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Development of polymorphic microsatellite loci for the design of management and conservation strategies of the critically endangered Barrens topminnow (*Fundulus julisia*, Williams & Etnier, 1982)

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Summary

The decline of the Barrens topminnow (*Fundulus julisia*), a small schooling fish endemic to the Barrens Plateau region of Middle Tennessee, is one of the most dramatic cases of species imperilment in eastern North America. Loss of undisturbed habitat, coupled with the introduction of the invasive Western Mosquitofish (*Gambusia affinis*) has led to the extirpation of 12 out of 14 natural populations since the 1980s. Identified is a set of 14 microsatellite loci to improve conservation and management strategies for the species. Four loci were cross-amplified using primers designed for congeneric taxa and 10 loci were developed from whole genome Illumina sequencing. Initial surveys in two populations suggest significant structuring of genetic variation and differing levels of heterozygosity among populations. These markers will contribute valuable information to ongoing conservation and management efforts.

1 | INTRODUCTION

The Barrens topminnow (*Fundulus julisia*) is one of the most critically endangered fish species in the state of Tennessee. When it was first described in the early 1980s (Williams & Etnier, 1982), 14 populations were known to exist throughout Barrens Plateau in middle Tennessee in three spring-fed watersheds: the Duck River, the Elk River, and the Caney Fork (Laha & Mattingly, 2007). These populations have declined drastically in number; currently only two natural and a handful of stocked populations remain. While loss of habitat has contributed to this decline, the most critical threat to the persistence of Barrens topminnows has been the widespread introduction of the invasive Western mosquitofish (*Gambusia affinis*; Laha, 2004; Laha & Mattingly, 2007). Despite their small size, mosquitofish are voracious predators and are known to harass local populations of many native fishes. In the case of Barrens topminnow, mosquitofish have been shown to prey on topminnow larvae and juveniles and harassment of adult topminnows by mosquitofish may reduce reproductive success (Goldsworthy & Bettoli, 2005; Laha & Mattingly, 2007).

The Barrens topminnow is currently not federally endangered but is under review for listing (USFWS, 2011). However substantial ongoing efforts are being made to recover this species. Non-profit organizations as well as state and federal agencies have implemented ongoing management strategies, which include captive propagation, stocking of juveniles, and annual monitoring of natural and introduced populations. These efforts could benefit from information regarding the structuring of genetic variation within and between natural, introduced, and captive populations. Currently little is known about the genetic structure of populations and the impact that captive breeding and stocking efforts may have had on levels of heterozygosity. One early study surveyed variation from RFLP and sequence data in the mitochondrial gene cytochrome b. Results from this study demonstrated low levels of genetic variation within populations and little evidence of substructure between drainages; however, sample sizes in this study were small (Strange & Lawrence, 2002). Population level analyses of variation at neutral nuclear markers such as microsatellites are most appropriate for determining the historical distinctiveness of populations and for informing the design of management strategies, which maximize levels of genetic variation.

2 | MATERIALS AND METHODS

We developed a set of 14 microsatellite markers for detecting and measuring population structure and for estimating levels of heterozygosity in natural, introduced, and captive populations of Barrens topminnow. Tested were 34 published primer sequences designed for two congeneric species, the mummichog (*F. heteroclitus*; 20 primer sets; Adams, Oleksiak, & Duvernell, 2005) and the blackstripe topminnow (*F. notatus*; 14 primer sets; Feldheim Kreiser, Schmidt, Duvernell, & Schaefer, 2014) for amplification of polymorphic microsatellites in the Barrens topminnow. PCR conditions were performed in 10-μl reactions containing 50–100 ng of template DNA, 0.5 μmol/L of each primer, 200 μmol/L each deoxynucleotidetriphosphate (dNTP), 0.3 U GoTaq hot start polymerase (Promega), 2.5 mmol/L $MgCl_2$, and 1× commercial buffer. Thermal cycling conditions were: 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. Candidate primer pairs were cloned using the Pgem-t easy vector cloning system (Promega) and sequenced on an ABI 3730XL sequencer (Applied Biosystems) using DNA from two Barrens topminnows. Sequences were compared to published GenBank sequences in order to verify amplification of targeted regions. Promising primer pairs were fluorescently labeled by the addition of 5′ fluorophores to the forward primer, and amplified products were electrophoresed on an ABI 3730 sequencer (Applied Biosystems). Resulting data were sized using the software Peak Scanner v2.0 (Thermo Fisher Scientific). Initial screenings were performed in 16 Barrens topminnows from two populations located in different drainage systems. Of the 34 primer pairs we examined, only four amplified consistently and produced variable sized products.

