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SHORT COMMUNICATION

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Cross-species conservation of a transposase-linked element enables genetic sexing of commercial populations of Russian sturgeon (*Acipenser gueldenstaedtii*)

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Summary

All-female culture of sturgeon is essential for efficient caviar production. However, Russian sturgeon (*Acipenser gueldenstaedtii*) does not exhibit external sexual dimorphism, and therefore, commercial farms apply gonadal endoscopy or ultrasound at the earliest age of 4–5 years to separate the sexes, with ~90% accuracy. Recently, a dominant genomic marker (*AllWSEX2*) has been found with association to femaleness in sturgeons. We developed a duplex PCR *(dAllWSEX2)* with the adjacent *bmp7* gene as an internal control, to validate an effective PCR. Robust amplification of control fragments was observed for all samples of our commercial *A. gueldenstaedtii* stock (n = 337). The *dAllWSEX2* assay was significantly associated with sex (n = 43, $p < 1.6 \times 10^{-8}$), yet four (18%) of the endoscopy-determined females were genetic males. To examine whether some females display a male genetic profile, we tested 96 egg-producing females, which were all verified as genetic females, indicating that the observed mismatches may be attributed to wrong sexing by endoscopy. Application of *dAllWSEX2* on 100 7-month-old fish showed no sex-dependent differences in body weight, indicating that weighing is not an applicable tool for sorting females at a young age. Sanger sequencing of the *bmp7* fragment revealed octaploidy and sex-independent variation, suggesting that the critical sex-determining region harboring *AllWSEX2* is small. In keeping with a model of a single-ploidy encoding female determination, *AllWSEX2* showed no variation despite being a transposase-linked repetitive element. Cross-species conservation of *AllWSEX2*, and absence of annotated sex-determination genes in this region suggests that, in sturgeons, the sex-determining mechanism is different from mechanisms identified in other fish.

KEYWORDS
AllWSEX2, aquaculture, sex identification, sex markers

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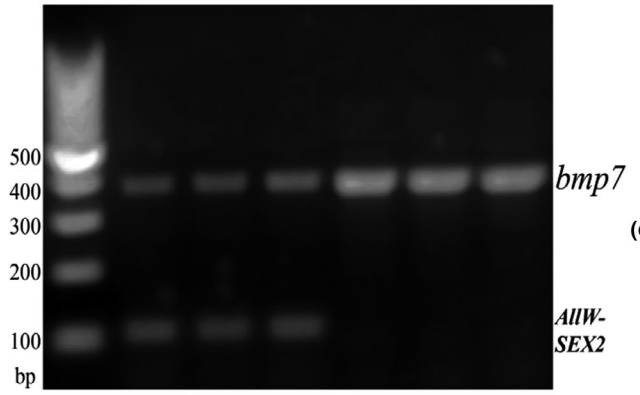
Intensive aquaculture of most fishes relies on controlling sex ratios. The preference for males or females is dependent on the specific farmed species (Beardmore et al., 2001; Martínez et al., 2014). A female mono-sex population of caviar sturgeon considerably enhances the economic benefits of caviar production (Dunham, 1990; Yom-Din & Degani, 2020). Currently, in commercial sturgeon farming, there is no technology to produce all-female populations apart from sexing and sorting. The sturgeon species that belong to the *Huso* and the *Acipenser* genera do not show external sexual dimorphism. Moreover, the level of plasma sex steroids is low until the beginning of gonadal development at the age of 3–4 years (Doroshov et al., 1997; Hurvitz et al., 2005). In Russian sturgeon, rare cases of intersex gonads have been found. The ovarian component of the intersex gonad is at the pre-vitellogenic stage as in normal females, and the testis component contains spermatids and mature spermatozoa as in similar normal 7-year-old males (Hurvitz et al., 2008; Jackson et al., 2006). However, in terms of estradiol and 11-ketotestosterone plasma levels, as well as expression levels of pituitary gonadotropin (βLH and αFSH), the fish with intersex gonads are closer phenotypically to normal males than to normal females, even though the testis part of the intersex gonad is smaller than the ovarian counterpart (Hurvitz et al., 2008; Jackson et al., 2006). This is yet another reason why steroid levels cannot be used as sex identification criteria. As a result, sexing is based on endoscopy or ultrasound examination of the gonads at the age of 4–5 years at the earliest (Hurvitz et al., 2008), with ~70–90% accuracy (Wuertz et al., 2019).

