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Molecular identification of water molds (oomycetes) associated with chum salmon eggs from hatcheries in Japan and possible sources of their infection

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Abstract
Oomycete infection of various freshwater animals, including salmonid eggs, causes significant economic damage to aquaculture worldwide. In this study, we detected oomycetes in infected chum salmon *Oncorhynchus keta* eggs at two hatcheries in northern Japan, in the source water used for egg incubation, and in the air at the hatcheries to clarify the source(s) of oomycete transmission using a DNA molecular marker. Seven oomycete taxa, belonging to Saprolegniaceae and Pythiaceae, were detected from the infected eggs. From the source water used for egg incubation and the air at the hatcheries, nine oomycete taxa, including those found on infected eggs, were detected, suggesting that both water and air are potential sources of oomycete transmission. There is no report of airborne transmission of these oomycetes detected in this study so far. Regarding protection and sterilization against oomycete infection in aquaculture hatcheries, not only water used at hatcheries but also the air in hatcheries may need to be considered hereafter.

Keywords Oomycetes · Chum salmon eggs · Saprolegniaceae · Pythiaceae · Internal transcribed spacer 2

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Introduction

Water mold infection has a negative impact on freshwater fishes, amphibians, and their eggs, as well as crustaceans, and is caused by oomycetes (Fisher et al. 1975; Noga 1993; Densmore and Green 2007; Phillips et al. 2008; Fernández-Benéitez et al. 2011; Bruno et al. 2013). Particularly, the major pathogens causing infection in fishes and their eggs are Saprolegniaceae belonging to oomycetes, such as *Saprolegnia*, *Achlya*, and *Aphanomyces* (Hatai 1980; van den Berg et al. 2013). Among the Saprolegniaceae, *Saprolegnia diclina* and *S. parasitica* are thought to cause significant damage to salmon hatcheries (van den Berg et al. 2013). In fact, economic losses to hatcheries because of *Saprolegnia* infections reach 10–50% worldwide (Phillips et al. 2008). In addition, the mortality of fish larvae caused by *Pythium*, belonging to the other oomycete family Pythiaceae, has also been reported in Japan (Miura et al. 2010).

To treat oomycete infection, malachite green was used in salmon hatcheries, but negative effects of malachite green have also been reported, such as the persistence of the medicine and carcinogenic effects on the organs of the treated fishes and their eggs. Thus, the use of malachite green has been banned worldwide since 2000 to ensure food hygiene (Miura et al. 2005; Sudova et al. 2007; Phillips et al. 2008). Some alternative chemical agents, such as bronopol, formalin, and hydrogen peroxide have been examined, but show lower effectiveness against water mold compared to malachite green and toxicity problems (Braidwood 2000; Ali et al. 2015). Therefore, in some chum salmon *Oncorhynchus keta* hatcheries in Japan, the staff manually removes the infected eggs individually.

Most hatcheries in Iwate Prefecture, northern Japan, were seriously damaged by the Great East Japan Earthquake and Tsunami on March 11, 2011. The number of released juvenile chum salmon decreased to 66%, and that of the returned chum salmon in 2011 and 2012 decreased to approximately 50% of that before the event (Ogawa 2014). In addition to the decreased numbers of eggs for artificial hatching because of the reduced number of returning adults, frequent water mold infection of eggs in hatcheries occurs in this area. However, little is known about the diversity of oomycetes causing water mold infection, as well as the possible sources of infection (such as water or air) in these hatcheries.

Oomycetes in salmon hatcheries are traditionally identified based on morphological observation (Kitancharoen et al. 1997; Hussein et al. 2001). However, molecular techniques using DNA markers, such as internal transcribed spacers (ITSs) of ribosomal RNA genes, have recently been used in many countries other than Japan (Lévesque and De Cock 2004; Diéguez-Uribeondo et al. 2009; Eissa et al. 2013; Sandoval-Sierra et al. 2014a, b). In the present study, using molecular techniques, the diversity and source of water molds infecting salmon eggs in hatcheries in northern Japan were investigated.

