

Status of the stippled studfish *Fundulus bifax*, a killifish endemic to the Tallapoosa River basin, USA

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ABSTRACT: The stippled studfish *Fundulus bifax*, which is endemic to the Tallapoosa River system of Alabama and Georgia, USA, has been considered at risk due to habitat degradation. Requisite habitat for this species is clean water over clean sand in small or large streams. *F. bifax* is apparently extirpated in Georgia, and appears increasingly uncommon in Alabama. This has resulted in a global ranking of N2N3, imperiled/vulnerable, from NatureServe. In 2008, 24 Tallapoosa River drainage sites in Alabama were visited in an effort to document the current status of this species. Many of these were locations where the fish has been collected since 1980, as documented in the University of Alabama Ichthyology Collection. At least 1 individual was found in each of 6 different creek systems in Coosa, Elmore, Randolph, and Tallapoosa Counties. This is a contraction of what has been considered this species' range, and appears to be the result of habitat degradation. To examine genetic variation between existing populations, and between 2 closely related species, 852 bases of the mitochondrial cytochrome *b* gene of 10 individuals were compared through neighbor-joining and Bayesian tree building, and through calculating genetic distance, *D*. Both analyses showed existing populations of *F. bifax* to be monophyletic, with low genetic variation between them. Neither of its putative closest relatives, *F. catenatus* and *F. stelleri*, was found to be more closely related to *F. bifax*. The future of the species is in doubt, with 6 disjunct populations being vulnerable to further habitat degradation and diminished gene flow.

KEY WORDS: Cytochrome *b* · Habitat degradation · Alabama

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INTRODUCTION

The stippled studfish *Fundulus bifax* (Fundulidae) is a species considered to be in slow decline (NatureServe 2010). It is included among a group of stream species known as 'narrow endemics' that are susceptible to habitat alterations due to growing human populations (Warren et al. 2000). The conservation status of *F. bifax* is poorly documented. According to NatureServe (2010), the global status of *F. bifax* is N2N3, imperiled/vulnerable, and the Alabama state status is S2, imperiled, while the International Union for the Conservation of Nature (IUCN) places the species on the Red List with the status of Near Threatened based on local rarity and an apparent range of 5000 km². The original range of the species was in the Piedmont of Alabama and

Georgia in the Tallapoosa River basin, USA (Fig. 1). Apparently, the species has been extirpated from Georgia and was last collected in the Little Tallapoosa River system in Carroll County in 1990 (Storey et al. 2003, see also http://georgiawildlife.com/sites/default/files/uploads/wildlife/nongame/pdf/accounts/fishes/fundulus_bifax.pdf). In Alabama, the species is known from the Tallapoosa River basin in Coosa, Elmore, Randolph, and Tallapoosa Counties, along with 1 stream in the Coosa River basin immediately to the west in Elmore County (Boschung & Mayden 2004). The species is found in medium-sized streams, typically along the edge of the current, and requires clean sand for successful reproduction, since spawning fish dive into the sand and deposit eggs that incubate there for several days until hatching (Scanlan 2008).

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Fig. 1. Alabama section of the Tallapoosa River watershed with Alabama counties indicated. Carroll County in Georgia is also indicated as the site of 2 historic *Fundulus bifax* collection sites. Inset shows the entire Tallapoosa River Basin shared between Alabama and Georgia (USA)

Fundulus bifax is part of the studfish clade, subgenus *Xenisma*, with 5 extant and 1 extinct species. This clade is considered to be monophyletic based on morphological data (Cashner et al. 1992). *F. bifax* was formerly included in *F. stellifer* until it was described as a species in 1988 based on differences in breeding coloration and pigmentation patterns (Cashner et al. 1988). Allozyme data showed *F. bifax* to be more closely related to *F. catenatus*, even though the range of *F. stellifer* immediately abuts that of *F. bifax*, while the nearest populations of *F. catenatus* are ~200 km to the north (Cashner et al. 1992).

The current project has 2 linked parts. The first part was intended primarily as a presence/absence survey of *Fundulus bifax* at historic location sites in Alabama based on holdings of the University of Alabama Ichthyology Collection (UAIC) in Tuscaloosa, Alabama. The Alabama section represents most of the historic range and may currently be the entire range. The second part of the project was to characterize the genetic diversity of *F. bifax* at the cytochrome *b* locus of mitochondrial DNA, as well as its

relationship with its 2 closest relatives, *F. catenatus* and *F. stellifer*.

