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Microfungi- like organisms developing on the eggs of pink salmon *Oncorhynchus gorbuscha*

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ABSTRACT

The authors investigated the growth of microfungi- like organisms on the eggs of pink salmon (*Oncorhynchus gorbuscha*) from Małki hatchery on Bystraja River, Kamchatka, Russia. Twenty two straminipilous organisms (fungus-like organisms) were found on eggs of females of pink salmon. The most commonly encountered species on the pink salmon eggs were *Saprolegnia parasitica*, *S. salmonis*, *S. ferax*, *S. diclina*, *S. hypogyna*, *Aphanomyces frigidophilus*, and *Dictyuchus monosporus*. However, such species as *Scoliolegnia asterophora*, *Leptomitus lacteus*, *Pythium diclinum* and *P. monospermum* are rare to salmonid fish.

KEYWORDS: microfungi, eggs, pink salmon

INTRODUCTION

Water pollution, intensive fishing and shrinkage of natural reproduction places are the main causes leading to a drop of population of many fish species including anadromous salmonids. Therefore, the significance of the fish farms, where artificial reproduction takes place, is increasing with each year. Mainly economically important fish species, which deliver valuable proteins [1] undergo farming. Economically, to the most precious fish species of the Far East belong the salmons of *Oncorhynchus* species. Six specimens of this species enter the river of Kamchatka and Sachalin for spawning. As the last years studies show, the number of populations entering rivers for spawning is constantly decreasing. It also relates to pink salmon population. Too intensive fishing and pollution of water environment (which is restricting the reproduction in fish) are concerned to be a cause of this process [2]. As a result of it, the artificial reproduction of those species in hatcheries becomes wide spread [3].

The salmon species belonging to *Oncorhynchus* genus, whose population decreased in recent years [4], enter the rivers of Far East, Alaska, Canada and West Coast of USA for spawning [5-8]. More than 20% of salmon from *Oncorhynchus* genus population enter the rivers of Kamchatka for spawning [9]. Pink and chum salmon are the main species caught in the Far East [7].

So far, the studies on some aquatic fungi growing on eggs of the *Oncorhynchus* species were performed by Taylor and Bailey [10], Czeczuga and Muszyńska [11], Kitancharoen *et al*. [12] and Kitancharoen and Hatai [13]. Saprolegniasis is the most problematic fungal infection of cultured salmonid fish in freshwater in many countries [14]. Some species of straminipilous organisms by infecting both wild and farmed mature salmonids as well as their eggs have a strong economical impact on the fish farming. So the search of new remedies preventing economic losses caused by fungi still lasts [15, 16].

According to Catalog of Fishes [17] *Oncorhynchus gorbuscha* occurs in Arctic and Pacific drainages

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from Mackenzie River delta, Northwest Territories, Canada to Sacramento River drainage, in California, USA; occasionally as far as La Jolla, southern California; also in northeast Asia. On Asia side, from North Korea to Jana and Lena drainages in Arctic Russia North, in Bering Sea on 40º N and from Bering Strait. In the last years pink salmon was recorded in the Revelva River, Hornsund area, SW Spitsbergen [18].

We have decided which fungus-like organisms species, being fish parasites are found to grow on the eggs of pink salmon- species whose population is dropping very rapidly last years.

MATERIAL AND METHODS

We have investigated infected eggs of pink salmon *Oncorhynchus gorbuscha* (Walbaum, 1792) which were obtained from Małki hatchery on Bystraja River in south Kamchatka, Russia. For the isolation of the fungi from eggs, 250 eggs covered with fungal mycelia were collected from the hatching trays and put into the thermos flask with physiological solution and were transported by air mail.