Next generation sequencing (NGS) of a genomic library was performed in order to obtain additional informative microsatellite loci. Muscle tissue from a single senescent Barrens topminnow that was a holdover from a captive propagation program at the Tennessee Aquarium in Chattanooga was used as a source for genomic DNA. DNA was extracted using a Qiagen Blood and Tissue kit following the manufacturer's protocol. An Illumina paired-end shotgun library was prepared by shearing 4 μg of DNA to an average insert size of 400 bp by sonication using an LE Series Covaris. Whole-genome 125-base pair, paired-end sequence reads were generated on 1/4 lane on an Illumina HiSeq, resulting in 63,617,464 reads. Resulting reads were trimmed using Trimmomatic 0.35 (Bolger, Lohse, & Usadel, 2014) and quality was checked using FastQC (Andrews, 2010). De novo genome assembly was performed by de Bruijn graph and SOAPdenovo2 software package (Luo et al., 2012) with default settings except that K-mer value was set at 59, resulting in 2,087,492 contigs with an average length of 529 bp. All contigs less than 500 bp length were filtered out prior to screening for microsatellites. Assembled contigs were screened for tri-nucleotide repeat sequences with 10 or more repeat units using the software program MSATCOMMANDER version 0.8.2 (Faircloth, 2008). MSATCOMMANDER uses PRIMER 3 (Untergasser et al., 2012) to simultaneously design flanking primers while searching for microsatellite repeats. The option to tag primers with an M13 tag (AGGGTTTTCCCAGTCACGACGTT) on one of the primers was selected. Our search resulted in a total of 732 loci with acceptable primer annealing sites. Assembled contigs for markers developed here are deposited in GenBank (accession #KX641157–KX641166).

For microsatellite genotyping, genomic DNA was extracted from fin clips from 53 topminnow sampled from two isolated natural populations (21 from Benedict Spring, Caney Fork drainage (type locality) and 32 from Pond Spring, Elk River Drainage) using the protocol by Wang and Storm (2006). All tissues used in this study were collected by collaborators at the Tennessee Aquarium in Chattanooga during routine monitoring. We selected 20 primer pairs from the NGS library for genotyping using the same PCR conditions described above except that a 0.5 μmol/L fluorescently labeled M13 primer was added to the PCR reactions. Of these, 10 primer sets produced robustly amplified PCR products of variable sizes. Combined with the four previously published, a total of 14 polymorphic loci were found to amplify consistently in Barrens topminnow. The PCR reactions were performed separately and products were then combined in four separate runs (PlexA, PlexB, PlexC, and PlexD). PCR products were analyzed by Molecular Cloning Laboratories (MC Laboratory: www.mclab.com) and manually scored using the software Peak Scanner 2.0 (Applied Biosystems). The number of alleles, observed and expected heterozygosities, exact tests for Hardy–Weinberg equilibrium, and log-likelihood ratio statistic (*G*-statistic) for linkage disequilibrium were estimated using Genepop 4.5.1 (Raymond & Rousset, 1995; Rousset, 2008) and corrected for multiple comparisons using the sequential Bonferroni approach (Rice, 1989). The probability of identity and measures of genetic differentiation (F_{ST}, and G'_{ST} (Hedrick, 2005)) were calculated using GenAlEx 6.5 (Peakall & Smouse, 2006).

3 | RESULTS

Results from this preliminary survey of 14 polymorphic loci indicated that levels of genetic variation differ greatly between Benedict Spring and Pond Spring populations. Benedict Spring samples were fixed for a single allele at 7 loci, with an average of 1.43 (range: 1–2) alleles per locus, while Pond Spring samples were polymorphic for 13 of the 14 surveyed loci, with an average of 3.64 (range: 1–7) alleles per locus (Table 1). The average expected heterozygosity (H_E) across all loci was 0.156 and 0.503 for Benedict Spring and Pond Spring, respectively. The average observed heterozygosity (H_O) across all loci was 0.150 and 0.470, for Benedict Spring and Pond Spring, respectively. Tests for deviation from Hardy–Weinberg equilibrium and for linkage disequilibrium were not significant after correcting for multiple tests. The combined probability of identity for the 14 loci was 1.31×10^{-1} and 3.54×10^{-8} for Benedict Spring and Pond Spring, respectively. Genetic differentiation between the two surveyed populations was high ($F_{ST} = 0.465$; $G'_{ST} = 0.777$).

