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FEATURE ARTICLE

Genus-specific quantitative PCR of thraustochytrid protists

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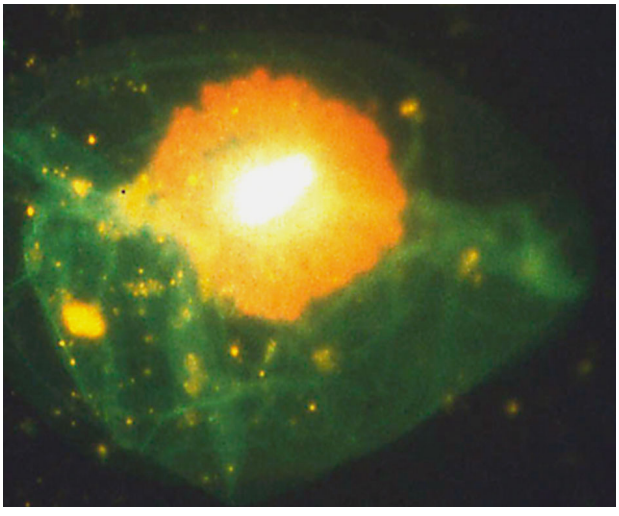
ABSTRACT: Thraustochytrids have the capability to recycle refractory organic matter, with a resulting impact on carbon cycling in coastal and open seawaters. The abundance of thraustochytrids has traditionally been estimated by acriflavine direct counting. However, this technique may lead to over- or underestimation. To accurately quantify the abundance of thraustochytrids, we developed a quantitative PCR (qPCR) system using 7 genus-specific primer sets targeting 7 genera (*Aurantiochytrium*, *Botryochytrium*, *Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia*) from the family Thraustochytriaceae. The high specificity was verified *in silico* and with culture strains of each genus. In addition, we applied this qPCR assay to test for the presence of thraustochytrids in coastal and open seawaters around Japan. We successfully detected the presence of *Aurantiochytrium* (in the range of 1.12×10^4 to 1.31×10^4 cells l^{-1}) and *Oblongichytrium* (in the range of 1.02×10^4 to 3.14×10^4 cells l^{-1}) in 8 surface water samples from around Satsuma-Iwojima (western Japan) and off the Karakuwa in Sanriku (eastern Japan). We obtained higher estimates using qPCR than the traditional acriflavine method in all cases, with a weak positive correlation between the 2 methods (r^2 = 0.495). Interestingly, we quantified thraustochytrids in 104 additional samples by direct count, but not by qPCR, possibly because of inhibition of the qPCR reaction and/or the presence of novel thraustochytrid groups. Although these trials are preliminary, our approach can provide the genus-specific value of abundance in the environment. It will also promote further advances in our understanding of thraustochytrid diversity.



Thraustochytrid protists (here: single cell stained with acriflavine) are an often overlooked part of the marine microbial food chain.

Photo: Takeshi Naganuma

KEY WORDS: Thraustochytriaceae · Stramenopile · qPCR · 18S rRNA gene · Abundance

INTRODUCTION

Thraustochytrids are estuarine/marine protists belonging to the family Thraustochytriaceae, of the class Labyrinthulomycetes within the kingdom Chromista (Cavalier-Smith et al. 1994, Honda et al. 1999, Cava-

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lier-Smith & Chao 2006). They have attracted attention by virtue of their biotechnological role in the production of omega-3 long-chain polyunsaturated fatty acids (PUFAs) such as docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Raghukumar 2008). They have the ability to decompose plant material, such as algal tissue and mangrove leaf litter, by means of extracellular cellulase (Sathe-Pathak et al. 1993, Bremer 1995, Nagano et al. 2011), with a resulting impact on carbon cycling in coastal and open seawaters, and may grow on terrestrial refractory organic substrates contained in river water (Kimura & Naganuma 2001). In addition, the bio-volume of thraustochytrids is ~10^3 times greater than that of bacterioplankton (Naganuma et al. 1998). Thus, they serve as potentially important food sources for picoplankton-feeders, thereby enhancing pelagic secondary production (Naganuma et al. 1998, Raghukumar & Damare 2011).

The abundance of thraustochytrids has been measured in many previous studies focusing on the direct enumeration of non-planktonic or planktonic thraustochytrids (Raghukumar & Schaumann 1993, Naganuma et al. 1998, Raghukumar et al. 2001, Naganuma et al. 2006), estimation of their biomass based on cellular carbon and nitrogen content and the C:N ratio (Kimura et al. 1999), estimation of the correlation between their abundance and environmental parameters (Kimura et al. 2001), and determination of the effect of river discharge on their distribution and abundance (Kimura & Naganuma 2001). These studies used a fluorogenic acriflavine dye to enumerate the thraustochytrids by direct detection. This technique relies on the fact that the wall and nucleus of thraustochytrid cells fluoresce differently (red and blue-green, respectively) under blue-light excitation. This dual fluorescence distinguishes thraustochytrids from other protists and detritus. However, the acriflavine count includes protozoan cysts, thereby leading to overestimation, and excludes thraustochytrid zoospores, leading to underestimation. In addition, there may be variation in results due to observer error. Kimura et al. (2001) pointed out that such over- or underestimation should be evaluated in future studies by using a more specific technique. To address this issue, Takao et al. (2007) developed a fluorescence *in situ* hybridization (FISH) method using an 18S rRNA-targeted fluorescent oligo-nucleotide probe for specific detection of thraustochytrids. Damare & Raghukumar (2010) used an internal transcribed spacer (ITS)-based *in situ* hybridization (ISH) technique to detect aplanochytrids (Labyrinthulomycetes). Although FISH and ISH are powerful tools, they require many hybridization steps and intensive microscopic work.

To address these limitations and provide a simple method that can be used to process multiple samples, we developed a quantitative polymerase chain reaction (qPCR) assay. Quantification by qPCR relies on detection of the increase in fluorescence from exponentially amplified DNA by a PCR involving a primer set and/or a fluorochrome-labeled probe designed to bind to the desired DNA locus. qPCR-based quantification provides a highly sensitive and specific system for the identification of target organisms. This method is increasingly being used in marine microbiological studies, such as in the detection of dinoflagellates (Bowers et al. 2000, Moorthi et al. 2006, Yamashita et al. 2011) and the thraustochytrid pathogen quahog parasite unknown (QPX) (Lyons et al. 2006, Liu et al. 2009). In a recent report, Bergmann et al. (2011) developed a qPCR assay for detection of the labyrinthulid *Labyrinthula zosterae* (Labyrinthulomycetes), known as the causative agent of eelgrass wasting disease. However, except for labyrinthulomycete pathogens, there are no published reports detailing qPCR detection of thraustochytrids. We developed and evaluated a new qPCR system with genus-specific primer sets targeting thraustochytrids and then used this assay to test for the presence of thraustochytrids in field samples.