The fungi were isolated according to Kitancharoen *et al*. [12]. The samples of eggs were washed several times in sterilized well water and egg membranes covered by fungal mycelia were separated. The egg membranes were placed in 30-40 mL sterilized well water in petri dishes and were incubated at temperature close to that of water at the hatchery origin (10-15ºC) for 2-3 days to induce zoospore formation. Simultaneously autoclave- sterilized hemp seeds (*Cannabis sativa* L.) were added to these dishes. The number of zoospores was estimated on inverted microscope. The germination type of zoospores was selected and classified according to Yuasa *et al*. [19]. Samples of 0.5 mL of the selected dilutions were spread on individual glucose-yeast extract (GY: 1% glucose from Hoffman La Roche Co., Switzerland; 0.25% yeast extract from Difco Laboratories, Detroit, USA and 1.5% agar from Hoffman La Roche Co., Switzerland) agar plates, air-dried and incubated at 10-15ºC for 24h. To prevent the growth of bacteria, ampicillin and streptomycin (Sigma Chemical Co., USA) at a concentration of 100 µg/mL each were applied to the selected dilation. Bacteria- free germinating thalli from single spores were then transferred to fresh GY agar. Twenty to thirty thalli from single spores were collected to determine the fungal species.

Formation of zoospores, antheridia and oogonia of aquatic straminipilous organisms growing on eggs was recorded. The methods used are described in detail by Seymour and Fuller [20] and Willoughby [21].

Carbohydrate and urease tests were performed on the genera *Achlya*, *Aphanomyces*, *Leptolegnia, Pythium* and *Saprolegnia* according to Yuasa and Hatai [22]. For the carbohydrate utilization test, the medium used to culture the fungal isolates was Yeast Nitrogen Base agar (Difco) and GY agar was used for the urease test. The isolates of the genus *Saprolegnia* were culture at 20ºC for 3 days, whereas the genera *Achlya* and *Aphanomyces* were cultured at 20ºC for 7days. For carbohydrate utilization test, Yeast Nitrogen Base broth (Difco) with 1% carbohydrate was prepared, adjusted to pH 7.2 and autoclaved at 110ºC for 10 min. For urease test GY broth with 1% urea was prepared, adjusted to pH 6.8 and filtered through a 0.45 µm Millipore filter (Whatman). Bromo thymol blue (BTB) and phenol red (PR) were used as indicators, added into Yeast Nitrogen Base broth and GY broth, respectively. Each test was performed in test tubes (1.5 mm x 20 mm) containing 5 mL of the medium. The tubes were incubated at 20ºC for 7 days. Positive reaction was determined by the change of BTB to yellow in the carbohydrate utilization test, and of PR to pink in the urease test. Additionally, isolation of *Aphanomyces* straminipilous pathogen was performed using methods recommended by Willoughby and Robers [23].

The basal medium was used in amino acid assimilation test. Medium preparation and indicator were as described for the carbohydrate assimilation test. Amino acids were added to a final concentration of 0.2% (w/v). Ten/ millimeter test tubes containing 2 mL of the medium were inoculated with fungal mycelia and the control tubes were prepared in the same manner as in the carbohydrate assimilation test. All tubes were incubated at 20ºC for 14 days. A positive result was determined by a change in the color of the medium to pink or purple, and a change to orange and yellow was considered to be a negative result.

The straminipilous organisms were identified using the following keys: Johnson [24], Seymour [25], Johnson *et al*. [26] and Pystina [27], and according to the works of the authors who were first to describe respective species. *Aphanomyces frigidophilus* was identified according to Kitancharoen and Hatai [28], *Saprolegnia salmonis* was identified according to Hussein and Hatai [29] and *S. shikotsuensis* according to Hatai *et al*. [30]. The systematics of straminipilous organisms was used according to Dick [31].

RESULTS

Twenty two straminipilous organisms including 18 belonging to the Saprolegniales, 3 to Pythiales and 1 to the Leptomitales were found to grow on the eggs of *Oncorhynchus gorbuscha* (Table 1). The *Achlya* and *Saprolegnia* genera were the most

Table 1. Microfungi- like organisms obtained from pink salmon eggs (n=250).

