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Tunic extract of the host ascidian attracts the causal agent of soft tunic syndrome, *Azumiobodo hoyamushi* (Kinetoplastea: Neobodonida)

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ABSTRACT: *Azumiobodo hoyamushi*, a kinetoplastid flagellate, is the causative agent of soft tunic syndrome, an infectious disease of the edible ascidian *Halocynthia roretzi*. The flagellate is thought to invade the tunic matrix via a damaged area of the tunic on the siphon wall. We hypothesized that the flagellate locates the tunic entry site by a chemotactic response to soluble substances diffused from the host ascidians. To investigate this hypothesis, we examined whether the flagellate shows a chemotactic response to tissue extracts (tunic and other tissues) from the host ascidian *H. roretzi*. We tested extracts from 5 tissues as well as hemolymph. Only the tunic extract showed significant positive chemotactic activity, and the activity decreased with increasing dilution. Furthermore, autoclaved tunic extract, extracts from diseased individuals, and extract from the styelid ascidian *Styela clava* also had chemotactic activity, although the activities were lower than that of tunic extract from healthy *H. roretzi*. Ultrafiltration of the tunic extract through a 3 kDa cutoff membrane completely abrogated the activity; the ultrafiltration retentate still showed activity. Thus, the soluble factors that attract the flagellate are present exclusively in the tunic extract, and the chemotactic factors are larger than 3 kDa. Our experiments also suggested that the tunic extract contains both heat-stable and heat-labile factors. We conclude that the flagellate locates the tunic entry site by chemotaxis toward soluble factors that diffuse from a damaged area of the tunic on the siphon wall.

KEY WORDS: Chemotaxis · *Halocynthia roretzi* · Kinetoplastid flagellate · Tunic extract

INTRODUCTION

The edible ascidian *Halocynthia roretzi* has a leathery integument called a tunic that entirely covers the epidermis, protecting the organism against predation, infection, and physical impact (Burighel & Cloney 1997). The tunic is composed of a cellulosic extracellular matrix, making it a unique tissue in metazoans; the gelatinous, cartilaginous, or leathery tunic matrix is comprised of cellulose fibers linked with polysaccharides and proteins (Van Daele et al. 1992, Lübbering-Sommer et al. 1996a,b). Furthermore, several types of tunic cells are embedded in the tunic matrix, serving various functions such as innate immunity and chemical defense (Hirose 2009).

Soft tunic syndrome is an infectious disease of *H. roretzi*, and outbreaks have been reported in ascidian aquaculture in Korea (Jung et al. 2001) and Japan

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(Kumagai et al. 2010). In diseased ascidians, the tunic becomes much softer and thinner than in healthy ascidians; the softened tunic finally tears and the individual dies. Consequently, soft tunic syndrome has caused substantial economic losses to ascidian aquaculture in both countries. During a recent outbreak in Japan, cumulative mortality reached 100% in 2 yr old ascidians growing on a culture-monitoring rope (Kumagai et al. 2010). Moreover, wild *H. roretzi*, *Pyura vittata*, and *Styela clava* have also been reported to be afflicted with soft tunic syndrome, in epizootic areas of the disease (Kumagai et al. 2013, 2014, Nam et al. 2015).

Azumiobodo hoyamushi, a kinetoplastid flagellate, is the causative agent of soft tunic syndrome (Kumagai et al. 2010, 2011, Hirose et al. 2012, Kim et al. 2014). This flagellate is exclusively found in the tunic matrix of diseased individuals and has never been observed in other tissues, even in heavily diseased individuals; therefore, the tunic is thought to be the sole site of infection (Kumagai et al. 2010). *A. hoyamushi* has been detected in an apparently healthy individual by means of quantitative PCR (Shin et al. 2014). The first symptom of the disease is the softening of the tunic of the branchial and atrial siphons, where flagellates can be detected in the early stage of infection (Kumagai et al. 2010). Although the tunic is usually overlaid by the tunic cuticle, which is dense enough to prevent infection by microorganisms, the tunic cuticle of the inner wall of siphons often shows signs of damage, even in healthy individuals, with the exception of juveniles <1 yr old (Hirose et al. 2014). This is consistent with the observation that following experimental exposure to the flagellate, no juvenile individuals became infected with *A. hoyamushi* but all older individuals did so (Hirose et al. 2014). Therefore, the flagellate has been proposed to enter the tunic matrix via damaged areas of the siphon wall tunic. A possible infection route is as follows: When host ascidians die, *A. hoyamushi* is released from the host tunic into the surrounding seawater. The flagellates are ingested by other healthy ascidians through the branchial siphons and then migrate into the tunic matrix via the damaged siphon wall tunic.

