

## Report on Genotyping of Black Bass from Kentucky

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### Executive Summary

- Two previously developed MassARRAY panels for assessing hybridization and genomic composition of Black Bass species were converted into a new GT-seq assay.
- Kentucky Department of Fish and Wildlife Resources sent 1,694 Black Bass samples to the Southeast Conservation Genetics Lab for genotyping.
- Genetic analyses were performed to test purity of Largemouth Bass versus Florida Bass and to test for hybridization among other Black Bass species.
- Results showed various levels of non-native Florida Bass introgression into native Largemouth Bass depending on sample collection location, but no apparent geographic pattern was seen in the data.
- No evidence of Alabama Bass introgression within sampled waterbodies was found.
- Results provide critical information for Black Bass management.
- The GT-seq assay is a new, cost-effective tool for Black Bass management that does not require specialized equipment.

## Introduction

Genetics management of aquatic natural resources helps ensure that management goals are met (Shafer et al. 2015; Holderegger et al. 2019; Hohenlohe et al. 2021; Willi et al. 2022). For example, genetics management can be used to monitor introgression of invasive species into native species, thereby allowing managers to design mitigation efforts (Leroy et al. 2018; Gibson et al. 2019; Berrebi et al. 2022). Hatchery efforts can benefit from genetics management, whether for sportfish management, conservation, or both. When managers pursue captive propagation, broodstock can be checked for genetic background to ensure that the genetic background of produced fish match management objectives. Otherwise, hatchery produced fish may be counterproductive to management goals (Taylor et al. 2019). Genetic data is required to monitor for non-native introgression in native sportfish and ensure that hatchery produced fish have the desired genomic background.

Black Basses of the genus *Micropterus* (Perciformes: Centrarchidae) are economically important sportfish (Chen et al. 2003; Driscoll et al. 2012; Driscoll and Myers 2014). In recent years, genetic data have revealed that *Micropterus* is comprised of numerous closely related species, some of which are capable of hybridizing (Zhao et al. 2018; Thongda et al. 2020; Kim et al. 2022). For example, for many years, Florida Bass were considered a subspecies of Largemouth Bass, but recent systematics work has clarified *Micropterus* taxonomy and demonstrated that both entities are valid, separate species level (Kim et al. 2022). The accepted scientific names for Florida Bass is *Micropterus salmoides* (Lacepède, 1802) and for Largemouth Bass is *Micropterus nigricans* (Cuvier, 1828) (Page et al. 2023). To effectively manage *Micropterus* genetics, several genetic assays have been developed. Two assays are of particular interest to managers in Kentucky: 1) an assay that distinguishes between Florida Bass (*M. salmoides*) and Largemouth Bass (*M. nigricans*) (Zhao et al. 2018) and 2) an assay that tests for introgression of Alabama Bass (*M. henshalli*) and other species into native *Micropterus* (Thongda et al. 2020). These assays are comprised of single nucleotide polymorphisms (SNPs) that were determined to be diagnostic for each respective species. They were designed to work with a Sequenom MassARRAY genotyping system (Zhao et al. 2018; Thongda et al. 2020), which requires specialized equipment not available in most genetics labs or core facilities. Furthermore, the number of SNPs that can be included on a single MassARRAY run, or assay, is limited. In contrast, newer technologies such as GT-seq offer genotyping solutions that do not require specialized equipment aside from Illumina sequencers that are present in virtually every genetics core facility (Campbell et al. 2015). GT-seq also allows for including more loci on a single assay (Campbell et al. 2015), potentially offering cost savings compared to genotyping with other platforms.

To help fisheries managers in Kentucky more effectively manage their *Micropterus* populations, we converted two MassARRAY assays into a single GT-seq assay that consists for two panels that can be analyzed separately: one for examining purity of Largemouth Bass versus Florida Bass and a second for testing introgression among species. After designing the assay, we used it to genotype Black Bass from reservoirs across the state of Kentucky as well as hatchery broodfish. Our results provide a snapshot of the genetic background of Black Bass in Kentucky reservoirs and lay the foundation for future management and propagation of Black Bass in Kentucky.

## **Material and Methods**

### *Sample Collection and DNA extraction*

Black Bass were collected by the Kentucky Department of Fish and Wildlife Resources (KDFWR) between September 2022 and May 2023 (Table 1). For each sampled fish, a fin clip was taken and placed in ethanol. Fin clips were sent to the Southeast Conservation Genetics Lab in Auburn, Alabama, which is a joint U.S Fish and Wildlife Service and Auburn University genetics research lab. DNA was extracted from fin clips with either a 96-well format Qiagen DNeasy Blood & Tissue Kit or a 96-well format Omega Bio-Tek Mag-Bind Blood & Tissue DNA Kit. Most extractions were done with the Qiagen kit because it resulted in extractions with higher yields and larger genome fragments. In total, 1,694 samples were analyzed.

### *GT-seq panel design and genotyping*

The MassARRAY primers for the 38 loci used in the Largemouth versus Florida Bass assay from Zhou et al. (2018) and for the 64 loci used for examining Black Bass introgression from Thongda et al. (2020) were downloaded from each publication's supplementary material. Each primer sequence had MassARRAY-specific nucleotides that we replaced with Illumina sequencer compatible adapter sequences (Tables 2, 3). The string of nucleotides targeting Black Bass genome regions were unchanged.

