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Development of DNA probe for a protistan parasite of tunicate *Halocynthia roretzi*

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Edible tunicate *Halocynthia roretzi,* one of the most commercially important aquatic organisms in Korea, has been killed by tunic softness syndrome since last decade. The intracellular protistan parasite observed by the transmission electron microscope in hemocytes of the tunicate was considered to be the causative agent of the mass mortality. The goal of the present work is to examine the characteristic features of the parasite by identifying the 18S rDNA sequences of the parasite. The experiments conducted include amplification of presumptive 18S rDNA from diseased tunicate tissues with UNonMet-PCR and sequencing the product. A preliminary phylogenetic analysis was performed on the presumptive parasite rDNA. A digoxigenin labeled DNA probe was designed on the basis of the sequences of rDNA. Dig-ISH assay was conducted to diagnose the protistan parasite. A PCR using UNonMet-PCR primer generated 595 bp SSU rDNA fragment. Subsequently, PCRs with primer pair expended this sequence to 1542 bp. This is the first partial sequences of SSU rDNA gene to be published on the protistan parasite that has presumed causing the mass mortality of tunicate. Since the Dig-ISH technique demonstrated the presence of infection in hemocytes on the all host tissues, the fragment was confirmed to be the intracellular protistan parasite SSU rDNA. A phylogenetic analysis suggested that the protistan parasite may be a unique eukaryote that is closely related to Apicomplexa.

Key words :Protistan parasite, Digoxigenin in situ hybridization, Tunic softness syndrome, Tunicate mortality, *Halocynthia roretzi*

Halocynthia roretzi is an important aquaculture species in Korea and Japan. It is an edible tunicate that has been used for a source of chondroitin. The mass mortalities of *H. roretzi* have occurred over the last decade along the southern and eastern coasts of Korea and those have caused serious economic affects. The moribund tunicates showed thin tunic characteristically and died of rupture of the tunic. Thus the disease is referred to as a tunic softness syndrome.

The description of a protistan parasite which is infected in the hemocytes of the tunicate is based on morphological features (Choi *et al*., 2006). The parasite is being intracellular and small (2 ㎛), the parasite infects only the hemocytes and causes the hemocytes to degenerate and lyse, which lead to mass mortality of

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the tunicate. Therefore, our understanding of the protistan parasite is important not only for proper diagnosis and control of its infection, but also for phylogenetic position. The protistan parasite may be understood more thoroughly if specific molecular diagnostic techniques are applied. However, development of specific molecular assays must precede the characterization of a diagnostic gene such as that coding for SSU rDNA (Hillis & Dixon 1991, Bower *et al*., 2004).

DNA probes and polymerase chain reaction (PCR) assays have been developed for numerous protistan parasites in commercially important aquatic organisms, including *Haplosporidium nelsoni* (Fong *et al*., 1993, Stokes & Burreson 1995, Stockes *et al*., 1995a*), Minchinia teredinia* (Stockes *et al*., 1995b), *Marteilia sydneyi* (Anderson *et al*., 1995), *Marteilia refringens* (Le Roux *et al*., 1999), *Mikrocytos roughleyi* (Adlard & Lester 1995, Meyer *et al*., 2005), *Mikrocytos macknin* (Carnegie *et al*., 2003) and *Bonamia ostreae* (Carnegie *et al*., 1999).

In this study, we cloned protistan parasite SSU rDNA from tunicate, *Halocynthia roretzi,* and developed a detection method by using in situ hybridization. In addition, a preliminary 18S rDNA phylogenetic examination of the parasite's affinities was performed.

Materials and Methods

1. Sample collection and histological processing

Cultured tunicates *Halocynthia roretzi* were collected from 3 farms at Tongyoung, southern coast of Korea, in February 2006. Tissues of the tunicates were fixed in Davidson's solution (Howard & Smith 1983) and were processed for paraffin-embedding and cut into 4 ㎛ thick sections. The sections were stained with Harris's hematoxylin and eosin (H& E) for light microscopic (Zeiss Axioskop2) examination. A consecutive (parallel) tissue section of each sample was mounted on a glass slide coated with aminoalkylsilane (Silane-Prep; Sigma Diagnostics, USA) for in situ hybridization.

