ABS ACTA BIOLOGICA SIBIRICA
A journal on the biodiversity of Siberia and the adjacent lands

Acta Biologica Sibirica 9: 13-21(2023)
doi: 10.5281/zenodo.7679902
http://journal.asu.ru

RESEARCH ARTICLE

Isolation and identification of *Saprolegnia* spp. from infected sturgeon caviar

Ekaterina P. Anokhina[1], Anna A. Tolkacheva[1], Nina A. Pryakhina[1], Mikhail Yu. Syromyatnikov[1], Olga S. Korneeva[1]

1 *Voronezh State University of Engineering Technologies, 19 Revolution Ave., Voronezh, 394036, Russia*

Corresponding author: Anna A. Tolkacheva (anna-biotech@yandex.ru)

Academic editor: R. Yakovlev | Received 4 January 2023 | Accepted 20 January 2022 | Published 30 January 2023

http://zoobank.org/D3FFCFF2-43BC-4B1C-9722-015477291754

Citation: Anokhina EP, Tolkacheva AA, Pryakhina NA, Syromyatnikov MYu, Korneeva OS (2023) Isolation and identification of *Saprolegnia* spp. from infected sturgeon caviar. Acta Biologica Sibirica 9: 13–21. https://doi.org/10.5281/zenodo.7679902

Abstract

Saprolegniosis is considered one of the most common fungal diseases in freshwater aquaculture, affecting eggs and fish of all ages, and is causing great economic losses worldwide. In sturgeon aquaculture, highest harm is caused by caviar saprolegniosis (byssus), a mycotic disease of caviar, which is characterized by damage through saprolegnium fungi during hatchery incubation. The main infectious agents are aquatic mold fungi of the genus *Saprolegnia* spp. A sample of water mold was isolated from infected eggs of a hybrid of Russian sturgeon (*Acipenser gueldenstaedtii*) with kaluga (*Huso dauricus*) with characteristic signs of the disease. Microscopic examination of an isolated oomycete revealed morphological features characteristic of *Saprolegnia* spp., but no oogonia or antheridia were found, which complicates further species identification. To identify the isolated pathogen, molecular tools such as PCR and sequencing of a DNA section including 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA were used to distinguish between different species of aquatic molds. Analysis of the obtained nucleotide sequence showed more than 99% identity with the previously known DNA sequences of *S. parasitica*. According to the results of phylogenetic analysis, the obtained nucleotide sequence was in the same group with the known sequences of *S. parasitica* and separated from other species belonging to *S. ferax*, *S. diclina*, *S. delica*, and *S. australis*.

Keywords

Aquaculture, phylogenetic analysis, saprolegniosis, sequencing

Copyright Ekaterina P. Anokhina et al. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Introduction

Sturgeons are valuable fish species of great biological and economic importance. More than 80% of existing sturgeon species are at risk of extinction due to late sexual maturity, long spawning periods in the wild, overfishing, and their environment pollution (Ciulli et al. 2020).

Sturgeon aquaculture is actively developing around the world and plays an important role in the restoration of natural populations and the production of fish and caviar, which is especially appreciated by consumers (Bronzi et al. 2014, 2017). A serious problem for sturgeon aquaculture is represented by various infectious diseases, including the fungal disease saprolegniosis, which is widespread among freshwater fish. The main infectious agents are aquatic molds of the genus *Saprolegnia* spp. (Thoen et al. 2011; Sandoval-Sierra et al. 2014a). Individuals of all ages, especially juveniles, and eggs during hatchery incubation are susceptible to this infection. Sturgeons are believed to be comparatively more disease resistant than other fish species (Radosavljevic et al. 2019), but the aquatic mold *Saprolegnia* spp. causes great damage during the incubation of sturgeon eggs (Jalilpoor et al. 2006; Ghiasi et al. 2010), in which damaged and unfertilized eggs are affected with gradual infection of live eggs. The death of eggs during this period can reach 70-90% (Radosavljevic et al. 2019).

