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RESEARCH ARTICLE

Genetic diversity and population structure in the Barrens Topminnow (*Fundulus julisia*): implications for conservation and management of a critically endangered species

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Abstract The Barrens Topminnow (*Fundulus julisia*) has undergone a rapid and dramatic decline. In the 1980s, at least twenty localities with Barrens Topminnows were known to exist in the Barrens Plateau region of middle Tennessee; currently only three areas with natural (not stocked) populations remain. The long-term survival of the Barrens Topminnow will depend entirely on effective management and conservation efforts. Captive propagation and stocking of captive-reared juveniles to suitable habitats have successfully established a handful of self-sustaining populations. However, very little is known about the genetic composition of source and introduced populations including levels of genetic diversity and structuring of genetic variation. Here we use both mitochondrial sequence data and genotypes from 14 microsatellite loci to examine patterns of genetic variation among ten sites, including all sites with natural populations and a subset of sites with introduced (stocked) populations of this species. Mitochondrial sequence analysis reveals extremely low levels of variation within populations and fixed differences between drainages. Microsatellite genotype data shows higher levels of genetic variability and a molecular signature consistent with a recent history of population bottlenecks. Measures of genetic diversity at microsatellite loci including allelic richness are similar within source and introduced populations. Bayesian assignment tests and analysis of molecular variation (AMOVA) support two distinct populations, consistent with drainage boundaries. Results from AMOVA analysis also suggest low levels of genetic connectivity between isolated populations within the same drainage. Here we propose two distinct evolutionary significant units (ESUs) and two management units that reflect this population substructure and warrant consideration in future management efforts.

Keywords Conservation genetics · Microsatellites · Mitochondrial DNA · Endangered species · *Fundulus julisia*

Introduction

The Barrens Topminnow (*Fundulus julisia*) is one of the most critically endangered fish species in the eastern United States (Williams and Etnier 1982; Jelks et al. 2008). The species is currently considered endangered by the state of Tennessee, was petitioned for federal listing in 2010, and is currently under status review (USFWS 2011; TN 2016). Prior to 1993, the species was known to occupy twenty locations throughout the Elk, Duck, and Collins River (Caney Fork) drainages in the Barrens Plateau region of Middle Tennessee. Currently only three areas are inhabited by natural populations, and a handful of reproducing introduced sites remain extant (Kuhajda et al. 2014) (Fig. 1). The species is restricted to springs and spring-fed creeks. Droughts and human disturbance of suitable habitats have negatively impacted Barrens Topminnow (BTM) populations. However their most serious threat has been the introduction of the Western Mosquitofish (*Gambusia affinis*), which impact BTM populations by predation on juveniles and by aggressive interactions with adults (Rakes 1989). Presence of Western Mosquitofish (WM) appears

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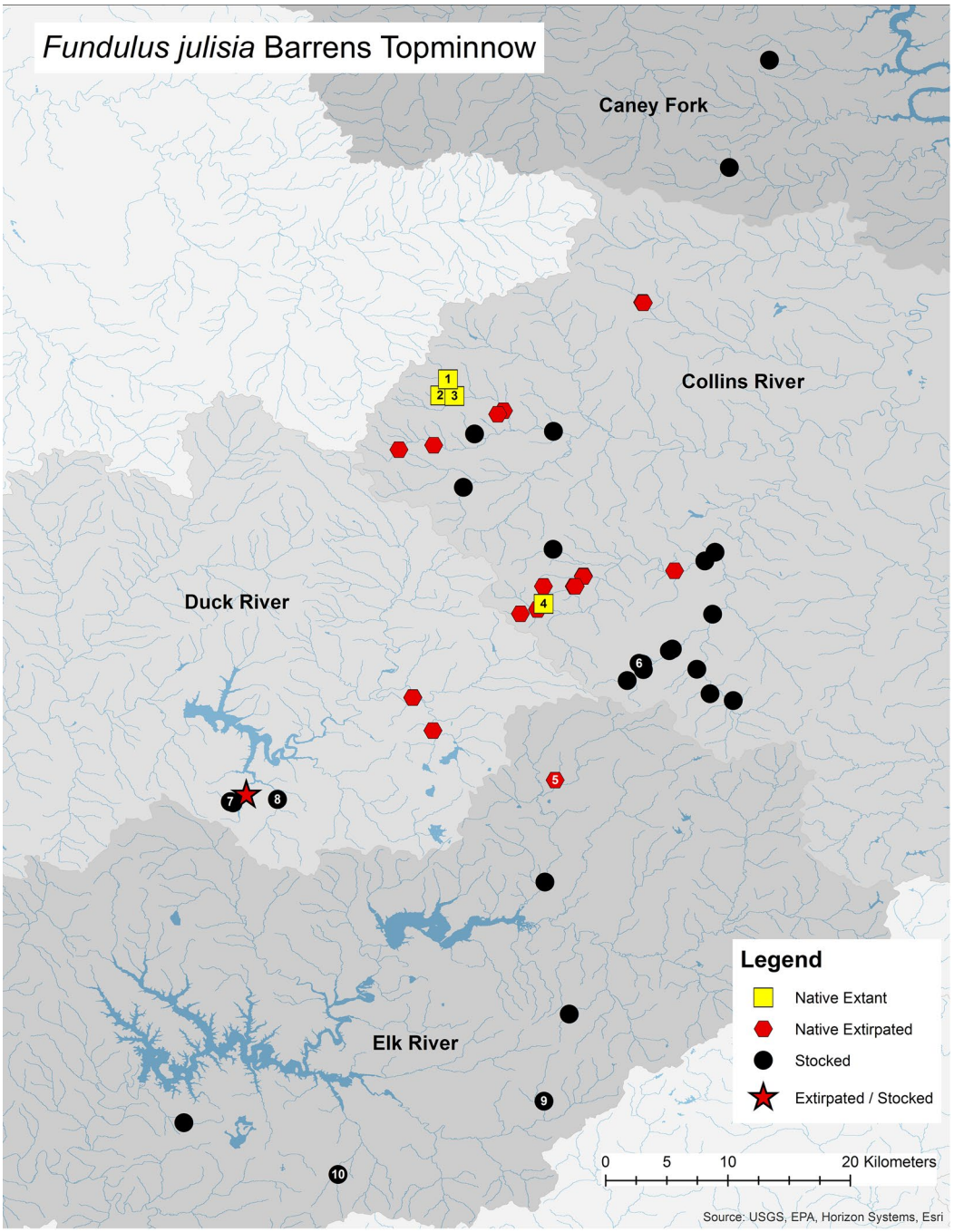