Taxa	On eggs	
	Number	%
Straminipila		
Peronosporomycetes		
Leptomitales		
1. *Leptomitus lacteus* (Roth) G. Agardh	18	7.2
Pythiales		
2. *Pythium diclinum* Tokun.	23	9.2
3. *P. monospermum* Pringsh.	56	22.4
4. *P. ultimum* Trow	37	14.8
Saprolegniales		
5. *Achlya klebsiana* Pieters	28	11.2
6. *A. oligocantha* de Bary	101	40.4
7. *A. racemosa* Hildebrand	98	39.2
8. *A. radiosa* Maurizio	23	9.2
9. *A. treleaseana* (Humphrey) Kauffman	41	16.4
10. *Aphanomyces frigidophilus* Kitanch. et Hatai	91	36.4
11. *A. laevis* de Bary	29	11.6
12. *Dictyuchus monosporus* Leitgeb	65	26.0
13. *Isoachlya anisospora* (de Bary) Coker	19	7.6
14. *Leptolegnia caudata* de Bary	52	20.8
15. *Saprolegnia australis* Elliott	34	13.6
16. *S. diclina* Humphrey	68	27.2
17. *S. ferax* (Gruith.) Thur.	118	47.2
18. *S. hypogyna* (Pringsh.) de Bary	54	21.6
19. *S. parasitica* Coker	132	52.8
20. *S. salmonis* Hussein et Hatai	124	49.6
21. *S. shikotsuensis* Hatai *et al.*	18	7.2
22. *Thraustotheca clavata* (de Bary) Humphrey	32	12.8

prevalent. The most commonly encountered species on the pink salmon eggs were *Saprolegnia parasitica* (132 eggs- 52.8%), *Saprolegnia salmonis* (124 eggs- 49.6%), *Saprolegnia ferax* (118 eggs- 47.2%), *Achlya oligocantha* (101 eggs- 40.4%), *Achlya racemosa* (98 eggs-39.2%) and *Aphanomyces frigidophilus* (91 eggs- 36.4%). Such species as *Leptomitus lacteus*, *Saprolegnia shikotsuensis*, *Isoachlya anisospora* and *Achlya racemosa* were also observed on some eggs. The results of carbohydrate, urease and amino acid utilization have been showed in Tables 2 and 3. All stated species from genus *Achlya*, *Aphanomyces*, *Leptolegnia* and *Saprolegnia* assimilated glucose and starch, but they did not assimilate arabinose and salicin (excepting species of the genus *Pythium*). Urease has been assimilated by specimens from *Saprolegnia*, *Leptolegnia* and *Pythium* genera. Six from 12 amino acids tested, namely, methionine, lysine, ornithine, phenyloalanine, leucine and glycine could not be assimilated by the investigated fungi.

DISCUSSION

The straminipilous organisms, which have been encountered on fish eggs, are mostly the representatives of Saprolegniales, especially species from *Achlya*, *Saprolegnia* and *Aphanomyces* genus. The most frequently encountered straminipilous organisms on the eggs of pink salmon included such species of the *Saprolegnia* genus as *S. parasitica*, *S. salmonis*, *S. ferax*. Those species are commonly encountered in Salmonidae [11, 14, 32-35]. *S. salmonis* were firstly described on the eggs of sockeye salmon in Japan, *Oncorhynchus nerka* [29]. In this survey *S. salmonis* was also found in masu salmon *Oncorhynchus masou* and chum salmon *Oncorhynchus keta* [36]. *Saprolegnia salmonis* was observed on eggs of sea trout *Salmo trutta* [34] and on eggs of whitefish *Coregonus lavaretus* [37, 38]. The most frequently encountered are: *Achlya oligocantha, A. radiosa* and *Aphanomyces frigidophilus*. Those species of *Achlya* genus were observed on the eggs of the coregonid, salmonid and cyprinid taxa and from other fish families [11, 32-34, 39, 40]. *Aphanomyces frigidophilus* was first detected on the eggs of Japanese charr, *Salvelinus leucomaenis* [28]. This species was also found on eggs of masu salmon [12] and sea trout [34]. Three species from *Pythium* genus have been observed on pink salmon eggs. *Pythium diclinum* was observed as a parasite on the eggs of few fish species in India [41]. We have also observed its growth on the eggs of crucian carp *Carassius carassius* [39] and on the skin of piranha *Pygocentrus nattereri* [42].

Pythium monospermum, known as a phyto- and zoosaprophyte [27], was isolated from eggs of rainbow trout in Japan [12]. *Pythium ultimum* was observed on the eggs of cyprinid taxa [43].

