

# Detection of two pathogenic marine ciliates *Ancistrum haliotis* and *A. crassum* (Ciliophora: Scuticociliatia) by fluorescence *in situ* hybridization

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## Abstract

The scuticociliatid ciliates *Ancistrum haliotis* and *A. crassum* are parasites that may cause high mortality in the cultured abalone *Haliotis* spp. and the bivalve *Ruditapes philippinarum*. Traditional identification with silver staining methods is hampered by their morphological similarities to closely related species and the complicated procedures of morphological analysis. We designed two SSU rRNA-targeted oligonucleotide probes labeled with a fluorochrome, and optimized the fluorescence *in situ* hybridization (FISH) protocols for identification of *A. halioti* and *A. crassum*, respectively. The assays resulted in a clear identification by strong fluorescence signals from the oligonucleotide probes. The method can be used for quick and accurate quantitative analysis of *A. haliotis* and *A. crassum* infections on host molluscs.

**Key words:** oligonucleotide probes, molecular methods, scuticociliates, SSU rRNA, abalone, mollusc

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## 1 Introduction

Ciliates assigned to the scuticociliate genus *Ancistrum* Maupas, 1883 are parasites of marine and freshwater molluscs (Chatton and Lwoff, 1949; Fenchel, 1965; Raabe, 1970; Xu and Song, 2003; Xu et al., 2015). Among these, *Ancistrum haliotis* Xu et al., 2015 is a pathogen that infects the gills and the mantle cavity of the abalone *Haliotis* spp., which is cultivated and has high economic importance in many countries (e.g., China, Japan, Australia). In case of mass occurrence, *A. haliotis* may cause high mortality in cultured abalones (especially juvenile) by weakening the respiratory function of the gill filaments and leading to secondary bacterial diseases. *Ancistrum crassum* Fenchel, 1965 is a parasite of the bivalves including *Ruditapes philippinarum*, *Caecella chinensis*, *Protothaca jadoensis* and *Saxidomus purpuratus*, which are also economical animals in China (Xu and Song, 2003). Like the case of *A. haliotis*, *A. crassum* can cause the mortality of *R. philippinarum*.

Morphological methods are the routine way to identify *Ancistrum* species, however, it has two significant limitations. First, *Ancistrum* spp. show morphologically high similarities to each other as well as other similar scuticociliates, which often concomitantly occur in the same host, e.g., *Uronema marinum*. The commonly used silver staining methods for morphological identification need considerable experience, and staining failure is often the case, making species identification difficult (Zhan et al., 2014, 2018). Second, these endoparasitic ciliates are hard to con-

trol when massive infection occurs in molluscs. Thus, early detection, quantification, and control (e.g., host animal isolation, changing the water, chemical treatment) of these infectious pathogens are crucial for mollusc aquaculture. There is a pressing need for a prompt and accurate diagnosis tool for these scuticociliates without the necessity of morphological identification.

FISH methods can circumvent the drawbacks of the morphological method (Stoeck et al., 2003; Zhan et al., 2014, 2018). FISH is based on the design of specific fluorochrome-labeled oligonucleotide probes that hybridize to a targeted sequence within an intact cell, resulting in colored signals that are detected with a fluorescence microscope (e.g., DeLong et al., 1989; Stoeck et al., 2003). FISH has been applied successfully to four pathogenic ciliates. Bourne et al. (2008) detected a scuticociliate associated with the coral disease brown band with two designed probes; and Zhan et al. (2014, 2018) designed three targeted probes to detect *Pseudocohnilembus persalinus*, *Boveria labialis* and *B. subcylindrica*, which infect the fish *Paralichthys olivaceus*, the sea cucumber *Apostichopus japonicus* and the bivalve *Atrina pectinata*, respectively.

