Peritrich ciliates are a large and highly diverse protistan group that so far comprises approximately 1,000 species (approximately 1/7 of all known ciliates) (28, 36, 37). Systematically, two monophyletic peritrich subclasses, the free-living Peritrichia (formerly the order Sessilida) and the parasitic Mobilia (formerly the order Mobiliida), have been recognized (28, 44). As the larger group, the Peritrichia comprises about 105 genera (28) and at least 800 described species (16). Most of the free-living peritrichs have a complex life cycle with cyst, free-swimming (i.e. telotroch), and sessile stages (4, 28, 42). Since only the sessile forms are generally morphologically identifiable, they have been recorded from a variety of environments, e.g. freshwater (14), brackish (5, 41), marine (24, 37), and soils (13); from plankton (3), and biofilm or periphyton of immersed substrates and animals (12, 20, 22, 42).

The peritrichs are responsible for water clarification by their bacterivory; hence, they are an ecologically important group in aquatic environments (9, 17, 29). The abundance of peritrichs is not often reported, and when planktonic, is difficult to interpret because they are either attached to algae in the plankton, or are in stages of telotroch, which are usually difficult to identify (see (28) and references therein). There are records of the temporal and spatial distribution of peritrich populations (41), while the community ecology of free-living peritrichs has been little studied.

During previous decades, diversity, phylogenetic and ecological studies of protists have increasingly relied upon small subunit ribosomal RNA (18S rRNA) gene sequences (6). Because there are numerous 18S rRNA gene sequences from diverse organisms in public databases, specific PCR primers or probes can be designed (and tested in silico) for the taxa of interest. Using specific PCR primers (33) and the quantitative real-time PCR (qPCR) assay, both the diversity and quantity (indicated by rDNA copy number) of the specific taxa in environmental samples can be assessed efficiently (19, 34, 40, 45); however, a specific molecular tool for the detection and quantification of peritrichs from environmental samples is not yet available.

In this study, we aimed to design and evaluate PCR primers to amplify partial 18S rDNA of free-living peritrichs (subclass Peritrichia sensu Zhan et al. 2009) (44) and to select a primer set for qPCR that specifically quantifies peritrich rDNA copies in environmental samples.

Materials and Methods

Sampling

Water samples were collected from the Guangdang River, a small river in Yantai, China (37°28’N, 121°28’E), on August 15, 2011. The 10-km-long river originates from a freshwater reservoir and flows into the Yellow Sea, with a great salinity gradient. Sampling was made at four sites, one freshwater (F), two brackish (B1 and B2), and one marine (M). Two liters of surface water were sampled at each site in the lower tidal period, filtered through 200-µm-pore mesh, and taken back to the laboratory within 1 h. Subsamples were maintained at room temperature and examined for live peritrich ciliates under a stereo microscope. Recognized cells were picked up with a micropipette and photographed in vivo under a microscope (BX51; Olympus, Tokyo, Japan). To collect eukaryotic biomass for molecular analysis, subsamples were gently filtered through 10-
µm-pore polycarbonate membranes (diameter 47 mm; Millipore, Bedford, MA, USA) until filtration performance became obviously lower. The water volumes filtered were recorded as 220, 540, 480 and 240 mL for sites F, B1, B2 and M, respectively. The membranes were placed immediately into 2 mL cryotubes and preserved at −80°C for DNA extraction. Salinity, pH, water temperature, chlorophyll a, and dissolved oxygen concentration were measured in situ with a multi-parameter probe (MSS; Hach, Loveland, CO, USA) (Table S1).

**DNA extraction, primer design and PCR**

Environmental DNA was extracted using the UltraClean Soil DNA Isolation Kit (MoBio, Solana, CA, USA). The concentration of the extracted DNA was checked using a NanoDrop 2000C spectrophotometer (Thermo, Winsted, DE, USA).

