Validation of a molecular sex marker in three sturgeons from eastern North America

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Abstract

Despite the importance of sex-specific information for sturgeon conservation and management, sex identification has been a major challenge outside of mature adults on spawning grounds. Recent work identified a sex-specific locus (*AllWSex2*) that appears to be broadly conserved across many Acipenserids, but the assay was not validated for all species within the family. We tested the *AllWSex2* marker in three sturgeon taxa (shortnose sturgeon *Acipenser brevirostrum,* Gulf sturgeon *A. oxyrhinchus desotoi*, and Atlantic sturgeon *A. oxyrhinchus oxyrhinchus*) from the Atlantic and Gulf of Mexico Coasts of North America to validate its use for sex identification. Our results indicate *AllWSex2* is conserved in all three taxa, presenting a new opportunity to derive sex-specific information from tissue samples, which are routinely collected from these taxa. We found high concordance (range: 97-100%) between genotypic and phenotypic/histological methods, suggesting the assay is broadly effective. However, the small amount of discordance between the methods (<3%) suggests further refinement may be possible.

Keywords: *AllWSex*2, Sturgeon, Genetic, Phenotypic, Sex, shortnose sturgeon (*Acipenser brevirostrum*), Gulf sturgeon (*A. oxyrhinchus desotoi*), Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*)

Introduction

Understanding sex-specific life history traits and sex ratios throughout a species' life cycle has important implications for demography and evolution (Sapir et al. 2008, Kahn et al. 2021). For instance, sex-specific migration patterns can impact vulnerability to human activities (Okamura et al. 2014). In addition, variation in life history between the sexes can be a major factor contributing to differences in breeding and adult sex ratios (Kahn et al. 2021). However, determining the sex ratio for a population can be difficult when sexes are disproportionally represented in time and space during different life stages and cannot be effectively sampled simultaneously (e.g., breeding population; Kahn et al. 2021). From an evolutionary perspective, when sex ratios deviate from 1:1, effective population size may be reduced (Wright 1938) as well. Such factors are particularly germane in sturgeons, as sex-specific exploitation (e.g., caviar fisheries) and life history differences may have important implications for aquaculture and conservation applications. Despite the importance of sex-specific behaviors and sex ratios, determining the sex of individuals collected throughout the life cycles of many species using visual methods alone remains challenging, particularly for immature individuals.

Sturgeon species can be challenging to assign sex as they exhibit minimal external sexual dimorphism (Vecsei et al., 2003; Wheeler et al., 2019) and mature at different rates among species and populations (Kynard et al., 2016; Hilton et al., 2016). To date, sex determination can be inferred using several phenotypic methods (e.g., examination of gonads during surgical implantation of transmitters, ultrasound, and gonad histology) (Kynard and Kieffer, 2002; Webb et al., 2019). Yet, current phenotypic methods to determine sex vary in accuracy, depending on the age of individuals sampled and the timing of sampling, and vary in the level of invasiveness

(reviewed by Webb et al. 2019). The latter is particularly relevant as most sturgeon species are of conservation concern and typically have restrictions on handling protocols (IUCN 2022).

Genetic-based methods of sex identification could provide a minimally invasive approach (e.g., Brunelli and Thorgaard, 2004; Brunelli et al., 2008) to facilitate the identification of both males and females throughout the sturgeon life cycle. Indeed, Kuhl et al. (2021) identified a sex-specific locus (*AllWSex2*) in several sturgeon species; however, no sturgeon species in North America were tested. Encouragingly, Scribner and Kanefsky (2021) recently tested the *AllWSex2* marker on lake sturgeon (*Acipener fulvescens*) and found that the locus could differentiate between the sexes. For many sturgeon species, sex-specific data are critical to inform management as stock assessment models rely on parameters such as growth rate, age of maturity, and spawning periodicity, which can vary between sexes (e.g., ASMFC 2017).

The objective of this study was to evaluate if the *AllWSex2* locus could differentiate male and female shortnose (*Acipenser brevirostrum*), Gulf (*A. oxyrhinchus desotoi*), and Atlantic (*A. oxyrhinchus oxyrhinchus*) sturgeons by assessing amplicon banding patterns in fish of known sex. Such validation is necessary before the marker can be applied to support the management of these three imperiled taxa.