MATERIALS AND METHODS

Stream survey

The UAIC provided a list of known *Fundulus bifax* collection sites based on their holdings. Fish collection was attempted at these sites in the Tallapoosa River basin both to ascertain the continued presence of the species at the site, and to collect specimens for DNA extraction. Streams were sampled using a 3.5 × 1.3 m seine with 3 mm mesh. At each site, 200 m of stream were sampled, if accessible. Captured fish were sacrificed immediately after capture with tricaine methanesulfonate (MS-222) and transferred to 95% ethanol. Individuals from Sofkahatchee Creek were provided by Joseph Scanlan.

Streams in the Alabama section of the Tallapoosa River basin with no historic record of *Fundulus bifax* collection were also sampled to determine the full range of the species. In particular, streams in Cleburne County, Alabama, to the north of historic collection sites in Alabama were sampled, since this area is where both the Tallapoosa and Little Tallapoosa Rivers enter Alabama from Carroll County, Georgia, where *F. bifax* has been found.

Extraction, amplification, and sequencing of DNA

DNA was extracted from white muscle using the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek), following the manufacturer's instructions for the Tissue DNA-Spin Protocol. Amplification of extracted DNA via polymerase chain reaction (PCR) was done by Oblique Bio of Huntsville, Alabama, using an ABI 9700 with a 96-well gold block. The PCR conditions consisted of 94°C for 5 min, 25 cycles of 94°C for 30 s, 50°C for 10 s, and 72°C for 45 s, with a final extension of 7 min at 72°C. PCR reaction reagents and volumes are proprietary information of Oblique Bio. Sequencing reactions were performed on an ABI 9700 with a 384 dual block. Sequence detection was performed on an ABI 3730xL system using a 50 cm array. Sequencing conditions were 96°C for 5 min, 10 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 1 min 15 s, followed by 10 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 1 min 45 s, followed by 10 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min 15 s. The following 2 primers were used for both PCR amplifica-

tion of the mitochondrial cytochrome *b* locus and sequencing of the PCR amplification product: L14724, 5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3' (Pääbo 1990) and H15915, 5'-AAC TGC CAG TCA TCT CCG GTA CAA GAC-3' (Irwin et al. 1991).

Analysis of DNA sequences and tree building

DNA sequences were examined for apparent errors such as gaps, and aligned with other sequences using Geneious Align in Geneious Pro v. 4.6.2 (Biomatters). Phylogenetic trees were built using Geneious. Neighbor-joining was used as a tree-building method with the Hasegawa-Kishino-Yana (HKY) genetic distance model with 1000 bootstrap resamples. Bayesian inference was also used to build a phylogenetic tree using MrBayes with the GTR nucleotide substitution method, gamma rate variation, a chain length of 1.1 million generations beginning with random trees and a subsampling frequency of 200 (Huelsenbeck & Ronquist 2001). To ensure accurate convergence, the

first 100 000 generations were treated as burn-in and discarded. The remaining trees were used to build a 50 % majority rule consensus tree. Molecular Evolutionary Genetics Analysis (MEGA) v. 5.1 was used to calculate both genetic distance (*D*) between individual sequences and the discrete gamma distribution, α , a maximum likelihood estimate of the gamma parameter for site rates (Tamura et al. 2011).

RESULTS

Stream survey

Fundulus bifax was found at 8 of 13 historic sites sampled between February and July 2008 (Table 1 and interactive map in the supplement at www.int-res.com/articles/suppl/n020p019_supp/). Three of these sites were in the Hillabee Creek system (including Broken Arrow Creek), although the species was not found in Josie Leg Creek, a tributary of Hillabee Creek. The downstream site in Channahatchee Creek produced a single fish, while the

Table 1. *Fundulus bifax*. Stream survey results reported as present or absent; ease of capture is rated on a scale of 1 to 5, with 1 being easy and 5 being difficult. Sites are broken down into sites where the species has previously been reported, and those within its reported range with no record of the species. Both sections are organized by county in alphabetical order