2. DNA extraction

Genomic DNA was extracted from mantle and gill of *Halocynthia roretzi* using a High pure PCR Template Preparation Kit (Roche Diagnostics, Germany) following the manufacturer's instructions on isolating nucleic acids from mammalian tissue protocol. The amount of DNA in all the samples was quantified using a ND-1000 (NanoDrop).

3. PCR amplification and sequencing of parasite 18S rDNA gene

Approximately 50 ng of genomic DNA were used for PCR amplification of the prototistan 18S rDNA region. We used the specific primer set (18S-EUK581-F (5'-GTGCCAGCAGCCGCG-3') and 18S-EUK-1134-F (5'-TTTAAGTTTCAGCCTTGCG-3') which was developed for amplification of the parasitic DNA by Bower *et al* (2004). In a final volume of 50 μl, the reaction mixture consisted of 1X PCR buffer (20 mM Tris-hydrochloride pH 8.4, 50 mM KCl and 1.5 mM $MgCl_2$), 200 μM of dNTP (Bioneer, Korea), 0.2 μM of the primers, and 2 U of Taq DNA polymerase (Bioneer, Korea). PCR was performed with an initial denaturation step for 5 min at 95℃ followed by 30 cycles of 30 sec of denaturation at 95℃, 30 sec of

annealing at 55℃ and 30 sec of extension at 72℃, the final elongation step at 72℃ for 5 min completed the reaction. The PCR products were electrophoresed on a 1.5 % agarose gel containing 0.1 ㎍/㎕ ethidium bromide and were visualized by using UV light.

Bands from the gel were cleaned up using QIAquick PCR purification kit (Qiagen, USA) and cloned using a TOPO TA Cloning Kit (Invitrogen, Canada), following the manufacturer's instructions. TOP 10 F' competent bacteria (Invitrogen, USA) were transformed, and plasmid extractions were carried out with a QIAprep Spin Miniprep Kit (Qiagen, USA), following the manufacturer's instructions. The nucleotide sequence was determined on an ABI 3730XL capillary DNA sequencer (Applied Biosystems, USA) using ABI prism Bigdye terminatior cycle sequencing ready reaction kit (Applied Biosystems, USA) by Solgent Company's sequencing service. The nucleotide sequences were aligned using Clustal W (Thompson *et al*., 1994) in BioEdit software and compared with the sequences in DDBJ/EMBL/Genbank using the BlastN program.

To characterize the parasite 18S full sequence, specific primer sets (upstream-f, 5'-ctacatggataaccgtggtaa-3', upstream-r 5'-ttcaaggcttatgcctgctt-3', downstream-f 5'-ct gtagagaagctgttcatc-3', downstream-r 5'-gtacaaagg gcagg gacgta-3') were designed to amplify a parasitic 18S ribosomal DNA. The generated PCR products were sequenced as described above and they were compared with all reported sequences in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov).

4. Phylogenetic analysis

The nucleotide sequences of 18S parasite of reported apicomplexa retrieved from the Swissprot, EMBL and GenBank databases were compared using the ClustalX program (Thompson *et al*., 1997). The accession numbers apicomplexa used in this analysis are Apicomplexa piroplasmida: *Babesia gibsoni* (DQ184507), *Babesia odocoilei* (AY237638), *Cytauxzoon felis* (AY679105), *Theileria bicornis* (AF499604), *Piropasmida* gen.sp. (AF158709), *Babesia* sp. (AY027816.1); Apicomplexa coccidian eucoccidiorida: *Adelina dimidiate* (DQ096835), *Adelina grylli* (DQ096836), *Adelina bambarooniae* (AF494059), *Hepatozoon felis* (AY628681), *Hepatozoon canis* (DQ439543); Apicomplexa coccidian eimeriida: *Cryptosporidium serpentis* (AF093500), *Cryptosporidium andersoni* (AB089285), *Cryptosporidium muris* (AF093498), *Cryptosporidium baileyi* (AF093495), *Cryptosporidium meleagridis* (EF179381), *Cryptosporidium parvum* (AF112576), *Frenkelia glareoli* (AF009245), *Cystoisispora* cf. *ohioensis* (AY618555.1), *Isospora belli* (DQ060683), *Besnoitia besnoiti* (AF109678), and unidentified symbiont (AF238266). A phylogenic tree of the parasite based on the nucleotide sequences was generated by the PHYLIP program (Felsenstein 1996), and distances were analyzed using the neighbor joining method. TreeView software (version 1.6.6.) was used to develop the radial tree. The reliability of the tree was assessed by 1000 bootstraps.