Materials and Methods

We assayed *AllWSex2* in samples of shortnose, Gulf, and Atlantic sturgeons using PCR-based approaches. The specific approach varied among the taxa; however, all used similar PCR conditions as those reported by Kuhl et al. (2021). Importantly, each lab used different but well-established methods to visualize PCR products. In general, the *AllWSex2* locus was evaluated in shortnose sturgeon using the original protocol (PCR and visualization of bands in agarose gels),

whereas Gulf and Atlantic sturgeon used protocols similar to Kuhl et al. (2021), but both included internal positive controls that were used to rule out failed reactions (which could falsely indicate a male individual) and PCR products were visualized on more sensitive instruments (bands in acrylamide gels for Gulf sturgeon, and peaks using capillary electrophoresis for Atlantic sturgeon). For each species below, we outline sampling details, specific molecular methods used, and how fish were sexed using phenotypic or histological characteristics. Congruence between sex identifications based on the *AllWSex2* locus and phenotypic characters was assessed to validate the molecular approach for each taxon.

Shortnose sturgeon (*Acipenser brevirostrum*)

Adult shortnose sturgeon (n = 53) were collected in the Hudson River by the New York (NY) State Department of Environmental Conservation. Fish were captured near Albany, NY, in April and May 2021 using anchored gill nets. Sex was determined by visual inspection of gonads when inserting acoustic transmitters. During standardized processing, a small section of the dorsal fin was removed from each specimen and preserved in 95% ethanol for later DNA extraction.

Genomic DNA was extracted from tissue samples using a DNA extraction kit following the manufacturer's instructions (Qiagen DNeasy Extraction kit; Qiagen, Germantown, MD). DNA concentrations were measured using a spectrophotometer (Nanodrop 2000). Scribner and Kanefsky (2021) provided known female and male lake sturgeon genomic DNAs, which served as positive controls. The *AllWSex2* locus was amplified with the protocol described in Kuhl et al. (2021) using a different DNA polymerase (Dream *Taq*; Thermo Fisher Scientific, Waltham, MA) in the reactions. Accordingly, 0.75x of the PCR buffer was used such that the final $MgCl_2$

concentration was 1.5 mM. PCR products were size-separated in a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Gulf sturgeon (*A. oxyrhinchus desotoi*)

Adult Gulf sturgeon were collected from the Choctawhatchee River, Florida (FL) in October of 2018 (n = 42) and 2021 (n = 17). The sex of the fish was visually determined in the field by inspection of the gonads during acoustic transmitter implantation post-spawn (2018) or via the presence of expressed gametes (2021). Gonad biopsies for the 2018 fish were also examined via histology. Tissue samples were collected using the same methods as in shortnose sturgeon.

DNA extraction methods for Gulf sturgeon were the same as for shortnose sturgeon. We multiplexed the *AllWSex2* locus with a microsatellite locus (AoxD165; Henderson-Arzapalo and King 2002) to provide an internal positive control for PCR amplification. The two loci were amplified in 12.5 µl reactions consisting of 1x *Taq* reaction buffer (New England Biolabs, Ipswich, MA), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 units of *Taq* polymerase, 0.16 µM of the M13 tailed (Schuelke, 2000) *AllWSex2* forward primer, 0.16 µM of the *AllWSex2* reverse primer, 0.08 µM of the M13 tailed AoxD165 primer, 0.08 µM of the AoxD165 reverse primer, 0.08 µM of the labeled M13 primer (Eurofins, Louisville, KY), 1 µl of 20-50 ng/µl DNA, and water to the final volume. PCR cycling conditions followed Dugo et al. (2004) with an annealing temperature of 56°C. Amplicons were visualized on acrylamide gels using a DNA sequencer (LI-COR 4300; LI-COR Inc., Lincoln, NE) and scored with gene imaging software (Gene Image IR v. 3.55;LI-COR). For a size standard, we used amplified fragments of the Lambda phage following methods our laboratory adapted from Wang et al. (2010).

Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*)

Adult Atlantic sturgeon (*n* = 95) were collected during the spring along the Atlantic coastline of Delaware (2017) and in the summer in the lower portions of Delaware Bay (2020). Delaware collections were made during non-spawning periods far from spawning grounds. For these adults, sex was visually assessed at the time of capture and then confirmed via histological examination of gonadal biopsies. York River adults (*n* = 61) were collected during the fall in the York River, Virginia (VA) (2018-2019). Collections were made over spawning habitat, and sex was determined by expressing gametes, when acoustic transmitters were implanted as part of a separate study (Hager et al. 2020), or histologically (Van Eenennaam and Doroshov, 1998). Tissue samples were collected using the same methods as described above.

