I. Methods

Sample Collection – Sampling for genetic analysis will be a collaborative effort between the University of Missouri – Columbia, The Missouri Department of Conservation, The Arkansas Game and Fish Commission, and Oklahoma State University. We will collect tissue samples of putative Neosho Smallmouth Bass (*M. d. velox*) out of various tributaries along the northeastern edge of the Arkansas River Basin where *M. d. velox* habitat is most suitable (Hubbs and Bailey 1940) and where it is considered unlikely to overlap with native Spotted Bass (*M. punctulatus*), including Indian Creek, Little Sugar Creek, Big Sugar Creek, Center Creek, Shoal Creek, the Elk River, Honey Creek, Sycamore Creek, Buffalo Creek, and the Illinois River. These samples will be considered pure *M. d. velox*. We will also collect fin clips of putative Northern Smallmouth Bass (*M. d. dolomieu*) from streams across its range, including portions of the Arkansas and Ouachita systems in Oklahoma and Arkansas, tributaries of the Missouri and Black Rivers in southeastern Missouri—namely the Meramec River, Current River, Big Piney River, Niangua River, and Tavern Creek, and from rivers throughout the northeastern United States, for interspecific genomic comparison. Geographic location information (UTMs or longitude/latitude) will be recorded at each site. Too assess genomic divergence among outgroups, we will also obtain several samples from *M. punctulatus* and Largemouth Bass (*M. salmoides*) from locations where hybridization can potentially occur. Samples were previously collected between June 7 and November 4, 2016; we will acquire additional samples in Spring and Summer, 2017. We will target more sites in Missouri than have been previously sampled to ascertain a clearer picture of Smallmouth diversity in this area.

Specimens will be captured through boat electrofishing or hook-and-line angling. Once fish are caught, we will remove fin clips from the upper portion of the caudal fin and preserve them either in 95 % EtoH or Longmire Buffer (Longmire et al. 1997). We will also collect a series of morphometrics for each specimen, including total length (mm), standard length (mm), orbital length (mm), head length (mm), body depth (mm) and number of soft dorsal rays. We will also measure the mass of each fish using a standard scale (specific kind of scale?). We will replace fish immediately in the water after tissue clips and all morphometrics are recorded.

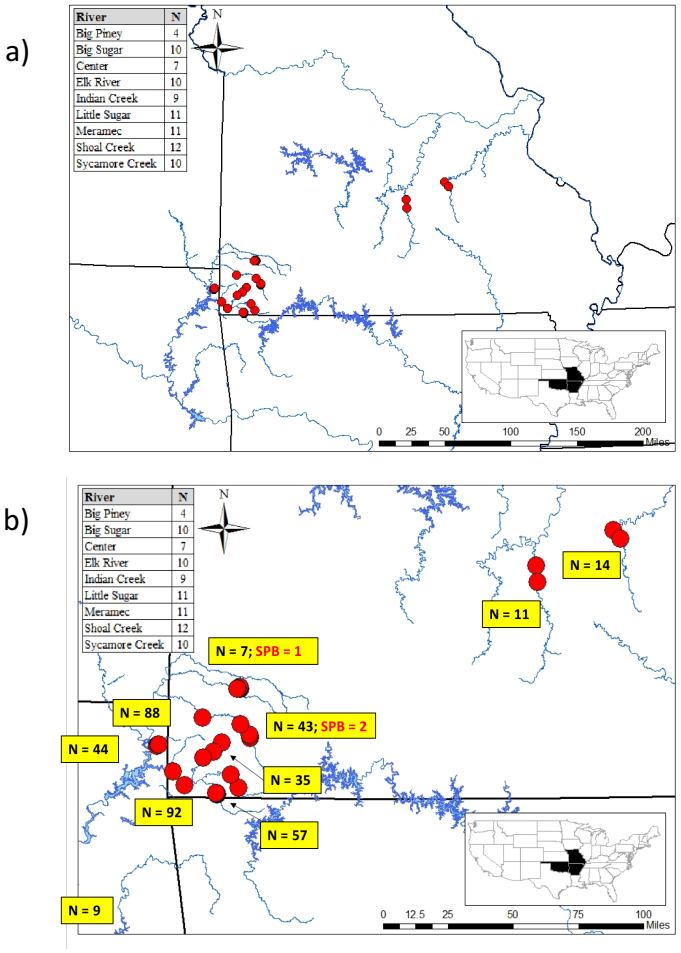

Figure 5. (a) Sample distribution. Red circles indicate sites where multiple samples have been collected. (b) Yellow boxes indicate the number of samples that have been collected from a given tributary; the number of Spotted Bass (SPB) that were collected from two of the listed sites are highlighted in red to emphasize the need for additional SPB sampling. Legends in the top left-hand corner of (a) and (b) indicate the tributaries

and associated sample sizes that were selected for preliminary microsatellite multiplex analysis. Samples were georeferenced and maps were designed in ArcMap.

