Short communication

The Sea Squirt *Styela clava* is a Potential Carrier of the Kinetoplastid *Azumiobodo hoyamushi*, the Causative Agent of Soft Tunic Syndrome in the Edible Ascidian *Halocynthia roretzi*

Akira Kumagai^{1*}, Keiichi Sakai¹ and Satoshi Miwa²

¹Miyagi Prefecture Fisheries Technology Institute
Kesennuma Fisheries Experimental Station,
Miyagi 988-0181, Japan

²National Research Institute of Aquaculture, Fisheries
Research Agency, Mie 516-0193, Japan

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ABSTRACT—The stalked sea squirt, *Styela clava*, was examined for the presence of the kinetoplastid, *Azumiobodo hoyamushi*, the causative agent of soft tunic syndrome in the edible ascidian, *Halocynthia roretzi*. Apparently healthy *S. clava* individuals were collected from a *H. roretzi* culture site, which was epizootic area of *A. hoyamushi*. After rearing for 50 days, the tunics of seven out of 48 specimens became softened and were found infected with *A. hoyamushi*. Healthy *H. roretzi* were experimentally infected with *A. hoyamushi* by rearing in water containing tunic from the diseased *S. clava*. Our results indicate that *S. clava* is a potential carrier of *A. hoyamushi*.

Key words: *Styela clava*, *Azumiobodo hoyamushi*, soft tunic syndrome, *Halocynthia roretzi*

The mass mortality of cultured ascidians, *Halocynthia roretzi*, associated with soft tunic syndrome has resulted in significant economic losses within the ascidian farming industry in Korea since 1995 (Jung *et al.*, 2001). In 2007, the disease was also observed in ascidian farms in Japan. The outbreaks in Japan occurred in areas where the spat had previously been introduced from Korea (Kumagai *et al.*, 2010). Since then, the disease has recurred annually at these farming sites, and the number of affected areas has increased (Kumagai *et al.*, 2010). The causative agent of soft tunic syndrome, a kinetoplastid flagellate,

Azumiobodo hoyamushi, was identified and isolated from diseased ascidians in Japan (Kumagai *et al.*, 2011; Hirose *et al.*, 2012). Recently, the flagellate has also been isolated from affected ascidians at Korean farming sites (Kim *et al.*, 2014).

The causative agents of several fish and shellfish diseases that cause serious problems within the aquaculture industry have been documented in wild animals that are closely related to the cultured species. Such wild species can act as disease carriers, causing outbreaks of the disease in farmed animals. For example, white spot syndrome virus (WSSV) was first introduced to a farming site of kuruma shrimp *Marsupenaeus japonicus* in Japan in 1993 by infected juvenile shrimps that were imported from China (Inouye *et al.*, 1994; Takahashi *et al.*, 1994). The disease rapidly spread to most of the shrimp farming sites throughout Japan, and the pathogen was later detected in a number of wild crustacean species (Maeda *et al.*, 1998; Momoyama, 2003). Similarly, outbreaks of bacterial cold-water disease (BCWD) have occurred in cultured ayu, *Plecoglossus altivelis*, in Japan since the mid-1980s (Wakabayashi *et al.*, 1994) and the causative bacterium has also been identified in other wild fish species (Iida and Mizokami, 1996; Izumi *et al.*, 2007).

The stalked sea squirt, *Styela clava*, is an ascidian that belongs to the same suborder (Stolidobranchia) as *Halocynthia roretzi*. The species inhabits the coasts of Japan at depths of 0 to 25 m. *Styela clava* attaches to rocks, wood, shells, or other hard surfaces and commonly occurs in colonies on the ropes or floats of facilities that culture edible ascidians. The body of *S. clava* is completely covered with a hard tunic (2–3 mm thick) so is hard and elastic, like *H. roretzi*. We evaluated the occurrence of *A. hoyamushi* in a population of *S. clava* collected from an ascidian culture facility that had previously experienced recurring outbreaks of soft tunic syndrome.

