

# Validating the Taxonomic and Distributional Status of the Neosho Smallmouth Bass (*Micropterus dolomieu velox*)

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## I. Introduction

The Black Basses (genus *Micropterus*) were originally described by Bernard Germaine de Lacépède in 1802 (Kassler et al. 2002). His taxonomic classifications were generally accepted, but they drew criticism for their insufficient detail and numerous inconsistencies regarding morphological characters (Henshall 1881). Dissatisfaction with the taxonomy encouraged Henshall (1881) and other contemporary naturalists to propose revisions that would build upon Lacépède's original descriptions. Taxonomists continued to reconfigure the classification of *Micropterus* over the next few decades. At one point, eight generic and 20 specific names were used to identify unique groups (Ramsey 1975). The genus was eventually consolidated by Jordan and Evermann (1898) into two broad, distinct taxa known as the Smallmouth (*M. dolomieu*) and Largemouth (*M. salmoides*) Basses. Hubbs and Bailey (1940) later reorganized the black basses as a tribe consisting of two separate genera: *Huro*, which housed the Largemouth Bass, and *Micropterus*, which included three species and five subspecies. In a later revision, *Huro* was absorbed into *Micropterus* (Bailey and Hubbs 1949). Biologists have worked to refine and understand the taxonomy of *Micropterus* ever since. New species— the Shoal Bass (*M. cataractae*), for example—have been named as recently as 1999 (Williams and Burgess 1999), and the former subspecies Alabama Bass (*M. henshalli*) was elevated to species status in 2008 (Baker et al. 2008). We currently recognize nine unique species (Shaw 2015).

Contention over Black Bass taxonomy can be partly attributed to their long-standing popularity as sport fishes. Aggressiveness, speed, and relatively large body size are all prized physical traits that make basses attractive to anglers. Because of their relative popularity, they are important for tourism, and, as such, they can positively impact local economies (Carey et al. 2011) and can be major assets in local communities throughout their native range (Brewer and Long 2015). Sport-fishing has prospered into a billion-dollar industry in the United States (USFWS 2006; Long et al. 2015). As participation in black bass fishing has increased, the need for conservation and management policy has become more pressing (Shaw 2015). Increased angling can lead to loss of unique subpopulations through unchecked fishery exploitation (Brown et al. 1981), pollution, habitat loss, and unwanted hybridization (Shaw 2015). (Smallmouth Bass (*M. dolomieu*) are highly managed throughout North America (Brewer and Orth 2015). Catch-and-release rules, harvest limitations, and hatchery supplementation for this species are all regulated in a way that seeks to maximize catch rate for anglers and minimize risk to fish populations (Copeland et al. 1996; Lyons et al. 1996; Slipke et al. 1998; Newman and Hoff 2000).

The status of *Micropterus* as a sport fish has contributed to ubiquitous stocking for the purpose of generating fishing opportunities (Robins and MacCrimmon 1974; Figure 2). This practice became common in the mid nineteenth century (Surber 1935) and continues today (Koppelman 2015). Nearly 3 million Smallmouth Bass were released in 1948 (Tunison et al. 1949), and more than 15 million Largemouth Bass were introduced into various reservoirs in 2005 (Siepker and Casto-Yertzy 2008). Stocking often involves the introduction of non-native forms. It is very common with *M. dolomieu*, which has been introduced throughout various

regions of the North America within (Stroud and Clepper 1975) and outside its native range (Brewer and Orth 2014). Species like *M. dolomieu* are ideal candidates for stocking because of their general robustness to changing environments (Taylor et al. 2016). However, this adaptability can have negative implications at the ecosystem level through invasion (Marchetti et al. 2004; Guenther and Spacie 2006), or, perhaps more notably, at the population level through unwanted hybridization.

Hybridization, defined as the mating of individuals of two different species or subspecies, is known to occur naturally (Koppelman 2015). When populations are subjected to anthropogenic influence through stocking, the process is accelerated (Koppelman 2015). Stocking-related cases of black bass hybridization often involve *M. dolomieu* (Morizot et al. 1991). Whitmore (1983), Edwards (1979), Whitmore and Butler (1982), and Morizot et al. (1991) reported hybridization between native Guadalupe Bass (*M. treculii*) and introduced *M. dolomieu*. Similarly, hybridization has been documented between *M. dolomieu* and Redeye Bass (*M. coosae*) (Turner et al. 1991; Pipas and Bulow 1998) as well as between Northern Smallmouth Bass (*M. d. dolomieu*) and Northern Largemouth Bass (*M. s. salmoides*) (Wheat et al. 1971; Whitt et al. 1971; Beaty and Childers 1980; Buck and Hooe 1986; Whitmore and Hellier 1998). Pierce and Van Den Avyle (1997), Pflieger and Fajen (1975), and Koppelman (1994) also demonstrated hybridization between *M. dolomieu* and Spotted Bass (*M. p. punctulatus*), noting that hybrid genotypes were seen in as high as 37% of individuals from intermixed populations in central Missouri.