For many motile aquatic pathogens, chemotaxis plays an important role in locating the host entry site (Hazen et al. 1982, Rand & Munden 1993, Paramá et al. 2004). We hypothesized that *A. hoyamushi* also locates damaged tunic sites by a chemotactic response; the parasite is attracted by soluble substances that diffuse from the damaged tunic. In this study, therefore, we tested the chemotactic activity of a tunic extract from the host ascidians for *A. hoyamushi* and partially characterized the active fractions.

MATERIALS AND METHODS

Azumiobodo hoyamushi

The cultured strain of *A. hoyamushi* used in this study was isolated from a diseased ascidian reared in Samenoura Bay, Miyagi Prefecture, Japan (Kumagai et al. 2011). The flagellate was cultured in maintenance medium (10% [w/v] minimum essential medium, 2.5% [v/v] fetal bovine serum, 5 mM HEPES [pH 7.2], 2 mM L-glutamine, 2 mM sodium bicarbonate, and 1% [v/v] penicillin–streptomycin in mixed solution [Nacalai Tesque] prepared in artificial seawater) at 15°C and subcultured by 10-fold dilution into fresh maintenance medium at 1 wk intervals. In the following experiments, we used flagellates that had been passaged 218 to 283 times.

Ascidians

Healthy, cultured individuals of *Halocynthia roretzi* (3 yr old) were collected from a farming site in Miyagi Prefecture where the disease has not been recorded. Based on the criteria for assessment of the symptoms of soft tunic syndrome (Kitamura et al. 2010), they were classified as Grade 0: healthy individuals from a population with no diseased individuals. Wild individuals of *Styela clava* (1 yr old) attached to hanging oyster culture lines were collected from an oyster farming site in the prefecture where ascidians have not previously been farmed.

Chemotaxis assays

Following subculture for 8 to 12 d in maintenance medium, a suspension of flagellates in sterilized artificial seawater (SASW) was prepared. The flagellate suspension was centrifuged at 500 × *g* for 1 min at 10°C. We discarded 90% of the supernatant volume and resuspended the pellet in an equal volume of SASW. We repeated these steps to remove possible chemotactic substances in the maintenance medium. The number of flagellates per 1 ml was determined using a Thoma hemocytometer (Sunlead Glass).

A Chemotaxicell (Kurabo) with a 3 µm pore size was used for the chemotaxis assay. Flagellates (4.31×10^4 to 3.71×10^5 cells) in 200 µl of seawater were

added to the upper chamber, and 600 µl of extract from ascidian tissue (described in the next subsection) was placed in the lower chamber. If the extract in the lower chamber contained a chemotactic factor(s), the flagellates would be expected to migrate into the lower chamber, passing through the 3 µm pores in the membrane that separated the upper and lower chambers. Although the flagellates have a maximum body width of 4.7 ± 0.95 µm (mean ± SD) (Hirose et al. 2012), they have been reported to pass thorough a 1 µm mesh nylon bag and to infect healthy ascidians (Kumagai et al. 2009). Therefore, the 3 µm pores of the Chemotaxicell membrane should be sufficiently large to permit the flagellates to migrate to the lower chamber in response to the chemotactic factor(s). After incubation for 30 min at 15°C, the number of flagellate cells in the lower chamber was determined using a Fuchs-Rosenthal hemocytometer (Sunlead Glass). The migration rate (%) was obtained by dividing the cell number in the lower chamber after incubation by the cell number in the upper chamber before incubation. We used SASW and healthy tunic extract as negative and positive controls, respectively.