Materials and methods

Collection of chum salmon eggs and environmental samples from hatcheries

Approximately 30 eggs of chum salmon *Oncorhynchus keta* infected with water mold were collected from incubation water tanks at two chum salmon hatcheries in the Iwate Prefecture, northern Japan: Hatchery A on December 24, 2014, November 19, 2015, and January 7, 2016 and Hatchery B on November 25, 2014, January 13, 2015, November 20, 2015, and January 8, 2016 (Table 1). To investigate the source(s) of water mold infection in these hatcheries, 4 L of

source water used for egg incubation on November 19, 2015 and January 7, 2016 at Hatchery A and on November 20, 2015 and January 8, 2016 at Hatchery B were collected. The water was from well or ground (fresh) water. In addition, 4 L distilled water in a bucket without a cover was left for 2 weeks near the egg incubation tanks to evaluate the possibility of aerial infection. The temperature of the source water used for egg incubation was measured at the time of the sample collection. The collected eggs and water were stored at − 80 °C and 4 °C, respectively.

Cultivation of oomycetes

Half (2 L) of each of the source water samples used for egg incubation and the water left for 2 weeks was concentrated to approximately 50 mL using a 0.22-μm filter. To cultivate oomycetes from these samples, sterilized hemp seeds as bait for oomycetes were added to each water sample in Petri dishes (Diéguez-Uribeondo et al. 2007). The hemp seed cultures were incubated for 2 weeks at 5, 10, and/or 15 °C based on the temperatures of incubation water tank at hatcheries A and B shown in Table 1. Hemp seeds covered by colonies of oomycetes were preserved at − 80 °C.

Sequencing

Genomic DNA was extracted from the water molds associated with eggs in the incubation tanks of the hatcheries and with hemp seeds in the cultures using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To determine the quality of the extracted DNA, the bacterial 16S rRNA and eukaryotic 18S rRNA genes were amplified using the universal primers 16S B27F (5′-AGAGTTTGATCCTGGCTCAG-3′)/16S U1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg et al. 1991) and 18SF (5′-AACC TGGTTGATCCTGCCAG-3′)/18SR (5′-AACCTGGTTGATCCTGCCAG-3′) (Ota et al. 2009), respectively. Next, the ITS 2 region of ribosomal RNA genes (ITS2) was PCR-amplified with the oomycete-specific primers Oom-Up5.8S (5′-TGCGATACGTAATG CGAATT-3′) and Oom-Lo28S (5′-ACTTGTTCGCTATCGGTCTCGCA-3′) (Tambong et al. 2006; Liu et al. 2014). The PCR was performed using TaKaRa Ex Taq (TaKaRa, Shiga, Japan) or HotStarTaq (Qiagen) under the following conditions: 30–40 cycles of denaturation at 94 °C

Table 1 Collection data of chum salmon eggs and environmental samples at two hatcheries. "White circle" indicates obtained samples

	Date of collection	Water tank temperature (°C)	Type of samples		
			Infected eggs	Water used for egg incubation in tank	Air in hatchery[a]
Hatchery A	Dec. 24, 2014	6.0	○		
	Nov. 19, 2015	12.7	○	○	○
	Jan. 7, 2016	7.8	○	○	○
Hatchery B	Nov. 25, 2014	14.7	○		
	Jan. 13, 2015	11.5	○		
	Nov. 20, 2015	15.2	○	○	○
	Jan. 8, 2016	12.8	○	○	○

[a] Water samples left for 2 weeks near the incubation tank of each hatchery were investigated

for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The PCR products obtained, approximately 800–900 bp in size, were cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) or pGEM T Easy using the pGEM T Easy system (Promega, Madison, WI, USA). The resulting clones were sequenced with a 3730xl Genetic Analyzer DNA AutoSequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The obtained sequences were subjected to blast searches. In addition, obtained *Saprolegnia* sequences were checked as described by Sandoval-Sierra et al. (2014b), in which *Saprolegnia* species with misidentified names in public DNA databases were carefully assessed by phylogenetic analyses.

