Plankton Benthos Res 2(2): 91–97, 2007

Plankton & Benthos Research

Fluorescence *in situ* hybridization using 18S rRNA-targeted probe for specific detection of thraustochytrids (Labyrinthulomycetes)

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Received 5 December 2006; Accepted 13 March 2007

Abstract: Thraustochytrids are cosmopolitan osmotrophic or heterotrophic microorganisms that play, especially in coastal ecosystems, important roles as decomposers and producers of polyunsaturated fatty acids (PUFA), and are also known to be pathogens of mollusks and seaweeds. However, because of shortcomings in the current methods for detection and enumeration of thraustochytrids, very little information is available concerning their natural dynamics and ecological roles. In this study, we propose a new method for detecting thraustochytrids using a fluorescent 18S ribosomal RNA (rRNA)-targeted oligonucleotide probe (Probe ThrFL1). Detection of thraustochytrids by means of the fluorescence *in situ* hybridization (FISH) technique with ThrFL1 was specific; the probe did not react with the other stramenopile organisms or with the dinoflagellate that was tested. Because of the high specificity and intense reactivity, the FISH protocol is expected to be a strong tool for examining ecological features of thraustochytrids.

Key words: *Aurantiochytrium*, Decomposer, FISH method, *Schizochytrium*, *Thraustochytrium*

Introduction

Thraustochytrids are cosmopolitan apochlorotic stramenopile protists classified in the class Labyrinthulomycetes within the kingdom Chromista (Cavalier-Smith et al. 1994, Leipe et al. 1994, Honda et al. 1999). Based on phylogenetic analysis of 18S ribosomal RNA gene sequences, Honda et al. (1999) suggested that thraustochytrid strains belong to either TPG (thraustochytrid phylogenetic group) which is a monophyletic group comprised of only thraustochytrid strains or LPG (labyrinthulid phylogenetic group) which includes all members of the family Labyrinthuraceae and some strains belonging to the family Thraustochytriaceae.

Ecologically, thraustochytrids have been frequently observed and/or isolated from seawater, sediments, algal and plant materials both as saprotrophs and parasites (Sparrow 1936, Gaertner 1979, Miller & Jones 1983). Their ubiquitousness and physiological capabilities to utilize a wide variety of organic substrates argue for their ecological importance as decomposers (Raghukumar et al. 2001). In addition, due to their high productivity of PUFAs such as docosahexaenoic acid and docosapentaenoic acid (Nakahara et al. 1996), they are considered remarkably important as food resources for higher organisms in marine systems (Raghukumar 1996, Kimura et al. 1999, Lewis et al. 1999). Because of these distinctive features of thraustochytrids, their ecological significance in coastal ecosystems has been highlighted (Raghukumar 2002).

However, the details of their natural dynamics and ecological roles *in situ* are almost unknown. In previous studies on thraustochytrids, a modified MPN (most probable number) technique using pine pollen as an adsorption substratum (Gaertner 1968) or an acriflavine direct detection (AfDD) technique (Raghukumar & Schaumann 1993) have frequently been used for their detection and/or enumeration. Use of the modified MPN technique enables estimation of relative abundance and diversity of thraustochytrids in a given habitat. However, this technique is accompanied with some disadvantages: 1) not all thraustochytrid species attach to the bait (pollen) and grow under the culture conditions used; 2) because this technique comprises a dilution

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procedure combined with baiting, predators, bacteria and/or viruses can affect the number of growable thraustochytrids; 3) the procedure is relatively time-consuming (Raghukumar & Schaumann 1993). AfDD technique, where thraustochytrids are identified based on the chemical characteristics of their layered cell wall composed of sulfated polysaccharides, is more rapid and quantitative than the modified MPN technique (Chamberlain 1980). Acriflavine hydrochloride stains the cell wall with orange-to-red fluorescence and the nuclei with yellow-to-green fluorescence. However, this also possesses disadvantages: 1) phylogenetic diversity is not reflected in the results given by this technique; 2) because the fluorescence strength varies with the amount of sulfated polysaccharides, this technique frequently overlooks the zoospores and young vegetative cells that have a very thin cell wall (Raghukumar & Schaumann 1993). Because of such problems, a new method for specifically and precisely detecting thraustochytrids is essential to further understand their *in situ* characteristics.