Expt 1: Tissue extracts

Healthy *H. roretzi* were dissected to obtain tunic, gill, mantle muscle, stomach, and hepatic gland tissues. All tissues were cut into small pieces (5 × 5 × 3 mm). Hemolymph was collected from the mantle through 23 gauge needles. Each tissue was incubated in SASW (5:1 solution volume to tissue wet weight) for 1 d at 15°C. Tissue suspensions were filtered through a 0.45 µm filter (Merck Millipore) to yield the extracts. Protein contents were measured using SuperKjel 1500 (ACTAC) based on a modified macro-Kjeldahl method. The protein content of each tissue ranged from 310 to 9130 mg ml^{-1} (data not shown). The chemotaxis assay was performed using these tissue extracts as test samples. Experiments using the same lot of test samples were replicated 8 times.

Expt 2: Tunic extract dilution series

The tunic extract was prepared following the procedure described in Expt 1. It was diluted with SASW using a 2-fold serial dilution method (1- to 16-fold). The chemotaxis assay was performed using these diluted tunic extracts as described in Expt 1. Experiments using the same lot of test samples were replicated 5 times.

Expt 3: Tunic extract from *Styela clava*

The chemotaxis assay was performed using tunic extract of *S. clava* prepared following the procedure described in Expt 1. Tunic extract of *H. roretzi* was used as the positive control. Experiments using the same lot of test samples were replicated 10 times.

Expt 4: Diseased tunic extract

To prepare diseased ascidians, we injected 2 ml of flagellate suspension (2.8×10^6 cells ml^{-1}) under the tunic matrix in the lateral sector of the body through 26 gauge needles. The 24 injected individuals were reared in 3 aquaria (25 l) containing aerated seawater at 17°C. After 20 d, 18 of the 24 individuals showed clinical symptoms of soft tunic syndrome. We selected 2 severely diseased specimens (Grade 3; see Kitamura et al. 2010) and cut their tunics into small pieces. Tunic extracts were prepared following the procedure used in Expt 1. To eliminate the possibility of flagellate contamination, the tunic extract was frozen for 24 h at −20°C to kill any contaminating flagellates (Kumagai et al. 2010) and centrifuged at 11 100 × *g* for 10 min at 10°C. A volume of supernatant equivalent to half the volume of the centrifuged extract was collected for use as the diseased tunic extract. Tunic extract of healthy individuals was used as the positive control. Experiments using the same lot of test samples were replicated 9 times.

Expt 5: Autoclaved tunic extract

Heat stability was evaluated by autoclaving tunic extract at 121°C for 15 min and then performing the chemotaxis assay as described in Expt 1. Experiments using the same lot of test samples were replicated 11 times.

Expt 6: 3 kDa cutoff ultrafiltration

A sample of tunic extract (4 ml) was applied to an Amicon Ultra-4 centrifugal filter device (Merck Millipore) with a 3 kDa cutoff membrane and centrifuged at 2800 × *g* for 60 min at 4°C. Following ultrafiltration, we collected approximately 3.8 ml of filtrate sample. Sub-

sequently, we added 3.8 ml of SASW to the 0.2 ml of retentate in the filter device to reconstitute the initial sample volume and centrifuged the sample again. After adding 3.8 ml of SASW, we obtained 4 ml of retentate solution. The chemotaxis assay was performed using the filtrate and the retentate. Experiments using the same lot of test samples were replicated 9 times.

Statistical analysis

We performed statistical analyses using Statcel4 (OMS Publishing). The non-parametric test was used for multiple comparisons because the Kolmogorov-Smirnov test did not support normality of the dataset for some groups. We used the Steel-Dwass test for pairwise comparisons among the groups and Steel's test for comparisons with controls.

RESULTS

Expt 1: Tissue extracts

The migration rate of the flagellates toward the tunic extract (12.1 ± 4.1 %, mean ± SD; n = 8) was significantly higher than that toward the negative control (SAWS) (1.9 ± 1.2 %; n = 8; $p < 0.01$, Steel's test; Fig. 1). The migration rate toward the hepatic gland extract (0.1 ± 0.1 %; n = 8) was significantly lower than that toward the control ($p < 0.01$, Steel's test). There were no significant differences between the other tissue extracts and the control.