Materials and Methods

Sampling and rearing of apparently healthy *S. clava*

The sampling was conducted in April 2010 at an *H. roretzi* culture facility that had experienced recurring disease outbreaks since 2008 (peak mortality = 50%). We visually observed the condition of approximately 100 *S. clava* individuals, but did not observe any individuals with typical clinical signs of soft tunic syndrome. Therefore, apparently healthy *S. clava* ($n = 48$, mean body weight = 27 g) that were attached to *H. roretzi* culture ropes were collected and transported on ice to the Miyagi Prefecture Fisheries Technology Institute (MPFTI). The sea squirts were reared in an aquarium filled with 50 L of aerated, running, pathogen-free seawater for 50 days. The experimental aquarium was supplied with seawater that was pumped from offshore

* Corresponding author
E-mail: kumagai-ak557@pref.miyagi.jp

of the laboratory and filtered through a sand filter. The absence of *A. hoyamushi* in the rearing water was confirmed in previous experiments (Kumagai *et al.*, 2010). The rearing water temperature ranged from 9°C to 16°C. Softening of the tunics were monitored by touching each specimen every 2 days. Dead *S. clava* were removed from the aquarium immediately to test for the presence of *A. hoyamushi*.

Testing for the presence of *A. hoyamushi* in dead *S. clava*

All dead *S. clava* ($n = 7$) were tested for the presence of *A. hoyamushi* using two methods, direct observation and PCR targeting against *A. hoyamushi* 18S rRNA. Direct observation was carried out as follows: the tunic (5 g) of each dead *S. clava* was cut into small pieces (approximately 3×1 cm) and each piece was put into a polyethylene bag (8.5×6 cm) containing 20 mL of sterilized seawater and incubated at 15°C overnight. After incubation, 100 μ L of the seawater from each bag was placed on a glass slide and observed under a light microscope for *A. hoyamushi* flagellates. For the PCR assay, the remaining seawater (1.5 mL) was centrifuged ($15,100 \times g$, 10 min) and DNA was extracted from the pellet using a DNeasy Blood and Tissue Kit (Qiagen). We performed PCR using primers for *A. hoyamushi* 18S rDNA, as described by Kumagai and Kamaishi (2013).

One of seven dead *S. clava* was histopathologically examined for *A. hoyamushi* according to the method of Kumagai *et al.* (2010). Two or more tunic pieces were cut from the dead animal and fixed in Davidson's fixative (Bell and Lightner, 1988) overnight. Tissues were then dehydrated in an ethanol series and embedded in paraffin wax via xylene. Sections were cut at 3 μ m and stained with Mayer's hematoxylin and eosin (H & E).

Infection trial

An infection trial was carried out at the MPFTI using the tunics of the dead *S. clava* ($n = 7$), which were all positive for the *A. hoyamushi* flagellate based on direct observation, as the infection source. The dead sea squirts were divided into two groups ($n = 3$ and 4). The tunics from each group were cut into small pieces (ca. 3×1 cm), pooled and put into a polyethylene net so that the net contained 20 g of tunic pieces. The mesh size of the net was 7.5×7.5 mm. Each net was then suspended in a 25 L aquarium containing 12 healthy ascidians *H. roretzi*, (2 years old, 160 g body weight) for 4 days. The aquarium was filled with 20 L of aerated seawater. The seawater was not changed for the first 4 days of the experiment. Five days after the start of the experiment, new seawater was introduced into the aquaria, and thereafter all ascidians were reared in 20 L of aerated, running seawater. In the control group, the tunics of healthy *H. roretzi* were

prepared as described above and suspended in an aquarium containing another 12 healthy animals. The healthy ascidians (Grade 0: see Kitamura *et al.*, 2010 for the criteria) were collected from a site at which the disease had not been detected. The rearing water temperature ranged from 16°C to 21°C. The experiment was conducted for 35 days, and the softening of the tunics was monitored by touching the ascidians every 2

Fig. 1. Gross view and histopathological sections (H & E stain) of diseased *Styela clava*. (A) Gross view. (B) Cross section of a diseased tunic containing of *Azumobodo hoyamushi* cells (*). (C) The diseased tunic at a higher magnification. Arrowheads indicate some of the *A. hoyamushi* cells.

Table 1. Experimental infection of healthy *Halocynthia roretzi* reared in seawater with tunic tissue of diseased *Styela clava*

Infection source (tunic pieces)	First appearance of clinical signs (day)* ¹	No. of diseased animals/ Total no. of animals
Diseased <i>S. clava</i> 1	8	9/12 (1)* ²
Diseased <i>S. clava</i> 2	8	8/12 (2)
Healthy <i>H. roretzi</i> (Control)	—	0/12

*¹ Days after the start of the experiment when softening of the tunic was first detected.

*² Numbers in parentheses indicates the numbers of ascidians that died because of factors other than soft tunic syndrome.

days. Dead ascidians were removed from the aquaria immediately. The presence of the *A. hoyamushi* flagellate was tested in all softened ascidians ($n = 17$ in total) by direct observation and PCR, as described above.