Although sequencing-based methods are another molecular way to detect ciliated pathogens, it has one significant limitation in aquaculture practice. Usually, there are various microorganisms including nonparasitic scuticociliates in the gills and the mantle cavity of the hosts, and in the waters and sediments of aquaculture systems. Most scuticociliates are small-sized (usu-

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ally 20–60  $\mu\text{m}$ ) and highly similar, and thus it is difficult to accurately isolate for sequencing. Alternatively, with the direct polymerase chain reaction (PCR) on the host tissues or the environmental samples, the target genes are often hard to be amplified when the cells are in low abundance. Furthermore, the sequencing-based methods can not obtain the number of the parasites. By contrast, FISH can visualize the target cells in any case (Stoeck et al., 2003; Zhan et al., 2014, 2018). Additionally, FISH is more convenient and much cheaper than the high-throughput sequencing.

In the present study, our aim is to develop a fast and user-friendly tool facilitating the identification and quantitative examination of *A. haliotis* and *A. crassum*. Here we designed two rRNA-targeted fluorochrome-labeled probes based on the small subunit rDNA (SSU rDNA) sequences, and tested the utility of these probes using FISH.

## 2 Materials and methods

### 2.1 Collection, isolation and identification

The host abalone *Haliotis discus hannai* Ino and the bivalve *R. philippinarum* were collected from two temporary culture ponds in Qingdao, China. Specimens of *A. haliotis* and *A. crassum* were isolated from the gills and mantle cavity of the host. The fluid of the mantle cavity was first examined for ciliates, and

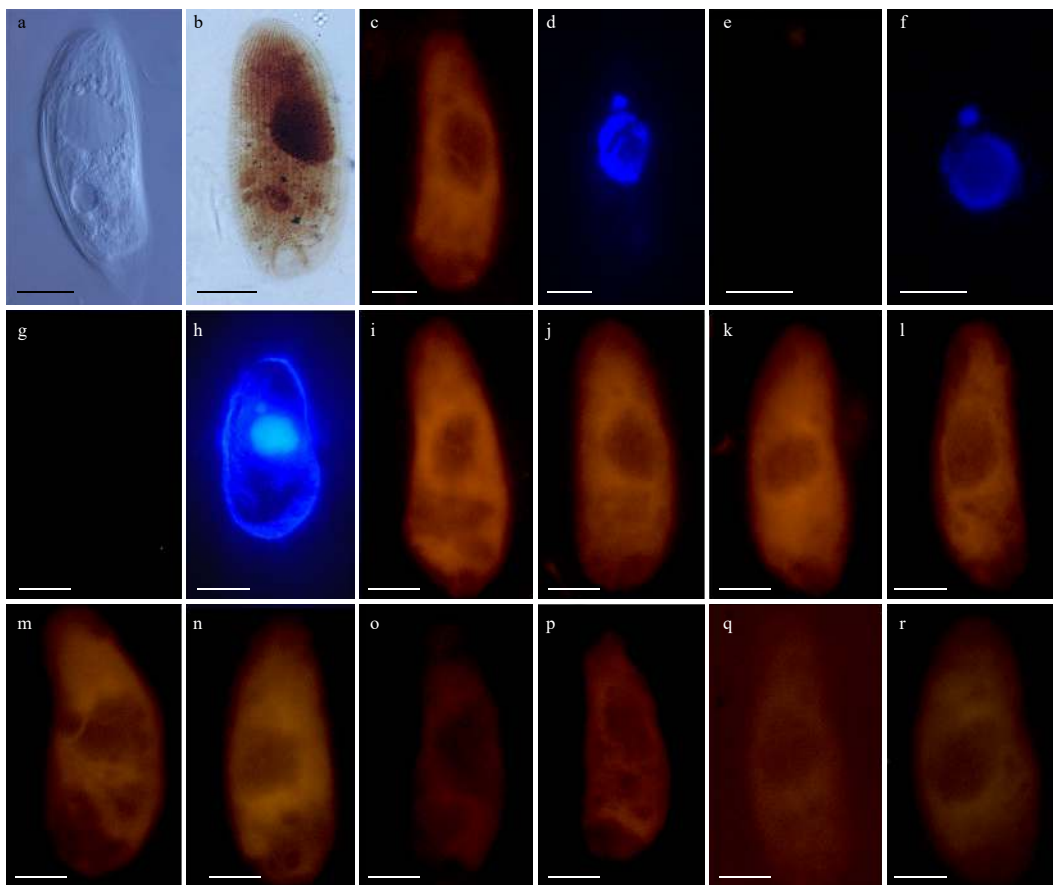
then the gills were removed and washed with sterile filtered (0.45  $\mu\text{m}$  pore size) seawater in a Petri dish. Ciliates were isolated with a micropipette and observed *in vivo* (Figs 1a and 2a) under a differential interference contrast microscope (Axio Imager Z2, Carl Zeiss GmbH). Protargol staining following Wilbert (1975) was used to reveal the ciliary and nuclear pattern for species identification (Figs 1b and 2b).