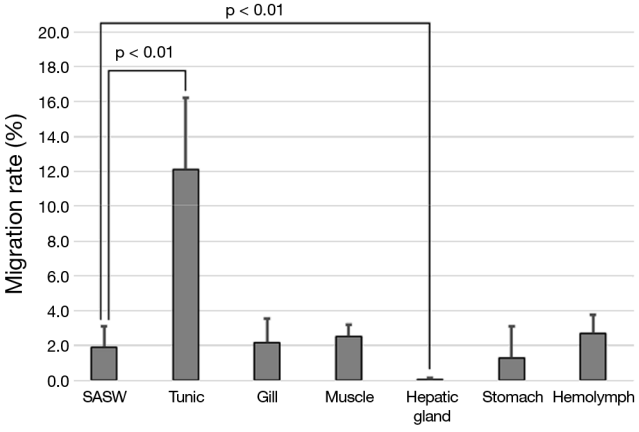

Fig. 1. Migration rates of *Azumiobodo hoyamushi* from the upper to the lower chamber, containing tunic, gill, muscle, hepatic gland, and stomach tissue extracts and hemolymph. Sterilized artificial seawater (SASW) was the negative control. Significant differences in migration rate between the experimental group and the negative control were determined by Steel's test. Error bars represent standard deviations

Expt 2: Tunic extract dilution series

The migration rates toward the undiluted tunic extract (12.6 ± 3.1 %, mean ± SD; n = 5), the 2-fold dilution (8.7 ± 1.4 %; n = 5), and the 4-fold dilution (5.0 ± 0.9 %; n = 5) were significantly higher than the migration rate toward the negative control (SASW) (2.4 ± 0.9 %; n = 5; $p < 0.05$, Steel's test; Fig. 2). Migration rates toward the 8-fold and 16-fold dilutions were not significantly different from the migration rate toward the control.

Expt 3: Tunic extract of *Styela clava*

The migration rates toward the tunic extracts from *Halocynthia roretzi* (13.8 ± 5.0 %, mean ± SD; n = 10) and *Styela clava* (5.8 ± 2.4 %; n = 10) were significantly higher than the migration rate toward the negative control (SASW) (1.5 ± 0.9 %; n = 10; $p < 0.01$, Steel-Dwass test; Fig. 3). The migration rate toward *H. roretzi* tunic extract was significantly higher than that toward *S. clava* tunic extract ($p < 0.01$, Steel-Dwass test).

Expt 4: Diseased tunic extract

The migration rates toward diseased tunic extract (7.8 ± 0.9 %, mean ± SD; n = 9) and the positive control (healthy tunic extract) (12.2 ± 1.9 %; n = 9) were significantly higher than the migration rate toward the negative control (SASW) (2.0 ± 1.1 %; n = 9; $p < 0.01$, Steel-Dwass test; Fig. 4). The migration rate toward diseased tunic extract was significantly lower than that toward the positive control ($p < 0.01$, Steel-Dwass test).

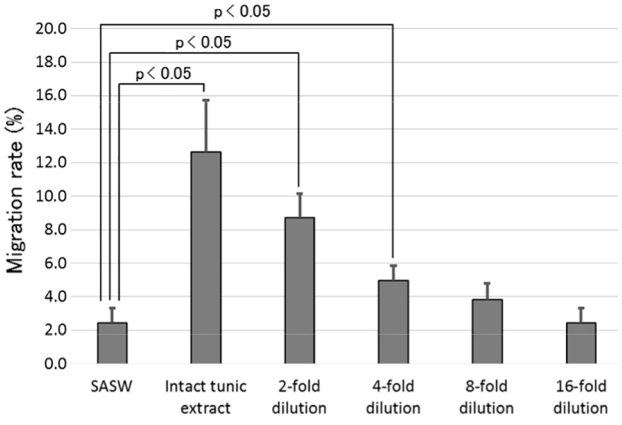

Fig. 2. Migration rates of *Azumiobodo hoyamushi* from the upper to the lower chamber, containing tunic extracts diluted 1- to 16-fold. Other details as in Fig. 1