Forward and reverse modified primers for all target loci were pooled into a single tube at 0.252  $\mu$ M concentration per primer. Initially, we followed the gt-seq protocol of Campbell et al. (2015), but initial runs resulted in a large proportion of primer dimers compared to target sequences. Therefore, we modified the protocol, which resulted in successful GT-seq library preps (see Appendix 1 for full protocol). To test whether the primer multiplex resulted in adequate sequencing coverage per locus, an initial batch of 92 samples from Paintsville Lake and 4 from Carr Creek Lake were multiplexed and sequenced on an Illumina MiSeq using a Micro flow cell with 2 x 150 bp chemistry. A second batch that

included the 1,598 remaining samples received from KDFWR was sequenced on an Illumina NovaSeq using an SP flow cell and 1 x 118 bp chemistry; for sequencing on the NovaSeq, the run included spiking a custom i5 index sequencing primer that was required because of differences in how i5 indexes are sequenced between the NovaSeq and MiSeq platforms.

Raw data were processed with fastp (Chen et al. 2018) using default parameters to remove adaptor sequences and low quality reads. Demultiplexing was done using the STACKS 2.62 program *process\_radtags* (Rochette et al. 2019). Although *process\_radtags* was designed for RADseq data, the program allows for 1 bp primer mismatch, which is not an option using standard GT-seq demultiplexing scripts. After demultiplexing, GT-seq loci were assembled with the script *GTseq\_Genotyper\_v3.pl* (available from <https://github.com/GTseq/GTseq-Pipeline/>). This script requires information on flanking regions of the target SNP for each locus and the two possible alleles that could be present at each locus. For loci in the introgression panel, this information was taken from the original publication (Thongda et al. 2020). For the Largemouth Bass versus Florida Bass panel, reference allele information was retrieved with *blastn* (Camacho et al. 2009) by comparing sequenced loci against reference transcriptomes from NCBI GenBank Transcriptome Shotgun Assembly database; this was required because information on which allele was indicative of Largemouth Bass versus Florida Bass was not provided in the original publication (Zhao et al. 2018).

The test GT-seq run performed on the MiSeq resulted in high sequencing success per sample. However, 8 of the original 38 loci used for the Largemouth versus Florida Bass assay failed to adequately sequence. This was likely a result of primer incompatibilities in the multiplex PCR reaction. However, 30 loci, if truly diagnostic as previously reported, should be suitable for making determinations on whether an individual is a “pure” Largemouth Bass, a “pure” Florida Bass, or an F1 hybrid.

#### *Purity and introgression analyses*

After assembly, genotypes for the Largemouth Bass versus Florida Bass assay loci were compiled into an excel spreadsheet with the script *GTseq\_GenoCompile\_v3.pl* (available from <https://github.com/GTseq/GTseq-Pipeline/>).

Following Thongda et al. (2020), the introgression analysis utilized a model-based approach to assign genomic admixture proportions to each individual. For this, we used the R (R Core Team 2022) package LEA (Frichot and François 2015) that assigns genomic background percentages of “pure” Black Bass species to each sampled individual. The analysis utilized reference genotypes for each Black Bass species from Thongda et al. (2020). The reference genotypes were combined with genotypes from

sampled individuals into a single dataset. Data were converted from the aforementioned excel spreadsheet to a PLINK (Chang et al. 2015) .ped file using GTseqTools v 0.1 (Mussmann 2023). ADMIXTURE coefficients were assigned to each individual using the sparse Non-Negative Matrix Factorization algorithm, implemented with the snmf function in LEA using  $K = 6$  and  $\alpha = 150$  as parameters. We chose a  $K$  of 6 because we were testing for ADMIXTURE among the following *Micropterus* species or species complexes: Alabama Bass, Largemouth Bass, Smallmouth Bass (*M. dolomieu*), Shoal Bass (*M. cataractae*), Spotted Bass (*M. punctulatus*), and Coosa Bass (*M. coosae*). An  $\alpha$  of 150 was used because it accurately grouped reference individuals together during a preliminary testing phase. For the introgression analysis, Largemouth Bass and Florida Bass are grouped together and all species within the Shoal Bass complex are grouped together (see Thongda et al. 2020 for more details). We ran the snmf function 300 times and cross-entropy criteria were calculated for each run. We reported the run with the lowest cross-entropy.

## Results

Assay design resulted in a GT-seq assay with a panel of 30 loci for assessing the purity of Largemouth Bass versus Florida Bass and a panel of 64 loci for assessing introgression among *Micropterus* species. Our analyses indicate that both panels are suitable for accomplishing objectives associated with genotyping Black Bass to assess genomic background for sportfish management.

