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# An awkward introduction: phylogeography of *Notropis lutipinnis* in its 'native' range and the Little Tennessee River

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Abstract – We evaluate the putative introduction of the yellowfin shiner, *Notropis lutipinnis*, in the Little Tennessee river basin. This species has only been noted in the Little Tennessee in the past several decades and appears to be expanding its range, even though there have been many potential historical pathways for dispersal from native drainages in Georgia, South Carolina, and North Carolina. We use a phylogeographic approach, examining sequence data from one mitochondrial and one nuclear locus, to determine the likely source of the population in the Little Tennessee. Our results suggest a complex history and cannot reject the possibility that *N. lutipinnis* is native to the Little Tennessee. Our data also indicate that particular drainages, including populations in the Altamaha and Flint Rivers, may be subject to local adaptation at the nuclear transferrin locus.

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Key words: *Notropis*; phylogeography; species introduction; transferrin; molecular evolution

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Species introductions represent one of the primary threats to the preservation of biodiversity. This threat occurs via both biological impacts on native species and by the diversion of resources from management agencies for study and control of invasive species (Pimentel et al. 2005). Although some species introductions are accidental, many introductions of fish species have been intentional with the goal of increased angling opportunities. Thus, the rainbow trout (*Oncorhynchus mykiss*) is one of the most widely distributed fish species – found on at least five continents – and has detrimentally affected a number of the systems it has been introduced to (Fausch et al. 2001). Additionally, fish may be introduced to a location via 'bait-bucket' releases when anglers transport potential bait fish from one drainage to another. This widespread anthropogenic redistribution of species may complicate the identification of biogeographic patterns and processes, particularly when purposeful (and thus documented) introductions are mixed with accidental ones, such as the accidental introduction of bigscale logperch (*Percina macrolepidota*) with largemouth bass (*Micropterus salmoides*) in northern California (Moyle 2002).

Fish species with unusual distributions, for example, those that are found in multiple river drainages thought to be long separated, are often considered to be introduced members of at least one drainage community. Identification of the source of a species introduction is then useful for management as well as biogeographic study because only the long-term members of the community are useful for reconstructing such geographic relationships (Mayden 1988). A variety of genetic methods can be used to identify

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source populations (Wares et al. 2005), but this can be difficult because it may require extensive sampling of remote regions (Ruiz et al. 2000) or the invasive population may comprise individuals from multiple sources (Kolbe et al. 2004). Consequently, the identification of source populations for introductions, especially fishes, is often unsuccessful. The probability of success may be increased where the putative introduction is on a regional – rather than global – scale, and such cases may be useful for developing improved techniques for reconstructing the sources and propagule pressure in an introduction.

The yellowfin shiner (*Notropis lutipinnis*; Jordan and Brayton, 1878) is native to Gulf and Atlantic slope drainages of the southeastern USA, principally the major drainages of Georgia and South Carolina (Wood & Mayden 1992). Within this range it is abundant and widely distributed. In the Little Tennessee drainage, however, this species has been considered 'introduced' for the past 40 years (Ramsey 1965; Johnston et al. 1995). Evidence for this assessment was based initially on its absence from museum collections (C. Johnston, pers. comm.) and its appearance in the 1990s in mainstem tributaries (W. McLarney, pers. comm.). This species is a good candidate for detection of the introduction source, because all potential populations are within ~300 km of the Little Tennessee sites where it has been collected. The true status of yellowfin shiner in the Little Tennessee drainage is uncertain for a variety of reasons. First, some species considered 'native' to the Little Tennessee are also found in rivers within the established native distribution of yellowfin shiners, whereas others with similar distributions are considered 'introduced' (Ramsey 1965; Swift et al. 1986). In addition, the literature may be contradictory, reporting some of these species as both native and introduced (Johnston et al. 1995, North Carolina Division of Water Quality 2007– see http://h20.enr.state.nc.us/esb/BAU.html). Consequently, clarification of the historical ecology of *N. lutipinnis* may be useful for examining the combined history of vicariance and stream capture that has shaped the southeastern aquatic biota.

Because of the relatively ancient geologic and hydrologic processes affecting biodiversity patterns in the southeastern USA, a phylogeographic approach to studying species introduction can be applied to establish the background pattern of genetic variation and differentiation among river drainages (Avise 1992; Baer 1998; Turner & Trexler 1998; Roe et al. 2001; Jones et al. 2006). In essence, gene tree data can be used to identify regional differentiation and enable one to 'assign' individuals of unknown origin to source regions (Wares et al. 2005). These techniques also allow the identification of allelic novelty in the sampled regions; an analysis of sequence data can represent complex evolutionary dynamics in part because of well-characterised models of sequence evolution (Hey et al. 1994). To this end, we surveyed patterns of genetic variation at one mitochondrial and one nuclear gene region across the distributional range of *N. lutipinnis* (Fig. 1). The goal of this multilocus survey is to help in the identification of source of the introduction, and to establish the historical basis for *N. lutipinnis* in the Little Tennessee. Our study also provides a reassessment of earlier work on this species, based on electrophoretic data, by Wood & Mayden (1992).

## Materials and methods

We made observations on the presence of *N. lutipinnis* in Coweeta Creek, NC (a tributary of the Little Tennessee) between 1983 and 2003 using both visual observations and quantitative electrofishing (Grossman et al. 1998). Coweeta Creek is typical of many relatively undisturbed fifth-order streams in the Southern Appalachian region. Observations were made in three permanent sites of varying lengths: a 37-m site (1983–1992; Grossman et al. 1998), a 30-m site (1984–1995; (Freeman et al. 1988; Grossman et al. 2006), and a 100-m site (1991–2003; G. D. Grossman, unpublished data). Sampling protocols and quantitative approaches are described in detail in Grossman et al. (1998, 2006).