Genomic DNA was isolated from tissue using DNA extraction reagents according to manufacturer's protocols (Gentra Puregene reagents; Qiagen, Germantown, MD). DNA concentrations were evaluated using a fluorometer (Qubit 2.0 Fluorometer; Thermo Fisher Scientific, Waltham, MA) and stock DNA was diluted to 25 ng/µl before PCR. The *AllWSex2* primers were added to an existing PCR multiplex containing 4 other sturgeon microsatellites. Multiplexing for genotypes and sex identification in the same reaction can save time, effort, and money. PCR conditions consisted of: 1X PCR master mix (Qiagen Multiplex PCR Master Mix; Qiagen, Germantown, MD), 0.15 µM of the labeled *AllWSex2* forward primer, 0.15 µM of the *AllWSex2* reverse primer, 1.5 µl of DNA, 0.1-0.35 µM of the other primers and water to the final volume of 15 µl. Amplifications were carried out using the following procedure: initial denaturing at 95°C for 15 min; 35 cycles of 94°C for 30 s, 58°C for 90 s, 72°C for 90 s; and a final extension at 72°C for 10 min. PCR products were then diluted and run on a genetic analyzer (Applied Biosystems 3500 Genetic Analyzer Foster City, CA) using an internal size standard

(LIZ-500, Applied Biosystems). Genotypes were size-scored based on electropherogram fluorescent-peaks visualized using genotyping software (Genemapper software v6.0; Thermo Fisher Scientific).

Results

Shortnose sturgeon females produced an amplicon at the expected length (~100 base pairs, bp), and males amplified a faint band at approximately 300 bp. These observed banding patterns matched the patterns in known lake sturgeon females and males described in Scribner and Kanefsky (2021). Three of the 53 lanes in the gel showed little DNA streaking, indicating potential failed PCRs. Given that no internal positive control was used, as in the original protocol, the assay was conducted twice to assess repeatability. In total, 94% (50/53) were scored the same – with the three individuals that were, potentially, failed PCRs resulting in the amplification of PCR products in the second attempt. Using genotypes from the second attempt, 98% (52/53) of phenotypic and genotypic sex identifications were concordant (Table S1, Supplemental information).

Female Gulf sturgeon amplicons were approximately 107 bp in size, whereas males did not amplify. While gonadal assessments, post-spawn, were generally consistent with genotypic assignment, three individuals identified as female via genotyping were scored differently in the field, and nine unknown fish were identified as male (Table S2). However, we found 100% congruence between genotypic and phenotypic assignments of sex via gonad histology in 2018 and in the field assignments in 2021 (Tables S2-S4).

Amplicons from female Atlantic sturgeon were approximately 106-107 bp in size (Figure S1) and usually very strong in intensity (>30,000 RFU, Relative Fluorescent Units). Most males did not amplify, but sometimes a weak peak at the same size was seen. If no peaks or

peaks weaker than 8000 RFU were seen, the animal was classified as male. Based on empirical data (see below and Figure S2), we developed an *ad hoc* criterion where individuals with peaks scored at 8,000-26,000 RFU were classified as Unknown. For York River spawning adults, all genotypic sex assignments were concordant with field observations except for one individual scored as Unknown (RFU = 24,831) (Table S5). Three individuals which were determined to be male using the laboratory approach were assigned as Male? (n = 2) or unknown (n = 1) in the field. Genotypic sex assignments for Atlantic sturgeon captured in the lower Delaware Bay and the Atlantic Ocean near Delaware were also highly concordant with phenotypic sex assignments. In all but one fish (RFU = 12,907, and therefore classified as Unknown) our molecular results matched field sex assignments (38/39; Table S6). In all cases where both methods were confident in sex assignments (76/76) the results matched. Finally, there was complete agreement between histological sex assignments and genotypic sex assignments (79/79; Table S7). Consistent with our *ad hoc* criteria, only one individual from the Delaware collections yielded ambiguous molecular results (12,907 RFU) but was independently assigned as female based on phenotypic characters (field observation and histology). The unknown sex assignment may have been due to poor PCR amplification or poor injection on the ABI instrument. For example, the individual from the Delaware collections, which we present as unknown (12,907 RFU), was later reanalyzed and produced 32,347 RFU. This suggests that it may be worthwhile to rerun ambiguous animals to clarify results. The genotypic assay was also able to assign sex to numerous animals where phenotypic observations were inconclusive or otherwise unavailable.

Discussion

Results indicate that the *AllWSex2* assay is a viable technique for inferring sex across all three sturgeon taxa, producing >97% concordance with phenotypic data. Importantly, among

231 those evaluated using histological techniques, results were 100% concordant. Validation of the
232 marker across three independent laboratories, which used different methods to amplify and
233 visualize PCR products, suggests the method is robust and broadly applicable. Notably, the
234 genotypic assay was also able to assign sex to numerous animals where phenotypic sex
235 assignments were unavailable.