We examined purity of Largemouth Bass versus Florida Bass for 1,385 successfully sequenced putative Largemouth Bass samples (Table 4). We considered any individual that had greater than 95% Largemouth Bass reference alleles as “pure” Largemouth Bass; any individual with greater than 95% Florida Bass reference alleles were considered “pure” Florida Bass. An individual was considered an F1 hybrid of Florida and Largemouth Bass if greater than 90% of loci were heterozygous. Our inferences about purity assume that all genotyped loci are diagnostic of Largemouth versus Florida Bass, as stated in Zhao et al. (2018). However, our results seem to indicate that not all loci from the Zhao et al. (2018) panel are diagnostic, and managers could consider individuals with a lower percentage of putative Largemouth Bass alleles as “pure” Largemouth Bass (see Discussion for more details).

Our analyses indicated that 485 of 1,380 individuals can be considered “pure” Largemouth Bass under a 95% threshold of having reference Largemouth Bass alleles. If the percentage is lowered to 90%, 931 individuals could be considered “pure” Largemouth Bass. No individuals were pure Florida Bass under either a 95% or 90% threshold. No individuals were F1 hybrids.

Even among individuals that were identified as pure Largemouth Bass, several loci of the Largemouth Bass versus Florida Bass assay were consistently heterozygous which suggests those loci may not be diagnostically useful for determining species. The percentage of sampled fish from each location that were identified as pure Largemouth Bass varied among lakes. Some lakes like Lake Beshear, Cave Run Lake, and Nolin River Lake had high percentages of pure Largemouth Bass, whereas others like Fishpond Lake and Greenbo Lake had no sampled fish that could be considered pure Largemouth Bass. No obvious geographical pattern was associated with lakes with relatively high percentages of Florida Bass alleles, but the four lakes with the highest percentage of FLB alleles were less than 200 acres in size. The locations with no pure Largemouth Bass are indicative of sites with recent Florida Bass introgression. We are unable to precisely determine the timing of hybridization events. However, in the absence of F1 hybrids, the introgression must have taken place more than one generation ago or is a result of introductions of introgressed Largemouth Bass or Florida Bass into those sites.

Genomic admixture analyses on all 1,678 successfully sequenced individuals indicated no genomic admixture among the vast majority of samples (Table 5). Genomic admixture, when present, was mostly between Largemouth Bass and Spotted Bass, but in four individuals, admixture was also seen between Smallmouth Bass and Largemouth Bass or between Smallmouth Bass and Spotted Bass. Two individuals from Martins Fork Lake were inferred to have only Coosa Bass ancestry. No sampled individual had any Alabama Bass ancestry.

## **Discussion**

The GT-seq panel developed here will have broad utility for Black Bass management as it can be used to distinguish purity of Largemouth Bass versus Florida Bass and more broadly test for introgression among Black Bass species. Importantly, the GT-seq assay combines two different panels (i.e., the Florida versus Largemouth Bass panel of Zhao et al. 2018 and the Black Bass Introgression panel of Thongda et al. 2020) into a single assay. The result is a useful genetic management tool that is more cost-effective and has broader utility than previously developed tools. Our Black Bass GT-seq panel is also more accessible as it does not require specialized equipment like a Sequenom MassARRAY genotyping machine.

Our results indicate that the degree to which Largemouth Bass have hybridized with Florida Bass varies across locations in Kentucky. In some locations, Florida Bass hybridization may have never occurred, especially if some loci are not diagnostic, as previously reported (see below). In areas with

Florida Bass introgression, we found no evidence of F1 hybrids, indicating that introgression of Florida Bass alleles into native Largemouth Bass populations is more complicated than a recent hybridization event. That said, some locations have clearly experienced Florida Bass introgression more recently than others, with sites having higher amounts of Florida Bass alleles likely experiencing more recent and/or larger influxes of Florida Bass. In the absence of repeated introduction of Florida Bass, heterozygosity is expected to decrease over time as backcrossing with native species occur. Thus, sites with lower amounts of heterozygosity could have experienced Florida Bass introductions farther in the past than those sites with higher amounts of heterozygosity. That said, complex hybridization histories resulting from multiple introductions and recombination could obscure such a pattern and further complicate trying to determine relative timing on Florida Bass introductions from genomics data alone.

Hybridization of non-native Florida Bass with native Largemouth Bass, resulting in introgression of Florida Bass alleles, could cause outbreeding depression in native Largemouth Bass populations by disrupting native genomic composition (Waples 1991). Past research indicated that outbreeding depression can cause lower resistance to disease, and lower physiological performance in Largemouth Bass (Cooke et al. 2001; Goldberg et al. 2005). Research on other fish species has also found susceptibility to outbreeding depression (e.g., Huff et al. 2011). Although we are not aware of a study that explicitly tested for effects of outbreeding depression on native Largemouth Bass from Kentucky, introgression with Florida Bass unnaturally (i.e., via human-mediated actions) changes the genomic composition of a native species. The result is introgressed individuals with unknown, but likely reduced, fitness.

When we designed the Largemouth Bass versus Florida Bass component of our GT-seq assay, we assumed that included loci had diagnostic alleles for either Largemouth Bass or Florida Bass. This was based on the original publication the loci came from and on previous use of the loci in a MassARRAY SNP panel for genetics management of Largemouth Bass populations in the southeastern U.S (Zhao et al. 2018). However, multiple loci were heterozygous for individuals that would otherwise have been 100% pure Largemouth Bass. This genetic pattern could be the result of a complex history of hybridization and backcrosses with some alleles selectively maintaining heterozygosity after it was introduced during a past introgression event. Alternatively, a small number of loci may not have diagnostic alleles for Largemouth Bass versus Florida Bass, contrary to past reports (e.g., Zhao et al. 2018).

