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Seasonal dynamics of culturable thraustochytrids (Labyrinthulomycetes, Stramenopiles) in estuarine and coastal waters

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ABSTRACT: Regular monitoring of estuarine and coastal areas at fixed points was carried out to investigate changes in biomass and species composition of thraustochytrids, which are colorless heterotrophs in the class Labyrinthulomycetes, Stramenopiles. Cell number counts using the most probable number (MPN) method with pine pollen baiting showed that 1 or 2 conspicuous peaks in abundance (termed 'thraustochytrid spikes') occurred between spring and late summer in most sampling years. Changes in thraustochytrid biomass had no correlation with phytoplankton abundance, although spikes seemed to occur after reductions in salinity and associated rapid rises of water temperature. The average cell density at the estuary site, excluding values during spike periods, was 4670 cells l^{-1}. Although thraustochytrid biomass was only 1.59% that of bacterial biomass, the fixed energy (as biomass) transferred directly from thraustochytrids to zooplankton was estimated to be 15.9% of that transferred from bacterioplankton via phagotrophic protists. This is because, per the theory of energy efficiency, energy transfer between trophic levels only creates 10% of the net production in the next trophic level relative to the first. The phylogenetic identification of established strains revealed an unexpectedly high diversity of thraustochytrids, including 10 unidentified lineages. A similar seasonal succession of phylogenetic groups was observed in each year of sampling. The differences in thraustochytrids isolated at each monitoring site and date suggest that habitat segregation may occur as a result of differences in environmental factors such as water temperature, salinity, and nutrient sources.

KEY WORDS: Thraustochytrids · Biomass · Seasonal succession · Phylogeny · Habitat segregation · Estuary · Microbial loop · Osmotrophs

INTRODUCTION

The microbial loop is believed to play an important role in the circulation of energy in aquatic ecosystems through the decomposition of organic matter (Azam et al. 1983). In terrestrial ecosystems, the eukaryotic true fungi, along with bacteria, are recognized as important decomposers. Although it has been shown that fungi decompose leaf litter in the aquatic habitat, the ecological impact of this group is comparatively small in the water column (Newell 2003, Kirchman 2008, Sridhar 2012, Wang et al. 2012). However, thraustochytrids have attracted attention as cryptic eukaryotic decomposers in the water column because of their biomass and ubiquitous existence in the marine environment (e.g. Raghukumar 2002).

Most members of the class Labyrinthulomycetes in the Stramenopiles are colorless unicellular or colonial osmotrophs (Patterson 1989, Porter 1989, Honda et al. 1999, Dick 2001, Adl et al. 2012, Anderson &

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Cavalier-Smith 2012, Gomaa et al. 2013). The Labyrinthulomycetes are characterized by the presence of the bothrosome system, which develops ectoplasmic nets that are thought to secrete degradative enzymes and absorbing nutrients (Coleman & Vestal 1987, Raghukumar et al. 1994, Bremer & Talbot 1995, Bongiorni et al. 2005b, Nagano et al. 2011). They have been reported from brackish and coastal waters, the open ocean, and deep seas in tropical to polar regions (Ulken 1986, Kimura et al. 1999, Raghukumar et al. 2001, Bongiorni et al. 2004, Naganuma et al. 2006). In traditional taxonomy, the class Labyrinthulomycetes is separated into 2 families, namely Thraustochytriidae and Labyrinthulidae (e.g. Olive 1975, Porter 1989). Recent molecular phylogenetic analyses revealed that the Thraustochytriidae is not a monophyletic group and consists of at least 3 groups, viz. aplanochytrids, oblongichytrids, and thraustochytrids sensu stricto (e.g. Yokoyama et al. 2007, Tsui et al. 2009, Anderson & Cavalier-Smith 2012). However, in this paper, the term 'thraustochytrids' is used to refer to members of all 3 groups. This is an ecologically functional rather than phylogenetic distinction, because it has been recognized that they have similar niches based on their morphological resemblance.

In previous reports, thraustochytrid biovolume and carbon biomass were 3 to 43% and 3.4 to 29.7%, respectively, of the biovolume and carbon biomass of bacterioplankton (Naganuma et al. 1998, Kimura et al. 1999). A recent study in Hawaii (USA) found that thraustochytrid biomass ranged from 0.79 to 281.0% of bacterial biomass in the water column (Li et al. 2013). Thraustochytrid cell or colony size is 85 to 21 400 times that of bacteria; therefore, thraustochytrids are preyed upon by larger predators (Naganuma et al. 2006). As a result, there are fewer steps in the food chain starting with thraustochytrids than in the microbial loop, and less energy is lost from the food chain. Therefore, the ecological effect of thraustochytrids is larger than that of the same volume of bacteria (Naganuma et al. 1998, 2006, Kimura et al. 1999, Raghukumar 2002). Moreover, thraustochytrids produce degradative enzymes (e.g. cellulase) that can process the recalcitrant organic matter of higher terrestrial plants, which is difficult to digest for most marine bacteria (Nagano et al. 2011). In fact, comparatively large numbers of thraustochytrids are observed in river discharge areas, and it has been suggested that they grow on terrestrial organic matter and play ecological roles that are different to those of bacteria (Kimura & Naganuma 2001).

Thraustochytrids grow quickly and accumulate high levels of squalene and polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and *n*-6 docosapentaenoic acid (DPA) (Nakahara et al. 1996, Lewis et al. 1999, Fan et al. 2000, Yokoyama & Honda 2007, Yokoyama et al. 2007, Kaya et al. 2011, Nakazawa et al. 2014). These lipids are recognized as important materials in the flow of energy in aquatic ecological processes, and higher trophic level fishes in particular require these PUFAs as essential nutrients (Tocher 2010). Thraustochytrids have been used to enhance the nutritional content of rotifers and *Artemia* that serve as food for marine fish larvae (Song et al. 2007, Yamasaki et al. 2007, Estudillo-de Castillo et al. 2009). The survival probability of the larvae increases when PUFA-enriched *Artemia* is used as food (Furuita et al. 1996).

Although it has been suggested that thraustochytrids have a certain amount of influence on the aquatic ecosystem, there is insufficient information to confirm this. Most biomass measurements have been conducted by individual research ship cruises or with low frequency sampling (e.g. Kimura et al. 1999, Raghukumar et al. 2001, Bongiorni et al. 2004). Because it is quite difficult not only to identify the species but also the genera based on morphological features, past data without molecular phylogenetic information should not be immediately accepted. Moreover, little information is available on thraustochytrid species composition and succession (Bongiorni & Dini 2002, 2004), and recent studies on the sequences of thraustochytrids in environmental DNA reported unknown labyrinthulid and thraustochytrid lineages (Collado-Mercado et al. 2010, Li et al. 2013). A species-monitoring investigation using quantitative PCR (qPCR) was also recently conducted, but thraustochytrids were detected in only a few samples using this method (Nakai et al. 2013).

In this study, we used culturing methods to regularly investigate cell density and phylogenetic group composition (species and/or genus) of isolates from the Shukugawa River mouth and inside Osaka Bay, Japan, at least once a month. Our aims were (1) to estimate the seasonal change in thraustochytrid biomass by the most probable number (MPN) method using pine pollen and to evaluate the influence of this change on the ecosystem, (2) to examine the seasonal succession of thraustochytrid phylogenetic groups and identify ecologically remarkable groups, and (3) to elucidate the relationship among thraustochytrid biomass, phylogenetic group composition, other organisms, and environmental factors and report this baseline information on thraustochytrid ecology.

MATERIALS AND METHODS

Sampling sites and collection

Sampling was conducted at 3 locations characterized by different environmental conditions at the east end of the Seto Inland Sea, Japan (Fig. 1). The first site was the Shukugawa River mouth (34° 43.35′ N, 135° 20.00′ E), where the water flows into Osaka Bay. Sampling of the Shukugawa River was carried out 100 times every 2 to 4 wk from 14 March 2008 to 19 September 2013. The other 2 sites were Station 8 (Stn 8; 34° 29.95′ N, 135° 10.73′ E) and Stn 15 (34° 36.00′ N, 135° 17.75′ E) in Osaka Bay. Stn 15 is about 15 km from the Yodo River, which contributes the largest quantity of fresh water to Osaka Bay. In contrast, Stn 8 is about 30 km from the Yodo River and receives little river water compared with Stn 15. Sampling in Osaka Bay was carried out monthly for 24 mo during cruises onboard the RV 'Osaka' (Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture) from 7 March 2011 to 1 February 2013.

Shukugawa River surface water was sampled after being filtered through a 2.0 mm mesh sieve to remove large debris (e.g. leaves). Surface water was collected at both Stns 8 and 15 in Osaka Bay (8S and 15S). Bottom water was collected from 31 m depth at Stn 8 (8B) and from 17 m depth at Stn 15 (15B), which equated to 2 and 1 m above the seabed, respectively. At all sampling sites, net samples were collected using a 20 µm mesh net (Rigo), and major plankton composition was observed under an inverted microscope (Olympus) on the sampling day. Vertical profiles of water temperature, salinity, and chlorophyll fluorescence were monitored using a compact CTD (JFE Advantech) at each site. Concentrations of chlorophyll *a* (chl *a*) were calibrated using the correlation between chlorophyll fluorescence observed with CTD casts and chl *a* concentrations determined with a spectrometer.