Since the ban on the use of malachite green dye in the fight against *Saprolegnia*, there have been no effective measures to control and combat this infection in aquaculture. Until now, there are performed active searches for new substances with fungicidal properties. As an alternative to malachite green, the antimycotic activity of a wide range of chemicals was studied (Ali et al. 2014; Hu et al. 2016; Tedesco et al. 2019), as well as of natural biologically active substances (Madrid et al. 2015; Tedesco et al 2020), bacterial isolates (Liu et al. 2015; Heikkinen et al. 2016; González-Palacios et al. 2019). Most of these compounds are either not very effective or have negative effects on fish health or the environment, which hinders their practical use in aquaculture.

Currently, effective control of caviar saprolegniosis remains one of the main tasks in sturgeon aquaculture. To develop effective and safe methods for the prevention and treatment of this disease, it is necessary to identify the type of the main causative agent of the infection. The aim of this study was to identify the pathogen *Saprolegnia* isolated from sturgeon eggs.

Materials and methods

Sampling and isolation of culture

The material for the study was caviar of hybrids of the Russian sturgeon (*Acipenser gueldenstaedtii*) with Kaluga (*Huso dauricus*) with signs of saprolegniosis, obtained

in the fish farm "Chernozem Sturgeon" in December 2020. A sample of infected caviar was washed with sterile distilled water, transferred to solid Saburo medium containing gentamicin (1 µg/ml) and incubated at 22° C for 5 days. To obtain a pure culture, repeated sequential cultivation (5 passages) was performed by cutting and transferring a small section of growing mycelium to a new dish using a sterile surgical blade, the dish was covered with a layer of sterile tap water 2 mm high. Incubation under a layer of water was carried out at 22° C for 5 days.

Microscopic examination

To study the morphology of the isolated culture, an oomycete preparation was prepared in a crushed drop and microscoped using a BiOptic B-200 microscope equipped with an Indus trial Digital Camera 3.0MP ½ COLOR USB2.0 APTINA CMOS SENSOR at 120x and 600x magnifications.

Molecular and phylogenetic analysis

Genomic DNA was isolated from colonies of microorganisms using the Proba-GS kit (DNA technology, Russia) according to the attached instructions. For molecular identification of fungi, the section of the internal transcribed spacer ITS was used. To amplify the DNA section, including the intergenic spacers ITS1 and ITS2, the following universal primers for eukaryotes were used: forward ITS1 TCCGTAGGTGAACCTGCGG, reverse ITS4 TCCTCCGCTTATTGATATGC [17].

The polymerase chain reaction was carried out using Taq polymerase on a Master cycler personal device (Eppendorf, Germany). The following components were mixed in a test tube: 5X reaction buffer - 5 µl; 10 mM dNTP - 1 µl; 10 µmol primer - 1 µl; 10 µmol reverse primer - 1 µl; 25 mM Mg2 + - 3 µl; DNA matrix - 2 µl; thermostable Taq polymerase - 2.5 units; deionized water - up to 25 µl. The following temperature cycles were used: 94° C 4 min, 35 cycles: 94° C 30 sec, 54° C 30 sec, 72° C 45 sec, final elongation 72° C 10 min.

Visualization of PCR products was performed by electrophoresis in 2% agarose gel on a TCP-20LM transilluminator at a wavelength of 312 nm. Ethidium bromide was used as a dye for nucleic acids. The size of the products was determined by comparison with DNA markers of known length (Evrogen, Russia).

Extraction from agarose gel and purification of the amplicon was performed using the commercially available CleanupStandard kit (Evrogen, Russia). Sequencing of the purified PCR products was performed on an Applied Biosystems 3500 genetic analyzer using the BigDyeTerminator v3.1 CycleSequencingKit. The primers used were the same primers as for PCR procedure (see above).

Phylogenetic analysis was performed using the MegaX program. Apodachlya minima was selected as the outgroup. Evolutionary discrepancy between groups was assessed using Kimura's 2-parameter model.