Fundulus julisia Barrens Topminnow
Caney Fork
Collins River
Duck River
Elk River
1
2
3
4
5
6
7
8
9
10
Legend
Native Extant
Native Extirpated
Stocked
Extirpated / Stocked
0
5
10
20 Kilometers
Source: USGS, EPA, Horizon Systems, Esri

◂**Fig. 1** Location of extant and extirpated sites with native populations and sites with stocked populations of the Barrens Topminnow *Fundulus julisia*. Ten sites sampled for genetic analyses included *1* McMahan Creek (n = 4), *2* Pedigo Highway (n = 20), *3* Pedigo Farm (n = 2), *4* Benedict Spring (type locality) (n = 22), *5* Pond Spring (n = 32), *6* Clayborne Spring (n = 19), *7* Collier Spring (n = 20), *8* Short Spring (n = 20), *9* Merkle (Big) Spring (n = 11), *10* Farris Spring (n = 10). *Inset* is illustration of male Barrens Topminnow in breeding colors

to preclude successful establishment of new BTM populations and results in the rapid extirpation of well-established sites (Rakes 1996; Goldsworthy and Bettoli 2006; Laha and Mattingly 2007; Bettoli and Goldsworthy 2011; Westhoff et al. 2013). Thus lack of suitable habitat, free of WM, has hindered recovery efforts. The loss of BTMs not only decrease the aquatic biodiversity of these systems but may negatively impact other rare fishes that occupy these springs and spring-fed creek habitats (Barrens Darter, *Etheostoma forbesi*; Redband Darter *E. luteovinctum*; Flame Chub *Hemitremia flammeae*; TN 2016) through disruption of ecosystem interactions. The long-term persistence of this species will depend on well-informed management and conservation planning that includes captive breeding and reintroduction efforts.

Persistence of the remaining extant populations of BTM is largely due to collaborative efforts of nonprofit conservation groups, university researchers, private landowners, and state and federal agencies. In 2001, a working group composed of The Tennessee Wildlife Resources Agency, the U.S. Fish and Wildlife Service, and non-profit organizations was assembled with the goal of preventing federal listing by protecting remaining wild populations, captive propagation, and establishment of a minimum of five populations in each of the Duck River, Elk River, and Caney Fork River drainages. Captive populations were established at Conservation Fisheries Inc. in Knoxville, TN and the Tennessee Aquarium in Chattanooga, TN (Goldsworthy and Bettoli 2006). These were initiated from brood stock collected from Pond Spring (Elk River drainage) and two sites (Hickory and Lewis/McMahan Creeks) in the Collins River watershed (Caney Fork drainage). A subset of fish reared in these facilities were transferred to the Dale Hollow National Fish Hatchery and used to establish a third captive population. Captive reared fish have been used as a source for reintroductions into suitable habitats within the BTM historical range. As a result of these efforts, more than 44,000 BTMs have been stocked at 27 sites in the headwaters of all three drainages (Fig. 1). However stocking efforts have had mixed success. Many introduced populations are not self-sustaining and are currently maintained by annual stocking of hatchery reared juveniles (Kuhajda et al. 2014). The BTM working group is currently focused on preserving the few remaining BTM sites where WM are not present. The group is also moving towards establishing ark populations, but information on BTM genetics is needed to properly manage captive populations.

Designing management plans and captive breeding programs for the effective recovery of endangered species requires information about levels of genetic diversity within populations and partitioning of genetic variation between populations. Source populations for reintroductions and captive populations should be selected in order to maintain unique evolutionary lineages and maximize total genetic diversity within populations (George et al. 2009). Translocations and captive propagation programs for BTM have attempted to keep populations from different sources separated, but little is known about the genetic diversity of source or stocked populations and their potential for long-term viability. Previous genetic work on the BTM is limited; restriction fragment length polymorphisms (RFLP) analysis of PCR products and allozyme electrophoresis studies have revealed little to no genetic variation within BTM populations with some divergence between drainages (Rogers and Cashner 1987; Strange and Lawrence 2002). However, inferences based on these results are limited as both RFLP and allozyme surveys reveal only a fraction of the underlying genetic variation at the nucleotide level. Sequence data from the mitochondrial genes and nuclear microsatellite genotypes are typically variable and informative for questions at the population level and are thought to reflect neutral processes. In combination, these markers provide a valuable tool for assessing levels of genetic diversity and patterns of historical distinctiveness.

Here we examined genetic variation at the mitochondrial control region and at 14 microsatellite loci in an effort to understand some of the genetic factors important for conservation and management of the BTM. Specifically, our objectives were to assess levels of genetic variation within and among nine isolated populations (10 sites) of BTM occurring in the Duck, Elk, and Caney Fork River Drainages. Measures of genetic variation were compared between stocked and natural populations in order to assess the maintenance of genetic variation in newly founded populations. Tests for population bottlenecks were performed in order to detect the impact of recent population declines on patterns of genetic variation. Information on partitioning of genetic variation between populations was used to identify and prioritize populations that should serve as a source for future propagation efforts and to differentiate unique evolutionary lineages that warrant separate management actions.

