FINAL REPORT

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South Carolina Department of Natural Resources

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Project Title – Identification and optimization of a disomic microsatellite panel for use in population genetics of Shortnose Sturgeon

Project Summary – The federally endangered Shortnose Sturgeon (*Acipenser brevirostrum*) was an initial member of the Endangered Species Act in 1973 largely due to overharvest for caviar, a shrinking population range, range-wide habitat degradation, and limited access to historic gravel bed spawning grounds due to the construction of dams. The use of genetic data to interpret patterns of population structure would vastly improve our ability to manage for population-specific threats and guide recovery efforts for the species. Currently, the only published genetic markers for Shortnose Sturgeon are mitochondrial markers and a set of polysomic microsatellites. The presumed hexaploid nature of Shortnose Sturgeon has made the development of standard disomic microsatellite markers challenging. Microsatellites remain the most affordable and tested method for analyzing genetic health and estimating population structure across a species' range. We utilized genomic sequencing from the Sterlet Sturgeon and Shortnose Sturgeon to identify a high number of microsatellites, which we screened to identify functionally disomic loci. A total of 693,511 conserved microsatellites were identified across both sturgeon genomes. The project team screened 664 of these microsatellites using Shortnose Sturgeon samples from across their range, with a total of 16 markers identified as putatively functionally disomic. Following further testing in two South Carolina data sets, the final multiplexed marker suite includes 13 microsatellites as well as the recently published sex identification marker. Project results suggest that our panel should be useful for genetically characterizing populations of Shortnose Sturgeon, although more research is needed to clarify underlying causes of observed deviations from Hardy–Weinberg Equilibrium (HWE) and potential presence of null alleles prior to interpretation of genetic data for management and conservation purposes. These observations would be expected in populations that experienced a severe historic bottleneck, which is consistent with our observed patterns in long-term N_e, contemporary N_{eb}, and Garza-Williamson Index (G-W) estimates. We did not detect genetic differences between the Winyah Bay and Savannah River data sets with the new marker panel; additional investigation into potential influences of movement patterns and collection biases are needed. From a genetic health perspective, project results would be consistent with a population of Shortnose Sturgeon that underwent a severe bottleneck historically and are slowly recovering. Supporting evidence includes contemporary N_{eb} estimates showing an increasing trend over long-term N_e estimates, a moderate level of retained genetic diversity, no indication of inbreeding, and low levels of outbreeding. Although further marker validation is

needed, we are highly optimistic that the new genetic tool will provide important information for future conservation and monitoring of Shortnose Sturgeon throughout their range. Our work has addressed specific goals and recommendations outlined in the NMFS Recovery Plan for Shortnose Sturgeon and the South Carolina State Wildlife Action Plan by providing improved tools for Shortnose Sturgeon conservation and recovery.

Introduction – Sturgeon species represent an ancient family of fishes dating back to the Triassic period 245-208 million years ago; their large size and unique armored morphology make them charismatic both in the wild and in education-based aquarium settings. The federally endangered Shortnose Sturgeon (*Acipenser brevirostrum*) was an initial member of the Endangered Species Act in 1973 largely due to overharvest for caviar, a shrinking population range, limited access to historic gravel spawning grounds due to the construction of dams, and range-wide habitat degradation (Vladykov and Greeley 1963, Kynard 1997). Shortnose Sturgeon is a non-game species with all American populations listed as Federally Endangered. The species is a typically large (up to 120 cm), long-lived (up to 60 years), and late-maturing (up to 18 years) member of Acipenseridae (Kynard 1997), which further complicates recovery. However, the species is an atypical sturgeon in that it is considered amphidromous and moves between its primary freshwater riverine or estuarine habitat and marine waters for reasons other than spawning (Bemis and Kynard 1997). Recent studies have found that individuals are moving through coastal waters much more frequently than previously thought (Peterson and Farrae 2011; Dionne et al. 2013; Post et al. 2014; Kynard et al. 2016). The use of genetic data to interpret patterns of population structure and gene flow would vastly improve our ability to manage for population-specific threats and guide recovery efforts for this species.

Currently, the only published genetic markers for population genetic analyses of the Shortnose Sturgeon are mitochondrial markers (Walsh et al. 2001; Quattro et al. 2002; Wirgin et al. 2010) and a set of polysomic microsatellites (Henderson and King 2012, King et al. 2014). The presumptive hexaploid nature of Shortnose Sturgeon (i.e. having six sets of chromosomes; Fontana et al. 2008; Havelka et al. 2013; Rajkov et al. 2014) has made the development and use of standard disomic microsatellite markers challenging. A hexaploid individual may inherit up to six different alleles at a given locus rather than the typical two observed in a diploid organism. Therefore, the only current microsatellite set is polysomic in nature (Henderson and King 2012, King et al. 2014). Even though microsatellites remain the most affordable and proven method for analyzing the population genetics of a species and estimating population structure across a species' range (e.g. Duckett 2016), polysomic microsatellites complicate population genetic studies because most statistical tests assume diploid alleles. As such, the available polysomic microsatellite set has not been widely adopted or used by researchers to analyze Shortnose Sturgeon populations, and population genetic research on this species remains scarce and outdated.

However, recent advancements in sequencing technology and automated data processing have led to the ability to search genomes to identify potentially thousands of microsatellites. The microsatellites can be screened to identify functionally disomic microsatellites, i.e. only two alleles are inherited per locus. Identification of functionally disomic markers in polyploids has been successful in a variety of species, such as the Sea Sandwort (Gravely et al. 2018), Snapping Shrimp (Gaynor et al. 2017), and Lake Sturgeon (Welsh and May 2006), which is a closely related species to Shortnose Sturgeon (Krieger et al. 2008).

The use of a standard, disomic microsatellite panel would allow for population genetic structure analyses and eventually, after more populations range-wide have been genetically characterized, potentially the assignment of unknown individuals to a population of origin. Recent studies have identified greater than expected movement of Shortnose Sturgeon between river systems (Peterson and Farrae 2011, Dionne et al. 2013; Post et al. 2014; Wippelhauser et al. 2015; Kynard et al. 2016). Therefore, there is a distinct need to be able to assign encountered individuals to a population of origin to fully understand the threats that individual population segments are facing.

Purpose and Objectives – The intent of our project was to identify and optimize a suite of disomic microsatellite loci to use for population genetic analyses of Shortnose Sturgeon populations to provide critical data for management of this protected species. Our project tasks included these objectives:

Objective 1: Microsatellite Identification - use whole genome sequencing technology to produce millions of DNA sequence reads for Shortnose Sturgeon and use bioinformatics to identify 400 microsatellite loci and develop PCR primers;

Objective 2: Screening, Selection, and Optimization of Microsatellites - screen up to 400 of these loci to find 15-20 functionally disomic microsatellites to incorporate into an optimized multiplexed marker suite; and

Objective 3: Genetic Assessment of Two Shortnose Sturgeon Populations - use the optimized suite of microsatellites to genotype 200 samples to assess the Savannah River and Winyah Bay populations of Shortnose Sturgeon.

Accomplishments –

Objective 1: Microsatellite Identification

Although new technologies (SNPs) are also being explored for their use in the evaluation of genetic population structure and gene flow patterns, these markers have neither yielded the significant increased power initially anticipated for these analyses (Duckett 2016, Flanagan and Jones 2019), nor, as a result of their unknown locations within the genomes coupled with their four character state limit, been demonstrated to meet the assumptions of neutral selection as

is required for all of the standardized analytical protocols for genetic population structure. The hexaploid nature of Shortnose Sturgeon would further complicate the use of SNPs as a population genetic tool. Therefore, in an effort to maximize cost and time efficiencies, our project team chose the tried and proven microsatellite technology for the development of a new genetic marker panel for Shortnose Sturgeon.

Our original proposed methodology included whole-genome sequencing of a Shortnose Sturgeon individual to identify 400 microsatellite loci from which we would develop polymerase chain reaction (PCR) primers for screening. Unfortunately, shortly after the onset of our project COVID-19 led to the closure of both our labs as well as many commercial and university sequencing centers nationwide. However, in late March 2020, Du et al. published the draft genome for Sterlet Sturgeon (*Acipenser ruthenus*), a closely-related sister species with a disomic genome. Given the anticipation of extended sequencing facility closures and the availability of the Sterlet Sturgeon genome, our team made the decision in early May 2020 to modify our proposed methodology in the interest of moving the project forward in a timely manner. In lieu of whole-genome sequencing and further delay, our team utilized the publicly available Sterlet Sturgeon genome and bioinformatics to identify microsatellite loci from Sterlet Sturgeon *in silico* for testing in Shortnose Sturgeon.