In *Micropterus*, a combination of biotic and abiotic mechanisms, such as courtship, color, or water turbidity, work to inhibit interbreeding, but they are not completely

reproductive barriers (Childers 1975). The findings of Pierce and Van Den Avyle (1997) are particularly significant given that *M. dolomieu* and *M. punctulatus* are often sympatric (Warren 2009) and the only sister taxa in *Micropterus* that inhabit overlapping geographic ranges (Near et al. 2003). Hybridization and subsequent genetic introgression between these species has been shown in at least two other studies (Koppelman 1994; Avise et al. 1997). Native endemic Smallmouth Basses are especially vulnerable to hybridization because of the relative frequency at which they have been introduced outside their native range (Lee et al. 1980). Also, *Micropterus* species are relatively recently-diverged; since empirical studies show that the capacity for hybridization tends to decrease as taxa become more genetically distinct (Darwin 1859; Bolnick and Near 2005), it is important to consider how this process can impact interspecific diversity.

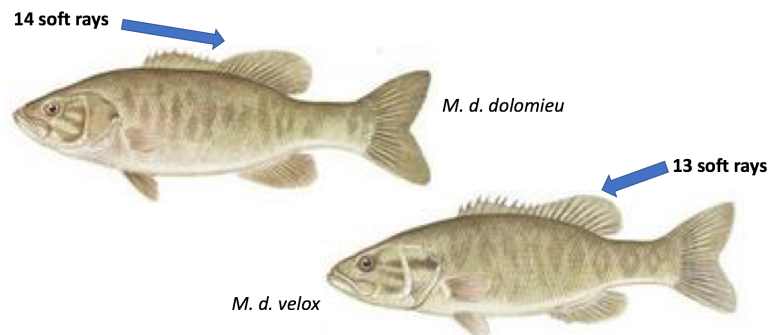
Factors associated with hybridization between congeners, such as the transfer of genetic information from one species or subspecies to another (introgression of non-native alleles) and backcrossing, can decrease the genetic integrity of a population (Barwick et al. 2006; Littrell et al. 2007). These effects can eliminate local adaptations that facilitate individual success in a given niche and help to maintain a species' evolutionary capacity (Koppelman 2015; Taylor et al. 2016). In extreme cases, hybridization can lead to total swamping of genetic variation in a subpopulation (Barwick et al. 2006) or potentially to outbreeding depression (Moyle et al. 1986; Altukhov and Salmenkova 1987; Stahl 1987; Philipp and Whitt 1991). In other words, by diluting a population's genetic makeup, it is possible to reduce overall fitness and increase vulnerability to factors such as parasites and pathogens (Hallerman 2003; Goldberg et al. 2005). Non-native black bass genotypes have altogether supplanted native

genotypes in some subpopulations (Barwick et al. 2006; Stormer and Maceina 2008; Leitner et al. 2015). Subpopulations with desirable qualities for recreational angling, such as catchability, size and aggressiveness, can be lost when unique genes are threatened (Thorpe and Koonce 1981). The risks associated with stocking have brought the need for taxonomic integrity to the forefront (Brewer and Long 2015). To prevent the potential negative impacts of hybridization on genetic diversity in bass populations and to avoid ambiguity in classification, it is important to understand the taxonomic and ecological relationships among and within species.