For genetic analysis, we collected specimens via electrofishing from Coweeta Creek (Little Tennessee) and Clear Creek (Savannah) and used specimens from museum and agency collections (Table 1). Whole fish were preserved in 95% undenatured ethanol. We digested ~1 mg lateral muscle or caudal fin tissue in PureGene (Gentra Systems) cell lysis buffer with Proteinase K, followed by precipitation isolation of genomic DNA and quantification on a Nanodrop spectrophotometer. We sequenced the mitochondrial cytochrome b region (mtCYTB) using universal primers Glu-5′ and CB2-3′ from Palumbi (1996) and the nuclear transferrin region (nTF) from Wares et al. (2004) and Wares (2009). The selected mitochondrial locus (cytochrome *b*) is one of the more variable and commonly used gene regions in fish population genetics, with the standard benefits of analysing mitochondrial data for such purposes (Avise et al. 1987), whereas the nuclear *transferrin* gene is also typically variable within and among species and may reflect distinct evolutionary pressures (Ford 2000). Annealing temperatures for PCR of the two gene regions were 45° and 50°, respectively, with all other PCR conditions as in Wares et al. (2004). Amplicons were prepared for direct cycle sequencing using the exonuclease–phosphatase reaction and BigDyes 3.1 sequencing kit (Applied Biosystems) as per Harley et al. (2006).

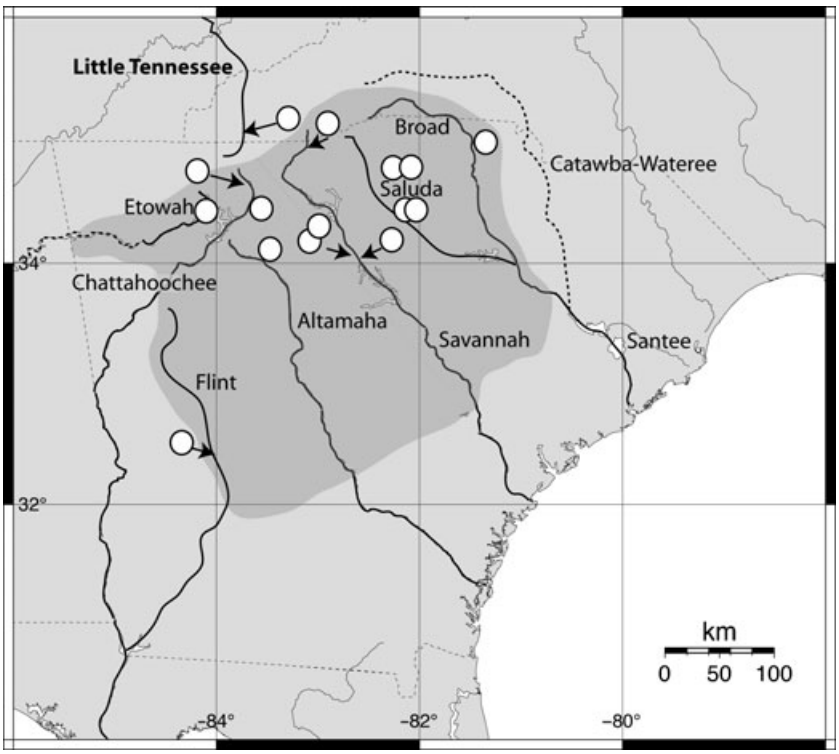


*Fig. 1.* River drainages in which *Notropis lutipinnis* was sampled. Solid lines represent rivers or drainages that specimens in Table 1 were collected (circles indicate collection locations, arrows indicate drainage connection where ambiguous); the dashed lines indicate places where rivers join higher-order rivers (Etowah) or rivers in which the sister species *Notropis chlorocephalus* can be found (Catawba-Wateree). Map was generated from Online Map Creation (http://www.aquarius.ifm-geomar.de/); river drainage location and sample population locations are approximate, with specific collection information available in Table 1. Native range of *N. lutipinnis* is shown in dark grey (based on Page & Burr 1991 and http://nas.er.usgs.gov).

Table 1. Sample sizes and locations for *Notropis lutipinnis*.

| Population | Latitude/longitude | Collection information | *n* (cyt b) | *n* (TF) |
|---|---|---|---|---|
| Coweeta (Little Tennessee) | 35°04′–83°27′ | PH(2006–7) | 28 | 52 |
| Flat Creek (Chattahoochee) | 34°44′–84°51′ | GMNH (BAP-1101; MMH-02-61) | 14 | 16 |
| Jenny (Chattahoochee) | 34°36′–83°49′ | GMNH (JCS-03-10) | 16 | 2 |
| Beaver (Flint) | 32°36′–84°11′ | GMNH (BAP1999Jun07) | 8 | 7 |
| Russell (Etowah) | 34°25′–84°04′ | GMNH (BJF-0503) | 9 | 8 |
| Oconee (Altamaha) | 34°19′–83°45′ | GMNH (BAP-1999-Jun19; BAP-1999-Jun10c) | 45 | 41 |
| Grove (Savannah) | 34°22′–83°36′ | GMNH (BAP1999Jun10b) | 9 | 10 |
| Beaverdam (Savannah) | 34°36′–83°32′ | GMNH (BAP1999Jun10a) | 18 | 11 |
| Double Branch (Savannah) | 34°11′–82°14′ | SC-DNR (MCS092203) | 2 | 3 |
| Clear Creek (Savannah) | 35°03′–83°18′ | MC004.50–55 | 6 | 0 |
| Laurel (Saluda/SC) | 34°46′–82°20′ | SC-DNR (MCS101305) | 5 | 8 |
| Broad Mouth (Saluda/SC) | 34°29′–82°20′ | SC-DNR (MCS101905) | 4 | 4 |
| Walnut (Saluda/SC) | 34°22′–82°08′ | SC-DNR (MCS101105) | 7 | 4 |
| Gilkey (Broad/SC) | 34°56′–81°28′ | SC-DNR (MCS04015) | 5 | 11 |

Following each population name is the river drainage in parentheses. Specimens were obtained from collections at Coweeta and Clear Creek; other specimens came from the Georgia Museum of Natural History Tissue Collection (GMNH) or monitoring collections of South Carolina Department of Natural Resources (SCDNR), with additional collection information indicated. Specific museum accessions and voucher specimens are available for GMNH samples.

We aligned and edited sequence data for ambiguities using CodonCode Aligner v. 2.01; low-quality data (Ewing & Green 1998) were scored as unknown, although ambiguous sites (PHRED scores 15–25) in the nTF locus were coded for subsequent haplotype analysis using PHASE 2.1.1 (Stephens et al. 2001). For this analysis, we excluded individuals with >25% informative sites missing, and four of 28 variable sites that did not fit the infinite-alleles model (only two character states). Of these excluded sites, three were parsimony-uninformative. We conducted the analysis in PHASE as per Sotka et al. (2004).