We used the software program Krait to scan and identify microsatellite loci across the Sterlet Sturgeon genome, which were then matched against other available sturgeon genetic datasets using NCBI's BLAST tool to identify loci that were highly conserved across the *Acipenser* genus. The resulting dataset included 945,491 purportedly highly conserved microsatellite loci for *Acipenser*. Using Krait, candidate microsatellites were then assessed for chromosome location to avoid over-representing certain areas of the genome and maintain independence of markers. Any loci that overlapped with other loci were excluded with zero tolerance as any overlap represents a potential polysomic microsatellite or a duplicate/reverse sequence read. Of loci with more than one *Acipenser* species BLAST matches, we designed primers for tri-, tetra-, penta-, and hexa-nucleotide microsatellite repeat motifs using Krait's Primer3 tool. The resulting primer set was further refined by removing duplicates, optimizing temperature stability (58-62˚C), and assessing GC content (<50%) to prevent non-specific binding, reducing the potential microsatellite primer pair pool to 298,511. All data were collated and concatenated in JMP 15 Pro. We chose to eliminate di- and tri-nucleotide repeats due to the high abundance of longer nucleotide repeats since longer motifs tend to score more easily in multiplexed panels and are more likely to be conserved across species. We assembled a final list of 102,239 tetra-, penta-, and hexa-nucleotide loci from which we selected a random subset of 329 primer pairs for evaluation in Shortnose Sturgeon.

During our continued testing of microsatellites identified from the Sterlet Sturgeon genome during Year 1 of our project, we also decided to proceed with the original plan of developing

microsatellites from whole-genome sequencing of a Shortnose Sturgeon individual to increase the strength of the final marker panel.

In December 2021, DNA from an individual Shortnose Sturgeon (extracted from an adult captured from the Savannah River in July 2019 and stored at -80° C) was submitted for short-read, whole-genome, low-coverage, next generation sequencing. DNA extraction, isolation, and whole genome sequencing was conducted by GENEWIZ. Sequencing was performed on an Illumina MiSeq using the V2 500 cycle sequencing kit with library preparation for 2 x 250 base pair paired-end sequencing. Data were received in January 2022. This sequencing resulted in 26,261,574 forward and reverse reads. Forward and reverse sequences were merged using default parameters in FLASH resulting in 20,503,683 reads ranging from 31 – 490 base pairs.

We used the program MSATCOMMANDER (Faircloth 2008) to identify microsatellites using our lab's standardized protocols. The automated software program searches for microsatellite repeat motifs and designs primers for PCR amplification of the identified microsatellites, greatly reducing the effort previously necessary for identifying and designing microsatellites via cloning. As MSATCOMMANDER searches through millions of individual DNA sequence reads, deep and complete genome sequencing needed for genome assembly is unnecessary, thereby further reducing the cost of microsatellite development. MSATCOMMANDER settings constrained the search to only tri-, tetra-, penta-, and hexa-nucleotide repeat motifs, PCR product sizes ranging from 100 – 400 base pairs, and primer melting temperatures ranging from 58°C – 62°C. Using these settings, we identified over 395,000 potential microsatellites in the Shortnose Sturgeon genome. After ensuring that duplicates were removed and selecting for ideal primer lengths and melting temperatures, we identified 335 tri-, tetra-, penta-, and hexa-nucleotide repeat motif microsatellites for further testing.

Objective 2: Screening, Selection, and Optimization of Microsatellites

We ordered an initial set of 52 primer pairs to verify amplification in Shortnose Sturgeon given our modified locus identification protocol from the Sterlet Sturgeon genome. West Virginia University (WVU) completed the initial screening of these 52 primer pairs using the DNA from eight Shortnose Sturgeon individuals in late October 2020 (Table 1). Shortnose Sturgeon genetic samples were selected to represent wide geographic and temporal ranges for the species from North Carolina to Georgia. The WVU team modified our original screening methodology to include designing primers with an M13 tail (Schuelke 2000) to allow the use of a fluorescently-labeled universal primer and capillary electrophoresis, which helps facilitate rapid screening and accurate allelic discrimination resulting in improved cost and time efficiencies of the microsatellite screening process. The entire project team met virtually to review results of the initial screenings to form consensus on results and determine a path forward. Of the initial 52 primer pairs tested, 13 (25%) appeared to be potential candidates. These best potential primers resulted in consistent high-quality amplification, were

polymorphic among tested individuals, and appeared to be disomic. Out of the remaining primer pairs, 15 (29%) appeared to be monomorphic, 12 (23%) resulted in poor amplification, 7 (13%) resulted in non-specific amplification, and 5 (10%) appeared to be polysomic.

Following successful amplification and promising polymorphism observed in the initial primer set, an additional 273 primer pairs with M13 tails were ordered in November 2020 and the WVU lab resumed screening these primers. Additional Shortnose Sturgeon samples were sent to WVU to have available for screening. These samples were selected to increase the detection of variation across the geographic and temporal range available in our SCDNR Genetic Tissue Collection (Table 1).

Table 1. Shortnose Sturgeon tissues samples selected for use in screening putative disomic functioning microsatellite primer pairs. The first eight samples were used for the initial screening and the second twenty individuals were used for subsequent screening by WVU.

Genetic ID	Collection Date	State	Collection Location	Total Length (mm)
Abr-00420	23-Jun-11	GA	Altamaha River	1045
Abr-00427	06-Jan-12	NC	Cape Fear River	982
Abr-00680	17-Mar-15	SC	Cooper River	1000
Abr-00711	18-Nov-15	SC	Lake Marion	945
Abr-00712	29-Apr-16	SC	Santee River	486
Abr-00790	19-May-17	SC	Edisto River	1015
Abr-00899	16-Feb-18	SC	Savannah River	895
Abr-00909	07-Jun-19	SC	Waccamaw River	1126
Abr-00190	28-Jul-10	GA	Altamaha River	877
Abr-00401	15-Jun-11	GA	Altamaha River	823
Abr-00417	23-Jun-11	GA	Altamaha River	1035
Abr-00526	21-Nov-13	SC	Santee River	790
Abr-00609	21-Apr-14	SC	Santee-Cooper System	882
Abr-00614	09-Feb-15	SC	Santee-Cooper System	880
Abr-00629	29-Feb-16	SC	Cooper River	1092
Abr-00667	17-Mar-15	SC	Cooper River	1128
Abr-00687	17-Mar-15	SC	Cooper River	1029
Abr-00689	30-Jul-13	SC	Edisto River	1015
Abr-00729	13-Feb-15	SC	Savannah River	969
Abr-00778	30-Nov-16	SC	Savannah River	872
Abr-00784	02-May-17	SC	Edisto River	779
Abr-00799	14-Aug-18	SC	Edisto River	836
Abr-00803	22-Jun-17	SC	Waccamaw River	960
Abr-00816	27-Jun-17	SC	Great Pee Dee River	1080
Abr-00850	08-Dec-16	SC	Savannah River	1048
Abr-00883	15-Jun-17	SC	Savannah River	1000
Abr-00888	27-Jun-17	SC	Savannah River	1039
Abr-00923	25-Jun-19	SC	Waccamaw River	964

The screening of the additional primer pairs was not as successful as the initial set. Following completion of screening of all 325 primer pairs derived from the Sterlet Sturgeon genome by WVU staff, 16 (5%) appeared to be good potential candidates. These top candidates were selected based on their consistent high-quality amplification, polymorphism among tested individuals, and apparent disomic behavior. From the remaining primer pairs, 19 (6%) were possible candidates with various flaws such as excessive stutter, possibly polysomic behavior, etc., 105 (32%) were monomorphic or fixed heterozygotes across all individuals tested, 89 (27%) showed non-specific amplification or were polysomic, and 96 (30%) had no to low amplification. Upon completion of screening, WVU shipped all primer pairs and tissue samples back to SCDNR to continue further testing.

SCDNR continued testing these original 325 primer pairs, plus 4 additional that had been ordered but not tested, using tissue samples of Shortnose Sturgeon from across their range (Table 2). In general, results were similar between labs with differences mostly due to primer pairs that did not amplify for WVU but did amplify for SCDNR under optimized protocols. The results indicated that 20 primer pairs (6%) appeared to be good potential candidates. Out of the remaining primer pairs, 35 (11%) were possible candidates with various flaws as listed above, 138 (42%) were monomorphic or fixed heterozygotes across all individuals tested, 76 (23%) showed non-specific amplification or were polysomic, and 60 (18%) had no to low amplification.

Table 2. Shortnose Sturgeon tissues samples selected for use in screening putative disomic functioning microsatellite primer pairs at SCDNR.

