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RESEARCH ARTICLE

Applicability of an in-house Chelex-100 DNA isolation method for extracting genetic material from sterlet (*Acipenser ruthenus*) embryos

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Abstract. Efficient, cost-effective DNA extraction methods are crucial for molecular research on sturgeon embryos given that substantial sample sizes must frequently be analyzed in short periods of time. The high lipid and carbohydrate contents of sturgeon embryo yolk sacs mean that obtaining genetic material of sufficient quality is challenging. The predominant methods used include tissue/cell lysis, organic extraction, purification on spin columns, and ethanol precipitation. However, these methods are expensive and time-consuming, which significantly limits the throughput of PCR-based molecular analyses. In the present study, we evaluated the usefulness of an in-house Chelex-100 DNA extraction method on sterlet (*Acipenser ruthenus*) embryos at the neurula developmental stage (48 hours post fertilization) and compared it with two other commercial silica membrane-based kits for isolating genetic material–NucleoSpin Tissue® (Macherey Nagel, Duren, Germany) and Sherlock AX (A&A Biotechnology, Gdynia, Poland). The yield and quality of nucleic acid, its suitability for PCR amplification, and the total cost and complexity of the extraction methods were evaluated and compared. Our results indicated that the in-house Chelex-100 is inexpensive and can be used as an effective high-throughput method of DNA isolation for sterlet embryos.

Keywords: comparative DNA extraction, DNA yield and purity, cost- and time-efficiency, PCR amplification quality, sturgeon genetics.

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Introduction

Comprehensive knowledge about the embryonic development of sturgeons is fundamental for effective hatchery management and the production of high-quality stocking material used in commercial aquaculture and for stocking open waters (Park et al. 2013, Wakchaure et al. 2015, Korentovich and Litvinienko 2018, Chandra and Fopp-Bayat 2021). Embryonic development is a difficult area of study, and the application of molecular techniques is essential in research conducted on sturgeons since they permit assessing gamete quality, identifying the genetic backgrounds of developmental disorders, analyzing gene expression profiles in fish embryos at early stages of development, verifying genome manipulation effectiveness, and monitoring selective breeding

programs (Ludwig 2006, Fopp-Bayat 2007, Bobe 2015, Yue and Wang 2017, Chandra and Fopp-Bayat 2021). Genetic screening is an essential tool when working with sterlet embryos to identify species and stocks/populations that play crucial roles in preventing the illicit trade of mislabeled or illegally harvested caviar trade and ensure compliance with legal regulations in sturgeon fisheries worldwide (Zhang et al. 2013, Boscari et al. 2014, Havelka et al. 2017).

The development of various molecular techniques based on analyses of short-sized DNA markers (up to about 600bp), such as microsatellites, single nucleotide polymorphisms (SNPs), qualitative trait loci (QTL), and mitochondrial DNA (mtDNA), has revolutionized biological sciences, including fisheries and aquaculture research (Maqsood and Ahmad 2017, Chandra and Fopp-Bayat 2021). Despite the fact that DNA-based analytical methods involving the polymerase chain reaction (PCR) are easy, inexpensive, sensitive, and highly reproducible, their throughput is limited by the DNA isolation step as it can be time-consuming and labor-intensive. Depending on the method used, DNA isolation can take several hours to complete, which can significantly slow down the overall PCR workflow. Moreover, the quality and yield of DNA obtained can also impact the efficiency and specificity of the PCR reaction (e.g., Fredricks et al. 2005, Di Pinto et al. 2007, Claassen et al. 2013, Yalçınkaya et al. 2017). Since genetic-based studies on fishes usually require large numbers of samples to be analyzed, the application of optimal cost- and time-effective methods of DNA extraction of sufficient quality is fundamental in molecular research on sturgeon embryos.

