Mycoscience (2007) 48:199–211
DOI 10.1007/s10267-006-0362-0

FULL PAPER

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Taxonomic rearrangement of the genus *Schizochytrium* sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for *Schizochytrium* and erection of *Aurantiochytrium* and *Oblongichytrium* gen. nov.

Received: November 25, 2006 / Accepted: December 13, 2006

Abstract The genus *Schizochytrium* sensu lato has been characterized by successive binary division of its vegetative cells. However, the molecular phylogeny strongly suggests that this genus is not a natural taxon, because the original and recorded strains that have been identified as *Schizochytrium* spp. separately form three well-supported monophyletic groups in the 18S rRNA gene tree. These three groups are clearly distinguishable by their combined morphological characteristics and the profiles of the polyunsaturated fatty acids and carotenoid pigments they contain, although these are hard to distinguish using only a single feature. Therefore, three different genera are proposed to accommodate these three groups, i.e., *Schizochytrium* sensu stricto, *Aurantiochytrium*, and *Oblongichytrium* gen. nov.

Key words Carotenoids · Heterokonts · Polyunsaturated fatty acids (PUFAs) · *Thraustochytrium* · Ultrastructure

Introduction

The class Labyrinthulomycetes (Levine and Corliss 1963; Olive 1975; Levine et al. 1980) is a member of the heterotrophic stramenopiles, which are characterized and distinguished from other fungoid organisms by the following characteristics: biflagellate zoospores possessing an anterior flagellum with mastigonemes (Amon and Perkins 1968; Kazama 1974; Perkins 1974), rhizoid-like ectoplasmic net elements produced by a unique organelle, the bothrosome (= sagenogen, sagenogenetosome) (Perkins 1972; Porter 1972, 1974; Moss 1980, 1985), and multilamellate cell walls composed of Golgi body-derived scales (Alderman et al. 1974; Perkins 1974; Porter 1974; Moss 1985). This class is composed of two families (Olive 1975; Porter 1989): Thraustochytriaceae Sparrow ex Cejp (Cejp 1959; see also Sparrow 1943, 1960), characterized by globose cells with ectoplasmic nets from a single bothrosome, and Labyrinthulaceae Cienk. (Cienkowski 1867; see also Haeckel 1868), having spindle-shaped cells gliding through channels of ectoplasmic nets produced from a number of bothrosomes.

Classification of six genera in the Thraustochytriaceae was based on the cell morphology of various stages during the life cycle. The type genus of this family is *Thraustochytrium* Sparrow emend. T.W. Johnson, which has been characterized by its globose sporangia with or without proliferous bodies and zoospore release caused by partial dissolution of the cell wall of the sporangia (Sparrow 1936; Johnson and Sparrow 1961). The other genera have subsequently been separated or newly erected on the basis of morphological features as follows: *Japonochytrium* Kobayasi et M. Ookubo is distinguished by the apophysis at its ectoplasmic nets (Kobayashi and Ookubo 1953); *Schizochytrium* S. Goldst. et Belsky emend. T. Booth et C.E. Mill. (i.e., *Schizochytrium* sensu lato) undergoes multiplication by binary cell division of vegetative cells (Goldstein and Belsky 1964); *Althornia* E.B.G. Jones et Alderman lacks the bothrosome and ectoplasmic nets (Jones and Alderman 1971); *Ulkenia* A. Gaertn. releases amoeboid cells before forming sporangia (Gaertner 1977); and *Aplanochytrium* Bahnweg et Sparrow emend. C.A. Leander et D. Porter is characterized by release of aplanospores and motility of vegetative cells (Bahnweg and Sparrow 1972; Leander and Porter 2000).

However, the genus-level classification has been problematical. The characteristic features overlap among some species of the genus *Thraustochytrium* (Booth and Miller 1968; Sparrow 1969; Alderman et al. 1974). It has been suggested that *Thraustochytrium* is a permissive and unarranged group, including morphologically diversified species (cf. Karling 1981). Also, amoeboid cells have been observed not only in *Ulkenia* but also in *Schizochytrium* sensu lato and in *Thraustochytrium* species under some culture conditions, and there is therefore some dispute as to the validity

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of the diagnostic characteristics for the genus classification (Raghukumar 1988a; Honda et al. 1998; Bongiorni et al. 2005). Moreover, Honda et al. (1999) clearly showed that none of these three genera formed a monophyletic group in the molecular phylogenetic tree of 18S rRNA genes, and it was therefore assumed by them that these genera were not natural taxa. Consequently, the currently used taxonomic criteria might need serious reconsideration, and the classification should be rearranged, if necessary.

One of the problematical genera, *Schizochytrium* sensu lato, is composed of the following five species: *S. aggregatum* S. Goldst. et Belsky, the type species of the genus, is characterized by the formation of large clusters of cells (Goldstein and Belsky 1964); *S. minutum* A. Gaertn. releases only two zoospores from one sporangium (Gaertner 1981); *S. octosporum* Raghuk. releases eight zoospores from one sporangium (Raghukumar 1988b); *S. mangrovei* Raghuk. does not form a zoosporangium, and each vegetative cell develops into zoospores (Raghukumar 1988a); and *S. limacinum* D. Honda et Yokochi is characterized by 16–32 clustered cells and amoeboid cells (Honda et al. 1998). However, in an 18S rRNA gene sequence tree, at least three species, *S. aggregatum, S. minutum*, and *S. limacinum*, were located in three independent lineages (Honda et al. 1999). This result means that the characteristic feature, that is, successive binary division of the vegetative cells, has been gained in several lineages. Conversely, it can be interpreted that the loss of vegetative cell division occurred in many thraustochytrid lineages classified as different genera, because the deeply branched groups, *Aplanochytrium* and *Labyrinthula* Cienk., also possess this feature (Leander and Porter 2001). It will therefore be necessary to assess how many phylogenetic lineages in the thraustochytrids with the ability of vegetative cell division are identified as *Schizochytrium* sensu lato.