Genetic ID	Collection Date	State	River	Total Length (mm)
Abr-00417	23 June 2011	GA	Altamaha River	1035
Abr-01017	6 May 2021	SC	Waccamaw River	597
Abr-01025	8 September 2020	SC	Edisto River	1030
Abr-01037	17 January 2018	DE	Delaware River	743
Abr-01056	14 August 2019	NY	Hudson River	695
Abr-01057	11 November 2016	ME	Penobscot River	712

In an attempt to improve time and cost efficiency, we developed a protocol for screening hundreds of microsatellites across many individuals simultaneously using an Illumina MiSeq Sequencing System that was acquired at the Hollings Marine Laboratory during the course of our project. Our initial run using the new protocol included 19 of the prior selected Sterlet Sturgeon-derived primers as well as 335 of the new Shortnose Sturgeon-derived primers and 24 individual sturgeon (Table 3). New primers for this initial run included tri-, tetra-, penta-, and hexa-nucleotide motifs, annealing temperatures between 59 – 61 °C, and a product length of less than 225 base pairs. We utilized available data analysis pipelines developed for screening/genotyping microsatellite loci in diploid organisms. Although the sequencing run successfully produced data, only 17 of the tested loci successfully genotyped in at least 10

individuals based on default parameters of the analysis pipeline. To validate the new potential sequencing screening analyses for Shortnose Sturgeon, we ordered primers with M13 tails to screen the most promising 37 loci identified via this new method on the GenomeLab GeXP for validation and comparison. Unfortunately, the MiSeq pipeline results did not translate well into promising disomic loci when viewed on the GenomeLab GeXP. Therefore, additional modifications and optimizations of this new MiSeq screening protocol for a polyploid species are needed, and we returned to our typical method of screening primers with M13 tails on our GenomeLab GeXP sequencing platform for visualization for the 335 selected Shortnose Sturgeon-derived primer pairs.

Table 3. Shortnose Sturgeon tissues samples selected for use in screening microsatellite primer pairs using the Illumina MiSeq at SCDNR.

Genetic ID	Collection Date	State	River	Total Length (mm)
Abr-00121	19-Oct-10	SC	Congaree River	299
Abr-00133	7-Jul-10	GA	Altamaha River	408
Abr-00427	6-Jan-12	NC	Cape Fear River	982
Abr-00523	18-Oct-12	SC	Santee River	831
Abr-00559	30-Jul-13	SC	Lake Marion	863
Abr-00591	1-Jan-10	SC	Savannah River	775
Abr-00615	10-Mar-15	SC	Santee-Cooper System	925
Abr-00711	18-Nov-15	SC	Lake Marion	945
Abr-00712	29-Apr-16	SC	Santee River	486
Abr-00817	6-Mar-18	SC	Great Pee Dee River	934
Abr-00940	13-Mar-18	SC	Cooper River	1003
Abr-00943	17-Mar-20	SC	Cooper River	815
Abr-00986	11-Dec-18	SC	Savannah River	980
Abr-00987	8-Jan-19	SC	Savannah River	1100
Abr-00988	8-Jan-19	SC	Savannah River	894
Abr-01024	16-Jul-20	SC	Edisto River	536
Abr-01043	17-Jan-18	DE	Delaware River	908
Abr-01050	17-Apr-14	NY	Hudson River	674
Abr-01055	3-Jul-19	NY	Hudson River	706
Abr-01063	4-Dec-17	ME	Penobscot River	738
Abr-01066	24-Sep-13	ME	Penobscot River	1145
Abr-01073	19-May-21	SC	Edisto River	793
Abr-01074	30-Jul-21	SC	Edisto River	730
Abr-01076	13-May-21	SC	Waccamaw River	1011

The screening of the Shortnose Sturgeon-derived primer pairs resulted in an additional 41 (8%) good potential candidates. Therefore, the combined screening efforts resulted in a total of 61 most promising primer pairs to visualize with more samples. These were amplified following standard protocols in single-primer reactions for 32 Shortnose Sturgeon samples from across their range (Table 4), separated and visualized on the GenomeLab GeXP, and scored by two independent readers. Afterwards, the readers compared notes on which microsatellite loci appeared to be disomic versus those that did not appear to be useful due to the presence of too many alleles, lack of consistent amplification, or excessive non-specific amplification. Following a rigorous evaluation, only 16 putative microsatellite loci were selected for further optimization.

These 16 loci were combined into three multiplex groups using the three dyes available to use on the GenomeLab GeXP: blue, green, or black (Table 5). The dye colors were selected so that initially observed allele ranges for each locus would not overlap amongst the loci sharing a dye color. Genotyping in multiplex groups improves cost-efficiency of PCR and visualization steps. We also incorporated the sturgeon sex marker, *AllWSex2* (Kuhl et al. 2021), which provides differentiation of male and female Shortnose Sturgeon (Sard et al. 2024) and will allow for sex identification of all individuals as they are simultaneously genotyped with our finalized microsatellite panel.

Final amplification conditions for each multiplexed panel included 1x Type-it Multiplex PCR Master Mix (Qiagen) without the Q-Solution, 0.33 µM forward and reverse primers, and 1 µL of 1:10 diluted DNA template in an 11 µL reaction. The thermal cycling protocol included an initial denaturation step at 95°C for five minutes, followed by 28 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 30 seconds, followed by a final extension step at 60°C for 30 minutes. All amplified products were run on a Beckman Coulter GenomeLab GeXP (Beckman Coulter, Inc., CA) along with a 400 bp size standard for genotype scoring to test for allele sizes and differences between males and females using established SCDNR protocols.

Table 4. Shortnose Sturgeon tissues samples selected for use in final screening of 61 microsatellite primer pairs at SCDNR.

Genetic ID	Collection Date	State	River	Total Length (mm)
Abr-00121	19-Oct-10	SC	Congaree River	299
Abr-00133	7-Jul-10	GA	Altamaha River	408
Abr-00143	9-Jul-10	GA	Altamaha River	426
Abr-00427	6-Jan-12	NC	Cape Fear River	982
Abr-00523	18-Oct-12	SC	Santee River	831
Abr-00559	30-Jul-13	SC	Lake Marion	863
Abr-00560	2-Oct-13	SC	Lake Marion	743
Abr-00591	1-Jan-10	SC	Savannah River	775
Abr-00615	10-Mar-15	SC	Santee-Cooper System	925
Abr-00711	18-Nov-15	SC	Lake Marion	945
Abr-00712	29-Apr-16	SC	Santee River	486
Abr-00816	27-Jun-17	SC	Great Pee Dee River	1080
Abr-00817	6-Mar-18	SC	Great Pee Dee River	934
Abr-00940	13-Mar-18	SC	Cooper River	1003
Abr-00943	17-Mar-20	SC	Cooper River	815
Abr-00986	11-Dec-18	SC	Savannah River	980
Abr-00987	8-Jan-19	SC	Savannah River	1100
Abr-00988	8-Jan-19	SC	Savannah River	894
Abr-01024	16-Jul-20	SC	Edisto River	536
Abr-01032	31-Aug-18	CT	Connecticut River	915
Abr-01033	4-Sep-18	CT	Connecticut River	995
Abr-01041	8-Dec-17	DE	Delaware River	326
Abr-01043	17-Jan-18	DE	Delaware River	908
Abr-01049	9-Oct-18	NY	Hudson River	880
Abr-01050	17-Apr-14	NY	Hudson River	674
Abr-01055	3-Jul-19	NY	Hudson River	706
Abr-01063	4-Dec-17	ME	Penobscot River	738
Abr-01066	24-Sep-13	ME	Penobscot River	1145
Abr-01067	28-Sep-21	SC	Waccamaw River	851
Abr-01073	19-May-21	SC	Edisto River	793
Abr-01074	30-Jul-21	SC	Edisto River	730
Abr-01076	13-May-21	SC	Waccamaw River	1011

Table 5. Multiplex panel, locus, repeat motif, fluorescent dye, number of alleles, allelic size range, and primer concentration (µM) for 16 microsatellite loci for Shortnose Sturgeon. The *AllWSex2* sex-specific marker was incorporated into Multiplex Panel 1. Number of alleles and size range are included from the final screening (Table 4) and the 200 samples from SC populations in the Savannah River and Winyah Bay. [D2=black; D3=green; D4=blue]

Multiplex panel	Locus	Repeat motif	WellRED dye	Number of alleles from final screen	Allelic size range (base pairs) from final screen	Number of alleles from SC pops.	Allelic size range (base pairs) from SC pops.	Primer concentration (µM)	Final status
1	ABR234	TTTA	D2	4	105 – 131	3	105 – 117	0.08	Included
	ABR116	TGTACT	D4	3	110 – 123	5	85 – 121	0.08	Included
	ABR663	AATGG	D3	10	133 – 210	--	--	0.04	Dropped (polysomic)
	ABR565	AAAT	D2	4	166 – 194	3	163 – 175	0.08	Included
	ABR453	AGG	D4	4	176 – 188	1	187	0.01	Dropped (fixed)
	AllWSex2	--	D3	--	--	--	--	0.05	Included
2	ABR437	AGG	D4	3	115 – 134	4	116 – 134	0.02	Included
	ABR541	ACAG	D3	7	121 – 339	4	119 – 135	0.10	Included
	ABR576	ACAG	D2	4	190 – 272	5	191 – 235	0.07	Included
	ABR695	ATATC	D4	4	190 – 202	3	192 – 202	0.02	Included
	ABR182	ATAA	D4	7	242 – 324	--	--	0.08	Dropped (poor amplification)
3	ABR670	ACAGT	D4	3	145 – 156	4	146 – 161	0.02	Included
	ABR557	AAAC	D3	4	156 – 162	4	158 – 182	0.05	Included
	ABR449	AAT	D2	5	164 – 177	8	134 – 185	0.07	Included
	ABR075	AACAA	D4	2	178 – 187	5	179 – 209	0.08	Included
	ABR143	TATG	D3	6	182 – 238	6	199 – 239	0.07	Included
	ABR202	TCTGTC	D2	4	213 – 226	3	214 – 226	0.30	Included