MATERIALS AND METHODS

Design of genus-specific PCR primers

We used the intercalation chemistry that employs the SYBR® Green I fluorochrome and designed the PCR primer sets based on specific regions of the 18S rRNA gene to differentiate 7 genera (*Aurantiochytrium*, *Botryochytrium*, *Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia*) belonging to the Thraustochytriaceae. We used 27 sequences obtained from the DDBJ/EMBL/GenBank databases as references (Table 1). Specific regions could not be determined in 3 additional genera (*Althornia*, *Japonochytrium*, and *Thraustochytrium*) for a variety of reasons, including the genus being phylogenetically diverse or the unavailability of a culture strain. Ten taxa of another Chromista group were also referenced as negative targets (Table 1). The obtained sequences were aligned using Clustal X 2.0 (Larkin et al. 2007) and manually edited by eye. The primer sequences were designed from regions specific to each target genus that allowed the elimination of non-target genera, and the threshold was set at 3 nucleotide mismatches.

Table 1. DDBJ/EMBL/GenBank accession numbers of 18S rRNA gene sequences used to design the genus-specific PCR primer sets. Asterisks (*) indicate scientific names according to Yokoyama & Honda (2007) and Yokoyama et al. (2007)

Taxon	Accession number	Sequence length (bp)
Genus *Aurantiochytrium*		
Aurantiochytrium limacinum NIBH SR21*	AB022107	1678
Aurantiochytrium mangrovei	DQ100293	1721
Aurantiochytrium sp. KH105*	AB052555	1755
Aurantiochytrium sp. mh0186	AB362211	1790
Aurantiochytrium sp. SEK 209	AB290574	1720
Aurantiochytrium sp. SEK 218	AB290573	1711
Aurantiochytrium sp. SEK 217	AB290572	1764
Genus *Botryochytrium*		
Botryochytrium radiatum SEK 353	AB355410	1699
Genus *Oblongichytrium*		
Oblongichytrium sp. SEK 347	AB290575	1774
Oblongichytrium sp. TN6	FJ821480	1798
Oblongichytrium sp. 8-7*	AF257317	1639
Oblongichytrium sp. 7-5*	AF257316	1635
Genus *Parietichytrium*		
Parietichytrium sarkarianum SEK 351	AB355411	1756
Genus *Sicyoidochytrium*		
Sicyoidochytrium minutum NBRC 102975*	AB290585	1733
Sicyoidochytrium sp. NBRC 102979*	AB183659	1711
Sicyoidochytrium minutum SEK 354	AB355412	1733
Genus *Schizochytrium*		
Schizochytrium sp. SEK 346	AB290578	1766
Schizochytrium sp. SEK 345	AB290577	1755
Schizochytrium sp. SEK 210	AB290576	1766
Schizochytrium aggregatum ATCC 28209	AB022106	1677
Schizochytrium sp. KK17-3*	AB052556	1793
Genus *Ulkenia*		
Ulkenia amoeboidea SEK 214*	AB290355	1790
Ulkenia profunda	L34054	1815
Ulkenia profunda BUTRBG 111	DQ023615	1762
Ulkenia sp. ATCC 28207*	AB022104	1760
Ulkenia visurgensis BURAAA 141	DQ100296	1812
Ulkenia visurgensis ATCC 28208	AB022116	1812
Other Chromista group		
Cafeteria roenbergensis	L27633	1718
Achlya bisexualis	M32705	1809
Phytophthora megasperma	X54265	1827
Hyphochytrium catenoides BR217	AF163294	1814
Chaetoceros debilis ch.4	AY229896	1739
Eucampia antarcia CCMP1452	AY485503	1632
Skeletonema costatum CCAP 1077/3	X85395	1798
Thalassiosira weissflogii CCAP1085/1	FJ600728	1764
Chattonera ovata C. Tomas Japan	AY788924	1781
Heterosigma akashiwo 893	AB217869	1806

Testing for primer specificity to culture strains using PCR and qPCR

To confirm that the designed primers matched 18S rRNA genes from the target genus rather than from non-target genera, we conducted a Primer-BLAST search (Altschul et al. 1997, Ye et al. 2012) against the NCBI non-redundant (nr) database with an input setting of 50 to 250 bp for the PCR product size. In addition, the search was also performed with an input setting of 50 to 5000 bp to examine the risks of unpredictable matching with other estuarine and marine organisms.

In addition to these database searches, we performed experimental confirmation by PCR using the above-mentioned primers against cultured strains of each genus: *Aurantiochytrium* sp. SEK 209 (NBRC-102614), *Botryochytrium radiatum* SEK 353 (NBRC104107), *Oblongichytrium* sp. SEK 347 (NBRC102618), *Parietichytrium sarkarianum* SEK 351 (NBRC104108), *Sicyoidochytrium* sp. MBIC11077 (NBRC102979), *Schizochytrium* sp. SEK 345 (NBRC-102616), and *Ulkenia amoeboidea* SEK 214 (NBRC104106). Samples of the culture (2–3 ml) were harvested during the exponential growth stage by centrifugation (6000 *g* ×, 5 min). The resultant cell pellets were suspended in 200 µl of phosphate-buffered saline (PBS, pH 7.2), then digested with 26 µl of 10% sodium dodecyl sulfate (SDS), 20 µl of 5 mg ml^{-1} lysozyme, and 40 µl of 25 mg ml^{-1} Proteinase K. DNAs in the solutions were extracted with phenol-chloroform-isoamyl alcohol (PCI; 25:24:1, v/v/v) and chloroform-isoamyl alcohol (CIA; 24:1, v/v), then precipitated by isopropanol in 0.3M sodium acetate. The DNA pellets were washed in 70% ethanol and then finally dissolved with 100 µl of sterile MilliQ water. A mixture (total volume: 20 µl) containing 0.5 µl of template DNA (dissolved as above), 1 µl of 10 pmol $µl^{-1}$ genus-specific forward and reverse primers, and the recommended volume of 5 units $µl^{-1}$ Ex *Taq* DNA polymerase, 10× Ex *Taq* buffer, 25 mM $MgCl_2$, and deoxynucleoside triphosphate (dNTP) mixture included in the Takara Ex *Taq* kit (Takara Bio) was subjected to conventional PCR to verify the

genus-specific amplicon from a designated thraustochytrid genus. The thermal-cycling protocol was: 1 cycle at 95°C for 5 min, 35 cycles at 95°C for 45 s, 60°C for 45 s, and 72°C for 30 s, followed by 1 cycle at 72°C for 5 min. PCR was conducted in a Takara PCR Thermal Cycler PERSONAL (Takara Bio). The annealing temperature was 58°C for *Aurantiochytrium* and 62°C for *Parietichytrium* and *Ulkenia*. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