The production of polyunsaturated fatty acids (PUFAs) by thraustochytrids, especially docosahexaenoic acid (DHA, C22:6, *n*-3), has recognized commercial use (Bowles et al. 1999; Lewis et al. 1999). Huang et al. (2003) showed that the PUFA profiles of strains in the monophyletic groups in the 18S rRNA gene tree were fundamentally similar and that each monophyletic group could be distinguished by its PUFA profile. The production of carotenoid pigments of the thraustochytrids has also received attention from the industry (Valadon 1976; Aki et al. 2003; Carmona et al. 2003; Yamaoka et al. 2004). These studies suggest that profiles of carotenoid pigments differ according to individual thraustochytrid strains.

In the present study, the following strategies were accomplished. As the first step, thraustochytrids were randomly collected from the field, and objective strains with binary-divided vegetative cells were selected. As the second step, the molecular phylogenetic positions of the strains were revealed by analyses of the sequences of their 18S rRNA gene. As the third step, selected strains were examined morphologically by light and electron microscopy, PUFAs, and carotenoid pigments. Finally, comparison of the entire set of data resulted in a general discussion on taxonomic rearrangement.

Materials and methods

Samples and cultivation

The examined strains and their sources are shown in Table 1. The original strains, shown by the prefix SEK, were isolated from seawater and mud collected at each site of Japan by pine-pollen baiting methods (Gaertner 1968). Cells were cultured at 25°C in medium-H (Honda et al. 1998).

Observation

Cells of each stage in the life cycle were cultured in both medium-H and seawater/pine-pollen cultures, which were proposed by Raghukumar (1988a) as a standard medium for identification. Light microscopic observations were by an BX60 (Olympus, Tokyo, Japan) fitted with a Nomarski interference differential contrast objective. These images were captured with an AxioCam HRc digital camera controlled by AxioVision software release 4.4 under the normal setting with no digital gain (Carl Zeiss, Hallbergmoos, Germany). For continuous observation, cells were transferred into a glass-bottomed culture dish (Meridian Instruments Far East, Tokyo, Japan) filled with fresh medium-H or seawater/pine pollen cultures. Cells started to develop after cultivation for 3–4 h and were observed with a CK40 (Olympus) or Axiovert 200 (Carl Zeiss) inverted microscope with a CCD camera CS900 (Olympus). Images were captured at one frame/2 s by Moto DV (Digital Origin, Palo Alto, CA, USA) on a Power Mac G4 (Apple Computer, Cupertino, CA, USA).

Vegetative cells were fixed in 2.5% glutaraldehyde and 0.1% sucrose in 0.1 M cacodylate buffer on ice for 5 h. After rinsing with the same buffer, materials were subsequently fixed in 1% OsO_4 for 12 h on ice, followed by washing in the buffer. The specimens were dehydrated in 30%, 50%, 70%, 90%, 95%, and 100% ethanol for 10 min each, followed by both ethanol-propylene oxide (PO) mixtures and pure PO twice for 10 min. Next, the specimens were embedded in pure epoxy resin (Quetol-651; Nisshin EM, Tokyo, Japan). The chamber was then placed in a 60°C oven where the resin was allowed to polymerize. Ultrathin sections were cut on a microtome (Leica Ultracut R; Leica, Wien, Austria) and were stained for 15 min with 4% uranyl acetate, followed by 5 min in Reynolds' lead citrate (Reynolds 1963). The sections were viewed under a JEM 2000FX (JEOL, Tokyo, Japan) transmission electron microscope.

Extraction of DNA

Cells were harvested after 3–4 days of culture. Total genomic DNA was extracted using the method suggested by the instructions in the Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and was purified using a phenol and chloroform/isoamyl alcohol protocol. To obtain the almost complete 18S rRNA gene, we used a polymerase chain reaction (PCR)

Table 1. The strains used in the molecular phylogenetic analyses with sequence accession numbers

