Identification of the pathogenic ciliate *Pseudocohnilembus persalinus* (Oligohymenophorea: Scuticociliatia) by fluorescence in situ hybridization

Zifeng Zhan\(^a,b\), Thorsten Stoeck\(^b\), Micah Dunthorn\(^b\), Kuidong Xu\(^a, \ast\)

\(^a\)Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
\(^b\)Department of Ecology, University of Kaiserslautern, Kaiserslautern 67653, Germany

Received 24 July 2013; received in revised form 31 August 2013; accepted 2 September 2013
Available online 22 October 2013

**Abstract**

Many scuticociliatid ciliates are regarded as devastating pathogens in aquaculture. Among these, *Pseudocohnilembus persalinus* is a facultative pathogen that often results in refractory diseases of mariculture fish. Although traditional silver staining methods have been successfully used to identify these ciliates, their identification is hampered by their small size and their morphological similarity to closely related species. We designed an alternative method of identification of *P. persalinus* using an SSU-rDNA targeted oligonucleotide probe labeled with a fluorochrome, and optimized in a fluorescence in situ hybridization (FISH) protocol. The assay results in a clear identification by strong fluorescence signals from the oligonucleotide probe. The method can be used for quick and early detection of *P. persalinus* infections on host fish, as well as other susceptible organisms in aquiculture water. It may also be used in studies of the geographical distribution of this scuticociliate.

© 2013 Elsevier GmbH. All rights reserved.

**Keywords:** Fluorescence in situ hybridization; Molecular methods; Oligonucleotide probes; Scuticociliates; SSU-rDNA

**Introduction**

Aquaculture is a booming business worldwide, and management of various refractory diseases, such as those caused by scuticociliates, is a challenging problem (Harikrishnan et al. 2010). Many scuticociliates—especially species within *Pseudocohnilembus, Uronema, Paralembus, Mamiensis* and *Philasterides*—are become serious opportunistic pathogens in cultured animals (Lynn 2008; Jung et al. 2010; Kim et al. 2004a,b; Paramá et al. 2006; Song et al. 2003; Sterud et al. 2000; Zhou et al. 2001). Despite their economic and ecological importance, species diagnoses for this group remain difficult (Harikrishnan et al. 2010; Song and Wilbert 2000, 2002; Paramá et al. 2006). There is a pressing need for a prompt and accurate identification of these facultative pathogens in diseased fish as well as in aquaculture farms.

Although morphological method is the routine way to identify scuticociliated ciliates, it has four significant limitations in aquaculture industry. First, scuticociliates largely look alike in vivo to non-taxonomists even after silver staining (Song and Wilbert 2000, 2002). Second, resting stages of these cells (cysts) are difficult to identify using light microscopy. Third, silver staining methods for morphological identifications are complicated and time-consuming. Fourth, worldwide only few experts in protistan taxonomy are available that can provide their expertise to the aquaculture industry. As a result, in several cases the ciliates involved could not be identified to species level (Dykova and Figueras 1994; Umehara et al. 2003; Yoshinaga and Nakazoe 1993).

Fluorescence in situ hybridization (FISH) methods can overcome these drawbacks. 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 some ciliates. Fried et al. (2002) designed four specific probes to identify Glaucoma scintillans; Stoeck et al. (2003) identified and visualized Euplotes species in environmental samples with two designed probes; Fried and Foissner (2007) set eight targeted probes to differentiate ciliate morphospecies, Glaucoma scintillans and Glaucomides bromelicola; and Bourne et al. (2008) detected the ciliate associated with the coral disease brown band.

As the first test case for a ciliate parasitizing fish, the scuticociliate Pseudocohnilembus persalinus Evans and Thompson, 1964 was chosen for the present study. Pseudocohnilembus persalinus is one of the most common facultative pathogens, which inhabits the waters and sediments of fishes, and affects the brains, ascites, gills and skin of the marine flounder Paralichthys olivaceus (Kim et al. 2004b, 2006; present study). It can also be found in the ovaries of the freshwater-reared rainbow trout Oncorhynchus mykiss (Jones et al. 2010). Here we designed a specific oligonucleotide probe based on the obtained small subunit rDNA (SSU-rDNA) sequence targeting the pathogen P. persalinus, tested this probe in FISH for its practicality and efficiency in the rapid and unambiguous identification of P. persalinus. This opens a way for researchers involved in aquaculture industry to rapidly and effectively identify ciliate parasites, help understand and prevent infections.