### 2.2 DNA extraction, PCR amplification, and sequencing

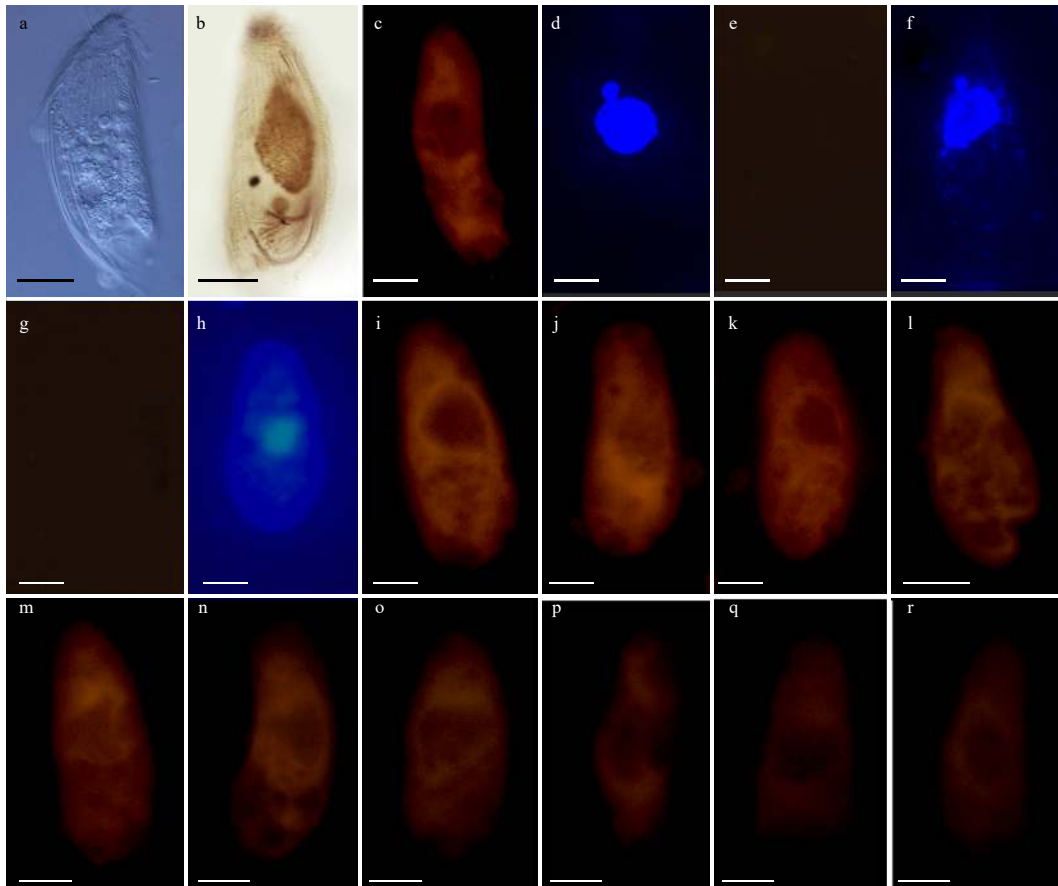
Ciliates were isolated using finely drawn glass pipettes, rinsed in filtered sterilized seawater, and transferred to 1.5 mL microcentrifuge tubes. DNA was extracted from 10 cells using the DNeasy tissue kit (Qiagen, Hildesheim, Germany). The amplification of SSU rDNA used the eukaryote universal EUK-A and EUK-B primers (Medlin et al., 1988). PCR cycling conditions, product purification and cloning followed Zhan et al. (2014). Sequencing was performed by Shanghai Sangon Biological Engineering and Technical Service Company (Shanghai, China) using an Applied Biosystems (ABI, Foster City, Canada) 3730 DNA Stretch Sequencer.