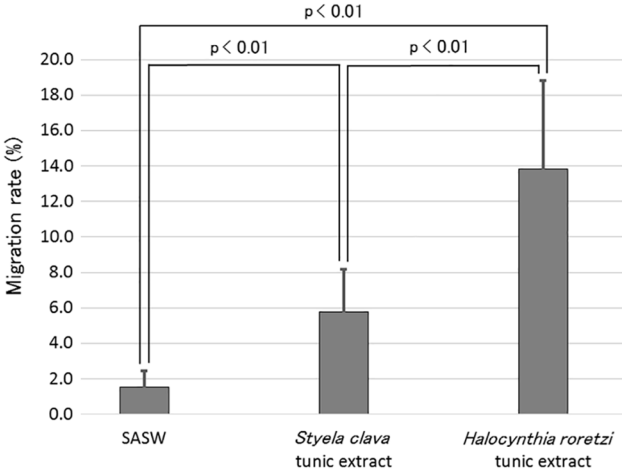

Fig. 3. Migration rate of *Azumiobodo hoyamushi* from the upper to the lower chamber, containing *Styela clava* tunic extract. *Halocynthia roretzi* tunic extract was the positive control, and sterilized artificial seawater (SASW) was the negative control. Significant differences in migration rate between groups were determined using the Kruskal-Wallis test followed by the Steel-Dwass test. Error bars represent standard deviations

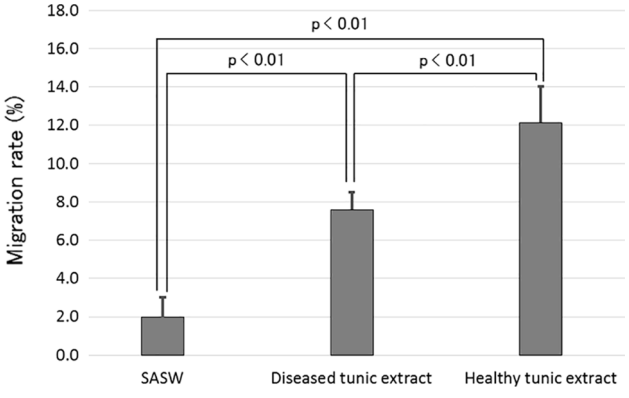

Fig. 4. Migration rate of *Azumiobodo hoyamushi* from the upper to the lower chamber, containing diseased tunic extract. Healthy tunic extract was the positive control, and sterilized artificial seawater (SASW) was the negative control. Other details as in Fig. 3

Expt 5: Autoclaved tunic extract

The migration rates toward the intact tunic extract (12.0 ± 5.1%, mean ± SD; n = 11) and autoclaved tunic extract (4.7 ± 2.5%; n = 11) were significantly higher than the migration rate toward the negative control (SASW) (2.2 ± 2.4%; n = 11; $p < 0.01$ and $p < 0.05$, Steel-Dwass test; Fig. 5). The migration rate toward intact extract was significantly higher than that toward autoclaved tunic extract ($p < 0.01$, Steel-Dwass test).

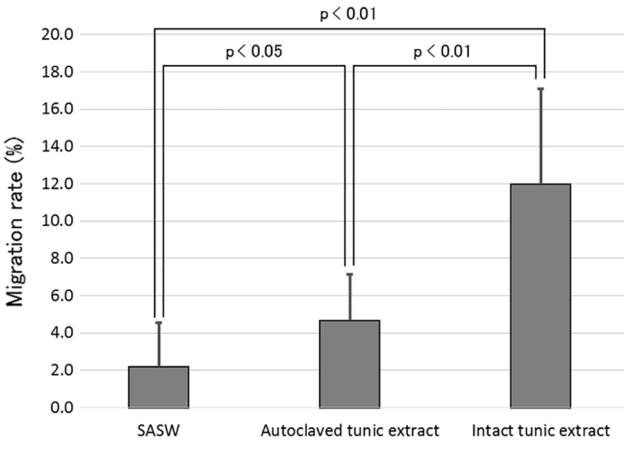

Fig. 5. Migration rate of *Azumiobodo hoyamushi* from the upper to the lower chamber, containing autoclaved tunic extract. Intact tunic extract was the positive control, and sterilized artificial seawater (SASW) was the negative control. Other details as in Fig. 3

