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Detection of the kinetoplastid *Azumiobodo hoyamushi*, the causative agent of soft tunic syndrome, in wild ascidians *Halocynthia roretzi*

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ABSTRACT: The occurrence of soft tunic syndrome in wild populations of the ascidian *Halocynthia roretzi* was monitored by diving at 5 to 6 sites in Miyagi Prefecture in Japan in summer 2010 and 2011. These sites were located at varying distances from farming sites at which the disease had previously been detected. All dead ascidians were collected, and their tunics were examined for *Azumiobodo hoyamushi,* the causative agent of soft tunic syndrome, using 18S rRNA PCR. In both years, <1% of wild ascidians we observed (18 out of 2100 in 2010, and 30 out of 3100 in 2011) were dead. The flagellates were only detected in 8 out of 18 dead ascidians from 3 sites in 2010, and 4 out of 30 from 2 sites in 2011. Healthy ascidians were successfully experimentally infected with the disease by immersing tunic samples from diseased wild ascidians into the rearing water. When apparently healthy ascidians collected from the wild population were reared for 40 d using pathogen-free water, the tunics of some ascidians became softened. The flagellates were detected in these individuals, which were diagnosed with soft tunic syndrome. Our results suggest that soft tunic syndrome affects the wild population of ascidians in Japan.

KEY WORDS: *Azumiobodo hoyamushi* · Soft tunic syndrome · Wild ascidian · *Halocynthia roretzi*

INTRODUCTION

Since 1995, mass mortality of cultured ascidians *Halocynthia roretzi* due to soft tunic syndrome has resulted in significant losses to the ascidian farming industry in Korea (Jung et al. 2001). In 2007, the disease was also documented in cultured ascidians in Miyagi Prefecture in Japan, an area where Korean spat was previously introduced (Kumagai et al. 2011). The diseased ascidians exhibit unique external symptoms in that the tunic, the outer covering of ascidians, becomes very soft and weak. Previously unidentified flagellates (10–14 × 2–3 µm) were observed in the softened tunic using light and electron microscopy and were confirmed experimentally as the causative agent of the disease (Kumagai et al. 2011). The unknown flagellate was originally described as *Azumiobodo hoyamushi* on the basis of the cytomorphology and molecular phylogeny (Hirose et al. 2012). According to the revised eukaryote classification (Adl et al. 2012), *A. hoyamushi* is a member of Neobodonida (Euglenozoa, Kinetoplastea, Metakinetoplastina).

Soft tunic syndrome has re-occurred annually at farming sites following an initial outbreak of the disease. Furthermore, the number of affected areas has increased since the disease was first detected (Kumagai et al. 2010). This has raised concerns regarding the spread to wild populations, because some diseases of fish and shellfish that cause problems in the aquaculture industry have subsequently also infected wild populations. For example, white spot syn-

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drome virus (WSSV) was first detected at kuruma prawn (*Penaeus japonicus*) farming sites in Japan in 1993 following the import of infected juvenile shrimps from China (Inouye et al. 1994, Takahashi et al. 1994). The disease was subsequently detected in wild-caught kuruma prawn in 1996 (Maeda et al. 1998, Mushiake et al. 1998). Similarly, outbreaks of bacterial cold-water disease (BCWD) in ayu (*Plecoglossus altivelis*) farms in Japan have been documented since the mid-1980s (Wakabayashi et al. 1994), but the disease was only found in wild ayu in 1993 in Hiroshima Prefecture (Iida & Mizokami 1996) and has subsequently spread throughout Japan (Inouye 2000).

We evaluated the occurrence of the soft tunic syndrome in wild populations of ascidians in the rocky shore near farming sites in 2010 and 2011. This encompassed the period before and after the Great East Japan Earthquake, which occurred on 11 March 2011. The resulting tsunami destroyed all ascidian farming facilities in Miyagi and Iwate Prefecture.

MATERIALS AND METHODS

Sampling of wild ascidians

We evaluated the presence of the disease in wild ascidian *Halocynthia roretzi* populations at 5 and 6 rocky shore sites in 2010 and 2011, respectively. Sites 1 to 5 were 0.3 to 4.0 km from the nearest ascidian farming site at which the disease was detected between May and July 2010. Site 6 was located in Sendai Bay, an area in which ascidians are not cultivated because of high water temperatures in summer. This site was 50 km from the nearest ascidian farming site at which the disease had been detected (Fig. 1). At each site, a diver conducted an annual survey for the disease by touching the tunic of 300 to 1100 individuals (surveys were conducted twice at Site 2 in 2011). All dead ascidians were collected to test for the presence of flagellates and infection. In addition, apparently healthy ascidians (n = 50) colonizing areas with dead individuals were collected at each site and reared at the Miyagi Prefecture Fisheries Technology Institute (MPFTI) in 2010. If dead ascidians were not found, apparently healthy individuals (n = 50) were randomly collected. All samples were transported on ice to the MPFTI. The surveys were conducted between June and August 2010 and between July and August 2011, since the outbreak of the disease was most severe in summer (Kumagai et al. 2010). The water depth at the sites ranged from 3 to 15 m.