Results

Rearing of apparently healthy *S. clava*

A dead animal was first observed 15 days after the start of the experiment. In total, 7 specimens (15%) were found dead during the 50 days rearing period. Tunics of the dead animals became softened, suggesting they had developed soft tunic syndrome (Fig. 1A). The affected tunics were softer and thinner than those of healthy individuals, but the condition was milder than in severely infected *H. roretzi* individuals; although the tunics lost its elasticity, there were no tears which are found in severe cases of affected *H. roretzi*. No clinical signs were found in the internal organs of the dead animals.

Presence of *A. hoyamushi* in dead *S. clava*

The flagellate was observed visually in all samples of the seawater that were incubated with the softened tunic of the dead *S. clava* ($n = 7$). The flagellate were fusiform, moving actively in seawater by using 2 flagella. The flagellates were also detected in all seven samples by PCR with the specific primers for *A. hoyamushi*. The presence of several flagellate cells was also confirmed by histological observation of the softened tunic of a dead *S. clava* individual (Fig. 1B, C).

Infection trial

When pieces of tunics from dead *S. clava* were immersed in the rearing water of healthy *H. roretzi*, 8 and 9 of 12 healthy ascidians (75% and 83%, respectively) were affected by the disease, exhibiting softened, weak tunics. The flagellates were detected in all 17 individuals by direct observation and PCR. There was no sign of the disease and no mortality in the control group. A few ascidians which died but still had hard tunics were thought to have died because of factors other than soft tunic syndrome (Table 1).

Discussion

The results of the present study strongly suggest that *A. hoyamushi* can be transmitted to wild populations of *S. clava* from neighboring populations of farmed *H. roretzi* that are affected by soft tunic syndrome. In addition, healthy *H. roretzi* were experimentally infected with *A. hoyamushi* in the infection test using diseased *S. clava* as the source of infection, indicating that *S. clava* is a potential carrier of *A. hoyamushi*.

Kumagai *et al.* (2013) showed that apparently healthy wild ascidians (*H. roretzi*) developed soft tunic syndrome while being reared in pathogen-free seawater in the lab, thereby demonstrating that the wild animals can be asymptomatic carriers of the pathogen. Similarly, we collected *S. clava* individuals that had no clinical signs of soft tunic syndrome and reared them in pathogen-free water. Several of these individuals later developed soft tunic syndrome. Thus, this approach appears to have utility for finding asymptomatic carriers of *A. hoyamushi* in both *H. roretzi* and *S. clava*.

Soft tunic syndrome reoccurred annually at ascidian farming sites in Japan following the initial outbreaks. Furthermore, the number of affected areas increased annually (Kumagai *et al.*, 2010) up until 2011, when the tsunami caused by the Great East Japan Earthquake destroyed all ascidian farming facilities. Ascidian culture was subsequently restarted using wild spat in December 2011. Although the first cohort is now 2 years old and susceptible to the disease (Kumagai *et al.*, 2010; Hirose *et al.*, 2014), there have been no signs of an outbreak to date. However, our results suggest that it is important to survey both cultured ascidians and wild *H. roretzi* and *S. clava*, as they may be carriers of the disease causative agent.

Styela clava is an invasive species that has been transported throughout the world (the USA, Canada, Europe, and Oceania) from the northeast Pacific Ocean (from the Sea of Okhotsk to Shanghai) during the last 80 years via ship fouling and oyster transfer. This species can handle adverse conditions or environmental stressors, affecting native biodiversity by replacing the indigenous competitive species (Clarke and Theriault, 2007). In addition to this problem, there is a major concern that *A. hoyamushi* may also spread worldwide and

affect native ascidians by the transportation of *S. clava* infected with the flagellate from epizootic areas in Japan and Korea. *Styela clava* is a seafood delicacy in South Korea. The consumer market for *S. clava* already exists in the Korean community in the USA (Karney and Rhee, 2009). Thus, the spread of *A. hoyamushi* through the commercial distribution of infected animals is also concerned.