Taxon	Strain[a]	Accession number	References
Genus *Aurantiochytrium*			
Aurantiochytrium limacinum	NIBH SR21^T (IFO 32693)	AB022107	Honda et al. 1999
Aurantiochytrium mangrovei	RCC893	DQ367049	–
Aurantiochytrium sp.	BURABQ 133	DQ023620	–
Aurantiochytrium sp.	SEK 217 (NBRC 103268)	AB290572	This study
Aurantiochytrium sp.	SEK 218 (NBRC 103269)	AB290573	This study
Aurantiochytrium sp.	SEK 209 (NBRC 102614)	AB290574	This study
Aurantiochytrium sp.	NIOS-4	AY705751	–
Aurantiochytrium sp.	FJN-10	AY773276	–
Aurantiochytrium sp.	NIBH N1-27	AB073308	Huang et al. 2003
Aurantiochytrium sp.	KH105	AB052555	Huang et al. 2003
	Uncultured thraustochytrid	DQ023610	–
Genus *Oblongichytrium*			
Oblongichytrium minutum	KMPB N-BA-77^T	AB022108	Honda et al. 1999
Oblongichytrium multirudimentale	KMPB N-BA-113	AB022111	Honda et al. 1999
Oblongichytrium sp.	SEK 347 (NBRC 102618)	AB290575	This study
Oblongichytrium sp.	7-5	AF257316	Mo et al. 2002
Oblongichytrium sp.	8-7	AF257317	Mo et al. 2002
Genus *Schizochytrium*			
Schizochytrium aggregatum	ATCC 28209	AB022106	Honda et al. 1999
Schizochytrium sp.	SEK 210 (NBRC 102615)	AB290576	This study
Schizochytrium sp.	SEK 345 (NBRC 102616)	AB290577	This study
Schizochytrium sp.	SEK 346 (NBRC 102617)	AB290578	This study
Schizochytrium sp.	KK17-3	AB052556	Huang et al. 2003
Schizochytrium sp.	NIBH N4-103	AB073309	Huang et al. 2003
Other strains			
Aplanochytrium kerguelense	KMPB N-BA-107	AB022103	Honda et al. 1999
Aplanochytrium minutum	n/a	L27634	Leipe et al. 1994
Aplanochytrium stocchinoi		AJ519935	Moro et al. 2003
Aplanochytrium sp.	PR1-1	AF348516	Leander et al. 2004
Aplanochytrium sp.	PR15-1	AF348518	Leander et al. 2004
Aplanochytrium sp.	SC1-1	AF348520	Leander et al. 2004
Japonochytrium sp.	ATCC 28207	AB022104	Honda et al. 1999
Labyrinthula sp.	AN-1565 (NBRC 33215)	AB022105	Honda et al. 1999
Labyrinthula sp.	L59	AB095092	Kumon et al. 2003
Labyrinthula sp.	L72	AB220158	Kumon et al. 2006
Labyrinthula sp.	f Sap 16-1	AF348522	–
Thraustochytrium agggregatum	KMPB N-BA-110^T	AB022109	Honda et al. 1999
Thraustochytrium aureum	ATCC 34304^T	AB022110	Honda et al. 1999
Thraustochytrium kinnei	KMPB 1694d	L34668	Cavalier-Smith et al. 1994
Thraustochytrium pachydermum	KMPB N-BA-114	AB022113	Honda et al. 1999
Thraustochytrium striatum	ATCC 24473^T	AB022112	Honda et al. 1999
Ulkenia profunda	#29 (Raghukumar)	AB022114	Honda et al. 1999
Ulkenia profunda	KMPB N 3077^T	L34054	Cavalier-Smith et al. 1994
Ulkenia radiata	#16 (Raghukumar)	AB022115	Honda et al. 1999
Ulkenia visurgensis	ATCC 28208^T	AB022116	Honda et al. 1999
	NIBH H1-14	AB073305	Huang et al. 2003
	BS1	AF257314	Mo et al. 2002
	BS2	AF257315	Mo et al. 2002
	NIOS-6 (A05-2)	AY705756	–
	C9G	AF474172	Anderson et al. 2003
	Fug1	AY870336	–
	QPX	AY052644	Stokes et al. 2002
Outgroup			
Bacillaria paxillifer	n/a	M87325	–
Ochromonas danica	n/a	M32704, J02950	–

[a]Abbreviations of the culture collections; ATCC, American Type Culture Collection; IFO, Institute for Fermentation, Osaka (Japan); KMPB, Kulturensammlung Mariner Pilze Bremerhaven, Alfred-Wegner-Institut für Polar und Meeresforschung (Germany); 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)
T, ex-type strain; n/a, not available

protocol with a thermostable DNA polymerase (Ex Taq DNA Polymerase; Takara, Ohtsu, Japan) and amplification primers SR1 and SR12 (Nakayama et al. 1996). We cut out the amplified band of about 1800 bases in 0.5% TBE-agarose gel and then extracted it using Gene Clean Turbo (Qbiogene, Baton Rouge, LA, USA). Nucleotide sequences were determined by the terminator method with 12 primers, 18S01–18S12, the same as those used by Nakayama et al. (1996), using an ABI PRISM 310NT Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular phylogenetic analyses

Determined sequences were added to the aligned sequence data set (Honda et al. 1999) through a profile alignment process using Clustal W version 1.74 (Thompson et al. 1994) and optically aligned. Other strain sequences were downloaded from the GenBank constructed by the National Center of Biotechnology Information (NCBI) (accession numbers are shown in Table 1). The phylogenetic trees were generated using the maximum-likelihood (ML) method (Felsenstein 1981) and the minimum-evolution (ME) method (Rzhetsky and Nei 1992, 1993). Both analyses were performed using PAUP* version 4.0b10 (Swofford 2003). In ML method analysis, the transition/transversion (ti/tv), the ratio of the HKY85 model, was estimated by maximizing the likelihood value for neighbor-joining (NJ) topology. The best tree was found by the heuristic search method. The bootstrap values were obtained from 100 resamplings. In the distance method analysis, the total distance of a tree was calculated using the ML parameter based on the ti/tv ratio that was estimated from NJ topology. The best tree was found by heuristic search. The bootstrap values were obtained from 1000 resamplings.