Material and Methods

Collection, isolation and identification

Specimens of the flounder Paralichthys olivaceus were collected from a mariculture pond in Qingdao, China, and maintained in the laboratory. Gills and lesions were removed from individuals displaying signs of parasitic infection and washed with sterile filtered (0.2 μm pore size) seawater in separate Petri dishes. Ciliates were manually collected from the Petri dishes and isolated using a micropipette, observed in vivo and then, for species identification, stained with protargol (Foissner 1991) and wet silver nitrate (Corliss 1953).

DNA extraction, PCR amplification, and sequencing

Ciliates were isolated for clonal culturing using finely drawn glass pipettes, rinsed in filtered sterilized seawater, and transferred to 1.5-ml microcentrifuge tubes. DNA was extracted from hundreds of cells using the DNeasy Tissue kit (Qiagen, Hilden, Germany). The amplification of SSU-rDNA used the eukaryote universal EUK-A and EUK-B primers (Medlin et al. 1988), with the following cycling conditions: 5:00 min at 98°C; 40 cycles of 1:00 min at 94°C, 1:00 min at 57°C, 2:00 min at 72°C; 10:00 min final extension at 72°C. The PCR products were purified using Qiagen’s MinElute PCR Purification Kit and were inserted into pGEM-T vector (Promega, Mannheim, Germany). The vectors were cloned with the TA cloning kit (Invitrogen, Darmstadt, Germany). Plasmids were isolated with Qiaprep Spin Miniprep Kit (Qiagen) from overnight cultures and PCR-amplified using M13F and M13R primers to screen for inserts of the expected size (ca. 1.8 kb in case of the SSU-rRNA fragment). Three colonies were sequenced bidirectionally from random positively screened plasmids by MWG Biotech (Ebersberg, Germany) using an Applied Biosystems (ABI, Foster City, CA) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit.

The length, GC (%) content, and GenBank accession number of the present SSU rRNA gene sequence of P. persalinus are as follows: 1700 base pairs, 45%, JQ956554. The new sequence was checked against the GenBank sequence collection by a standard nucleotide–nucleotide BLAST search, which was found to be 99% identical to the closest sequences, all SSU-rDNA of P. persalinus from different geographic origins (GenBank AY835669, AY551906, and GU584096) and 96% identical to the next closest sequence, SSU rDNA of P. longisetus (GenBank FJ899594).

Sequences for all other ciliates used in this analysis were from the GenBank database. Sequences were aligned with MUSCLE v3.7 (Edgar 2004) with default parameters and the alignment was refined manually to excise highly variable regions using BIOEDIT (Hall 1999). The final alignment comprised 20 taxa and 1666 nucleotide positions. The GTR-I-C evolutionary model was the best-fitted model selected by AIC as implemented in Modeltest v.3.4 (Posada and Crandall 1998) for the alignment. ML analyses were carried out in RAXML-HPC v7.2.5 (Stamatakis et al. 2008), with node support from a majority-rule consensus tree of 1000 bootstrap replicates. BI was carried out using MrBayes v3.2.1 (Huelsenbeck and Ronquist 2003). Posterior probability was estimated using four chains running 10,000,000 generations sampling every 100 generations. The first 25% of sampled trees were considered burn-in trees and were discarded prior to constructing a 50% majority-rule consensus trees. Phylogenetic trees were visualized with MEGA v.4 (Tamura et al. 2007).

Design and test of the specific oligonucleotide probe

A set of probes (nucleotides, 18–22; GC contents, 50–60%; nucleotide-nucleotide Tm, 50–60°C) was designed using the probe design tool as implemented in the ARB software package for the SSU-rDNA sequence of the present P. persalinus and those retrieved from GenBank (GenBank numbers AY835669, AY551906, and GU584096) (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, the probe Pspe210 targeted the helix 12 region of SSU-rDNA was chosen that fulfilled the general criteria of potentially successful probes (Pernthaler et al. 2001). The probe Pspe210 (5′-ATGATTTATCCCAGAGGG-3′) had no mismatches with the SSU-rDNA of all *P. persalinus* isolations and the environmental sample *P. sp.*, but exhibited at least a 2-bp mismatch with other close sequence in GenBank (Table 2). Other probes used included the universal Cy-3-labeled eukaryotic probe Euk1209R (5′-GGGATCACAGACCTG-3′) (Giovannoni et al. 1988) and its Cy-3-labeled complement as a nonsense probe.