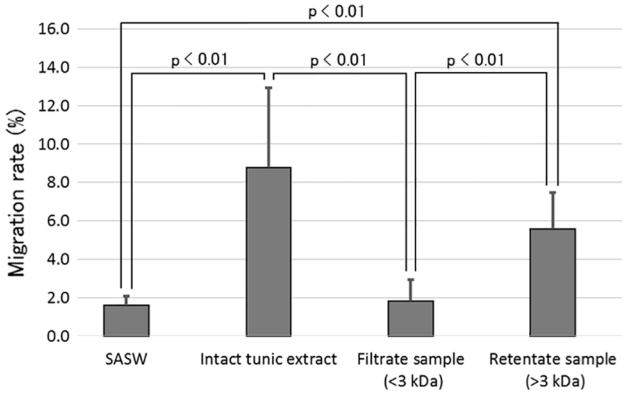

Fig. 6. Migration rate of *Azumiobodo hoyamushi* from the upper to the lower chamber, containing 3 kDa ultrafiltration samples: filtrate or retentate. Intact tunic extract was the positive control, and sterilized artificial seawater (SASW) was the negative control. Other details as in Fig. 3

Expt 6: 3 kDa cutoff ultrafiltration

The migration rates toward intact tunic extract (8.8 ± 4.2%, mean ± SD; n = 9) and the filtration retentate (>3 kDa) (5.6 ± 1.9%; n = 9) were significantly higher than those toward the filtrate (<3 kDa) (1.8 ± 1.1%; n = 9) and the negative control (SASW) (1.6 ± 0.5%; n = 9; $p < 0.01$, Steel-Dwass test; Fig. 6). No significant differences were observed between the migration rates toward the filtrate and SASW or between the retentate and intact tunic extract ($p > 0.05$, Steel-Dwass test).

DISCUSSION

Some motile aquatic pathogens are known to show positive chemotaxis toward host tissues. For example, *Aeromonas hydrophila*, the causative bacterium of red-sore disease, which causes ulcers on the surface of infected fish, shows positive chemotaxis toward the surface mucus of largemouth bass *Micropterus salmoides* (Hazen et al. 1982). Zoospores of *Saprolegnia diclina*, a water mold that infects the eggs of freshwater fish, show positive chemotaxis toward chorionic membrane extracts from live eggs of brook trout *Salvelinus fontinalis* (Rand & Munden 1993). The histophagous ciliate *Philasterides dicentrarchi* shows positive chemotaxis toward serum components of turbot *Scophthalmus maximus* (Paramá et al. 2004). Because this ciliate invades internal organs through lesions on the gills or skin from which serum diffuses, positive chemotaxis toward serum probably plays an important role in permitting the ciliate to locate the lesions for host entry. In these pathogens, positive chemotaxis to the host tissue is likely to enhance infection.

Azumiobodo hoyamushi showed significant positive chemotaxis toward tunic extract of the host ascidian *Halocynthia roretzi*, but no significant difference in migration rate was observed between SASW and other tissue extracts, except hepatic gland extract. The flagellate showed significant negative chemotaxis to the hepatic gland extract, likely because the extract contains substances unfavorable to *A. hoyamushi*, such as digestive enzymes. In addition, the chemotactic activity of the tunic extract gradually decreased with increasing dilution. Moreover, when we placed the tunic extract and the flagellates in the upper chamber and SASW in the lower chamber, the migration rates toward SASW was 0.59 ± 0.25% (mean ± SD; n = 3). This result supports our conclusion that the high migration rate toward the lower chamber was caused by a chemotactic response, not by random migration or geotaxis. These findings indicate that only the tunic extract contains a soluble factor(s) that attracts the flagellate to the host ascidians. This is consistent with observations that the flagellates are found exclusively in the tunic (Kumagai et al. 2010).