FISH techniques using fluorescently labeled oligonucleotide probes complementary to unique target sites on the rRNA have been revealed to be useful for specifically detecting microorganisms without cultivation and particular equipment (Stahl et al. 1988, DeLong et al. 1989, Amann et al. 1990). Actually, they are widely used for analyzing the population composition of natural bacterial communities (Schönhuber et al. 1997, Juretschko et al. 1998, Schmid et al. 2000, Juretschko et al. 2002). Recently, Sako et al. (2004) and Hosoi-Tanabe & Sako (2005) established a FISH protocol optimized for toxic dinoflagellates and evaluated its efficiency.

In this study, we aimed to establish a FISH method using an 18S rRNA-targeted fluorescent oligo-nucleotide probe that is specifically reactive to TPG thraustochytrid strains.

Materials and Methods

Organisms

Clonal strains of thraustochytrids and other microorganisms used in this study are shown in Table 1. All thraustochytrid strains are free from bacterial contamination. They were grown at 20°C in 10×medium-H (Honda et al. 1998). Phytoplankton strains were grown at 20°C in modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987); light conditions were set at 12 hL : 12 hD cycle at 50 μmol photons m^{-2} s^{-1} with cool white fluorescent illumination.

Fluorescent probe

To design a TPG-specific probe, 18S rRNA gene sequences of 26 eukaryotic microorganisms were aligned, 13 of which were of phylogenetically distant members of TPG thraustochytrid strains (Table 2). The 18S rRNA gene sequences are derived from NCBI. They were automatically aligned using ClustalW (Thompson et al. 1994), and then manually refined. Consequently, the probe ThrFL1 (5′-GTC GAC AAC TGA TGG GGC AG-3′) having a nucleotide sequence complementary to a region of thraustochytrid 18S rRNA: nucleotide 282-301 of *Aurantiochytrium* sp. NIBH N1-27 (formerly *Schizochytrium* sp., see Yokoyama &

Table 1. List of eukaryotic microorganism strains and their reactivity with the probe ThrFL1 tested by fluorescence in situ hybridization.

Taxon	Strain	Original locality	Reactivity**
Thraustochytriaceae (TPG)	*Aurantiochytrium* sp. NIBH N1-27*	Nakaminato Harbor, Ibaragi, Japan	+
	Aurantiochytrium limacinum NIBH SR-21 (IFO 32693)*	Colonia, Yap Island, Micronesia	+
	Aurantiochytrium sp. MBIC 11066*	Iriomote Island, Okinawa, Japan	+
	Aurantiochytrium sp. NBRC 102976 (MBIC 11072)*	Iriomote Island, Okinawa, Japan	+
	Aurantiochytrium sp. NBRC 102614 (SEK 209)*	Kobe Habor, Hyogo, Japan	+
	Schizochytrium sp. NBRC 102615 (SEK 210)*	Okinawa Island, Okinawa, Japan	+
	Schizochytrium sp. SEK 0213*	Iriomote Island, Okinawa, Japan	+
	Thraustochytrium aureum ATCC 34304	Woods Hole, Massachusetts, USA	+
	Ulkenia minuta NBRC 102975 (MBIC 11071)*	Iriomote Island, Okinawa, Japan	+
	Thraustochytriaceae sp. SEK 0211*	Ishigaki Island, Okinawa, Japan	+
	Thraustochytriaceae sp. SEK 0212*	Ishigaki Island, Okinawa, Japan	+
	Thraustochytriaceae sp. SEK 0214*	Hiroshima Bay, Hiroshima, Japan	+
Bacillariophyceae	*Eucampia zodiacas* AREZ	Ariake sea, Fukuoka, Japan	−
	Chaetoceros debilis Ch48	Ariake sea, Saga, Japan	−
Raphidophyceae	*Heterosigma akashiwo* H93616	Hiroshima Bay, Hiroshima, Japan	−
	Chattonella ovata COTJ-1	Tajiri Harbar, Hiroshima, Japan	−
Dinophyceae	*Karenia mikimotoi* GmH5	Hiroshima Bay, Hiroshima, Japan	−

*Scientific names are according to Yokoyama & Honda in press. ** Stainability of each strain with ThrFL1 was determined by epifluorescence microscopy; +, cells were intensively fluorescence stained; −, cells were not stained. Abbreviations: ATCC, American Type Culture Collection (USA); MBIC, Marine Biotechnology Institute Culture Collection (Japan); NBRC, NITE (National Institute of Technology and Evaluation)- Biological Resource Center (Japan); NIBH, National Institute of Bioscience and Human Technology (Japan); SEK, Laboratory of Systematics and Evolution at Konan University (Japan)

Table 2. Theoretical specificity of the designed probe (ThrFL1).