To confirm taxonomic identification of study tissues, we aligned sequence data with data from the following sister species: *Notropis chlorocephalus*, M. Cashner, unpublished data; *Notropis chrosomus* GenBank AF352262 and U01321; *Notropis blennius* AF117170-1; *Notropis potteri* AF117192-3; *Nocomis biguttatus* AY486057; *Nocomis micropogon* AF452077; *Clinostomus funduloides* (J. P. Wares, unpublished data). Six individuals from the Coweeta (NC) population were found to carry a mitochondrial haplotype identical to that of *C. funduloides* (J. P. Wares and R. Miller, unpublished data) and were

excluded from the subsequent analysis. In addition, a small number of 'native' range specimens were also found to either group with *Clinostomus* or *Nocomis* and were excluded as misidentified or ambiguous as well. These individuals are not included in Table 1.

We analysed sequence data for phylogenetic structure at both gene regions using PAUP* v.4.0b10 (Swofford 2002). In each case, the data were analysed under a simple parsimony criterion with gaps coding as missing data (in the case of indels in the nTF intron regions). We used a heuristic search with tree bisection reconnection branch swapping and simple sequence addition. Support for phylogenetic structure was assessed using 1000 nonparametric bootstrap replicates of the data at each locus; majority-rule consensus of the best 1000 parsimony trees (maximum trees held to 1000 due to computation limitations) was also calculated. Results are represented as midpoint-rooted phylograms due to inherent problems with rooting intraspecific gene trees (Castelloe & Templeton 1994) and similar representation to haplotype networks (Wares et al. 2001).

Net nucleotide divergence (Nei & Li 1979) was estimated among major drainages at each locus using dnaSP v.4.10.4 (Rozas & Rozas 1999), along with $\pi_A$:$\pi_S$ ratios within drainages and $k_A$:$k_S$ between drainages at the nTF locus. We calculated Tajima's *D* (Tajima 1989) for all drainages at both loci, pooling populations within drainages to evaluate whether either sequenced data set deviated from the assumption of neutral evolution and constant population size. To evaluate heterogeneity and structure within and among river drainages, the analysis of molecular variance (AMOVA) on pairwise distance was performed using Arlequin v.3.1 (Excoffier et al. 2005) with sample sites grouped by drainages; pairwise population $F_{st}$ was also calculated at this point. We excluded sites with >20% missing or ambiguous data from the analysis. We attempted to identify source population(s) for the Little Tennessee via both phylogenetic similarity (i.e., whether LT haplotypes are grouped with haplotypes from particular source regions) and minimal pairwise nucleotide divergence with other populations. Pairwise genetic differentiation ($F_{st}/1 - F_{st}$) was regressed against log (km) between sites (Euclidean distance) and the regression examined with a Mantel test using GenAlEx v.6.0 (Peakall & Smouse 2006) as an examination of whether sites are longitudinally differentiated as suggested by Wood & Mayden (1992).

## Results

We first observed yellowfin shiners in Coweeta Creek in 1999, 16 years after we began sampling in the creek. Yellowfin shiners were present only in the 100-m site during the drought years of 1999–2002, although they were not observed in the 30-m site during the similarly severe drought of 1985–1988. Yellowfin shiners were never abundant, ranging from 1 to 2 present per survey of the 100-m site. Nonetheless, in sites approximately 0.6 km downstream, yellowfin shiners were observed in higher abundances as early as 1996, though their abundances declined between 1996 and 1998 (G. Grossman & M. Farr, unpublished data).

The final mtCYTB data set (GenBank accessions FJ604893–FJ605094) included 479 aligned nucleotides; congeneric and other outgroup species. Of the retained sequence data, 56 were phylogenetically informative, whereas 384 characters were invariant. The best set of maximum-parsimony trees required 158 steps; a representative tree is presented in Fig. 2. This tree has high majority-rule consensus (>85%; most >95%) at all nodes among 1000 best trees but no bootstrap support >50% at any node. Surprisingly, the Coweeta Creek (NC) population harbours 21 distinct haplotypes (in 28 individuals), 17 of them not shared with any other population. Many of these haplotypes were located near the 'root' of the midpoint-rooted tree set, intermediate or basal to a clade that is predominantly comprised of individuals from the South Carolina drainages and a clade dominated by Georgia specimens. Missing/ambiguous sites had little effect on haplotype placement; we obtained similar topologies from both reduced and jackknifed data sets. In both cases, some individuals with the most 'missing' data share haplotypes with individuals in more 'derived' portions of the tree. In addition, many individuals near the root have the least amount of missing data, with no statistical difference among populations as to the level of missing data at either end of the sequence data set (J. P. Wares, unpublished data).

The nTF data set (GenBank FJ604860–FJ604892) comprised 562 characters; after reduction to haplotype data using the best-fit results from PHASE, there were 33 distinct haplotypes, with 24 variable characters (15 parsimony informative), for the analysis. The nTF sequence data set was partitioned into intron (at both ends of the data set) and exon regions (TBLASTX of a 195-bp ORF from sites 250–444 has 86% identity and *E*-value of 1e−25 with GenBank accession AF457152, *Cyprinus carpio* transferrin; 84% identity and *E*-value of 2e−25 with accession AF518744, *Carassius auratus*). The best set of parsimony trees (combining intron and exon data) required 38 steps; a representative tree is shown in Fig. 3, with high consensus among the trees (and similar topology when only exon data are analysed) but no strong bootstrap support. We observed two distinct clades, recovered in 100% of the MP trees and generally representing different