Materials and methods

Samples

A total of nine populations (10 sites) were sampled for this study (Fig. 1). These include the three remaining natural

populations in the Collins River of the Caney Fork drainage: Benedict Spring (site 4; type locality) in Hickory Creek system, and Pedigo Spring [including both Pedigo Farm (site 3) and Pedigo Highway sites (site 2)] and McMahan Creek (site 1) in the Lewis/McMahan Creek system. Also included is the recently extirpated natural population from Pond Spring in the Elk River drainage (site 5). No Duck River BTMs are extant in the wild or in captivity; all sites within this drainage are stocked with Collins or Elk River progeny. In addition, samples were collected from five introduced populations; these were selected to include recipient populations from each of the natural sources. These include Clayborne Spring (site 6; Benedict Spring brood stock), Collier Spring (site 7; Pedigo Spring brood stock), Merkle Spring (site 9; Pond Spring brood stock), Farris Spring (site 10; Pond Spring brood stock), and Short Spring (site 8; Benedict and Pedigo Farm brood stock). Fin clips were obtained during routine monitoring of populations by collaborators at the Tennessee Aquarium in Chattanooga, Tennessee. Fin clips from Pond Spring fish were collected by USFWS in 2011 prior to the extirpation of this population between 2012 and 2013 (Zuber and Mattingly 2012; Kuhajda et al. 2014). Tissue was stored in 95% ethanol and kept at −20 °C prior to extraction of DNA.

Molecular techniques

Mitochondrial control region

A total of 60 individuals from eight localities distributed in the headwaters of the Duck, Elk, and Collins (Caney Fork) River drainages were sequenced for a portion of the mitochondrial control region. Sampled population included the following: Benedict Spring (n = 10), Clayborne Spring (n = 4), McMahan Creek (n = 4), Pedigo Farm (n = 18), Short Spring (n = 6), Farris Spring (n = 3), Merkle Spring (n = 4), and Pond Spring (n = 11). Early analyses of control region sequence data showed little or no variation within drainages and were uninformative for fine scale population differentiation. For this reason, Pedigo Farm was selected to represent the Elk River drainage and Pedigo Highway and Collier Spring were excluded from the mitochondrial analyses (Pedigo Highway is located adjacent to Pedigo Farm and Collier Spring was stocked with Pedigo Farm fish). DNA was isolated from BTM fin clips using a modification of the protocol by Wang and Storm (2006) and was diluted to a concentration of 50–100 ng/µl for use in PCR. Primers for amplification of a 690 bp region of the mitochondrial control region were designed from a sequence alignment of four congeneric species, *Fundulus heteroclitus, F. sciadicus, F. olivaeus*, and *F. lima*. Sequences for forward and reverse primers were FJCR89F 5′ GCT CCC AAA GCT AGG ATT CTC-3′, and FJCR862R 5′ ACC CCC TAA AAG CCG AGA GG-3′, respectively. PCR conditions were performed in 10-µl reactions containing 50–100 ng of template DNA, 0.5 µM of each primer, 200 µM each dNTP, 0.3 U GoTaq hot start polymerase (Promega), 2.5 mM MgCl2, and 1× commercial buffer. Thermal cycling conditions were as follows: 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 75 s, and ending with 72 °C for 10 min. PCR products were sequenced in both directions using Sanger Sequencing on an ABI 3730XL automated sequencer. Sequence chromatograms were imported and visualized using SEQUENCHER version 5.2 (Gene Codes Corp. Ann Arbor, MI, USA). Consensus sequences were exported to the program Bioedit (Hall 1999) and the alignment was adjusted by eye.

Microsatellites

We identified 14 microsatellite loci that are polymorphic within the BTM. Primers and techniques used for determining microsatellite genotypes followed Hurt and Harman (2017). PCR reactions were combined for multiplexing following amplification and 1 µl of MCLAB orange size standard was added to each reaction. Fragment analysis was performed on an ABI 3730XL genetic analyzer (MCLAB, San Francisco, CA, USA). Electropherograms were manually scored using the program PEAKSCANNER 2.0 (Applied Biosystems).

Statistical analysis

Mitochondrial control region

Measures of genetic variation for mitochondrial data including gene diversity and nucleotide diversity were estimated for populations using ARLEQUIN 3.5.2 (Excoffier and Lischer 2010). Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was also performed in ARLEQUIN in order to assess the amount of variance due to differences between drainages, and differences between populations within drainages.

Microsatellites

Deviations from Hardy–Weinberg equilibrium due to genotyping errors resulting from stuttering, large allele dropout, and null-alleles was evaluated using the software MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) (1000 randomizations). Departures from Hardy–Weinberg equilibrium and genotypic disequilibrium for microsatellite data were examined using the Markov chain Monte Carlo approximation of Fisher's exact test as implemented in GENEPOP 4.2 (Rousset 2008). Tests for Hardy–Weinberg and genotypic disequilibrium were evaluated after Bonferroni correction

for multiple tests. Standard measures of genetic diversity including allele frequencies, the number of alleles per locus, observed and expected heterozygosity values, unbiased heterozygosity, private alleles, and inbreeding coefficient (F_{IS}) were estimated using GENALEX 6.5 (Peakall and Smouse 2006). Allelic richness was calculated for each population using the program FSTAT 2.9.3 (Goudet 1995). A one-tailed permutation test was performed to test the hypothesis that source population had higher allelic richness than did introduced populations using the program FSTAT (1000 permutations).