PUFA composition analyses

Each strain of our own isolates was previously cultured in medium-H for 2–3 days at 25°C. The cell suspension was spread on the agar-dGPY medium containing 2 g glucose, 1 g poly-peptone, 0.5 g yeast extract, and 15 g agar per liter of a half-salt concentration of artificial seawater and then incubated for 7–10 days at 25°C. The cell growths were gathered by scraping a small spatula across the surface and dried at 105°C for 3 h. The fatty acids were directly transmethylated from dried cells with 10% methanolic HCl and methylene chloride (Shimizu et al. 1988). The methyl-esterified fatty acids were extracted with *n*-hexane, and the resultant extracts were applied to a gas/liquid chromatograph (GC-17A; Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (25 m × 0.25 mm i.d.; GL Science, Tokyo, Japan); a temperature program rising from 180° to 220°C in increments of 4°C/min was used. Peaks were identified using authentic standards of the following fatty acid methyl esters according to Nakahara et al. (1996) and Yokochi et al. (1998): arachidonic acid (AA, 20:4 *n*-6), eicosapentaenoic acid (EPA, 20:5 *n*-3), docosapentaenoic acid (DPA, 22:5 *n*-3 and *n*-6), and docosahexaenoic acid (DHA, 22:6 *n*-3). Each PUFA production was calculated from each peak area of the chromatogram relative to the peak area of an internal standard.

Carotenoid composition analyses

Each strain was inoculated into a 500-ml conical flask containing 200 ml medium-H and then incubated with reciprocal shaking at 120 rpm for 2–3 days at 25°C. The growth cells were harvested by centrifugation at 2500 rpm for 15 min. The pellets were freeze-dried on liquid nitrogen. The endopigments were extracted by suspending the cells in 1 ml acetone and crushing in a mortar, after which the pigment solutions were filtrated through an EkicrodiscR 3 CR filter (0.45 μm × 3 mm; Gelman Science, Tokyo, Japan). The resulting solutions were loaded into a HPLC equipped with a UV-VIS detector (SPD6AV; Shimazu, Kyoto, Japan), which was set at 450 nm. Astaxanthin, phoenicoxanthin, canthaxanthin, echinenone, and β-carotene were identified using the peaks of the standard sample and the data of previous studies (Carmona et al. 2003).

Taxonomy

According to comparison of all the data sets shown later, the following three genera, including two new genera, are recharacterized or established in the Thraustochytriaceae, Labyrinthulales, and Labyrinthulomycetes.

Schizochytrium S. Goldst. et Belsky emend. R. Yokoyama et D. Honda

Thallus thin-walled, globose, pale yellow. Cells possessing only β-carotene as carotenoid pigment and possessing ca. 20% arachidonic acid as the fatty acids. Colonies large by continuous binary cell divisions. Ectoplasmic nets well developed. Zoospores biflagellate heterokont; reniform to ovoid. 18S rRNA gene sequence distinct. Resting spores not observed.

Type species: *Schizochytrium aggregatum* S. Goldst. et Belsky

Oblongichytrium R. Yokoyama et D. Honda, gen. nov.

Thallus pariete tenui, globosus, lutescens. Cellulae cum pigmentis dictis "Canthaxanthin," "β-carotene," et cum acido pingui comparate abundanto "*n*-3 docosapentaenoic acid" dicto et pauco "*n*-6 docosapentaenoic acid" dicto. Coloniae comparate evolventes per fissionem binariam cellulae diadosae. Reticulum ectoplasmaticum conspicue evolvens. Zoosporae ellipticae vel oblongae, biflagellatae, heterokontae. 18S rRNA genetica distincta. Sporae perdurantes non observatae.

Thallus thin-walled, globose, pale yellow. Cells possessing canthaxanthin, β-carotene, and possessing comparatively abundant *n*-3 docosapentaenoic acid and little *n*-6 docosapentaenoic acid as the fatty acids. Colonies large by continuous binary cell divisions. Ectoplasmic nets well developed. Zoospores biflagellate heterokont; elliptical to oblong. 18S rRNA gene sequence distinct. Resting spores not observed.

Etymology: *oblongus* = oblong, *chytrion* = pot, referring the oblong zoospores.

Type species: ***Oblongichytrium minutum*** (A. Gaertn.) R. Yokoyama et D. Honda, comb. nov.

Basionym: *Schizochytrium minutum* A. Gaertn., Veröff Inst Meeresforsch Bremerh 19:68, 1981.

Oblongichytrium multirudimentale (S. Goldst.) R. Yokoyama et D. Honda, comb. nov.

Basionym: *Thraustochytrium multirudimentale* S. Goldst., Am. J. Bot. 50:273, 1963.

Other species that we believe belong to *Oblongichytrium*: ***Oblongichytrium octosporum*** (Raghuk.) R. Yokoyama et D. Honda, comb. nov.

Basionym: *Schizochytrium octosporum* Raghuk, Trans. Br. Mycol. Soc. 90:273, 1988.

Aurantiochytrium R. Yokoyama et D. Honda, gen. nov.