#### Smallmouth Bass (*M. dolomieu*)

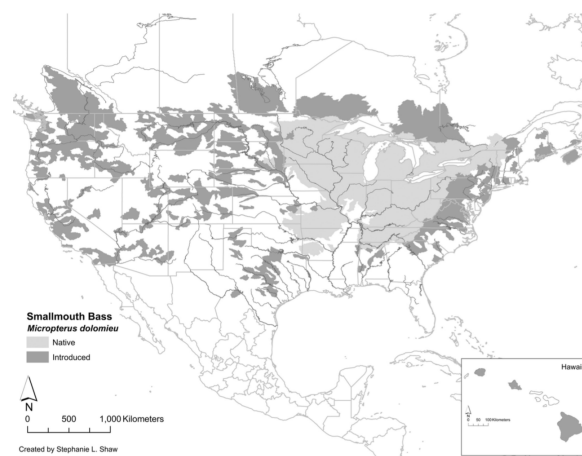
*Morphology* – Two subspecies of *M. dolomieu*, the Northern Smallmouth Bass (*M. dolomieu dolomieu*) and the Neosho Smallmouth Bass (*M. dolomieu velox*), provide an informative model for assessing relatively recent genetic divergence. The subspecies can be identified by their differential morphology and non-overlapping native ranges. Hubbs and Bailey (1940) describe *M. d. velox* as having a combined total of 21 to 23 soft dorsal fin rays and dorsal spines, a straight, slender predorsal contour, a protruding lower mandible, developed teeth along the tongue, and dark, broad, less elevated bars in juveniles. In contrast, they characterize *M. d. dolomieu* as having 24 to 25 total soft dorsal rays and dorsal spines, a bulky and more rotund predorsal contour, a non-protruding lower mandible, a lack of teeth along the tongue, and dark, slender, and elevated bars (Hubbs and Bailey 1940). In their study, nearly 80 percent of observed samples could be identified as one or the other based on these meristics (Hubbs and Bailey 1940). Individuals can be distinguished predominately by the slight variation

in the number of soft dorsal fin rays, of which *M. d. dolomieu* usually has 14 while *M. d. velox* usually has 13 (Stark and Echelle 1998; Figure 1).



**Figure 1.** Morphological comparison of Northern Smallmouth Bass (*M. d. dolomieu*; top left) and Neosho Smallmouth Bass (*M. d. velox*; bottom right). Arrows point to groupings of soft dorsal rays, of which *M. d. dolomieu* generally exhibit 14 while *M. d. velox* generally exhibit 13 (Figure from Hubbs and Bailey 1940).

*Distribution* – Geographically, these subspecies occupy a significant swath of the North American Midwest, with native habitat extending from the southern edge of the Arkansas River Basin to the Saint-Lawrence Great Lakes system near the northern border of the United States (Brewer and Orth 2014; Figure 2). *M. d. dolomieu* is found north and east of the Mississippi River and is not known to inhabit the Central Interior Highlands. *M. d. velox* is restricted to the lower Ozark Highlands ecoregion (Stark and Echelle 1998; Nigh and Schroeder 2002).



**Figure 2.** Known distribution of Smallmouth Bass (*Micropterus dolomieu*) in North America. Light grey shaded regions represent the native geographic range of *M. dolomieu*, while dark grey regions indicate areas where both variants of Smallmouth Bass have been introduced (from Brewer and Orth, 2014).

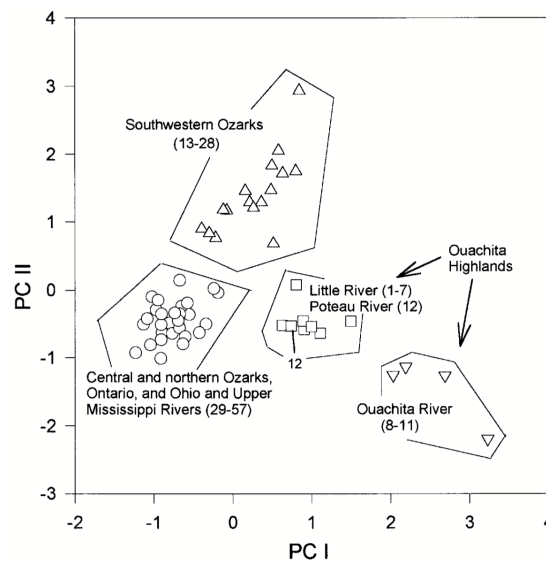
*Habitat* – *M. d. velox* typically occurs in geological landscapes dominated by soluble limestone and defined by a mixture of smooth and rugged, woody plains (Brewer and Long 2015). The combination of geology and the geography location expose *M. d. velox* to slow-moving streams (Dauwalter and Fisher 2007) that carry limited sediment loads. Although *M. d. velox* has been observed to inhabit turbid, sediment-heavy areas, it is generally found in clearer waters (Brewer and Long 2015). Additionally, *M. d. velox* is limited in its distribution by water temperature (Brewer and Long 2015). Only a few studies have tested the tolerance of *M. d. velox* to changing temperatures, but it is hypothesized that individuals alter their temperature preferences over the course of their lifetime, moving from warmer to cooler pools (Brewer 2013b).