We performed qPCR of the above-mentioned DNA extracts from 7 genera. A mixture (total volume: 20 µl) containing 0.5 µl of template DNA (dissolved as described above), 0.4 µl of 10 pmol μl^{-1} genus-specific forward and reverse primers, and 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG with ROX™ (Invitrogen) was analyzed in an ABI PRISM 7000 (Applied Biosystems) using the following thermal protocol: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 2 min, and 40 cycles at 95°C for 15 s and 60°C for 30 s. Following qPCR, the melting profiles of the PCR product versus temperature (dissociation curves) were obtained for each sample to check for the occurrence of positive amplification of target DNA and absence of primer–dimers.

Quantification of thraustochytrid cells using qPCR

The cell numbers for each culture were counted under a light microscope, and culture aliquots equivalent to 5000, 10 000, 100 000, and 1 000 000 cells were each filter-trapped onto a 0.22 µm pore size Sterivex filter (Millipore). The filter units were then stored at –20°C for a period >1 d. The units were thawed before DNA extraction and the cells lysed as described by Somerville et al. (1989). The crude lysates were used for DNA preparation by PCI extraction, CIA extraction, and isopropanol precipitation as described above. The DNA pellets were finally dissolved with 100 µl of sterile MilliQ water. Triplicate aliquots of 0.5 µl (each equivalent to 25, 50, 500, or 5000 cells $reaction^{-1}$) were retrieved and subjected to qPCR using each genus-specific primer set, with sterile MilliQ water as a non-template control or a negative control. In addition, to determine whether each primer set produces a signal from non-target genera, a 5000 cell-equivalent DNA derived from each of the other genera was also subjected to qPCR. These qPCR measurements were characterized by 2 interrelated parameters: (1) the cycle threshold (C_t) value, i.e. the number of cycles at which the reaction crossed the specified fluorescence threshold; and (2) the normalized reporter signal (Rn), which is calculated as the ratio of the fluorescence of the reporter dye (SYBR® Green I) divided by the fluorescence of the passive reference dye (ROX™). The larger the amount of starting target DNA, the earlier a significant increase in Rn is observed, leading to a decrease in the C_t value. The change in Rn, or delta Rn (ΔRn), was plotted against the cycle number of the reaction.

Application of qPCR in testing seawater samples

Seawater samples were collected at 88 sites around the islands of Koshiki-jima, Satsuma-Iwojima, and Tanega-shima (southwestern Japan), in the Seto Inland Sea (western Japan) during cruises of the RV 'Toyoshio-maru', Hiroshima University, in April 2008, May 2010, April 2011, and March and April 2012, and off the Karakuwa Peninsula in Sanriku (eastern Japan) during a cruise of the RV 'Tansei-maru', Japan Agency for Marine-Earth Science and Technology (JAMSTEC), in August 2011. In this study, qPCR only successfully quantified the thraustochytrid cell numbers in 8 of the 212 seawater samples, viz. those collected at sites around Satsuma-Iwojima and off the Karakuwa Peninsula (Fig. 1). (A detailed list of all 212 samples is given in Table S1 in the Supplement at www.int-res.com/articles/suppl/m486p001_supp.pdf). Samples were collected using a conductivity, temperature, and depth (CTD) profiling rosette equipped with Niskin bottle samplers at between 1 and 4 depths (surface to 210 m), depending on the water depth at the site.

From the 93 seawater samples collected in 2008, a 2 l water sample was filtered through a 0.22 µm pore size Sterivex filter (Millipore) using a peristaltic pump. The DNA was then extracted from the Sterivex housing as described above. From the 119 samples collected in 2010 to 2012, a 20 l water sample was prefiltered with a 500 µm mesh, then filtered through a 0.22 µm pore size Steripak-GP20 filter (Millipore) using a peristaltic pump. The DNA was extracted from this Steripak filter using PCI and CIA, as described by Frias-Lopez et al. (2008). Each DNA pellet was finally dissolved with 100 to 500 µl of sterile MilliQ water. Prior to qPCR, the initial screening for confirmation of the existence of thraustochytrids was performed by conventional PCR. Amplicons from the DNA extract with a minimum of 3 $cells\ reaction^{-1}$ could be reliably detected on an agarose gel by ethidium bromide staining. Therefore, this initial screening was used to select the samples applicable for our qPCR system to quantitate thraustochytrid cells rang-