Taxon	Organisms	Accession no.	Target sequence**						
			5'-CTG	CCC	CAT	CAG	TTG	TCG	AC-3'
Kingdom Chromista									
class Labyrinthulomycetes									
family Thraustochytriaceae (TPG)	*Aurantiochytrium* sp. NIBH N1-27*	AB073308	–––	–––	–––	–––	–––	–––	––
	Aurantiochytrium limacinum NIBH SR-21 (IFO 32693)*	CS186227	–––	–––	–––	–––	–––	–––	––
	Aurantiochytrium sp. MBIC 11066*	AB290587	–––	–––	–––	–––	–––	–––	––
	Aurantiochytrium sp. NBRC 102976 (MBIC 11072)*	AB183657	–––	–––	–––	–––	–––	–––	––
	Aurantiochytrium sp. NBRC 102614 (SEK 209)*	AB290574	–––	–––	–––	–––	–––	–––	––
	Schizochytrium sp. NBRC 102615 (SEK 210)*	AB290576	–A–	–––	–––	–––	–––	–––	––
	Schizochytrium sp. SEK 0213*	AB290354	–A–	–––	–––	–––	–––	–––	––
	Thraustochytrium aureum ATCC 34304	AF391543	–––	–––	–––	–––	C––	–––	–T
	Ulkenia minuta NBRC 102975 (MBIC 11071)*	AB290585	–––	–––	–––	–––	C––	–––	–T
	Thraustochytriaceae sp. SEK 0211*	AB290352	–––	–––	–––	–––	–––	–––	––
	Thraustochytriaceae sp. SEK 0212*	AB290353	–––	–––	–––	–––	–––	–––	––
	Thraustochytriaceae sp. SEK 0214*	AB290355	T––	–––	––A	–––	–––	–––	––
	labyrinthulid quahog parasite X (QPX)	AY052644	–––	–––	T––	–––	–––	–––	TA
family Thraustochytriaceae (LPG)	*Oblongichytrium multirudimentalis**	AB022111	–––	–––	T––	–––	C––	–––	–T
	Aplanochytrium kerguelense	AB022103	–––	–––	T––	–––	C––	–––	–T
family Labyrinthulaceae (LPG)	*Labyrinthula* sp. AN 1565 (NBRC 33215)	AB022105	–––	A––	T––	–––	C––	–A–	–T
class Bicosoecida	*Cafeteria roenbergensis*	L27633	–––	–––	T––	–––	C–T	–––	–T
class Oomycetes	*Achlya bisexualis*	M32705	–––	–––	T––	–––	C–T	–G–	–T
	Phytophthora megasperma	X54265	–––	–––	T––	–––	C–T	–G–	–T
class Hyphochytriomycetes	*Hyphochytrium catenoides*	AF163294	–––	–––	T––	–––	C–T	–G–	–T
class Bacillariophyceae	*Chaetoceros debilis*	AY229896	–––	–––	T––	–––	C–T	–GA	CG
	Eucampia antarcia	AY485503	–––	A––	T––	–––	T–T	C––	––
	Skeletonema costatum	DQ897642	–––	–––	T––	–––	C–T	GG–	–T
class Raphidophyceae	*Chattonera ovata*	AY788924	–––	–––	T––	–––	C–T	CG–	–T
	Heterosigma akashiwo	AB217869	–––	–––	T––	–––	C–T	CG–	–T
Kingdom Protozoa									
class Dinophyceae	*Karenia mikimotoi*	AF009131	–––	–––	T––	–––	C–T	–G–	–T

*Scientific names are according to Yokoyama & Honda in press. ** This column shows the mismatch bases for target sequence; –, means match for target sequence. Strains shown above the broken lines were tageted by the probe ThrFL1.

Honda (in press); [DDBJ accession number AB073308]) was designed setting the threshold at two nucleotide mismatches (Table 2). We excluded the Labyrinthulid quahog parasite X (QPX) from target organisms in this study, because this strain is the only member having a three base mismatch among the TPG thraustochytrid strains. The probe (Invitrogen™) was 5′ end labeled with fluorescein isothiocyanate (FITC) and used in the following experiments.

