteri*) examined in this study. Alternatively, relationships among outgroup taxa were not strongly supported by cyt *b* sequences, and resolution of their relationships will require sampling additional DNA sequences or additional taxa.

There was strong support in both maximum-parsimony and maximum-likelihood analysis for a sister-group relationship between *N. candidus* and *N. shumardi*. This was not surprising because *N. candidus* formerly was considered to be a form of *N. shumardi* in the Mobile Bay basin [subsequently elevated by Suttkus (1980) to species status]. These two species typically inhabit turbid waters of main channels of large rivers (Gilbert and Bailey, 1962; Suttkus, 1980); *N. candidus* is endemic to the Mobile Bay drainage of Alabama and Mississippi (Suttkus, 1980), whereas *N. shumardi* is widespread in major tributaries of the Mississippi basin and drainages of the Texas Gulf coastal slope (Lee et al., 1980). The allopatric distributions and sister-group relationship of these two species suggests a historical drainage connection between the Mississippi Basin and the Mobile Bay drainage. Mayden (1987) noted that the Mobile Bay included relatives of species present in the Mississippi Basin and hypothesized that divergence between species in the two regions was initiated by splitting of a connection between the Tennessee River and Mobile Bay drainage. Results of this study are consistent with this hypothesis. Moreover, *N. candidus* and *N. shumardi* exhibit distribution patterns that are nearly identical to those of two other big-river species pairs: *Scaphirhynchus platorhynchus* and *Scaphirhynchus suttkusi* (Williams and Clemmer, 1991), and *N. edwardraneyi* and *N. blennius* (Suttkus and Clemmer, 1968). Although the specific nature and timing of a historical connection between the Tennessee River and Mobile Bay systems remains controversial (Starnes and Etnier, 1986), the relationships between *N. candidus* and *N. shumardi* and the other big-river species pairs supports the notion that the connection probably involved a large river (Mayden, 1987, 1988).

Phylogenetic analysis of subgenus Notropis.—Exclusion of *N. candidus* and *N. shumardi* from subgenus *Notropis* raises the question of monophyly of the remaining 14 species currently placed in the subgenus. Maximum parsimony and nonparametric bootstrapping indicated monophyly of a subset (eight species of subgenus *Notropis*). K-H tests, however, indicated that, after exclusion of *N. candidus* and *N. shumardi,* relationships suggested by maximum parsimony provided no better fit to the data than monophyly of the remaining 14 species of subgenus *Notropis*. Alternatively, maximum likelihood supported monophyly of the remaining 14 species of subgenus *Notropis*. Jackknife analysis indicated that this hypothesis was unaffected by any one outgroup taxon.

Jackknife analysis also indicated that both maximum-parsimony and maximum-likelihood estimates of relationships among species of sub-

genus *Notropis* were sensitive to sampling of outgroup taxa. Swofford et al. (1996) suggested that sensitivity of a topology to the most divergent taxa (i.e., outgroups) in phylogenetic analysis is an indication that systematic error is influencing the analysis. Analysis of cyt *b* data revealed heterogeneity in both nucleotide composition and rate of substitution among outgroup taxa. These two are both potential sources of systematic error (Kuhner and Felsenstein, 1994; Lockhart et al., 1994), and the greater sensitivity exhibited by maximum parsimony to jackknifing is consistent with its presumed greater sensitivity to the negative affects of systematic error as compared to maximum likelihood (Huelsenbeck, 1995). Consequently, differences between maximum-parsimony and maximum-likelihood topologies when all taxa were used may be the result of differences in sensitivity to heterogeneity in nucleotide composition and substitution rate among the outgroup taxa, that is, to systematic error.

Regardless of the method of analysis (maximum parsimony or maximum likelihood) or use of outgroup taxa, phylogenetic analysis of cyt *b* indicated strong support for the following hypothesis of relationships: ((*N. rubellus, N. suttkusi), (N. amoenus, N. stilbius,* ((*N. atherinoides, N. oxyrhynchus*), (*N. amabilis, N. jemezanus*)))). This hypothesis is largely incompatible with relationships hypothesized by Coburn (1982). For example, taxa placed by Coburn (1982) in *N. atherinoides* species group (*N. atherinoides* and *N. amabilis*), *N. shumardi* species group *(N. oxyrhynchus* and *N. jemezanus*), and *N. photogenis* species group (*N. amoenus*) were placed together based on phylogenetic analysis of cyt *b* sequences. However, the phylogenetic hypothesis generated from cyt *b* sequences is consistent with Amemiya and Gold (1990) and Coburn and Cavender (1992) in placing *N. oxyrhynchus* (formerly of the *N. shumardi* species-group) as sister to *N. atherinoides,* and Dowling and Brown (1989) and Coburn and Cavender (1992) in placing *N. rubellus* and *N. girardi,* respectively, in subgenus *Notropis.* Placement of *N. girardi* by Coburn and Cavender (1992) as a relative of *N. candidus, N. shumardi,* and *N. jemezanus* was not supported by analysis of cyt *b* sequences.