Thallus pariete tenui, globosus, aurantiacus. Cellulae cum pigmentis dictis "astaxanthin," "phoenicoxanthin," "canthaxanthin," "β-carotene," et cum acido pingui pauco "arachidonic acid" dicto, et cum acido pingui dominanto "docosahexaenoic acid" dicto. Coloniae comparate parvae per fissionem binariam cellulae diadosae. Reticulum ectoplasmaticum non evolvens comparate. Zoosporae reniformes vel ovoideae, biflagellatae, heterokontae. 18S rRNA genetica distincta. Sporae perdurantes non observatae.

Thallus thin-walled, globose, orange. Cells possessing astaxanthin phoenicoxanthin, canthaxanthin, and β-carotene and possessing minor arachidonic acid and dominant docosahexaenoic acid as the fatty acids. Colonies small by continuous binary cell divisions. Ectoplasmic nets not well developed. Zoospores biflagellate heterokont; reniform to ovoid. 18S rRNA gene sequence distinct. Resting spores not observed.

Etymology: *aurantius* = orange in color, *chytrion* = pot, referring the color of the thallus.

Type species: ***Aurantiochytrium limacinum*** (D. Honda et Yokochi) R. Yokoyama et D. Honda, comb. nov.

Basionym: *Schizochytrium limacinum* D. Honda et Yokochi in Honda et al. Mycol. Res. 102:441, 1998.

Aurantiochytrium mangrovei (Raghuk.) R. Yokoyama et D. Honda, comb. nov.

Basionym: *Schizochytrium mangrovei* Raghuk, Trans. Br. Mycol. Soc. 90:627, 1998.

Results

Light microscopic morphology

Diads and tetrads were observed in the life cycles of all studied isolates (Fig. 1). A settled zoospore transformed into some sporangia through binary cell division. This type of multiplication had previously been characterized as belonging to the genus *Schizochytrium* sensu lato (Goldstein and Belsky 1964).

Molecular phylogeny of 18S rRNA gene sequences

Almost the entire length of the 18S rRNA gene sequences was determined and deposited in the DNA Data Bank of Japan (DDBJ) (see Table 1), and the alignment was deposited in TreeBASE (matrix accession number: M3311 at http://treebase.org/). We used 1235 sites for comparison among all operational taxonomic units without the gaps and ambiguous sites. The strains of *Schizochytrium* sensu lato separated into three independent monophyletic groups (Fig. 2). The clade of *Aurantiochytrium* diverged with a long branch from the internal node, including strains of *A. limacinum* NIBC SR21, *Aurantiochytrium* sp. SEK 209, *Aurantiochytrium* sp. SEK 217, and *Aurantiochytrium* sp. SEK 218, and was supported by 100% bootstrap values in both ML and ME analyses. The clade of *Schizochytrium* sensu stricto, including strains of *S. aggregatum* ATCC 28209, *Schizochytrium* sp. SEK 210, *Schizochytrium* sp. SEK 345, and *Schizochytrium* sp. SEK 346, was also supported by 100% bootstrap values in both analyses. The clade of *Oblongichytrium*, including strains of *O. minutum*, *O. multirudimentale*, and *Oblongichytrium* sp. SEK 347, was supported by 90% and 100% bootstrap values in ML and ME analyses, respectively. The members of the genus *Labyrinthula* formed a monophyletic group supported by 100% bootstrap values in both analyses. Members of the genus *Aplanochytrium*

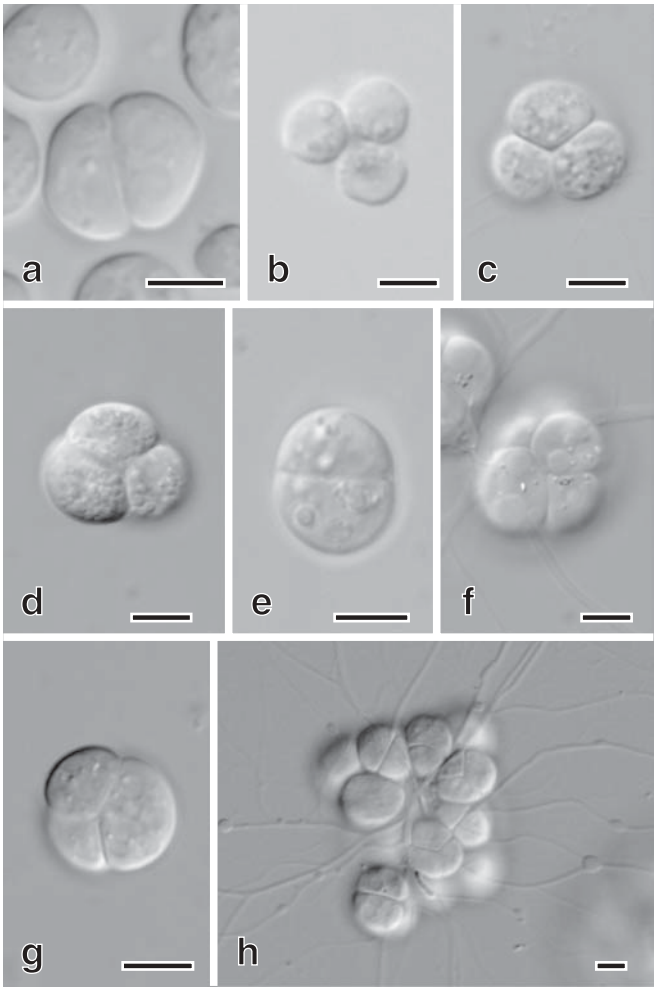

Fig. 1. Light micrographs showing cluster of cells via binary cell division on the medium-H. **a** *Aurantiochytrium limacinum*; **b** *Aurantiochytrium* sp. SEK 209; **c** *Aurantiochytrium* sp. SEK 217; **d** *Schizochytrium aggregatum*; **e** *Schizochytrium* sp. SEK 210; **f** *Schizochytrium* sp. SEK 326; **g** *Oblongichytrium* sp. SEK 347; **h** *Oblongichytrium* sp. SEK 347. *Bars* 5 μm