**FISH staining**

FISH was used to visualize the ciliate *P. persalinus* both in mass culture samples and mixed with other test species of Scuticociliatia and *Condylostoma* sp. as the negative controls. Cells were fixed with 50% Bouin’s solution (Fried et al. 2002; Stoeck et al. 2003) and filtered onto 1.2-μm-pore-size white Isopore membrane filters (25 mm; Millipore GmbH, Schwalbach/Ts., Germany) using low underpressure. The filter was then washed five times with 1 ml of filtered sterile water. The basic hybridization followed the protocol of Stoeck et al. (2003). In short, membranes were covered with hybridization buffer. Formamidie concentrations ranged from 0% to 50% (the results with every 5% growth were shown) for Pspe210-probe testing, and 30% for the universal eukaryote probe Euk1209R and the nonsense probe. Probe concentrations were 50 ng/μl for each experiment.

All hybridizations were conducted at 46 °C for 3 h, after which membranes were floated into prewarmed washing buffer at 48 °C for 15 min to remove excess and unbound oligonucleotide probes. After rinsing filters with distilled water and 80% ethanol, the membranes were allowed to air dry for a few minutes in the dark before counterstaining the filters for 3 min with 50 μl of 4′,6-Diamidino-2-Phenylindole (DAPI) solution (1 μg/ml), and incubated for 3 min. Subsequently, filter sections were washed again for several seconds in distilled water and 80% ethanol to remove unspecific staining. Air-dried filters were mounted in Vecta Shield mountant (Burlingame, CA) before being viewed and imaged on an epifluorescence microscope (Axioskop Plus, Carl Zeiss GmbH). To allow direct signal comparison, microscope settings, such as excitation power, pinhole diameter, detection gain, amplifier offset, time exposure (10 ms for Cy3 signals, 0.4 ms for DAPI signals), and filter sets for fluorescence image acquisition were kept the same for all tests.

**Results and Discussion**

**Morphological identification**

*Pseudocohnilembus persalinus* was originally described as a free-living ciliate, and reported from the Great Salt Lake in Utah, alkaline soil in Australia and Namibia, and marine water in China (Evans and Thompson 1964; Foissner et al. 2002; Pomp and Wilbert 1988; Song and Wilbert 2000). Three populations were recently isolated from the diseased olive flounder *Paralichthys olivaceus* in Korea, from the mantle cavity of the mollusc clam *Ruditapes philippinarum* in China, and from the ovarian fluid of freshwater-reared adult rainbow trout *Oncorhynchus mykiss* in Idaho, USA (Jones et al. 2010; Kim et al. 2004a,b; Xu and Song, 1999).

The specimens isolated from the infected olive flounder *P. olivaceus* in Qingdao are morphologically similar to previous populations (Table 1). Briefly, the ciliate is about 27–40 × 10–18 μm in vivo, ellipsoidal to slightly pyriform with an anteriorly narrowed end and posterior rounded end. The large globular macronucleus is located in the mid-body. There is one contractile vacuole with a single excretory pore located at end of the third kinety (Fig. 1C). There are 10 or 11 somatic kineties with cilia 6–9 μm long, composed of dikinetids in the anterior 2/3 of the body. The caudal cilium is about 10 μm long. Membranelle 1 is single-rowed, terminating anteriorly to paroral membrane and parallel to membranelle 2; membranelle 2 is also one-rowed and about 4/5 of length of the buccal field; membranelle 3 is very small, located immediately posterior to membranelle 2 (Fig. 1E, F).

The morphology of our isolate matches the original description of *P. persalinus* as well as those reported from East Asia and Australia (Table 1). Thus, the identification is assured. To note: Foissner et al. (2002) described the subspecies *P. persalinus hexakineta* with merely six or seven somatic kineties from saline soil in Namibia; by contrast, all other populations of the species have 9–11 somatic kineties (Table 1).