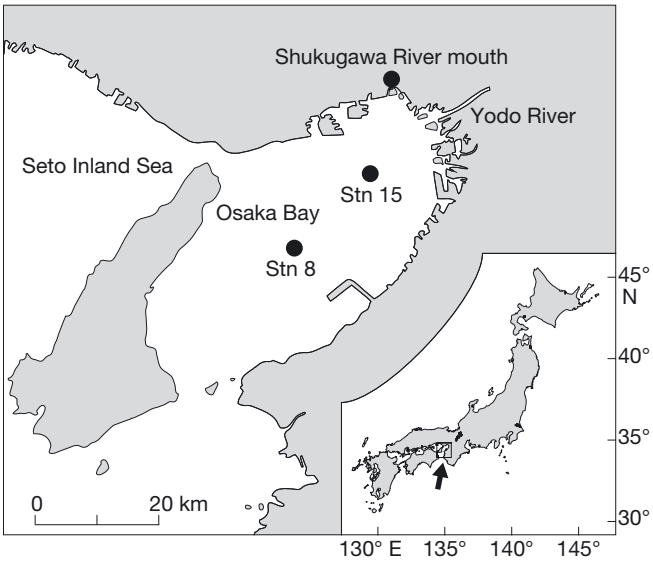


Fig. 1. Locations of the 3 sampling sites (black circles): Shukugawa River mouth and Stns 8 and 15 in Osaka Bay at the east end of the Seto Inland Sea, Japan

Estimation of thraustochytrid cell density by the MPN method

Thraustochytrid cell numbers in the seawater samples collected were estimated by the MPN method using the pine pollen baiting culturing approach (modified from Gaertner 1968). Samples were processed as soon as possible following collection. First, each sample was diluted 10-fold 6 times in screw-capped tubes. Each diluted sample was replicated 3 times. All of the replicates were brought to a constant volume of 9 ml with sterile seawater containing pine pollen and incubated at 15, 20, or 25°C (equal to the water temperature at the time of sampling) for 1 wk. After the 1 wk incubation, pine pollen was spread on d-GPY agar plates with an antibiotic medium that consisted of 0.2% glucose, 0.1% poly-peptone, 0.05% yeast extract, and 1.5% agar in 50/50 seawater/distilled water with antibiotics (0.02% chloramphenicol until 2 December 2009; 0.01% streptomycin sulfate and 0.01% ampicillin sodium salt from 28 December 2009). Because thraustochytrid zoospores can recognize chemical compounds, particularly polysaccharides, amino acids, and leaf extracts (Fan et al. 2002, Tsui et al. 2012), thraustochytrids were selectively attracted to the pollen and formed colonies on the medium. Therefore, it was easy to confirm the presence of thraustochytrids in each of the diluted samples by microscopy. Thraustochytrid cell numbers with the lower and upper detection limit (95% confidence levels) were estimated from the positive samples in which colonies were detected using the computer program mpn.exe (Klee 1993).

The MPN method using pine pollen likely underestimates cell number compared with the acriflavine direct detection (AfDD) method using epifluorescence microscopy (Raghukumar & Schaumann 1993, Raghukumar 2002, Li et al. 2013, Nakai et al. 2013; Table 1). In some samples, no thraustochytrids were detected, i.e. there was no settlement on the pine pollen or no growth on the agar plate with antibiotics. However, the MPN method obtains reliable data regarding

Table 1. Past reports of thraustochytrid abundance estimated by the most probable number (MPN) and acriflavine direct detection (AfDD) methods. All abundance values are reported as colony-forming units (MPN) or cells (AfDD) l^{-1} unless otherwise noted

Detection method and habitat Sampling site	Abundance Maximum	 Minimum	 Average	Reference
MPN - water				
Atlantic Ocean off Portugal	488	1		Gaertner (1982)
Antarctic Ocean	>100	<5		Bahnweg & Sparrow (1974a,b)
Sargasso Sea	115	ca. 5		Ulken (1979)
Fladen Ground Area, North Sea	320	1		Raghukumar & Gaertner (1980)
Arabian Sea	1.10×10^4	<1		Raghukumar (1985)
Arabian Sea	$>1.10 \times 10^3$	<6		Raghukumar et al. (1990)
Mouth of the Zuari estuary in Goa, India	348	14		Raghukumar et al. (1990)
Arabian Sea	1.00×10^3	0		Raghukumar et al. (2001)
Liqurian Sea (NW Mediterranean)			130 ± 100 (SD)	Bongiorni & Dini (2002)
MPN - sediment				
Atlantic Ocean off Portugal	4.38×10^4			Gaertner (1982)
Port Klangm, Malaysia	4.57×10^4	9.13×10^3		Ulken (1986)
Fladen Ground Area, North Sea	7.30×10^4	1.46×10^4		Raghukumar & Gaertner (1980)
Liqurian Sea (NW Mediterranean)			$6.10 \pm 5.30 \times 10^4$ (SD)	Bongiorni & Dini (2002)
Liqurian Sea (NW Mediterranean)			$6.10 \pm 5.30 \times 10^4$ (SD)	Bongiorni & Dini (2004)
AfDD - water				
Arabian Sea	1.31×10^6	0		Raghukumar et al. (2001)
Equatorial Indian Ocean	6.75×10^5			Damare & Raghukumar (2008)
Seto Inland Sea, Japan	5.60×10^4	2.10×10^3	1.03×10^4	Naganuma et al. (1998)
Seto Inland Sea and Hyuga Nada area, Japan	4.52×10^4	2.50×10^3	1.59×10^4	Kimura et al. (1999)
Seto Inland Sea and Hyuga Nada area, Japan	2.14×10^4	86	3.11×10^3	Kimura et al. (2001)
Offshore area of the Shimanto River	3.60×10^3	900	2.50×10^3	Kimura & Naganuma (2001)
Greenland and Norwegian Seas	2.30×10^5	<810	3.10×10^4	Naganuma et al. (2006)
Hawaii Ocean	6.30×10^5	Undetectable		Li et al. (2013)
AfDD - sediment				
North Sea	5.40×10^6 g^{-1}	1.00×10^5 g^{-1}		Raghukumar & Schaumann (1993)
Eastern Ligurian Sea (NW) Mediterranean			$4.23 \pm 0.29 \times 10^4$ (SD)	Bongiorni et al. (2004)
Fish farm in the Bay of Pachino, Italy	1.35×10^4 g^{-1}	710 g^{-1}		Bongiorni et al. (2005c)

changes in cell density if suitable dilutions and replicates are prepared (Bongiorni & Dini 2002). Moreover, the AfDD method cannot identify the thraustochytrid phylogenetic groups present. In contrast, living strains are easily established in the MPN process by isolating single cells from agar plates. Determination of the strain sequences provides phylogenetic information, which will be useful for future investigations such as physiological tests under culture conditions. Another reason for selecting the MPN method in this study was to avoid overestimation caused by misidentification and counting of dead cells. The estimated abundance values indicate the minimum cell numbers converted from colony-forming units of MPN.

Isolation and identification of thraustochytrids

Using a glass capillary rod under an inverted microscope, a maximum of 24 single colonies per sample were taken from the agar plates during the MPN process and put onto a 24-well plate with d-GPY liquid medium and antibiotics. After approximately 5 d of incubation at 15, 20, or 25°C, the isolates were transferred to test tubes.

Approximately 10 isolates were established and 5 were randomly selected for sequencing from each seawater sample. The isolates were identified to the highest taxonomic resolution possible by 18S rRNA gene phylogenetic analysis. Total genomic DNA was extracted by a modified CTAB extraction method as described by Murray & Thompson (1980). Roughly the full length of the 18S rRNA gene sequence (ca. 1800 bp) was amplified using PCR with the primers SR01 (Nakayama et al. 1996) and SR12L1 (5′-CCT TGT TAC GAC TTC ACC TTC C-3′; this study). One-third of the sequence from the head side of the 18S rRNA gene (ca. 600 bp) was determined using the primers SR01 or SR05 (Nakayama et al. 1996). The sequenced region contained relatively high variation and had sufficient resolution to analyze the phylogenetic position. Determined sequences were added to the aligned sequence data set (Yokoyama et al. 2007), aligned with a profile alignment process using the

ClustalX 2.1 software program (Larkin et al. 2007), and finally refined manually. The phylogenetic tree was generated by the neighbor-joining (NJ) method using the distances of the Tamura–Nei model with the pairwise deletion option as the gap treatment in MEGA 5.2.2 (www.megasoftware.net). Bootstrap values were obtained from 1000 resamplings. See Fig. 3 for abbreviations of phylogenetic group names used in this study. One to 3 isolates were selected from each phylogenetic group (with 2 exceptions), and the nearly complete 18S rRNA gene sequences (ca. 1600 bp) were determined. These sequences were submitted to GenBank under accession numbers AB973502–AB973563 (see Table S1 in the supplementary material available at, and via link from, www.int-res.com/articles/suppl/a074p187_supp.pdf).