Site	County	Latitude (°N)	Longitude (°W)	Present?	Ease of capture
Sites with previous collections					
Elkahatchee Creek at State Route 22	Coosa	32.9050	86.0103	Yes	2
Gold Branch at State Route 63	Elmore	32.6929	86.0071	No	
Channahatchee Creek at Highway 63	Elmore	32.6697	86.0373	No	
Channahatchee Creek at Highway 229	Elmore	32.6501	85.9469	Yes	5
Sofkahatchee Creek at County Road 338	Elmore	32.6300	86.1833	Yes	1
Cornhouse Creek at County Road 15	Randolph	33.2260	85.5411	Yes	1
Hillabee Creek at State Route 22	Tallapoosa	32.9846	85.8614	Yes	1
Hillabee Creek at Sanford Road	Tallapoosa	33.0664	85.8805	Yes	1
Broken Arrow Creek at Harlan Road	Tallapoosa	33.0753	85.9095	Yes	2
Josie Leg Creek at Lashley Road	Tallapoosa	33.0023	85.8328	No	
Sandy Creek at County Road 34	Tallapoosa	32.7857	85.6842	No	
Emuckfaw Creek at State Route 49	Tallapoosa	32.9952	85.7492	Yes	4
Sweetwater Creek at Sweetwater Creek Road	Tallapoosa	32.9922	85.6947	No	
Sites with no previous collections					
Emuckfaw Creek at Hodgescreek Road	Clay	33.1000	85.6833	No	
Tallapoosa River at Highway 46	Cleburne	33.6168	85.4738	No	
Dynne Creek at County Road 36	Cleburne	33.5642	85.5508	No	
Locheloooge Creek at County Road 5	Cleburne	33.5075	85.5691	No	
Chulafinnee Creek at County Road 8	Cleburne	33.5396	85.6283	No	
Fishhead Creek at Highway 431	Cleburne	33.5111	85.6429	No	
Verdin Creek at County Road 46	Cleburne	33.5667	85.4500	No	
Little Tallapoosa River at County Road 49	Cleburne	33.4947	85.3379	No	
Knokes Creek at County Road 10	Cleburne	33.5233	85.4086	No	
Hurricane Creek at County Road 15/26	Randolph	33.1752	85.5969	No	
Cohobadiah Creek at County Road 615	Randolph	33.4905	85.4618	No	

upstream site was flooded behind a beaver dam and was inaccessible for sampling. Multiple fish were easily captured in the Hillabee Creek sites, Cornhouse Creek, and Elkahatchee Creek, as well as in Sofkahatchee Creek (J. Scanlan pers. comm.). One site, Sweetwater Creek, produced few fish of any species and showed evidence of severe stream degradation from timber harvesting.

Eleven sites with no previous *Fundulus bifax* collection records were sampled in an effort to find new stream populations (Table 1). Most of these sites were to the north of known sites in Cleburne County, Alabama, following the Tallapoosa River upstream towards Georgia in an attempt to find intermediate populations between the 2 known sites in Georgia and those in Alabama. Most of these sites showed obvious degradation from human uses, and none produced *F. bifax*. One site at the Tallapoosa River at Highway 46 was sampled and had the right habitat, including sandy substrate, but with heavy algal growth covering the sand. A site at the Little Tallapoosa River at County Road 49 was unsuitable habitat, with muddy water and deep unconsolidated sediments.

DNA sequencing

Sequences of the cytochrome *b* mitochondrial locus were successfully generated from 10 *Fundulus bifax* from 5 sites. The same locus was also sequenced from an *F. catenatus* from the Paint Rock River in north Alabama, an *F. stellifer* from the Conasauga River in east Tennessee, and an *F. escambiae* from Alligator Creek in the western panhandle of Florida. *F. escambiae* was included as an outgroup. For analytical purposes, a sequence of 852 bases was used from all 13 fish, from position 30 to position 881 in the 1140-base gene. DNA sequences used are available through GenBank, accession numbers KC204750–KC204762.

Tree building

Fig. 2 shows a phylogenetic tree built using neighbor-joining with the HKY model and 1000 bootstrap replications. The 10 *Fundulus bifax* se-

quences formed a shallow clade with 100% bootstrap support. *F. stellifer* rather than *F. catenatus* is weakly supported as a sister taxon in a clade with all of the *F. bifax*. Within *F. bifax*, there is weak support for a clade of 2 of the Elkahatchee specimens along with the only Channahatchee specimen, all on the southwestern edge of the species' known range..