Flagellate examination

The tunics (5 to 10 g ind.$^{-1}$) of the dead ascidians were cut into small pieces (ca. 3 × 1 cm) that were placed into a polyethylene bag (8.5 × 6 cm) containing 20 ml of sterilized seawater and incubated at 15°C overnight. Following incubation, 100 µl of the seawater from each bag was microscopically examined to confirm the presence or absence of the flagellates (direct observation). The remaining seawater (1.5 ml) was centrifuged (15 100 × *g*, 10 min) and DNA was extracted from the pellet using a DNeasy Blood

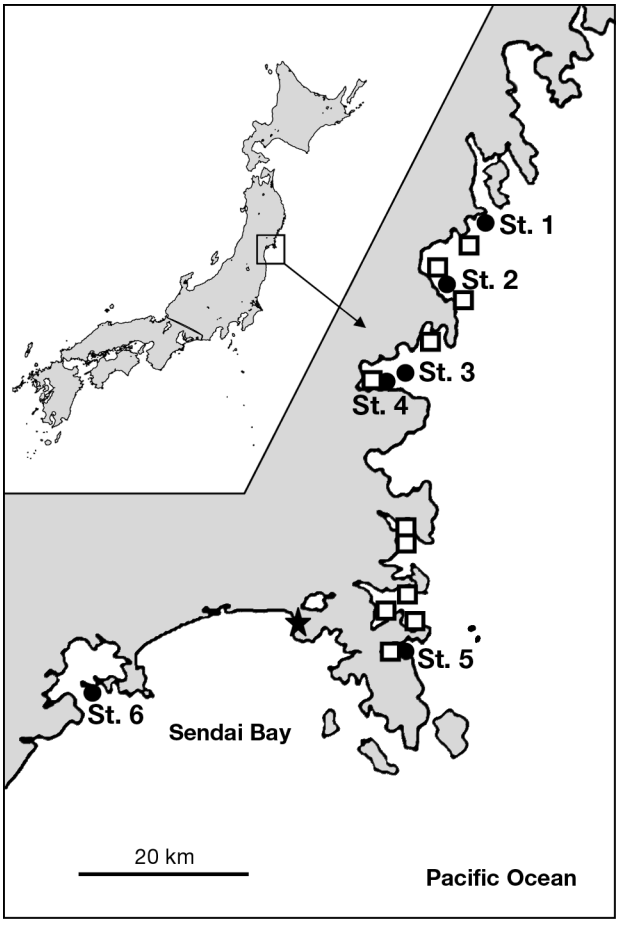

Fig. 1. Locations of sample collection and ascidian *Halocynthia roretzi* farming sites where the disease occurred along the coast of Miyagi Prefecture, Japan. (●): Sample collection sites; (□): ascidian farming sites where the disease occurred in 2010; (★): Miyagi Prefecture Fisheries Technology Institute, where the experimental infections were conducted

and Tissue Kit (Qiagen). We performed PCR using primers for the 18S rRNA, as described by Kumagai & Kamaishi (2013).

Infection test

Infection tests were carried out at the MPFTI. We used pooled ascidian tunic tissue from individuals (collected at Sites 2 and 4 in 2010) that were positive for the flagellate (based on direct observation) as an infection source. The experimental aquaria were supplied with seawater that was pumped from offshore of the laboratory and filtered through a sand filter. The pieces of tunic tissue from diseased ascidians (n = 2 [20 g] from Site 2, n = 1 [12 g] from Site 4) were placed into a polyethylene net (mesh size 7.5 × 7.5 mm) and suspended in a 25 l aquarium containing 12 healthy ascidians for 5 d. The aquarium was filled with 20 l of seawater that was aerated, but not exchanged, for the first 5 d of the experiment. Fresh seawater was introduced into the aquaria 6 d after the start of the experiment, and thereafter all ascidians were reared in 20 l of aerated, running seawater. We also set up a control in which the tunics (20 g) of healthy ascidians (n = 2) were treated in a similar manner and suspended in an aquarium containing 12 healthy animals. The healthy ascidians were collected from a site at which the disease had not been detected. The rearing water temperature ranged from 19.7 to 24.6°C. The experiment was conducted for 30 d, and the softening of the tunics was monitored by touching the ascidians every 2 d. Dead ascidians were removed from the aquaria immediately. The presence of the flagellate was tested in all softened ascidians (n = 15 in total) by direct observation and 18S rRNA PCR, as described above.