Upon examination of Zhao et al. 2018, it appears that all Largemouth Bass samples used to identify putatively diagnostic loci came from only two sites, one in Illinois and one in Minnesota, USA, but the methods of Zhao et al. 2018 are slightly unclear; it's possible the loci were only tested on

samples from a source lake in Illinois. Yet, Largemouth Bass have a much larger range (Kim et al. 2022), meaning that natural genetic variation within Largemouth Bass may have been overlooked when putatively determining alleles to be species specific. Thus, we think the most likely explanation for the often-heterozygous loci is that the loci are not diagnostic for Largemouth Bass. However, given that we cannot confidently reject either explanation without much greater geographic sampling, we decided to report all loci in the Largemouth Bass versus Florida Bass Panel. As discussed earlier, if these often-heterozygous loci are not diagnostic, then the percentage of pure Largemouth Bass in these samples would be higher than we reported. Managers and other interested parties should interpret results, specifically Table 4, with these issues in mind.

Results of the Black Bass introgression panel, which lumps Largemouth and Florida Bass together, indicated that the vast majority of the 1,678 sequenced samples had genomic backgrounds indicative of only one species, or species complex in the case of Shoal Bass or Largemouth and Florida Bass. Unlike in some other southeastern states, Alabama Bass do not appear to have invaded Kentucky. Nevertheless, non-native species can jump waterways suddenly and spread rapidly once established. Thus, there is still a non-zero risk to native Bass in Kentucky from Alabama Bass introgression. We think that continued monitoring is appropriate, especially in waterbodies that are most geographically proximate to locations that are known to harbor Alabama Bass.

## **Conclusions**

The newly developed Black Bass GT-seq assay is a cost-effective tool for genetics management of Black Bass. We anticipate that the assay will be useful for sportfish management for years to come. The GT-seq assay demonstrated that Kentucky has waterbodies that both harbor a high percentage of pure Largemouth Bass and waterbodies that have seen Florida Bass introgression. In contrast, we observed no evidence of Alabama Bass introgression with native Kentucky species.

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Table 1: Sample Information for Sequenced Fish

Lake	Lab Code	Number of Samples	Bass Species^
Paintsville Lake	PVL	100	Largemouth
Minor Clark Hatchery	MCH	50	Largemouth
Carr Creek Lake	CRC	50	Largemouth
Fishpond Lake	FPL	50	Largemouth
Highsplint Lake	HSL	50	Largemouth
Yatesville Lake	YVL	50	Largemouth
Lake Barkley	BAR	50	Largemouth
Barren River Lake	BRL	50	Largemouth
Lake Cumberland	CBL	50	Largemouth
Cedar Creek Lake	CCL	50	Largemouth
Guist Creek Lake	GCL	50	Largemouth
Kentucky Lake	KEN	50	Largemouth
Kincaid Lake	KIN	50	Largemouth
Lake Malone	LMA	50	Largemouth
Laurel River Lake	LRL	50	Largemouth
Greenbo Lake	GBL	50	Largemouth
Green River Lake	GRL	50	Largemouth
Grayson Lake	GRY	50	Largemouth
Herrington Lake	HER	50	Largemouth
Jacobson Park Lake	JCP	50	Largemouth
Mill Creek Lake	MIL	50	Largemouth
Cave Run Lake	CRL	50	Largemouth
Lake Beshear	BES	50	Largemouth
Taylorsville Lake	TVL	50	Largemouth
Fishtrap Lake	FTL	50	Largemouth
Nolin River Lake	NRL	50	Largemouth
Rough River Lake	RRL	50	Largemouth
Lake Barkley	BAR	50	Spotted or Smallmouth
Lake Cumberland	CBL	50	Spotted or Smallmouth
Herrington Lake	HER	50	Spotted or Smallmouth
Kentucky Lake	KEN	44	Spotted or Smallmouth
Laurel River Lake	LRL	50	Spotted or Smallmouth
Martins Fork Lake	MF	50	Spotted or Smallmouth

^All samples were included in Black Bass species ID and genomic admixture analyses. Only Largemouth Bass were tested for Florida Bass introgression



**Table 2: List of 30-SNP multiplex primers for GT-Seq Panel to disntguish between Largemouth Bass and Florida Bass (5' to 3')**