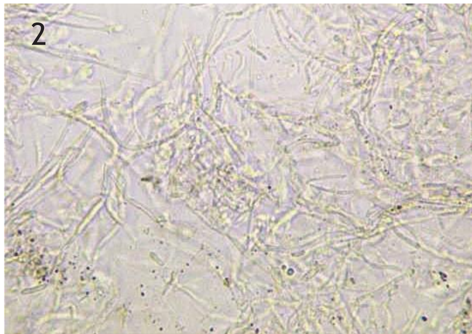
Result

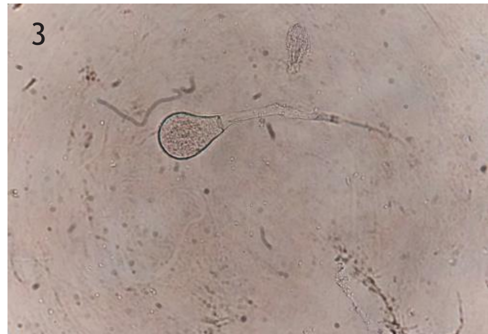
Microscopic examination

The mushroom cultured on Sabouraud solid medium showed the characteristic cultural characteristics of *Saprolegnia*. After 5 days of incubation, abundant growth of white round colonies with a felt structure was observed (Figure 1).

Microscopic examination revealed branched non-septic mycelium, thin hyphae, young and proliferating clavate zoosporangia with spherical zoospores (Figures 2, 3). Oogonia and antheridia were not observed.

Figure 1. Cultural features of the isolated oomycete under a layer of sterile water.





Figures 2–3. 2 – Morphological features of *Saprolegnia* oomycete, magnification 120x. **3** – Morphological features of Saprolegnia oomycete, magnification 600x.

Molecular analysis

A fragment from the DNA of a fungal culture, presumably Saprolegnia, was amplified and sequenced using primers specific to the DNA section including 18S rRNA, ITS1, 5.8S rRNA, ITS2, and 28S rRNA.

As a result of sequencing this region, a 715 bp sequence was obtained: GCTCCCCCCACAACACCCCACGTGATGTACTCTTTATGAGGCTTT-GCGCTGCCCTTGTGGCAGCTAGCCGAAGGTTTCGCAAGAAGCCGAT-GTCAATTTGAATCCTTTTTAAAATACGACTGATCAAAACTGCAGA-TAGAAATATCTGCATGCAATTGAAATACAACTTTCAACAGTGGAT-GTCTAGGCTCGCACACCGATGAAGAACGCTGCGAACTGCGATACG-TAATGCGAATTGCAGAATTCAGTGAGTCATCAAAATTTTGAACGCAT-ATTGCACTTCCGGGTTAGTCCTGGGAGTATGTTTGTATCAGTGTCCGT-GAACACAAACTTGTTTCATTTCTTGATTGGGATGGAGCAGACTGTGAAG-GTCTTGTAATTACAAGTCCTTTTAAACGACGGTACCTATGCGTCCTAGT-GAGATGTATTATTTAAAGGTATGCCTGCGCTCCTTTCGAAAGTCTTGT-GTGGCGGCACACAGCACTCAAAGAGAGAGCAAATCGCGGTAGTTTT-GCTTGTACTTCGGTACGAGTGGACACATATTGCTTTTTGTGATTTCT-GCGAGTCTGTTGTCAAAGTACAAGGCACGTAAGGAGAGTTGGTAT-GCTGGTGCATTTCTTGGCGTATGGAGGCAAATTGGGAATTCAATC-CAATTTGGACCTGATATCAAACAAGACTACCCGCTGAACTTAAGCATAT-CAATAAGGCGGAGGAA.

Analysis of the obtained nucleotide sequence using the international GenBank database showed more than 99% identity with previously registered DNA sequences of *S. parasitica*, including 18S rRNA, ITS1, 5.8S rRNA, ITS2, and 28S rRNA (GenBank accession numbers: MG597031.1, MH458749.1, KX494868.1, JN400038.1, etc.).

