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Molecular sexing of lake sturgeon

Kim T. Scribner [a,b,c,*], Jeannette Kanefsky [a]

[a] Department of Fisheries and Wildlife, Michigan State University, 480 Wilson Rd, East Lansing, MI 48824, USA
[b] Department of Integrative Biology, Michigan State University, 252 Farm Ln., East Lansing, MI 48824, USA
[c] Ecology, Evolution, and Behavior Program, 103 Giltner Hall, 293 Farm Lane, East Lansing, MI 48824, USA

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ABSTRACT

Demographic data including characterizations of population sex and age composition are of fundamental importance for effective management, especially for numerically depressed Great Lakes populations of imperiled species such as lake sturgeon (*Acipenser fulvescens*). The goal of this paper was to extend a recently reported Acipenserid-derived PCR-based genotyping test to determine sex of lake sturgeon. We demonstrate that the recently reported AllWSex2 primers amplified the female sex-specific region in lake sturgeon of known sex, consistent with a ZZ/ZW mode of inheritance. Sanger sequencing of female lake sturgeon PCR products matched published sequences from female sterlet (*Acipenser ruthenus*), showing 100% query cover and 98% (63/64) identity. The ability to provide a rapid, cost-effective, and unambiguous determination of sex for lake sturgeon will allow managers to determine compositional estimates of sex ratios during any season, and for individuals at any age or size, which is of great utility for species characterized by delayed sexual maturity and lacking external sexual dimorphisms.

Introduction

Demographic data including sex and age composition for Great Lakes species of conservation concern, including imperiled lake sturgeon (*Acipenser fulvescens*) would allow estimation of the abundance of adults of reproductive age and expectations of recruitment, which are critical for management. Demographic parameters can be particularly difficult to estimate for species with delayed sexual maturity (McGuire et al., 2019; Hamel et al., 2020), and where external sex-diagnostic morphological features are lacking (Webb et al., 2019).

Among sturgeon and paddlefish species, the timing of gonadal histological and hormonal development varies greatly, and assessment methods employed in wild populations and in captivity (e.g., aquacultural operations) vary widely. Methods commonly employed for sex determination in Acipenserid fishes generally include ultrasound, assays of plasma sex steroids, endoscopy, and gonadal biopsy (review in Webb et al., 2019). For lake sturgeon in the Great Lakes, ultrasound (Chiotti et al., 2016), histological examination (McGuire et al., 2019), and sex hormone assays (Craig et al., 2009) have been described. The accuracy of sex determination can vary greatly depending on season and individual age/size (Webb and Doroshov 2011). Gonadal tissue differentiation in lake sturgeon is slow, and histology is not reliable until 3–4 years of age (McGuire et al., 2019).

Until very recently the sex determining mechanism in sturgeon and paddlefish, including investigations in lake sturgeon was unknown despite the application of multiple molecular methods of detection (Keyvanshokooh et al., 2007, McCormick et al., 2008, Hale et al., 2010). Recently, an evolutionarily conserved region consistent with a ZZ/ZW (female heterogametic) system was described (Kuhl et al., 2020). Sex in five Eurasian and one North American species (Atlantic sturgeon; *Acipenser oxyrinchus*) were evaluated and confirmed using a PCR-based assay. Lake sturgeon and other members of North American clades (Peng et al., 2007) were not evaluated by Kuhl et al. (2020).

The goal of this paper was to extend a recently reported Acipenserid-derived PCR-based genotyping test to determine sex in lake sturgeon and to validate that the region amplified is homologous to the region characterized in other sturgeon species that were recently evaluated (Kuhl et al., 2020).

Materials and methods

Study area

Adult lake sturgeon including individuals of known and unknown sex were collected during late April through late May

* Corresponding author at: Department of Fisheries and Wildlife, Michigan State University, 480 Wilson Rd, East Lansing, MI 48824, USA.
E-mail address: scribne3@msu.edu (K.T. Scribner).

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2020 from the Black River population in the northern lower peninsula of Michigan (detailed descriptions provided in Larson et al., 2020). All males and the majority of females could be accurately sexed by direct expression of gametes. Several suspected females were provisionally sexed based on visual inspection of genitalia and gross anatomical features. Fin clips (approx. 1 cm^2) were collected from all adults and used for DNA extraction and molecular sex determination.