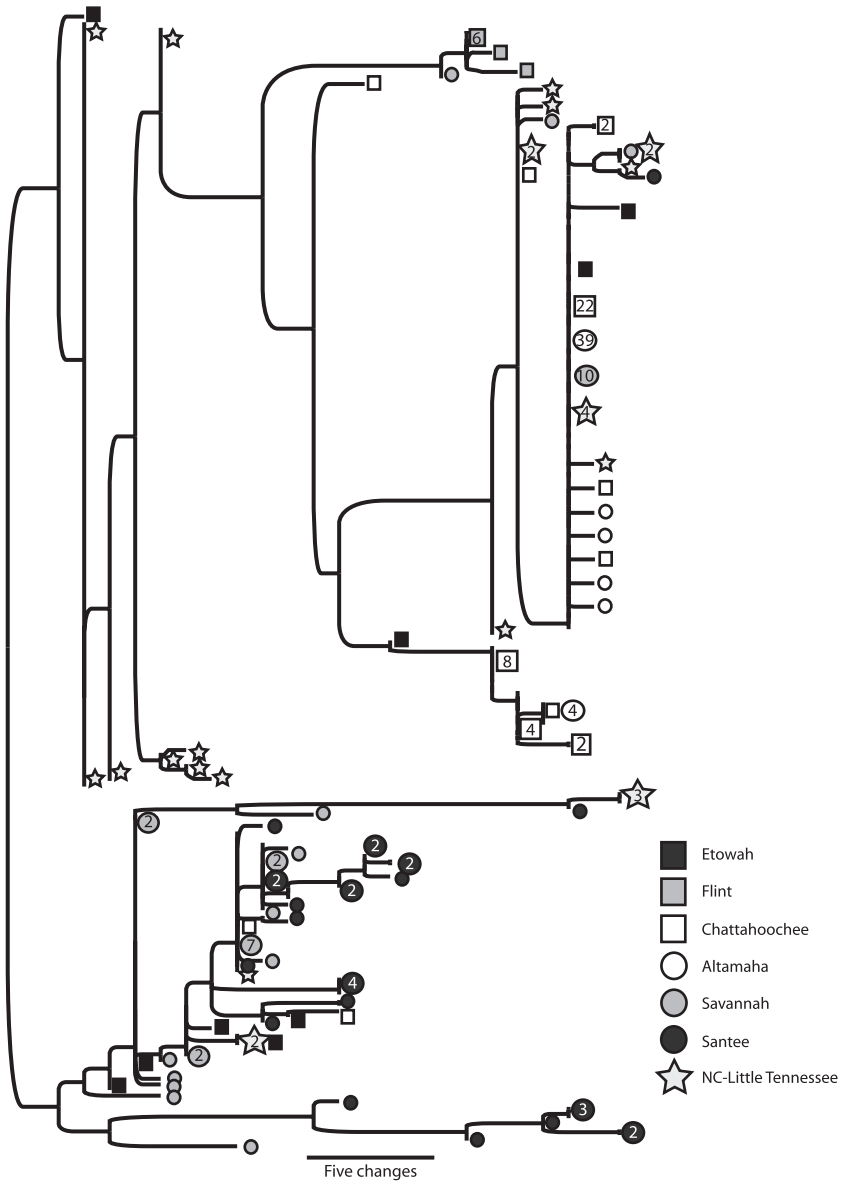


*Fig. 2.* A representative MP phylogram ($N = 1000$) for the mtCYTB gene region in *Notropis lutipinnis*. Minimum consensus for all nodes across 1000 trees is 85%, with 134 of 200 internal nodes recovered in 100% of maximum-parsimony trees. The number of individuals sharing a haplotype from a given sampled river drainage is indicated by the numbers within symbols (see key), with no number indicating a single individual.

sample sites. Clade A comprised 94% individuals from the Altamaha and Flint drainages, whereas 97% of the populations in the B clade are from non-Altamaha/Flint drainages. Examination of particular substitutions in MacClade 4.08 (Maddison & Maddison 1992) indicated that there were six nonsynonymous substitution events in the exon region in clade A and seven in clade B, with some substitutions occurring multiple times. From the Coweeta (NC) population, eight distinct haplotypes were recovered, with five of them not shared with any other sampled location.

We represented net divergence among drainages as $d_A$ (Nei & Li 1979), averaged over all sequences and populations within a river drainage. A small number of sequences with large (>25%) amounts of missing data were excluded to provide the greatest overlap of aligned nucleotides for comparison; these excluded sequences were uniformly distributed across sampled locations. At the mtCYTB locus, mean diversity within basins ($\pi = 0.0384$ average across five river drainages) was an order of magnitude higher than the mean divergence among basins (mean $d_A = 0.0066$, Table 2), indicating significant levels of ancestral polymorphism shared among most basins. Similarly, at the nTF locus, mean diversity within basins (average $\pi = 0.0033$) was much higher than the mean among-basin divergence of 0.0017 (Table 3). Tajima's $D$ values for each of the seven drainages are shown in Table 4 and indicate no significant deviation from the null model for the mtCYTB data; at least a small portion of the nTF-coding sequence is significantly positive (0.751, $P < 0.05$) in the Altamaha