The biology of pink salmon has been already well known [44, 45]. It is anadromous species which inhabits ocean and mountain and piedmont rivers with moderate to fast current and gravel bottom. It spends 18 months in the sea, after which spawning migration to the natal river or stream occurs. Because the species is less certain of its homing and there is a certain degree of wandering, streams as much as 640 km from natal stream may be used. Upon emerging from the gravel, fry immediately moves downstream and remain inshore for a few months before going out to the sea. The period, in which pink salmon stays in mountain and piedmont rivers or streams is the most dangerous, when it is about fungal infection. Environmental conditions in those kinds of water bodies fulfill the infectional conditions of fungal species. In this environment, there is also plenty of food- substrate such as dead representatives of pink salmon after spawning and left eggs. It refers to all species of Pacific Ocean salmons.

Fungus- like organisms including Saprolegniales occur in water reservoirs in well oxygenated layers. The oxygen optimum for the genus *Achlya* ranges from 12.0 to 15.0, while for the genus *Saprolegnia* the range is 8.0 to 12.0 mg O_2 l^{-1} [46]. Moreover, the optimum temperatures for those genera oscillate between 0.0 and 10.0ºC. Such thermal and oxygen conditions can be found at the spawning sites of salmonid species. The fungi species, developing on dead representatives of salmon or on eggs, leave in the environments spores from which the hyphae of this fungal species will develop on dead specimens or eggs of the next generation of Pacific Ocean salmons. The presence of "homing instinct" in representatives of *Oncorhynchus* suggests that in certain part of the river or stream where the spawning takes

Table 2. Carbohydrate and urease assimilation by the microfungi- like organisms isolated from pink salmon eggs.

Species	Fru	Glu	Ara	Man	Gal	Xyl	Sor	Raf	Suc	Mal	Lac	Mel	Cel	Tre	Sta	Dex	Rha	Gly	Sal	Ure
Achlya klebsiana	+	+	-	+	x	x	x	+	+	+	+	+	+	+	+	+	+	+	-	-
A. radiosa	+	+	-	+	x	x	x	+	+	+	+	+	+	+	+	+	+	+	-	-
A. treleaseana	+	+	-	+	x	x	x	+	+	+	+	+	+	+	+	+	+	+	-	-
Aphanomyces frigidophilus	-	+	-	-	x	x	x	-	-	x	x	-	-	-	+	-	-	-	-	-
A. laevis	-	+	-	-	x	x	x	-	-	-	x	-	-	-	+	-	-	-	-	-
Leptolegnia caudata	+	+	x	+	-	x	x	-	-	+	-	+	+	+	+	+	-	+	-	+
Pythium monospermum	+	+	-	+	+	x	x	+	+	+	+	+	+	+	+	+	+	+	+	+
P. ultimum	+	+	-	+	+	x	x	+	+	+	+	+	+	+	+	+	+	+	+	+
Saprolegnia australis	+	+	-	+	x	x	x	-	-	+	x	-	+	+	+	+	-	+	-	+
S. diclina	+	+	-	+	x	x	x	-	-	+	x	-	+	+	+	+	-	+	-	+
S. ferax	+	+	-	+	x	x	x	-	-	+	x	-	+	+	+	+	-	+	-	+
S. hypogyna	+	+	-	+	-	-	x	-	-	+	-	+	+	+	+	+	-	+	-	+
S. parasitica	+	+	-	+	x	x	x	-	-	+	x	-	+	+	+	+	-	+	-	+
S. salmonis	+	+	-	+	x	x	x	-	-	+	x	-	+	+	+	+	-	+	-	+
S. shikotsuensis	+	+	-	+	x	x	x	-	-	+	x	-	+	+	+	+	-	+	-	+

"+" positive; "-" negative; "x" no growth.
Abbreviations: Fru- Fructose, Glu- Glucose, Ara- Arabinose, Man- Mannose, Gal- Galactose, Xyl- Xylose, Sor- Sorbose, Raf- Raffinose, Suc- Sucrose, Mal- Maltose, Lac- Lactose, Mel- Melibiose, Cel- Cellobiose, Tre- Trehalose, Sta- Starch, Dex- Dextrin, Rha- Rhamnose, Gly- Glycerol, Sal- Salicin, Ure- Urease.

Table 3. Amino acid assimilation by the microfungi- like organisms isolated from pink salmon eggs.