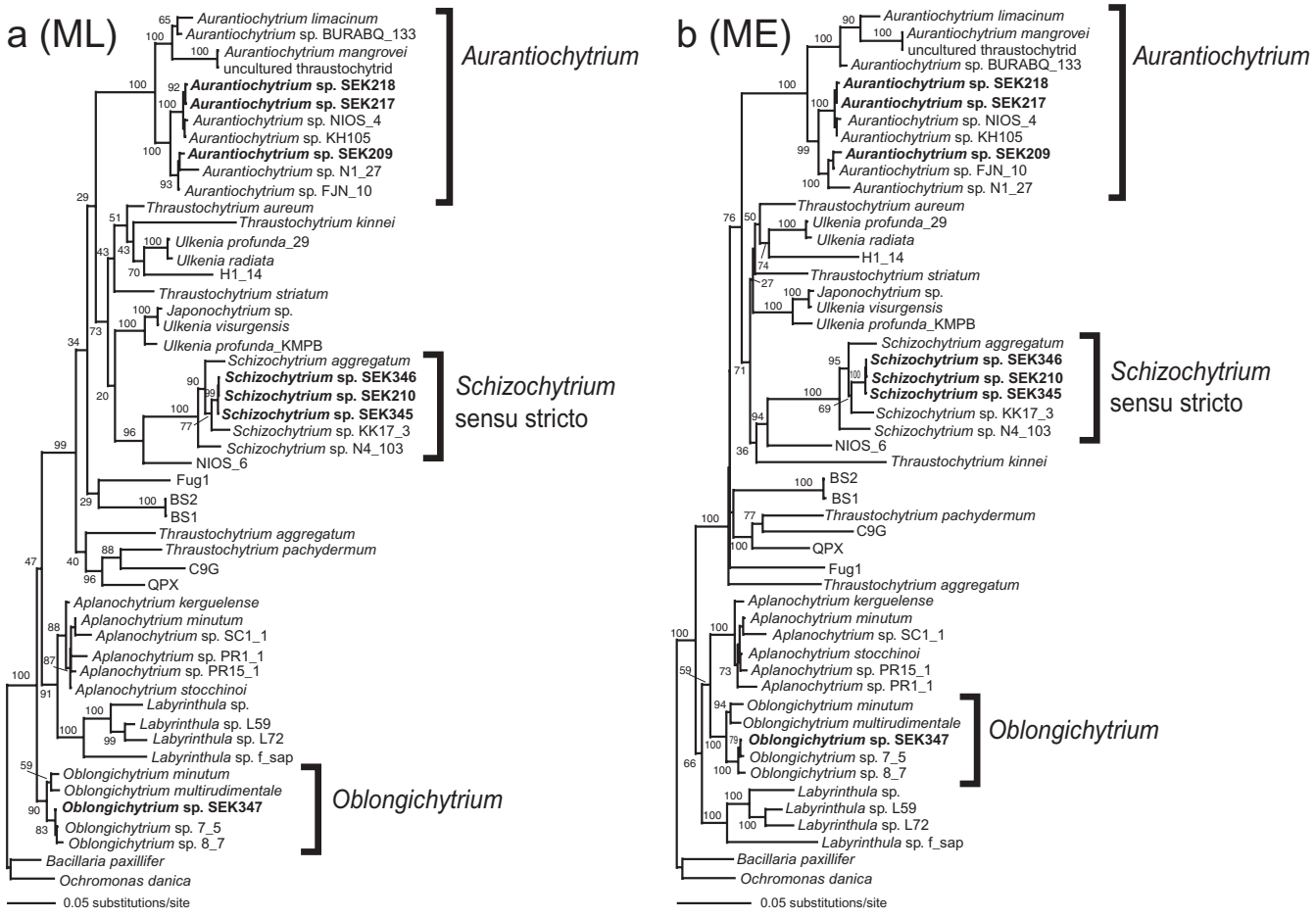

Fig. 2. Phylogenetic trees of the Labyrinthulomycetes using 18S rRNA gene with *Bacillaria paxillifer* and *Ochromonas danica* as outgroup (51 operational taxonomic units, 1235 nucleotide sites). **a** The best maximum-likelihood (ML) tree (log-likelihood = –133766.32608) on the HKY85 model (ti/tv ratio = 1.071671). **b** The best minimum-evolution (ME) tree constructed from distances estimated by the ML method with HKY85 model (ti/tv ratio = 1.071671). The *numbers at each internal branch* show the bootstrap values (%) for the nodes calculated by 100 and 1000 replicates on ML and ME analyses, respectively. *Bold characters* indicate our original isolates, whose sequences were determined in this study

also formed a monophyletic group and made a sister relationship with the clade of *Labyrinthula*. The clade of *Oblongichytrium* spp. branched out deeply from the basal node near *Labyrinthula* and *Aplanochytrium* clades.