Rearing of apparently healthy ascidians

Apparently healthy ascidians collected at Sites 1 to 4 (n = 42 per site) in 2010 were reared at the MPFTI using pathogen-free water. These 42 ascidians were divided into 3 groups (n = 14 per group) and each group was reared in 20 l of aerated, running seawater (19.7 to 24.6°C) for 40 d. The control groups consisted of 12 healthy ascidians that were collected from a site where the disease had not occurred. We monitored for softening of the tunics by touching the ascidians every 2 d. We tested for the presence of flagellates in all dead ascidians (n = 15) by direct observation and using the 18S rRNA PCR assay, as described above.

RESULTS

Flagellate examination

In 2010, 18 dead ascidians (0.9%, 18 out of 2100) were found at Sites 1, 2, 4, and 5. These included animals whose tunics were still hard, but atrophied, and animals that only had a tunic around appendages for attachment to the substrate, without internal organs. Hence, the typical clinical signs of soft tunic syndrome were not observed in these individuals. Live flagellates were found in 5 out of 18 (28%) individuals examined by direct observation. In contrast, the flagellate was detected in 8 out of 18 (44%) individuals when the seawater was tested using 18S rRNA PCR. Although the flagellate was detected in samples collected in 3 of the 6 sites (Sites 2, 4, and 5), the prevalence of infection within the wild population was low (0.4%, 8 out of 2100) (Table 1).

In 2011, 30 dead ascidians (1.0%, 30 out of 3100) were collected at Sites 1, 2, 3, 5, and 6. The clinical signs exhibited by these individuals were similar to those collected in 2010, and samples with the typical clinical signs of the disease were not found. The flagellates were detected in only 1 out of 30 individuals (3%) during microscopic observation and in 4 out of 30 individuals (13%) by 18S rRNA PCR. The flagellate was detected only in samples collected at Sites 2 and 5, and the prevalence of infection (0.1%, 4 out of 3100) was lower than in 2010 (Table 1).

All the dead ascidians in which the PCR test for the flagellates was positive possessed a tunic only around their appendages, and thus these ascidians had probably died long before we collected them. In contrast, 55 and 89% of dead wild ascidians tested negative for *Azumiobodo hoyamushi* using primers for the 18S rRNA in 2010 and 2011, respectively (Table 1). These individuals possessed a complete hard, but atrophied, tunic and are thought to have been affected by factors other than soft tunic syndrome, such as stress due to of the presence of bryozoans or damage from the tsunami.

Infection test

When pieces of tunic from diseased animals were immersed in the rearing water of healthy individuals, 3 and 12 out of 12 healthy ascidians (25 and 100%, respectively) were subsequently affected by the disease, exhibiting softened, weak tunics. *Azu-*

Table 1. Detection of *Azumiobodo hoyamushi* in wild populations of *Halocynthia roretzi* ascidians in Miyagi Prefecture, Japan. Only the dead ascidians were collected and examined for *A. hoyamushi*. Distance is the distance from the nearest ascidian farming site at which the disease occurred. NE: not examined

Site	Distance (km)	Month	Diving observation		No. of ascidians positive for *Azumiobodo hoyamushi*/ No. of ascidians examined	
			No. of ascidians examined	No. of dead ascidians	Direct observation	18S rRNA PCR
2010						
1	4.0	Jul	300	4	0/4	0/4
2	0.3	Jul	300	3	2/3	3/3
3	0.4	Jun	800	0	NE	NE
4	0.5	Jun	300	3	1/3	1/3
5	0.4	Aug	400	8	2/8	4/8
Total			2100	18	5/18 (28%)	8/18 (44%)
2011						
1	4.0	Aug	500	8	0/8	0/8
2	0.3	Jul, Aug	1000[a]	12[a]	0/12	3/12
3	0.4	Aug	400	1	0/1	0/1
4	0.5	Aug	300	0	NE	NE
5	0.4	Jul	500	6	1/6	1/6
6	50	Aug	400	3	0/3	0/3
Total			3100	30	1/30 (3%)	4/30 (13%)

[a]The surveys were conducted in July and August; values represent the total from the 2 surveys