Marker Selection – We will obtain primer sequence sets for 11 of 12 known *M. d. velox* microsatellite loci, including *Mdo1*, *Mdo2*, *Mdo3*, *Mdo4*, *Mdo5*, *Mdo6*, *Mdo7*, *Mdo8*, *Mdo9*, and *Mdo10* (Malloy et al. 2000; Table 1) and *Lma21* (Colbourne et al. 1995; Table 1) to assess the level of genetic diversity between native *M. d. velox* and non-native congeners. We will omit the locus designated *Mdo11* from our analyses due to previously-discovered heterozygote deficiency (Malloy et al. 2000). These microsatellites are polymorphic, and their associated primers are known to amplify microsatellite loci in *M. punctulatus*, making them informative in comparing genetic signatures across multiple taxa.

Table 1. Allele counts, annealing temperature, fluorescence dye labels, minimum allele size, maximum allele size, and nucleotide primer sequences for 11 *M. d. velox* microsatellite loci.

Name	allele #	Anneal T (°C)	Tag	Min. allele size	Max. allele size	Primer Sequence
Lma 21	6	55	Pet	154	182	*CAGCTCAATAGTTCTGTCAGG ACTACTGCTGAAGATATTGTAG
Mdo 1	6	55	Vic	200	220	*GCTCTTCCCAGTGGTGAGTC ATCTCAGCCCATACCGTCAC
Mdo 2	6	55	Ned	187	207	*GCCCTTTCATATTGGGACAA CTGCTCTGGCGTACATTTCA
Mdo 3	6	55	Ned	125	145	*AGGTGCTTTGCGCTACAAGT CTGCATGGCTGTTATGTTGG
Mdo 4	3	55	Vic	132	152	*TCTGAACAACTGCATTTAGACTG CTAATCCCAGGGCAAGACTG
Mdo 5	2	55	Fam	190	210	*CAGGTTCCCTCTCACCTTCA ATGGTCTCACCAGGGACAAA
Mdo 6	3	55	Pet	140	160	*TGAAATGTACGCCAGAGCAG TGTGTGGGTGTTTATGTGGG
Mdo 7	2	55	Vic	162	182	*TCAAACGCACCTTCACTGAC GTCACTCCCATCATGCTCCT
Mdo 8	8	55	Fam	210	230	*GTGAGGACCAGCCAAAATGT GGAAGATTGAGGTCCCAACA

Mdo 9	5	55	Fam	116	136	*TTTGATGGGCGTTTTGTGTA GACCGGTCCTGCATATGATT
Mdo 10	2	55	Pet	91	111	*GTGTCTCCGTGTGTTGATGG AACACCAGAGGCAAACAAGC

DNA Isolation and Microsatellite Multiplexes – We will isolate nuclear DNA from fin clips using the Spin-Column protocol for Purification of Total DNA from Animal Tissue in the DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD). DNA concentrations (ng/uL) in each extract will be quantified using a ND-1000 Spectrophotometer. We will amplify microsatellite fragments through polymerase chain reaction (PCR) in three multiplex reactions based on optimal annealing temperatures—previously determined by running each individual locus on a gradient PCR—and allele sizes at each locus using an Eppendorf™ Thermocycler. Multiplexes will be run in 8 uL reactions and will consist of 1X Platinum® PCR Multiplex Master Mix (Applied Biosystems, Inc., Foster City, California), 0.4uM reverse primers, 0.4uM fluorescently labeled forward primers, 0.8mM BSA, 1X GC enhancer (Applied Biosystems, Inc., Foster City, California), and 1.5 uL template DNA. Apart from differing annealing temperature settings, we will use the following parameters for PCR amplification: 95 °C for 15 minutes, 35 cycles of 94 °C for 30 seconds, optimal annealing temperature for 90 seconds, and 72 °C for 1 minute, and 1 cycle of 60 °C for 30 minutes. PCR products will be held at 4 °C until they are removed for gel electrophoresis imaging. The Multiplex 1 reaction will include *Mdo4*, *Mdo5*, *Mdo6*, and *Mdo1* run at an optimal annealing temperature of 54.1 C. Multiplex 2 will include *Mdo7*, *Mdo8*, *Mdo9*, *Mdo10*, and *Mdo2* run at an optimal annealing temperature of 55.4 C. Multiplex 3 will include *Lma21* and *Mdo3* run at an optimal annealing temperature of 52.8 °C (Table 2).