**SSU-rDNA gene sequences and topology**

The SSU-rDNA sequences of *P. persalinus* AY551906 (Kim et al. 2004a,b) and AY835669 (Jung unpubl.) isolated from *Paralichthys olivaceus* in Korea, and the one GU584096 from the ovary of the freshwater-reared rainbow trout *Oncorhynchus mykiss* (Jones et al. 2010), were found to be 99% identical to new sequence here from *P. olivaceus* in China. These differences are base substitutions (Table 3). In the SSU-rDNA trees, all isolates of *P. persalinus* formed a separate clade branching early in the *Pseudocohnilembus* clade with high supported notes (Fig. 2). Based on the morphological data and the present SSU-rDNA topology, we conservatively accepted the assignment of all the isolations to *P. persalinus*. 
<table>
<thead>
<tr>
<th>Character</th>
<th>Qingdao population</th>
<th>Qingdao population</th>
<th>Utah population</th>
<th>Idaho population</th>
<th>South Korea population</th>
<th>Australia population</th>
<th>Namibia population</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenBank Number</td>
<td>JQ956554</td>
<td>–</td>
<td>–</td>
<td>GU584096</td>
<td>AY551906</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Habitats</td>
<td>Yellow Sea</td>
<td>Yellow Sea</td>
<td>Great Salt Lake (salt water)</td>
<td>Marine</td>
<td>Alkaline soil</td>
<td>Alkaline soil</td>
<td></td>
</tr>
<tr>
<td>Length of buccal field: cell length</td>
<td>ca 1/2</td>
<td>ca 1/2</td>
<td>ca 1/2</td>
<td>-</td>
<td>ca 1/2</td>
<td>ca 1/2</td>
<td>ca 1/2</td>
</tr>
<tr>
<td>Number of somatic kineties (SK)</td>
<td>10–11</td>
<td>10–11</td>
<td>9–11</td>
<td>8–9</td>
<td>8–11</td>
<td>9–10</td>
<td>6–7</td>
</tr>
<tr>
<td>Number, contractile</td>
<td>1</td>
<td>1</td>
<td>1 (sedom 2)</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of basal bodies in SK1</td>
<td>15–17</td>
<td>15–18</td>
<td>15–20</td>
<td>15–18</td>
<td>15–18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Position of CVP, located near the end of somatic kinety</td>
<td>SK3</td>
<td>SK3</td>
<td>SK3 (when two, then near Sk3 &amp; SK4)</td>
<td>–</td>
<td>SK3</td>
<td>SK3</td>
<td>SK3</td>
</tr>
</tbody>
</table>

All measurements in μm. –, Data not available.

<sup>a</sup>Data based on protargol impregnated specimens.

<sup>b</sup>Data based on Chatton-Lwoff silvered specimens.
Fig. 1. *Pseudocohnilembus persalinus* from life (A and B) and after protargol (E) and wet silver nitrate impregnation (C, D, F, G). A. Ventral view of a cell. Arrow indicates the caudal cilium. B. Right side view. Arrow points to the contractile vacuole. C. Right side view showing the contractile vacuole pore (arrow) and cytopyge (arrowhead). D. Left side view of a cell. E. Ventral view showing the membrane 1 – paroral membrane complex (arrowhead), membrane 2 (double-arrowhead), and membrane 3 (arrow). F. Right ventral side view showing the membrane 1 (arrowhead), membrane 2 (double-arrowhead), membrane 3 (arrow), and paroral membrane (double-arrow). G. Right side view. Scale bars = 10 μm.

Table 2. The sequence alignment of probe Pspe210 evaluated with the probe match tool of the ARB software package (the species with more than three mismatches with target probe sequence are not showed).