Optimization of staining procedure

Basically, the staining procedure for thraustochytrids involves (1) collection of cells, (2) fixation, (3) dehydration, (4) hybridization, (5) washing and (6) epifluorescent microscopic observation. To optimize the FISH protocol with the oligonucleotide probe ThrFL1, the type and concentration of fixation buffer, concentration of denaturing reagent (formamide) in hybridization buffer, temperature and time for the hybridization process were investigated, where *Aurantiochytrium* sp. NIBH N1-27 was used as the test strain. For each test, the best staining conditions were determined based on the shape and stainability of thraustochytrid cells, which were judged by epifluorescence microscopic observations, and then these were used in the subsequent experiment. Following each staining procedure, samples were placed in an aliquot of antifade (Mowiol 4-88 mounting medium containing 0.2% p-phenylenediamine) on a slide

and observed using epifluorescence microscopy on a Zeiss Axiovert 200M (Carl Zeiss, Inc.) microscope with FITC or DAPI filter sets (Carl Zeiss, Inc.). Images were photographed using a Zeiss Axiocam HRc camera (Carl Zeiss, Inc.). The filter-trapped cells were stained by 4′, 6-diamidino-2-phenylindole (DAPI, 1 μg mL^{-1}) for 15 min and also observed by epifluorescence microscopy.

Specificity test

To check the specificity of the probe ThrFL1, we performed experiments using the optimal FISH protocol for exponentially growing cells of the other thraustochytrid and phytoplankton strains shown in Table 1.

Results and Discussion

Optimization of the hybridization protocol

One ml of exponentially growing cell culture, collected by centrifugation (2,000×*g*, 3 min) or filtration using 0.01% poly-L-lysin (Wako Ltd.)-treated 1.2 μm pore size filters (Nuclepore) was used for optimization of the FISH procedure. Initially, 1× or 5×PBS (phosphate buffered saline) [1×PBS: 137 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.5] was used as the fixation buffer. However, use of PBS resulted in spontaneous precipitation of insoluble salts (e.g. calcium phosphate); the filter was choked with the precipitate and cells were not efficiently trapped onto the filter. Thereafter, the suitability of 1×PBS, 25 mM TBS (137 mM NaCl, 2.68 mM KCl, 25 mM Tris, pH 7.4), and 125 mM TBS with 4% paraformaldehyde as a fixation buffer was examined, where the formamide concentration in the hybridization buffer, temperature and time for hybridization were set at 40%, 39°C and 30 min, respectively (see below). Except for 5×PBS, for all tested buffers, high fluorescence intensity of tested cells was observed. However, use of 1×PBS or 25 mM TBS caused remarkable cell collapses in the thraustochytrid cells tested. The buffer concentration is unlikely to be significant in determining fluorescence intensity, but is important for stabilizing cell shape. Consequently, we concluded that 125 mM TBS is the most suitable fixation buffer (Table 3 a–d).

Next, we optimized the formamide concentration in the hybridization buffer and the hybridization temperature. The theoretical hybridization temperature (melting temperature [*Tm*]) of the oligonucleotide probe ThrFL1 is 72°C (*Tm*=81.5+41×GC ratio−675 / primer length); it was designed setting the threshold at two nucleotide mismatches compared to the 13 thraustchytrid strains (Table 2). Considering this, the rough annealing temperature was estimated to be 67°C under native conditions (without formamide). Because an increase in formamide concentration decreases *Tm* at a rate of 0.7°C per 1% (McConaughy et al. 1969), optimization experiments for the hybridization protocol were conducted under the following conditions (formamide concentration-annealing temperature): 5%−63.5°C, 20% −53.0°C, 30%−46.0°C and 40%−39.0°C (Table 3e–h). Under the 5%−63.5°C conditions, the resultant fluorescence was very weak; cells were likely damaged by the high temperature of incubation. In contrast, when using all the other conditions tested, thraustochytrid cells were clearly fluorescence-stained (Fig. 1). These results indicate that the combination of formamide concentration–hybridization temperature is not so crucial in determining the staining efficiency as long as it is set within the proper range. Among the combinations tested, the 30%–46.0°C condition provided the most stable shaped and clearly fluorescence-labeled cells.

Suitable incubation times for hybridization were also examined by setting it at 5, 15, 30 and 60 min, where the best conditions for fixation and hybridization were

Table 3. Comparison of FISH protocol conditions.