Table 2. Microsatellite Multiplexes for Fragment Analysis.

Multiplex	Annealing Temperature (°C)	Loci Amplified (Primer Names)
1	54.1	*Mdo4, Mdo5, Mdo6, Mdo1*
2	55.4	*Mdo7, Mdo8, Mdo9, Mdo10, Mdo2*
3	52.8	*Lma21, Mdo3*

We will run 5 uL of each PCR product on a 2% agarose gel stained with GelStar (Lonza) and view amplicons using a Fotodyne gel imager. Amplified PCR products will be diluted 1:10 with ddH_2O and sent in 2 uL aliquots for fragment analysis on an ABI 3730xl DNA analyzer (Thermo Fisher Scientific, Waltham, MA) at the University of Missouri DNA Core Facility with added 500LIZ size standard. We will visualize the alleles present in each sample at each locus using GeneMarker v. 1.97 (Kellander et al. 2002).

Preliminary Analysis – We have selected 94 DNA samples from various sites within our sample distribution for a preliminary assessment of polymorphism among putative *M. d. velox* (Table 3). We chose sites in multiple streams and across the ranges of both *M. d. velox* and *M. d. dolomieu* in order to approximate an accurate representation of genetic diversity. We chose equal sample sizes (*N*) to the best of our ability, but *N* was dependent on the number of samples available at a given site (Table 3).

Table 3. Sample sizes (*N*) for all waterways represented in preliminary fragment analysis. *N* was equally distributed to the best of our ability, but exact sample sizes were dependent on the number of samples available at a given site.

River	*N*
Big Piney	4
Big Sugar	10
Center Creek	7
Elk River	10
Indian Creek	9
Little Sugar	11

Meramec River	11
Shoal Creek	12
Sycamore Creek	10
Lake Fabre, Quebec	5
Big Rideau Lake, Ontario	5

We will amplify all microsatellite loci (Table 1) for our selected DNA samples in 3 individual 96-well plates (one plate for each multiplex; Table 2) using polymerase chain reaction at the parameters listed in the *DNA Isolation and Microsatellite Multiplexes* section above. We will include one positive control and one negative control along with our 94 template DNA extracts for each multiplex. We will select one 8-well row, including the positive and negative controls, to image on a 2% agarose gel for each plate to check for contamination. PCR products will be diluted 1:10 with ddH_2O to prevent over-fluorescence in fragment analysis. Diluted products will be sent in 2 uL aliquots for analysis on an ABI 3730xl DNA analyzer (Thermo Fisher Scientific, Waltham, MA) at the University of Missouri DNA Core Facility. We will visualize fluorescence of microsatellite alleles using GeneMarker v. 1.97 (Kellander et al. 2002). To enumerate and identify alleles consistently across multiplexes and across all loci, we will score alleles automatically using Panel Editor in GeneMarker. Potential peaks that register below 200 fluorescence units will be considered erroneous and not included in downstream analyses.

Genomic Analyses – We will estimate genomic divergence among pure *M. d. velox* and non-native conspecifics (*M. d. dolomieu*) and congeners (*M. punctulatus* and *M. salmoides*) using single nucleotide polymorphisms (SNPs). With next-generation sequencing (NGS) technologies and the increased availability of methods that apply NGS to genotyping non-

model organisms (Davey et al. 2011; Elshire et al. 2011), we will be able to more precisely determine levels of Smallmouth Diversity in the Interior Highlands.

Specifically, we will Analysis of Molecular Variance (Weir and Cockerham 1984) and Bayesian methods within STRUCTURE (Pritchard et al. 2000) to detect differentiation among pure *M. d. velox, M. d. dolomieu*, *M. punctulatus*, and *M. salmoides*. Usjng SNPs from putative pure *M. d. velox* as a reference genotype, we will also be able to ascertain genomic proportions from *M. d. velox* and non-native conspecifics and congeners within hybrids. These analyses will allow us to determine levels of genetic introgression through hybridization in the Interior Highlands and will enable us to pinpoint potential broodstock for stocking *M. d. velox*.

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