Phylogenetic analyses

The ITS2 region sequences of oomycetes detected in this study were separately aligned with the corresponding sequences from phylogenetically diverse Saprolegniaceae and Pythiaceae using MEGA 7 (Kumar et al. 2016) and MAFFT (Katoh and Standley 2013). The alignments of Saprolegniaceae and Pythiaceae were trimmed by removing ambiguously aligned regions using trimAl version 1.3 with the option "automated1" (Capella-Gutiérrez et al. 2009). The maximum likelihood (ML) phylogenetic trees and corresponding bootstrap support values (1000 replicates) were calculated using MEGA 7 software for the resulting datasets (40 taxa/419 positions for Saprolegniaceae and 34 taxa/682 positions for Pythiaceae). Initial trees for the heuristic search were obtained automatically by applying the neighbor joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with the superior log-likelihood. For these datasets, Bayesian analyses were performed using MrBayes v3.2.5 (Ronquist et al. 2012). Six parallel metropolis-coupled Markov chain Monte Carlo (MCMCMC) runs, each consisting of three heated chains and one cold chain with default chain temperatures, were run for 1,000,000 generations. Log-likelihood scores and trees with branch lengths were sampled every 1000 generations. The first 250,000 generations were excluded as burn-in, and the remaining trees were summarized to obtain Bayesian posterior probabilities. Convergence of parallel MCMCMC runs was judged by the average SD of split frequencies. For both ML and Bayesian analyses, the most appropriate models selected with PhyML 3.0 (Lefort et al. 2017) were applied. The sequences detected in this study showing identity of more than 98% with those of described species were identified at the species level. The sequences determined in this study were deposited into the DDBJ/EMBL/GenBank databases under accession nos. LC369091–LC369095, LC369097–LC369099, and LC369101–LC369103.

Results

Identification by phylogenetic analyses

Phylogenetic analyses of the Saprolegniaceae sequences determined in this study revealed four taxa of the genus *Saprolegnia* (*S. australis*, *S. diclina*, *S. ferax*, and *S. parasitica*) and one taxon of the genus *Aphanomyces* (*A. laevis*) at the species level, while two taxa of the genus *Saprolegnia* and one taxon of genus *Leptolegnia* were not identified at the species level (hereafter, referred to as *Saprolegnia* sp. 1, *Saprolegnia* sp. 2, and *Leptolegnia* sp. 1,

respectively) (Fig. 1). Among Pythiaceae, one taxon of the genus *Pythium* (*P. monospermum*) was identified at the species level, while two taxa of this genus were not identified at the species level (hereafter, referred to as *Pythium* sp. 1 and *Pythium* sp. 2) (Fig. 2).