Bacterial cell staining and counting

Seawater collected for bacterial cell counting was immediately fixed in 2.5% glutaraldehyde (Nacalai Tesque). Bacterial cells were collected from 0.5 to 1 ml of the fixed samples on brown color polycarbonate filters (Isopore™ Membrane Filter, Merck Millipore; pore size 0.22 µm; diameter 25 mm). Bacterial cells were then stained with 1 µg ml^{-1} 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) and washed twice with 1 ml autoclaved and 0.2 µm-filtered seawater. The filters were air-dried. Bacterial cells were counted in 50 fields of 50 µm × 50 µm by epifluorescence microscopy under UV excitation.

Analysis of particulate organic carbon and nitrogen, and dissolved organic carbon at the Shukugawa River mouth

Immediately after sampling, a 100 ml seawater sample was filtered through a pre-combusted glass fiber 25 mm Whatman GF/F filter (450°C, 2 h; GE Healthcare). The filter for particulate organic carbon (POC) and particulate organic nitrogen (PON) was then incubated at 60°C overnight and was kept in a desiccator until analysis. POC and PON were measured with a CHN analyzer (Model MT-5, Yanaco). Filtered seawater samples were kept at –20°C until analysis of dissolved organic carbon (DOC). DOC was measured with a total organic carbon analyzer (TOC-VCSH, Shimadzu). POC and DOC samples were measured from 7 December 2010 to 19 September 2013 (n = 37) and from 4 April 2012 to 19 September 2013 (n = 19), respectively.

RESULTS

Thraustochytrid cell density and environmental data at the Shukugawa River mouth

In total, 100 samples were obtained over 6 yr at the Shukugawa River mouth (Table S3 in the supplementary material). Thraustochytrids were found on all sampling days and the cell density ranged from 40 cells l^{-1} (28 December 2009) to 266 400 cells l^{-1} (1 May 2008 and 19 August 2013) (Table 2). A pattern of high cell density in summer and low cell density in winter was seen in all years. At least 1 or 2 peaks in cell density were observed in 2008, 2010, 2012, and 2013 between spring and summer (Fig. 2), when thraustochytrid cell density reached 47 470 to 266 400 cells l^{-1} (Table 2). In contrast, no peak was observed in either 2009 or 2011. Average cell density excluding peak periods was 4670 cells l^{-1} (n = 93).

In contrast, bacterioplankton cell density ranged from 2.86×10^8 cells l^{-1} (7 December 2011) to 1.08×10^{10} cells l^{-1} (4 June 2009). Average bacterioplankton cell density was 2.03×10^9 cells l^{-1} (n = 98; Table 2). Bacterioplankton abundance was similar to a previous report in an estuary in Japan (Kimura & Naganuma 2001). Water temperature ranged from 6.3°C (19 January 2010) to 30.9°C (19 August 2013), with an average of 19.3°C. Salinity ranged from 3.9 (15 July 2010) to 31.6 (4 September 2008) PSU with an average of 22.9 PSU. Changes in salinity were extreme because the sampling site is in the Shukugawa River mouth. Remarkably high chl *a* concentrations of 139.8 µg l^{-1} (27 May 2008) and 202.5 µg l^{-1} (4 June 2009) were recorded during *Heterosigma akashiwo* blooms. Excluding these blooms, chl *a* concentrations ranged from 1.28 µg l^{-1} (13 May 2010) to 49.9 µg l^{-1} (3 July 2013) with an average of 10.4 µg l^{-1}, and remained relatively constant during monitoring except during these 2 blooms.

Thraustochytrid phylogenetic group composition at the Shukugawa River mouth

In total, 932 isolates were obtained, and the partial 18S rRNA gene sequences of 435 new isolates were determined. Isolates were classified according to phylogenetic groups from the labyrinthulomycete 18S rRNA gene NJ tree. The molecular phylogenetic analyses revealed that at least 24 phylogenetic groups, including the new isolates, inhabit the Shukugawa River mouth (Fig. 3, Tables S1 & S3 in

Table 2. Thraustochytrid and bacterioplankton density and carbon biomass with water temperature (Temp), salinity (Sal), and chl *a* concentration. Ratio: ratio of thraustochytrid to bacterioplankton carbon biomass (%); Stns 8 and 15 in Osaka Bay show data for surface (S) and bottom (B) waters

		Environmental data			Thraustochytrids		Bacterioplankton		Ratio
		Temp (°C)	Sal (PSU)	Chl *a* (µg l^{-1})	Density (cells l^{-1})	Biomass (µg C l^{-1})	Density (× 10^8 cells l^{-1})	Biomass (µg C l^{-1})	
Shukugawa River mouth									
Average (including spikes)		19.3	22.9	13.68	13729	2.27	20.34	61.44	3.60
Average (excluding spikes)		18.8	23.1	13.45	4670	0.77	19.50	58.90	1.59
Min (including spikes)		6.3	3.9	1.28	40	0.01	2.86	8.63	0.04
Max (including spikes)		30.9	31.6	202.50	266400	43.96	107.52	324.72	79.57
Max (excluding spikes)		30.5	31.6	202.50	26640	4.40	107.52	324.72	12.19
Thraustochytrid spikes									
1 May 2008		20.9	21.2	10.41	266400	43.96	36.33	109.73	40.06
24 July 2008		30.5	21.0	46.36	47470	7.83	55.95	168.96	4.64
6 May 2010		21.4	12.1	9.02	103600	17.09	17.68	53.41	32.01
17 Aug 2010		30.6	13.6	15.49	103600	17.09	50.64	152.94	11.18
6 June 2012		21.7	27.7	16.43	47470	7.83	19.18	57.94	13.52
1 July 2012		23.7	24.0	9.69	103600	17.09	20.61	62.25	27.46
19 Aug 2013		30.9	24.4	9.51	266400	43.96	18.29	55.24	79.57
Osaka Bay									
Stn 8S	Average	17.7	30.9	2.35	122	0.02	4.96	14.99	0.22
	Min	8.7	22.2	–3.93	0	0.00	1.05	3.18	0.00
	Max	28.0	32.4	16.82	475	0.08	20.72	62.58	1.38
Stn 8B	Average	17.5	32.1	–2.15	47	0.01	4.05	12.23	0.07
	Min	8.5	31.5	–3.84	0	0.00	0.50	1.51	0.00
	Max	27.3	32.6	0.08	475	0.08	14.86	44.88	0.42
Stn 15S	Average	18.0	28.1	11.43	509	0.08	9.64	29.13	0.27
	Min	8.0	20.4	–8.84	0	0.00	0.61	1.83	0.00
	Max	29.0	30.8	61.10	4747	0.78	31.40	94.84	1.23
Stn15B	Average	17.1	32.0	–0.72	45	0.01	4.64	14.01	0.07
	Min	8.5	31.4	–3.43	0	0.00	0.57	1.73	0.00
	Max	27.2	32.5	7.20	266	0.04	17.26	52.11	0.52

the supplementary material). The isolates formed 7 phylogenetic groups with no previously identified strains or environmental DNA clones (Collado-Mercado et al. 2010, Li et al. 2013); these were temporarily named unidentified thraustochytrid 1, 2a, 2b, 3a, 4, 6, and 7. Phylogenetic groups that appeared at comparatively high frequencies were referred to as major phylogenetic groups. The 7 major phylogenetic groups were named unidentified thraustochytrid 1, 2a, and 2b, *Schizochytrium* sp., and *Oblongichytrium* sp. 1, 2, and 3 (Figs. 3 & 4). *Oblongichytrium* sp. was divided into 5 phylogenetic groups called *Oblongichytrium* sp. 1 to 5 (Fig. 3, Table S1). The seasonal succession of phylogenetic group composition of the isolates followed a similar trend every year (Fig. 2).

The phylogenetic groups isolated from each peak were different. In 2008, the isolates detected at significantly high cell density on 1 May were composed of 3 isolates of *Aurantiochytrium* sp. 2, 1 of *Thraustochytrium* aff. *striatum*, and 1 of thraustochytrid 1. The isolates forming the second peak on 24 July were composed of 7 isolates of *Aurantiochytrium* sp. 2. In 2010, the isolates in the first peak on 6 May were composed of 6 isolates of thraustochytrid 2a and 4 of *Oblongichytrium* sp. 1. The isolates in the second peak on 17 August were composed of 3 isolates of thraustochytrid 2b, 3 of *Botryochytrium* sp., and 2 of *Schizochytrium* sp. In 2012, the isolates forming the peak on 6 June comprised 2 isolates of *Oblongichytrium* sp. 2, 2 of thraustochytrid 7, and 1 of *Oblongichytrium* sp. 1. On 1 July, the isolates in the peak were 2 isolates of *Sicyoidochytrium* sp., 2 of thraustochytrid 7, and 1 of *Oblongichytrium* sp. 1. In 2013, the isolates forming the peak on 19 August were composed of 3 isolates of thraustochytrid 2b, 1 of *Schizochytrium* sp., and 1 of *Thraustochytrium* aff. *caudivorum*. It should be noted that the antibiotics used in the agar plates for isolation were changed beginning in December 2009 as described in 'Materials and methods'.