Objective 3: Genetic Assessment of Two Shortnose Sturgeon Populations

Our original objective included genotyping 100 samples each from the Santee-Cooper River System and Winyah Bay populations of Shortnose Sturgeon. Upon discussion with project collaborator William Post (SCDNR Diadromous Fishes Section Manager), it was determined that it would be more beneficial to genotype fish from the Savannah River instead of the Santee-Cooper River System to help inform other management concerns. Therefore, 100 samples each were identified from the Population Genetics Tissue Collection from the Savannah River and Winyah Bay populations. These samples were collected from 2004 – 2022 and included adults and juveniles, many of which had corresponding telemetry data to maximize the information gained from this genotyping effort (Appendix Table A1). There were 9 duplicate samples unintentionally included in the sample set from Winyah Bay. After identification, we used these duplicates as internal controls to assess genotyping accuracy and verify that genotyping the same individual more than once would result in the same genotype. Therefore, a total of 91 individual fish from Winyah Bay were included in genotyping efforts.

After initial genotyping, we dropped 3 of the 16 microsatellite loci (Table 5) from further consideration and analysis in this dataset. LocusABR663 exhibited substantial polysomy, ABR453 had only a single fixed allele, and ABR182 exhibited very poor amplification. Although these three loci did not work well for the current dataset, it may be worth evaluating these markers (especially ABR453) more extensively in a more geographically diverse sample set in the future.

Locus validation of the microsatellite panel was conducted by testing for adherence to Hardy-Weinberg equilibrium (HWE), linkage disequilibrium, and the presence of genotyping artifacts. Examinations for departures from HWE and linkage disequilibrium between locus pairs were performed using the program GENEPOP 4.7.2 (Raymond and Rousset 1995) with default parameters. Departures from HWE were also estimated using CERVUS 3.0.7 (Kalinowski et al. 2007). The frequency of null alleles at each locus was evaluated in GENEPOP. Significance levels for all simultaneous analyses were adjusted using a sequential Bonferroni correction (Holm 1979, Rice 1989). The combined non-exclusion identity probability of the microsatellite panel was calculated in CERVUS.

To estimate the degree of genetic differentiation and patterns of gene flow between the Winyah Bay and Savannah River populations, we evaluated the spatial genetic variation between the rivers with pair-wise comparisons of R_{ST} in ARLEQUIN and exact tests for allelic (genic) distributions with GENEPOP using default parameters. The Bayesian clustering program *Structure* 2.3.4 (Pritchard et al. 2000) was used to observe individual genetic clustering over a range of populations (*K*), 1 – 5. Run parameters were set at 10,000 burn-in repetitions followed by 100,000 Markov chain Monte-Carlo repetitions, with and without location information included as a prior. Basic molecular diversity indices were calculated for each river system using

ARLEQUIN and GenAlEx 6.5 (Peakall and Smouse 2006, 2012), including number of alleles per locus (N_a), genetic diversity (H_o, H_E; Nei 1987), inbreeding coefficient (F_{IS}; Weir and Cockerham 1984), and Garza-Williamson Index (G-W). Effective population size (N_e) was estimated using both long-term and contemporary methods. Long-term estimates were calculated using heterozygosity-based methods (Ohta and Kimura 1973) with both a step-wise mutation model (SMM) and an infinite alleles model (IAM). The SMM predicts that at mutation-drift equilibrium, N_e is represented by [(1/1-HE)2-1]/8μ. Although the SMM is likely the most appropriate algorithm for microsatellite data, it is sensitive to mutational modeling; therefore, N_e was also estimated under the IAM using the equation Ne=H/4μ(1-H). The most commonly used mutation rate in fishes (μ=5x10-4; Estoup and Angers 1998) was used for both model estimations.

Contemporary (parental generation) estimates of N_{eb} (effective number of breeding adults) were estimated for each river system using the single-sample programs LDN_e 1.31 (Waples 2006). As data from multiple cohorts is included, in reality these contemporary estimates represent something in between N_e and N_{eb}. Genetic drift generates non-random associations among unlinked loci; LDN_e analyzes this linkage disequilibrium between a set of loci to determine contemporary N_{eb} for a single time point. Minimal allele frequencies for inclusion were set at default values (0.01, 0.02, and 0.05) assuming a random mating model.

A total of 81 and 57 Shortnose Sturgeon were successfully genotyped from Winyah Bay and the Savannah River, respectively, at 10 or more microsatellite loci. These sample sizes increase to 97 and 79 samples, respectively, when considering genotyping at 7 or more microsatellite loci. The genotypes of all duplicate samples within the Winyah Bay data set matched 100%; one set of duplicates was removed for subsequent analyses. Within each sample set, locus validation analyses resulted in four departures from HWE equilibrium that were consistent by locus across both populations and the combined dataset (Table 6), and none of the 78 pairwise comparisons indicated deviations from linkage disequilibrium (Table 7). Null allele frequencies ranged 0 – 0.83 across the locus panel, with only five having allele frequencies >0.02 (Table 6). Although deviations from HWE and the presence of null alleles are not optimal for a genetic panel, the processes associated with a severe population bottleneck followed by population recovery could also produce similar metrics that are independent of selective processes (HWE) or non-amplifying alleles. Given Shortnose Sturgeon was listed as endangered in 1973 and has been afforded protection under the Endangered Species Act since that time, we have chosen to retain all 13 loci in the final microsatellite panel for analyses of our current data sets. Further research into the potential influence of these mechanisms on the new genetic panel would be beneficial, and additional sample processing across a wider geographic range is highly recommended prior to interpretation of genetic data for management and conservation purposes. The combined non-exclusion identity probability for the new marker panel was high (7.82×10^{-6}) suggesting there is sufficient power to identify individual Shortnose Sturgeon.

The sex marker accurately assigned sex based on the samples for which phenotypic assignment was available. Overall, there were 78 females, 18 males, and 4 undetermined (positive control amplification failed) fish from Winyah Bay, and 45 females, 43 males, and 12 undetermined from the Savannah River (Table A1). Prior to interpretation of these data as being representative of wild sex ratios, further evaluation of collection efforts should be considered to determine the potential for collection bias in the capture of Shortnose Sturgeon included in the current project.

The Winyah Bay and Savannah River data sets were not significantly genetically different from each other based on allele frequency distributions ($p = 0.34$) and $R_{ST} = 0.004$ (Table 8). The two datasets were similar in terms of allelic diversity (3.5 – 4.0 alleles/locus), genetic diversity (H_e: 0.416 – 0.423), and low levels of inbreeding (< 0.000). The identification of a single population was also supported by the analyses in *Structure*, which indicated that the Shortnose Sturgeon from Winyah Bay and Savannah River were best fit by one genetic cluster. The result is also in agreement with prior genetic studies suggesting variable gene flow patterns within the southeastern U.S. (Quattro et al. 2002; Wirgin et al. 2005 and 2010; King et al. 2014). The lack of differentiation may be explained by the inclusion of both juveniles and adults in the data set, the latter of which are known to inhabit one estuary before making a spawning migration in another (personal communication W. Post, E. Waldrop). A more targeted analysis approach using only juvenile samples that may still be in their natal estuary, would be an ideal way to evaluate the presence of genetic population structure without the confounding influence of subadult/adult movement behaviors. However, given the infrequent capture of juvenile Shortnose Sturgeon, an alternative approach could use only adults with documented spawning migration in specific rivers for genetic evaluation.

Although LDN_e did not produce quantitative estimates of contemporary effective number of breeders for any of the data sets, the consistent negative estimates generated for all default allele frequencies are indicative of 'large' effective sizes (Table 8). In such cases, the lower bounds of the confidence intervals are useful for comparative and conservative evaluation purposes. For all data sets, LDN_e produced a quantitative lower confidence internal using the minimum 0.05 allele frequency. Long-term effective population size estimates ranged from ~300 – 500 for the data set and were notably lower than the lower bound of contemporary estimates for both the Savannah River and combined data set. The G-W Index for all data sets were very low (~0.2), confirming the sampled Shortnose Sturgeon experienced a severe population bottleneck. From a genetic health perspective, collectively these patterns would be consistent with a population of Shortnose Sturgeon that underwent a severe bottleneck historically and are slowly recovering. Supporting evidence includes contemporary N_{eb} estimates showing an increasing trend over long-term N_e estimates, a moderate level of retained genetic diversity, no indication of inbreeding, and low levels of outbreeding detected (negative F_{IS} values).