The design of specific primers for the subclass Peritrichia was based on alignment of the 18S rRNA gene sequences of 105 eukaryotic organisms (49 peritrichs, 36 non-peritrich ciliates, and 20 non-ciliate protozoa) retrieved from GenBank (NCBI). Four candidate peritrich-specific primers targeting the conserved regions were newly designed (Table 1). The two reverse primers (Peri1004R and Peri1403R) were paired with the eukaryote-specific primer EukA (30) to amplify longer fragments of 18S rDNA, and short fragments were amplified with two forward primers (Peri974F and Peri979F) (Fig. 1).

**Cloning, RFLP analysis and sequencing**

Since longer 18S rDNA fragments tend to reveal deeper phylogenetic positions of the environmental peritrich sequences obtained, the closely related sequences identified by BLAST as well as the 18S rRNA genes of morphologically identified representatives were retrieved from GenBank. Sequences were aligned using Muscle, and a neighbor-joining (NJ) tree was constructed with MEGA 4.0 (38) based on the Kimura 2-parameter distance model. The tree was rooted with Tetrahymena and 240 mL for sites F, B1, B2 and M, respectively. The membranes were placed immediately into 2 mL cryotubes and preserved at −80°C for DNA extraction. Salinity, pH, water temperature, chlorophyll a, and dissolved oxygen concentration were measured in situ with a multi-parameter probe (MSS; Hach, Loveland, CO, USA) (Table S1).

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**Primers newly designed and tested for the amplification of peritrich 18S rRNA genes**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Length</th>
<th>GC%</th>
<th>Tm (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EukA</td>
<td></td>
<td>AACCTGGTTGATCTCCGCCAGT</td>
<td>21</td>
<td>52.4</td>
<td>58.9</td>
<td>Medlin et al., 1988</td>
</tr>
<tr>
<td>Peri974F</td>
<td></td>
<td>GGAAACTCATCAGGCAAGAGATT</td>
<td>25</td>
<td>42</td>
<td>54.9–57.3</td>
<td>This study</td>
</tr>
<tr>
<td>Peri979F</td>
<td></td>
<td>CCTCATACGGCARCAAGAGATT</td>
<td>20</td>
<td>42.5</td>
<td>49.3–52.3</td>
<td>This study</td>
</tr>
<tr>
<td>Peri1004R</td>
<td></td>
<td>TCTCTTAAATCTCTTGCGCTGATG</td>
<td>24</td>
<td>41.7</td>
<td>51.7–56.6</td>
<td>This study</td>
</tr>
<tr>
<td>Peri1403R</td>
<td></td>
<td>GGGGCTTGCTGACACCTGTT</td>
<td>19</td>
<td>50</td>
<td>51.8–54.5</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Summary of the clone library analysis of the four sampling sites

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>F</th>
<th>B1</th>
<th>B2</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clones checked</td>
<td>193</td>
<td>168</td>
<td>168</td>
<td>168</td>
</tr>
<tr>
<td>No. of RFLP types</td>
<td>19</td>
<td>11</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>No. of peritrich clones obtained</td>
<td>30</td>
<td>7</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>No. of peritrich sequences</td>
<td>10</td>
<td>4</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>No. of peritrich OTUs (^a)</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) OTU sequence similarity cutoff 99%.