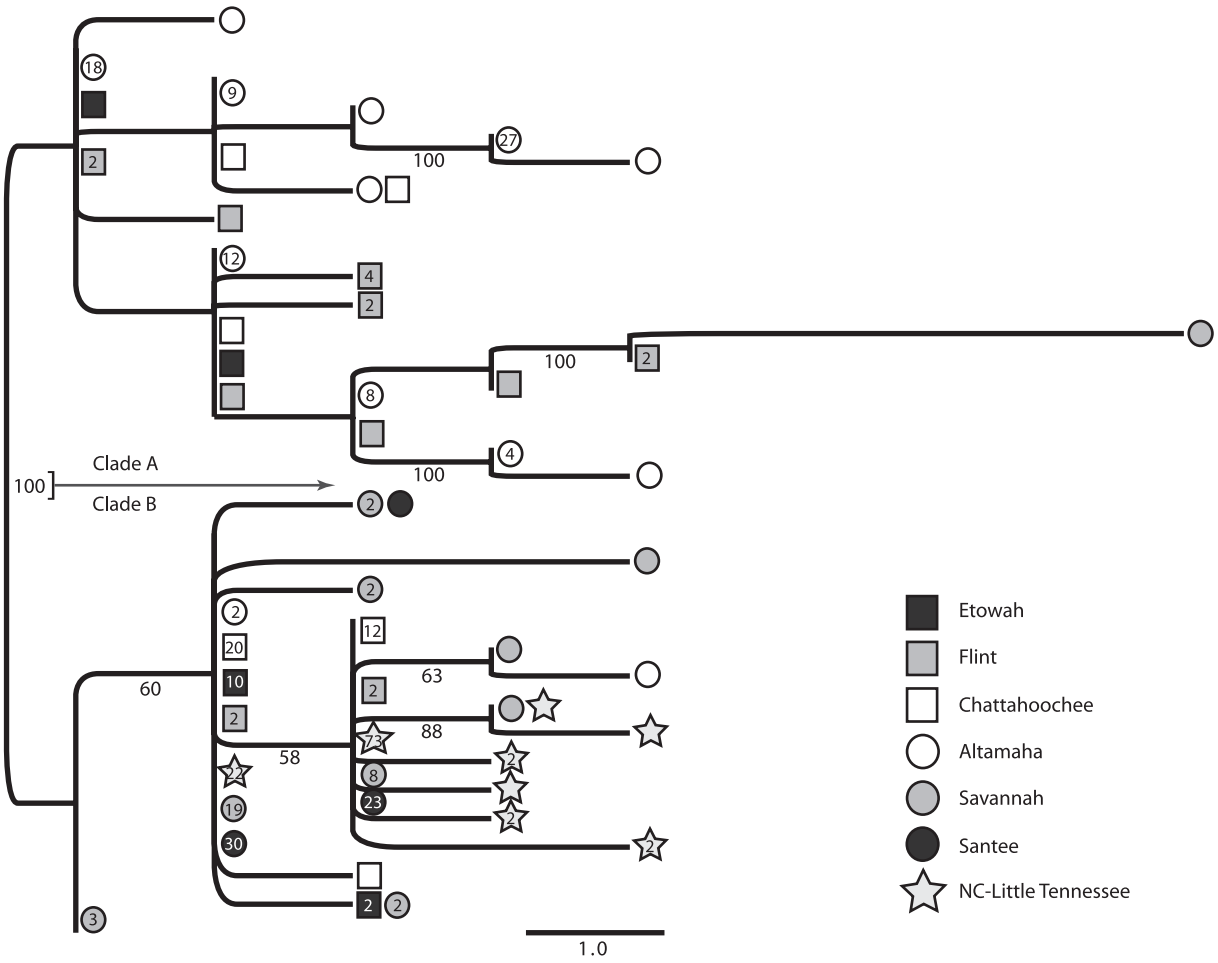


*Fig. 3.* A representative MP tree ($N = 1000$) for the nTF gene region (inferred haplotypes) in *Notropis lutipinnis.* Clades A and B are recovered in 100% (strict consensus) of MP trees. Other branches with >50% consensus are indicated by numbers under branch. The number of individuals sharing a haplotype from a given sampled river drainage are indicated by numbers in symbols (see key), with no number indicating a single individual.

population, suggesting either dramatic demographic changes or additional diversity maintained by balancing the selection of some form.

The AMOVA of the mtCYTB data indicated strong and significant divergence among populations ($F_{st} = 0.443$, $P < 0.001$; $F_{sc} = 0.364$, $P < 0.001$) but not significant divergence between drainages predominantly in Georgia (Etowah, Chattahoochee, Altamaha, Flint), South Carolina (Savannah, Santee), and North Carolina (Little Tennessee), with $F_{ct}$ only 0.124. Results were very similar at the nTF locus with a high $F_{st}$ (0.584, $P < 0.001$), high $F_{sc}$ (0.508, $P < 0.001$), and a nonsignificant $F_{ct}$ (0.154). When the structure was changed to reflect the gene tree in Fig. 3 (a clade containing the Altamaha and Flint populations and the second clade comprising all other populations), among-group variation increased from 15.4% to 65.3% and within-group variation dropping from 42.9% to 5.3%, with concomitant changes in $F_{st}$(0.706), $F_{sc}$(0.153) and among-group $F_{ct}$(0.653), all significant ($P < 0.05$).

Within-drainage population differentiation in the Chattahoochee, Savannah, and Santee drainages also was high at the mtCYTB locus; AMOVA including all population data separated into sites and individual locations grouped by drainage indicated 77.8% of variation was among populations within groups ($F_{sc} = 0.666$, $P < 0.001$ and $F_{st} = 0.611$, $P < 0.001$). As with the among-drainage pairwise $F_{st}$ values (Table 2), almost all populations exhibit significant pairwise $F_{st}$ values, with the exception of Coweeta (Little Tennessee), Etowah ($F_{st} = -0.025$), Beaverdam ($F_{st} = -0.013$), and Doublebranch ($F_{st} - 0.022$) sites. Thus, overall differentiation among sites was very high, with only a small set of comparisons sharing large amounts of genetic variation with the Little Tennessee population. Overall diversity at mtCYTB was high in each river drainage; haplotype diversity (H) in the Little Tennessee population was $1.000 \pm 0.009$, although in other drainages $H$ ranged from 0.893 to 0.998, with a diversity across all presumed native drainages $H = 0.992 \pm 0.002$.

Table 2. Polymorphism and divergence at the mtCYTB locus among sample locations.