Table 6. Marker validation statistics for the new microsatellite marker panel for Shortnose Sturgeon. Hardy-Weinberg Equilibrium results for Winyah Bay, Savannah River, and combined datasets as calculated in GENEPOP and CERVUS. Null allele frequency estimated by GENEPOP on the combined dataset.

Locus	Winyah Bay (GENEPOP)	Savannah (GENEPOP)	Combined (GENEPOP)	Combined (CERVUS)	Null Allele frequency
ABR234	Significant	Significant	Significant	Significant	0.00
ABR116			Significant		0.83
ABR565	Significant	Significant	Significant	Significant	0.00
ABR437	Significant	Significant	Significant	Significant	0.00
ABR541	Significant	Significant	Significant		0.22
ABR576					0.00
ABR695					0.22
ABR670					0.00
ABR557					0.00
ABR449	Significant		Significant		0.22
ABR075	Significant	Significant	Significant	Significant	0.02
ABR143			Significant		0.01
ABR202					0.44
# Samples	89	97	186	186	186

Table 7. Linkage disequilibrium results for Shortnose Sturgeon microsatellites tested by GENEPOP. No values were statistically significant after sequential Bonferroni corrections.

Locus 1	Locus 2	p-value	Locus 1	Locus 2	p-value	Locus 1	Locus 2	p-value
ABR075	ABR143	0.181	ABR437	ABR143	0.112	ABR565	ABR557	0.194
ABR075	ABR202	0.597	ABR437	ABR557	0.184	ABR565	ABR670	0.208
ABR116	ABR695	0.062	ABR437	ABR576	0.281	ABR565	ABR075	0.221
ABR116	ABR541	0.130	ABR437	ABR075	0.493	ABR565	ABR695	0.289
ABR116	ABR576	0.191	ABR437	ABR541	0.790	ABR565	ABR541	0.416
ABR116	ABR670	0.239	ABR437	ABR202	0.835	ABR565	ABR449	0.498
ABR116	ABR565	0.346	ABR437	ABR449	0.928	ABR565	ABR143	0.553
ABR116	ABR557	0.361	ABR437	ABR670	0.929	ABR565	ABR576	0.589
ABR116	ABR075	0.654	ABR437	ABR695	0.978	ABR576	ABR075	0.092
ABR116	ABR202	0.669	ABR449	ABR075	0.342	ABR576	ABR449	0.306
ABR116	ABR437	0.715	ABR449	ABR143	0.457	ABR576	ABR202	0.318
ABR116	ABR143	0.770	ABR449	ABR202	0.483	ABR576	ABR557	0.466
ABR116	ABR449	0.773	ABR541	ABR143	0.021	ABR576	ABR143	0.612
ABR143	ABR202	0.545	ABR541	ABR557	0.073	ABR576	ABR695	0.710
ABR234	ABR541	0.050	ABR541	ABR576	0.123	ABR576	ABR670	0.904
ABR234	ABR143	0.118	ABR541	ABR449	0.444	ABR670	ABR202	0.036
ABR234	ABR565	0.136	ABR541	ABR670	0.611	ABR670	ABR557	0.049
ABR234	ABR075	0.153	ABR541	ABR202	0.622	ABR670	ABR075	0.206
ABR234	ABR116	0.232	ABR541	ABR695	0.729	ABR670	ABR143	0.507
ABR234	ABR695	0.276	ABR541	ABR075	0.732	ABR670	ABR449	0.512
ABR234	ABR449	0.361	ABR557	ABR075	0.079	ABR695	ABR202	0.153
ABR234	ABR202	0.688	ABR557	ABR143	0.093	ABR695	ABR075	0.190
ABR234	ABR576	0.708	ABR557	ABR202	0.701	ABR695	ABR670	0.388
ABR234	ABR557	0.754	ABR557	ABR449	0.771	ABR695	ABR449	0.459
ABR234	ABR670	0.813	ABR565	ABR202	0.089	ABR695	ABR143	0.477
ABR234	ABR437	0.927	ABR565	ABR437	0.100	ABR695	ABR557	0.833

Table 8. Molecular diversity metrics for Shortnose Sturgeon captured in Winyah Bay and the Savannah River using the final microsatellite panel. The reported R_{ST} is not statistically significant (p = 0.34) when calculated with 999 permutations in GenAlEx (same value and non-significance was also found in Arlequin and GENEPOP). All LDN_e estimates were negative, indicating a large effective population size; parametric confidence intervals are reported for 0.05 lowest allele frequencies.

Population	H_o	H_E	N_a	F_{IS}	GW	R_{ST}	Long Term N_e		Contemporary N_e
							SMM	IAM	LDN_e CI
Winyah Bay	0.502	0.423	3.5	-0.154	0.21	0.004	502	367	332.6 – ∞
Savannah	0.500	0.416	4.0	-0.161	0.17		467	347	581.8 – ∞
Combined	0.501	0.417	4.4	-0.157	0.19	--	484	357	543.8 – ∞

H_o observed heterozygosity
H_E expected heterozygosity
N_a number of alleles
F_{IS} inbreeding coefficient
GW Garza-Williamson modified Index
R_{ST} fixation index

Conclusions – Following the screening of 329 microsatellite loci from the Sterlet Sturgeon genome and an additional 335 loci from the Shortnose Sturgeon genome, we identified a total of 16 loci that appeared disomic for Shortnose Sturgeon. These loci were multiplexed with the newly developed sex marker into a genetic panel for Shortnose Sturgeon that was tested for validation in both the Winyah Bay and Savannah River populations. Project results suggest that our final 13 locus microsatellite panel should be useful for genetically characterizing populations of Shortnose Sturgeon, although more research is needed to clarify underlying causes of the observed deviations from HWE and putative presence of null alleles which is highly recommended prior to interpretation of genetic data for management and conservation purposes. These patterns would be expected in populations that experienced a severe historic bottleneck, which is consistent with our observed patterns in long-term N_e, contemporary N_{eb}, and G-W estimates. We did not detect genetic differences between the Winyah Bay and Savannah River data sets with the new marker panel. The lack of detected population structure may be attributable to a combination of the life history of Shortnose Sturgeon (i.e. movement between rivers) and capture biases associated with collection. As these samples covered a range of years and life stages, they possibly represented a combination of individual fish from different populations; although the limited sample size used in our evaluation here was not able to detect unique genetic groups within the combined data set. From a genetic health perspective, project results would be consistent with a population of Shortnose Sturgeon that underwent a severe bottleneck historically and are slowly recovering. Supporting evidence includes contemporary N_{eb} estimates showing an increasing trend over long-term N_e estimates,

a moderate level of retained genetic diversity, no indication of inbreeding, and low levels of outbreeding detected. As we further test and begin to implement our new microsatellite panel on additional samples, we will continue to optimize the balance of primer ratios and reaction conditions to maximize the panel's efficiency. We anticipate the number of observed alleles and allelic range to expand with the inclusion of higher sample sizes across their geographic range (see Table 5). Although further validation is needed, we are highly optimistic that the new genetic tool will provide important information for future conservation and monitoring of Shortnose Sturgeon throughout their range.

Significant deviations – The COVID-19 pandemic and associated logistical issues substantially impacted our project activities during the first year, which led to a shifting of our approach and timeline but no modifications to our project objectives. As a potential alternative approach, we began testing microsatellites identified in the Sterlet Sturgeon genome; however, following completion of the screening of these putative markers, we decided to revert to our initial workplan of identifying/testing microsatellites generated from whole genome sequencing of the Shortnose Sturgeon genome to increase the power of the final marker panel. We also attempted to incorporate new sequencing technologies to improve screening efficiencies, but further work developing those analysis pipelines is needed before it can be reliably used in tool development.

Estimated Federal Cost – The entire project budget ($234,943) has been expended to complete project activities.

Recommendations – The grant has been completed; close the grant.

Prepared by – Daniel Farrae and Tanya Darden

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Literature Cited

Bemis W, Kynard B (1997) Sturgeon rivers: an introduction to Acipensiform biogeography and life history. Environmental Biology of Fishes 48: 167–183.

Dionne P, Zydlewski G, Kinnison M, Zydlewski J, Wippelhauser G (2013) Reconsidering residency: characterization and conservation implications of complex migratory patterns of shortnose sturgeon (*Acipenser brevirostrum*). Canadian Journal of Fisheries and Aquatic Sciences 70(1): 119–127.

Duckett D (2016) Influences of statistical power on studies of population genetic structure and empirical population genetic structure analysis of the shortfin mako (*Isurus oxyrinchus*). College of Charleston Masters Thesis, 68 pp.

Du K, Stöck M, Kneitz S, Klopp C, Woltering JM, Adolfi MC, Feron R, Prokopov D, Makunin A, Kichigin I, Schmidt C (2020) The sterlet sturgeon genome sequence and the mechanisms of segmental rediploidization. Nature Ecology & Evolution 4:841-52.