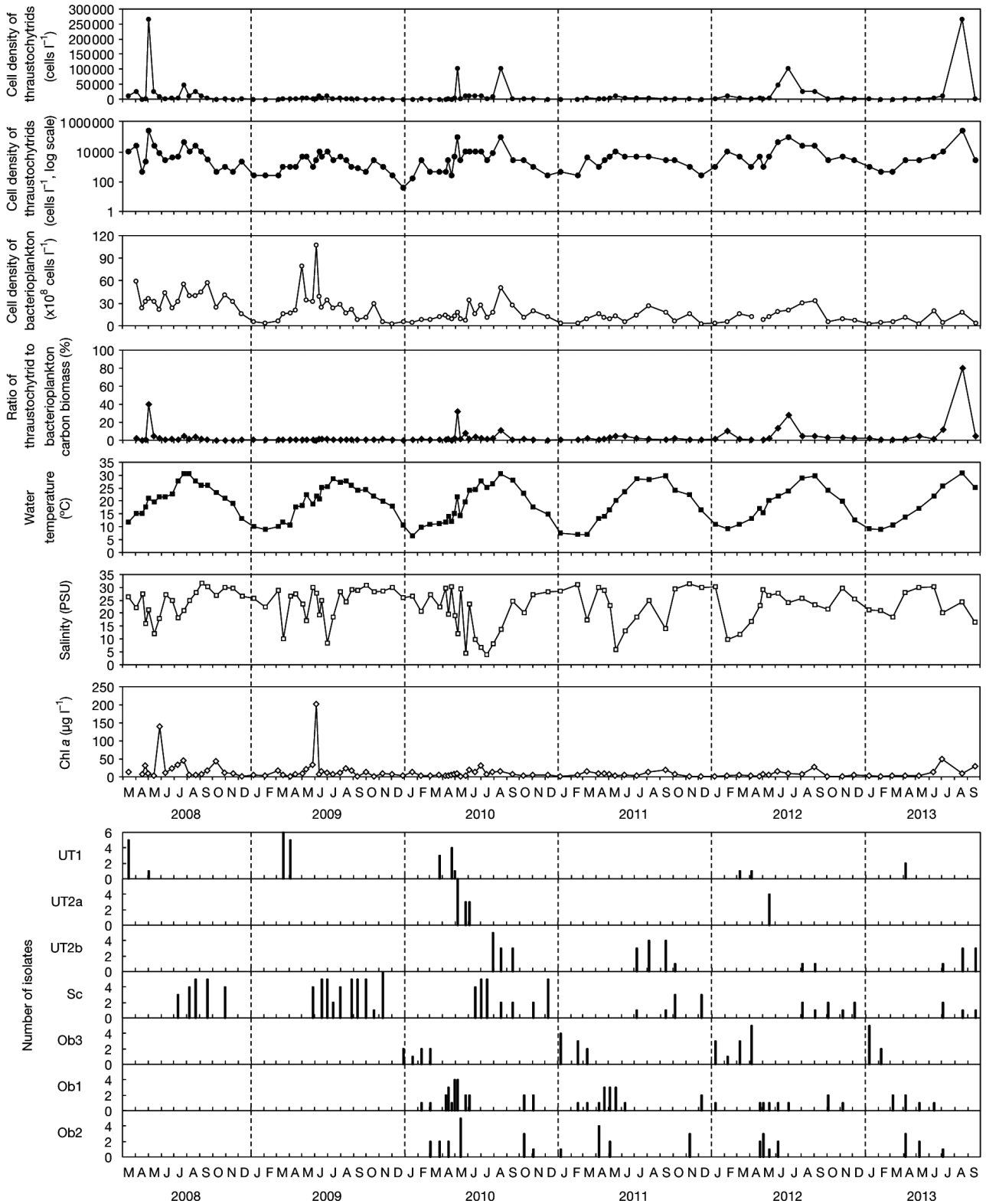


Fig. 2. Monitoring data from the Shukugawa River mouth. Sampling was carried out 100 times once or twice a month from March 2008 to September 2013. The line graphs show thraustochytrid and bacterioplankton cell density, ratio of thraustochytrid to bacterioplankton carbon biomass, and environmental data (water temperature, salinity, and chl *a* concentration). Bars show the number of isolates sequenced for each major phylogenetic group. Abbreviations of the phylogenetic groups are shown in Fig. 3

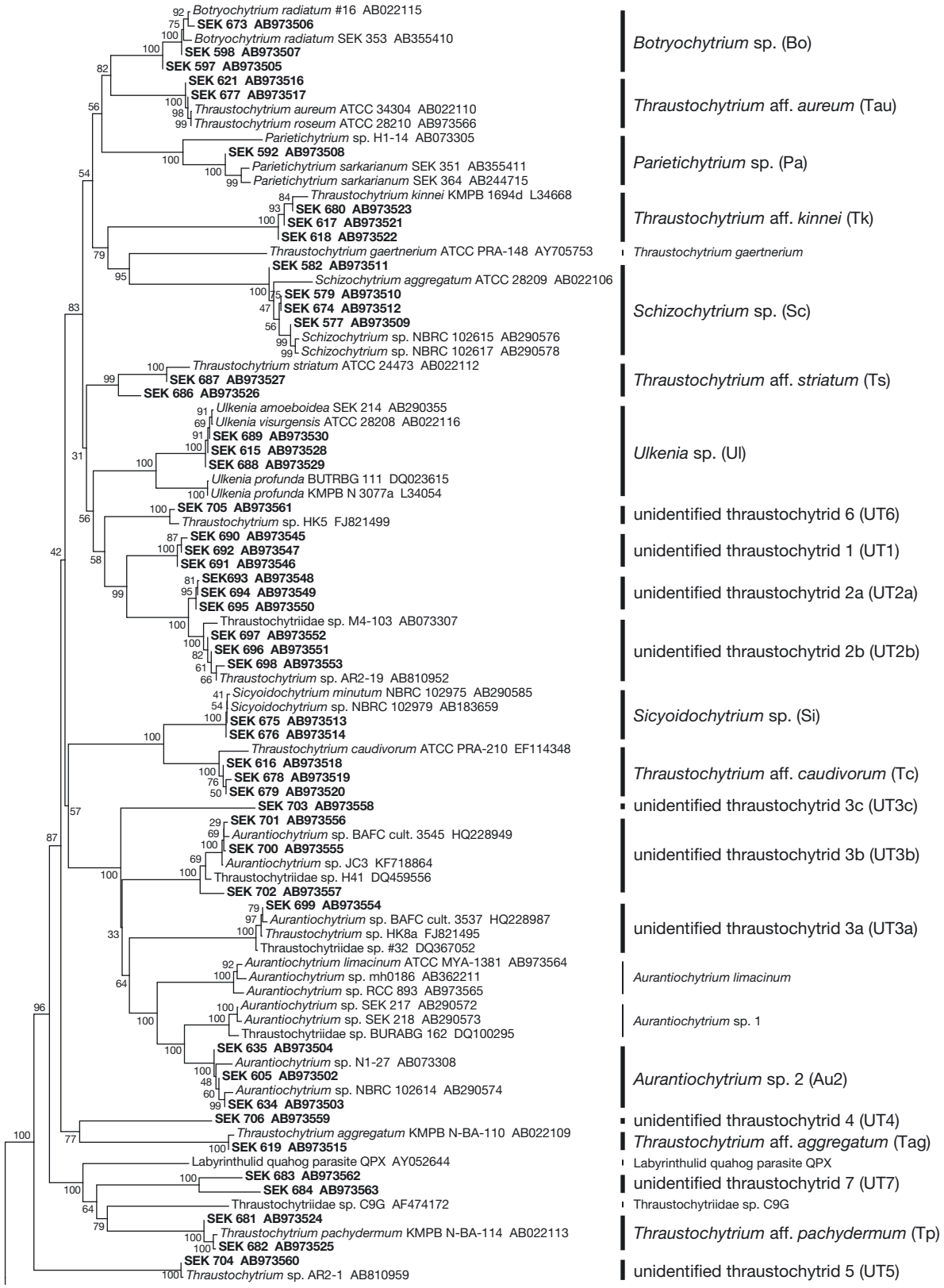


Fig. 3. Labyrinthulomycete phylogenetic tree, including the original strains from this study using the 18S rRNA gene. Bold vertical lines indicate phylogenetic groups from this study. The sequences analyzed are shown in Tables S1 and S2 in the supplementary material. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values were obtained from 1000 resamplings. All strains whose names start with SEK (**bold**) were sequenced in this study. Abbreviations of the phylogenetic groups are shown in parentheses