Laboratory procedures

DNA was extracted from fin clips from 11 adult lake sturgeon (*Acipenser fulvescens*) individuals using a DNeasy Blood and Tissue kit (QIAGEN, Germantown, MD) following the manufacturer's protocols. Assayed individuals included 5 fish unambiguously identified as male, 4 fish unambiguously identified as female, and 2 fish tentatively identified as female in the field at the Black River in Michigan. DNA samples from these individuals were tested with the female sex-specific primers amplifying the AllWSex2 marker developed by Kuhl et al. (2020) to determine if the primers would assign sex properly in lake sturgeon, a species not assayed in that study. PCR reactions were carried out as described in Kuhl et al. (2020), and contained 2 μl of DNA at a concentration of 20 ng/μl and 23 μl of master mix (2.5 μl of 10× ChoiceTaq Buffer--containing 15 mM $MgCl_2$, 2.5 μl of 2 mM dNTPs, 1.25 μl each of the AllWSex2 forward and reverse primers at 10 pmol/μl, 15.4 μl of diH_2O and 0.1 μl of ChoiceTaq (Denville Scientific, Metuchen, NJ) at 5U/μl) for a total volume of 25 μl. Cycling conditions were as follows: an initial denaturation step of 2 min @ 95 °C, followed by 35 cycles of 1 min @ 94 °C, 45 s @ 56 °C and 45 s @ 72 °C, and a final extension step of 5 s @ 72 °C. PCR products were run on a 1.5% agarose gel along with a 100 bp DNA ladder (Invitrogen, Waltham MA) and stained with ethidium bromide for visualization.

M13-tagged forward and reverse AllWSex2 primers were then used to amplify the female sex-specific region in the same 11 lake sturgeon individuals in order to allow Sanger sequencing of the fragments. The PCR products were cleaned using the QIAquick PCR Purification kit (QIAGEN, Germantown, MD), quantified, diluted to a concentration of 10 ng/μl and sequenced with M13 forward and reverse primers at the Michigan State University Research Technology Support Facility on an ABI 3730xl 96-capillary DNA sequencer (Applied Biosystems, Waltham, MA). Sequences were examined, reduced to a common length, and aligned using MEGA version 6 (Tamura et al., 2013). The resulting sequences were queried against the GenBank database using the BLAST algorithm of Altschul et al. (1990).

Results

Primer test on lake sturgeon males and females

In the 6 sturgeon species Kuhl et al. (2020) tested with the AllWSex2 primers, female individuals consistently produced a single amplification product slightly larger than 100 bp in size. This was also true for the four individuals of lake sturgeon identified as female in the field tested here. In addition, the two fish tentatively identified as female in the field (F?) also produced single bands slightly larger than 100 bp in size (Fig. 1). The five lake sturgeon individuals identified as male in the field showed amplification products as well: a faint band slightly less than 300 bp in size, which was distinguishable from the amplification pattern produced in the female lake sturgeon (Fig. 1). Non-specific amplification was also observed in males of some of the sturgeon species tested by Kuhl et al. (2020) (*A. oxyrinchus*, *A. ruthenus* and *Huso huso*).

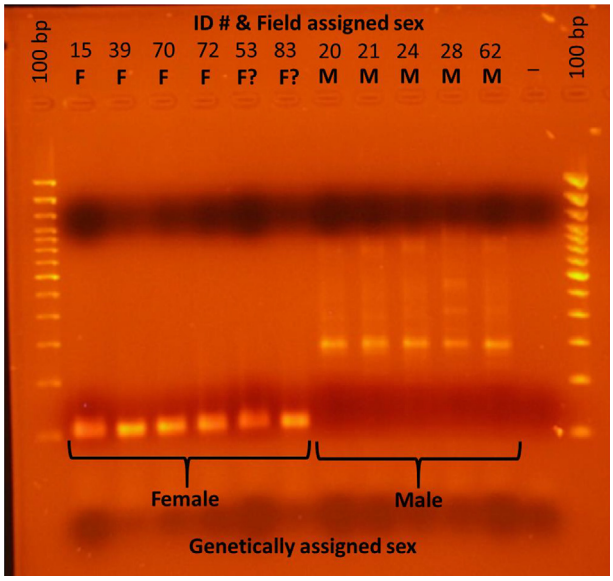

Fig. 1. Ethidium bromide stained agarose gel showing PCR products for 2020 Black Lake male and female lake sturgeon amplified using the Acipenserid sexing primers described by Kuhl et al. (2020). Under field assigned sex, M indicates an animal unambiguously identified as male, F indicates an animal unambiguously identified as female and F? indicates an animal tentatively identified as female.