Estoup P, Angers B (1998) Microsatellites and minisatellites for molecular ecology: theoretical and empirical considerations. In: Advances in Molecular Ecology (ed. Carvlho, G.) pp. 55-86. NATO Science Series, IOS Press, Amsterdam.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47-50. http://cmpg.unibe.ch/software/arlequin3/

Faircloth B (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Molecular Ecology Resources 8: 92–94.

Flanagan S, Jones A (2019) The future of parentage analysis: from microsatellites to SNPs and beyond. Molecular Ecology 28(3): 544-567.

Fontana F, Congiu L, Mudrak V, Quattro J, Smith T, Ware K, Doroshov S (2008) Evidence of hexaploid karyotype in Shortnose Sturgeon. Genome 51: 113–119.

Gaynor K, Solomon J, Siller S, Jessell L, Duffy J, Rubenstein D (2017) Development of genome- and transcriptome-derived microsatellites in related species of snapping shrimps with highly duplicated genomes. Molecular Ecology Resources 17: e160–e173.

Gravely M, Sage G, Talbot S, Carlson M (2018) Development and characterization of 12 polymorphic microsatellite loci in the sea sandwort, *Honckenya peploides*. Journal of Plant Research 131: 879–885.

Havelka M, Hulák M, Bailie D, Prodöhl P, Flajšhans M (2013) Extensive genome duplications in sturgeons: new evidence from microsatellite data. Journal of Applied Ichthyology 29: 704–708.

Henderson A, King T (2012) Development of polysomic microsatellite markers for characterization of population structuring and phylogeography in the Shortnose Sturgeon (*Acipenser brevirostrum*). Conservation Genetics Resources 4(4): 853–859.

Holm S (1979) A simple sequential rejective multiple test procedure. Scandinavian Journal of Statistics 6:65–70.

Kalinowski S, Taper M, Marshall T (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099-1006.

King T, Henderson A, Kynard B, Kieffer M, Peterson D, Aunins A, Brown B (2014) A Nuclear DNA Perspective on Delineating Evolutionarily Significant Lineages in Polyploids: The Case of the Endangered Shortnose Sturgeon (*Acipenser brevirostrum*). PLoS ONE 9(8): e102784. doi:10.1371/journal.pone.0102784

Krieger J, Hett A, Fuerst P, Artyukhin E, Ludwig A (2008) The molecular phylogeny of the order Aciepnseriformes revisited. Journal of Applied Ichthyology 24(Suppl. 1): 36–45.

Kuhl H, Guiguen Y, Höhne C, Kreuz E, Du K, Klopp C, Lopez-Roques C, Yebra-Pimentel ES, Ciorpac M, Gessner J, Holostenco D (2021) A 180 Myr-old female-specific genome region in sturgeon reveals the oldest known vertebrate sex determining system with undifferentiated sex chromosomes. Philosophical Transactions of the Royal Society B 376: 20200089.

Kynard B (1997) Life history, latitudinal patterns, and status of the shortnose sturgeon (*Acipenser brevirostrum)*. Environmental Biology of Fishes 48: 319–334.

Kynard B, Bolden S, Kieffer M, Collins M, Brundage H, Hilton E, Litvak M, Kinnison M, King T, Peterson D (2016) Life history and status of Shortnose Sturgeon (*Acipenser brevirostrum* LeSueur, 1818). Journal of Applied Ichthyology 32(suppl. 1): 208–248.

NMFS (1998) Final recovery plan for the Shortnose Sturgeon, *Acipenser brevirostrum*. Prepared by the Shortnose Sturgeon Recovery Team for National Marine Fisheries Service, 104pg.

Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York, NY, USA.

Ohta J, Kimura M (1973) A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genetic Research 22:201-204.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295.

Peakall R, Smouse PE (2012) GENALEX 6.5: genetic analysis in Excel. Population genetic software for teaching and research- an update. Bioinformatics 28: 2537–2539.

Peterson D, Farrae D (2011) Evidence of metapopulation dynamics in Shortnose Sturgeon in the southern part of their range. Transactions of the American Fisheries Society 140(6): 1540–1546.

Post W, Darden T, Peterson D, Loeffler M, Collier C (2014) Research and management of endangered and threatened species in the Southeast: riverine movements of shortnose and Atlantic sturgeon. Final Report to NMFS.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959.Rajkov J, Shao Z, Berrebi P (2014) Evolution of polyploidy and functional diploidization in sturgeons: microsatellite analysis in 10 sturgeon species. Journal of Heredity 105(4): 521–531.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86(3):248–249.

Rice W (1989) Analyzing tables of statistical tests. Evolution 43(1):223–225.

Sard NM, Kreiser BR, Pendleton RM, Lubinski BA, Johnson RL, Fox DA, Van Eenennaam JP, Kahn JE, Hager C, Higgs AL, Kazyak DC (2024) Validation of a molecular sex marker in three sturgeons from eastern North America. Conservation Genetics Resources 16 173–177.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18:233–234.

Quattro J, Greig T, Cokendall D, Bowen B, Baldwin J (2002) Genetic issues in aquatic species management: the Shortnose Sturgeon (*Acipenser brevirostrum*) in the southeastern United States. Conservation Genetics 3(2):155–166.

Walsh M, Bain M, Squiers T, Waldman J, Wirgin I (2001) Morphological and genetic variation among Shortnose Sturgeon *Acipenser brevirostrum* from adjacent and distant rivers. Estuaries 24(1):41–48.

Waples R (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conservation Genetics 7:167-184.

Welsh A, May B (2006) Development and standardization of disomic microsatellite markers for lake sturgeon genetic studies. Journal of Applied Ichthyology 22:337–344.

Weir B, Cockerham C (1984) Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.

Wippelhauser G, Zydlewski G, Kieffer M, Sulikowski J, Kinnison M (2015) Shortnose Sturgeon in the Gulf of Maine: Use of Spawning Habitat in the Kennebec System and Response to Dam Removal, Transactions of the American Fisheries Society, 144(4):742-752.

Wirgin I, Grunwald C, Carlson E, Stabile J, Peterson D, Waldman J (2005) Range-wide population structure of shortnose sturgeon (Acipenser brevirostrum based on sequence analysis of the mitochondrial DNA control region. Estuaries 28(3):406-421.

Wirgin I, Grunwald C, Stabile J, Waldman J (2010) Delineation of discrete population segments of Shortnose Sturgeon *Acipenser brevirostrum* based on mitochondrial DNA control region sequence analysis. Conservation Genetics 11(3):689–708.

Vladykov V, Greeley J (1963) Order Acipenseroidei, Pgs 24–60 *in* Olsen VH (editor) Fishes of the western North Atlantic, part III. Memoirs of the Sears Foundation for Marine Research, New Haven, CT.

Appendix

Table A1. Shortnose sturgeon samples genotyped from the Savannah River and Winyah Bay populations. *indicates duplicate samples