4 | DISCUSSION

Despite long-term management and conservation efforts to restore Barrens topminnow, the population dynamics and genetic structure

TABLE 1 Primer sequences and summary statistics for microsatellite loci isolated from two populations of *Fundulus julisia* (Benedict Spring (n = 21) and Pond Spring (n = 32)

Locus	Forward Primer	Repeat motif	Allele size range	N_A BS,PS	H_O BS,PS	H_e BS,PS	p_{HWE} BS,PS
Plex A							
Fuju001	F:GCCGTCTCATTGTTGCCTC R: AGCATGGCAGAGGTAGCTG[a]	$(ATT)_{16}$	448–454	2, 3	0.250, 0.429	0.358, 0.527	0.214, 0.424
Fuju002	F:GCCTTGTGGTTATGGCTCG R: CCTCCTTTCTCACATGGGC[a]	$(ATT)_{15}$	426–432	1, 4	0.000, 0.645	0.000, 0.691	—, 0.444
Fuju004	F:CTGACGACATGCTAGTGTCC R: CAACCTGGGTGGGTCCTTC[a]	$(ATT)_{15}$	460–472	1, 2	0.000, 0.281	0.000, 0.289	—, 1.000
Fuju013	F:ACCCGCAAGACTTCTGGAC R: CCGATGTTGCTTCAGTTCTTTG[a]	$(AAT)_{16}$	311–336	2, 5	0.158, 0.500	0.149, 0.504	1.000, 0.344
Plex B							
Fuju005	F: AGCAGAACTGGAGCGTTTC[a] R:GTTGCGTCTGTTCCAGGATG	$(GAT)_{15}$	166–194	1, 5	0.000, 0.333	0.000, 0.364	—, 0.632
Fuju012	F: CGCAGAAGTAGTTAGACGCC[a] R:GCCGTAGTCCCGCAAATTC	$(ACT)_{18}$	191–212	2, 3	0.278, 0.444	0.386, 0.617	0.260, 0.020[b]
Fuju014	F: CTGCATGTACACAAGCTACC[a] R:CATCACCCTGAACCAGAGC	$(ATT)_{17}$	459–493	1, 4	0.000, 0.643	0.000, 0.560	—, 0.283
Plex C							
FhCA-1	F:FAM-GTCCATGCAATGTCGTTCAC R:GAGGCCAGAAACGCATACAT	(CA)13	174–182	1, 3	0.000, 0.520	0.000, 0.597	—, 0.675
Fuju020	F: GGTGGTTGATGCAGACCAAG[a] R:GGGCCGAAGTCAAATCTCAAG	$(ATC)_{15}$	443–449	2, 3	0.316, 0.240	0.341, 0.280	1.000, 0.484
FhCA-21	F:PET-GGTCATTATGGAAAACAGCAACAGATC R:GCTCACTGACACACTGGATTTGGTAGA	$(CA)_{27}$	185–189	1, 2	0.000, 0.423	0.000, 0.491	—, 0.683
Plex D							
FhATG-B103	F:FAM-CGGAGCATTGTGATTGTGTTGTTTT R:CCGGGGGACACTTATATGAAATCAGA	$(ATG)_{34}$	288–326	2, 5	0.571, 0.667	0.511, 0.715	0.675, 0.161
FhATG-B5	F:PET-GGCACATTAATTACCTGGAAACAT R:CTGGGTCACATTCCTCGGTCTG	$(ATG)_{17}$	329–347	2, 1	0.524, 0.000	0.438, 0.000	0.611, —
Fuju008F	F:AGCCATCCAGTTCTGATCTAAAG R: GGAGGTGAGCTACGTTCCC[a]	$(AAT)_{14}$	188–215	1, 7	0.000, 0.955	0.000, 0.804	—, 0.970
Fuju018F	F:AATGAGGCTTCGCTTGCAG R: CATCGGTTTACCCGCCTATG[a]	$(ATT)_{15}$	173–182	1, 4	0.000, 0.500	0.000, 0.608	—, 0.518

N_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; P_{HWE}, p-value for Hardy–Weinberg equilibrium for Benedict Spring (BS) and Pond Spring (PS), respectively.

[a]Primers were tagged with a 5′M13 universal sequence (AGGGTTTTCCCAGTCACGACGTT) and labeled using four different fluorescent labels.

[b]Not significant after Bonferroni correction.

of this species remain poorly understood. Preliminary results from our survey of both Benedict and Pond springs indicate that levels of heterozygosity vary substantially between populations and that structuring of genetic variation among drainages is significant. However, a comprehensive survey of all remaining natural, introduced, and captive populations is needed. The loci characterized here will provide a valuable tool for enhancing management strategies and planning future reintroductions that will maximally preserve the remaining genetic variation in this highly threatened species.

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