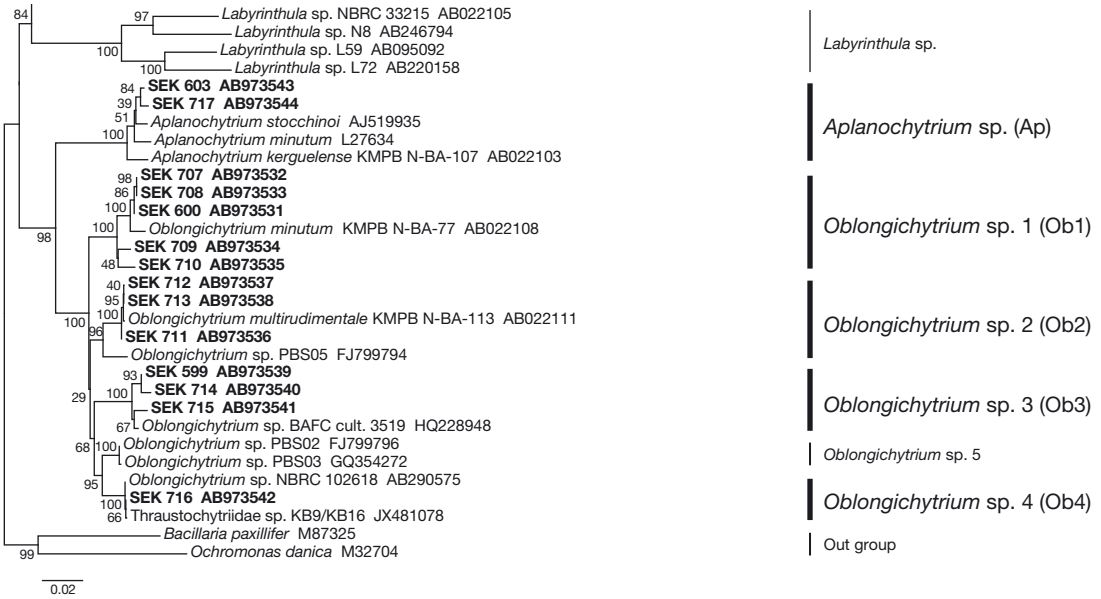


Fig. 3 (continued)

Correlation between thraustochytrid abundance and environmental parameters at the Shukugawa River mouth

Correlation coefficients were calculated among thraustochytrid abundance, bacterioplankton abundance, water temperature, salinity, chl *a* concentration, POC, PON, and DOC at the Shukugawa River mouth, excluding data during thraustochytrid peaks. While thraustochytrid abundance was well correlated with POC and DOC, there was no relationship with chl *a* concentration (Table 3, Fig. S1 in the supplementary material). This phenomenon suggests that the most abundant thraustochytrids in the river mouth consumed POC and DOC derived from non-phytoplankton as their nutrient sources. Average POC (from 7 December 2010 to 19 September 2013) and DOC (from 4 April 2012 to 19 September 2013), excluding the peak values, at the Shukugawa River mouth were 2.07 and 3.36 mg l^{-1}, respectively. We estimated the ratio of POC derived from phytoplankton from the regression line of chl *a* concentration and POC at the Shukugawa River mouth using the method of Kimura et al. (2001) (Fig. 5). The slope of the minimum model ($y = ax + b$) was 0.099 (Fig. 5). The maximum model, in which we assume that all POC is phytoplanktonic and set the *y*-intercept to 0 ($y = a'x$), gives a slope of 0.16 (Fig. 5). Using the average concentration of chl *a* (8.51 µg l^{-1}), phytoplankton biomass was calculated as 0.84 and 1.36 mg C l^{-1} for the minimum and maximum models, respectively, and accounted for 40.6 and 65.7% of the average concentration of POC (2.07 mg C l^{-1}). The average thraustochytrid carbon biomass was estimated to be 7.59×10^{-4} mg l^{-1} (from 7 December 2010 to 19 September 2013, excluding the peak values) based on a cellular carbon content of 1.65×10^{-4} µg C $cell^{-1}$ as reported by Kimura et al. (1999). Because the ratio of thraustochytrid carbon biomass to average total POC was 0.037%, thraustochytrid POC in total POC can be ignored. The average bacterioplankton carbon biomass was 0.033 mg l^{-1} (from 7 December 2010 to 19 September 2013, excluding the peak values) based on a cellular carbon content of 30.2 fg C $cell^{-1}$ (Fukuda et al. 1998), and the ratio of carbon in bacterioplankton biomass to average total POC was 1.59%. The microzooplankton and net-zooplankton carbon were negligible at 0.03 to 1.4% and 0.6 to 1.7%, respectively (Uye et al. 1996, Uye & Shimazu 1997). Therefore, it was estimated that the POC derived from non-phytoplankton was 35 to 60% at the Shukugawa River mouth.

Thraustochytrid cell density and environmental data at Osaka Bay

Sampling was carried out 24 times over 2 yr (Table S4 in the supplementary material). The cell density of thraustochytrids in samples from Stns 8S, 8B, 15S, and 15B varied from 0 to 475 cells l^{-1} (avg.

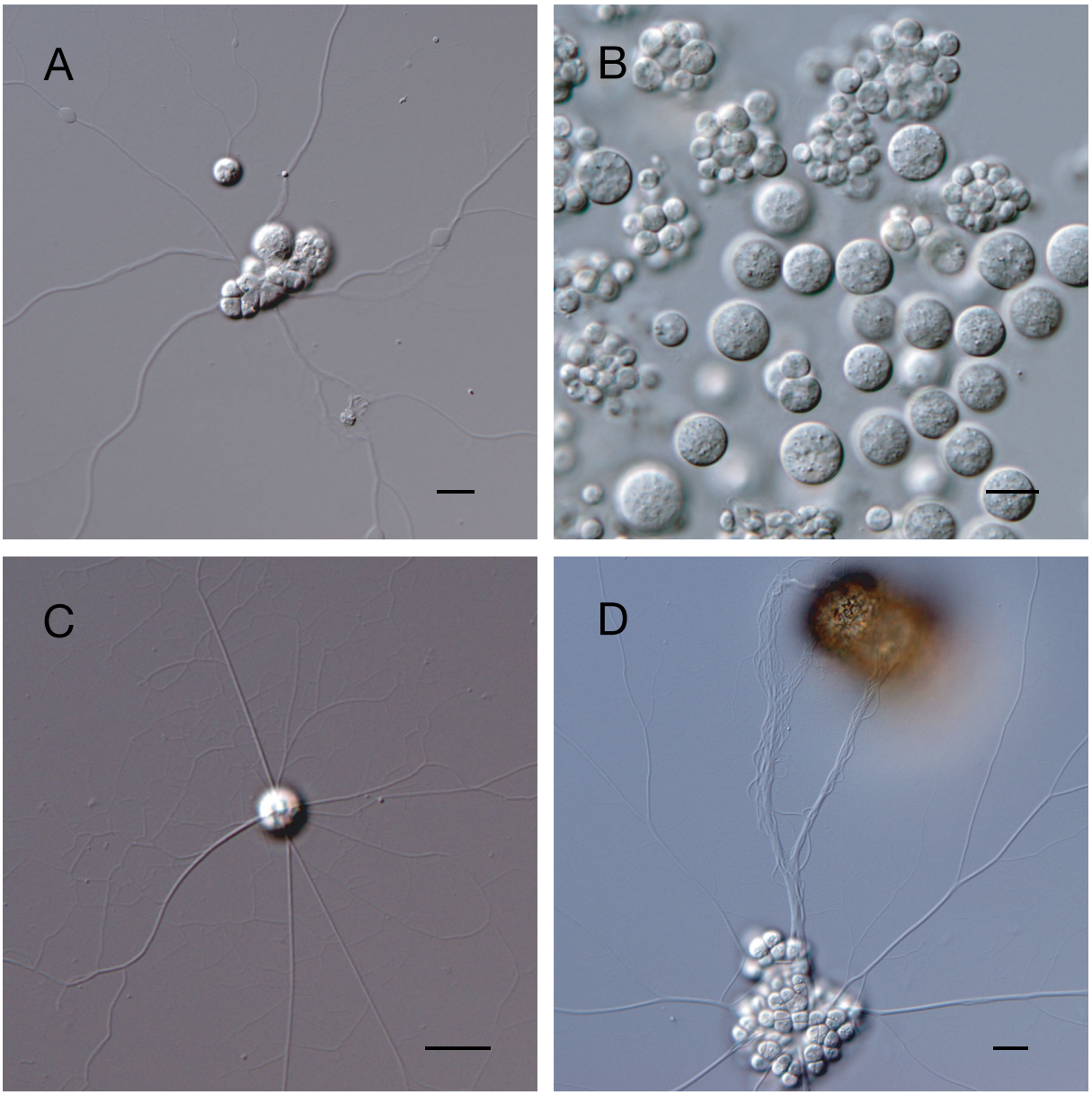


Fig. 4. Thraustochytrid cells. (A) *Schizochytrium* sp. (SEK 579), (B) unidentified thraustochytrid 1 (SEK 691), (C) unidentified thraustochytrid 2a (SEK 694), and (D) *Oblongichytrium* sp. 1 (SEK 600) with strongly extended ectoplasmic nets to pine pollen. SEK 691 was cultured in dGPY medium, and SEK579, SEK694, and SEK600 in sterilized seawater with pine pollen. All scale bars = 10 µm

122 cells l^{-1}), 0 to 475 cells l^{-1} (avg. 47 cells l^{-1}), 0 to 4747 cells l^{-1} (avg. 509 cells l^{-1}), and 0 to 266 cells l^{-1} (avg. 45 cells l^{-1}), respectively (Fig. 6, Table 2). Thraustochytrid densities at Osaka Bay were equivalent to those of previous reports using the MPN method (Gaertner 1968) (Table 1). Only 1 peak in cell density was observed each year at 15S. The highest cell densities in 2011 and 2012 were 4747 cells l^{-1} (1 August 2011) and 2664 cells l^{-1} (3 September 2012), respectively. However, the peaks were conspicuously lower than those of the Shukugawa River mouth.