The high proportion of males in the meiotic gynogens of different sturgeon species indicates a ZW/ZZ sex-determining system in this group of fish (reviewed by Wuertz et al., 2019). Although much effort has been directed towards genomic and transcriptomic characterization of the genetic factors underlying sex determination in sturgeons, the causative genes have remained elusive due to the complex genomes of these fish (Chen et al., 2016; Degani et al., 2019), of which the Russian sturgeon has developed octaploidy (Fontana et al., 2001). Transcriptome studies of the gonads have been conducted in the Russian sturgeon (Chen et al., 2016; Degani et al., 2019; Hagihara et al., 2014). As sexual maturation is earlier in males than in females, the gonad and fin transcription profiles of 29 known and sex-related genes of males (4 years old) and females (7 years old) have been compared. In non-gonadal tissues of Russian sturgeon, the expression of these genes is similar among the sexes at 4 years old (Degani et al., 2021). Nevertheless, by analyzing the genome variation of five female and five male Russian sturgeon, it has been shown that 536 and 339 short segments are unique to females and males respectively (Hajouj, 2020). Recently, using whole genome sequencing, sex-specific differences have been detected in chromosome 4 of sterlet (*Acipenser ruthenus*), and a female sex-specific marker (*AllWSEX2*) has been designed (Kuhl et al., 2021). *AllWSEX2* is a dominant marker, and in accordance with a WZ/ZZ sex-determining system, its PCR product is amplifiable using female DNA only. This marker has been effective for genetic sexing in multiple sturgeon species including *Acipenser sturio*, *Acipenser oxyrinchus*, *Huso huso*, *Acipenser baerii*, *Acipenser gueldenstaedtii*, and *Acipenser fulvescens* (Kuhl et al., 2021; Scribner & Kanefsky, 2021). In the present study, we modified the *AllWSEX2* assay to include a positive control for an efficient and reliable sex identification tool in Russian sturgeon.

As detailed in Materials and Methods (Appendix S1), we were able to amplify and sequence a 109-bp PCR fragment orthologous to the sterlet's *AllWSEX2* (98% identity to CACTIG010000179.1:61,246,236–344) from *A. gueldenstaedtii* females (Figure 1). A BLASTN search against 10 Mbp spanning *AllWSEX2* indicated that this fragment sequence is derived from a highly repetitive element, with seven significant matches (expect value <1e-21, identity >85%) within the analyzed interval (CACTIG010000179.1:56,246,236–66,246,236). Moreover, BLASTX analysis against the vertebrate NR protein database indicated that it was placed 380 bp to the 5′ of the initiation codon of transposable element Tcb1 transposase (95% identity and similarity to Sequence ID: ACV85768, CACTIG010000179.1:61,246,724–7741). However, the chromatogram sequence was uniform with no SNPs, suggesting that it is from a single genomic position on a single chromosome (Figure 2a). Using PCR, it was evident that *AllWSEX2 is* amplified in females only. Thus, to validate PCR effectivity also in males, we introduced an internal control amplicon of the *bmp7* gene, which is annotated to the sex specific region-containing chromosome (CACTIG010000179:60,248,693–9,033), less than 1 Mbp from *AllWSEX2*. This amplification product, when extracted from agarose gel and sequenced showed that this gene has multiple copies (Figure 2b,c). Investigation of copy-number variation based on Tide analysis for individual fish that had a shift in their sequence (Brinkman et al., 2014; Seroussi, 2021), and peak ratio examination of four SNPs for other individual fish (Fig. S1, Seroussi, 2021) suggested that this gene is in keeping with an octaploid model (Figure 2b,c). In these panels of Figure 2, typical chromatograms of two individuals exemplify the observed chromosomal complexity at the *bmp7* gene. All *bmp7* copies had a similar size (384 ± 1 bp); and although this gene is located near *AllWSEX2*, no sex specific pattern was found within the genotypes of eight individuals (four males, four females, Table S2). Thus, our amplification product was adequate as an internal control for indicating the presence of template DNA of all the sex chromosome paralogs, and as an effective PCR.