The subspecies is often constrained by these elements of the environment even though its specific habitat preferences can vary depending on life stage and time of year (Todd and Rabeni 1989; Livingstone and Rabeni 1991). *M. d. velox*, like *M. d. dolomieu*, is migratory; individuals travel long distances for overwintering or homing purposes (Webster 1954; Langhurst and Schoenike 1990; Larimore 1952; Fajen 1962). *M. d. velox* mirrors *M. d. dolomieu* in several aspects of its ecology, most notably in habitat choice for spawning (Brewer and Long 2015). However, few studies have compared their ecological adaptation. Regardless, the combination of historical and ecological processes—that is, the processes that have resulted in the geographic isolation among the basses—may have contributed to genetic variation between *M. d. velox* and *M. d. dolomieu* (Borden and Krebs 2009).

*Genetics* – Morphological and ecological differences between *M. d. velox* and *M. d. dolomieu* are subtle (Near et al. 2003; Miller 1975; Mabee 1993). Bailey (1956) determined that

morphology between the forms was not sufficiently distinct to warrant taxonomic distinction. Hoyt (1973) further suggested that interspecific stocking had altered meristic traits since their original descriptions, ultimately concluding that *M. d. velox* and *M. d. dolomieu* could not be classified as separate subspecies.

Several genetic studies, however, support genetic divergence between *M. d. velox* and *M. d. dolomieu* (Stark 1995; Stark and Echelle 1998). Stark and Echelle (1998) found in their analysis of 33 allozyme loci that Smallmouth Basses from the Interior Highlands ecoregion could be classified into three clades, including *M. d. velox*, the Ouachita Smallmouth Bass (currently unnamed), and a separate clade consisting of individuals from multiple streams in the upper Ozark Highlands. This separation was shown by principal component analysis based on allele frequencies (Figure 3).

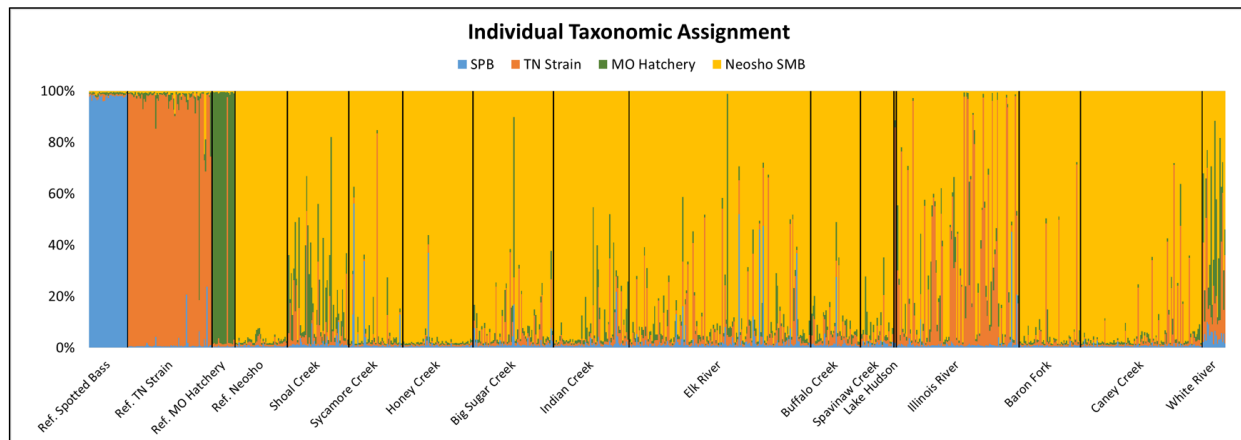


**Figure 3.** Principal component analysis showing arcsine-transformed allele frequencies among Smallmouth Bass subpopulations in the Interior Highlands (from Stark and Echelle, 1998). *M. d. velox* populations are represented by the Southwestern Ozarks cluster, the Ouachita lineage is represented by both Ouachita Highlands clusters, and *M. d. dolomieu* is represented by the Central and Northern Ozarks cluster.