Locus ID	Forward GT-seq Primer	Reverse GT-seq Primer	Largemouth Bass Genotype	Florida Bass Genotype
Contig11272	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAAGGAAGGCGATGCTTCTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTTTGAGCAGCAGATCAAGG	G	A
Contig12388	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTTCTTTCCAGCGACGTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGGAGGACATGTTGTA AAC	A	T
Contig1240	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAACGGTTTGGACTCAACAGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTCAAATTGGAGTTGAGGG	G	A
Contig13020	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAAGGAGTGATGTTTGATGGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTCAAAGTCAGAATCATC	T	C
Contig15950	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGAGAAGCTGCAGAGGAATC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTGTACAAAAGTTGCGGG	G	A
Contig16665	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGAACCCTGCCTTAGATTAC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGTGCATGCAAATCTGATG	C	A
Contig18101	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAAAAGATGTCCTCTCTGCG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGGCCAGAAGAGAAAACCAC	A	C
Contig1826	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCAAAGAGAGCGAGAGGTAG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTATGTTACACTCTGCTC	A	T
Contig18667	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGATCACCTGTGAAAACTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTGCAGAGAATTCAGAGC	T	C
Contig19961	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTGATGAACAACCCGCTTAGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGAAAGAGTCCAGCAGCCAG	G	A
Contig20908	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGTATCACCCAAATCTGCCG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGTGAGTGCCTGAGACCAAAC	C	T
Contig21621	CGACAGGTTCAGAGTTCTACAGTCCGACGATCATAAGCGTGGATGACTCAGC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGTAGGCCAATAGGATGGTG	T	C
Contig21676	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGGGATTTGAAAATGAGCACAC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAAGAGTCCAGAGACTTTG	T	C
Contig21917	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTTACAGATACAGAGCGCC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTGTTGGGTGGATGGATTGG	A	G
Contig2242	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGGATAGACATTGAGCACAGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCATCAGGCACGAGTACATC	G	A
Contig22709	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAAAGGAGCCATCACCAGAAG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGTCTGGGTTGTCCACTTTG	C	A
Contig22803	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCAACACTGTTCTCTTTCTC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCTGTCTGGCAGCTCTTC	C	A
Contig23008	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTTCTCCACAGCCTCATAAG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGCAGTTCTAAATGGCATC	A	C
Contig2635	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTACTGGACAAGACATGGTGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGATTGACTGTCAGTGCTC	G	A
Contig28601	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTTCTACTGTAAAGTTAGGC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCAATGGCCACTTAAGGAG	G	A
Contig31979	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAATGAATGCACAGGCTTGTC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGCTAAGATGTATTCACAG	A	T
Contig3296	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGTGAGTAAACAGGAAGACGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGTGTTGTGGTCTGCATAGC	C	A
Contig3379	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCCAGCATTCTGTTGTTACC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGTAGGCCTTCAAACACAGAG	G	C
Contig3616	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCCAGTCTTTGAACCCTGTC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTGCTTTTAGCTGGTGTCTG	C	T
Contig4919	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGTAGGCCAAGCGTTAAAAGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTTTGTAAAGTGCCACC	G	T
Contig4936	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTACTTCCCCGCCATTACCAC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCGACCTGTGCCAAAAAGC	A	G
Contig6127	CGACAGGTTCAGAGTTCTACAGTCCGACGATCATGTGAAAAGCATGTACTGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAGGAATTGCCTTTGACTG	C	G
Contig6920	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCCACCTTTCTTGAACTCC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCATCAAGGTAGCAAAGGAG	T	C
Contig9758	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTGTCTGCACAGCTGCTTCTC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCCCCGAGGAATTTAAAAG	T	G
Contig9870	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCTTAGAATCTGTCCCTCTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGCCAATGATTTTGTGAGC	T	C

**Table 3: Black Bass Introgression Primers**

Locus ID	GT-seq Forward Primer	GT-seq Reverse Primer	SNP Alleles
MpRETP16597TP20237T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCTTACCTGCAGCTTTCAGAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAACATACTGCAGCTGCAGGG	G/T
MpRETP17639A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCAGTACTGTATGCGCAACCTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGATGCTTCTGTGCTTATTCCG	A/G
MpRETP29431T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCTCGCGTGAAACACACAAAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGTTGGAGAAGACAGATGGG	C/T
MpRETP30468C	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTTGTCTGCAGTTTCAGAGCG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACTTGACAATTTTCAGGCC	C/G
MpRETP5605T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCGCAGGTTTAAGCAAGTGCAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTCATCCTGCCATGTTTCCTC	C/T
MpRETP8149T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCAGCCTGAGATTGCCAAGATG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGGCTGAAGTCATTCTGGTC	C/T
MpRETP9363A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCGCGTGACAATCAGGTCTTTT	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGCGTCTTATTTGTGGATGGG	A/T
MpSHTP18868C	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCAGTTCAACCTGCAGGCAAAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGAGACCTGCTCCACTTTTTTC	C/T
MpSMBTP16142A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTTTTCTACTCTCCTTCTGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTCAGCAGTGGGACCAAATTC	C/A
RETP10556A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCGGCTGCTCACATAAACACAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGCAGCACATGAAATCAACAC	G/A
RETP13743C	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCAGCTAACTGGCTTTCTCTC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGAGCCTGCTCGTAACCTTG	C/G
RETP3097A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCTGGTTTCCCTGACAACATC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGTCAAACGCAGTCCGAGAG	G/A
RETP3652A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCGTACTCAACTCTACAGCTC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGTTCACTTCTGCAGGAAGCTC	T/A
RETP4454T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTGAAGCGTGGAGACAGAGTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGTTTGTGCTCTTGTCTGGG	C/T
RETP4504A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCAATCACAGCTGGTCATGCAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACCACCAGTCATTGTTAGAG	C/A
RETP4592A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCACACGCTCAGCCACGCACA	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTTTATGTCCTGCTCCGCTC	C/A
RETP4714A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTCATCTGCTGCTTTGGCGTC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAAGAAGCAGATGCTGGAGAG	T/A
RETP4763G	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCACCCAGCCAATATGATCCAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCAAAGTAGTGGTGCAGGTTG	A/G
RETP4967G	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCCTGTTGCACACATAAACCC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAATGGACAACCTGGGACTTGG	G/C
RETP5033T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTTACAAAATGTCTGCAGAGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAAGGCCTAGTAACAGTCCAG	T/A
RETP5089T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTATCTGAGCCAGCGAGTC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTTGGAGAGCCAGCTGGTAG	G/T
RETP5103T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCAACCTGTGTTCTGCAGAGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACACCGGCATAGAGTAAAC	T/C
RETP5172C	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCTGTGTGATCTGTAATCTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCTGGTTTTATAGCCGTGGTC	C/A
RETP5306A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCGAAATTCGGCACTTGAGGAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGCTTGAAGTTTGGAGGTCAC	A/T
RETP5317T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCAGAGGCAACAACCTGCTGAGG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACGTCCCTTTTCCCCTTG	C/T
RETP5360T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCTGCCTGTCAGCATGTTACTC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTAGGCACAGGTTAATGAAGC	C/T
RETP5475A	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCCGAGTGGATGGTAATGTTT	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGTGTTGCGATGAAGCTTCAG	A/G
RETP7076G	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCACTGCAGATGGTGAGAAGGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGTGCAGAGAGAGCTGACATT	G/T
RETP7594T	CGACAGGTTCCAGAGTTCTACAGTCCGACGATCAACATCTGTCTCTGCCGCTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGCTTATCAGAGGGACACTTG	T/C