Available DNA isolation methods can differ greatly in total cost, complexity of use, and in the quantity and quality of the genetic material extracted. The high lipid and carbohydrate contents of sturgeon embryo yolk sacs mean obtaining sufficient-quality genetic material is a challenging process. The methods usually applied are tissue/cell lysis, organic extraction, purification on spin columns, and ethanol precipitation. Silica membrane-based extraction methods produce high-quality DNA, but they are expensive and time-consuming, which means less throughput than DNA isolation methods based on phase separation (Abdel-Latif and Osman 2017, Yalçınkaya et al. 2017). Silica membrane-based and precipitation extraction methods might be also less effective in isolating mitochondrial DNA and can cause the mechanical shear of the genetic material isolated (Guo et al. 2009). On the other hand, DNA isolation methods based on phase separation, such as the in-house Chelex-100 technique, usually provide higher DNA yields in a shorter time, but they are less effective in removing contaminants than commercial silica membrane-based methods of DNA extraction.

Our study aimed to evaluate the effectiveness of the in-house Chelex-100 method for isolating genetic material from sterlet (*Acipenser ruthenus* L.) embryos and its suitability for downstream PCR amplification of short-sized DNA fragments. The yield and quality of the extracted DNA, and its suitability for PCR amplification, as well as the total cost and complexity of the phase separation-based DNA extraction method were evaluated and compared with two commercial kits: (2) NucleoSpin Tissue® (Macherey Nagel, Duren, Germany) and (3) Sherlock AX (A&A Biotechnology, Gdynia, Poland).

Materials and Methods

Compliance with ethical standards

This study was conducted in strict accordance with Polish regulations (Act of the Polish Parliament of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes, Journal of Laws 2015, item 266). The research was conducted on fish embryos, and special permission was not required for the experiments. None of the experiments described in this article involved human participants.

Fish material

The present study was conducted on sterlet embryos at the neurula developmental stage (48 hours post

fertilization). A total of 30 embryos were collected from a fish farm in Wąsosze (southern Poland). Each sterlet embryo was preserved in 96% ethanol and stored at room temperature until DNA isolation.

DNA extraction

Ten randomly selected embryos were used for DNA extraction using three different methods: (1) in-house Chelex-100, and two commercial kits: (2) NucleoSpin Tissue® (Macherey Nagel, Duren, Germany) and (3) Sherlock AX (A&A Biotechnology, Gdynia, Poland). Entire embryos were used for DNA extraction.

In the Chelex-100 method, DNA was isolated based on the standard protocol. Embryonic tissues were placed in 500 µl of a mixture of 10% Chelex-100 (Biorad, USA) solution containing 60 µg of Proteinase K (Sigma, Japan) (final concentration 0.1 µg/µl). Next, samples were incubated at 55°C for 2 h, and then centrifuged at 376 ×g for 10 min. The genetic material present in the supernatant (200 µl) was carefully transferred to new 1.5 ml tubes and stored in a refrigerator (4°C) until further examination of its content, purity, and suitability for PCR amplification.

DNA was isolated with NucleoSpin Tissue® (Macherey Nagel, Duren, Germany) and Sherlock AX (A&A Biotechnology, Gdynia, Poland) commercial silica membrane-based kits in accordance with the manufacturers' protocols that included sample lysis with proteinase K and subsequent DNA purification with filtration columns. The Sherlock AX kit comprised two types of filtration columns and an additional precipitation stage. After isolation, the DNA was suspended in TE buffer and stored in a refrigerator (4°C) until later examinations of DNA content and purity and PCR amplification tests.

Evaluation of the overall performance of DNA extraction methods

The DNA isolation methods tested were assessed based on four criteria: (1) DNA yield, (2) DNA quality, (3) suitability of the genetic material extracted for PCR amplification of short sized DNA fragments (200-350bp), and (4) complexity and price. DNA yield was determined with a NanoDropTM 2000 spectrophotometer (Thermo Scientific, Willington, USA).

The quality of the DNA extracted was assessed based on its integrity and purity. To determine DNA integrity, the isolated samples were loaded onto 1.5% agarose gel (Sigma-Aldrich, St. Louis, USA) stained with ethidium bromide (0.05 mg/ml) and subjected to electrophoresis for 30 minutes at 5 V per every cm of the distance between the anode and the cathode. Gels were visualized under UV light and photographed in G-box (Syngene, UK). The purity of the extracted DNA was determined by measuring the absorbance ratio of A260/280 and 260/230.