Fig. 3 shows a phylogenetic tree built using MrBayes. This tree also shows *Fundulus bifax* as a shallow clade. A strongly supported clade of an Elkahatchee fish and the only Channahatchee fish is present, with 2 other Elkahatchee fish (1 and 4 in Fig. 3) slightly separated from all other individuals. *F. stellifer* is not in a clade with *F. bifax*, but is slightly more distant from *F. bifax* than *F. catenatus* in what is effectively an outgroup clade.

Genetic distance

D was calculated among all 13 sequences. The range of distance between the 10 *Fundulus bifax* sequences was small, ranging from 0.000 to 0.018. The one *F. bifax* that was consistently at the greatest distance from others sampled was the individual from Channahatchee Creek at the southwestern edge of the species' range, with a range of 0.008 to 0.018. The other 2 subgenus *Xenisma* species were further removed from *F. bifax*, with the distance between *F. catenatus* and all *F. bifax* sequences being 0.062 to 0.073 and that between *F. stellifer* and



Fig. 2. Neighbor-joining tree with the HKY genetic model and 1000 bootstrap replications. *Fundulus escambiae* was designated as the outgroup. Channahatchee, Cornhouse, Elkahatchee, Hillabee, and Sofkahatchee are collection sites of *F. bifax*, numbers following site names refer to multiple individuals from those sites; *F. catenatus* and *F. stellifer* are the 2 species most closely related to *F. bifax*; *F. escambiae* is more distantly related. Numbers at nodes represent bootstrap support for the node. A distance scale (percentage of sequence divergence) is shown at the bottom

lappoosa River (S. J. Rider pers. comm.). The species has not been observed in Georgia since 1990 (Storey et al. 2003, see also http://georgiawildlife.com/sites/default/files/uploads/wildlife/nongame/pdf/accounts/fishes/fundulus_bifax.pdf), and efforts reported here failed to find either the fish or even suitable habitat for it in Cleburne County, Alabama, intermediate to historically known Alabama and Georgia populations. The Little Tallapoosa River also appears to be degraded habitat unsuitable for *F. bifax*, with a heavy sediment load and little clean sand necessary for successful spawning. The few known populations in the middle and lower Tallapoosa basin along with the 1 Coosa basin population currently define the species' range.

Four of the creeks in which *Fundulus bifax* was found appear to support healthy populations, based on ease of capture: Cornhouse Creek in Randolph County, Hillabee Creek in Tallapoosa County, Elkahatchee Creek in Coosa County, and Sofkahatchee Creek in Elmore County. A single fish was collected in Channahatchee Creek in Elmore County, and 2 fish were collected in Emuckfaw Creek in Tallapoosa County. All 6 of these creeks appear to be relatively pristine, with clear water and abundant clean sand that this species needs both for feeding and reproduction. These 6 creeks all run through relatively undisturbed land, with woodlands or well-managed farms making up much of their drainage.

Both phylogenetic tree-building methods used, neighbor-joining and MrBayes, resolved *Fundulus bifax* as a monophyletic clade with low internal genetic variation in the cytochrome *b* gene. The only evidence of genetic variation and structuring was a clade of an Elkahatchee fish and the Channahatchee fish, from the southwestern edge of the species' current range. Unfortunately no genetic data are available for the extirpated Georgia population(s), so it may never be known what genetic diversity has been present in the species. Neither tree building nor analysis for *D* showed either *F. catenatus* or *F. stellifer* to be clearly more closely related to *F. bifax*. Neighbor-joining placed *F. stellifer* in a clade with all of the *F. bifax*, but this was only very weakly supported, with bootstrap support of just over 0.5. The range of values for *D* between *F. catenatus* and *F. bifax* of 0.062 to 0.073 was slightly lower than that between *F. stellifer* and *F. bifax*, 0.067 to 0.079. Small sample size precludes any strong conclusion about those relationships, but *F. bifax* was clearly differentiated from its 2 congeners. Interestingly, the genetic distance between

F. catenatus and *F. stellifer* of 0.056 shows them as being more closely related to each other than to *F. bifax*, which may be more remote from other *Xenisma* species than previously thought.