ShTP20328A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTGGCAAGCACAACACCAAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTTCTACATCCTCTGGCTCG	A/G
ShTP6906C	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGCCTTTCATACTTTGGCCC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGCCTTCACTGCATTGATTC	T/C
ShTP9651T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCCACAGAGGATGAGTGAGTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTGACTCATGTGAAATGGTGG	C/T
ShTP9781T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTAAGAGCCGCAGAGTTTCC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCAAACAGCAGCAGGAGGTC	T/C
SMRETP23527A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAAATAGCAGGAGGAAGGTTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTACAGGTAACAGTGTGCCTC	G/A
SMRETP25045A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCATGACCACTGCATAACCCAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCTCTGTTCCACGGTCTTTTC	A/G
SMRETP6977C	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCAGATGGAGACAGTTGCTTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCTGTGCACTGTTCTGAAATG	C/A
SMSHtP1235G	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTAACTGTGCAGACACAGAGG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTGCCGGTATGCATACATCAG	G/A
SMSHtP13277T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGACGTAGATGAGCCCGTTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACACAAGAACAGAGTCGTGG	T/C
SMSHtP21440A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCAATCATCACACCCTGAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCGGATTTTGTCTGCCTCTGC	A/G
SMSHtP5127T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGCAGAGCTTGTTGAAAACC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGAGTTGTAACCCAACCTGTC	T/A
SMSHtP5873G	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGCTGCCTTTCTGTAAGTCC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACAGTCACAGGAGAAGGTC	G/A
SMSHtP7448C	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGATGGCTGCAGATTGAACAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGATGTGTGTAACGTGACC	C/A
SPMhRETP11109C	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGCAGCATCCTGAAGTCGTC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGTGTGTACTAGTACTCGGG	C/T
SPMhRETP1192T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCTGAACAGACTGCAGAATC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTACTTGTCTGTCTACACAG	A/T
SPMhRETP12009T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGAGTGAGCGTTGGAACGAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGGACTGCAGTACAACATGTG	A/T
SPMhRETP1486CG	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTTACCCTCTCAATCTCGTGG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTATGTGGTTCAGACAGAGTG	C/G
SPMhRETP15885T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCCTGCAGCTGTAACAACAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGCACATTTTCAGCACATTG	G/T
SPMhRETP18151G	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGGACTGAGGGTGTCAACAAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGTGTGTCAGGCAGACTGACTTT	G/A
SPMhRETP18435T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAACATATCTGCAGGAGTCGG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAAAAGTTGCTGCCTGCTCTG	C/T
SPMhRETP18863TP20134T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCTTAATCCCTGCAGGCAAAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCCATCAACAGCAGCACTATC	C/T
SPMhRETP21822TP22590A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAAGCTGTCAGTCACCTGGAG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGGTGAAGCTGGATCAGTGG	G/A
SPMhRETP24673A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCAGCGTTATCTCCTACTTTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTATTACCTTGTCTGTTGTGTG	G/A
SPMhRETP26317T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGGGTATGTCTGTGAAATGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCGCCTTCCAGTTTTCTTGTG	C/T
SPMhRETP28164T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCAGCAGATTAATCCCTGAGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGTAAGTGTGACCACCAGAAG	G/T
SPMhRETP2910G	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGTCGCGTTTGGTGCAATTTG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTGACAGATCCACTCAGAGC	G/C
SPMhRETP3437TC	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTTGAAGTGCAGACTCACAGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTACAGGCTTCATGATCAGGG	T/C
SPMhRETP4105A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCAGAGACAGCCAGTCAGAAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTGTGCGAACAACGTTCCGG	C/A
SPMhRETP6034TP13641T	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCCTGCAGAGTTGTGAAGAAC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTAGCGTTTCAGAATTTCTCC	C/T
SPMhRETP6389C	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTGTAGATGTTGAGTCTGCCG	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCATCAAACGGCTTGCCGAC	C/T
SPMhRETP7161A	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGCAGATGTGCTGCAATTTCC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGAGAGACAACGTCAGCAGAT	G/A
SPMhRETP7458TP15274C	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTATCGCTCCGGTTTGATTGC	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTTGAATGAGGCTGCAGCTGAG	C/T