5. In situ hybridization

To determine the origin of the presumptive parasitic sequence, Dig-ISH was used. A specific primer set was designed based on the presumptive parasitic sequence: HALO-F (5'-CTGCAGAGAAGCTGTTCATC-3') and HALO-R (5'-GACTAGAGATTGGAGGAACT-3'). A

nucleotide probe which was synthesized with PCR DIG Probe Synthesis kit (Roche, Germany) was generated by polymerase chain reaction with the primer set, following the manufacturer's instructions.

In Situ Hybridization Core kit (BioGenex, USA) was used for the Dig-ISH assay. The procedure of Dig-ISH was based on the manufacturer's instructions. Tissue section adhered to aminoalkylsilane coated slides were deparaffinized in xylene (2×5 min), rehydrated through a descending ethanol (100, 95, 70% for 5 min), washed in sterile deionized water treated with 0.1% DEPC (2 ×5 min). The section was permeabilised by covering with proteinase K solution and incubated at room temperature for 10 min. Proteolysis was halted with a wash in sterile deionized water treated with 0.1% DEPC (2×5 min). The section was post-fixed with PBS containing 1% formaldehyde for 10 min at room temperature. The section was washed in sterile deionized water treated with 0.1% DEPC (2×5 min). A hybridization mixture was prepared by diluting the probe in the provided hybridization buffer to 40-50 ng/㎖. Hybridization involved placing 50 ㎕ of the Hybridization Mixture onto the section and covering it with a plastic coverslip. And also hybridization buffer without probe was used as negative control. Then, the section was placed on a heated slide moat at 80℃ for 5 min and cooled to 37℃ and incubated at 37℃ for 3-6 h. Following the hybridization, the coverslip was

Fig. 1. Partial sequences of 18 S ribosomal DNA of the tunicate parasite and *Adelina grylli.* The primers are marked by boxed.

gently removed, and the tissue section was washed in 2× PBS plus 0.1% Tween-20 (2×5 min) and 1× PBS plus 0.1% Tween-20 (2×3 min). The section was covered with enough drops of Power Block Reagent (1×5 min) provided, drained and covered with Anti-Probe antibody (1×20 min) at room temperature. The section was rinsed with 1× PBS plus 0.1% Tween-20 (2×3 min). The section was covered with Streptoavidin-Alkaline phosphatase conjugate and incubated at 37℃ for 5 min. The section was rinsed with 1× PBS plus 0.1% Tween-20 (2×3 min) and the activation buffer provided at room temperature for 1 min followed by 2 X 5 min wash in the washing buffer provided. The section was covered with 200 μl of nitroblue tetrazolium salt (NBT)/5-bromo-4chloro-3indolyl phosphate (BCIP) colour reaction solution (Roche Diagnostics) diluted 1:50 in washing buffer, and incubated for 2 h in a dark humid chamber. The section was rinsed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) to stop colour reaction. The section was counterstained for 1 min in 0.05% Bismark Brown Y and destained in 95% ethanol (2×5 min), finally cleared in xylene (3×15 sec). Glass coverslip was mounted with Permount (Fisher Scientific, USA). Slides were examined on light microscopy (Zeiss Axioskop2).