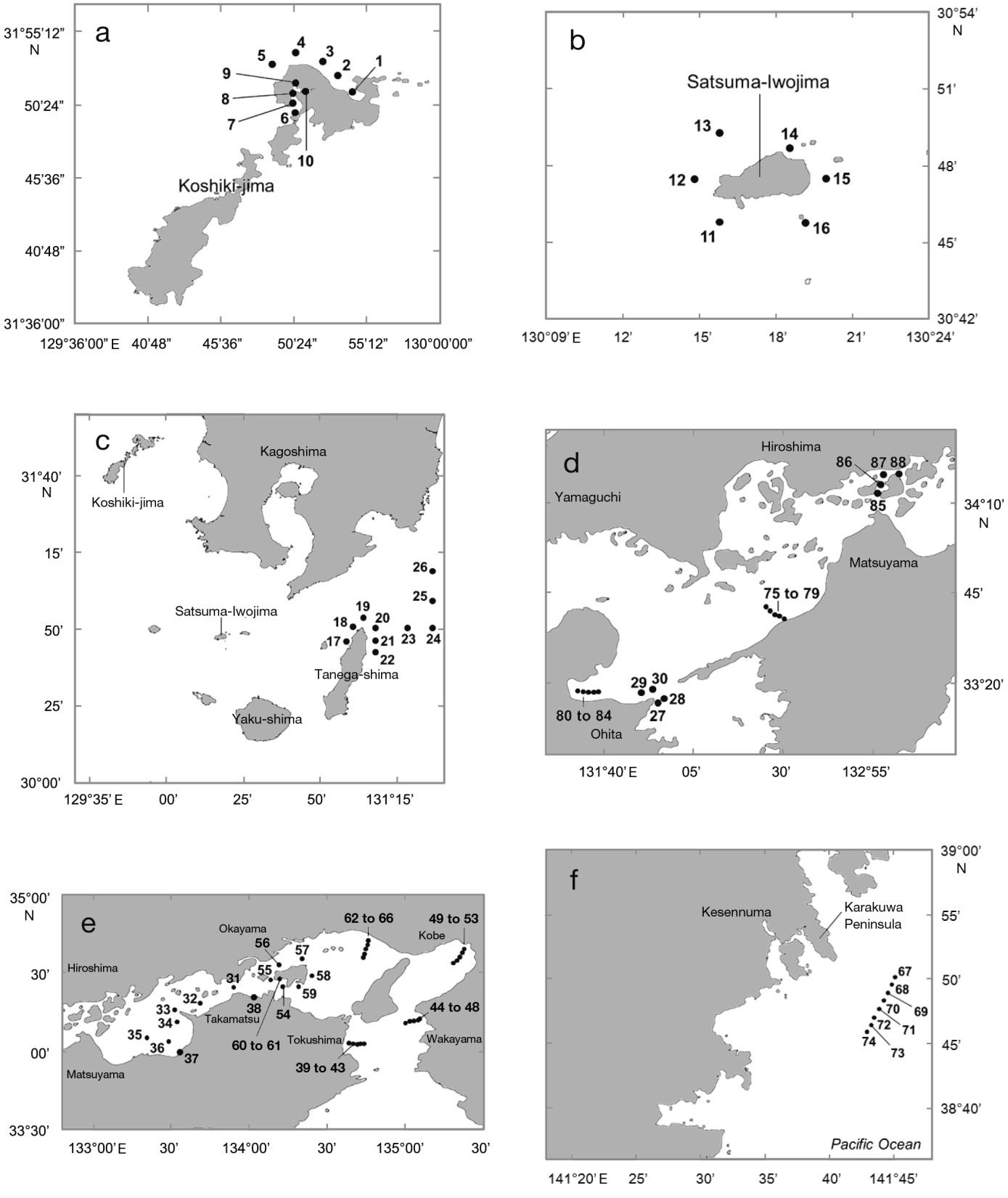

Fig. 1. Locations of sample collection in the area surrounding (a) Koshiki-jima, (b) Satsuma-Iwojima, and (c) Tanega-shima, (d,e) in the Seto Inland Sea, and (f) off the Karakuwa Peninsula in Sanriku

Table 2. Sequences and amplicon sizes of genus-specific PCR primer sets

Genus	Name	Sequence	Amplicon size (bp)
Aurantiochytrium	Aur-F	5′-CTACGGTGACTATAACGGGTG-3′	120
	Aur-R	5′-GTGGAGTCCACAGTGGGTAA-3′	
Botryochytrium	Bot-F	5′-ATGTGAGTGCGATAGCTTTCG-3′	92
	Bot-R	5′-CGATTGCCTTCACACAAAAATG-3′	
*Oblongichytriu*m	Obl-F	5′-GAGCCTTCGGGTTCGTGT-3′	93
	Obl-R	5′-AACGATATGGATCCCATGCC-3′	
Parietichytrium	Par-F	5′-TTCGTAAGAGAACCAAATGTGG-3′	164
	Par-R	5′-GCCATGCAAACCAACAAAAT-3′	
Sicyoidochytrium	Sic-F	5′-ACGAGGAAAAAGTCCTTATCCG-3′	235
	Sic-R	5′-TACGCTACATCAAACTTTCATCC-3′	
Schizochytrium	Sch-F	5′-AATTCCCATGATTGTGCGTTGTGT-3′	172
	Sch-R	5′-CCCGAGGGCTATGCGATTCGCTC-3′	
Ulkenia	Ulk-F	5′-GGGCTAAGCCTACTCTTTCTG-3′	168
	Ulk-R	5′-CTGGTCCGTCCTACCAATACTT-3′	

ing from 25 to 5000 cells reaction^{-1}. This step was included to reduce the effort and cost associated with qPCR. A mixture (total volume: 20 µl) containing 0.5 µl of template DNA, pooled primers comprising 0.7 µl of 10 pmol µl^{-1} of each forward and reverse primer, and the recommended volume of 5 units µl^{-1} Ex *Taq* DNA polymerase, 10× Ex *Taq* buffer, 25 mM $MgCl_2$, and dNTP mixture included in the Takara Ex *Taq* kit (Takara Bio) was used in the conventional PCR. The thermal-cycling protocol consisted of 1 cycle at 95°C for 2 min, 35 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 40 s, followed by 1 cycle at 72°C for 10 min. This cycle number (35 cycles) was chosen based on the C_t values and ΔRn values obtained during the qPCR amplification of standard DNA. In the preliminary experiments with pooled primers, some losses in PCR performance were observed. We therefore reduced the primer concentration and optimized the PCR reaction conditions using control DNAs. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. For samples that tested positive using the mix of primers, we performed PCR using each forward and reverse primer to determine the presence or absence of each genus.

For samples that tested positive during conventional PCR-based screening, we conducted qPCR using the primer sets described above. A mixture (total volume 20 µl) containing 0.5 µl of template DNA, 0.4 µl of 10 pmol µl^{-1} genus-specific forward and reverse primers, and 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG with ROX™ (Invitrogen) was analyzed in an ABI PRISM 7000 (Applied Biosystems), as described above, but with a decrease in the number of PCR cycles from 40 to 35 to minimize potential interference by non-specific amplification. DNA extracts from the culture strain of the target genus, each corresponding to 25, 50, 500, or 5000 cells reaction^{-1}, were loaded for every run to serve as quantification standards (all performed in triplicate). The abundance of each genus was determined based on these culture-based standardizations.

For the 8 samples for which thraustochytrids were quantified by qPCR, we tested for interference due to other co-extracted compounds (e.g., organic acids or polysaccharides) or from filter-trapped particles in the qPCR by adding 50 or 500 cell equivalents of DNA from the culture strains to every sample as internal standards. The additional increase in the qPCR signal corresponding to the additional DNAs was recorded. When interference was suspected, the samples were diluted sufficiently to diminish interference. In addition, the 6 samples in which thraustochytrid cells were found and strong amplification was observed by conventional PCR but not qPCR were checked for amplification inhibition in the same manner. To compare the results of qPCR with traditional acriflavine counts, 125 of the 212 samples were counted with acriflavine using epifluorescence microscopy following the method described by Raghukumar & Schaumann (1993). Briefly, particles in a water sample of 10- to 100 ml were collected on an isopore membrane filter (Millipore; pore size 0.2 µm, diameter 25 mm). The particles on the filter were stained with 4 ml of 0.2 µm filtered 0.05% acriflavine in 0.1 M citrate buffer (pH 3.0) for 4 min and then rinsed with 75% isopropanol for 1 min. Thraustochytrid cells were counted in 100 microscopic fields, and each count was duplicated.