The suitability of the extracted DNA for PCR analysis was assessed by amplifying the *Spl-106* microsatellite DNA marker (F: CACGTGGATGCGAGAAATAC, R: GGGGAGAAAACTGGGGTAAA) developed by McQuown et al. (2000). The PCR assay was performed in a reaction volume of 25 µl comprising 1x PCR reaction buffer (50 mM KCl, pH 8.5, Triton X-100), 0.4 µM of each primer, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 3.3 mM of $MgCl_2$, and 0.55 units of Go Taq Flexi DNA Polymerase (Promega, Madison, WI, USA). All PCR assays were conducted in triplicate, using exactly 40 ng of template DNA, and included negative controls without template DNA. PCR was conducted in a Mastercycler gradient thermocycler (Eppendorf, Germany) under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, annealing at 57°C for 30 s, elongation at 72°C for 30 s, and a final elongation step at 72°C for 10 min. PCR conditions are described in detail in Fopp-Bayat et al. (2015). PCR efficacy was quantified as the proportion of successful PCR amplifications determined by agarose gel electrophoresis.

The complexity and cost of the DNA isolation methods tested were determined based on the number of single actions, including vortexing, in their protocols, and the overall cost of DNA extraction per

sample. The total cost of DNA extraction was expressed in PLN based on the price of DNA extraction reagents, excluding plastic materials.

Statistical analysis

The collected data were subjected to Shapiro-Wilk and Levene's tests to check for the normality of distribution and the homogeneity of variance. Significant differences in the values of the analyzed parameters were determined with one-way ANOVA and Dunnett's post-hoc test. For the final assessment, the averaged values of the parameters measured for each DNA isolation method were expressed as relative values and summarized.

Results

In general, all of the tested methods supported successful extraction of genomic DNA from sterlet embryos. However, the techniques analyzed differed in DNA yield and the quality and suitability of the material extracted for PCR. The samples isolated with NucleoSpin Tissue® and Sherlock AX commercial kits revealed a single, sharp band of high-molecular-weight DNA. In contrast, the samples isolated with in-house Chelex-100 revealed shearing and non-specific bands stemming from contamination and lower DNA integrity (Fig. 1).

The Chelex-100 method was characterized by the highest DNA yield ranging from 160.50 to 273.10 ng μl^{-1} (mean = 207.68 ng μl^{-1}). The DNA yields of the commercial isolation kits ranged from 5.10 to 20.60 ng μl^{-1} (mean = 10.59 ng μl^{-1}) for NucleoSpin Tissue® and 4.20 to 24.80 ng μl^{-1} (mean = 15.64 ng μl^{-1}) for Sherlock AX, which were significantly ($P < 0.05$) lower in comparison to the Chelex-100 extraction method. No significant differences ($P < 0.05$) in DNA yield were observed between the commercial kits (Fig. 2).

The samples extracted with the Sherlock AX kit were characterized by the highest DNA purity (mean A280/A260 = 1.89 and A280/A230 = 2.32), which was slightly higher ($P<0.05$) than in the samples isolated with the NucleoSpin Tissue® kit (mean A280/A260 = 1.54 and A280/A230 = 1.97). DNA purity was significantly lowest ($P < 0.05$) in the samples isolated with the Chelex-100 method (A280/A260 = 0.93 and A280/A230 = 0.29) (Fig. 2).

The PCR amplification efficiency of the *Spl-106* microsatellite locus was equal (100%, $P > 0.05$) for DNA templates isolated with each extraction method tested. Sharp amplicons with a length of about 200–350 bp were clearly distinguishable in all DNA templates (Figs. 1 and 2).