Results

1. Amplification and sequencing of presumptive parasite SSU rDNA

PCR generated three amplification products from the diseased tunicates *Halocynthia roretzi*, showing a 595 bp product of predicted size among the three amplification products (550-850 bp). The 595 bp product was cloned and sequenced. Subsequently, PCRs with a primer pair expended this sequence to 1542bp. The 18S rDNA sequence of the product is shown in Fig 1 and registered in GenBank (Accession No EF558768). Comparisons of the sequence using BLAST confirmed that the sequence

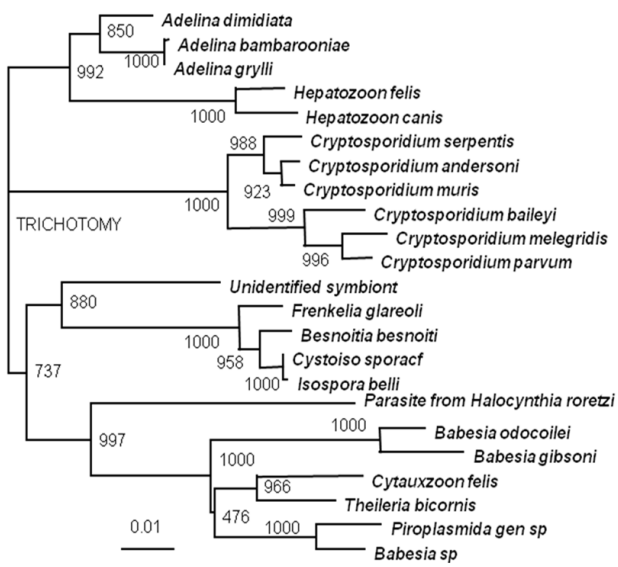

Fig. 2. Phylogenetic tree of apicomplexea parasite and protistian parasite from *Halocynthia roretzi* based on 18S ribosomal RNA sequences

belongs to apicomplexa 18S rDNA and bears the closest resemblance to *Cardiosporidium cionae* (Table 1).

The 18S ribosomal DNA sequence identities between the parasite from tunicate *Halocynthia roretzi* and the reported apicomplexa ranged from 58.08-86.01%: apicomplexa coccidian to 72.32-85.76%: apicomplexa piroplasmida (Table 1).

2. Phylogenetic analysis

A phylogenetic tree analysis based on the SSU rDNA gene of protistan parasite from tunicate *Halocynthia roretzi* and other members of apicomplexa showed that this parasite was not clustered with piroplasmida or coccidia (Fig. 2).

3. Dig-ISH

The digoxigenin-labeled DNA probe which was a non-purified PCR product hybridized hemocytes in the fixed tissue sections. The hybridized hemocytes were assumed to be infected with presumptive intrahemocytic paramyxean parasite (Fig. 3-7). Hypertrophied and stained dark blue, the infected hemocytes were shown in all the tissues including the mantle, reproductive tissue, intestine, gill, and muscle.

Table 1. Percentage of identities among 18S rDNA Gene sequences

	Apicomplexa											
	Coccidia									Piroplasmida		
	eucoccidirida			eimeriida								
	A.dimidiata	A.grylli	H. felis	C.baileyi	C.serpentis	F.glareoli	I.belli	B.besnoiti	T.gondii	B.gibsoni	C.felis	P.sp
Parasite from *Halocynthiaroretz* (1-1542)	85.95	86.01	82.96	58.08	65.66	84.95	84.99	84.67	84.99	72.32	85.76	84.76
A.dimidiata (273-1846)	100	95.79	90.48	76.10	77.69	88.30	89.17	88.65	88.89	71.17	85.50	86.36
A.grylli (112-1624)		100	91.08	76.09	80.56	88.14	88.49	88.42	88.46	73.78	86.12	85.78
H.felis (129-1649)			100	71.73	76.79	84.97	85.13	94.99	85.10	71.92	84.80	85.05
C.baileyi (133-1577)				100	94.43	82.17	85.49	85.70	86.01	82.31	84.06	82.98
C.serpentis (133-1586)					100	86.36	86.62	85.81	86.41	83.29	85.12	83.94
F.glareoli (72-1584)						100	96.04	96.30	96.24	73.58	84.41	84.75
I.belli (124-1629)							100	97.81	97.68	74.67	84.92	86.65
B.besnoiti (133-1636)								100	98.54	74.05	85.05	85.32
T.gondii (133-1633)									100	72.51	84.68	85.75
B.gibsoni (134-1594)										100	85.97	88.10
C.felis (133-1614)											100	90.59
P.sp (110-1587)												100