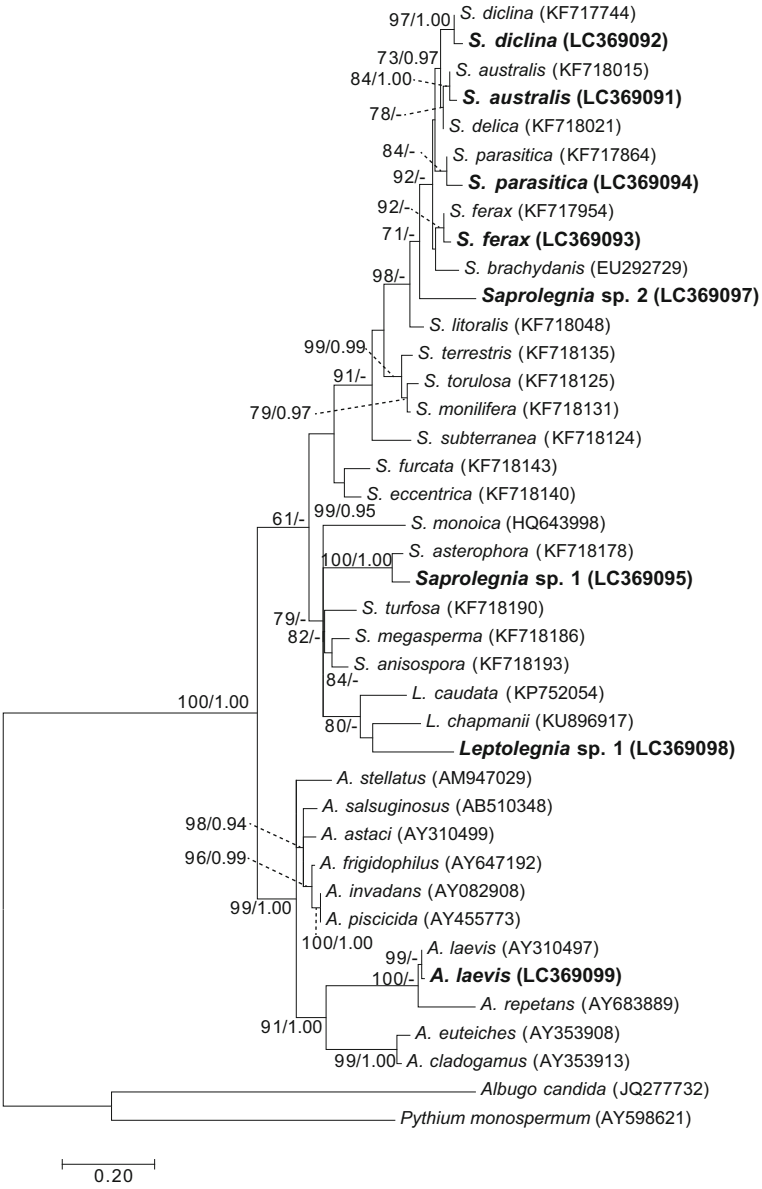

Fig. 1 Maximum likelihood (ML) phylogeny of internal transcribed spacer 2 region of ribosomal RNA genes from Saprolegniaceae (*Saprolegnia*, *Leptolegnia*, and *Aphanomyces*). *Pythium monospermum* (Pythiaceae) and *Albugo candida* (Albuginaceae) were used as outgroups to root the tree. ML bootstrap probabilities and Bayesian posterior probabilities for bipartitions with over 65% and 0.90 support, respectively, are shown. Sequences determined in this study are shown in bold. DDBJ/ EMBL/GenBank databases accession numbers are shown in parentheses

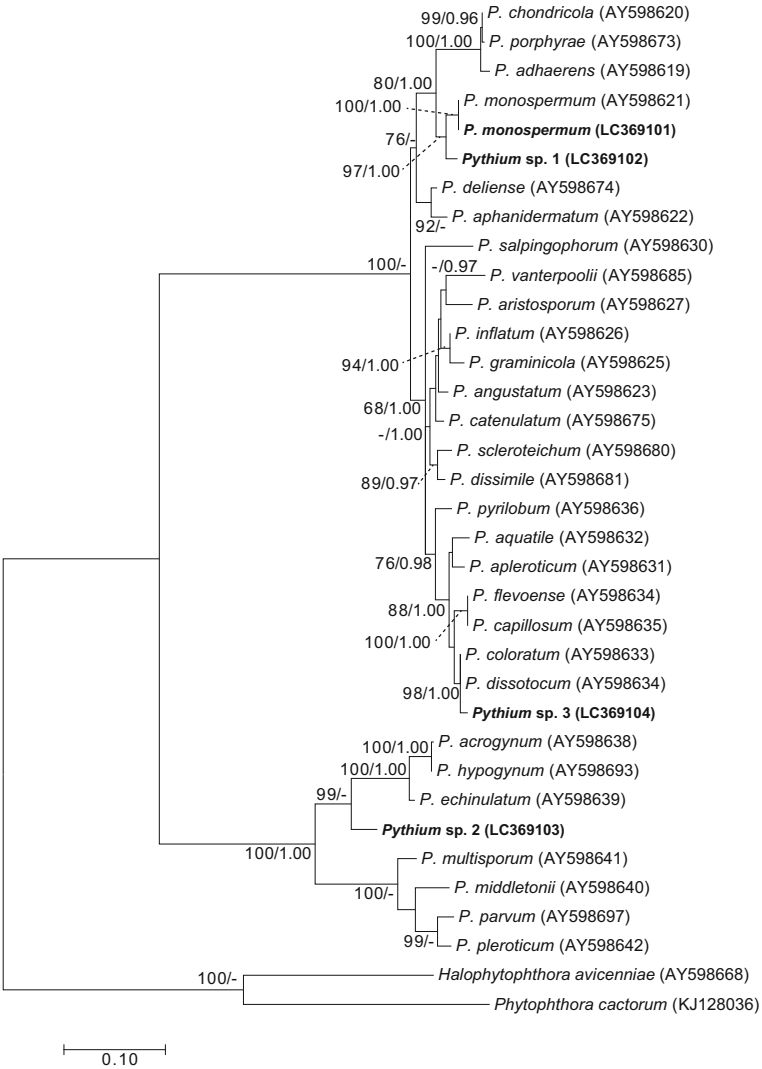

Fig. 2 Maximum likelihood (ML) phylogeny of internal transcribed spacer 2 region of ribosomal RNA genes from Pythiaceae. *Halophytophthora avicenniae* (Pythiaceae) and *Phytophthora cactorum* (Pythiaceae) were used as outgroups to root the tree. ML bootstrap probabilities and Bayesian posterior probabilities for bipartitions with over 65% and 0.90 support, respectively, are shown. Sequences determined in this study are shown in bold. DDBJ/EMBL/GenBank databases accession numbers are shown in parentheses