Most of the diversity within the Smallmouth Basses can be traced to the Interior Highlands in central North America (Malloy 2000; Stark and Echelle 1998). Until the past two decades, though, most genetic studies have investigated hybridization between *M. d. dolomieu* and non-native congeners in populations east of the Mississippi River (Whitmore and Butler 1982; Whitmore 1983; Whitmore and Hellier 1988; Morizot et al. 1991; Koppelman 1994), and investigation of ecoregions west of the Mississippi River with high endemic diversity have been lacking. With the discovery of high diversity in the Interior Highlands, it has become imperative to assess the effects of stocking on *M. d. velox* in its native tributaries of the Arkansas River Basin. There has been recent interest in stocking Grand Lake o' the Cherokee's—an 18,800 ha impoundment in Northeastern Oklahoma and within the range of *M. d. velox*—with a Tennessee “lake strain” broodstock to increase angling opportunities for the surrounding community (Taylor et al. 2016). Taylor et al. 2016 conducted an analysis of the genetic structure of Interior Highland Smallmouth Bass using 7 microsatellite loci to determine the degree of introgression of non-native alleles from the Tennessee lake strain, which had previously been introduced in Lake Tenkiller and Broken Bow Lake, Oklahoma in the 1990s (Boxrucker et al. 2004). Their overall goal was to ascertain potential *M. d. velox* broodstock that could be introduced into Grand Lake to maintain the existing genetic diversity in *M. d. velox* populations (Taylor et al. 2016). They identified hybridization in all populations that they tested in Oklahoma and Missouri, although the degree of mixing varied considerably (Taylor et al. 2016; Figure 4). Intermixing was reported between *M. d. velox* and two other clusters, including lake strain and an MO Hatchery strain of unknown origin (Taylor et al. 2016). However, due to a relatively small sample size from Missouri streams and a relatively small collection of reference

*M. punctulatus*, it is difficult to draw strong conclusions about hybridization in *M. d. velox* across their entire range. Because of the possibility that intermixing non-native subpopulations can lead to hybridization and loss of co-adapted traits (Koppelman 2015), Taylor et al. (2016) demonstrated the need for additional investigation into the genetic diversity among Smallmouth Bass.



**Figure 4:** Genotype assignment proportions for 873 Smallmouth Bass individuals (represented by individual bars) sampled from the Interior Highlands ecoregion. Assignment estimates were calculated in STRUCTURE (Pritchard et al. 2000) using allele frequencies for the four reference taxonomic groups (Spotted Bass (SPB), Tennessee Strain (TN Strain), MO Hatchery, and Neosho SMB)). Within sites (horizontal axis), individual genotypes are organized such that left-to-right corresponds approximately to the upstream-to-downstream direction in the sampled tributary. (Figure and caption adapted from Taylor et al. 2016).

*Management Implications* – Fish biologists and management professionals have begun to focus their efforts toward black bass conservation in recent years (Tringali et al. 2015). However, without a proper understanding of genetic or morphological variation among species and subspecies, irreparable dilution of genetic diversity within certain subpopulations by introducing non-native forms may be possible (Nelson and Soule 1987; Echelle 1991). The genetic identities of Smallmouth Bases have already been affected by interspecific stocking (Birdsong et al. 2015). To understand the potential impacts of this practice on *M. d. velox*, it is important to evaluate the distinctions of *M. d. velox* and *M. d. dolomieu* as novel subspecies.

Because of the viability of hybrid offspring in *Micropterus*, the genus does not conform to the Biological Species Concept. Taxonomic classification should therefore be assessed using the Ecological (Van Valen 1976) and Evolutionary (Simpson 1961) Species Concepts. More specifically, *M. d. dolomieu* and *M. d. velox* must be evaluated against the criteria that they represent separately evolving lineages with their own “unitary roles and tendencies” (Evolutionary Species Concept; Simpson 1961), and that they occupy unique, adaptive zones in which they are “minimally different from that of any other lineage in [their] range” (Ecological Species Concept; Van Valen 1976).

The gaps in our knowledge of morphological and genetic diversity in *M. d. velox* are disproportionate to the management and conservation efforts that may be required to preserve their diversity (Brewer and Long 2015). With a more complete understanding of *M. d. velox* taxonomy and, more specifically, its relatedness to conspecific groups, it will be easier to conserve its endemic diversity. For these reasons, it is necessary to validate the taxonomic and distributional status of *M. d. velox*. We propose to investigate the taxonomic and distributional status of *M. d. velox* using molecular tools. More specifically, through microsatellite and genomic analyses, we seek to 1) determine the taxonomic status of the subspecies, and 2) determine the delimited range of the diverged, unique form should it exist.