Discussion

Mass mortality of cultured *Halocynthia roretzi* has cost about 20 million dollars annually in Korea since the last decade. The mortality begins each year in late November and continues with the highest mortality in April. The similar mortality of cultured tunicate has occurred in February 2007 in Japan (according to personal communication). The cause of mortality of *Halocynthia roretzi* in Japan is being investigated. The diseased tunicate in Japan showed same symptoms like Tunic softness syndrome described by Choi *et al* (2006). The seeds of *H. roretzi* were supposed to be introduced from Korea to Japan because the seed production of *H. roretzi* was not enough in Japan from 2004.

Molecular data of ribosomal DNA genes are commonly used for classifying species and strains that are originally erected on the basis of morphological and life history. However, characterizing the ribosomal DNA of a new protistan parasite of unknown taxonomic affiliation by PCR is difficult because the universal eukaryotic PCR primers amplify the host DNA as well as the parasite DNA (Bower *et al*., 2004). Therefore, we used the protist-specific primer set which was developed by Bower *et al*. (2004) for amplification of the 18S rDNA of the protistan parasite from the infected tunicate. The protist-specific primer set produced a presumptive parasitic DNA ranging in size from about 550 bp to 850 bp from some samples. The parasite DNA was successfully separated from the host DNA by applying UNonMet-PCR.

DIG-ISH made it possible to detect and localize the intrahemocytic paramyxean parasite within histological sections. Presumptive intrahemocytic paramyxean parasite-specific probe was hybridized to hemocytes in histological sections that were presumed to be intrahemocytic paramyxean parasite cells (Choi *et al*., 2006). This phenomenon suggests that the PCR product produced by UNonMet-PCR might be the intrahemocytic parasitic DNA.

SSU rDNA genes are highly conserved among the species in general. A phylogenetic analysis of 18S rDNA of the protistan parasite showed that the homology of the 18S rDNA of the parasite is closely related to Apicomplexean. Despite the morphological evidence suggested by Choi *et al*. (2006), a comparative study of partial SSU rDNA sequences of the protistan parasite in tunicate suggests that the taxonomic position of this protistan parasite belongs to Apicomplexa (Ciancio *et al*., 2008).

The 2007 outbreak of tunicate mortality in Japan strongly calls for a joint epizootiological survey of Japan and Korea since an international search for proper diagnostic methods for the protistan parasite is certainly important enough for the interests of both countries. Hence the ISH assay and PCR described here provide information on the protistan parasite SSU rDNA, which may be useful in identifying the parasite.

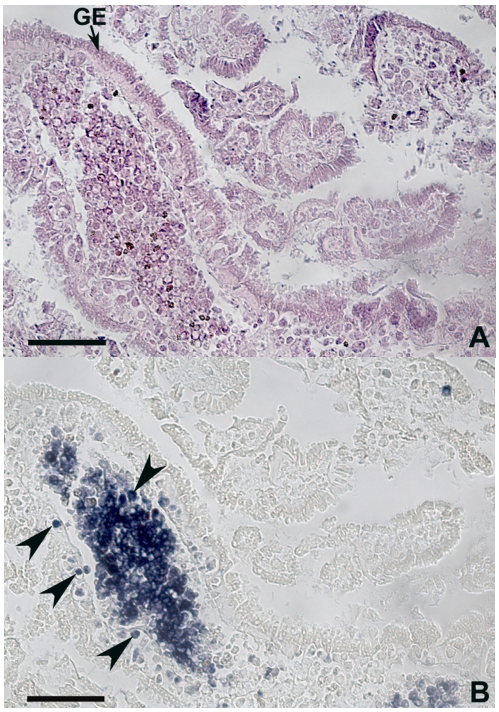

Fig. 3. Gill of *Halocynthia roretzi* stained with H-E (A) and ISH (B) on consecutive sections. Hemocytes infected in the gill are stained by Dig-labeled probe (arrow heads). GE: gill epithelium. Bar is 200 ㎛