Our improved DNA-based assay (Figure 1a) showed the internal control fragment for all 377 samples,

(a)



(b) Association analysis between *AllWSEX2* and sex in 4.5-year-old fish.

dAllWSEX2 \ Sex[a]	Female	Male	*P*[b]
Positive (female)	18	0	1.6×10^{-8}
Negative (male)	4	21	

[a] Sample was of 43 individuals for which sex was determined by gonadal endoscopy.

[b] *P* for binomial test using hypothesized probability of 0.5 for an error.

(c) Distribution of sex following identification by *dAllWSEX2* and gonadal-endoscopy assays in 7-month-old fish.

Sex \ Assay	*dAllWSEX2*	Endoscopy	p^a
Female	18	22	0.22
Male	25	21	

[a] Pearson's chi-squared test.

FIGURE 1 A duplex PCR assay for sex identification in sturgeon (*dAllWSEX2*). (a) DNA was extracted from a random sample of six caudal fins (three females and three males) and used as a template for PCR amplification with primer mix (Primer-pair#1 and #2, Appendix S1). Products were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. On the left, a molecular size marker was applied (M). (b) Table presenting analysis of association between *AllWSEX2* and sex. (c) Table presenting distribution of sex identified by *dAllWSEX2* and gonadal-endoscopy assays

suggesting that it is robust and efficient (Table S1). *AllWSEX2* was significantly associated with sex (Figure 1b, $p < 1.6 \times 10^{-8}$, Table S1). However, four (18%) of the endoscopy-determined females were found to be genetic males. Nonetheless, the hypothesis that the assigned sex distributions of 4.5-year adults by *dAllWSEX2* and gonad endoscopy were similar, was not rejected (Figure 1c, $p < 0.22$). To examine whether some females display a male genetic profile, we sampled an additional 96 females with fully developed egg-producing ovaries (Table S1). All these females showed the *AllWSEX2* female genotype, suggesting that all previous mismatches may be attributed to wrong sexing by endoscopy. Employing the genetic assay to study the relationship between weight and sex at an early age, we observed no significant association (*t*-test, $p = 0.89$) between these traits for a random sample of seven-month-old fish consisting of 52 females (average weight 67.7 ± 3 g) and 48 males (average weight 65.4 ± 3.2 g).

In this study, we validated the specificity of *AllWSEX2* for sex identification in our *A. gueldenstaedtii* stock, providing an important confirmation for the observation of Kuhl et al., 2021. We also improved the genetic assay to include *bmp7* as an internal control for an effective PCR, which is crucial when absence of amplification for *AllWSEX2* represents the male genotype (Figure 1a). The sturgeon genome octaploidy may explain the high efficiency and robust PCR results for *bmp7* gene, this being due to multiple target sequences (Table S1). Furthermore, in addition to the advantage of genetic analysis at a young age, our results indicate that *dAllWSEX2* is more reliable than gonadal endoscopy, which is currently being used by commercial farms. It is noteworthy that gonadal endoscopy is likely to be error-prone due to the intersex gonads (Hurvitz et al., 2008; Jackson et al., 2006).

We further used the assay to study the relationship between weight and sex at a young age. We hypothesized that on the one hand, at this age, after the initial larval mortality, the population is stabilized and the fish sizes allow safe fin clipping, but on the other hand, some anatomical differences between the sexes may have emerged (Grandi et al., 2007). However, no association was found between sex and weight in 7-month-old fish, indicating that weight is not useful for commercial sexing at young ages, in agreement with a previous report for undifferentiated stages (Hurvitz et al., 2007).