The in-house Chelex-100 was the cheapest DNA extraction method. The total estimated application cost of this method per sample was approximately three and six times lower compared to the Sherlock

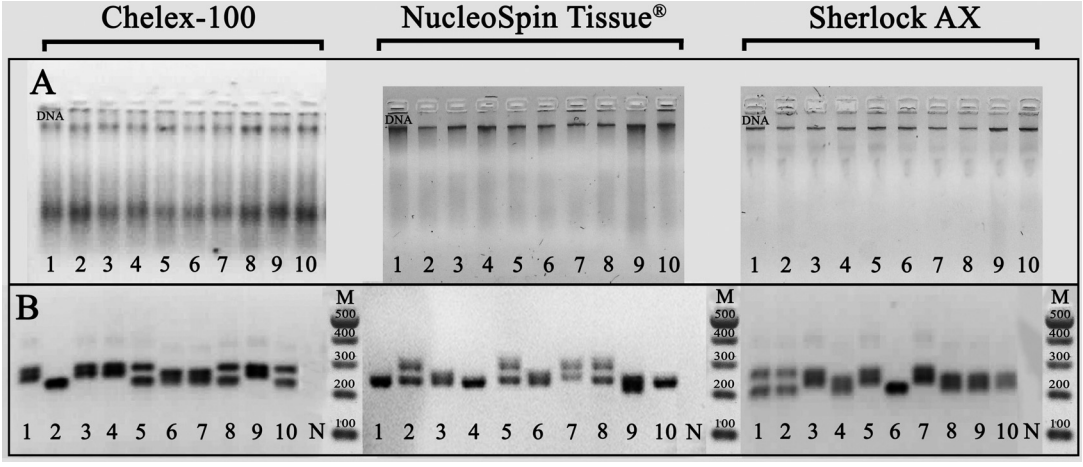

Figure 1. Agarose gel electrophoresis of extracted DNA (A) and the results of PCR amplification involving DNA templates extracted from sterlet embryos with each method tested (B). N: Negative control, M: 100 bp DNA ladder (A&A Biotechnology s.c., Poland).

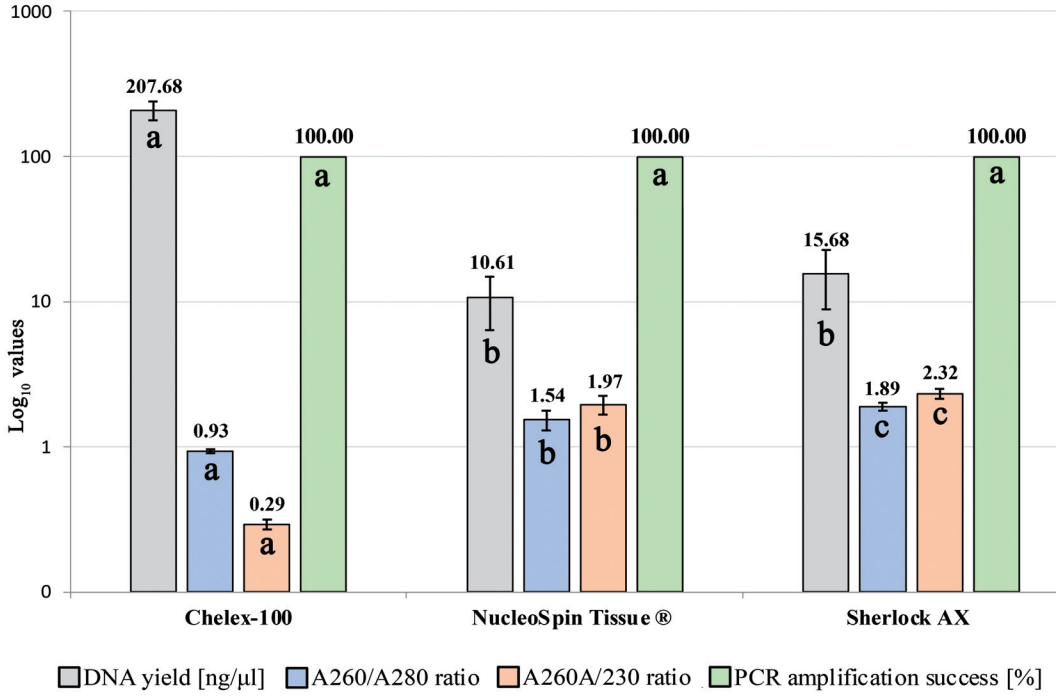

Figure 2. Comparison of mean DNA yield, DNA purity (A260/A280 and A260/A260 ratios), and PCR amplification success. Different letters indicate significant differences ($P < 0.05$) in the parameters of DNA samples extracted from sterlet embryos analyzed with each method tested.