### 2.3 Design and test of the specific oligonucleotide probe

A set of probes (nucleotides, 18–22; GC contents, 50%–60%; nucleotide-nucleotide  $T_m$ s, 50–60°C) was designed using the probe design tool as implemented in the ARB software package



**Fig. 1.** *Ancistrum haliotis* (a–d, i–r), *A. crassum* (e, f) and *Uronema marinum* (g, h), cells *in vivo* (a) and after protargol impregnation (b), simultaneous fluorescence *in situ* hybridization (FISH) and 4', 6'-Diamidino-2-Phenylindole (DAPI) staining (c–r). For a–d, cells of *A. haliotis* *in vivo* (a), after protargol impregnation (b), hybridized with EUK1209R probe, with which only the Cy3-dye-derived light emission (orange red) is recorded (c), stained with the nonsense probe and DAPI, showing only the nucleus in blue (DAPI-derived light emission, d). For e–f, same cell of *A. crassum* stained with the Cy3-labeled probe Ah1030 (e) and DAPI (f). For g–h, same cell of *U. marinum* stained with probe Ah1030 (g) and DAPI (h). For i–r, *A. haliotis* stained with the Cy3-labeled probe Ah1030. The formamide concentration is 0%–45%, and the figures with every 5% increase in concentration are shown. Scale bar=15  $\mu\text{m}$ .



**Fig. 2.** *Ancistrum crassum* (a–d, i–r), *A. haliotis* (e, f) and *Uronema marinum* (g, h), cells *in vivo* (a), after protargol impregnation (b), simultaneous fluorescence *in situ* hybridization (FISH) and 4', 6'-Diamidino-2-Phenylindole (DAPI) staining (c–r). For a–d, cells of *A. crassum in vivo* (a), after protargol impregnation (b), hybridized with EUK1209R probe, with which only the Cy3-dye-derived light emission (orange red) is recorded (c), stained with the nonsense probe and DAPI, showing only the nucleus in blue (DAPI-derived light emission, d). For e–f, same cell of *A. haliotis* stained with the Cy3-labeled probe Ac699 (e) and DAPI (f). For g–h, same cell of *U. marinum* stained with probe Ac699 (g) and DAPI (h). For i–r, *A. haliotis* stained with the Cy3-labeled probe Ac699. The formamide concentration is 0%–45%, and the figures with every 5% increase in concentration are shown. The optimal formamide concentration is 20%. Scale bar=15  $\mu\text{m}$ .

for the SSU rDNA sequence of *A. haliotis* and *A. crassum* (Ludwig et al., 2004). Generated probes were checked against the GenBank sequence collection by a standard nucleotide-nucleotide BLAST search and were compared to an accessibility map of the SSU rRNA of *Saccharomyces cerevisiae* for hints of probe target sites with promising high signal intensities (Behrens et al., 2003). From the original probe candidates, probe Ah1030 (5'-GCTGATGGAGCTAATATGACCC-3') for *A. haliotis* and probe Ac699 (5'-ACAAATTCGTCGTAATCG-3') for *A. crassum* fulfilled the general criteria of potentially successful probes (Pernthaler et al., 2001). Other probes used included the universal Cy-3-labeled eukaryotic probe Euk1209R (5'-GGGCATCACAGACCTG-3') (Giovannoni et al., 1988) and its Cy-3-labeled complement as a nonsense probe.