Genetic ID	Collection ID	Collection Date	State	River	Total Length (mm)	Population	Molecular Sex
Abr-00693	MS-246	5-Nov-13	SC	Savannah	501	Savannah	Male
Abr-00705	MS-285	7-Mar-14	SC	Savannah	1196	Savannah	Female
Abr-00707	MS-288	14-Mar-14	SC	Savannah	1065	Savannah	Female
Abr-00714	SH11	17-Dec-14	SC	Savannah	843	Savannah	Male
Abr-00715	SH12	17-Dec-14	SC	Savannah	815	Savannah	Male
Abr-00716	SH13	17-Dec-14	SC	Savannah	860	Savannah	Male
Abr-00717	SH14	17-Dec-14	SC	Savannah	1066	Savannah	Female
Abr-00725	SH23	13-Feb-15	SC	Savannah	1170	Savannah	Female
Abr-00726	SH24	13-Feb-15	SC	Savannah	1150	Savannah	Female
Abr-00731	SH32	6-Mar-15	SC	Savannah	584	Savannah	Female
Abr-00732	SH34	20-Mar-15	SC	Savannah	1040	Savannah	Female
Abr-00736	SH39	20-Mar-15	SC	Savannah	548	Savannah	Male
Abr-00741	SH47	2-Jun-15	SC	Savannah	975	Savannah	Female
Abr-00747	SH67	6-Aug-15	SC	Savannah	1010	Savannah	Female
Abr-00750	SH8	11-Nov-14	SC	Savannah	915	Savannah	Male
Abr-00751	SH82	22-Sep-15	SC	Savannah	875	Savannah	Female
Abr-00752	SH84	22-Sep-15	SC	Savannah	803	Savannah	Female
Abr-00753	SH86	22-Sep-15	SC	Savannah	860	Savannah	Unknown
Abr-00754	SH9	11-Nov-14	SC	Savannah	423	Savannah	Unknown
Abr-00755	SH91	5-Nov-15	SC	Savannah	841	Savannah	Male
Abr-00760	SH-103	3-Dec-15	SC	Savannah	911	Savannah	Female
Abr-00769	SH-146	17-Jun-16	SC	Savannah	463	Savannah	Female
Abr-00770	SH-160	21-Jun-16	SC	Savannah	551	Savannah	Male
Abr-00771	SH-161	21-Jun-16	SC	Savannah	500	Savannah	Male
Abr-00772	SH-169	22-Jun-16	SC	Savannah	512	Savannah	Female
Abr-00773	SH-172	27-Jun-16	SC	Savannah	430	Savannah	Male
Abr-00774	SH-183	9-Nov-16	SC	Savannah	899	Savannah	Male
Abr-00775	SH-184	9-Nov-16	SC	Savannah	1008	Savannah	Female
Abr-00776	SH-187	30-Nov-16	SC	Savannah	885	Savannah	Male
Abr-00778	Sh-189	30-Nov-16	SC	Savannah	872	Savannah	Male
Abr-00779	Sh-190	30-Nov-16	SC	Savannah	921	Savannah	Male
Abr-00850	SH-202	8-Dec-16	SC	Savannah	1048	Savannah	Male
Abr-00851	SH-204	8-Dec-16	SC	Savannah	667	Savannah	Male
Abr-00852	SH-205	8-Dec-16	SC	Savannah	1002	Savannah	Female
Abr-00866	SH-240	25-Apr-17	SC	Savannah	627	Savannah	Male
Abr-00867	SH-241	25-Apr-17	SC	Savannah	433	Savannah	Unknown
Abr-00868	SH-242	25-Apr-17	SC	Savannah	436	Savannah	Unknown
Abr-00869	SH-243	25-Apr-17	SC	Savannah	422	Savannah	Male
Abr-00870	SH-245	25-Apr-17	SC	Savannah	407	Savannah	Male
Abr-00871	SH-246	25-Apr-17	SC	Savannah	413	Savannah	Unknown
Abr-00872	SH-247	25-Apr-17	SC	Savannah	376	Savannah	Unknown
Abr-00873	SH-250	2-May-17	SC	Savannah	1021	Savannah	Female
Abr-00877	SH-254	2-May-17	SC	Savannah	446	Savannah	Unknown
Abr-00878	SH-261	2-May-17	SC	Savannah	409	Savannah	Unknown
Abr-00879	SH-263	2-May-17	SC	Savannah	424	Savannah	Female

Abr-00880	SH-266	2-May-17	SC	Savannah	405	Savannah	Unknown
Abr-00888	SH-281	27-Jun-17	SC	Savannah	1039	Savannah	Female
Abr-00889	SH-283	8-Aug-17	SC	Savannah	1040	Savannah	Female
Abr-00890	SH-285	26-Oct-17	SC	Savannah	1004	Savannah	Female
Abr-00893	SH-290	9-Jan-18	SC	Savannah	909	Savannah	Female
Abr-00894	SH-291	9-Jan-18	SC	Savannah	613	Savannah	Unknown
Abr-00895	SH-292	1-Feb-18	SC	Savannah	933	Savannah	Female
Abr-00900	SH-300	23-Feb-18	SC	Savannah	910	Savannah	Female
Abr-00961	SH-308	6-Apr-18	SC	Savannah	515	Savannah	Male
Abr-00963	SH-310	6-Apr-18	SC	Savannah	648	Savannah	Female
Abr-00964	SH-311	6-Apr-18	SC	Savannah	883	Savannah	Male
Abr-00965	SH-313	17-Apr-18	SC	Savannah	701	Savannah	Male
Abr-00967	SH-333	30-May-18	SC	Savannah	560	Savannah	Male
Abr-00969	SH-350	12-Jul-18	SC	Savannah	565	Savannah	Male
Abr-00970	SH-361	18-Jul-18	SC	Savannah	701	Savannah	Female
Abr-00973	SH-364	31-Aug-18	SC	Savannah	1018	Savannah	Female
Abr-00975	SH-377	7-Dec-18	SC	Savannah	807	Savannah	Male
Abr-00976	SH-378	11-Dec-18	SC	Savannah	1010	Savannah	Female
Abr-00985	SH-387	11-Dec-18	SC	Savannah	1088	Savannah	Female
Abr-00986	SH-388	11-Dec-18	SC	Savannah	980	Savannah	Female
Abr-00987	SH-389	8-Jan-19	SC	Savannah	1100	Savannah	Female
Abr-00988	SH-390	8-Jan-19	SC	Savannah	894	Savannah	Male
Abr-00989	SH-391	8-Jan-19	SC	Savannah	1093	Savannah	Female
Abr-00996	SH-409	25-Apr-19	SC	Savannah	591	Savannah	Male
Abr-00997	SH-422	6-May-19	SC	Savannah	557	Savannah	Male
Abr-00998	SH-423	6-May-19	SC	Savannah	575	Savannah	Female
Abr-00999	SH-440	13-May-19	SC	Savannah	429	Savannah	Unknown
Abr-01000	SH-441	13-May-19	SC	Savannah	454	Savannah	Unknown
Abr-01001	SH-442	13-May-19	SC	Savannah	631	Savannah	Male
Abr-01004	SH-471	21-Oct-19	SC	Savannah	1085	Savannah	Female
Abr-01005	SH-474	31-Oct-19	SC	Savannah	1024	Savannah	Female
Abr-01008	SH-477	31-Oct-19	SC	Savannah	971	Savannah	Male
Abr-01010	SH-479	1-Nov-19	SC	Savannah	837	Savannah	Female
Abr-01013	SH-493	16-Dec-19	SC	Savannah	975	Savannah	Female
Abr-01014	SH-499	29-Jan-20	SC	Savannah	382	Savannah	Male
Abr-01015	SH-500	29-Jan-20	SC	Savannah	375	Savannah	Female
Abr-01222	SH-501	29-Jan-20	SC	Savannah	388	Savannah	Male
Abr-01223	SH-502	29-Jan-20	SC	Savannah	410	Savannah	Male
Abr-01224	SH-503	30-Jan-20	SC	Savannah	413	Savannah	Female
Abr-01225	SH-509	16-Nov-20	SC	Savannah	900	Savannah	Female
Abr-01226	SH-512	2-Dec-20	SC	Savannah	706	Savannah	Male
Abr-01227	SH-513	2-Dec-20	SC	Savannah	821	Savannah	Female
Abr-01228	SH-515	2-Dec-20	SC	Savannah	735	Savannah	Male
Abr-01229	SH-516	4-Dec-20	SC	Savannah	676	Savannah	Male
Abr-01230	SH-524	16-Apr-21	SC	Savannah	576	Savannah	Male
Abr-01231	SH-525	16-Apr-21	SC	Savannah	421	Savannah	Female
Abr-01232	SH-526	16-Apr-21	SC	Savannah	493	Savannah	Female
Abr-01233	SH-527	19-Apr-21	SC	Savannah	693	Savannah	Female
Abr-01241	SH-570	12-May-21	SC	Savannah	628	Savannah	Male
Abr-01243	SH-573	25-May-21	SC	Savannah	1034	Savannah	Female
Abr-01246	SH-578	19-Oct-21	SC	Savannah	824	Savannah	Male
Abr-01248	SH-581	20-Oct-21	SC	Savannah	878	Savannah	Male
Abr-01249	SH-591	26-Oct-21	SC	Savannah	1022	Savannah	Male