SPMhRETP8383G

CGACAGGTT CAGAGTTCTACAGTCCGACGATCACAATCTGCAGCACACAAGG

GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCAACAGGATCTGTCACAATG

G/C

SPMhRETP8469A

CGACAGGTT CAGAGTTCTACAGTCCGACGATCAAGAATGGCATGCACTCAGG

GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCAGTGCCGGTGTGTTTTATG

G/A

SPMhRETP8660C

CGACAGGTT CAGAGTTCTACAGTCCGACGATCATGTGTGTTACTGCAGCACC

GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTCCCATCACAACCTTTCACACC

T/C

























Table 5: Results of genetic admixture analyses. Values are the proportion of genomic background for each individual that matches reference genomic backgrounds

Samples	Alabama Bass	Coosa Bass	Largemouth Bass	Shoal Bass	Smallmouth Bass	Spotted Bass
BAR_10.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_11.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_1.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_12.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_13.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_14.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_15.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_16.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_17.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_18.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_19.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_20.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_2.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_22.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_24.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_25.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_26.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_27.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_28.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_29.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_30.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_31.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_3.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_32.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_33.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_34.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_35.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_36.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_37.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_38.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_39.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_40.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_41.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_42.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_43.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_44.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_45.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_46.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_47.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_48.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_49.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_50.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_51.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_5.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_52.1	0.000	0.000	0.889	0.000	0.110	0.000
BAR_53.1	0.000	0.000	0.390	0.000	0.000	0.610
BAR_54.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_55.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_56.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_57.1	0.000	0.000	0.275	0.000	0.000	0.725
BAR_58.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_59.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_60.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_61.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_6.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_62.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_63.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_64.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_65.1	0.000	0.000	0.310	0.000	0.000	0.690
BAR_66.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_67.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_68.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_69.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_70.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_71.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_7.1	0.000	0.000	1.000	0.000	0.000	0.000
BAR_72.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_73.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_74.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_75.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_76.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_77.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_78.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_79.1	0.000	0.000	0.000	0.000	0.000	1.000
BAR_80.1	0.000	0.000	0.000	0.000	0.000	1.000

















































YVL_46.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_47.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_48.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_49.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_50.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_53.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_6.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_7.1	0.000	0.000	1.000	0.000	0.000	0.000
YVL_9.1	0.000	0.000	1.000	0.000	0.000	0.000



## Conservation Genetics Program Southeast Conservation Genetics Program

### Standard Operating Procedure (SOP) *GTSeq*

#### Materials:

Sequel Prep plates  
Unlabeled Primers (Fwd and Rvs)  
PCR Plus Master Mix (Qiagen)  
Unlabeled barcode primers (i5-well position and i7-plate designation)  
AMpure XP beads  
Magnetic rack  
Lo-Bind 2.0mL tubes  
Lo-Bind 0.5mL tubes  
Lo-Bind 1.7mL tubes  
96-well plates  
Filter Pipette tips all sizes  
Reservoirs for chemicals  
Project layout spreadsheet

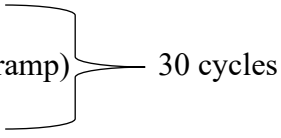
#### Methods:

1. Open Project layout excel sheet. Fill in information on green tabs (GT and qPCR, print both pages).

#### **PreAMP PCR**

2. Make PreAmp PCR master mix
  - a. Amount per sample
    - i. Qiagen 2X PCR Plus MM 3.5 $\mu$ L
    - ii. Primer mix (0.252 $\mu$ M) 0.5 $\mu$ L
    - iii. ddH<sub>2</sub>O 1.0 $\mu$ L
3. Label PCR plates: Plate Name, PreAmp, Date.
4. Add 5 $\mu$ L of PCR master mix to all wells of labeled plates.
5. Add 2 $\mu$ L of ExoSAP treated DNA.
6. Seal, vortex, and briefly centrifuge plate.