In the female lake sturgeon, an identical 64 nucleotide sequence (primers excluded) was obtained from the PCR products of all 6 individuals. This sequence was queried against the GenBank nucleotide collection and found to be most similar in sequence (100% coverage, 94% identity) to a predicted mRNA sequence (Accession number XM_034058592) from an *Acipenser ruthenus* genome assembly (BioProject Accession number PRJNA635364; Du et al., 2020) (Supplemental Table S1). The sequence was then queried separately against the GenBank Sequence Read Archive for 31 pooled male (SRX9341540) and for 30 pooled female (SRX9341541) *Acipenser ruthenus* (starlet) individuals produced by Kuhl et al. (2020). The top matching reads obtained from male sterlet showed 100% query cover and 94% (60/64) identity. However, the top matching reads from female sterlet showed 100% query cover and 98% (63/64) identity. The sequence was also compared to the region from a female sterlet reference genome assembly (BioProject Accession number PRJEB35912; CACTIG010000179.1: 61246236–61246344) identified by Kuhl et al (2020) as containing the AllWSex2 marker (Electronic Supplementary Material (ESM) Fig. S1). This region also showed 98% (63/64) identity to our lake sturgeon sequence, and was identical to the reads from the female sterlet showing 98% identity to our lake sturgeon sequence. In the male lake sturgeon, amplification with the M13-tagged AllWSex2 primers produced very faint PCR products slightly larger than 300 bp in size (corresponding to the size of the male product in Fig. 1 with the added length of the M13 sequences), but failed to produce usable sequence.

Discussion

Identification of sex for non-reproductive sturgeon is of particular importance for species conservation and management. Size at age is a fundamental requirement for estimation of important life history features such as size and age at sexual maturity and longevity (Hamel et al., 2020). Despite widespread conservation efforts, demographic information sex ratios and age structure are difficult to determine, particularly during the non-spawning season, largely because lake sturgeon and other Acipenseriform species generally lack external sexually dimorphic morphological

features (Webb et al., 2019). As demonstrated in Fig. 1, based on highly conserved sequence of the sex-specific region, managers now have a rapid, cost-effective, and unambiguous PCR-based method for determining sex of lake sturgeon of any age/size.

A genetic sex marker allows for sex determination from a small minimally invasive blood sample, biopsy, or fin clip, which can be taken quickly and with minimal impact to individual sturgeon. Other methodologies are comparatively more invasive. Gonad biopsies/visualizations are highly invasive. Ultrasound instruments are expensive, and are not universally diagnostic during all life stages. Hormonal sexing requires blood collection and processing by professionals with the technical expertise. The ability to obtain information on sex will be extremely important for studies where capture takes place during non-spawning times of the year when gametes cannot be expressed.

Based on our sequence analysis of the region flanked by the amplification primers, the female specific AllWSex2 marker sequence is highly conserved between lake sturgeon and sterlet, showing only 1 nucleotide difference between the 2 species. It would be of interest to test the AllWSex2 primer pair for female specific amplification in all extant sturgeon species, particularly those belonging to clades other than those tested by Kuhl et al. (2020), to determine if they are functional across the entire sturgeon phylogeny. Sequencing the marker in these species would also provide an independent estimate of the level of its sequence divergence across the Acipenseridae for a region that is not confounded by gene duplication events. In particular, testing on member(s) of the genus *Scaphirhynchus* and on species belonging to the "Pacific" clade of sturgeons, a group first identified by Ludwig et al. (2001) and supported by subsequent phylogenetic studies (for example: Peng et al., 2007; Krieger et al., 2008; Luo et al., 2019; Shen et al., 2020; Sheraliev and Peng, 2020) would be informative. In addition, it would be instructive to test them on paddlefish (*Polyodon spathula*) to ascertain if their conservation and utility extends to the Polyodontidae, the sister family of the Acipenseridae (sturgeon).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jglr.2021.03.015.

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