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References

- Bell, T. A. and D. V. Lightner (1988): A handbook of normal penaeid shrimp histology. Allen Press, Lawrence, KS.
- Clarke, C. L. and T. W. Theriault (2007): Biological synopsis of the invasive tunicate *Styela clava* (Herdman 1881). *Can. Manuscr. Rep. Fish. Aquat. Sci.*, 2807, vi + 23 p.
- Hirose, E., A. Nozawa, A. Kumagai and S. Kitamura (2012): *Azumiobodo hoyamushi* gen. nov. et sp. nov. (Euglenozoa, Kinetoplastea, Neobodonida): the pathogenic kinetoplastid of the soft tunic syndrome in the ascidian aquaculture. *Dis. Aquat. Org.*, **97**, 227–235.
- Hirose, E., A. Kumagai, A. Nawata and S. Kitamura (2014): *Azumiobodo hoyamushi*, the kinetoplastid causing soft tunic syndrome in ascidians, may invade through the siphon wall. *Dis. Aquat. Org.*, **109**, 251–256.
- Iida, Y. and A. Mizokami (1996): Outbreaks of coldwater disease in wild ayu and pale chub. *Fish Pathol.*, **31**, 157–164.
- Inouye, K., S. Miwa, N. Oseko, H. Nakano, T. Kimura, K. Momoyama and M. Hiraoka (1994): Mass mortalities of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: Electron microscopic evidence of the causative virus. *Fish Pathol.*, **29**, 149–158. (in Japanese with English abstract)
- Izumi, S., S. Ouchi, T. Kuge, H. Arai, T. Mito, H. Fujii, F. Aranishi and A. Shimizu (2007): PCR-RFLP genotypes associated with quinolone resistance in isolates of *Flavobacterium psychrophilum*. *J. Fish Dis.*, **30**, 141–147.
- Jung, S. J., M. J. Oh, T. Date and S. Suzuki (2001): Isolation of marine birnavirus from sea squirts *Halocynthia roretzi*. In: "The biology of ascidians" (ed. by H. Sawada, H. Yokosawa and C. C. Lambert) Springer-Verlag, Tokyo, pp. 436–441.
- Karney, R. and W. Rhee (2009): Market potential for *Styela clava*, a non-indigenous pest invading New England coastal waters. *Aquat. Invas.*, **4**, 295–297.
- Kim, H. J., J. S. Park, K. H. Park, Y. K. Shin and K. I. Park (2014): The kinetoplastid parasite *Azumiobodo hoyamushi*, the causative agent of soft tunic syndrome of the sea squirt *Halocynthia roretzi*, resides in the East Sea of Korea. *J. Invert. Pathol.*, **116**, 36–42.
- Kitamura, S., S. Ohtake, J. Y. Song, S. J. Jung, M. J. Oh, B. D. Choi, K. Azumi and E. Hirose (2010): Tunic morphology and viral surveillance in diseased Korean ascidians: soft tunic syndrome in the edible ascidians, *Halocynthia roretzi* (Drasche), in aquaculture. *J. Fish Dis.*, **33**, 153–160.
- Kumagai, A., A. Suto, H. Ito, T. Tanabe, K. Takahashi, T. Kamaishi and S. Miwa (2010): Mass mortality of cultured ascidians *Halocynthia roretzi* associated with softening of the tunic and flagellate-like cells. *Dis. Aquat. Org.*, **90**, 223–234.
- Kumagai, A., A. Suto, H. Ito, T. Tanabe, J. Y. Song, S. Kitamura, E. Hirose, T. Kamaishi and S. Miwa (2011): Soft tunic syndrome in the edible ascidian *Halocynthia roretzi* is caused by a kinetoplastid protist. *Dis. Aquat. Org.*, **95**, 153–161.
- Kumagai, A. and T. Kamaishi (2013): Development of polymerase chain reaction assays for detection of the kinetoplastid *Azumiobodo hoyamushi*, the causative agent for soft tunic syndrome in the ascidian *Halocynthia roretzi*. *Fish Pathol.*, **48**, 42–47.
- Kumagai, A., H. Ito and R. Sasaki (2013): Detection of the kinetoplastid *Azumiobodo hoyamushi*, the causative agent of soft tunic syndrome, in wild ascidians *Halocynthia roretzi*. *Dis. Aquat. Org.*, **106**, 267–271.
- Maeda, M., T. Itami, N. A. Furumoto, O. Hennig, T. Imamura, M. Kondo, I. Hirono, T. Aoki and Y. Takahashi (1998): Detection of penaeid rod-shaped DNA virus (PRDV) in wild-caught shrimp and other crustaceans. *Fish Pathol.*, **33**, 373–380.
- Momoyama, K. (2003): Detection of white spot syndrome virus from small penaeid shrimp species caught in the western Seto Inland Sea. *Fish Pathol.*, **38**, 81–85.
- Takahashi, Y., T. Itami, M. Kondo, M. Maeda, R. Fujii, S. Tomonaga, K. Supamattaya and S. Boonyaratpalin (1994): Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). *Fish Pathol.*, **29**, 121–125.
- Wakabayashi, H., T. Toyama and T. Iida (1994): A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. *Fish Pathol.*, **29**, 101–104.