	Fixation Buffer	Buffer (Formamide %)	Hybridization Temperature (°C)	Time (min)	Reactivity*
a	5 BS	40	39	30	+**
b	1 BS	40	39	30	±
c	25 mM TBS	40	39	30	±
d	125 mM TBS	40	39	30	+
e	125 mM TBS	5	63.5	30	−
f	125 mM TBS	20	53	30	+
g	125 mM TBS	30	46	30	++
h	125 mM TBS	40	39	30	+
I	125 mM TBS	30	46	5	+
j	125 mM TBS	30	46	15	+
k	125 mM TBS	30	46	30	+
l	125 mM TBS	30	46	60	+

* Stainability of each stain with probe ThrFL1 was determined by epifluorescence microscopy. '++, Cells with stabilized shape were intensively fluorescence stained; +, Cells were intensively fluorescence stained; ±, Cell lysis occurred; −, Cells were not stained. ** precipitation of insoluble salts was observed.

used; 125 mM TBS for fixation buffer; formamide concentration=30%; hybridization temperature=46.0°C (Table 3 i–l). No significant difference in the resulting cell shape or fluorescence intensity was found among the tested conditions; thus, we concluded that 5 min hybridization is long enough to obtain a reasonable result. However, in the case of staining filter-trapped cells, the possibility should be noted that the hybridization buffer temperature in a small glass petri dish may not reach the target temperature within 5 min of starting incubation; therefore, we consider 15 min to be the optimum hybridization period.

Integrating the results of the optimization experiments, the best FISH protocol for thraustochytrid detection was as follows: (1) cells were collected from 1 mL of logarithmically growing culture by centrifugation (2000×*g*, 3 min) or filtration using a 1.2-μm-pore-size filter (Nuclepore) treated with 0.01% poly-L-lysin (Wako LTD); (2) the collected cells were fixed in 1 mL of 125 mM TBS containing 4% paraformaldehyde for 5 min, and subsequently dehydrated in an ethanol series (50% and 80% ethanol, each for 2 min); (3) the cells were added to 1 mL of hybridization buffer (30% formamide, 0.1% SDS/5×SSC [83 mM NaCl+ 83 mM sodium citrate, pH 7.0]) and denatured at 70°C for 3 min; then, immediately placed on ice; (4) with the addition of 40 μL of probe ThrFL1 solution (100 ng μl^{-1}) denatured at 98°C for 3 min and quickly chilled on ice, hybridization was allowed to proceed at 46°C for 15 min; (5) the cells were washed twice with 1 mL of 5×SSC at 46°C for 5 min and observed as mentioned above. This protocol is very quick, taking only one hour.