Oomycetes associated with infected eggs at the hatcheries

In Hatchery A, two Saprolegniaceae taxa and three Pythiaceae taxa were identified from the infected eggs (Table 2). *Saprolegnia* sp. 1 was detected in December 2014, November 2015, and January 2016 samples. *Aphanomyces laevis* and *Pythium* sp. 1 were detected in December 2014. *Pythium monospermum* was found in November 2015. *Pythium* sp. 1 and *Pythium* sp. 2 were identified in January 2016. In Hatchery B, three Saprolegniaceae taxa and two Pythiaceae taxa were detected from the infected eggs. *Saprolegnia parasitica* was detected in November

2014. *Pythium monospermum* and *Pythium* sp. 1 were identified in January 2015, while *P. monospermum* was detected in November 2015. *Saprolegnia ferax* and *A. laevis* were found in January 2016.

Oomycetes associated with water used for egg incubation in tank and water left for 2 weeks

In Hatchery A, six taxa (*S. australis*, *S. ferax*, *Saprolegnia* sp. 1, *Saprolegnia* sp. 2, *Pythium* sp. 1, and *Pythium* sp. 2) were detected from hemp seed cultures at 5 °C with tank water used for egg incubation in January 2016 (Table 3). From the hemp seed cultures at 5 °C and 10 °C with water left for 2 weeks near the incubation tanks, nine taxa (*S. australis*, *S. diclina*, *S. ferax*, *S. parasitica*, *Saprolegnia* sp. 1, *Saprolegnia* sp. 2, *Leptolegnia* sp. 1, *Pythium* sp. 1, and *Pythium* sp. 2) were detected at Hatchery A in January 2016. *Saprolegnia* sp. 2 and *Pythium* sp.1 were found only from the hemp seed culture at 5 °C, while *S. diclina*, *S. ferax*, *S. parasitica*, and *Leptolegnia* sp. 1 were found only from the culture at 10 °C. At Hatchery B, one taxon (*Saprolegnia* sp. 2) was detected from hemp seed cultures at 15 °C with water used for egg incubation from the tank in November 2015. One taxon (*S. ferax*) and five taxa (*S. australis*, *S. diclina*, *S. ferax*, *S. parasitica*, and *Leptolegnia* sp. 1) were detected from the hemp seed culture at 15 °C with water left for 2 weeks in November 2015 and from that at 10 °C in January 2016 at Hatchery B, respectively.

Discussion

In this study, *Saprolegnia ferax*, *S. parasitica*, *Aphanomyces laevis*, and *Pythium monospermum* were identified from eggs infected by water molds. These species, except for *A. laevis*, were previously reported to infect eggs of salmonid fishes at Japanese hatcheries (Kitancharoen et al. 1997). *A. laevis* often occurs as a dominant species in Japanese lake water (Suzuki 1960), but this species was also found on eggs of a salmonid fish in Poland (Czeczuga et al. 2005).

In the present study, the sequences of not only Saprolegniaceae taxa but also Pythiaceae taxa (*P. monospermum* and *Pythium* sp.1) were frequently recovered. Kitancharoen et al.

Table 2 Oomycetes species detected from chum salmon eggs infected with water mold. "+" and "–" indicate detected and undetected, respectively

	Hatchery A			Hatchery B			
	Dec. 24, 2014	Nov. 19, 2015	Jan. 7, 2016	Nov. 25, 2014	Jan. 13, 2015	Nov. 20, 2015	Jan. 8, 2016
Saprolegnia ferax	–	–	–	–	–	–	+
S. parasitica	–	–	–	+	–	–	–
Saprolegnia sp. 1	+	+	+	–	–	–	–
Aphanomyces laevis	+	–	–	–	–	–	+
Pythium monospermum	–	+	–	–	+	+	–
Pythium sp. 1	+	–	+	–	+	–	–
Pythium sp. 2	–	–	+	–	–	–	–