RESULTS AND DISCUSSION

PCR specificity

The sequence and amplicon size of the qPCR primer sets designed to amplify each 18S rRNA gene of 7 thraustochytrid genera are given in Table 2. Some primer sets had mismatches with target thraustochytrid sequences ranging from 1 to 5 bases.

Aurantiochytrium sp. SEK 209 (AB290574) had a 1-base difference with our *Aurantiochytrium*-specific reverse primer; *Oblongichytrium* sp. SEK 347 (AB290575) had a 1-base difference with our forward primer; and *Ulkenia profunda* (L34054) had a 5-base difference (2 in the forward primer and 3 in the reverse) with our primers. The desired amplifications against each genus were simulated in the Primer-BLAST database search. Mismatched regions were also found in the search results. For example, *Aurantiochytrium* sp. B013 (JF266573) had a 4-base difference (2 in the forward primer and 2 in the reverse) with our primers; and *Parietichytrium* sp. BAFCcult 3109 (HQ228977) had a 2-base difference with our forward primer.

In addition, Primer-BLAST results suggested that the reverse primer for detection of *Parietichytrium* attaches to 2 regions in each genome of the coelacanth *Latimeria menadoensis* (AC215904), the nematode *Caenorhabditis elegans* (Z68116), and the bacterium *Acinetobacter* sp. ADP1 (CR543861) and produces unexpected PCR products. In contrast, the forward primer did not match with these 3 sequences. The *L. menadoensis* had a 4-base difference (1 in the one region and 3 in the other region; predicted product size: 231 bp), the *C. elegans* had a 6-base difference (3 in the one region and 3 in the other region; 1148 bp), and the *Acinetobacter* sp. had a 7-base difference (2 in the one region and 5 in the other region; 3148 bp) with the reverse primer. These product sizes were easily distinguishable from the predicted product size of 164 bp for *Parietichytrium*. Moreover, because the *C. elegans* sequence has a mismatch at the 3′-end of the reverse primer, we predict that PCR amplification will not proceed. While our primers were not perfect in all instances, they matched the designated genera in the database. Furthermore, we used conventional PCR to confirm positive PCR amplification of the target thraustochytrid genus and negative amplification of other non-target genera (Fig. 2).

qPCR quantification of 7 thraustochytrid genera

The relationship between C_t values and a logarithmic plot of cell numbers of each thraustochytrid genus (25, 50, 500, 5000 cells reaction^{-1}) yielded a strong linear correlation (Fig. 3). Linear regression fits between cell numbers of standard genus (X) and the corresponding C_t values (Y) for these runs are described in Fig. 3. Despite several attempts, we were unable to quantify cell numbers in filtered samples with <25 cells reaction^{-1} using the current qPCR

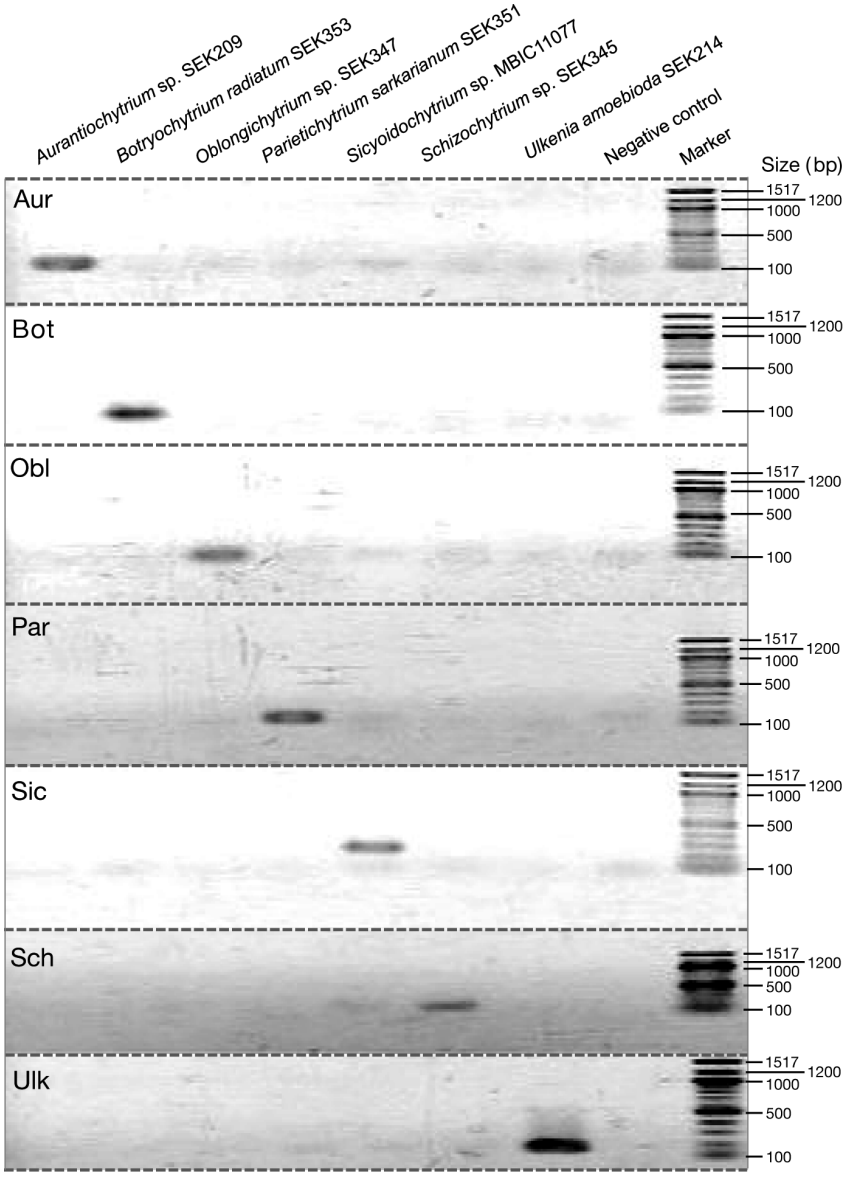

Fig. 2. Agarose gel electrophoresis of PCR-amplified products with genus-specific primer sets against target and non-target DNA solutions. Aur, Bot, Obl, Par, Sic, Sch, and Ulk represent *Aurantiochytrium*-, *Botryochytrium*-, *Oblongichytrium*-, *Parietichytrium*-, *Sicyoidochytrium*-, *Schizochytrium*-, and *Ulkenia*-specific primer sets, respectively, that were used for each amplification

assay (data not shown). In addition, in the 4 assays for *Botryochytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia*, the amplification efficiencies were high, ranging from 90.8 to 107.1%. The efficiencies of the other 3 primer sets ranged from approximately 70.4 to 81.5%; this suggests that there is room for improvement. However, all assays designed in this study had comparable levels of detection limits as described below.