| Population | Cow (LT) | Flat (CHA) | Jny (CHA) | Russ (ETO) | Bvr (FLN) | Oco (ALT) | Clear (SAV) | Grove (SAV) | Bvdm (SAV) | Doub (SAV) | BrMth (SAL) | Laur (SAL) | Waln (SAL) | Gilk (BRD) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Coweeta (LT) | 3.14 | 0.66 | 3.34 | −0.99 | 10.64 | 1.83 | 4.56 | 18.23 | −0.99 | −2.03 | 3.46 | 6.31 | 4.43 | 4.47 |
| Flat (CHAT) | *0.16* | 4.57 | 7.94 | 4.30 | 16.09 | −0.05 | 13.98 | 32.98 | 2.76 | −0.25 | 13.63 | 17.09 | 11.51 | 10.65 |
| Jenny (CHAT) | 0.36 | 0.49 | 11.58 | 5.85 | 12.10 | 8.75 | 13.58 | 30.88 | 8.11 | 2.46 | 12.15 | 13.16 | 11.54 | 10.32 |
| Russell (ETO) | **−0.22** | 0.42 | 0.36 | 8.39 | 11.43 | 6.03 | 2.16 | 26.07 | −0.12 | −3.19 | 3.56 | 5.50 | 1.86 | 1.89 |
| Beaver (FLN) | 0.80 | 0.83 | 0.59 | 0.70 | 0.89 | 17.24 | 17.68 | 37.69 | 14.29 | 10.18 | 16.80 | 18.23 | 17.41 | 16.15 |
| Oconee (ALT) | 0.43 | **0.00** | 0.67 | 0.68 | 0.90 | 2.05 | 16.60 | 34.21 | 4.32 | 1.28 | 16.04 | 19.61 | 14.12 | 13.16 |
| Clear (SAV) | 0.62 | 0.80 | 0.60 | *0.27* | 0.96 | 0.90 | 0.33 | 31.69 | 4.70 | 1.50 | 4.13 | 5.78 | 2.52 | 2.75 |
| Grove (SAV) | 0.73 | 0.76 | 0.68 | 0.65 | 0.77 | 0.88 | 0.71 | 20.28 | 30.43 | 17.53 | 30.42 | 31.91 | 28.30 | 26.97 |
| Beaverdam (SAV) | **−0.17** | *0.24* | 0.41 | **−0.02** | 0.63 | 0.50 | *0.31* | 0.68 | 11.34 | −3.67 | 5.65 | 8.57 | 2.65 | 2.22 |
| Double (SAV) | **−0.04** | **0.20** | **0.17** | **−0.43** | *0.85* | *0.61* | *0.63* | *0.45* | **−0.50** | 11.00 | 1.25 | 3.95 | 1.07 | 0.55 |
| Br. Mouth (SAL) | 0.52 | 0.68 | 0.50 | *0.28* | 0.74 | 0.85 | *0.41* | 0.60 | *0.34* | **0.00** | 18.50 | −1.95 | 2.93 | 2.55 |
| Laurel (SAL) | 0.61 | 0.72 | 0.52 | *0.35* | 0.76 | 0.87 | *0.47* | 0.63 | 0.42 | **0.18** | **−0.13** | 15.10 | 6.41 | 6.36 |
| Walnut (SAL) | 0.58 | 0.73 | 0.55 | *0.22* | 0.89 | 0.86 | 0.54 | 0.68 | *0.20* | *0.36* | **0.31** | *0.45* | 3.57 | 0.39 |
| Gilkey (BRD) | 0.59 | 0.72 | 0.50 | *0.20* | 0.91 | 0.86 | 0.65 | 0.64 | **0.14** | **0.31** | **0.24** | **0.41** | **0.10** | 2.90 |

On the diagonal is $\pi$ (average pairwise differences within population). Above diagonal are net nucleotide divergences $d_A$, with standard deviation <0.001 in all comparisons. Below diagonal are pairwise $F_{st}$ values, with significance $0.01 < P < 0.05$ indicated by italics; $P > 0.05$ by bold (all others $P < 0.01$).

Table 3. Polymorphism and divergence at the nTF locus among sample locations.

| Population | Cow (LT) | Flat (CHA) | Jny (CHA) | Russ (ETO) | Bvr (FLN) | Oco (ALT) | Clear (SAV) | Grove (SAV) | Bvdm (SAV) | Doub (SAV) | BrMth (SAL) | Laur (SAL) | Waln (SAL) | Gilk (BRD) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Coweeta (LT) | 0.72 | 0.16 | 5.17 | 3.60 | 0.68 | 4.37 | | 0.27 | 0.30 | 0.79 | 0.03 | −0.01 | −0.01 | 0.36 |
| Flat (CHAT) | 0.18 | 0.73 | 4.49 | 2.91 | 0.17 | 3.69 | | 0.02 | 0.01 | 0.30 | 0.01 | 0.02 | 0.02 | 0.02 |
| Jenny (CHAT) | 0.85 | 0.78 | 2.59 | −0.23 | 3.40 | 0.94 | | 3.97 | 4.57 | 4.73 | 4.86 | 4.92 | 4.92 | 4.59 |
| Russell (ETO) | 0.85 | 0.82 | **0.03** | 4.00 | 2.06 | −0.67 | | 2.43 | 3.07 | 3.17 | 3.30 | 3.36 | 3.36 | 3.03 |
| Beaver (FLN) | 0.46 | 0.15 | 0.62 | *0.59* | 1.63 | 2.95 | | 0.13 | 0.08 | 0.23 | 0.36 | 0.42 | 0.42 | 0.08 |
| Oconee (ALT) | 0.75 | 0.65 | 0.27 | **−0.13** | 0.55 | 2.48 | | 3.17 | 3.85 | 3.94 | 4.07 | 4.13 | 4.13 | 3.80 |
| Clear (SAV) | | | | | | | | | | | | | | |
| Grove (SAV) | 0.24 | **0.03** | 0.64 | *0.57* | *0.07* | 0.57 | | 2.05 | 0.07 | 0.32 | 0.10 | 0.12 | 0.12 | 0.07 |
| Beaverdam (SAV) | 0.28 | **0.02** | 0.74 | 0.77 | *0.06* | 0.64 | | *0.04* | 0.96 | 0.24 | 0.09 | 0.11 | 0.11 | 0.00 |
| Double (SAV) | 0.52 | *0.29* | 0.67 | **0.71** | **0.08** | 0.61 | | **0.09** | **0.18** | 0.67 | 0.47 | 0.52 | 0.52 | 0.15 |
| Br. Mouth (SAL) | **0.04** | **0.01** | 0.77 | *0.85* | 0.25 | 0.65 | | **0.06** | **0.10** | 0.47 | 0.53 | −0.05 | −0.05 | 0.12 |
| Laurel (SAL) | **−0.02** | **0.02** | 0.72 | *0.81* | 0.23 | 0.63 | | **0.03** | **0.10** | *0.48* | **−0.09** | 0.54 | −0.07 | 0.16 |
| Walnut (SAL) | **−0.02** | **0.02** | 0.72 | *0.81* | 0.23 | 0.63 | | **0.03** | **0.10** | *0.48* | **−0.09** | **−0.14** | 0.54 | 0.16 |
| Gilkey (BRD) | 0.35 | **0.03** | 0.79 | 0.87 | 0.09 | 0.65 | | *0.05* | **0.01** | *0.29* | *0.21* | *0.27* | *0.27* | 0.40 |

On the diagonal is $\pi$ (average pairwise differences within population). Above diagonal are net nucleotide divergences $d_A$, with standard deviation <0.001 in all comparisons. Below diagonal are pairwise $F_{st}$ values, with significance $0.01 < P < 0.05$ indicated by italics; $P > 0.05$ by bold (all others $P < 0.01$). No samples from Clear Creek were sequenced at the nTF locus.