Table 3 Oomycetes species detected from environmental samples in hatcheries and chum salmon eggs. "+" and "–" indicate detected and undetected, respectively

	Water used for egg incubation in tank							Air in hatchery[a]						
	Hemp seed culture							Hemp seed culture						
	Hatchery A				Hatchery B			Hatchery A				Hatchery B		
	Nov. 19, 2015		Jan. 7, 2016		Nov. 20, 2015	Jan. 8, 2016		Nov. 19, 2015		Jan. 7, 2016		Nov. 20, 2015		Jan. 8, 2016	
Water temperature of oomycete cultivation (°C)	10	15	5	10	15	10	15	10	15	5	10	10	15	10	15
Saprolegnia australis	–	–	+	–	–	–	–	–	–	+	+	–	–	+	–
S. diclina	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
S. ferax	–	–	+	–	–	–	–	–	–	–	+	–	+	+	–
S. parasitica	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
Saprolegnia sp. 1	–	–	+	–	–	–	–	–	–	+	+	–	–	–	–
Saprolegnia sp. 2	–	–	+	–	+	–	–	–	–	+	–	–	–	–	–
Leptolegnia sp. 1	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
Pythium sp. 1	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–
Pythium sp. 2	–	–	+	–	–	–	–	–	–	+	+	–	–	–	–

[a] Water samples left for 2 weeks near the incubation tank of each hatchery were investigated

(1997) reported that the genus *Saprolegnia*, *Leptolegnia*, and *Achlya* of the Saprolegniaceae family were dominant, while the genus *Pythium* was not dominant on infected eggs of salmonid fishes. Czeczuga et al. (2014) also rarely found *P. monospermum* on eggs from a salmonid fish in Poland. However, based on our findings, oomycetes other than Saprolegniaceae such as *P. monospermum* dominantly colonized when no Saprolegniaceae species were present (see infected eggs of Hatchery B). It remains unclear which environmental factors, such as water temperature, are significantly related to the occurrence of *Pythium* and Saprolegniaceae species.

Among the oomycete taxa detected from the infected eggs, sequences of *S. ferax*, *S. parasitica*, *Saprolegnia* sp. 1, *Pythium* sp. 1, and *Pythium* sp. 2 were retrieved from the water used for egg incubation in the tanks and water left near the incubation tanks at both hatcheries. These findings suggest that both water for egg incubation and air are sources of water mold transmission. Regarding airborne transmission, in oomycete species infecting to terrestrial plant (such as *Peronospora tabacina* and *Phytophthora infestans*), aerial dispersion of their zoospores has been reported (Aylor 2003; Glais et al. 2014), like bacteria and fungi (Yanong and Erlacher-Reid 2012). Although there is no report about the airborne transmission of oomycete species detected in this study, it is possible that the zoospores of water molds infecting salmon eggs can also float in the air as aerosols.

In salmonid hatcheries, water used for incubation is known to be a source of oomycete invasion (Bruno et al. 2011). To prevent invasion from this route, disinfection of the water for incubation has been attempted by ultraviolet (UV) light, ozone, and hydrogen peroxide (Heikkinen et al. 2013). Markedly, UV irradiation experiments performed by Vlasenko (1969) and Kimura et al. (1980) showed that zoospores of oomycetes (*Saprolegnia*) were more sensitive than their hyphae. Based on the results found in the present study, it is highly likely that the air in hatcheries, as well as the water for incubation, is a route of oomycete invasion. In public human health, to reduce airborne disease transmission by bacteria and

spores of fungi, the effectiveness of UV air filters and air ultraviolet germicidal irradiation (UVGI) in the indoor environment has been reported (Kujundzic et al. 2006, 2007; Xu et al. 2011; Kim and Kang 2018). Thus, such treatments could also be applied to control the possible airborne transmission of oomycete zoospores in hatcheries, which may be a new viewpoint to address the problem of oomycete infection.

The sequences of *Pythium monospermum* and *A. laevis* detected from the infected eggs were not recovered from the source water used for egg incubation and water left near the incubation tank. These species may be very rare in our samples for analyzing the infection sources. Otherwise, we cannot completely exclude the possibility that these two taxa were transmitted by artificial contamination at the time of egg collection (Yanong and Erlacher-Reid 2012).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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