Phylogenetic analysis using the Kimura's 2-parameter model showed that the obtained nucleotide sequence was in the same group with the known sequences of *S. parasitica* and separated from other species belonging to *S. ferax*, *S. diclina*, *S. delica* and *S. australis* (Figure 4, phylogenetic tree).

Discussion

The oomycete strain isolated from infected eggs of a hybrid of Russian sturgeon with kaluga was identified as *S. parasitica* based on morphological characteristics and genetic analysis. The caviar affected by water mold was characterized by the presence of abundant white fluffy mycelium on the surface. The isolated pathogen showed typical cultural and morphological features characteristic of *Saprolegnia* spp. as described in early works (Shin et al. 2017; Ke et al. 2009). Species identification of *Saprolegnia* spp. according to morphological characteristics is a difficult task. Several typical morphological features of asexual and reproductive organs of repro-

duction are used for the classical identification of Saprolegnia (Sandoval-Sierra and Diéguez-Uribeondo 2015). In our study, no oogonia or antheridia (reproductive structures) were found in the isolated mold even after a long incubation period, which coincides with the data obtained by other authors (Eissa et al. 2013). The most reliable approach to the classification and identification of the genus *Saprolegnia* is the use of molecular methods of analysis that can reliably distinguish between different types of aquatic mold fungi (Sandoval-Sierra et al. 2014b).

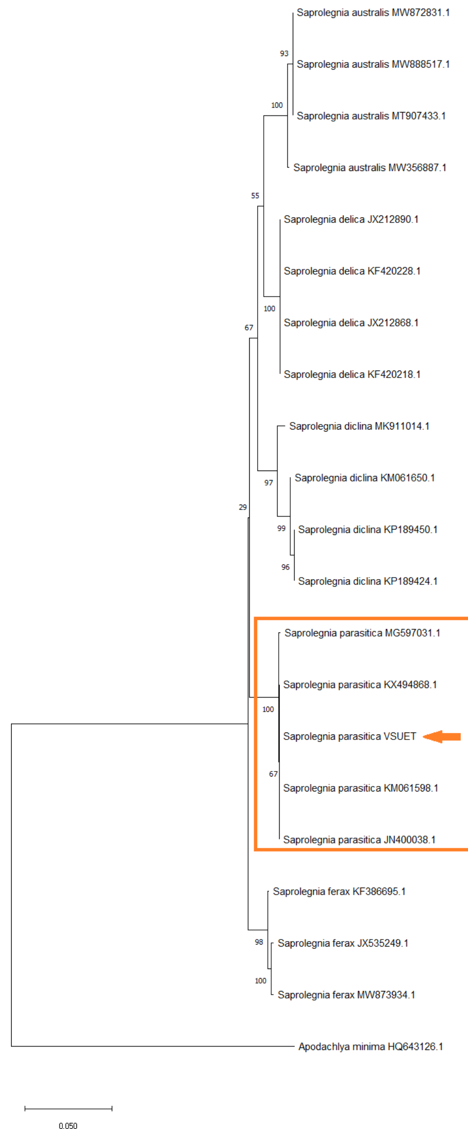


Figure 4. Phylogenetic tree based on DNA sequence including 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA in species of the genus *Saprolegnia*. The arrow marks the obtained nucleotide sequence.

The most informative genetic markers for eukaryotic identification are the non-coding internal spacers ITS1 and ITS2, due to the relatively high variability of their sequences (Sandoval-Sierra and Diéguez-Uribeondo 2015). These non-coding sections are located between the 18S and 28S genes; the 5.8S gene is between ITS1 and ITS2. Thus, to identify the isolated oomycete, a genetic analysis of a DNA region containing 18S rRNA, ITS1, 5.8S rRNA, ITS2, and 28S rRNA was carried out. The comparison of this nucleotide sequence with known *Saprolegnia* DNA sequences from the international GenBank database and phylogenetic analysis confirmed that the isolated water mold strain belongs to *S. parasitica*. Our results are consistent with the data of early studies (Jalilpoor et al. 2006; Ghiasi et al. 2010), according to which *S. parasitica* is the most frequently found species infecting sturgeon eggs. In general, in freshwater aquaculture, this pathogen species accounts for the largest proportion of infections and mortality of fish eggs (Songe et al. 2016). The identification of the saprolegniosis pathogen isolated from the caviar of the Russian sturgeon-kaluga hybrid will serve as the basis for the development of effective and safe methods for the prevention and treatment of this disease in sturgeon aquaculture.