Results

Specificity of newly designed primers

The in silico check using probeCheck and BLAST against GenBank confirmed that none of these newly designed primers matched members of the subclass *Mobilia* and other organisms, and that all the peritrich 18S rDNA sequences at present were targeted very well across lineages except for a few species. Peri974F and/or Peri979F matched perfectly with 46 (93.9%) peritrich sequences belonging to genera such as *Astylozoan, Carcanchum, Epicarchesium, Epistyli*, *Ophrydium, Opisthonecta, Pseudovorticella, Telotrochidium, Vaginicola, Vorticella, Zoanthinum* and *Zoothamnopsis*, but mismatched with three peritrich sequences at 2–4 sites: *Epistyli galea* (AF401527), *Campanella umbellaria* (AF401524), and *Opcerularia microdiscum* (AF401525). Non-peritrich sequences differed from the primer at at least 3 sites. Peri1004R showed perfect matches with 7 peritrich sequences, 1 mismatch at the 5’ primer end with 39 peritrich sequences, and 3–4 mismatches with *E. galea* (AF401527), *C. umbellaria* (AF401524), and more than 4 mismatches with *O. microdiscum* (AF401525) and non-peritrichs. Peri1403R showed perfect matches with 47 peritrich sequences including *E. galea* (AF401527), *C. umbellaria* (AF401524), but showed 1 mismatch with *O. microdiscum* at the 5’ primer end and an uncultured bacterium (DQ298151) at the 3’ primer end, and no less than 3 sites for other non-peritrich sequences.

The primer pairs EukA/Peri1004R, EukA/Peri1403R, Peri974F/Peri1403R and Peri979F/Peri1403R all produced positive amplifications on genomic DNAs of peritrich isolates, and generally non-amplification of non-peritrich ciliates (Table S2). One microliter of template DNA or about 1/20 of genomic DNA of a single isolated peritrich cell of *Vorticella* sp. was enough to yield bright specific bands with 35 PCR cycles (data not shown). Using cloning and sequencing we also confirmed that the primer set Peri974F/Peri1403R was highly peritrich-specific, hence it is suitable for qPCR and reliable for environmental surveys (see Materials and methods).

Compared with Peri974F/Peri1403R, PCR with the primer set EukA/Peri1403R produced a longer fragment of 18S rDNA, but its specificity was much lower, as less than 10% clones examined (n=697) were peritrichs (Table 2). Nevertheless, it was shown that peritrich-specific clones can be successfully screened out by primers Peri974F/Peri1403R whose amplicons are semi-nested within that of EukA/Peri1403R.

Peritrich ciliate composition and distribution along the Guangdong River

A total of 697 clones from 4 libraries of the river samples were screened, and 67 peritrich clones and 23 peritrich sequences were identified and obtained, 28% sequences were found to be chimeras, and 11 OTUs were finally determined (Table 2; Fig. 2). Peritrich OTUs were seldom shared among
sites, except for one (OTU7) that was found at both sites B1 and M (Fig. 2). In the library of the freshwater site, 6 OTUs were detected; the most frequently found (70%) sequences belonged to the OTU2, which clustered with *Epistylis hentscheli* and *E. plicatilis* in the NJ tree; OTU3 was the second most abundant, accounting for 13% sequence abundance. OTU6 was clustered with an uncultured ciliate species (HQ219427) previously detected in a limnetic and eutrophic freshwater lake in France (31). Other OTUs (OTU1, 4 and 5) in the freshwater library were detected only once. Among these, OTU1 clustered with OTU3 at 53% support. OTU4 grouped with *Vorticellidae* (*Carchesium polypinium*) and *Ophyrididae* with moderate bootstrap support (75%). The phylogenetic position of OTU5 was very close to OTU6.

For the two brackish sites, there were more OTUs (4 vs 1) in B2 than B1 (Table 2). OTU7 was represented by 7 clones in B1, clustering with *Zoothamnium nii* with 100% bootstrap support. The B2 library was dominated by OTU8 and OTU9 (relative abundance 39% and 39%), which likely belong to a population of *Opisthodonecta minima* (EF417834, sequence similarity about 99%) and the family *Epistylididae*, respectively. OTU11 was similar to OTU9 and belonged to *Epistylididae*. OTU10 was close to OTU1 and 3. Among these OTUs, the taxonomic positions of OTU1, 3 and 10 could not be resolved in our phylogenetic analyses.

For the morphological observation of water samples, we only found two morphospecies *Epistylis* sp. and *Zoothamnium* sp. at freshwater and brackish sites, respectively (Fig. S1).