Morphological characteristics

Morphological features are summarized in Table 2. Colonies of the vegetative cells of *Aurantiochytrium* (*A. limacinum* NIBH SR21, *Aurantiochytrium* sp. SEK 209, *Aurantiochytrium* sp. SEK 217) are dispersed and do not form large colonies. Their ectoplasmic nets are undeveloped in both nutrient-enriched media and seawater/pine pollen culture (Fig. 3a–c). Vegetative cells do not form large colonies and are mostly dispersed as single cells in these liquid media, but occasionally old vegetative cells and mature sporangia do not separate and form clusters with ectoplasmic nets. The zoospores are ovoid in shape (*A. limacinum* NIBH SR21, 5.0–7.0 × 6.0–8.5 µm; *Aurantiochytrium* sp. SEK 209, 2.5–3.0 × 4.0–6.0 µm; *Aurantiochytrium* sp. SEK 217, 2.0–3.0 × 4.0–5.0 µm in Fig. 3d–f). Under ultrastructural observations, there were electron-dense bodies (EDB) around the nuclei of all strains (Fig. 3g–i). Paranuclear bodies (PA) were also observed (Fig. 3j–l). A PA composed of a complex of inflated smooth endoplasmic reticulum cisternae has been previously demonstrated in other genera, namely, *Aplanochytrium yorkense* (F.O. Perkins) C.A. Leander et D. Porter (Perkins 1973), *Ulkenia visurgensis* Ulken emend. A. Gaertner (Moss 1980, 1985), and *Ulkenia amoeboidea* Bahnweg et Sparrow (Raghukumar 1982a,b). A typical vegetative cell has a nucleus, a vacuole, a Golgi body, and mitochondria with tubular cristae (Fig. 3l).

The vegetative cells of *Schizochytrium* sensu stricto (*S. aggregatum* ATCC 28209, *Schizochytrium* sp. SEK 210, *Schizochytrium* sp. SEK 346) form large colonies with well-developed ectoplasmic nets in both seawater/pine pollen culture and enriched media (Fig. 4a–c). The zoospores are ovoid in shape, similar to those of the *Schizochytrium* sensu lato strains (*S. aggregatum* ATCC 28209, 2.5–3.0 × 4.0–7.5 µm; *Schizochytrium* sp. SEK 210, 2.5–3.0 ×

Table 2. Morphological features of *Aurantiochytrium*, *Oblongichytrium*, and *Schizochytrium* sensu stricto strains

	Genus Aurantiochytrium			*Genus Schizochytrium*			*Genus Oblongichytrium*
	A. limacinum	*A.* sp. SEK209	*A.* sp. SEK217	*S. aggregatum*	*S.* sp. SEK210	*S.* sp. SEK346	*O.* sp. SEK347
Colony	Small	Small	Small	Large	Large	Large	Large
Ectoplasmic nets	Slightly developed	Undeveloped	Undeveloped	Well developed	Well developed	Well developed	Well developed
Size of a zoospore (μm)	5–7 × 6–8.5	2.5–3 × 4–6	2–3 × 4–5	2.5–3 × 4–7.5	2.5–3 × 4–5	3.5–4 × 4.5–6.5	3.5–5 × 7–8.5
Shape of zoospore	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Narrow, elliptical
Number of releasing zoospores	8–32	8–32	8–16	16–64	16–64	16–64	4–67
Cell wall remains after zoospore release	–	–	+	+	+	+	+
Amoeboid cells	+	–	–	–	–	–	–
Electron-dense body	ud	+	+	+	+	+	+
Para-nuclear body	ud	+	+	+	+	+	+

ud, undetermined

4.0–5.0 μm; *Schizochytrium* sp. SEK 346, 3.5–4.0 × 4.5–6.5 μm in Fig. 4d–f). Under ultrastructural examination, EDB (Fig. 4g–i) and PA (Fig. 4j–l) were also observed. A typical vegetative cell has a nucleus, a vacuole, a Golgi body, kinetosome, and mitochondria with tubular cristae (Fig. 4m).

The vegetative cells of *Oblongichytrium* sp. SEK 347 form large colonies with more well-developed ectoplasmic nets compared to those of *Aurantiochytrium* strains in both seawater/pine pollen culture and enriched media (Fig. 5a). The zoospores are a narrow elliptical shape (3.0 × 7.0 μm; Fig. 5b) and are only released when their sporangia are transferred from the agar cultivation to liquid media. Under ultrastructural observations, EDB (Fig. 5c) and PA (Fig. 5d) were observed in vegetative cells. A typical vegetative cell has a nucleus, mitochondria with tubular cristae, vacuoles, and a Golgi body (Fig. 5d).

PUFA and carotenoid composition

The PUFA profiles of arachidonic acid (AA, C20:4, *n*-6), eicosapentaenoic acid (EPA, C20:5, *n*-6), docosapentaenoic acid (DPA, C22:5, *n*-3 and *n*-6), and docosahexanoic acid (DHA, C22:6, *n*-3) were compared in three genera and other strains including isolates that have reported in Huang et al. (2003). Figure 6 shows that all examined strains of *Aurantiochytrium* contained less than 5% AA and about 80% DHA, whereas all the *Schizochytrium* sensu stricto strains contained about 20% AA. *Oblongichytrium* sp. SEK 347 accumulated about 20% of *n*-3 DPA, which is unique among the thraustochytrid strains examined.