The ΔRn curves for the 7 genera and primer sets are summarized in Fig. 4. The graphs for the *Aurantiochytrium*, *Botryochytrium*, *Oblongichytrium*, *Schizochytrium*, and *Sicyoidochytrium*-specific primers clearly illustrate a significant increase in ΔRn of each target genus in comparison with the 5000 cell-equivalent DNAs derived from 1 of the other 6 non-target genera. Our data also suggest that for these 5 genera, the increase in the non-specific ΔRn may have occurred in later PCR cycles (typically beyond 33 to 35 cycles; Fig. 4). Based on the thermal dissociation curve analysis of the qPCR amplicons, such an increase in ΔRn would likely result from the amplification of non-target DNA. In addition, for the *Sicyoidochytrium*-specific primer set, a weak peak from the formation of primer-dimers occurred, and the signal was recognized after 35 cycles as described in the

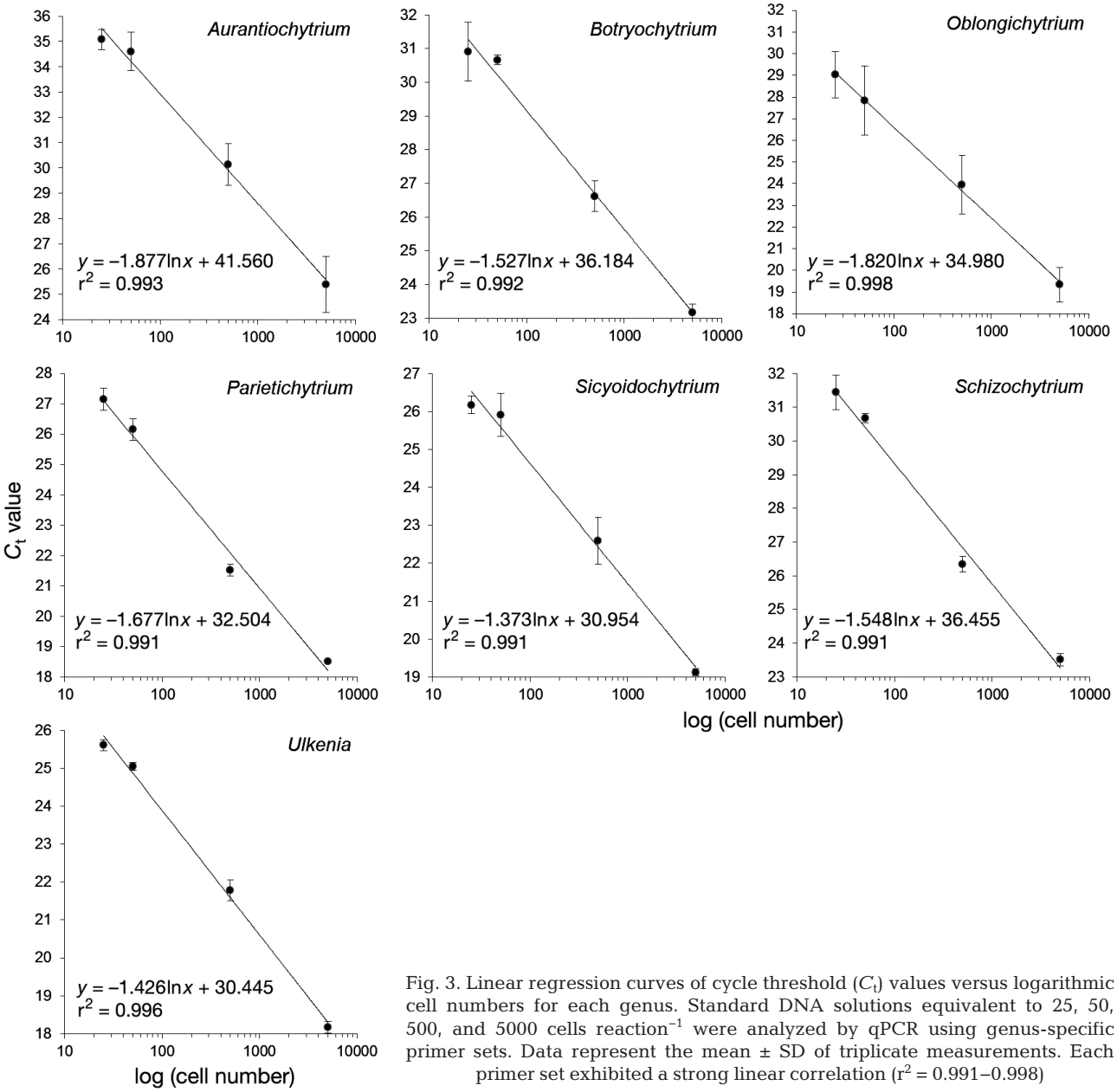

Fig. 3. Linear regression curves of cycle threshold (C_t) values versus logarithmic cell numbers for each genus. Standard DNA solutions equivalent to 25, 50, 500, and 5000 cells reaction^{-1} were analyzed by qPCR using genus-specific primer sets. Data represent the mean ± SD of triplicate measurements. Each primer set exhibited a strong linear correlation (r^2 = 0.991–0.998)

non-template control (NTC) reaction result (Fig. 4). However, in our qPCR system, the ΔRn for the 25 cells reaction^{-1} as the detection limit was observed in C_t values ranging from 26 to 32 cycles (Fig. 3). To minimize the effect of such signals, the number of PCR cycles was reduced from 40 to 35 during analysis of the field samples. Thus, the signals derived from non-specific amplification or primer-dimers have no real influence on our results. In the case of *Aurantiochytrium*, the C_t value (35.1 ± 0.4 SD; n = 3) for the 25 cell equivalent could not be discriminated from the signal of the non-target DNA amplified. Conversely, for the *Parietichytrium* and *Ulkenia*-specific primers, the increase in ΔRn derived from the amplification of non-target genera DNA appeared to occur relatively quickly, after 27 cycles. This cycle number is similar to the C_t values for the *Parietichytrium* 25 cell equivalent (27.1 ± 0.3, mean ± SD; n = 3) and the *Ulkenia* 25 cell equivalent (25.6 ± 0.1; n = 3) samples (Fig. 3). However, in the case of non-target genera not being detected in the sample by conventional PCR-based screening, the non-specific ΔRn is considered to have no real effect on the qPCR results. We emphasize that prior to the qPCR assay, we performed conventional PCR to determine the presence or absence of each thraustochytrid genus. This was necessary to confirm background genera that could affect the quantification in qPCR.