**Quantifying peritrich ciliates using rDNA copy numbers as an indicator**

Specific PCR products were identified by melting curve analysis and a reproducible distinct melting point (Tm) of 78.39°C was observed from all standards. The linear relationship between the Ct (cycle threshold) and the log of rDNA copy number was \(C_T = 3.5187 + 0.999 \times \log_{10}(\text{rDNA copies µL}^{-1})\) + 32.035, with an amplification efficiency of 92.4% and R² of 0.999 (Fig. 3). Melting curves of environmental samples were almost identical to those of the standards during the qPCR assay (Tm=79.97°C), except for the marine sample, for which melting curves with a peak of primer dimers were observed (Fig. S2). According to the Ct values obtained (23, 26, and 24), the peritrich rDNA gene copy numbers were calculated as 112.40±7.00, 5.72±1.08 and 29.91±3.30 (×10⁴ liter⁻¹) in the F, B1 and B2 samples (Fig. 4). The non-specific amplicons of the marine samples were confirmed to be primer dimers by running electrophoresis in a 1.5% agarose gel (data not shown), so we could not quantify peritrich sequences in the marine sample.

According to the standard curve, the minimum detection sensitivity was 96 copies of 18S rDNA (Ct value of 29.97 ± 0.20), corresponding to rDNA extracted from 0.0006 cells of *Vorticella* sp., as we had estimated the rDNA copy number per cell of this species to be 160,000 (unpublished data).

Correlation among richness, abundance and environmental factors showed that water salinity was better coupled with peritrich OTU, sequence and rDNA copy numbers than other factors (Table S1). Their coefficients ranged from −0.81 to −0.90, indicating a negative effect of salinity on peritrich community diversity and abundance.

**Discussion**

Although most ciliates including peritrichs can be easily observed under a microscope, their identification has been based on their morphology, infraciliature and silverline system revealed using silver staining methods (15), which, however, is difficult for non-specialists and time-consuming for ecological and biogeographic studies. Recent studies have also shown that morphological markers underestimate peritrich diversity (18, 43). This demonstrated the importance of using molecular tools to characterize the diversity of both isolated and environmental peritrichs.

Phylum-specific primers for ciliates have been developed and successfully used for soil ecosystems (8, 26, 33); however, for aquatic samples where highly diverse protists occur, some non-ciliates would also be targeted by these primers, resulting in substantial decreases of PCR specificity (8). In order to reveal both the diversity and quantity of ecologically important ciliate groups in aquatic environments, specific primers for ciliate taxa in lower ranks (e.g. classes, orders, families etc.) with highly specific performance may be one of the options. For example, order-specific primers have been used for studying clone library analysis (7) and denaturing gradient gel electrophoresis (DGGE) profiling (39) of marine planktonic oligotrich and choreotrich ciliates. In this study we extend and expand this strategy by designing and testing primers specifically and developed a qPCR assay for characterizing peritrich ciliates.
With the four samples from the Guangdang River, we showcase the good specificity of the primer pair Peri974F/Peri1403R, and the nested approach for studying peritrich community from environmental samples. It should be noted that species in the order Operculariida (e.g. Epistylis galea, Campanella umbellaria, and Opercularia microdiscum, see [43]; Fig. 2) have several mismatches mostly at the 5’ end with these primers. We did not detect any sequences closely related to or placed basal to known species/taxa with low bootstrap values (<10%), as shown by the results of clone sequencing (Table 2). However, taking these clones with known sequence information as templates, we demonstrated that colony PCR with the primer set Peri974F/Peri1403R was only able to amplify the clones of peritrich sequences, not the non-peritrichs (e.g. algae). This suggests that the primer set Peri974F/Peri1403R outperforms EuKa/Peri1403R in specifically recovering peritrich sequences from environmental DNA, although the length of PCR products is relatively shorter (about 441 bp).