The chemotactic activity of diseased tunic extract was lower than that of healthy tunic extract, indicating that the quantity of the chemotactic factor(s) decreases during softening of the tunic. We prepared the 2 extracts using the same quantity of tissue (0.2 g ml^{-1}). Because diseased tunic is much thinner than healthy tunic, it should contain a lower quantity of the chemotactic factor(s) per unit area than healthy tunic. Accordingly, *A. hoyamushi* was more strongly attracted by healthy ascidians than by diseased ones. This would aid dispersal of the flagellates.

Tunic extract of the styelid ascidian *Styela clava* also attracted the flagellate, although the migration rate was lower than that toward *H. roretzi* tunic extract. Thus, *A. hoyamushi* may also be attracted by other ascidians. This is consistent with previous reports of tunic softening in *S. clava*; Kumagai et al. (2014) reported tunic softening caused by *A. hoyamushi* in *S. clava* from Japan, and Nam et al. (2015) detected *A. hoyamushi* by PCR assay from the wild ascidians *Pyura vittata*, *S. clava*, and *Styela plicata*, as well as from *H. roretzi,* collected in Korea. The presence of chemotactic activity in multiple ascidians suggests that homologues of the chemotactic factor(s) present in *H. roretzi* tunic extract are present in the tunic extracts of related species. Species-specific differences among the homologues may underlie the differences in chemotactic activity among species. Performing chemotaxis assays for tunic extract from various ascidian species would provide valuable information to estimate the range of the potential host species for *A. hoyamushi.*

The chemotactic factor(s) present in the tunic extract was partially characterized by means of heat treatment (autoclaving) and ultrafiltration. Whereas heat treatment of the tunic extract significantly decreased chemotactic activity, the extract retained partial but still significant activity. We hypothesize that the tunic extract contains multiple chemotactic factors comprised of both heat-stable and heat-labile compounds. Moreover, ultrafiltration with a 3 kDa cutoff membrane demonstrated that the chemotactic factors in the tunic extract appear to be larger than 3 kDa, because the chemotactic activity of the tunic extract was completely abrogated by ultrafiltration. Although the activity of the retentate was lower than the activity of the tunic extract before ultrafiltration (positive control), the Steel-Dwass test indicated that the difference was not significant. It is possible that some of the chemotactic substances were adsorbed onto the filtration membrane, decreasing their concentration. Alternatively, the filtrate may contain some low molecular weight subsidiary factors that increase its activity; low molecular weight compounds, such as amino acids, monosaccharides, and lipids, have been identified as chemotactic factors for aquatic pathogens and parasitic kinetoplastids of insects (Hazen et al. 1984, Rand & Munden 1993, O'Toole et al. 1999, Barros et al. 2006). The factors underlying the species-specific and tissue-specific

chemotaxis of *A. hoyamushi* remain to be fully characterized, but it seems clear that the tunic-specific chemotactic factors are primarily high molecular weight compounds.

Thus, soluble factors that attract the flagellate are present exclusively in tunic extract, and the chemotactic factors are larger than 3 kDa. Our experiments also suggested that tunic extract contains both heat-stable and heat-labile factors. Healthy tunic extract showed higher chemotactic activity than softened tunic extract. Although tunic extract from *S. clava* also had chemotactic activity, its activity was significantly lower than that of the *H. roretzi* extract. Based on this evidence, we hypothesize that the infection process is as follows. Flagellates are released from the tunic of a heavily diseased and/or dead host into the surrounding seawater. Some of the flagellates form temporal cysts (Nawata et al. 2015). The flagellates and temporal cysts are taken up by other ascidians through their branchial siphons. There, the temporal cysts become flagellates, because tunic extract also shows activity to induce excystment of *A. hoyamushi* (Nawata et al. 2015). It is possible that the chemotactic factors and excystment factor(s) are the same molecules. The flagellates chemotactically locate the damaged tunic emitting the chemotactic factors and migrate into the tunic matrix via the damaged siphon wall tunic. Considering the significant difference in chemotactic activity observed between healthy and diseased tunic tissue, flagellates preferentially infect healthy hosts. It is possible that the flagellates are also taken up by other ascidian species, such as *S. clava*, in response to the chemotactic factors diffused from the tunic, resulting in infection.

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