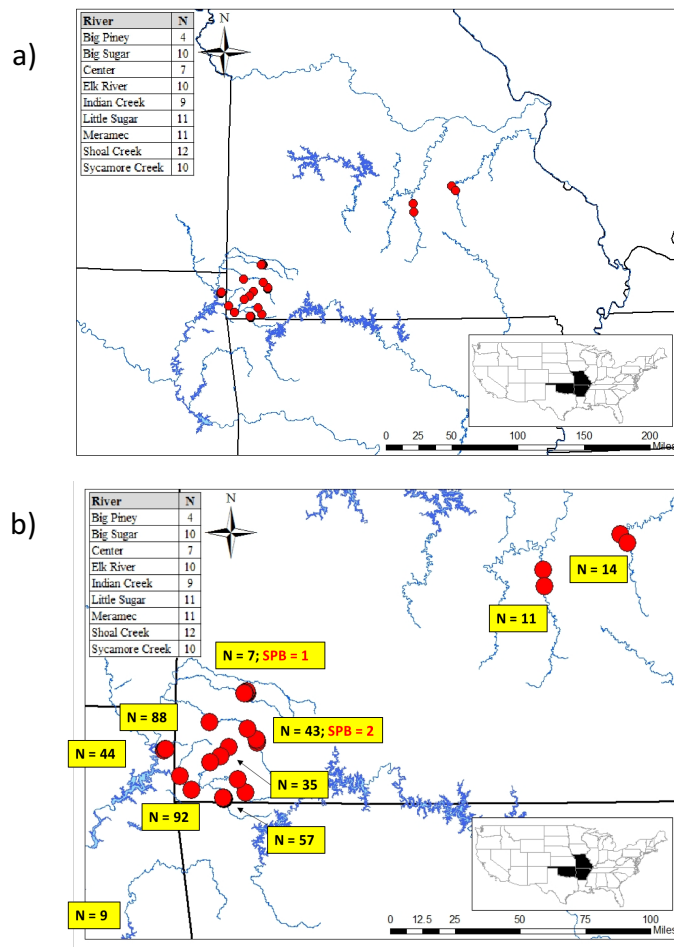
## II. Methods

*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. To 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
<i>Lma 21</i>	6	55	Pet	154	182	*CAGCTCAATAGTTCTGTCAGG ACTACTGCTGAAGATATTGTAG
<i>Mdo 1</i>	6	55	Vic	200	220	*GCTCTTCCCAGTGGTGAGTC ATCTCAGCCCATACCGTCAC
<i>Mdo 2</i>	6	55	Ned	187	207	*GCCCTTTCATATTGGGACAA CTGCTCTGGCGTACATTTCA
<i>Mdo 3</i>	6	55	Ned	125	145	*AGGTGCTTTGCGCTACAAGT CTGCATGGCTGTTATGTTGG
<i>Mdo 4</i>	3	55	Vic	132	152	*TCTGAACAACCTGCATTTAGACTG CTAATCCCAGGGCAAGACTG
<i>Mdo 5</i>	2	55	Fam	190	210	*CAGGTTCCCTCTCACCTTCA ATGGTCTCACCAGGGACAAA
<i>Mdo 6</i>	3	55	Pet	140	160	*TGAAATGTACGCCAGAGCAG TGTGTGGGTGTTTATGTGGG
<i>Mdo 7</i>	2	55	Vic	162	182	*TCAAACGCACCTTCACTGAC GTCACCTCCCATCATGCTCCT
<i>Mdo 8</i>	8	55	Fam	210	230	*GTGAGGACCAGCCAAAATGT GGAAGATTGAGGTCCCAACA
<i>Mdo 9</i>	5	55	Fam	116	136	*TTTGATGGGCGTTTTGTGTA GACCGGTCCTGCATATGATT
<i>Mdo 10</i>	2	55	Pet	91	111	*GTGTCTCCGTGTGTTGATGG AACACCAGAGGCAACAAGC

*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/μL) 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	<i>Mdo4</i> , <i>Mdo5</i> , <i>Mdo6</i> , <i>Mdo1</i>
2	55.4	<i>Mdo7</i> , <i>Mdo8</i> , <i>Mdo9</i> , <i>Mdo10</i> , <i>Mdo2</i>
3	52.8	<i>Lma21</i> , <i>Mdo3</i>

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<sub>2</sub>O 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.

<b>River</b>	<b><i>N</i></b>
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<sub>2</sub>O 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*. Using 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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