#### 2.4 FISH staining

FISH was used to visualize *A. haliotis* and *A. crassum* both in field samples and mixed samples with both species as well as *U. marinum* that concomitantly occurred in the same host mollusc; the latter species served as a negative control. The basic hybridization followed the protocol of Stoeck et al. (2003) and Zhan et al. (2014). In short, cells were washed down from the gills and the

mantle cavities with filtered seawater, and the sample water was fixed with Bouin's solution and filtered onto 1.2  $\mu\text{m}$  pore size white Isopore membrane filters (25 mm; Millipore GmbH, Schwalbach/Ts., Germany). The membranes were covered with hybridization buffer at 46°C for 2–3 h, which was followed by 15 min of washing with preheated washing buffer at 48°C. After this, the cells were washed with distilled water, counterstained with DAPI (4', 6'-Diamidino-2-Phenylindole). The membranes were viewed and imaged under an epifluorescence microscope (Axio Imager Z2, Carl Zeiss GmbH) using the Rhod and DAPI filters. To allow direct signal comparison, microscope settings, such as excitation power, pinhole diameter, detection gain, amplifier offset, time exposure (55 ms for Cy3 signals, 48 ms for DAPI signals), and filter sets for fluorescence image acquisition were kept the same for all tests. Formamide concentrations ranged from 0% to 45% (the results with every 5% growth are shown in Figs 1i–r, 2i–r) for Ah1020- and Ac699-probe testing, and 30% for the universal eukaryote probe Euk1209R and the nonsense probe.

In order to assess the hybridization rates with the probes Ah1030 and Ac699, a total of 10, 20 and 30 cells of *A. haliotis* and *A. crassum* were added respectively to each 100 mL culture water,

and were quantified with visualized red fluorescence signal count. The environmental water contained ciliates such as *Euplotes* spp. and *Pseudokeronopsis* sp., but no *Ancistrum* species were present. We also quantified the hybridization rate with probe Ah1030 from a mix of *A. haliotis* (30 cells) and a culture of *U. marinum* (10 mL).

### 3 Results and discussion

The length, GC content (%), and GenBank accession number of the present SSU rRNA gene sequence of *A. haliotis* and *A. crassum* are as follows: 1 755 base pairs, 44%, HQ445963; 1 646 base pairs, 46%, MF407351. The new sequences were checked against the GenBank sequence collection by a standard nucle-

otide-nucleotide BLAST search, which were found to be at least 98% identity to the known *Ancistrum* sequences. The probes Ah1030 and Ac699 evaluated with the probe match tool of the ARB software package and the GenBank BLAST tool show that they are specific to *A. haliotis* and *A. crassum*, respectively. Probe Ah1030 has at least 3 mismatches to other species of *Ancistrum* as well as those like *Cyclidium glaucoma*. The GenBank BLAST shows that it has 14 mismatches to the host *Haliotis discus hannai* (Table 1). Probe Ac699 has 2 mismatches to other species of *Ancistrum*, and 3 mismatches to those like *C. glaucoma* (Table 1). The GenBank BLAST shows that it has at least 5 mismatches to the host *R. philippinarum* and 7 mismatches to *H. discus hannai* (Table 1).

**Table 1.** Mismatches of the sequence alignments including the close related species and the hosts

Sequence accession number	Target alignment for probe Ac699	Target alignment for probe Ah1030
	5'-UGUUUAAGGCAGGCAUUAGC-3'	5'-GGGUCAUUAUAGCUCCAUCAGC-3'
<b><i>Ancistrum crassum</i> MF407351</b> , HM236340, JQ956537, JQ956538	*****	*****CC*****T*****
<b><i>A. haliotis</i> HQ445963</b>	*****GC****	*****
<i>A. japonicum</i> JQ956536	*****GC****	*****CC*****T*****
<i>A. mytili</i> JQ956535	*****GC****	*****CC*****T*C****
<i>Cyclidium glaucoma</i> DQ442852, DQ442853, DQ442854	*****GCA***	*****CC*****T*****
<i>C. glaucoma</i> HQ445963, KF256817, JQ956553	*****GCA***	***TCA*****T*****
<i>C. varibonneti</i> KF256817	*****GCA***	***TCA*****T*****
Uncultured Scuticociliatia clone JQ692043, JQ692041, EU446379	*****GCA***	***TCA*****T*****
<i>Ruditapes philippinarum</i> AM774568, JN807342, EF426293	*C*C*A*****C*(GGC)C**	*(A)*(TT)*CT**_*G*_**GGCGG**
<i>Ruditapes philippinarum</i> JN807341	*C*C*A*****C*C**	*A*T*GC*T*A*T*A**GGCGG**
<i>Haliotis discus hannai</i> KY485141, KY485142	*****A******(CAG)CCC**	*(C)G(TT)*C**_***C*A***(GG)*GGG**-