Bacterioplankton cell density ranged from 0.5 to 31.4 × 10^8 cells l^{-1} across sites. Bacterioplankton abundance was similar to that of previous reports in the Seto Inland Sea (Naganuma et al. 1998). Changes in water temperature were similar at all sampling points. Water temperature ranged from 8.0 to 29.0°C, with an average of approximately 17–18°C (Fig. 6, Table 2, Table S4). At Stn 15S,

Table 3. Correlation coefficients of thraustochytrids (Thra), bacterioplankton (Bac), water temperature (Temp), salinity (Sal), chl *a* concentration (Chl), particulate organic carbon (POC), particulate organic nitrogen (PON), and dissolved organic carbon (DOC) at the Shukugawa River mouth, excluding data during spikes in abundance of thraustochytrids. $^{*}p < 0.05$; $^{**}p < 0.01$

	Thra	Bac	Temp	Sal	Chl	POC	PON	DOC
Thra	1							
Bac	0.357**	1						
Temp	0.373**	0.401**	1					
Sal	−0.296**	0.055	−0.139	1				
Chl	0.060	0.527**	0.208*	−0.032	1			
POC	0.589**	0.399*	0.358*	−0.247	0.762**	1		
PON	0.458**	0.360*	0.334	−0.102	0.798**	0.952**	1	
DOC	0.815**	0.768**	0.747**	0.114	0.220	0.343	0.267	1

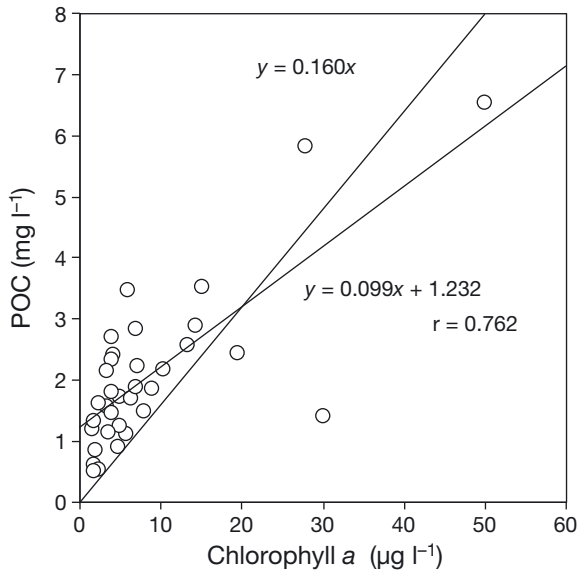


Fig. 5. Correlation between chl *a* concentration and particulate organic carbon (POC) at the Shukugawa River mouth, not including data during spikes in thraustochytrid abundance. If the *y*-intercept is forced through 0, the slope is 0.16

salinity was influenced by rainfall and ranged from approximately 20.4 to 30.8 PSU. At Stn 8S, salinity was comparatively stable (29.6–32.4 PSU), except after a typhoon which reduced salinity to 22.2 PSU. In contrast, salinity at Stns 8B and 15B was stable at ca. 32 PSU. Relatively large changes in chl *a* concentration caused by fluctuations in diatom populations were observed at Stn 15S only; these ranged from 0 (5 July 2011) to 61.1 µg l^{-1} (1 August 2011). Changes in thraustochytrid abundance at Stns 8S and 15S were weakly related to water temperature and bacterioplankton abundance, respectively (Fig. 6, Tables S5 & S6 in the supplementary material).

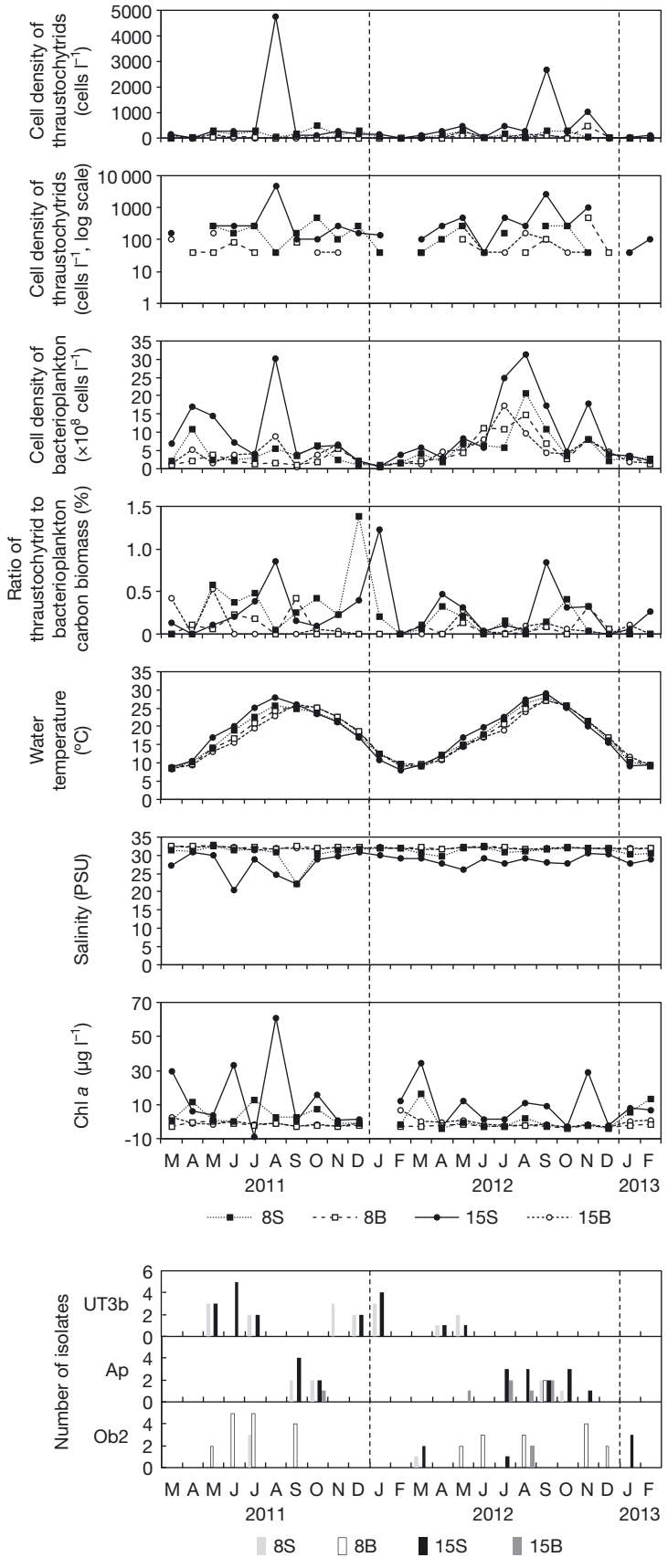
Thraustochytrid phylogenetic group composition at Osaka Bay

The number of isolated strains (numbers of 18S rRNA gene sequences are in parentheses) at the Osaka Bay sampling Stns 8S, 8B, 15S, and 15B were 71 (46), 56 (37), 113 (71), and 20 (16), respectively. The molecular phylogenetic analyses revealed at least 16 phylogenetic groups in the bay including 8 unidentified isolates, which were temporarily called unidentified thraustochytrid 2a, 2b, 3b, 3c, 4, 5, 6, and 7 (Fig. 3, Tables S1 & S4). Stns 8S and 15S yielded similar phylogenetic groups including 3 major groups as follows: unidentified thraustochytrid 3b, *Aplanochytrium* sp., and *Oblongichytrium* sp. 2 (Fig. 6). The phylogenetic groups isolated at Stns 8B and 15B were also similar; these were *Oblongichytrium* sp. 2 and *Aplanochytrium* sp. Because the number of isolates from bottom water was low, it was not possible to determine seasonal succession of the phylogenetic groups at Stn 8B or 15B. The phylogenetic group composition of the isolates forming each peak was different at Stn 15S. The isolates detected at significantly high cell density in 2011 were composed of 5 isolates of thraustochytrid 2b, whereas the isolates forming the peak in 2012 comprised 2 isolates of *Aplanochytrium* sp. and 1 of *Thraustochytrium* aff. *caudivorum*.

DISCUSSION

Thraustochytrid spikes

The regular monitoring of thraustochytrid biomass at the Shukugawa River mouth in this study revealed 1 or 2 conspicuous peaks in abundance between spring and summer in most sampling years (Fig. 2). Similar but smaller-scale peaks were also observed in Osaka Bay (Fig. 6). Peaks at both locations were never observed simultaneously, and the phylogenetic group composition of the isolates differed between the river mouth and the bay area, suggesting that the occurrences of these peaks are independent events. Additionally, similar peaks were observed in preliminary monitoring from 2006 to 2007 in Ago Bay (34° 18.10′ N, 136° 51.12′ E), a brackish water body located in central Japan (D. Honda unpubl. data). This coincidence in peaks observed at independent sites suggests that these thraustochytrid



spikes probably occur as a general phenomenon of brackish and coastal regions.