The population structures of 2 other Tallapoosa River endemics which have been studied previously offer a comparison for the possibility of higher historic genetic diversity in *Fundulus bifax*. The Tallapoosa darter *Etheostoma tallapoosae* is currently found from the Georgia headwaters of the river downstream as far as the fall line. Based on sequencing of the mitochondrial control region and cytochrome *b* sequences, Brogdon et al. (2003) identified genetic structure within this species with at least 4 identifiable management units. Three of these management units are in the same area of the lower Tallapoosa basin as most of the *F. bifax* collections sites reported herein. The Tallapoosa shiner *Cyprinella gibbsi*, also with a current distribution from the Georgia headwater of the Tallapoosa River to the fall line, was found to have a similar population structure to *E. tallapoosae* based on the analysis of mitochondrial ND4L sequences (Connelly et al. 2006). This similar distribution of genetically distinct populations within these 2 species suggests that they share a common biogeographic history (Connelly et al. 2006). Although also a Tallapoosa endemic, *F. bifax* is more habitat-specific than these 2 species (author's pers. obs.), so it may have responded in different ways to historical changes in the Tallapoosa River basin. In particular, *F. bifax* requires extensive clean benthic sand for successful reproduction.

Fundulus bifax does indeed seem to be in a slow, steady decline in its range. Although apparently common in 4 streams, there appears to be little genetic diversity among these populations. Such low genetic diversity may make these populations less able to respond to future environmental perturbations through natural selection (Allendorf & Luikart 2007). This threat from low genetic diversity is higher because these populations are now either largely or entirely isolated both through habitat fragmentation including the impoundment of the Tallapoosa River that historically connected most of them. The current conservation status of the species, N2N3 of NatureServe (imperiled/vulnerable) globally, S2 (imperiled) for Alabama, and Near Threatened with the IUCN, are very optimistic ratings because the species is apparently extirpated in Georgia and now has a range of much less than 5000 km². The species needs more attention as a declining species than it currently receives.

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LITERATURE CITED

- Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell Publishing, Malden, MA
- Boschung HT, Mayden RL (2004) Fishes of Alabama. Smithsonian Institution, Washington, DC
- Brogdon SM, Tabit CR, Kral LG (2003) Population structure of the Tallapoosa darter (*Etheostoma tallapoosae*). Southeast Nat 2:487–498
- Cashner RC, Rogers JS, Grady JM (1988) *Fundulus bifax*, a new species of the subgenus *Xenisma* from the Tallapoosa and Coosa River systems of Alabama and Georgia. Copeia 1988:674–683
- Cashner RC, Rogers JS, Grady JM (1992) Phylogenetic studies of the genus *Fundulus*. In: Mayden RL (ed) Systematics, historical ecology, and North American freshwater fishes. Stanford University Press, Stanford, CA, p 421–437
- Connelly HM, Tabit RT, Kral LG (2006) Population structure of the Tallapoosa shiner (*Cyprinella gibbsi*) and the Tallapoosa darter (*Etheostoma tallapoosae*). Southeast Nat 5:85–92
- Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755
- Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene of mammals. J Mol Evol 32:128–144
- NatureServe (Hammerson G) (2010) *Fundulus bifax*. In: IUCN Red List of Threatened Species. Version 2012.1. Available at www.iucnredlist.org (accessed on 28 June 2012)
- Pääbo S (1990) Amplifying ancient DNA. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, p 159–166
- Scanlan J (2008) Notes on the captive spawning of *Fundulus bifax*. Am Currents 34:9–11
- Storey JC, Straight CA, Freeman BJ, Peterson J, Irwin ER, Freeman MC (2003) Distribution of endemic and imperiled fauna of the Tallapoosa River System of Georgia. In: Hatcher KJ (ed) Proc 2003 Georgia Water Resources Conference, April 23–24, 2003 University of Georgia. Institute of Ecology, The University of Georgia, Athens, GA
- Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739
- Warren ML Jr, Burr BM, Walsh SJ, Bart HL Jr and others (2000) Diversity, distribution, and conservation status of the native freshwater fishes of the southern United States. Fisheries 25:7–29
- Yang Z (1996) Among-site rate variation and its impact on phylogenetic analyses. Trends Ecol Evol 11:367–372

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