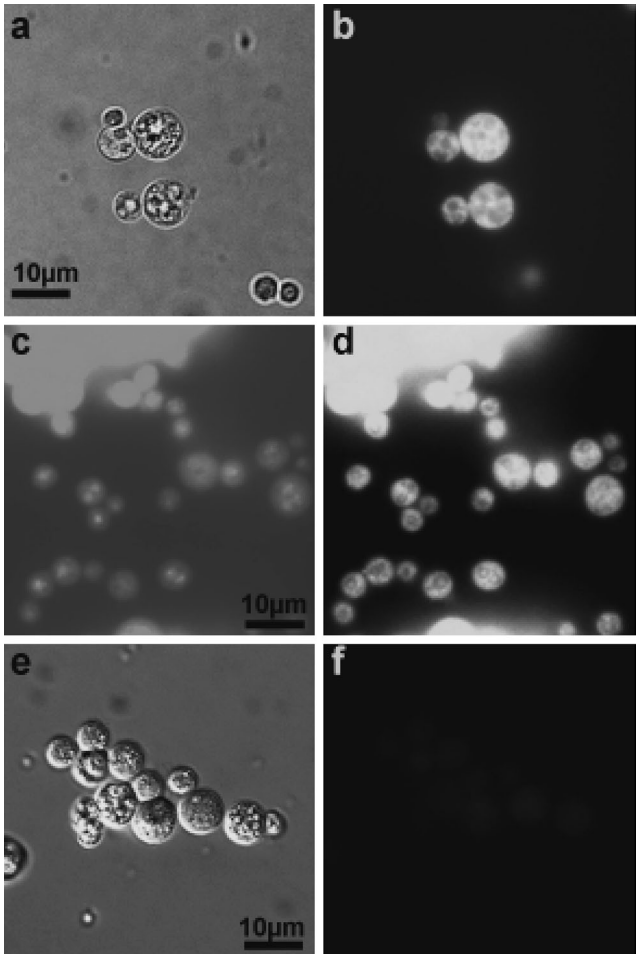

Fig. 1. *Aurantiochytrium* sp. NIBH N1-27 cells stained with a thraustochytrid-specific probe ThrFL1 (a, b), DAPI and ThrFL1 (c, d), and those not stained (e, f). Cells were collected by centrifugation (a, b, e, f) or filtration (c, d). Photographs were taken by optical microscopy (a, e), epifluorescence microscopy using UV (c) and B-excitation filters (b, d, f).

FISH performance

By means of FISH with the optimized protocol using the probe ThrFL1, *Aurantiochytrium* sp. NIBH N1-27 cells were intensively and uniformly stained by green fluorescence (Fig. 1b, d). Without the probe, no fluorescent signal was detected (Fig. 1e, f). By comparing the DAPI-stained and ThrFL1-stained samples, the nuclear area, strongly stained with DAPI, was found to be less intensively stained with the probe ThrFL1 (Fig. 1c, d). These observations suggest that cytoplasmic rRNA molecules in the thraustochytrid cells positively hybridized with the 18S rRNA-targeted probe ThrFL1. All of the TPG strains tested in this study, having 0–2 mismatches to the sequence of probe ThrFL1, were positively stained; on the other hand, diatoms, raphidophytes and the dinoflagellate that was tested (having≥5 base mismacthes) were not stained (Table 1, 2). However, the reactivity of probe ThrFL1 against QPX, LPG thraustochytrid strains and several of the microorganisms shown in Table 2 (having 3–4 base mismatches) were not determined due to their inaccessibility. Therefore, the ThrFL1 probe could possibly react positively with these organisms, especially those having 3 base mismatches: QPX and LPG thraustochytrids. Because of these results, we conclude that the FISH technique established in this study is useful for detecting thraustochytrids, at least TPG thraustochytrids.

Further optimization will be required to apply the FISH technique to natural samples; e.g. how to avoid nonspecific staining, how to increase the FISH sensitivity for stationary phase cells, and how to apply it for enumeration. Especially, low stainability of stationary phase thraustochytrid cells is remarkable (data not shown); this may reflect a decrease in the abundance of rRNA inside the cell (Anderson et al. 1999). Several methods are considered to increase the sensitivity of FISH: using a brighter fluorochrom such as Cy3 (Wessendorf & Brelje 1992), a multiple-labeled polyribonucleotide probe (DeLong et al. 1999) or signal amplification with reporter enzymes (catalyzed reporter deposition [CARD]-FISH) (Lebaron et al. 1997, Schönhuber et al. 1997).

Summary

We established a FISH protocol for detection of thraustochytrids, especially TPG thraustochytrids. Because this protocol doesn't require any cultivation procedure, it is expected to overcome the disadvantages involved in the modified MPN method, the results of which can be affected by the growth characteristics of host organisms and growth inhibition of them caused by predators, bacteria and/or viruses. Since the probe ThrFL1 hybridizes to cytoplasmic rRNA molecules, physiologically active cells (zoospores and young vegetative cells) are detected more intensely by this method, while these are difficult to detect using the AfDD technique due to the thinness of their cell walls. The appearance of young vegetative cells and/or zoospores may be related to the physiological status of the thraustochytrid population; hence, their detection is important to estimate the population dynamics. Considering their ubiquitousness, it is most likely that thraustochytrids comprise more than one subgroup having different ecological characteristics. When the phylogeny of thraustochytrids is more intensively examined, it may be possible to design new probes that distinguish thraustochytrid strains at the subgroup level. The FISH technique established in this study will be of use to further understand the ecological characteristics of thraustochytrids, especially concerning their population composition and dynamics.

Acknowledgements

This work was supported by the Kato Memorial Bioscience Foundation and the Asahi Glass Foundation. Authors are grateful to Dr. Toro Nakahara and Dr. Toshihiro Yokochi (National Institute of Advanced Industrial Science and Technology, Japan) who kindly provided the thraustochytrid strains. We wish to thank Dr. Shoko Hosoi-Tanabe (Research Center for Inland Seas, Kobe University) and Dr. Yoshihiko Sako (Laboratory of Marine Microbiology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University) for their technical advice concerning the FISH method.

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