Thraustochytrid spikes occurred for short periods of time. Repeated weekly sampling from April to May 2010 showed that the peaks lasted less than 2 wk. To clarify the cause of the rapid increase in the number of thraustochytrid cells, both chl *a* concentration and phytoplankton composition were considered. Blooms of *Heterosigma akashiwo*, *Skeletonema* spp., *Chaetoceros* spp., and *Prorocentrum* spp. were observed in the monitoring area, but thraustochytrid spikes were not strongly correlated with these. Although Gaertner (1979) showed that thraustochytrids parasitize living diatoms, we found little correlation between phytoplankton and thraustochytrid abundance.

In contrast, salinity and water temperature were likely related to thraustochytrid spikes. A reduction in salinity was observed either during the same period or just prior to the occurrence of each spike. Low salinity in coastal water indicates that the area has received river discharge. Thus, one explanation may be that thraustochytrids grow on terrestrial organic matter supplied by river discharge (Kimura & Naganuma 2001). It is also possible that physical changes in the seawater environment and the supplied terrestrial organic matter act as the stimulus to release zoospores; in fact, thraustochytrids tend to release zoospores after physical changes in their environment take place (e.g. Honda et al. 1998, Yokoyama & Honda 2007).

At the river mouth, seawater temperature rose rapidly after the rainy period in the spring and summer. Spikes in cell abundance in spring and summer corresponded with seawater temperatures exceeding 20°C and 30°C, respectively (Fig. 2). Bloom-forming microalgae often have a cyst stage from which swarmers simultaneously hatch following a rise in seawater temperature. Blooms are then formed by rapid and repeated cell division (Anderson 1980, Imai 1989). The observed spikes may be caused by germination of thraustochytrid cysts; however, there

Fig. 6. Surface and bottom water monitoring data at Stns 8 and 15 in Osaka Bay. Sampling was carried out 24 times once a month from March 2011 to February 2013. Line graphs show thraustochytrid and bacterioplankton cell density, ratio of thraustochytrid to bacterioplankton carbon biomass, and environmental data (water temperature, salinity, and chl *a* concentration). 8S and 8B (15S and 15B) are surface and bottom water at Stn 8 (Stn 15), respectively. Bars show the number of the isolates for each major phylogenetic group. Abbreviations of the phylogenetic groups are shown in Fig. 3

are no reliable reports of a cyst stage in thraustochytrids or of temperature activation of thraustochytrid cells.

Conversely, a viral infection is a possible reason for the rapid decrease in the number of thraustochytrid cells. It is known that blooms of several microalgal species, e.g. *Heterosigma akashiwo* and *Heterocapsa circularisquama*, come to an end because of viral infections (Nagasaki et al. 1994, Tomaru et al. 2007, 2008). Two kinds of viruses infecting thraustochytrids have already been isolated, viz. a single-stranded RNA virus from *Aurantiochytrium* sp., and a double-stranded DNA virus from *Sicyoidochytrium* sp. (Takao et al. 2005, 2006, 2007), both of which destructively lyse the host cells. Viral infection may explain why thraustochytrid spikes only occurred every other year. Some microalgae form large-scale blooms every other year. As the virus particles are scattered on the seabed in large quantities by the bloom, microalgae may be unable to increase in the following year because of infection (Tomaru et al. 2007).

Thraustochytrid biomass

As described in 'Results', the average cell numbers of thraustochytrids at the river mouth were >10 times higher than those in the bay area. However, average total organic carbon (TOC) at the river mouth was 5.43 mg C l^{-1} in this study (Table S3 in the supplementary material), which is only twice as large as that of Osaka Bay and its surrounding coastal area (2.72 mg C l^{-}; Kimura et al. 2001). Previous studies of thraustochytrid abundance in the water column using the MPN method with pine pollen baiting have reported 5 to 1000 cells l^{-1} in littoral regions worldwide (Table 1). For example, averages of 68 and 173 cells l^{-1} were measured in an Indian Ocean estuary (Raghukumar et al. 1990), and 1.3 ± 1(SD) × 10^2 cells l^{-1} were found in the coastal Mediterranean Sea (Bongiorni & Dini 2002). Thus, thraustochytrid densities at the Shukugawa River mouth were substantially higher than those previously reported. It is possible that high thraustochytrid biomass in the estuarine environment is caused not only by the quantity of organic matter, but also by the quality of decomposing objects as a nutrient source. Thraustochytrids are comparatively abundant in coastal regions with low-salinity seawater, where river discharge has an influence (Kimura & Naganuma 2001). In particular, terrestrial organic matter in the form of litter and detritus is constantly supplied to estuaries, and thraustochytrids have been observed on plant material such as litter, pollen, sea grass, and macroalgae (Miller & Jones 1983, Raghukumar et al. 1992, Bremer 1995, Leander et al. 2004, Wong et al. 2005). We suggest that there are species of thraustochytrids that specialize and have adapted to the estuarine environment (see next section).

The carbon contents of thraustochytrid and bacterioplankton cells have been estimated to be 1.65 × 10^{-4} µg $cell^{-1}$ and 30.2 fg $cell^{-1}$, respectively (Fukuda et al. 1998, Kimura et al. 1999). The average thraustochytrid and bacterial carbon biomass values of the whole period at the river mouth, excluding thraustochytrid spikes, were 0.77 and 58.9 µg C l^{-1}, respectively (Table 2). The average ratio of thraustochytrid to bacterial cell carbon biomass in each sample was 1.59% (0.04–12.19%). In the microbial loop, bacteria are food for phagotrophic protists, which in turn become food for higher trophic zooplankton such as copepods; consequently, the energy flow from bacteria via the protists is incorporated into the grazing food chain that begins with the phytoplankton (Azam et al. 1983). The ecological efficiency of energy transport among lower trophic marine organisms is estimated to be 10 to 20% (Ryther 1969). On the assumption that the ecological efficiency is 10%, it was estimated that 1% of bacterial energy is transferred to copepod-like zooplankton after 2 trophic steps. However, thraustochytrid cell size ranges from 5 to 20 µm, which corresponds to that of small phagotrophic protists. Moreover, thraustochytrids often form colonies under culture conditions with oligotrophic media, which correspond to the condition of the natural environment, and thraustochytrids possibly attach to larger particles by their ectoplasmic nets (Goldstein 1963, Gaertner 1968, Raghukumar 1988, Honda et al. 1998, Bongiorni et al. 2005a; Fig. 4). Most of them might be an ideal size (ca. 5–50 µm) for copepod-like zooplankton to feed on (Uye 1986, Hansen et al. 1994). In this case, 10% of thraustochytrid energy is transferred to the zooplankton after only 1 trophic step. Therefore, although thraustochytrid biomass was only 1.59% of bacterial biomass at the river mouth, the fixed energy (biomass) transferred directly from thraustochytrids to zooplankton was estimated to be 15.9% of that transferred from bacterioplankton via phagotrophic protists. In contrast, the ratio of thraustochytrid to bacterial biomass during the spikes was 4.64 to 79.57%; that is, the potential effect of thraustochytrids on zooplankton was approximately 0.5 to 8 times that of bacteria during the spikes. Moreover, the MPN method with pine pollen baiting underestimates cell numbers, because certain thraustochytrids

are not detected if they do not settle onto the pine pollen or grow on the agar plate with antibiotics. Thus, it is likely that the ecological effect of thraustochytrids during spikes in abundance is very strong in the marine ecosystem, although they may also play an important role at other times.

Many marine fishes possess high amounts of PUFAs such as eicosapentaenoic acid (EPA) and DHA. However, they cannot synthesize enough PUFAs from short-chain fatty acids and must obtain EPA and DHA from their food, i.e. zooplankton and phytoplankton (Bell & Tocher 2009, Tocher 2010, Meesapyodsuk & Qiu 2012). In fact, it has been shown that the survival ratio of cultured fish larvae fed EPA- or DHA-enriched *Artemia* is higher than that of larvae cultured without these PUFAs (Furuita et al. 1996). Thraustochytrids accumulate high amounts of DHA in their cells; thus, they may play an important role as DHA suppliers in the ecosystem. Many fish larvae were observed in the river mouth from spring to early summer. During the same period, thraustochytrid spikes indicated high thraustochytrid biomass and therefore possibly increased DHA for fish larvae. However, this connection remains unverified, and data on the ecological connection in the food chain between the fishes, thraustochytrids, and zooplankton, such as the rotifers and copepods, is necessary.

Seasonal dynamics and distribution

In this study, the regular monitoring suggested seasonal changes in thraustochytrid phylogenetic group composition of the isolates at the Shukugawa River mouth and in Osaka Bay. Based on the 18S rRNA gene phylogeny, the isolated strains were separated into 27 phylogenetic groups, which included 10 unidentified thraustochytrid lineages (Fig. 3, Table S1). These groups appeared at the river mouth in the following order: thraustochytrid 1 (March to May), thraustochytrid 2a (May to June), *Schizochytrium* sp. (June to December), thraustochytrid 2b (July to September), *Oblongichytrium* sp. 1 and 2 (October to July), and *Oblongichytrium* sp. 3 (December to April) (Fig. 2). The strains were isolated from seawater samples that had water temperature and salinity conditions specific to each of these phylogenetic groups (Fig. 7). For example, the members of thraustochytrid 1, 2a, and 2b were isolated from seawater samples at 10–15°C, 20–25°C, and 25–30°C, respectively. The members of *Schizochytrium* sp., which appeared over a comparatively long period (early summer to winter), were isolated from seawater with broad salinity at a high temperature (>22°C), but they were also isolated from a high-salinity environment at a low temperature (<22°C; Fig. 7). In contrast, in the surface water in the bay area, *Aplanochytrium* sp. appeared from summer to fall (July to November), and thraustochytrid 3b appeared in spring (April to July) and winter (November to January; Fig. 6). Members of both *Aplanochytrium* sp. and thraustochytrid 3b were mainly isolated from high salinity seawater at 25–30°C and 10–25°C, respectively (Fig. 7). Thus, there appears to be a seasonal succession of thraustochytrid phylogenetic groups according to seawater temperature and salinity.