Acknowledgments

This work was supported by the Russian Foundation for Basic Research, project № 20-08-01149 A.

References

Ali SE, Thoen E, Evensen O, Skaar I (2014) Boric acid inhibits germination and colonization of *Saprolegnia* spores *in vitro* and *in vivo*. PLoS One 9 (4): e91878. https://doi.org/10.1371/journal.pone.0091878

Bronzi P, Rosenthal H (2014) Present and future sturgeon and caviar production and marketing: A global market overview. Journal of Applied Ichthyology 30 (6): 1536–1546. https://doi.org/10.1111/jai.12628

Bronzi P, Chebanov M, Michaels JT, Wei Q, Rosenthal H, Gessner J (2019) Sturgeon meat and caviar production: Global update 2017. Journal of Applied Ichthyology 35: 257–266. https://doi.org/10.1111/jai.13870

Ciulli S, Volpe E, Sirri R, Tura G, Errani F, Zamperin G, Toffan A, Silvi M, Renzi A, Abbadi M, Biasini L, Pretto T, Emmanuele P, Casalini A, Sarli G, Serratore P, Mordenti O, Mandrioli L (2020) Multifactorial causes of chronic mortality in juvenile sturgeon (Huso huso). Animals (Basel) 10: 1866. https://doi.org/10.3390%2Fani10101866

Eissa AE, Abdelsalam M, Tharwat N, Zaki M (2013) Detection of *Saprolegnia parasitica* in eggs of angelfish *Pterophyllum scalare* (Cuvier-Valenciennes) with a history of decreased hatchability. International Journal of Veterinary Science and Medicine 1: 7–14. https://doi.org/10.1016/j.ijvsm.2013.04.001

Ghiasi M, Khosravi AR, Soltani M, Binaii M, Shokri H, Tootian Z, Rostamibashman M, Ebrahimzademousavi H (2010) Characterization of *Saprolegnia* isolates from Persian sturgeon (*Acipencer persicus*) eggs based on physiological and molecular data. Journal of Medical Mycology 20: 1–7. https://doi.org/10.1016/j.mycmed.2009.11.005

González-Palacios C, Fregeneda-Grandes JM, Aller-Gancedo JM (2019) Biocontrol of saprolegniosis in rainbow trout (*Oncorhynchus mykiss* Walbaum) using two bacterial isolates (LE89 and LE141) of *Pseudomonas fluorescens*. Journal of Fish Diseases 42 (2): 269-275. https://doi.org/10.1111/jfd.12928

Heikkinen J, Tiirola M, Mustonen SM, Eskelinen P, Navia-Paldanius D, von Wright A (2016) Suppression of *Saprolegnia* infections in rainbow trout (*Oncorhynchus mykiss*) eggs using protective bacteria and ultraviolet irradiation of the hatchery water. Aquaculture Research 47 (3): 925–939. https://doi.org/10.1111/are.12551

Hu K, Ma RR, Cheng JM, Ye X, Sun Q, Yuan HL, Liang N, Fang WH, Li HR, Yang XL (2016) Analysis of *Saprolegnia* parasitica transcriptome following treatment with copper sulfate. PLoS One 11 (2): e0147445. https://doi.org/10.1371/journal.pone.0147445

Jalilpoor J, Mosouleh SA, Masoumzadeh M (2006) Fungal flora in *Acipenser persicus* eggs with particular emphasis on *Saprolegnia* sp. (Oomycetes) and mortality during mass incubation at the Shahid Behesti hatchery. Journal of Applied Ichthyology 22: 265–268. https://doi.org/10.1111/j.1439-0426.2007.00965.x