236 Despite the robustness of the marker, it may be possible to improve the assay, as there
237 was a small proportion (<3%) of samples where the *AllWSex2* marker yielded ambiguous results.
238 Most samples used for this study were sexed with high confidence (i.e., via histology; see tables
239 in Supplement 1), but errors in the process cannot be completely ruled out. Biological
240 mismatches between genotypic and phenotypic sex are another possible explanation for the
241 occasional inconsistencies in our assignments (e.g., Wedekind, 2017). However, our results
242 might also be explained by variation in priming regions of the locus among species tested. While
243 uncertainty in sex identification can be accounted for in models, the original primers designed by
244 Kuhl et al. (2021) could potentially be further optimized in North American sturgeons. In
245 addition, the original assay did not include an internal positive control which could result in
246 incorrect sex assignment. Also, the original assay was designed for visualization on traditional
247 gels, not with primers attached to fluorescent dyes that can be visualized with more sensitive
248 techniques (e.g., electropherograms).

249 The effectiveness of the *AllWSex2* locus for molecular sex identification in the focal
250 species presents new scientific opportunities that can support conservation and aquaculture (e.g.,
251 production of only females) efforts. Sex ratios can be assessed in spawning populations. Even if
252 phenotypic sex is obtained, genotypic sex could provide additional data to help improve
253 estimates of sex ratios and other sex-associated metrics (e.g., Brunelli et al. 2010). For instance,

the application of the *AllWSex2* locus is particularly useful, as most sturgeon (except for males near spawning) are difficult to sex using non-invasive procedures. Sample sizes of known females are often quite small, with many more "suspected" females (i.e., failure of an individual to express milt when the abdomen is massaged during the spawning season). In addition, when combined with telemetry data, the timing and periodicity of spawning migrations can be assessed for each sex, and sex-specific-migration patterns can be resolved. Given that tens of thousands of genetic samples from shortnose, Gulf, and Atlantic sturgeons have been collected and archived over the last several decades, there is ample opportunity to study a range of sex-specific questions. While improvements for the assay may be possible, the suggested changes described above should not deter the immediate use of the assay, which represents an effective tool to assess sex in the three focal taxa.

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Data availability

Data associated with this manuscript are published in the supplement.

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Supplemental information

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Table S1. Comparison of molecular sex assignments with field assignments for shortnose sturgeon (*Acipenser brevirostrum*).

	Field Sex		
Genetic Sex	**Female**	**Male**	**Grand Total**
Female	22	0	22
Male	1	30	31
Grand Total	23	30	53

Table S2. Comparison of molecular sex assignments with field sex assignments via inspection of gonads during acoustic tag implementation for Gulf sturgeon (*A. oxyrhinchus desotoi*) captured in the Choctawhatchee River in 2018.

	Field Sex					
Genetic Sex	**Female**	**Male**	**Female?**	**Male?**	**Unknown**	**Grand Total**
Female	11	1	3	2	0	17
Male	0	4	0	12	9	25
Grand Total	11	5	3	14	9	42

Table S3. Comparison of molecular sex assignments with gonad histological sex assignments for Gulf sturgeon (*A. oxyrhinchus desotoi*) captured in the Choctawhatchee River in 2018.

	Gonad Histological Sex					
Genetic Sex	**Female**	**Male**	**Female?**	**Male?**	**Unknown**	**Grand Total**
Female	17	0	0	0	0	17
Male	0	25	0	0	0	25
Grand Total	17	25	0	0	0	42

Table S4. Comparison of molecular sex assignments with field sex assignments based on expressed gametes for additional Gulf sturgeon (*A. oxyrhinchus desotoi*) captured in the Choctawhatchee River in 2021.

	Field Sex					
Genetic Sex	**Female**	**Male**	**Female?**	**Male?**	**Unknown**	**Grand Total**
Female	13	0	0	0	0	13
Male	0	4	0	0	0	4
Grand Total	13	4	0	0	0	17

Table S5. Comparison of molecular sex assignments with field sex assignments for Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*) captured in the York River, Virginia.

	Field Sex					
Genetic Sex	**Female**	**Female?**	**Male**	**Male?**	**Unknown**	**Grand Total**
Female	14	5				19
Male			38	2	1	41
Unknown		1				1
Grand Total	14	6	38	2	1	61

Table S6. Comparison of molecular sex assignments with sex assignments based on visual inspection of gonads in the field for Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*) captured in the Atlantic Ocean off the coast of Delaware. Underlined values indicate discrepancies between field and genotypic sex assignments.