<table>
<thead>
<tr>
<th>Organism</th>
<th>GenBank/EMBL sequence accession number</th>
<th>Probe sequence (3′–5′) GGGAGACCCCTATT TAGTA</th>
<th>Target sequence (5′–3′) CCCTCTGGGATAA ATCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudocohnilembus persalinus</em> AY551906, GU584096, AY835669</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ciliate sp.1 AB505509</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ciliate sp.2 AB505510</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudocohnilembus hargisi</em> AY833087, AY212806</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| *Pseudocohnilembus longisetus* FJ899594 | | | *
| *Pseudocohnilembus marinus* Z22880 | | | *
| *Parauronema longum* HM236338 AY212807 | | | *
| *Uronema elegans* AY103190 | | | *
| Entodiscus borealis AY541687 | | | *
| Glaucanema trihyrne GQ214552 | | | *
| Ciliate sp. 3 AF290081 | | | *
| Ciliate sp. 4 AY876050 | | | *
| Pleuronema sinica EF486864 | | | *

Only nucleotides different to the target sequence (mismatches) of *P. persalinus* are shown.
The most likely ML tree and its branch lengths are shown. The Bayesian (BI) tree inferred using MrBayes and the ML tree are nearly identical in topology. Node support is as follow: ML bootstrap/BI posterior probability. N reflects minor differences in topology that could not be represented on the present tree. The new sequence is highlighted in boldface. Support <50% is shown as -. -

**Probe evaluation and FISH staining**

The Pspe210 probe evaluated with the probe match tool of the ARB software package shows that it is specific to *P. persalinus* (Table 2). The probe binds to other populations of *P. persalinus* with no mismatches. Besides two mismatches to the homologous target sites of two unclassified scuticociliated species, the Pspe210 has at least three mismatches to other species belonging in *Pseudocohnilembus*, and at least three mismatches to other ciliates like *Uronema elegans* (GenBank AY103190) and *Entodiscus borealis* (GenBank AYS41687) (Table 2). The GenBank BLAST shows that it binds to other populations of *P. persalinus* and the environmental sample *Pseudocohnilembus* sp. (GenBank FN430390) with no mismatches, and has at least one mismatch to other organisms like zebrafish (GenBank FP102983) and *Mus musculus* (GenBank AC168091). Based on the GenBank BLAST result, we suggest that the environmental sample *Pseudocohnilembus* sp. (GenBank FN430390) be *P. persalinus*.

Fluorescence in situ hybridization with the probes EUK1195 and Pspe210 resulted in the presence of a red fluorescence signal for *P. persalinus* (Fig. 3A, C, G–Q), clearly distinguishable from the faint autofluorescence signal achieved with negative-control hybridizations using the Pspe210 probe to hybridize the untargeted ciliates *Condylostoma* sp. and Scuticociliatia sp. (Fig. 3D, E), and the nonsense probe (Fig. 3F). The FISH approach also provided some morphological information such as body shape, macro and micronuclei shape and number of micronuclei (e.g. Fig. 3A–C). This will help verification of morphotypes in mixed taxa samples. The signal intensity on the probe Pspe210 became weaker when the formamide (FA) concentration increased in the hybridization buffers (Fig. 3G–Q), and the fluorescence signals with more than 20% FA (Fig. 3L–Q) were weaker than those of the positive control (Fig. 3C). Therefore, 20% of formamide in the hybridization is the optimal concentration for the stringency of probe Pspe210 (Fig. 3K).

For the test of the probe specificity, using congeners of *Pseudocohnilembus* instead of Scuticociliatia sp. and *Condylostoma* sp. as negative control is a much convincing practice. However, *Pseudocohnilembus* is a genus consisting of about 10 species, and all except *Pseudocohnilembus persalinus* are rare species (Song et al. 2003). We tried to sample other species of *Pseudocohnilembus* for control since the start of the work, but failed. This is often the case for certain infrequent ciliate groups, for instance, that in Bourne et al. (2008). Following Fried and Foissner (2007), we evaluated the probe not only by the ARB software package but also the GenBank BLAST tool to show the probe specificity. Previous studies have already shown the power of the method for specific detection. Nonetheless, we keep in mind that the probe is still in need of consolidation with the support of the isolation and/or sequencing of congeners of *Pseudocohnilembus*.

As a test case using probes for the identification of pathogenic ciliates in aquaculture, the present study utilized the probe Pspe210 to unambiguously detect and identify *P. persalinus* (Figs 2, 3). Our study demonstrates that the FISH method is able to quickly and accurately identify the potential ciliate parasite involved in cases of fish sickness without the necessity of morphological identification. This study sets the framework for a long-term project that will result in a set of oligonucleotide probes, which will identify all major ciliates and also other protistan parasites, impacting aquaculture.