Table 2. *Azumiobodo hoyamushi* infecting *Halocynthia roretzi*. Test for infection of healthy individuals following rearing in seawater incubated with tunic tissue of diseased wild ascidians. Appearance of clinical signs is the day softening of the tunic was first detected after the start of the experiment. Numbers in parentheses indicate the number of ascidians that died because of factors other than the soft tunic syndrome

Infection source	Water temperature (°C)	Appearance of clinical signs (Day)	No. of diseased/ Total no. of ind.
Diseased (Site 2)	24.1–24.6	9	3/12 (1)
Healthy (control)		–	0/12 (1)
Diseased (Site 4)	19.7–23.4	10	12/12
Healthy (control)		–	0/12

Table 3. *Azumiobodo hoyamushi* infecting *Halocynthia roretzi*. Observations of disease following the rearing of apparently healthy wild ascidians. Appearance of clinical signs is the day softening of the tunic was first detected after the start of the experiment. Numbers in parentheses indicate the number of ascidians that died because of factors other than the soft tunic syndrome

Site	Water temperature (°C)	Appearance of clinical signs (Day)	No. of diseased/ Total no. of ind.
1	24.1–24.6	—	0/42 (20)
Control		—	0/14 (1)
2	24.1–24.6	5	7/42 (28)
Control		—	0/14 (1)
3	19.7–24.1	—	0/42 (4)
Control		—	0/14
4	19.7–24.1	11	8/42 (7)
Control		—	0/14

miobodo hoyamushi was detected in all 15 individuals by direct observation and using the 18S rRNA PCR assay. There was no sign of disease in the control group and very low mortality (Table 2).

Rearing of apparently healthy ascidians

The tunics of 7 and 8 out of 42 apparently healthy ascidians (17 and 19%) that had been collected at Sites 2 and 4, respectively, became softened. These individuals were considered to have developed soft tunic syndrome. The flagellate was observed in samples from all these individuals during microscopic observations. The flagellates were also detected in all 15 samples by 18S rRNA PCR. There was no sign of the disease in individuals collected at Sites 1 and 3 and in the control groups. At each site, 10 to 67% of ascidians with hard and atrophied tunics were thought to have died because of the stress of high temperature during sampling (Table 3).

DISCUSSION

We found no evidence of mass mortality in wild populations of ascidians despite their proximity to farming sites at which outbreaks of soft tunic syndrome had previously occurred. However, *Azumiobodo hoyamushi* was detected with low prevalence in dead ascidians in 2010 and 2011, indicating that a low proportion of wild ascidians (<1%) had died of soft tunic syndrome.

The disease has re-occurred annually at several farming sites following an initial outbreak, suggesting that the flagellate had become established in many ascidian farming areas in Japan (Kumagai et al. 2010). In addition, the number of affected areas has increased annually (Kumagai et al. 2010). This is probably due to dispersion of the flagellates from the torn tunics of diseased ascidians in local currents. We detected *Azumiobodo hoyamushi* in dead, wild ascidians collected near farming sites at which outbreaks of the disease have been confirmed but not from individuals collected in Sendai Bay, an area that is free of ascidian culture. Based on this, we speculate that the disease derived from cultured ascidians is being transmitted to wild ascidian populations.

Even after the 2011 tsunami destroyed all ascidian farming facilities, *Azumiobodo hoyamushi* was detected in wild population of ascidians. Following the tsunami, many of the rearing units at these culture facilities were retrieved from the seafloor and incinerated, because they were likely to be infection sources. However, the present results indicated that the disease still persisted in the wild populations of ascidians after this event.

Kumagai et al. (2009) demonstrated that apparently healthy ascidians collected from cultured ascidian lots affected with the disease were asymptomatic carriers of the pathogen. Therefore, we collected both apparently healthy ascidians and dead individuals to improve our sensitivity of detection for the flagellate. The disease was found only in healthy ascidians that were colonizing areas with dead individuals in which the flagellate was detected. Because the disease tends to spread sequentially from affected ascidians to neighboring individuals in ascidian farms (Kumagai et al. 2010), we suspect that the flagellates may also infect horizontally among colonies of wild ascidians. In this study, we demonstrated the presence of asymptomatic carriers by rearing apparently healthy ascidians.

The ascidian farming industry in Miyagi Prefecture was restarted using spat from wild ascidians in December 2011. Given that the industry is likely to expand rapidly, it is important to monitor for re-occurrence of the disease at farming sites. Our results suggest that *Azumiobodo hoyamushi* derived from wild populations of ascidians may be a cause of soft tunic synrome in cultured populations.

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