	Field Sex					
Genetic Sex	**Female**	**Female?**	**Male**	**Male?**	**Unknown**	**Grand Total**
Female	52	1		2	5	60
Male			24	2	8	34
Unknown	1					2
Grand Total	53	1	24	4	13	95

Table S7. Comparison of molecular sex assignments with gonad histological sex assignments for Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*) captured in coastal waters near Delaware. Unknown sex fish were from biopsy samples that did not contain any gonadal tissue.

	Gonad Histological Sex			
Genetic Sex	**Female**	**Male**	**Unknown**	**Grand Total**
Female	55		5	60
Male		24	10	34
Unknown	1			1
Grand Total	56	24	15	95

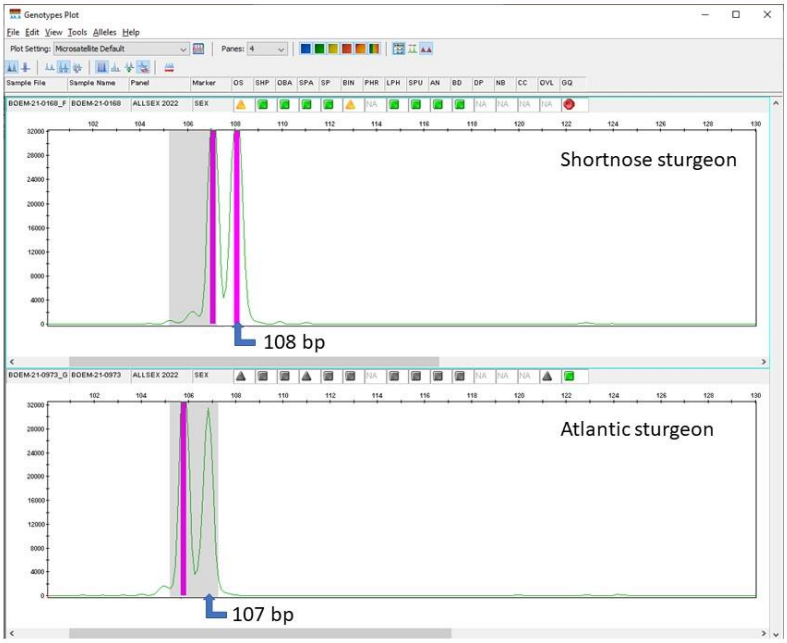

Figure S1. Sample electropherograms for shortnose (*Acipenser brevirostrum*) and Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*). The amplicon generated for female shortnose sturgeon was 107-108 base pairs in length, whereas the amplicon produced for female Atlantic sturgeon was 106-107 bp. The magenta bands indicate very high fluorescence levels. Most amplification products seen on the genetic analyzer (3500 Genetic Analyzer ;Applied Biosystems) showed a split peak profile due to incomplete adenylation.

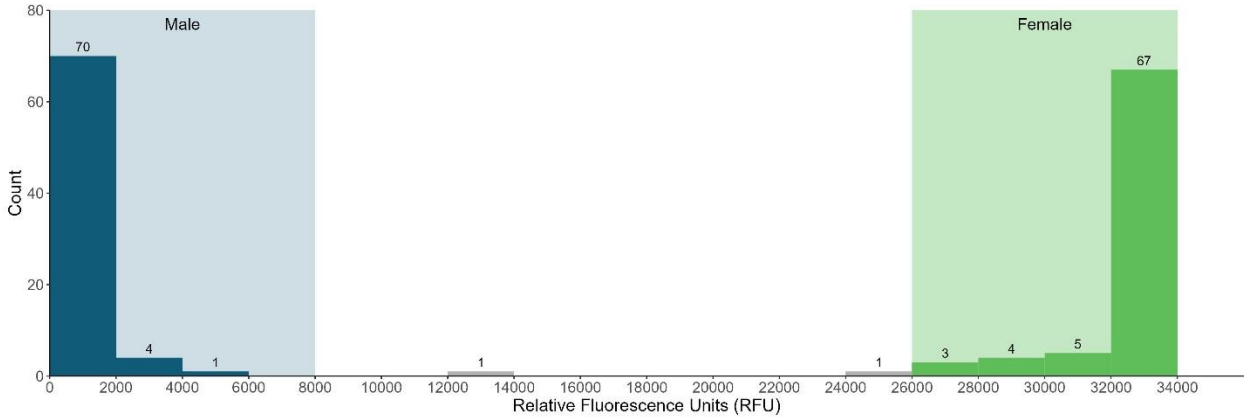

Figure S2. Histogram of Relative Fluorescent Unit (RFU) scores for 156 Atlantic sturgeon (*A. oxyrhinchus oxyrhinchus*) genotyped at the *AllWSex2* locus using capillary electrophoresis.