Additionally, *P. persalinus* has been found in many localities (e.g. America, Australia, Korea and China), and in different habitats (e.g. saline soil, salt and fresh water), but molecular data of only four isolations are known (Evans and Thompson 1964; Foissner et al. 2002; Jones et al. 2010; Kim et al. 2004a,b; Pomp and Wilbert 1988; Song and Wilbert 2000; Xu and Song 1999 and the present study). The FISH probe designed here has the potential to be used for confirming the geographical distribution and detecting the possible dispersal of this facultative pathogen.

**Comparison with the Sanger and next-generation sequencing**

Although the Sanger sequencing method is another molecular way to identify *Pseudocohnilembus persalinus*, it has one significant limitation in aquaculture practice. Usually, there are often various ciliate species including *P. persalinus* in the waters and sediments of aquaculture systems. Most scuticociliates are small-sized (usually 20–50 μm) and morphologically quite similar, and thus usually difficult to isolate (with accurate identification) for Sanger sequencing. Alternatively, with the direct sequencing on environmental samples, the genes are often hard to be amplified by PCR when the cells are in low abundance. By contrast, FISH can visualize the target cells in any case.
Table 3. Nineteen variable sites of the alignment of SSU rRNA gene sequences of *Pseudocohnilembus persalinus*-populations.

<table>
<thead>
<tr>
<th>P. persalinus-populations</th>
<th>178</th>
<th>189</th>
<th>351</th>
<th>625</th>
<th>677</th>
<th>705</th>
<th>802</th>
<th>1026</th>
<th>1034</th>
<th>1044</th>
<th>1327</th>
<th>1333</th>
<th>1335</th>
<th>1366</th>
<th>1375</th>
<th>1377</th>
<th>1700</th>
</tr>
</thead>
<tbody>
<tr>
<td>JO956954</td>
<td>A</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>AY551906</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>AY835669</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>GU584096</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>G</td>
<td>G</td>
</tr>
</tbody>
</table>

Fig. 3. Simultaneous fluorescence *in situ* hybridization (FISH) and 4′, 6-Diamidino-2-Phenylindole (DAPI) staining of *Pseudocohnilembus persalinus* (A–C, F–Q) and other test ciliates (D and E). A. *P. persalinus* probed with EUK1195 probe. Only the Cy3-dye-derived light emission (red) is recorded. The formamide (FA) concentration is 30%. B. The cell counterstained with DAPI. Only the nucleus is shown in blue. C. Superimposition of (A) with 30% transparency on (B). Both channels were recorded separately by Cy3-dye-derived light emission (red) and DAPI-dye-derived light emission (blue). D. Superimposition of *Condylostoma* sp. stained with the Cy3-labeled probe Pspe210 and DAPI. The FA concentration is 20%. E. Superimposition of Scuticociliatia sp. stained with the Cy3-labeled probe Pspe210 and DAPI. The FA concentration is 20%. F. Superimposition of *P. persalinus* stained with the nonsense probe and DAPI. The FA concentration is 20%. G–Q. Superimposition of *P. persalinus* stained with the Cy3-labeled probe Pspe210 and DAPI. The FA concentration is 0–50%, and the figures with every 5% increase in concentration are shown. Scale bars = 10 μm.
Our aim is to establish a fast and user-friendly tool facilitating qualitative and quantitative examination of facultative ciliate pathogens in aquaculture systems. By using a fluorescence microscope, ordinary workers can easily screen whether the potential pathogens exist or not. In this point of view, the FISH method is more convenient and much cheaper than the next-generation sequencing. Furthermore, the gene sequencing methods could not obtain the number of ciliate cells in environments; knowing the quantity is an important issue for the control of pathogens in aquaculture.

Acknowledgements

We thank Anke Behnke for technical advice. Support came from the National Natural Science Foundation of China (No. 41076102) to K.X., the China Postdoctoral Science Foundation (grant 2013M531655) to Z.Z., the Deutsche Forschungsgemeinschaft (DFG grant STO414/3-2) to T.S., and the Alexander von Humboldt Foundation and DFG (grant DU1319/1-1) to M.D.

References


Foissner, W., Agatha, S., Berger, H., 2002. Soil ciliates (Protozoa, Ciliophora) from Namibia (Southwest Africa), with emphasis on two contrasting environments, the Etosha Region and the Namib Desert. Denisia 5, 1–1459.