It has been well documented that BTM populations have experienced rapid declines in numbers due to alteration of habitat and establishment of WM populations over the last 30 years (Rakes 1996; Goldsworthy and Bettoli 2006; Laha and Mattingly 2007; Bettoli and Goldsworthy 2011; Westhoff et al. 2013). The only remaining native population that has not been inhabited by WM is McMahan Creek; all other populations have likely experienced population declines within the last few decades. Such recent declines may leave a molecular signature on patterns of allelic diversity on polymorphic loci. First, there is a transient excess of heterozygosity at selectively neutral loci (lasting 0.25–2.5 times $2N_e$ generations) relative to expectations under drift-mutation equilibrium (Cornuet and Luikart 1996). Second, as a result of the loss of rare alleles, recent population bottlenecks will show a modal shift away from an L-shaped distribution of allele frequency categories (lasting a few dozen generations) (Luikart et al. 1998). We tested for evidence of recent population decline using the program BOTTLENECK (Piry et al. 1999; Chiucchi and Gibbs 2010). A test for heterozygosity excess was performed using the Wilcoxon signed rank test ($\alpha = 0.05$). We ran 1000 iterations using the Two-Phase model (Cornuet and Luikart 1996; Luikart and Cornuet 1998). Two different values were used for percent stepwise mutations (p_{smm}); these are $p_{smm} = 0.90$ and $p_{smm} = 0.78$. The variance of mutational sizes (σ) was set at 3.1. Parameter values were selected based on the best estimated values from empirical studies (Peery et al. 2012). We also tested for a mode-shift in allele frequency distributions to detect a more recent bottleneck (Luikart et al. 1998). Due to small sample size McMahan Creek was not included in these analyses.

Hierarchical partitioning of genetic variation within and between populations and between drainages was examined using analysis of molecular variance (AMOVA) as implemented in the software ARLEQUIN (Excoffier and Lischer 2010). Estimates of pairwise F_{ST} values were also obtained using ARLEQUIN and significance values were generated using 1000 permutations according to Excoffier et al. (1992).

The software STRUCTURE v2.3.4 (Pritchard et al. 2000) was used to estimate q, the proportion of each individual's genome assigned to each group (K). Simulations ran for 100,000 steps (following a 20,000 step burn-in period) and three independent runs were performed for each of 1–10 groups. STRUCTURE analyses were performed using the admixture model with correlated allele frequencies. Optimal K values were determined using the ΔK method (Evanno et al. 2005) as performed by Structure Harvester (Earl 2012). Graphical depictions of STRUCTURE results were generated using DISTRUCT (Rosenberg 2004). Finally, the software program POPTREE2 (Takezaki et al. 2010) was used to construct an unrooted neighbor-joining tree using D_A distance (Takezaki and Nei 1996) with bootstrap support (1000 replicates).

Results

Mitochondrial control region

PCR amplification of the mitochondrial control region produced an approximately 690-bp product in all 60 individuals selected for sequence analysis. Genetic variation of haplotypes was extremely low. The resulting alignment identified one indel and one polymorphic site for a total of three haplotypes among all eight populations examined (Genbank Accession MF158052 - MF158054). All populations within the Caney Fork and Duck River drainages were fixed for the same haplotype. Elk River drainage populations were polymorphic for two unique haplotypes and did not share the Duck River/Caney Fork drainage haplotype. Gene diversity and nucleotide diversity for the Elk River samples were 0.233 and 0.003, respectively. Pairwise F_{ST} values for Elk River/Caney Fork was 0.944. Results from hierarchical AMOVA, which partitioned individuals by population and then by river drainage showed that the majority of the genetic variance (90.93) can be attributed to difference between drainages (Table 1). Only 1.59% of the genetic variance was attributed to differences between populations within drainages and the remaining 7.48% of the variance was found within the Elk River sites.

Microsatellites

A total of 178 individuals were genotyped at 14 microsatellite loci. Analysis using Micro-Checker identified four instances of heterozygote deficiency. A possibility of null alleles was found for *Fuju12* in Pond Spring and Short Spring and for *Fuju20* in Merkle Spring. The possibility of genotyping error due to stuttering was found for *Fuju13* in Short Spring, however careful re-examination of electropherograms showed that genotyping error was unlikely. Pairwise tests for genotypic disequilibrium were not significant after Bonferroni correction. Tests for Hardy–Weinberg

Table 1 Hierarchical analyses of molecular variation (AMOVA) for mitochondrial control region sequence (above) and microsatellite genotype data (below) from Barrens Topminnow, *Fundulus julisia*

Loci	Source of variation	Variance	% Total	Φ-statistics	*p*-value
Mitochondrial control region	Among drainages	0.302	90.93	$\varphi_{CT}=0.909$	=0.0098
	Among populations within drainages	0.005	1.59	$\varphi_{SC}=0.175$	=0.1525
	Within populations	0.025	7.48	$\varphi_{ST}=0.925$	<0.0001
Microsatellites	Among drainages	0.422	20.84	$\varphi_{CT}=0.208$	=0.0342
	Among populations within drainages	0.453	22.36	$\varphi_{SC}=0.282$	<0.0001
	Within populations	1.150	56.80	$\varphi_{ST}=0.432$	<0.0001

Hierarchical subdivision includes drainages within species, populations within drainages, and individuals within populations

equilibrium identified no instances of heterozygote deficiency after Bonferroni correction for multiple tests. None of the loci demonstrated heterozygote deficiency consistently across multiple populations and samples that failed to amplify were rare indicating that null homozygotes were not common. Therefore all loci were included for further analyses.

Microsatellite analyses revealed moderate to low levels of genetic variability across the ten examined sites (Table 2; Fig. 1). All 14 loci were polymorphic however the average number of alleles was low, ranging from one to five alleles per locus per population. Allelic diversity was lowest for the Benedict Spring and Clayborne Spring populations. Benedict Spring was fixed for a single allele at seven of the 14 loci examined and averaged 1.50 alleles per locus. Clayborne Spring was fixed at six loci and averaged 1.57 alleles per locus. Pond Spring and McMahan Creek had the highest allelic diversity, with an average of 3.57 and 3.79 alleles per locus respectively. Average expected heterozygosity values were lowest for Benedict Spring and Clayborne Spring ($H_e=0.159$ and 0.166, respectively) and was highest for Merkle Spring ($H_e=0.553$). Pond Spring had the highest number of private alleles (A_p); ($A_p=9$). The inbreeding coefficient within populations (F_{IS}) was lowest for Collier Spring ($F_{IS}=-0.258$), indicating an excess of heterozygotes from expectations under Hardy–Weinberg equilibrium, but this value was not significant after Bonferroni adjustment for multiple tests. No significant difference was detected for allelic richness in native sites versus introduced sites. The average allelic richness across native sites was 1.904 and for introduced sites was 2.139 ($p=0.781$).