7. Place on thermal cycler and run program: GTSEQ30

- a. 95.0°-15:00
  - b. 95.0°-00:30
  - c. 57.0°-00:30 (5% ramp)
  - d. 72.0°-2:00
  - e. 72.0°-00:30
  - f. 4° hold
- 
- 30 cycles

8. Take 3 uL from first or last two samples on plate for Qubit and gel

NOTE: Discard PreAmp-PCR plate after Barcoding step or if needed,

### Dilute and Barcode

8. Dilute PreAmp-PCR (1:10) by adding 63µL nuclease-free H<sub>2</sub>O directly to PreAmp-PCR.

9. Seal, vortex, and briefly centrifuge plate.

10. Make i7 working stock (10uM) if none is available (you will need a different i7 for each plate that is being sequenced):

- a. Nuclease-free H<sub>2</sub>O      90µL
- b. i7 (100µM).              10µL

11. Label a new lo-bind centrifuge tube with i7 index number (one for each plate).

12. Make i7 master mix

- a. Amount per sample
  - i. Qiagen 2X PCR Plus MM      5.0µL
  - ii. 10uM Primers i7              1.0µL

13. Vortex and briefly spin down i7 master mix.

14. Label PCR plates: Plate Name, i7 index #, Date.

15. Add 6µL of i7 cocktail to all wells of labeled plates.

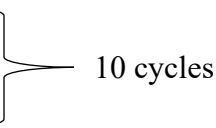
16. Add 3µL of diluted PreAmp-PCR product to all wells of barcode plate.

17. Thaw, mix gently, and briefly spin i5 barcode plate.

18. Add 1µL i5 barcode to each well. (Use 1 box of tips)

19. Seal, vortex, and briefly centrifuge plate.

20. Place on thermal cycler and run program: gtseq2

- a. 95.0°-15:00
  - b. 95.0°-00:10
  - c. 65.0°-00:30
  - d. 72.0°-00:30
  - e. 72.0°-5:00
  - f. 4.0°-hold
- 
- 10 cycles

NOTE: If complete evaporation after cycling is observed, add 10µL nuclease-free H<sub>2</sub>O to rehydrate.

## Normalization

21. Label new SequelPrep Normalization plates: Plate Name, Seq, i7 index, Date
22. If you have a partial plate, aliquot liquids to wells that will have DNA only, for all steps below.
23. Binding Step
  - a. Add 10 $\mu$ L of SequelPrep Normalization Binding Buffer to SequelPrep Normalization plate.
  - b. Transfer ALL content of Barcoded DNA to SequelPrep Normalization plate. (Use new tips for each well.)
  - c. Seal, vortex, and briefly centrifuge.
  - d. Incubate plate at room temperature for minimum of 1 hour. Do not disturb plate.
24. Wash Step
  - e. Aspirate all liquid from wells and discard. (Try not to scratch the bottom of wells. You do not have to change tips, you can use same tips for each column.)
  - f. Dispense 50 $\mu$ L SequelPrep Normalization Wash Buffer.
  - g. Using multichannel pipette (set to 55 $\mu$ L) and 1 set of tips, flush well by pipetting up and down twice and then completely aspirate the buffer from the wells and discard liquid.
  - h. Invert and tap the plate on paper towels to remove any residual wash buffer from the wells. It is okay if a small amount of wash buffer (1-3 $\mu$ L) is present still.
25. Elution Step
  - i. Add 20 $\mu$ L SequelPrep Normalization Elution Buffer to each well.
  - j. Seal, vortex, and briefly centrifuge.
  - k. Incubate at room temperature for 5 minutes. At this stage, plate can be stored overnight.

NOTE: SequelPrep provides just enough wash buffer – do not take more than you need

## Pooling and Size Select

26. Remove AMPure XP beads from fridge and allow to come to room temperature for 30 minutes.
27. Take all liquid from each well of a single normalized plate and combine into a strip tube.
28. Transfer all normalized DNA from strip tubes to two 2.0 mL lo-bind tubes and label: “NP” + the name of the plate. Vortex and spin down tubes. Store tubes at 4 °C or -20 °C for long term.
29. Remove long sequences with AMPure XP beads
  - a. Vortex AMPure XP beads very well.
  - b. Add 0.5X beads into each tube and mix well by pipetting. Avoid bubbles
  - c. Incubate at room temperature for 5 minutes.

- d. Transfer tubes to magnetic stand and let sit for 4 minutes.
  - e. Keeping tubes on magnetic stand, transfer supernatant to 2<sup>nd</sup> set of 2.0mL lo-bind tubes.
  - f. Discard tubes with beads
30. Leave short sequences
- g. Again vortex AMPure XP beads.
  - h. Add up to 1.0X (another 0.5X) AMPure XP beads, and mix well by pipetting.
  - i. Incubate at room temperature for 5 minutes.
  - j. Place on magnetic stand for 4 minutes.
  - k. Keeping tubes on magnetic stand, remove and discard supernatant.
31. Wash step (keep tube on magnetic stand for entire wash)
- l. Mix FRESH 80% Ethanol:
    - i. Amount per 8 plates
      - 1. 2450µL ethanol (200 proof)
      - 2. 1050µL nuclease-free H<sub>2</sub>O.
  - m. Add 500µL 80% Ethanol tubes containing beads, incubate, for 30 seconds.
  - n. Remove and discard supernatant.
  - o. Repeat wash (step o and p).
  - p. Remove tubes from magnetic stand and wait 5 minutes to dry on bench top.
32. Ready for long term storage
- q. Label 0.5mL lo-bind tubes e.g. with plate name.
  - r. Add 28µL EB to tubes containing beads and mix well by pipetting.
  - s. Place on the magnetic stand for 2 minutes.
  - t. Remove/transfer supernatant to newly labeled tube.