Table 4. Tajima's *D* for each drainage at mtCYTB and nTF locus.

| Population | *D* (mtCYTB) | *D* (nTF) |
|---|---|---|
| Altamaha | −1.083 | 0.751† |
| Chattahoochee | 0.491 | −0.763 |
| Etowah | 1.205 | −0.848 |
| Flint | −0.939 | 0.734 |
| NC | −0.033 | −0.533 |
| Savannah | 0.329 | −1.507‡ |
| SC combined | 0.435 | 0.385 |

Although overall values are not statistically significant based on tests in DNAsp and/or Arlequin, rolling averages indicate a significantly positive value ($P < 0.05$) for a 100-bp window within the exon portion of the nTF gene region in the Altamaha population (†), and a marginally significant ($P < 0.10$) negative value for a window in the exon of the Savannah population (‡).

At the nTF locus, pairwise population differentiation represented a pattern distinct from the mitochondrial data. The Coweeta population was significantly divergent from all populations except for those in the Saluda drainage; those three populations themselves were not significantly differentiated from several other sites, including Flat Creek (Chattahoochee) and two of the Savannah populations (Table 3). This association could be meaningful because the South Carolina sites harbour less intra-population diversity than the Georgia sites, and the net nucleotide divergence of the Coweeta population was effectively zero for the Saluda sites. Nevertheless, five of eight distinct nTF haplotypes identified from the Coweeta site were unique to that location, though the sample size is much higher at this site. At neither the mtCYTB nor nTF locus was differentiation significantly correlated with Euclidean distance; a Mantel isolation-by-distance test produced a nonsignificant ($P > 0.10$) correlation of only 0.49 for mtCYTB, and was much lower at the nTF locus.

## Discussion

Despite sampling fish from every major drainage in which *N. lutipinnis* is found – including a sampling effort for distinct sites and number of individuals (average population $n = 15.2$ for mtCTYB, 13.6 for nTF) that is commensurate with better-sampled studies reviewed by Muirhead et al. (2008) – we were unable to identify the likely source(s) for the Little Tennessee population of this species. Although there are statistical similarities (based on the frequency of closely related haplotypes between populations) between the Little Tennessee population and sites in the Etowah and Savannah rivers (based on mtCYTB) or between the Little Tennessee population and sites in the Saluda river (based on nTF), at each locus there is a high level of endemic diversity associated with the Little Tennessee population. There were additional inter-drainage comparisons that did not differ statistically (e.g., Savannah and Etowah, Chattahoochee, and Altamaha), and the overall high level of diversity within and among all sites suggests the long-term sorting of a highly polymorphic ancestral species range. Although the two loci analysed in this study suggest no consistent identification of the origin of the Little Tennessee population, it is always possible that additional sampling effort in the presumed native range could recover the 'unique' haplotypes found in the Little Tennessee (Wares et al. 2005; Muirhead et al. 2008). Negative results in the analysis of species introductions are difficult to interpret because they cannot falsify hypotheses of a species being native or introduced; even 10-fold increases in sampling effort may not resolve the origin of unique diversity found in a population presumed to be introduced (Blakeslee, A. M. H., Byers, J. E. & Lesser, M. P. 2008. Resolving cryptogenic histories using host and parasite genetics. Molecular Ecology: 17: 3684–3696).

However, our results suggest that it is also worth examining the available information regarding the introduced status of yellowfin shiner. Phylogenetic placement of many unique mitochondrial haplotypes at an intermediate position in the mtCYTB haplotype network suggests that the species may not be invasive in the Little Tennessee river. A large number of congeneric and confamilial species have long been considered native to the entire range explored in this study, including the Tennessee River basin (Ramsey 1965). This suggests that 'native' status is not biogeographically improbable; yet this species has been considered 'introduced' based on missing information in historical collections, anecdotal evidence, and recent surveys in which yellowfin shiners were first noted for upstream sites in the Little Tennessee drainage. Our own monitoring data on this species suggests there may be some environmental correlates (e.g., repeated severe droughts) with the recent appearance of *N. lutipinnis* in tributary sites, and other monitoring studies have indicated that even if native, the range of this species within the Little Tennessee drainage has increased substantially over the past two decades (B. McLarney, pers. comm.).

In addition to information pertinent to the introduced status of yellowfin shiner, the nTF data seem to exclude any association between the Little Tennessee population and sites in the Altamaha and Flint drainages. Previous evidence has suggested that transferrin evolves in accordance with diversifying selection in natural fish populations as a response to selective pressures by microbial pathogens (Ford 2000, 2001), and recent work shows that there is considerable diversity at this locus among populations of *N. lutipinnis* that is consistent with positive selection (Wares, 2009). The strength of differentiation

between the Altamaha and Flint populations and all other populations suggests more than historical lineage sorting. Consequently, regardless of the fact that many of our samples represent a single site in a given drainage, nTF sequence data strongly indicate an evolutionarily significant divergence between all other sampled populations and those sampled in the Altamaha and Flint; whether this represents historical divergence or environmental differentiation is yet to be resolved.

Overall, the evidence for selection driving this differentiation among populations in *N. lutipinnis* appears somewhat weak (Table 3). For example, comparisons of the Altamaha sequence data with those from other populations produced a $k_A$:$k_S$ ratio close to 1, a result consistent with neutral evolution (Wayne & Simonsen 1998; but see Kryazhimskiy & Plotkin 2008). However, many of the replacement substitutions recovered on the gene tree in Fig. 3 are not fixed within or among populations (not shown); only a single population (Etowah) had zero nonsynonymous substitutions segregating in the sample relative to the number of synonymous substitutions, with all other populations having relatively high $\pi_A$:$\pi_S$ ratios (Table 3). The general discordance between the mtCYTB and nTF data suggests environmental processes could be influencing the dynamics of gene flow in this species, and provides preliminary insights into how this system may be further explored for methods to distinguish environmental, stochastic, and biological factors in generating the observed diversity in this species (Endler 1982; Haydon et al. 1994).