Sanger sequencing of our internal control gene *bmp7* matched an octaploid model displaying an allele pattern which was disassociated from sex. In contrast, the *AllWSEX2* sequence showed no variation, implying that there was only one chromosome containing the sex-determining region, despite the sturgeon genome octaploid complexity. Assuming that *bmp7* is located within an equivalent of 1 cM of *AllWSEX2* also in Russian sturgeon, the lack of genetic association between sex and this gene within the analyzed stock suggests a sex determination system in which the underlying variation is a short nucleotide sequence within a chromosomal region that readily undergoes genetic recombination. Occurrence of frequent recombination is typical of undifferentiated sex chromosomes similar to those of sturgeons (Du et al., 2020; Kuhl et al., 2021). In such scenarios, association with sex may be limited to <1000 bp around the causative mutation (Curzon et al., 2021; Fan et al., 2021). However, there are no known annotated sex-determining genes in such proximity to *AllWSEX2*. Thus, deciphering the mechanism that drives sex determination in sturgeon remains a challenge. *AllWSEX2* itself is a repetitive element within the putative promoter that regulates transcription of Tc1-like transposons in fish (Krasnov

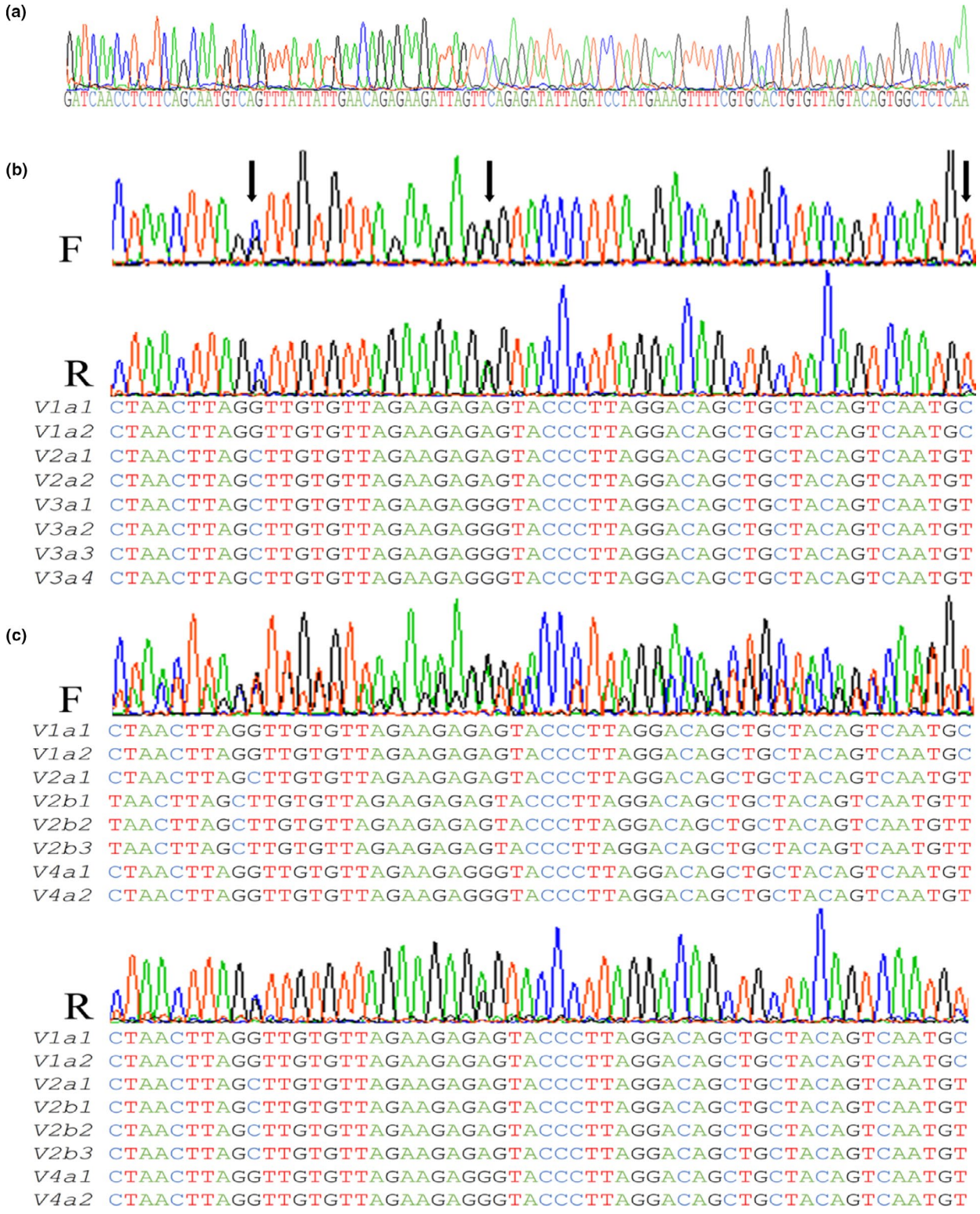


FIGURE 2 Sanger chromatograms of PCR amplicons from *Acipenser gueldenstaedtii* chromosome 4. DNA samples extracted from fin clips of individuals (Table S3) were PCR amplified and sequenced from both forward (F) and reverse (R) orientations. (a) The *AllWSEX2* sequence (female #4, using Primer-pair#1, Appendix S1). (b) Chromatogram of the *bmp7* amplicon (male #6, using Primer-pair#2, Appendix S1). Below the chromatogram, the predicted nucleotide sequences are presented in accordance with the peak-ratio analysis at the positions indicated by arrows. (c) Chromatogram of *bmp7* amplicon (male #5, using Primer-pair#2, Appendix S1). Below the chromatogram the predicted nucleotide sequences are presented in accordance with decomposition analysis using the Tide software