The aims of this study were primer design and protocol optimization rather than application; however, the results provide an interesting insight into the diversity and composition of peritrich ciliates in water samples with distinct environmental gradients. With these peritrich-specific primers and sequencing, we detected 11 peritrich OTUs in about 1.5 L of water samples from the Guangdang River. The local (alpha-) diversity of peritrich ciliates seems to be low. This may be due to strong environmental gradients, especially salinity differences. Our data from the Guangdang River samples also showed that peritrich diversity generally decreased from the freshwater site to the marine site (Table 2, S1). Is peritrich diversity in water samples mainly controlled by salinity, other environmental factors or food supply? Is peritrich diversity higher in freshwater than in marine habitats? These ecological/biogeographic questions warrant further studies with dedicated experimental design and analyses.

From the Guangdang River samples we obtained several peritrich rRNA gene sequences (e.g. OTU1, 3, and 10), which are placed basal to known species/taxa with low bootstrap values (<50%) in the NJ tree. This does not necessarily mean that these sequences represent new species or new taxa of peritrichs, because among the 800 described peritrich species there is only a small proportion whose 18S rRNA genes are available from public databases. Nevertheless, it can be deduced that these OTUs could be rare in environmental samples and hence have frequently “escaped” morphological observation.

Quantitative PCR has advantages such as high accuracy and throughput and has been employed for quantifying eukaryotic groups such as diatoms, dinoflagellates, and picoeukaryotes (19, 34, 45). Using the primers Peri974F/Peri1403R, we have also developed a qPCR assay for quantifying peritrich rDNA copy numbers in environmental samples. The ratio of copy numbers generated using qPCR (116:5:32) is roughly consistent with the ratio of peritrich clone numbers (30:7:18) at least for the samples (F, B1 and B2) from the Guangdang River, although the water volumes filtered differed among samples (220, 540 and 480 mL). Furthermore, only 1 peritrich clone was detected from the 168 selected clones of library M, and the low abundance of peritrichs at this site was verified by the qPCR result (i.e. below the detection limit of qPCR). For the Vorticella sp. used for generating standard curves in the qPCR assay, we had estimated the rDNA copy number per cell to be 160,000 (more data on rDNA copy numbers from a variety of ciliate species are in preparation). The high sensitivity (lower detection limitation of about 0.0006 cells), specificity, reproducibility, and relatively low cost of using SYBR Green fluorescence enabled it become a practical tool for the detection and quantification of peritrichs in various environments.

The protocol for DNA extraction may be a key factor that affects the result of qPCR in our study. Some environmental DNA will inevitably be lost during the procedure in the use of either the UltraClean Kit or any other commercial kits for DNA extraction. This will lead to underestimation of the rDNA copy numbers, and hence the quantity of the taxa in question; however, the effect will be minimized if DNA samples are extracted and processed in parallel, and will be no problem in ecological studies in which many samples are compared.

With these peritrich-specific primers and the developed qPCR protocol, additional applications are possible. For instance, the internal transcribed spacers (ITS) and 28S rRNA genes have been suggested as barcodes for fungi, and for biogeographic analysis (32, 46). Our peritrich-specific forward primers can be paired with other primers targeting the ITS, 5.8S and 28S rRNA genes, thus yielding longer fragments which could be useful in population genetics or biogeographic studies. qPCR has also been used for assessing the gene copy number within a given genome (25, 40). Likewise, the qPCR assay could also be employed to assess rDNA copy numbers per peritrich cell from species to species, which might be helpful in calculating peritrich abundance in environmental samples given that the total rDNA copy number is known and the species composition of the sample is well identified.

In conclusion, we report here for the first time the development of specific primers for characterizing the peritrich ciliate community in environments using traditional PCR and real-time quantitative PCR assays. The DNA-based approach circumvents the difficulties of morphological identification and allows the detection of peritrichs of all development stages. The molecular approach also enables the analysis of many samples from different environments, which will undoubtedly stimulate further ecological and biogeographic researches on these organisms. Also, our study illustrates the potential of taxon-based molecular tools to reveal the diversity and quantity of other ciliate groups in freshwater, marine, and terrestrial ecosystems.
Acknowledgements

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References