We found evidence for a population bottleneck in 7 out of the 9 study sites examined (Table 3). The mode-shift test detected recent population bottlenecks in two populations, Benedict Spring and Farris Spring. Tests for a historical population bottleneck using the Wilcoxon sign-rank test ($p_{smm}=0.9$, $\sigma=3.1$) were significant for six populations including Benedict Spring, Pond Spring, Pedigo Farm,

Table 2 Standard measures of genetic diversity for ten sampled locations of Barrens Topminnow *Fundulus julisia*

Site	*N*	N_a	A_R	H_o	H_e	uH_e	F_{IS}	A_p
Benedict (4)[a]	22	1.500 ± 0.275	1.354 ± 0.112	0.155 ± 0.055	0.159 ± 0.053	0.163 ± 0.054	0.017 ± 0.055	0
Pond (5)[a]	32	3.571 ± 0.501	2.347 ± 0.196	0.453 ± 0.060	0.491 ± 0.057	0.499 ± 0.058	0.084 ± 0.036	7
McMahan (1)[a]	4	3.786 ± 0.114	1.525 ± 0.186	0.202 ± 0.080	0.194 ± 0.066	0.224 ± 0.077	−0.036 ± 0.108	0
Pedigo H. (2)[a]	20	3.143 ± 0.467	2.124 ± 0.236	0.439 ± 0.084	0.387 ± 0.070	0.397 ± 0.072	−0.120 ± 0.051	2
Pedigo F. (3)[a]	20	3.143 ± 0.443	2.171 ± 0.210	0.475 ± 0.068	0.436 ± 0.063	0.448 ± 0.065	−0.100 ± 0.050	2
Clayborne (6)	19	1.571 ± 0.137	1.374 ± 0.111	0.153 ± 0.048	0.166 ± 0.053	0.171 ± 0.054	0.047 ± 0.040	2
Collier (7)	20	2.500 ± 0.310	1.987 ± 0.190	0.496 ± 0.094	0.387 ± 0.069	0.397 ± 0.071	−0.258 ± 0.068	0
Farris (10)	10	3.714 ± 0.266	2.600 ± 0.143	0.528 ± 0.065	0.539 ± 0.038	0.568 ± 0.040	0.045 ± 0.071	1
Merkle (9)	11	3.500 ± 0.442	2.641 ± 0.223	0.570 ± 0.065	0.553 ± 0.055	0.581 ± 0.058	−0.061 ± 0.083	4
Short (8)	20	2.857 ± 0.312	2.095 ± 0.178	0.412 ± 0.068	0.429 ± 0.061	0.441 ± 0.063	0.054 ± 0.061	1
Total	178	2.707 ± 0.608	2.022 ± 0.179	0.388 ± 0.025	0.374 ± 0.022	0.389 ± 0.023	−0.033 ± 0.021	19

Numbers in parentheses indicate site locations on Fig. 1

Summary statistics are given as mean ± standard error

N sample size, N_a average number of alleles, A_R allelic richness, H_o average observed heterozygosity, H_e average expected heterozygosity, uH_e average unbiased expected heterozygosity, F_{IS} Inbreeding coefficient, A_p number of private alleles. Pedigo H. and Pedigo F. refer to Pedigo Highway and Pedigo Farm, respectively

[a]Indicate natural sites, all other sampled sites are introduced

Collier Spring, Merkle Spring, and Sharp Spring. Excess heterozygosity was not detected in Benedict Spring when alternative parameter values were used ($p_{smm}=0.78$, $\sigma=3.1$). However, in general changes to parameter settings for the Wilcoxon test for excess heterozygosity had very little effect on results.

Genetic variation of microsatellites genotypes was highly structured by populations, both within and between drainages. AMOVA analysis for the combined 14 microsatellite loci showed that 20.84% of the variance in allele frequencies could be attributed to differences between drainages. Populations within drainages accounted for 22.36% of the variance and over half of variance was found within populations (Table 1). Pairwise F_{ST} values ranged from 0.001 to 0.668 and were significant for all but four comparisons (Table 4). Non-significant F_{ST} values all corresponded to comparisons between introduced populations and their respective source population. Only two introduced/source population pairs had significant pairwise F_{ST} values. These include Collier Spring which was stocked with Pedigo Farm brood stock ($F_{ST}=0.098$) and Short Spring which was stocked with a mixture of Benedict Spring and Pedigo Farm brood stock ($F_{ST}=0.257$ and 0.223, respectively).

Bayesian cluster analysis using the ΔK method to determine optimal K values suggested that the most likely number of clusters was two, although ΔK was also high for K = 3 clusters (Fig. 2). In the K = 2 graph, individuals from Elk River drainage assigned to one cluster,

Table 3 Results of the mode-shift test and Wilcoxon sign-rank test for heterozygosity excess within 9 sampled sites of Barrens Topminnow, *Fundulus julisia*