Individuals of *Oblongichytrium* sp. 2 were isolated at high frequencies from both areas, but each of the other major groups was observed primarily in 1 area. Correlations between thraustochytrid abundance and POC and DOC, but not chl *a*, suggest that most thraustochytrids in the river mouth consumed POC and DOC derived from non-phytoplankton as their nutrient sources. POC derived from non-phytoplankton made up 35 to 60% of POC at the river mouth, of which terrestrial organic matter was likely the major element with comparatively little marine detritus. Although the present study did not measure POC and DOC in Osaka Bay, Kimura et al. (2001) reported that in Osaka Bay and the surrounding coastal area, thraustochytrids consumed non-phytoplankton-derived POC comprising terrestrial organic matter and marine detritus. Our investigation found that the thraustochytrid phylogenetic group composition of the isolates in the bay area was different to that of the river mouth, despite the fact that POC in both the river mouth (this study) and the bay area (Kimura et al. 2001) was similarly derived from non-phytoplankton sources. We suggest that this difference in thraustochytrid composition was either caused by differences in the quality or composition of non-phytoplanktonic POC between the river mouth and the bay or by other unknown factors. The fluctuating seawater salinity and the continuous supply of terrestrial organic matter at the river mouth are the most conspicuous differences between these habitats. The *Schizochytrium* sp. strains that often appeared in the river mouth had well-developed ectoplasmic nets, possibly attaching to the particles, under culture conditions (Fig. 4A) and possessed high cellulase activity (Nagano et al. 2011). Bongiorni & Dini (2002, 2004) also observed an abundance of *S. aggregatum* near a river mouth in the Mediterranean Sea. The *Oblongichytrium* sp. 1 strains isolated from the Shukugawa River mouth extended massive ectoplasmic nets to the pine pollen under culture conditions (Fig. 4D).

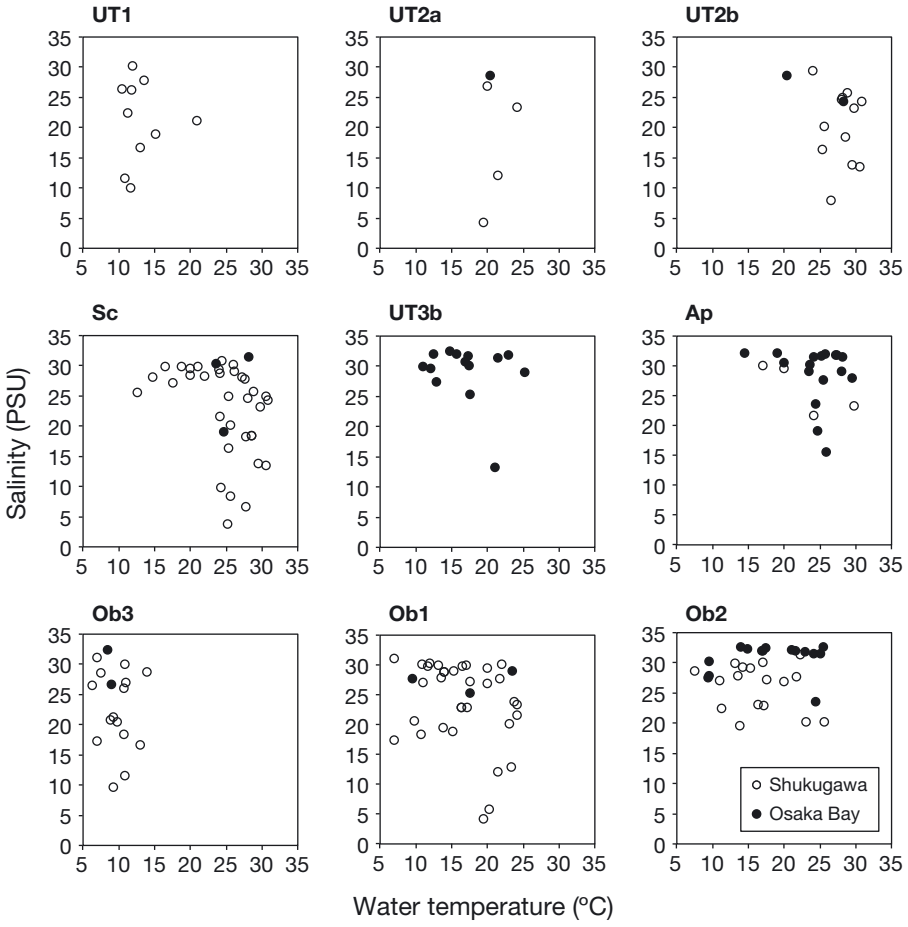


Fig. 7. Plots of water temperature and salinity of collected seawater samples at the time of isolation of each phylogenetic group. White and black circles correspond to the Shukugawa River mouth and Osaka Bay, respectively. Abbreviations of the phylogenetic groups are shown in Fig. 3

These thraustochytrids are probably highly adapted to the estuarine environment, and we suggest that habitat segregation occurs between the river mouth and the bay.

CONCLUSIONS

This study is the first report in which the biomass dynamics and phylogenetic group composition of thraustochytrids were investigated by regular monitoring using the MPN method with pine pollen at fixed points. Based on the results, we conclude that repeated thraustochytrid spikes in abundance and a seasonal succession cycle occur in both the estuarine and the coastal areas. Additionally, we speculate that thraustochytrids have a large ecological impact in these areas. In particular, the cell density results revealed that carbon biomass during thraustochytrid spikes was >50% of that of the bacterioplankton. The identification of established strains in each sample revealed unexpectedly high thraustochytrid diversity, including 10 unidentified lineages. To date, thraustochytrid ecological knowledge has led us to assume that all species have the same nutrient strategy and occupy the same niche. However, our study suggests that habitat segregation of thraustochytrids occurs between estuaries and coastal areas. Additionally, the seasonal succession of phylogenetic groups appears to be driven by differences in environmental factors including water temperature, salinity, and nutrient sources (Fig. 8). To understand the true ecological impact of thraustochytrids, further work is needed clarifying the more specific relationships of each thraustochytrid phylogenetic group with both its nutrient source and its predators.

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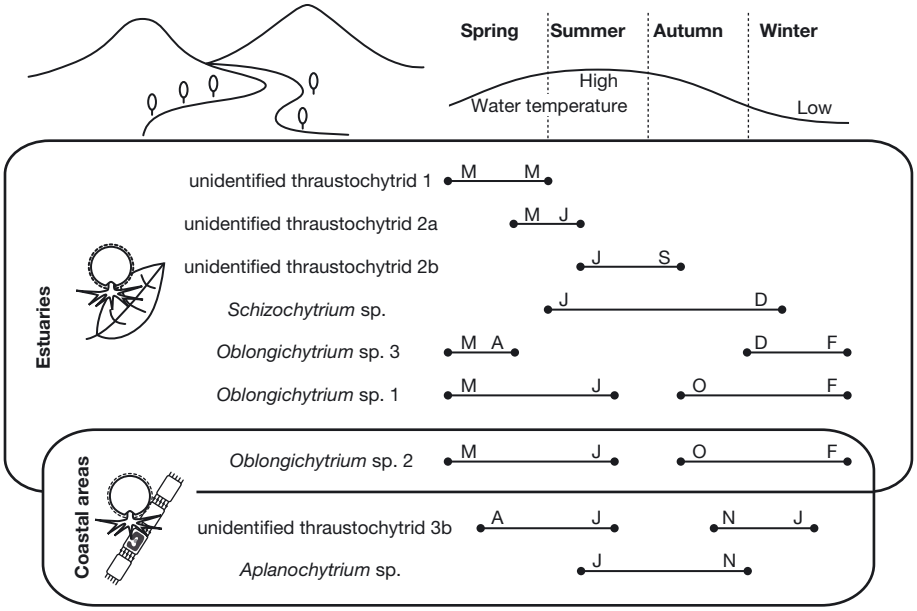

Fig. 8. Summarized schematic of thraustochytrid seasonal dynamics and distribution. Hypothetical habitat segregation between estuaries and the coastal areas as well as seasonal succession of the major phylogenetic groups of thraustochytrids are shown. Only *Oblongichytrium* sp. 2 (Ob2) appeared at both estuaries and the coastal areas. The nutrient sources of the major phylogenetic groups at estuaries and the coastal areas are expected as terrestrial organic matter (e.g. fallen leaves) and detrius derived from the sea (e.g. dead diatoms), respectively. Letters represent months of the year

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