Coburn and Cavender (1992) placed *N. shumardi, N. candidus, N. jemezanus,* and *N. girardi* into a clade based on a pronounced enlargement of the olfactory apparatus. Except for *N. oxyrhynchus,* which possesses a moderately enlarged olfactory apparatus, all other species of subgenus *Notropis* possess relatively small olfactory apparatus (Coburn, 1982, pers. comm.). With the exception of *N. candidus* and *N. shumardi,* none of the species with an enlarged olfactory apparatus were closely related in any of the phylogenies inferred from cyt *b* sequences. Independent development of an enlarged olfactory apparatus in *N. jemezanus, N. oxyrhynchus,* and *N. girardi* within subgenus *Notropis,* and in *N. candidus* and *N. shumardi* outside of subgenus *Notropis,* clearly suggests that increased olfactory capabilities have arisen repeatedly in the evolution of these species. Huber and Rylander (1992) conducted a detailed analysis of relationships among various aspects of brain morphology and turbidity of preferred habitat of North American cyprinids and found that enlargement of brain structures associated with olfaction (olfactory bulb and facial lobe) was correlated with turbidity of preferred habitat. They hypothesized that species adapted to turbid environments should possess increased taste, hearing, and olfaction capabilities relative to species adapted to clearer waters. Interestingly, *N. jemezanus, N. oxyrhynchus,* and *N. girardi* (species that possess an enlarged olfactory apparatus) tend to prefer more turbid waters (Huber and Rylander, 1992). With the exception of *N. atherinoides,* which can be found in water of varying turbidity, all other species of subgenus *Notropis* generally prefer waters of low turbidity (Lee et al., 1980; Huber and Rylander, 1992) and possess a relatively small olfactory apparatus.

It appears that homoplasy associated with specialization for life in turbid habitats is in part responsible for the conflict between morphological and molecular hypotheses of relationships for subgenus *Notropis.* Enlargement of the olfactory apparatus could be viewed as a trophic specialization, as it could enhance food capture, or at least food identification, particularly in turbid waters. Interestingly, replicated patterns of trophic specialization have lead to significant conflict between morphological and molecular hypotheses in other fish lineages (Martin and Birmingham 1998; Rüber et al. 1999; Hanel and Sturmbauer, 2000). Collectively, these findings question the general utility of morphological characters associated with trophic specialization for inferring phylogenies.

Materials Examined

Traditional taxonomy {in braces}, collection localities, drainages (in parentheses), and catalog number of the voucher specimen [in brackets] are listed below. Where required, all collections were made under valid scientific collecting permits. Specimens of *N. girardi* were collected in 1984 prior to being listed (federally threatened) in 1998. Voucher specimens for most col-