Site	Mode-shift	L	TPM ($p_{smm}=0.9$, $\sigma=3.1$)			TPM ($p_{smm}=0.78$, $\sigma=3.1$)		
			Obs LH_{exc}	Exp LH_{exc}	p	Obs LH_{exc}	Exp LH_{exc}	p
Benedict (4)[a]	Shifted	7	5	3.13	0.039*	5	3.15	0.055
Pond (5)[a]	Normal	13	10	7.03	0.003*	10	6.99	0.003*
Pedigo H. (2)[a]	Normal	12	7	6.51	0.285	8	6.46	0.212
Pedigo F. (3)[a]	Normal	12	10	6.54	0.039*	10	6.56	0.039*
Clayborne (6)	Normal	8	5	3.72	0.273	5	3.72	0.273
Collier (7)	Normal	11	8	5.80	0.006*	8	5.80	0.006*
Farris (10)	Shifted	14	7	8.01	0.428	8	8.09	0.404
Merkle (9)	Normal	13	12	7.18	<0.001*	12	7.16	<0.001*
Short (8)	Normal	13	10	6.85	0.040*	10	6.76	0.034*

Numbers in parentheses indicate site locations on Fig. 1

L the number of polymorphic loci, *TPM* two-phase model, p_{smm} proportion of mutations following a strict stepwise mutation model, σ variance in the size of non-stepwise mutations, LH_{exc} number of loci with heterozygosity excess, *p* *p*-value from Wilcoxon sign-rank test for heterozygosity excess

*Indicates significant results ($p<0.05$)

[a]Indicate natural sites, all other sampled sites are introduced

Table 4 Pairwise F_{ST} values (below diagonal) and *p*-values (above diagonal) for 10 collected sites of Barrens Topminnow, *Fundulus julisia*, as calculated by ARLEQUIN (1000 permutations)

	Benedict	Pond	McMahan	Pedigo H.	Pedigo F.	Clayborne	Collier	Farris	Merkle	Short
Benedict (4)[a]	–	0.0000	0.0000	0.0000	0.0000	**0.3436**	0.0000	0.0000	0.0000	0.0000
Pond (5)[a]	0.5365	–	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0049**	**0.0029**	0.0000
McMahan (1)[a]	0.6634	0.4673	–	0.0000	0.0000	0.0010	0.0000	0.0000	0.0010	0.0010
Pedigo H. (2)[a]	0.5469	0.3976	0.2792	–	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Pedigo F. (3)[a]	0.5513	0.3401	0.2939	0.0744	–	0.0000	0.0000	0.0000	0.0000	0.0000
Clayborne (6)	**0.0007**	0.5271	0.6676	0.5294	0.5420	–	0.0000	0.0000	0.0000	0.0000
Collier (7)	0.5899	0.4142	0.3393	0.0978	0.1222	0.5771	–	0.0000	0.0000	0.0000
Farris (10)	0.5873	**0.0618**	0.3919	0.3339	0.2775	0.5713	0.3548	–	**0.0537**	0.0000
Merkle (9)	0.5720	**0.0639**	0.4104	0.3556	0.2977	0.5559	0.3685	**0.0193**	–	0.0000
Short (8)	0.2573	0.3727	0.2552	0.1662	0.2233	0.2560	0.2309	0.3262	0.3372	–

Numbers in parentheses indicate site locations on Fig. 1

Bolded F_{ST} values represent non-significant comparisons after Bonferroni corrections ($\alpha<0.0011$)

[a]Indicate natural sites, all other sampled sites are introduced

and individuals from Caney Fork and Duck River drainages assigned to the second cluster. Assuming three clusters (K = 3), Elk River populations were assigned to one cluster. Pedigo Highway, Pedigo Farm, McMahan Creek and Collier Spring individuals were assigned to a second cluster. Benedict Spring and Clayborne Spring were assigned to a third cluster, and Short Spring displayed admixture between clusters two and three.

The neighbor-joining tree resolved three well-supported groups (Fig. 3). The first group was composed of three populations from the Elk River drainage including Pond Spring, Merkle Spring, and Farris Spring (bootstrap = 100). The second group was composed of Benedict Spring, Clayborne Spring (both in the Caney Fork drainage), and Short Spring (Duck River). The third group was composed of Collier Spring (Duck River), Pedigo Farm, and Pedigo Highway (Caney Fork drainage).

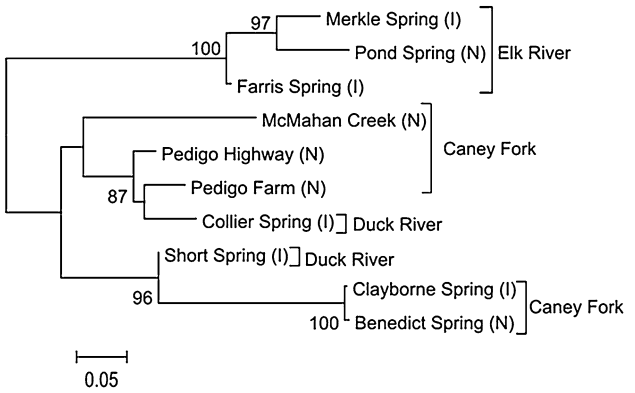
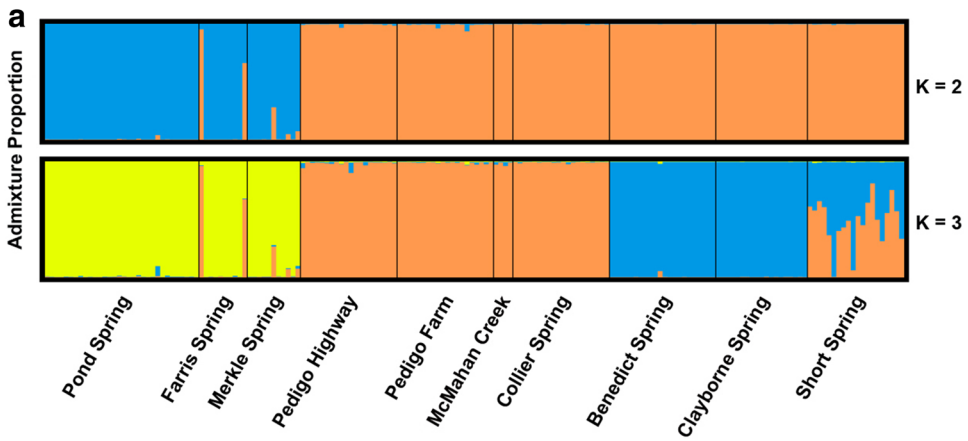