Species	Met	Asp	Lys	Glu	Orn	Phe	Arg	Leu	Ala	His	Cys	Gly
Achlya klebsiana	-	+	-	+	-	x	+	-	+	-	-	-
A. radiosa	-	+	-	+	-	x	+	-	+	-	-	-
A. treleaseana	-	+	-	+	-	x	+	-	+	-	-	-
Aphanomyces frigidophilus	x	-	-	+	-	x	-	-	+	-	+	-
A. laevis	x	-	-	+	-	x	-	-	+	-	x	-
Leptolegnia caudata	-	+	-	+	-	-	-	-	+	-	-	-
Pythium monospermum	-	-	-	-	-	x	-	-	+	+	x	-
P. ultimum	-	-	-	-	-	-	-	-	+	-	-	-
Saprolegnia australis	-	+	-	+	-	-	+	-	+	+	-	-
S. diclina	-	+	-	+	-	-	+	-	+	+	-	-
S. ferax	-	+	-	+	-	-	+	-	+	+	-	-
S. hypogyna	-	+	-	+	-	-	-	-	+	-	-	-
S. parasitica	-	+	-	+	-	-	-	-	+	-	-	-
S. salmonis	-	+	-	+	-	-	+	-	+	+	-	-
S. shikotsuensis	-	+	-	+	-	-	+	-	+	+	-	-

"+" positive; "-" negative; "x" no growth.
Abbreviations: Met- Methionine, Asp- Asparagine, Lys- Lysine, Glu- Glutamine, Orn- Ornithine, Phe- Phenylalanine, Arg- Arginine, Leu- Leucine, Ala- Alanine, His- Histidine, Cys- Cysteine, Gly- Glycine.

place, constant interactions between population of pink salmon and water fungi are being created. So, in different rivers and streams occur various types of fungal infections. Beside common cosmopolitan species of *Saprolegnia ferax* or *S. parasitica* developing on eggs of all fish species, the development of rare fungal species in particular water reservoirs has been stated. In our study these are: *Leptomitus lacteus*, *Isoachlya anisospora* or *Thraustotheca clavata*.

Of great significance were the environmental conditions of a respective water course during the spawning period and growth of salmonid juveniles (before they enter the sea) and how much do the stressogenic factors affect both-reproduction and growth of the fish [47]. Those factors include the influence of hydrology and waterway distance in a large river [9, 48], water pollution [49], food availability and quality [50]. To this group of factors belong also: viral, bacterial and fungal infections [51, 52], mycosporean parasite *Parvicapsula minibicornis* [53, 54], sea lice *Lepeoptheirus salmonis* [55-59], plerocercoid of *Diphyllobthrium nihonkainse* [60] and nematode *Anisakis* sp. [61]. Stressogenic factors in representatives of *Oncorhynchus* species cause not only changes in cortisol concentration [62] and lower body weight growth [63] and flesh colour [64] but first of all they have an influence on the quality of the gametes. According to Campbell *et al*. [65] stress reduces the quality of gametes produced by rainbow trout. Stressed representatives produce less gametes, which are also smaller and contain less carotenoids [34, 66, 67]. As it is known, the carotenoids increase disease resistance of organisms [68]. Therefore, in fish aquacultures, especially those from *Oncorhynchus* species, the most valuable are the populations which tolerate stressogenic factors well and are immune to viral and bacterial infections [69] and also to mycotic ones [70].

As described by Churikov and Gherrett [71] the pink salmon spawns in habitats which were repeatedly and profoundly affected by Pleistocene glacial advances. A strict two- year life cycle of pink salmon has resulted in two reproductively isolated broadlines, which spawn in alternating years and evolved as temporal replicates of the same species. Nowadays, the genome of pink salmon specimens is labile. Genetic changes in pink salmon were fund during acclimatization of specimens of the American populations [72] and of the Asian populations [73-75]. This phenomenon allows spreading of occurrence territory of this species [18] and select the forms which will tolerate stressogenic factors and will be immune to viral, bacterial and mycotic infections. As already known, those infections cause often huge losses among aquarium fish species [76], in natural reservoirs aquacultures [3, 35, 77] and in wild populations [78].

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