et al., 2005). Nevertheless, it shows a remarkable cross-species conservation associated with the female phenotype (Kuhl et al., 2021; Scribner & Kanefsky, 2021); and this cross-species conservation is especially intriguing because given that the divergence time of *A. gueldenstaedtii* and *A. ruthenus* is estimated to be over 60 Mya (Peng et al., 2007). Although in vertebrates, Tcb1 transposases are usually inactive (Krasnov et al., 2005), they are known to be actively transcribed in sturgeons and were identified as candidate sex-related genes based on transcriptome sequencing of *A. gueldenstaedtii* gonads (Chen et al., 2016). In this sturgeon, transposable element transposase and transposase-related genes are expressed at a very high level in males, but less in female (Chen et al., 2016). Thus, a possible function of *AllWSEX2* and the transposase-like sequence located to the 3′ of *AllWSEX2* should be further studied, including the possibility of their involvement in female transposon silencing. However, repression of transposable elements has been shown to participate in sex determination only in invertebrates (Yang et al., 2021); and transposable elements are over-expressed in testes of fish with no obvious association to sex determination (Dechaud et al., 2021).

Our findings support large-scale implementation of the *ALLWSEX2* as marker for sexing in *A. gueldenstaedtii* aquaculture. Such application requires further modifications and adaptations for field conditions. These modifications have already been implemented in a pilot run at Caviar Galilee and are showing satisfactory specificity and scalability (data not shown).

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CONFLICT OF INTEREST

Ben-Naim N., Domovitz R., and Hurvitz A. are involved in sturgeon aquaculture at Caviar Galilee.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. The Russian sturgeon is protected by the international convention CITES. However, Caviar Galilee operates under full control of the CITES authority in Israel—The Authority for the Protection of Nature and National Parks.

DATA AVAILABILITY STATEMENT

All the data relevant to the study are included in the article or uploaded as supplementary information.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1 Fish; Duplex PCR for a control fragment (genetic sexing); Young and mature fish sampling and weighing; Sex identification of 4.5-year-old adults using endoscopy; DNA extraction, separation and sequencing of PCR products; and Statistics.

Figure S1 Output of Tide software for male individual #5.

Table S1 Data set of samples used in experiments.

Table S2 *Acipenser gueldenstaedtii* nucleotide sequences.

Table S3 Genotype octaploid models for eight sturgeon individuals for the *bmp7* amplicon.

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