Fig. 3 Unrooted neighbor-joining tree for the 10 sample sites of Barrens Topminnows *Fundulus julisia* based on D_A distances from 14 microsatellite loci



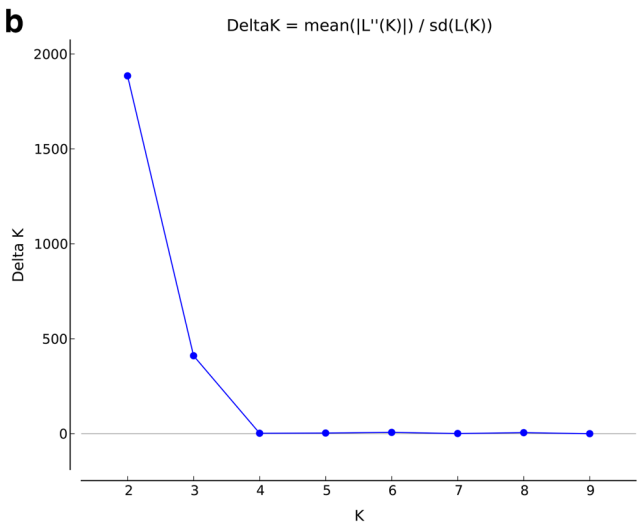


Fig. 2 **a** Bayesian clustering and individual assignment as calculated with STRUCTURE using K = 2 and K = 3 (K = the number of clusters) for 10 different samples sites of Barrens Topminnow, *Fundulus julisia*. Each vertical bar represents an individual. **b** Delta K for different values of K for 10 sampled sites of Barrens Topminnows

Discussion

The long-term outlook for the recovery of the BTM can be improved by implementing management strategies that integrate information on genetic diversity and population structure. Alteration of habitat from anthropogenic factors, naturally occurring droughts, and establishment of invasive WM continue to cause dramatic declines in BTM numbers. As it currently stands, the persistence of this species is entirely dependent on successful captive propagation and establishment of new populations into suitable habitats and maintaining ark population. The selection of founding individuals for captive populations, introduced populations, and supplementation of stocked populations from natural sites is critical, as this establishes the genetic characteristics for the future of the entire species. Ideally, captive populations should maximize genetic diversity within populations while maintaining unique evolutionary lineages. The results of the present survey of genetic variation at mitochondrial and microsatellite loci have important implications for founding and maintaining ark populations and for planning future introductions.

Genetic variation within populations

Many threatened and endangered species are genetically depauperate in comparison to related species that are demographically stable. Analyses of mitochondrial sequence data in the BTM showed lower levels of genetic variation than what has been found in surveys of other *Fundulus* taxa that are more widespread. From 60 sequenced individuals only three mtDNA haplotypes were identified and no variation was found within the entire Collins River system (Caney Fork drainage). For comparison, a study of mtDNA control region sequences in the congeneric Mummichog (*F. heteroclitus*) from the Elizabeth and York Rivers in Virginia identified 46 unique mitochondrial haplotypes from 208 sequenced individuals (Mulvey et al. 2003). Genetic variation at mitochondrial loci may not be reflective of the variation present in the nuclear genome. In addition to small effective population size, selective forces can also reduce variation at mitochondrial genes. Although mtDNA evolves rapidly and should therefore maintain higher levels of genetic variation, the mitochondrial genome lacks recombination and is inherited as a single haplotype so that purifying selection against deleterious alleles at any mitochondrial gene will reduce genetic variation at all mitochondrial loci through hitchhiking (Bazin et al. 2006).

Nuclear microsatellite markers showed much greater variation within and between populations than mitochondrial control region sequences. However, microsatellite variation in the BTM was still less than what has been found in related *Fundulus* species. In Mummichog, allelic richness ranged from 1.0 to 10.0 alleles per population per locus and the average H_e was 0.77 for populations spread across the southeastern United States (Adams et al. 2006). In Blackstripe Topminnow (*F. notatus*), allelic richness ranged from 6 to 50 alleles per locus and average H_e was 0.47. Collectively these results suggest that genetic variation in both the mitochondrial and nuclear genomes has been negatively affected by dwindling population numbers, but some variability has been retained.

Sudden reductions in population size will result in loss of allelic variability and eventually a reduction in heterozygosity. Our analysis using the program BOTTLENECK suggests that recent population bottlenecks may explain the low levels of genetic variability found in Benedict Spring (the type locality for the BTM). This population had substantially lower levels of heterozygosity and allelic variability than the other surveyed sites (except for Clayborne Spring which was stocked from Benedict Spring brood stock). Benedict Spring showed an excess of alleles at intermediate frequency compared to what is expected for a population of stable size, a signature consistent with a recent bottleneck event. This pattern may reflect recent drought events in the Cumberland Plateau. Benedict Spring has dried up three times since 2006; each time between 120 and 1000 fish were removed from the spring and housed until water returned to the site. A mode shift was also detected for Farris Spring which may reflect a founding event for the population. Farris Spring is a stocked site in the Elk River drainage that was initiated in 2004 from 200 Pond Spring stock fish. The program BOTTLENECK also detected evidence of historical size reductions in six sites including Pond Spring, Pedigo Farm, Collier Spring, Merkle Spring and Short Spring. The native Pond Spring population has been declining in numbers since 2011 due to habitat degradation and the establishment of WM and is now thought to be extirpated (Watts and Mattingly 2009; Zuber et al. 2010; Zuber and Mattingly 2011, 2012; Kuhajda et al. 2014). Pedigo Farm (also native) has experienced a decline in numbers of BTM following invasion of WM that occurred during winter flooding in 2004–2005 (Goldsworthy and Bettoli 2005). Collier Spring, Merkle Spring, and Short Spring are all stocked sites and patterns of allelic diversity may reflect founder events.