lection localities were deposited in the Texas Cooperative Wildlife Collection (TCWC) at Texas A&M University. *Notropis amabilis* {subgenus *Notropis*}, Johnson Creek, Texas, (Neches River), [TCWC: 10865.01]; *N. amoenus* {subgenus *Notropis*}, Deep River, North Carolina, (Cape Fear), [TCWC: 10864.01]; *N. ariommus* {subgenus *Notropis*}, Clinch River, Tennessee, (Clinch River), [TCWC:8295.03]; *N. atherinoides* {subgenus *Notropis*}, Cahaba River, Alabama, (Cahaba River), [TCWC:8291.01]; *N. atherinoides* {subgenus *Notropis*}, Blue River, Oklahoma, (Red River), *N. atherinoides* {subgenus *Notropis*}, Huron River, Michigan, (Huron River); *N. atherinoides* {subgenus *Notropis*}, Red River, Oklahoma, (Red River); *N. boops* {*N. texanus* species group}, Briar Creek, Oklahoma, (Red River), [TCWC: 10863.01]; *N. candidus* {subgenus *Notropis*}, Black Warrior River, Alabama, (Black Warrior River), [TCWC:7989.01]; *N. chrosomus* {subgenus *Hydrophlox*}, Gurley Creek, Alabama, (Black Warrior River), [TCWC: 7248.01]; *N. edwardraneyi* {subgenus *Alburnops*}, Black Warrior River, Alabama, (Black Warrior River), [TCWC: 7988.01]; *N.* girardi {subgenus *Notropis*}, South Canadian River, Oklahoma, (Arkansas River) [TCWC:7663.01]; *N. jemezanus* {subgenus *Notropis*}, Pecos River, New Mexico, (Pecos River), [TCWC:10867.01] *N. longirostris* {*N. dorsalis* species group}, Red Creek, Mississippi, (Pascagoula River), [TCWC: 10859.01]; *N. nubilus* {subgenus *Hydrophlox*}, Lee Creek, Arkansas, (Arkansas River); *N. oxyrhynchus* {subgenus *Notropis*}, Brazos River, Texas, (Brazos River), [TCWC:10862.01]; *N. perpallidus* {subgenus *Notropis*}, Kiamichi River, Oklahoma (Red River) [TCWC:8432.01]; *N. photogenis* {subgenus *Notropis*}, Scioto River, Ohio, (Scioto River); *N. photogenis* {subgenus *Notropis*}, Clinch River, Tennessee, (Clinch River); *N. potteri* {subgenus *Alburnops*}, Red River, Oklahoma, (Red River) [TCWC:8433.02]; *N. rubellus* {subgenus *Notropis*}, Susquehanna River, New York, (Susquehanna River); *N. rubellus* {subgenus *Notropis*}, Loch Alpine Creek, Michigan, (Huron River); *N. scepticus* {subgenus *Notropis*}, Deep River, North Carolina, (Cape Fear), [TCWC:7981.02]; *N. scepticus* {subgenus *Notropis*}, Second Broad River, North Carolina, (Santee River), [TCWC: 7983.02]; *N. shumardi* {subgenus *Notropis*}, Brazos River, Texas, (Brazos River), [TCWC: 10862.02]; *N. stilbius* {subgenus *Notropis*}, Cahaba River, Alabama, (Cahaba River), [TCWC: 10860.02]; *N. stilbius* {subgenus *Notropis*}, Schultz Creek, Alabama, (Cahaba River); *N. suttkusi* {subgenus *Notropis*}, Blue River, Oklahoma, (Red River), [TCWC:10861.01]; *N. suttkusi* {subgenus *Notropis*}, Kiamichi River, Oklahoma, (Red River), [TCWC:8429.02]; *N. telescopus* {subgenus *Notropis*}, Clinch River, Tennessee, (Clinch River), [TCWC:8295.04]; *N. telescopus* {subgenus *Notropis*}, North Fork, Holsten River, Virginia, (Holsten River); *N. texanus* {*N. texanus* species-group}, Wolf River, Mississippi, (Gulf River), [TCWC:8289.02]; *N. volucellus* {*N. volucellus* species group}, Cahaba River, Alabama, (Cahaba River), [TCWC:10860.01]. Nucleotide sequences were deposited in GenBank (AF352261–AF352290).

Acknowledgments

We thank R. E. Broughton, R. C. Cashner, T. E. Dowling, D. A. Etnier, W. M. Howell, W. J. Matthews, and W. C. Starnes for assistance in obtaining the species examined in this study and K. Vaughn for assistance with cataloging voucher specimens. We also thank T. E. Dowling for kindly providing cytochrome *b* sequences of two representatives of *Notropis rubellus*; L. R. Richardson, L. B. Stewart, L. R. Cervantez, and R. A. Dworak for laboratory assistance; K. A. Dunn, R. L. Honeycutt, J. Rice, A. Rooney, D. L. Swofford, T. F. Turner, T. Ward, and Z. Yang for valuable discussions; and D. Rowe for valuable assistance in a variety of areas. We are grateful for the comments of three anonymous reviewers. Research was supported in part by a National Science Foundation Doctoral Dissertation Improvement Grant (DEB-9700717), in part by a Thomas Slick Research Fellowship, and in part by the Texas Agricultural Experimental Station under Project H-6703. This paper represents contribution number 95 of the Center for Biosystematics and Biodiversity at Texas A&M University.

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