Abr-01250	SH-595	27-Oct-21	SC	Savannah	858	Savannah	Male
Abr-01251	SH-596	27-Oct-21	SC	Savannah	963	Savannah	Male
Abr-00072	K-906	16-Mar-04	SC	Waccamaw	1012	Winyah Bay	Female
Abr-00073	K-908	27-Apr-04	SC	Waccamaw	517	Winyah Bay	Unknown
Abr-00100	MS-124	21-Apr-11	SC	Great Pee Dee	1025	Winyah Bay	Unknown
Abr-00101	MS-125	21-Apr-11	SC	Great Pee Dee	888	Winyah Bay	Male
Abr-00102	MS-055	14-Apr-11	SC	Waccamaw	1005	Winyah Bay	Female
Abr-00103	MS-026	6-Apr-11	SC	Waccamaw	916	Winyah Bay	Unknown
Abr-00104	MS-135	24-May-11	SC	Great Pee Dee	956	Winyah Bay	Male
Abr-00796	X411	26-Feb-18	SC	Waccamaw	1044	Winyah Bay	Female
Abr-00801	PD010	22-Jun-17	SC	Waccamaw	903	Winyah Bay	Female
Abr-00802	PD011	22-Jun-17	SC	Waccamaw	973	Winyah Bay	Female
Abr-00803	PD012	22-Jun-17	SC	Waccamaw	960	Winyah Bay	Female
Abr-00804	PD013	22-Jun-17	SC	Waccamaw	957	Winyah Bay	Female
Abr-00805	PD014	22-Jun-17	SC	Waccamaw	946	Winyah Bay	Male
Abr-00806	PD020	22-Jun-17	SC	Waccamaw	974	Winyah Bay	Female
Abr-00807	PD021	27-Jun-17	SC	Waccamaw	1031	Winyah Bay	Female
Abr-00808	PD022	27-Jun-17	SC	Waccamaw	945	Winyah Bay	Female
Abr-00809	PD023	28-Jun-17	SC	Waccamaw	943	Winyah Bay	Female
Abr-00810	PD024	28-Jun-17	SC	Waccamaw	947	Winyah Bay	Female
Abr-00811	PD025	28-Jun-17	SC	Waccamaw	914	Winyah Bay	Male
Abr-00812	PD027	28-Jun-17	SC	Waccamaw	1004	Winyah Bay	Female
Abr-00813	PD029	28-Jun-17	SC	Waccamaw	822	Winyah Bay	Female
Abr-00814	PD030	28-Jun-17	SC	Waccamaw	855	Winyah Bay	Female
Abr-00815	PD031	28-Jun-17	SC	Waccamaw	768	Winyah Bay	Female
Abr-00816	C-055	27-Jun-17	SC	Great Pee Dee	1080	Winyah Bay	Unknown
Abr-00817	PD075	6-Mar-18	SC	Great Pee Dee	934	Winyah Bay	Male
Abr-00818	PD103	2-May-18	SC	Waccamaw	799	Winyah Bay	Female
Abr-00819	PD107	29-May-18	SC	Waccamaw	735	Winyah Bay	Female
Abr-00820	PD108	29-May-18	SC	Waccamaw	830	Winyah Bay	Male
Abr-00821	PD109	29-May-18	SC	Waccamaw	950	Winyah Bay	Female
Abr-00822	PD111	30-May-18	SC	Waccamaw	743	Winyah Bay	Male
Abr-00823	PD112	30-May-18	SC	Waccamaw	775	Winyah Bay	Female
Abr-00824	PD115	30-May-18	SC	Waccamaw	795	Winyah Bay	Female
Abr-00825	PD120	4-Jun-18	SC	Waccamaw	912	Winyah Bay	Male
Abr-00826	PD121	5-Jun-18	SC	Waccamaw	937	Winyah Bay	Female
Abr-00827	PD122	6-Jun-18	SC	Waccamaw	805	Winyah Bay	Female
Abr-00828	PD123	11-Jun-18	SC	Waccamaw	942	Winyah Bay	Female
Abr-00829	PD126	12-Jun-18	SC	Waccamaw	1050	Winyah Bay	Female
Abr-00830	PD128	13-Jun-18	SC	Waccamaw	841	Winyah Bay	Female
Abr-00831	PD129	13-Jun-18	SC	Waccamaw	866	Winyah Bay	Female
Abr-00832	PD130	13-Jun-18	SC	Waccamaw	975	Winyah Bay	Female
Abr-00833	PD132	14-Jun-18	SC	Waccamaw	872	Winyah Bay	Male
Abr-00834	PD136	14-Jun-18	SC	Waccamaw	930	Winyah Bay	Female
Abr-00835	PD141	28-Aug-18	SC	Waccamaw	791	Winyah Bay	Female
Abr-00836	PD156	28-Aug-18	SC	Waccamaw	1026	Winyah Bay	Female
Abr-00837	PD157	28-Aug-18	SC	Waccamaw	1070	Winyah Bay	Female
Abr-00838	PD158	28-Aug-18	SC	Waccamaw	1009	Winyah Bay	Female
Abr-00839	PD161	30-Aug-18	SC	Waccamaw	870	Winyah Bay	Female
Abr-00840	PD166	4-Sep-18	SC	Waccamaw	960	Winyah Bay	Female
Abr-00841	SM040	17-Apr-17	SC	Waccamaw	954	Winyah Bay	Female
Abr-00842	SM066	24-May-17	SC	Waccamaw	1230	Winyah Bay	Female
Abr-00843	SM086	8-Jun-17	SC	Waccamaw	975	Winyah Bay	Female

Abr-00844	SM093	13-Jun-17	SC	Waccamaw	941	Winyah Bay	Female
Abr-00845	SM094	13-Jun-17	SC	Waccamaw	957	Winyah Bay	Male
Abr-00846	SM095	13-Jun-17	SC	Waccamaw	1051	Winyah Bay	Female
Abr-00847	SM099	14-Jun-17	SC	Waccamaw	948	Winyah Bay	Female
Abr-00848	SM100	14-Jun-17	SC	Waccamaw	1004	Winyah Bay	Female
Abr-00902	PD172	4-Sep-18	SC	Waccamaw	970	Winyah Bay	Female
Abr-00903	PD219	6-Jun-19	SC	Waccamaw	811	Winyah Bay	Female
Abr-00904	PD220	6-Jun-19	SC	Waccamaw	900	Winyah Bay	Female
Abr-00905	PD221	6-Jun-19	SC	Waccamaw	1066	Winyah Bay	Male
Abr-00906	PD222	6-Jun-19	SC	Waccamaw	1044	Winyah Bay	Female
Abr-00907	PD227	7-Jun-19	SC	Waccamaw	1012	Winyah Bay	Female
Abr-00908	PD228	7-Jun-19	SC	Waccamaw	971	Winyah Bay	Female
Abr-00909	PD229	7-Jun-19	SC	Waccamaw	1126	Winyah Bay	Female
Abr-00910	PD230	7-Jun-19	SC	Waccamaw	995	Winyah Bay	Female
Abr-00911	PD231	7-Jun-19	SC	Waccamaw	962	Winyah Bay	Female
Abr-00912	PD232	7-Jun-19	SC	Waccamaw	789	Winyah Bay	Female
Abr-00913	PD239	9-Jun-19	SC	Waccamaw	1094	Winyah Bay	Female
Abr-00914	PD253	10-Jun-19	SC	Waccamaw	1041	Winyah Bay	Female
Abr-00915	PD254	10-Jun-19	SC	Waccamaw	884	Winyah Bay	Female
Abr-00916	PD255	10-Jun-19	SC	Waccamaw	781	Winyah Bay	Male
Abr-00917	PD259	19-Jun-19	SC	Waccamaw	941	Winyah Bay	Female
Abr-00918	PD260	19-Jun-19	SC	Waccamaw	859	Winyah Bay	Female
Abr-00919	PD269	24-Jun-19	SC	Waccamaw	800	Winyah Bay	Male
Abr-00920	PD270	24-Jun-19	SC	Waccamaw	941	Winyah Bay	Female
Abr-00921	PD271	24-Jun-19	SC	Waccamaw	869	Winyah Bay	Female
Abr-00922	PD274	24-Jun-19	SC	Waccamaw	790	Winyah Bay	Female
Abr-00923	PD284	25-Jun-19	SC	Waccamaw	964	Winyah Bay	Female
Abr-00924	PD286	25-Jun-19	SC	Waccamaw	862	Winyah Bay	Female
Abr-00925	SA181	10-Jun-19	SC	Waccamaw	790	Winyah Bay	Female
Abr-00954	PD323	24-Jun-20	SC	Waccamaw	994	Winyah Bay	Female
Abr-00955	PD332	26-Aug-20	SC	Waccamaw	849	Winyah Bay	Female
Abr-01017	JW099	6-May-21	SC	Waccamaw	597	Winyah Bay	Female
Abr-01067	JW362	28-Sep-21	SC	Waccamaw	851	Winyah Bay	Female
Abr-01068	PD487	14-Jun-21	SC	Waccamaw	769	Winyah Bay	Male
Abr-01069	PD491	15-Jun-21	SC	Waccamaw	937	Winyah Bay	Female
Abr-01070	PD492	15-Jun-21	SC	Waccamaw	754	Winyah Bay	Male
Abr-01071	PD495	16-Jun-21	SC	Waccamaw	959	Winyah Bay	Male
Abr-01072	PD508	17-Jun-21	SC	Waccamaw	768	Winyah Bay	Female
Abr-01076	PD374	13-May-21	SC	Waccamaw	1011	Winyah Bay	Female
Abr-01203*	PD259	19-Jun-19	SC	Waccamaw	941	Winyah Bay	Female
Abr-01204*	PD260	19-Jun-19	SC	Waccamaw	859	Winyah Bay	Female
Abr-01205*	PD269	24-Jun-19	SC	Waccamaw	800	Winyah Bay	Male
Abr-01206*	PD270	24-Jun-19	SC	Waccamaw	941	Winyah Bay	Female
Abr-01207*	PD271	24-Jun-19	SC	Waccamaw	869	Winyah Bay	Female
Abr-01208*	PD274	24-Jun-19	SC	Waccamaw	790	Winyah Bay	Female
Abr-01209*	PD284	25-Jun-19	SC	Waccamaw	964	Winyah Bay	Female
Abr-01210*	PD286	25-Jun-19	SC	Waccamaw	862	Winyah Bay	Female
Abr-01211*	SA181	10-Jun-19	SC	Waccamaw	790	Winyah Bay	Female
Abr-01221	JW468	12-Jan-22	SC	Sampit	876	Winyah Bay	Male