Recent population declines may not be entirely to blame for the observed low levels of genetic variability. Specialized spring habitat requirements may naturally limit population sizes and migration between creek systems in the BTM. This species requires permanent springs and spring-fed creeks which were probably common during moister climates (Pleistocene and early Holocene). However such habitats are now limited, largely seasonal, and fragmented (Rakes 1996). Natural population fluctuations, limits to population size, and limited movement between

populations may contribute to the low levels of genetic variation observed across all BTM populations, as has been seen in another southeastern spring endemic, the Watercress Darter *Etheostoma nuchale* (Fluker et al. 2010).

Finally, an important goal for reintroduction programs is that stocked sites maintain the genetic diversity that was present in the original source population. Reintroduced sites are frequently initiated from a small number of founder individuals; such founder effects can have negative impacts on the genetic diversity of introduced sites. Fortunately, our results suggest that stocked populations of BTMs evaluated here have retained the majority of the genetic variability present from their original source populations (Table 2). Pond Spring was the source for the brood stock that were used to establish Farris and Merkle Spring. The average allelic richness (R) is slightly higher for Merkle Spring and Farris Spring than for Pond Spring ($R = 2.64$, 2.60, and 2.35, respectively). Similarly Pedigo Farm was the source for the brood stock that founded Collier Spring; R is slightly less for Collier Spring than for Pedigo Farm ($R = 1.39$ and 2.17, respectively). Finally, Benedict Spring and Clayborne Spring both have similarly low levels of allelic variability ($R = 1.35$ and 1.37, respectively), and Benedict Spring was the source of brood stock for Clayborne Spring. Furthermore, results from the permutation test showed no significant difference in allelic richness between native and stocked sites. These results suggest that these introduced sites are providing a genetic safety net should the remaining natural populations continue to decline.

Divergence between populations and units of conservation

The identification of discrete population units is necessary so that monitoring, management, and conservation efforts can be effectively targeted. Evolutionarily Significant Units or ESUs are defined as historically isolated groups of populations that are on independent evolutionary trajectories (Moritz 1994). Molecular results coupled with distribution and habitat data suggest that Elk River populations should be considered as a separate ESU. Elk River populations did not share mitochondrial haplotypes with the Caney Fork populations; this finding is consistent with PCR-RFLP data at the mitochondrial cytochrome B gene showing that Elk River populations possessed a unique RFLP type that was distinguishable from all other Caney Fork populations (except for a now extirpated population at Charles Creek) (Strange and Lawrence 2002). Microsatellite results also support this recommendation. Pairwise F_{ST} values separating Elk River populations from all other populations were high and in the neighbor-joining tree Elk River populations grouped together with 100% bootstrap support. The only natural site within the Elk River drainage, Pond Spring, is now likely extirpated. Fortunately, the two introduced sites in the Elk River, Merkle Spring and Farris Spring, appear to have retained genetic diversity from Pond Spring brood stock.

Sites within the Caney Fork drainage are likely not sufficiently differentiated to qualify as distinct ESUs as they all share a single mitochondrial haplotype, but may be considered as separate management units (MUs) based on divergence at microsatellite loci. Moritz (1994) defines MUs as "populations that do not show reciprocal monophyly for mtDNA alleles, yet have diverged in allele frequency and are significant for conservation". Results from our microsatellite analysis including STRUCTURE plots, pairwise F_{ST} values, and the neighbor-joining tree were used to identify two MUs within the Collins River system (Caney Fork drainage). The first MU includes Benedict Spring, the type locality for the species, and Clayborne Spring, which was stocked from Benedict Spring brood stock. These are in the Hickory Creek watershed, a tributary to the Barren Fork. The second MU includes two sites with native populations, Pedigo Farm and Pedigo Highway, and Collier Spring (Duck River drainage), which was stocked from Pedigo brood stock. The Pedigo sites (and the McMahan Creek site) are in the Witty Creek watershed, a tributary to the South Prong of the Barren Fork. These watersheds (Hickory and Witty Creeks) are separated by 23 km of river habitat not favorable to BTMs and likely acts as a barrier to migration. Unfortunately we were not able to obtain large enough sample sizes to make recommendations regarding McMahan Creek. The STRUCTURE plot indicates McMahan Creek groups with Benedict Spring, however this was not well supported in the neighbor-joining tree. Geographically McMahan Creek should group with the Pedigo sites as they are all part of the Witty Creek watershed and are only separated by <2 river km of favorable spring-fed creek habitat.

There are no historical records of BTMs for Short Spring and no brood stock was ever available from the Duck River drainage. This site has been stocked with BTMs from Pedigo and Benedict Spring, and a few specimens (ten) from Pond Spring brood stock. Based on both mitochondrial and microsatellite results, the current population appears to have retained Pedigo and Benedict Spring alleles. Therefore it is advisable that this site be managed as part of the Caney Fork drainage.

Conclusion

Management and conservation of endangered species is enhanced by information on the partitioning of genetic variation in extant natural populations. Such information is

often not available for even the most critically endangered species. Due to urgency, population recovery efforts must proceed without prior knowledge of underlying genetic structure. In the BTM, population monitoring and reintroduction efforts over the last 15 years have been key to the persistence of this species. The results from this study will be used by U.S. Fish and Wildlife Service in the status review for potential listing under the ESA and will also be used for preparation of a recovery plan for BTM. Our study provides a baseline for understanding patterns of neutral genetic variation in both managed and natural sites. Results presented here suggest that introduced sites have retained similar levels of genetic variation as their source populations. In the case of the Elk River populations, these stocked sites are likely the only remaining source of Elk River genetic diversity. Continued monitoring, propagation, and stocking efforts should incorporate genetic information in their design and implementation.

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