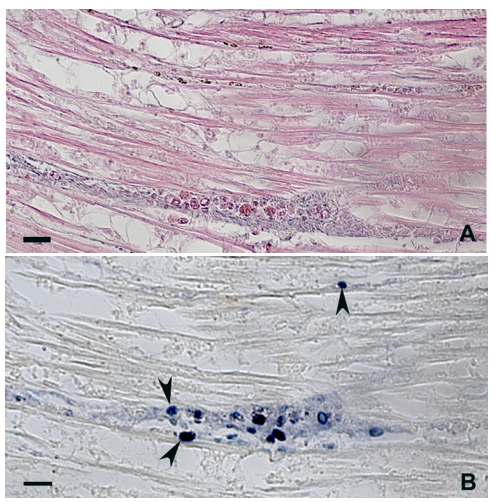

Fig. 4. Muscle tissues of *Halocynthia roretzi* stained with H-E (A) and ISH (B) on consecutive sections. Hemocytes in muscle tissue are stained by Dig-labeled probe (arrow heads). Bar is 100 ㎛

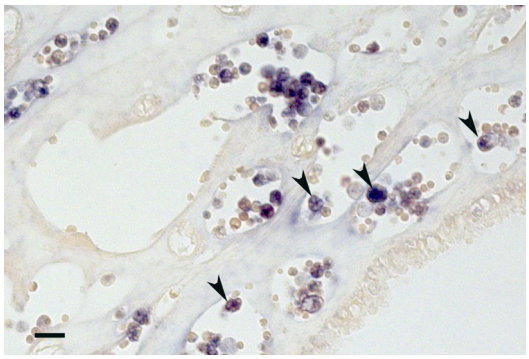

Fig. 5. Mantle tissues of *Halocynthia roretzi* stained with ISH. Hypertrophied hemocytes in the mantle tissue are stained by Dig-labeled probe (arrows). Bar is 20 ㎛

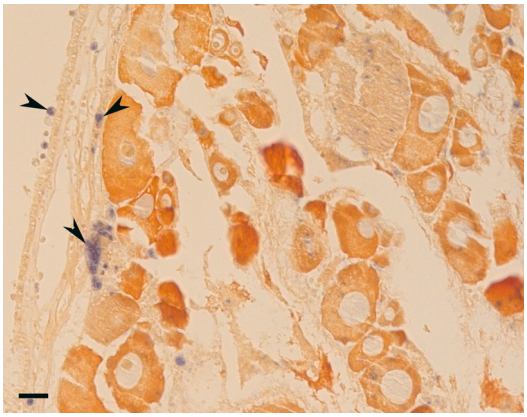

Fig. 6. Ovarian oocytes in the reproductive tissue are not stained by Dig-labeled probe but the infected hemocytes are stained (arrow heads). Bar is 100 ㎛

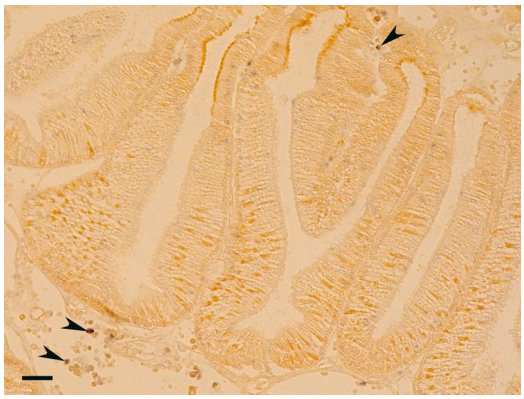

Fig. 7. Intestinal epithelium is not stained by Dig-labeled probe but the infected hemocytes are stained (arrow heads). Bar is 100 ㎛

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