AX and NucleoSpin Tissue® kits, respectively. Additionally, the in-house Chelex-100 DNA isolation method was the least laborious, requiring approximately three to four times fewer number of steps in its protocol compared to the two other DNA extraction methods tested (Fig. 3).

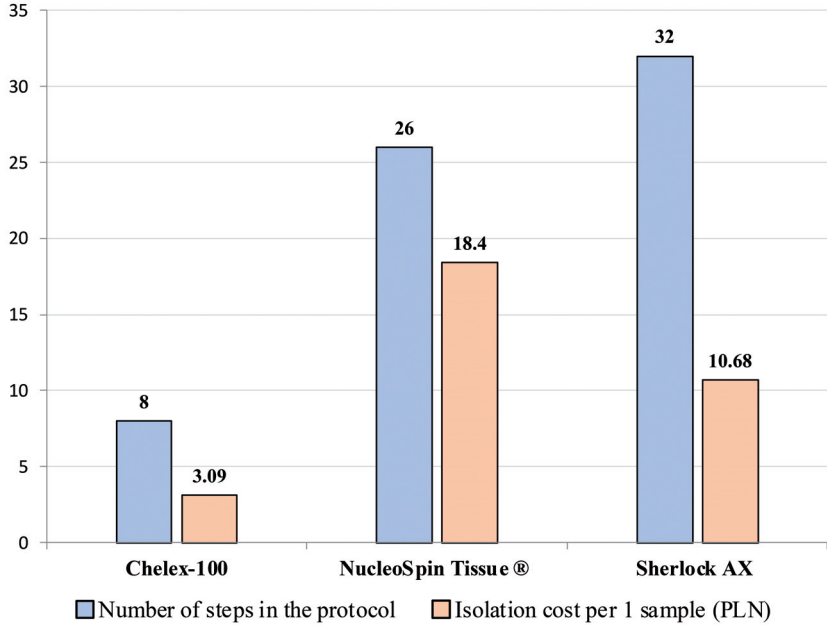

Figure 3. Number of steps in the protocols and total cost per sample of the DNA isolation methods compared.

Discussion

Molecular analyses have revolutionized modern fisheries and aquaculture by offering powerful tools for studies of population genetics, species and stock identification, parentage and pedigree validation, management of selection programs, and verification of the effectiveness of genome manipulations (Maqsood and Ahmad 2017, Chandra and Fopp-Bayat 2021). Genetic analyses of sturgeons at the embryonic level support the determination of genetic similarities and differences within fish species and stocks and facilitate

effective parentage analyses to obtain progeny characterized by favorable traits such as desirable phenotype, resistance to disease, high survivability and growth rate, faster maturation, and improved quality of meat and gametes (Chandra and Fopp-Bayat 2021). Moreover, molecular analyses of sturgeon embryos support the validation of the results of germ cell transplantation in sperm production, gene banking, and surrogate reproduction (Psenicka et al. 2015, Fatira et al. 2018, Kashiwagi et al. 2020).

Despite the fact that all existing DNA extraction methods provide genetic material that is suitable for PCR, real-time PCR amplification, and restriction digestion, most of these techniques require long incubation times, numerous precipitation steps, and ethanol washes, which can decrease the total yield of genomic DNA. Moreover, in addition to sufficient DNA purity, the total concentration of DNA in the sample also plays a pivotal role in molecular analyses (Holden et al. 2003, Abouseadaa et al. 2015). In the present study, DNA yield was significantly higher with the Chelex-100 DNA method (mean value = 207.68 ng μl^{-1}) than with the NucleoSpin Tissue® and Sherlock AX techniques (mean value = 10.59-15.64 ng μl^{-1}). The DNA yields of the silica membrane-based methods were lower than those reported by other authors (Hajibabaei et al. 2005, Zetzsche et al. 2008, Dittrich-Schrodero et al. 2012, Schiebelhut et al. 2017), which indicates that the presence of fat and carbohydrates in sterlet embryos decreases extraction efficacy. Chelex-100 was the only method that supported the acquisition of large quantities (mean concentration of DNA = 207.86 ng μl^{-1}) of genetic material for molecular analyses that require higher concentrations of genetic material, such as restriction enzyme digestion. This observation emphasizes the importance of suitable DNA isolation methods for different types of tissues and downstream molecular analysis techniques.