Ke XL, Wang JG, Gu ZM, Li M, Gong XN (2009) Morphological and molecular phylogenetic analysis of two *Saprolegnia* sp. (Oomycetes) isolated from silver crucian carp and zebra fish. Mycological Research 113 (5): 637–644. https://doi.org/10.1016/j.mycres.2009.01.008

Liu Y, Rzeszutek E, van der Voort M, Wu CH, Thoen E, Skaar I, Bulone V, Dorrestein P C, Raaijmakers JM, de Bruijn1 I (2015) Diversity of aquatic *Pseudomonas* species and their activity against the fish pathogenic oomycete *Saprolegnia*. PLoS One 10(8): e0136241. https://doi.org/10.1371%2Fjournal.pone.0136241

Madrid A, Godoy P, González S, Zaror L, Moller A, Werner E, Cuellar M, Villena J, Montenegro I (2015) Chemical characterization and anti-oomycete activity of *Laureliopsis philippianna* essential oils against *Saprolegnia parasitica* and *S. australis*. Molecules 20 (5): 8033–8047. https://doi.org/10.3390%2Fmolecules20058033

Radosavljevic V, Milicevic V, Maksimović-Zorić J, Veljović L, Nesic K, Pavlović M, Ljubojević-Pelić D, Marković Z (2019) Sturgeon diseases in aquaculture. Archivos de Medicina Veterinaria 12 (1): 5–20. https://doi.org/10.46784/e-avm.v12i1.34

Sandoval-Sierra JV, Diéguez-Uribeondo J (2015) A comprehensive protocol for improving the description of *Saprolegniales* (Oomycota): two practical examples (*Saprolegnia aenigmatica* sp. nov. and *Saprolegnia racemosa* sp. nov.). PLoS One 10 (7): e0132999. https://doi.org/10.1371/journal.pone.0132999

Sandoval-Sierra JV, Martín MP, Diéguez-Uribeondo J (2014) Species identification in the genus *Saprolegnia* (Oomycetes): defining DNA-based molecular operational taxonomic units. Fungal Biology 118 (7): 559–578. https://doi.org/10.1016/j.funbio.2013.10.005

Sandoval-Sierra JV, Latif-Eugenin F, Martín MP, Zaror L, Diéguez-Uribeondo J (2014) *Saprolegnia* species affecting the salmonid aquaculture in Chile and their associations with

fish developmental stage. Aquaculture 434: 462–469. https://doi.org/10.1016/j.aquaculture.2014.09.005

Shin S, Kulatunga DCM, Dananjaya SHS, Nikapitiya C, Lee J, De Zoysa M (2017) *Saprolegnia parasitica* isolated from rainbow trout in Korea: characterization, anti-saprolegnia activity and host-pathogen interaction in zebrafish disease model. Mycobiology 45 (4): 297–311. https://doi.org/10.5941/myco.2017.45.4.297

Songe MM, Willems A, Wiik-Nielsen J, Thoen E, Evensen O, van West P, Skaar I (2016) *Saprolegnia diclina IIIA* and *S. parasitica* employ different infection strategies when colonizing eggs of Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 39 (3): 343–352. https://doi.org/10.1111/jfd.12368

Tedesco P, Fioravanti ML, Galuppi R (2019) In vitro activity of chemicals and commercial products against *Saprolegnia parasitica* and *Saprolegnia delica* strains. Journal of Fish Diseases 42: 237–248. https://doi.org/10.1111/jfd.12923

Tedesco P, Beraldo P, Massimo M, Fioravanti ML, Volpatti D, Dirks R, Galuppi R (2020) Comparative therapeutic effects of natural compounds against *Saprolegnia* spp. (Oomycota) and *Amyloodinium ocellatum* (Dinophyceae). Frontiers in Veterinary Science 7: 83. https://doi.org/10.3389/fvets.2020.00083

Thoen E, Evensen O, Skaar I (2011) Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. Journal of Fish Diseases 34: 601–608. https://doi.org/10.1111/j.1365-2761.2011.01273.x