Note: Probe Ac699 (5'-ACAAATTCGGTCCGTAATCG-3') is targeted for *A. crassum*, and probe Ah1030 (5'-GCTGATGGAGCTAATATGACCC-3') is designed for *A. haliotis*. \* represents same nucleotide to the target alignment, - gap in the alignment, and () insert in the alignment. Only nucleotides different to the target alignment (mismatches) are shown. New sequences are bold. The scuticociliates with more than 4 mismatches with target alignments are not shown.

Fluorescence *in situ* hybridization with the probes EUK1209R and Ah1030 resulted in the presence of an orange-red fluorescence signal in the cytoplasm for *A. haliotis* (Figs 1c, i-r), clear distinction from the faint autofluorescence signal achieved with negative-control hybridizations using the Ah1030 probe to hybridize the untargeted ciliates *A. crassum* and *U. marinum* (Figs 1e, g), and the nonsense probe (Fig. 1d). The FISH approach also provided some morphological information such as the shapes of the body, macronucleus and micronucleus (Figs 1c, d, h). This helped to verify morphotype identity in mixed taxa samples. The signal intensity of the probe Ah1030 became weaker when the formamide (FA) concentration increased in the hybridization buffers (Figs 1i-q), and the fluorescence signals with more than 25% FA (Figs 1o-r) were weaker than those of the positive control (Fig. 1c). Therefore, 25% of formamide in the hybridization was the optimal concentration for the stringency of probe Ah1030 (Fig. 1n). In the check of the rate of hybridization with probe Ah1030, all the cells of *A. haliotis* presented orange-red fluorescence signals by the FISH protocol with 25% FA concentration, which indicated the rate was 100%. The uniform time exposure with 48 ms was too long for *U. marinum* that their some bodies were artificially stained by the little remaining DAPI (e.g., Fig. 1h).

Fluorescence *in situ* hybridization with the probes EUK1209R and Ac699 resulted in the presence of an orange-red fluorescence signal in the cytoplasm for *A. crassum* (Figs 2c, i-r, t), clear

distinction from the faint autofluorescence signal achieved with negative-control hybridizations using probe Ac699 to hybridize the untargeted ciliates *A. haliotis* and *U. marinum* (Figs 2e, g), and the nonsense probe (Fig. 2d). The signal intensity of probe Ac699 became weaker when the formamide (FA) concentration increased in the hybridization buffers (Figs 2i-r), and the fluorescence signals with more than 20% FA (Figs 2n-r) were weaker than those of the positive control (Fig. 2c). Therefore, 20% of formamide in the hybridization is the optimal concentration for the stringency of probe Ac699 (Fig. 2m). In the check of the rate of hybridization with probe Ac699, only one cell in the trial with 30 targeted ciliates did not present the orange-red fluorescence signal by the FISH protocol with 20% FA concentration, which indicated the rate was at least 99.6%.

The present study developed the probes Ah1030 and Ac699 to unambiguously detect and identify *A. haliotis* from the abalone and *A. crassum* from the bivalve (Figs 1 and 2). Furthermore, due to the high hybridization rates (96.6%–100%) in our test cases, rapid quantification can be achieved in order to determine the intensity of *A. haliotis* and *A. crassum* infections. Our study demonstrates that the FISH method enables the rapid and accurate identification and enumeration of *A. haliotis* and *A. crassum*.

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