Histone and non-histone proteins and inhibitors present in samples or introduced during genetic material extraction can disrupt DNA polymerase, restrict enzyme activity, and decrease the efficiency of enzymatic reactions (Couch and Fritz 1990, Di Pinto et al. 2007, Claassen et al. 2013, Schiebelhut et al. 2017, Abdel-Latif and Osman 2017, Yalçınkaya et al. 2017). An A260/A280 absorbance ratio higher than 1.8 is indicative of high-purity DNA that is free of proteins and phenols. However, values higher than 2.0 can indicate sample contamination with RNA, therefore, the required value of the A260/A280 absorbance ratio for the DNA template used for scientific research is known to vary between 1.8 and 2.0 (Khosravinia et al. 2007, Abdel-Latif and Osman 2017). In turn, the A260/A230 ratio is indicative of DNA contamination with carbohydrates and chemical reagents such as phenol, guanidine, glycerol, EDTA, and chloroform. The A260/A230 ratio of pure nucleic acids is higher than 1.8 (Khosravinia et al. 2007, Abdel-Latif and Osman 2017, Yalçınkaya et al. 2017). In the present study, DNA purity exceeded 1.8 in most samples extracted with the commercial Sherlock AX kit, and it was determined at 1.6–1.8 in the samples isolated with NucleoSpin Tissue® kit, which indicates the lower efficacy of DNA purification of the latter. Although the data available indicate that the critical values of DNA purity for enzymatic reactions (A260/A280 and A260/A230 ratios) were below 1.4 and 1.0, respectively, the genetic material extracted with the Chelex-100 DNA method was successfully amplified, and in this respect, this technique did not differ significantly from the NucleoSpin Tissue® or Sherlock AX commercial kits. Nevertheless, the suitability of the DNA template obtained for PCR must be evaluated in the future by amplification for longer DNA fragments and with the application of different techniques, such as LAMP.

High-throughput DNA isolation methods are needed for molecular studies that require many individuals and multiple molecular analysis techniques (Garrick et al. 2015, Huang et al. 2016, Ruggeri et al. 2016). DNA is extracted from fish tissues with various protocols, and the methods applied differ in complexity, extraction time, and cost. In this study, the Chelex-100 DNA isolation method was less laborious and expensive than the silica membrane-based NucleoSpin Tissue® and Sherlock AX commercial kits. The present findings confirm the applicability of the

Chelex-100 technique as a cheap, high-throughput method of DNA extraction from sterlet embryos.

Conclusions

Molecular analyses are a powerful tools for various studies related to fisheries and aquaculture. Given that genetic-based studies on fishes typically involve analyzing large numbers of samples, the use of cost- and time-effective DNA extraction methods that provide sufficient quality is crucial in molecular research on sturgeon embryos. In the current study, the in-house Chelex-100 DNA isolation method was found to be a cheap, efficient alternative to silica membrane-based commercial kits. Our results indicated that the Chelex-100 method provided large quantities of genetic material suitable for molecular analysis techniques. Despite the significantly lower purity of DNA isolated compared to silica membrane-based commercial kits, no significant differences in PCR amplification efficiency were observed relative to the samples isolated with the Chelex-100 method. Therefore, the in-house Chelex-100 extraction method can be considered an alternative to commercial DNA extraction kits, particularly for large-scale molecular studies that require a high throughput of DNA isolation. However, it is necessary to assess in future studies the suitability of the DNA template obtained for PCR by amplifying longer DNA fragments and employing various techniques, such as LAMP.

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Author contributions. D.F.-B. designed and performed the experiments. T.R. conducted a part of the experiments. M.K. analyzed the data and prepared graphic presentations. All authors were involved in writing and editing the manuscript.

Competing interest statement. The authors declare no conflicts of interest.

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