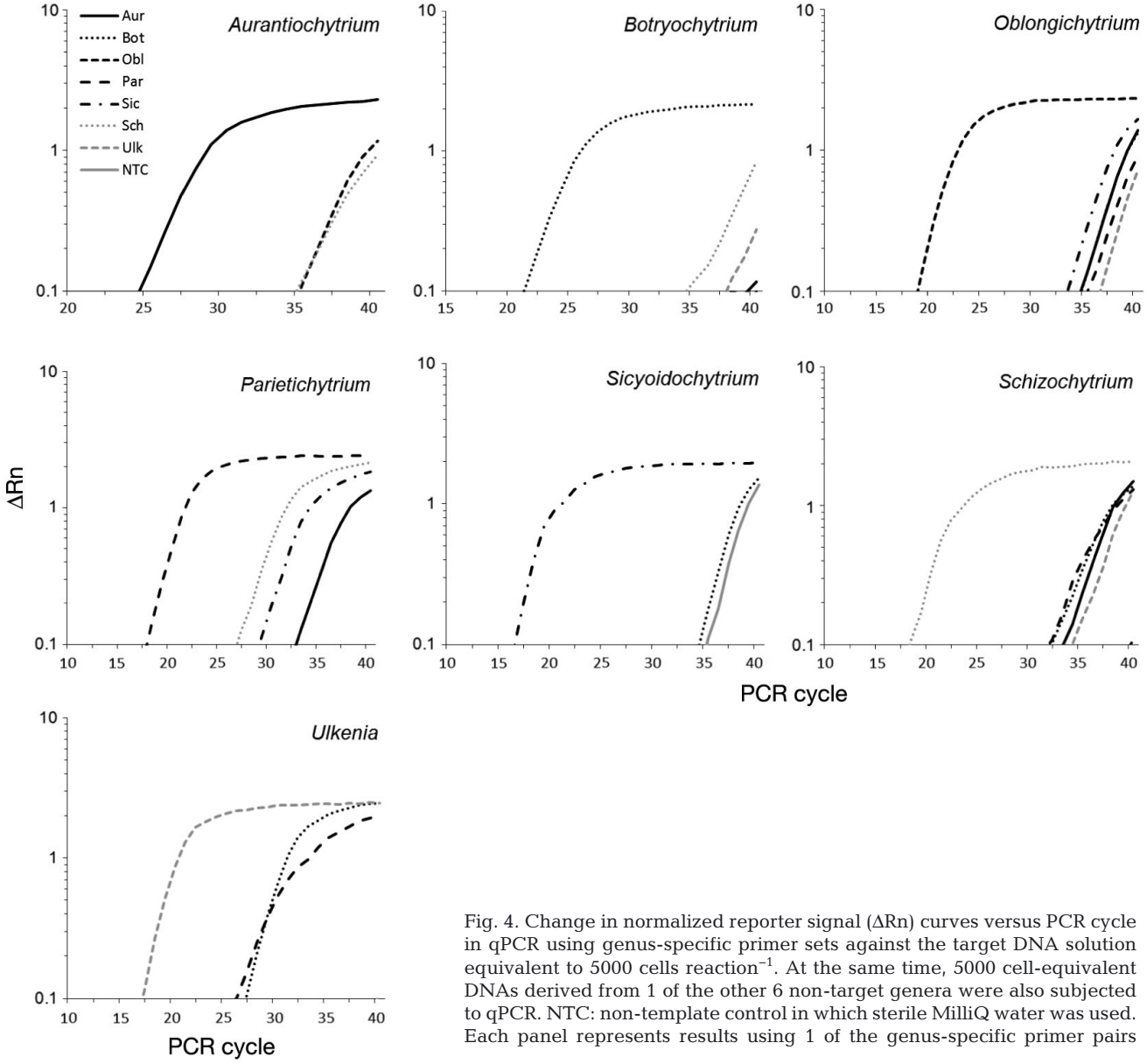

Fig. 4. Change in normalized reporter signal (ΔRn) curves versus PCR cycle in qPCR using genus-specific primer sets against the target DNA solution equivalent to 5000 cells reaction^{-1}. At the same time, 5000 cell-equivalent DNAs derived from 1 of the other 6 non-target genera were also subjected to qPCR. NTC: non-template control in which sterile MilliQ water was used. Each panel represents results using 1 of the genus-specific primer pairs

Table 3. Thraustochytrid cell numbers estimated from traditional acriflavine counts and qPCR. –: no data; ND: not detected

Sample no.	Site no.	Area description	Depth (m)	Direct count estimates ($\times 10^3$ cell l^{-1})	qPCR estimates ($\times 10^3$ cell l^{-1}) *Aurantiochytrium*	*Oblongichytrium*
April 2008						
I1-1	11	Around Satsuma-Iwojima Island, southwestern Japan	1	16.0 ± 6.4	11.2 ± 1.5	15.3 ± 1.8
I1-2	11		70	–	ND	31.4 ± 3.6
I2-1	12		1	3.4 ± 3.3	ND	15.9 ± 1.1
I4	14		Surface water	6.6 ± 9.1	13.1 ± 1.6	10.2 ± 2.3
I5-1	15		1	3.1 ± 3.6	ND	12.8 ± 1.2
I6-1	16		1	8.0 ± 7.9	ND	14.6 ± 2.4
August 2011						
St16-3	69	Off Karakuwa Peninsula in Sanriku, eastern Japan	1	9.6 ± 1.1	ND	15.9 ± 1.8
St16-6	72		1	5.3 ± 3.2	ND	17.9 ± 1.9

Based on our results, the determination limit was 25 cells reaction^{-1} for 6 genera (*Botryochytrium*, *Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia*), and 50 cells reaction^{-1} for 1 genus (*Aurantiochytrium*). Given the appropriate dilution covering the standard range, the quantitative results obtained by our qPCR analyses were acceptable, as we obtained a linear relationship between cell numbers and C_t values for all genera (Fig. 3).