Although little is yet known about the biology or genetics of yellowfin shiners, Wood & Mayden (1992) used electrophoretic techniques to demonstrate the likely presence of two primary and reciprocally monophyletic lineages of *N. lutipinnis*: one in the Santee-Broad basin of North Carolina and South Carolina, and the other in the Savannah, Altamaha, and Chattahoochee basins of South Carolina and Georgia. Our data suggest that the divergence between these two regional groups is not complete, although our mitochondrial data resemble the pattern recovered by Wood & Mayden (1992). This pattern is not consistent with the typical 'coastal drainage' phylogeographic patterns of Avise (1992), in which Gulf and Atlantic slope drainages are deeply isolated from each other (Baer 1998). However, it is consistent with studies of ancient stream capture events [e.g., between the Savannah and the Chattahoochee (Swift et al. 1986); in the southern Appalachian region (Lydeard et al. 1991; Mayden & Matson 1992; Wood & Mayden 1992; Voss et al. 1995; Kozak et al. 2006)]. Although there is little direct evidence of these ancient connections, the comparison of genetic variation in numerous species may yield more complete explanations.

Wood & Mayden (1992) also suggested that the somewhat longitudinal correlation with genetic distance among populations in their data was consistent with an ancient northeastward-flowing drainage hypothesised by White (1953). However, this proposed drainage lay in Miocene sediments, which are much more ancient than the shared allelic variation among yellowfin shiner populations would indicate. Further analyses indicate that our data are not consistent with the spatially limited vicariance hypothesis proposed by Wood & Mayden (1992); a Mantel isolation-by-distance test produced a nonsignificant correlation of only 0.49. This test of differentiation based on Euclidean distances is of course a very weak examination of this idea given the complexity of the habitat of yellowfin shiners. Yet in addition, Fig. 2 demonstrates that although sites in Georgia tend to be differentiated from sites in South Carolina, with the Savannah mostly intermediate in relationship, the most westward samples from the Etowah tend to cluster with the samples in South Carolina. In general, the Etowah site tends to generate the furthest-outlying points in the isolation by distance analysis (not shown), suggesting that the data are consistent with a more complex model of historical equilibrium movement and exchange (McRae 2006). Alternatively, our data may suggest that more consideration should be given to the native status of the Etowah population, which has been called into question (Burkhead et al. 1997; see http://nas.er.usgs.gov/queris/FactSheet.asp?speciesID=602).

Further work will be necessary to clarify the geographic differentiation of yellowfin shiner populations. A number of non-exclusive interpretations may apply to the gene tree data shown here for *N. lutipinnis*, including: (i) lineage sorting and ancestral polymorphism (Avise 1994; Bulgin et al. 2003), and (ii) contemporary gene flow – both within and among basins due to natural processes or anthropogenic environmental changes (Slatkin 1985; Scott & Helfman 2001). We are beginning to explore the use of microsatellite markers developed for cyprinid minnows (Turner et al. 2004) to obtain a more complete assessment of inter-drainage gene flow and/or ancestral polymorphism in this and related species, and comparisons of larger numbers of markers will also promote our understanding of which markers or genes are responding to environmental influences and demographic ones (Luikart et al. 2003).

The high genetic diversity found across the broad geographic range of *N. lutipinnis* – whether in the North Carolina samples or in the presumed native range – may encompass some long-isolated lineages that are no longer in demographic contact with one

another. The sister species, *N. chlorocephalus*, is found in the easternmost drainages of the ancestral range of *N. lutipinnis* (Wood & Mayden 1992), and a strongly divergent clade is found in our Flint River sample as well (Fig. 2). This range-wide diversity should be a strength in identifying the putative source for a species introduction, which may – depending on the mechanism of introduction – incorporate diversity from multiple sources. Kolbe et al. (2004) identified a comparable level of range-wide diversity across the native populations of *Anolis sagrei*, and the greatest diversity was found within the introduced populations in Florida. However, haplotypes from these introduced populations were consistently shared with source-range populations, providing clear indication that multiple sources were responsible for the introduction, while our phylogenetic signal is more evolutionarily ambiguous.

Freshwater fishes tend to be anomalous among introduced species in that there are generally a greater number of species introductions from different watersheds (or portions of watersheds) on the same continent, rather than the exotics coming from different global regions (Scott & Helfman 2001; Sax et al. 2005; Rahel 2007). This process has, of course, led to a dramatic homogenisation of freshwater fish communities (Rahel 2000; Gherardi 2007). Although much work is being carried out to evaluate the factors leading to invasion success for freshwater fishes (Fausch et al. 2001; Kolar & Lodge 2002; Jeschke & Strayer 2005; Ruesink 2005; García-Berthou 2007), genetic analysis aids in documenting the role of diversity and propagule pressure in successful introductions (Wares et al. 2005). Our somewhat conflicting results illustrate the difficulties inherent in determining whether a population is invasive (Kolbe et al. 2004; Wares et al. 2005; Wares & Blakeslee 2007; Muirhead et al. 2008), especially if the introduction comes from multiple sources (Kolbe et al. 2004). In conclusion, it appears less likely that yellowfin shiner are invasive in the Little Tennessee than originally thought, although it is also possible that we did not sample the source population(s). Even if native, long-term collections made by multiple investigators indicate that yellowfin shiners are clearly undergoing a widespread range expansion in the Little Tennessee drainage (Johnston et al. 1995) – a demographic pattern typical of an invasive species.

This study marks an updated multilocus phylogeographic treatment of the broadly distributed minnow *N. lutipinnis*, and provides new insights into the historical and adaptive biology of southeastern fishes. Although we cannot disprove the alien status of this species in the Little Tennessee river basin, our genetic data were not consistent with typical patterns for recent invaders (Wares et al. 2005). The degree of geographic differentiation among populations of this species suggests both an ancient distribution in the southern Appalachians and large and persistent effective population sizes in historical populations (Wakeley & Hey 1997; Bulgin et al. 2003). Our study also generated new hypotheses of the role of adaptive processes in divergence among different river drainages, and suggests that further study of the interactions between native fishes and their abiotic and biotic (including microbial) environment is warranted. Such interactions may have played a role in the remarkably high diversity of aquatic organisms in the southeastern USA. Further synthesis of the comparative phylogeographic diversity of southeastern aquatic species should be a next step in considering ecosystem-level management of regional diversity.

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## Biosketch

Author contributions: G.G. and J.W. conceived the idea for this study; C.S. and J.W. collected and analysed the data; M.C. and G.G. carried out the field and systematic expertise; and J.W. and G.G. led the writing of this paper.

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