Using qPCR to enumerate thraustochytrid cells in the marine environment

Using our qPCR assay, we enumerated planktonic thraustochytrid cells in field samples. We successfully detected the presence of *Aurantiochytrium* (in the range of 1.12×10^4 to 1.31×10^4 cells l^{-1}) and *Oblongichytrium* (in the range of 1.02×10^4 to 3.14×10^4 cells l^{-1}) in 8 surface-seawater samples from around Satsuma-Iwojima and off the Karakuwa Peninsula in Sanriku (Table 3). However, the other 5 genera (*Botryochytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia*) were not quantifiable in any of the samples. If the number of PCR cycles was increased, our qPCR assay may have detected and quantified a smaller number of thraustochytrid cells (as illustrated in Fig. 3). However, in this case the disadvantage resulting from an increase in the non-specific ΔRn in later PCR cycles, as described in Fig. 4, would be relatively conspicuous.

Of the 8 qPCR-positive samples, we tested 7 using the traditional acriflavine count, which yielded lower estimates for cell abundance in all cases (Table 3), with a weak positive correlation ($r^2 = 0.495$; Fig. 5; raw count data are given in Table S1 in the Supplement). We speculate that the acriflavine count excludes thraustochytrid zoospores because of the fact that zoospores of most thraustochytrid species lack a cell wall (Moss 1986). In addition, very small cells (<5 µm) are not easily distinguished because the cell wall-associated red fluorescence is weaker than the

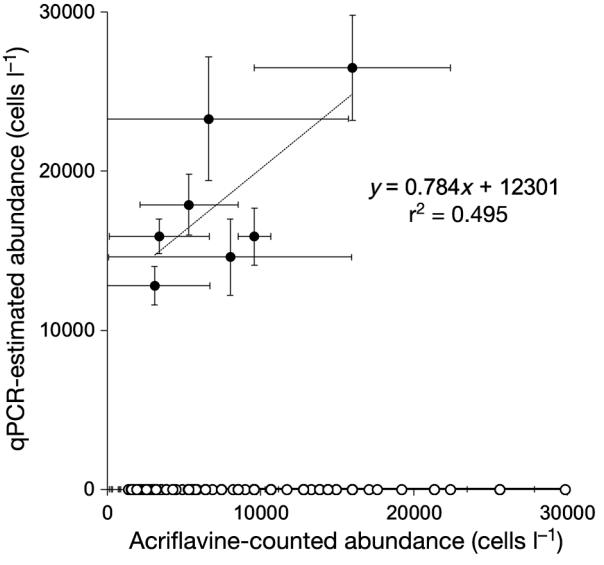

Fig. 5. Correlation between traditional microscopic and qPCR-derived estimates for thraustochytrid abundance. For the samples (Sites 11 and 14) in which more than 1 genus was detected by qPCR, the sum of the value for each genus was defined as a qPCR-derived estimate. Only 7 of the 212 water samples were quantified by both direct count and qPCR (●). There was a weak positive correlation between the 2 methods ($r^2 = 0.495$). In an additional 104 samples, thraustochytrid cells were quantified by direct count (○); 6 of these samples exhibited strong bands when amplified by conventional PCR screening but could not be quantified by qPCR. The 7 samples containing more than 3×10^4 cells l^{-1} are not included

nucleus-associated green fluorescence. Thus, these cells are often not counted, leading to underestimation. Taken together, our observations suggest that qPCR provides a more accurate estimate of the abundance of these zoospores and very small cells.

Thraustochytrid cells were found in 104 additional samples by direct count. The abundance of thraustochytrids ranged from $1.37 \pm 1.22 \times 10^3$ cells l^{-1} (Site 20, around Tanega-shima, southwestern Japan) to $7.68 \pm 0.85 \times 10^4$ cells l^{-1} (Site 53, Osaka Bay, Seto Inland Sea, western Japan; Table S1). Prior studies reported an average thraustochytrid abundance of 10^3 to 10^4 cells (Naganuma et al. 1998, Kimura et al. 1999, 2001) in the coastal Seto Inland Sea and adjacent open waters. Our direct count estimates of abundance were consistent with prior studies. However, for these samples, thraustochytrids could not be quantified by qPCR, although 6 did produce strong bands when amplified by conventional PCR (Table S1). We suspect that contamination with non-target DNAs derived from smaller microorganisms and/or other co-extracted compounds, such as organic acids or polysaccharides, inhibits the amplification reaction. This is consistent with the decrease in signals we observed for the 500 cell internal standard used in the qPCR runs with the 6 samples above (data not shown). Given this, we recommend further evaluation of methods for filtration to remove picoeukaryotes and bacteria. Moreover, recent culture-independent studies revealed that thraustochytrid 18S rRNA gene sequences in the environment were more phylogenetically diverse than expected (Collado-Mercado et al. 2010). There is the possibility that the presence of these novel thraustochytrid groups could not be detected, which may have affected our results.

Properties of the qPCR developed in the present study

We have developed and applied a SYBR® Green qPCR method for the identification and quantification of 7 thraustochytrid genera. Our initial objective was to estimate genus-specific abundances of the family Thraustochytriaceae, all of which were based on the qPCR results. Therefore, our qPCR system has targeted the specific regions of 18S rRNA gene from representative strains for each genus. Expressing this parameter as the copy number of an rRNA gene would be more accurate, but less insightful for ecological purposes. Thus, we calculated cell numbers using the formula obtained from C_t values versus cell numbers of cultured strains. The qPCR-based cell number was determined based on each independent genus standard. Therefore, our culture-based standardizations should give independent levels of detection and should not be affected by differences in the rRNA gene copy numbers among the genera. In contrast, since the environmental samples may contain multiple and/or unknown species of each genus, there was the potential that the copy number variations within each genus affect the qPCR estimates. Zhu et al. (2005) studied the copy number variations of 18 algal strains belonging to different phylogenetic groups and suggested that qPCR could be used to monitor specific narrow groups since the range of rRNA gene copy numbers was quite restricted. Thus, the difference in copy number within genus does not seem to present a significant obstacle to the determination of cell number based on the culture-based standardizations. Nevertheless, accurate quantification that takes the copy number variations into consideration is desirable, as this has not been conclusively determined so far. Although the trials for natural samples are still preliminary, our molecular approach can provide the genus-specific value of abundance in the environment. It could also help advance our understanding of thraustochytrid diversity.

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