The ketocarotenoids profiles of β-carotene, echinenone, canthaxanthin, phoenicoxanthin, and astaxanthin were compared (Table 3). These pigments are intermediates of the synthetic pathway of astaxanthin from β-carotene. The strains of *Aurantiochytrium* possess all the aforementioned pigments, resulting in orange colonies. Because the strains of *Schizochytrium* sensu stricto only contain β-carotene, the colonies are light yellow. *Oblongichytrium* sp. SEK 347 accumulates canthaxanthin and β-carotene, but not astaxanthin and phoenicoxanthin. The other strains showed different carotenoid profiles; that is, *Japonochytrium* sp. possesses only astaxanthin and β-carotene, whereas *Thraustochytrium striatum* Joa. Schneid. contains all five pigments.

Discussion

The genus *Schizochytrium* sensu lato was characterized by the successive binary divisions of the vegetative cells (Goldstein and Belsky 1964). This characteristic is easily recognized by continuous observation and colony morphology. This characteristic feature also is observed without regard to growth media, whereas the other morphological features under light microscopy are influenced by media (Booth and Miller 1968; Goldstein 1973; Kazama et al. 1975). However,

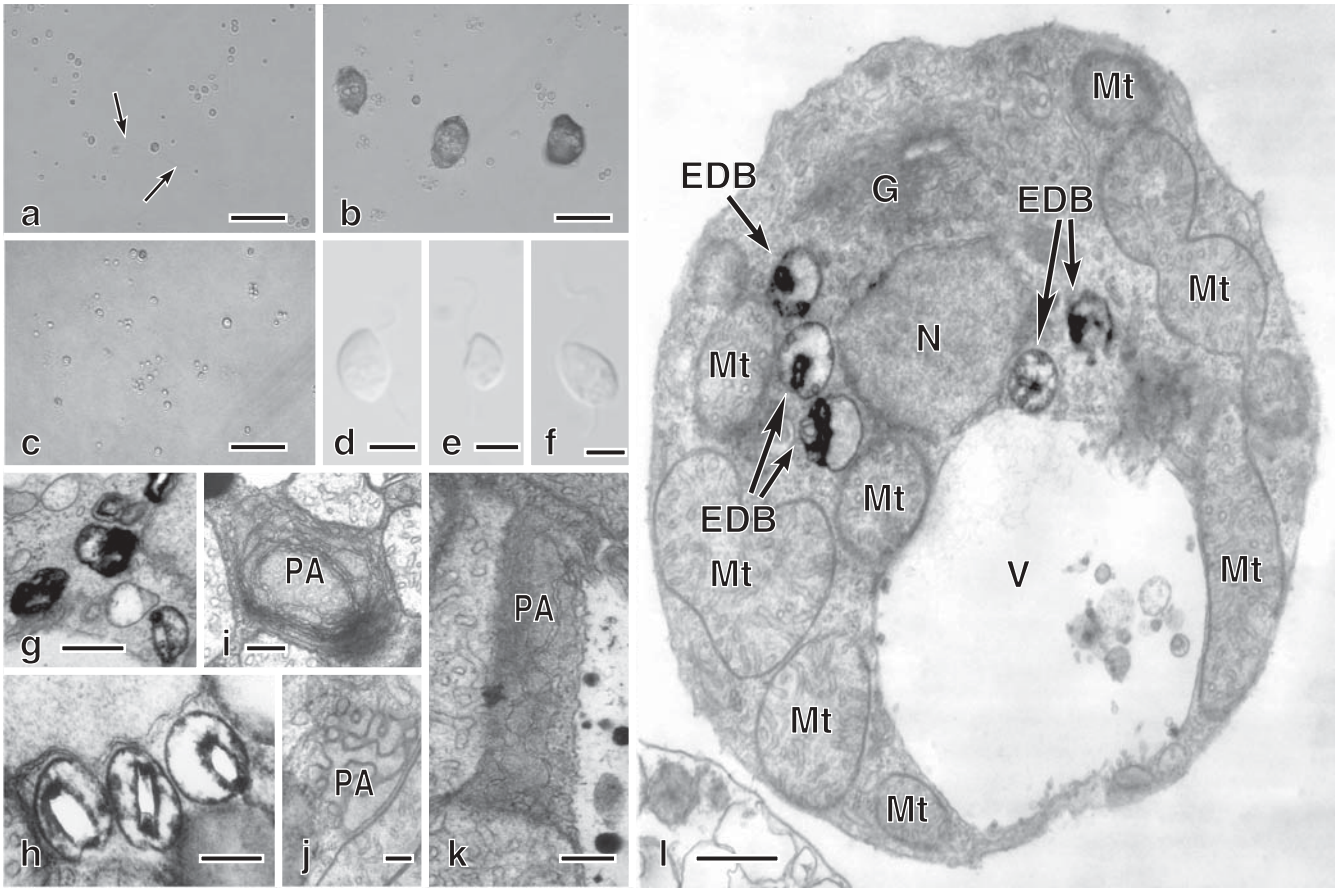

Fig. 3. Micrographs of the genus *Aurantiochytrium* spp. **a–c** Light micrographs showing cells rarely with ectoplasmic net elements (*arrows*) spread in seawater/pine pollen cultures. **d–f** Light micrographs of the ovoid-shaped zoospore. **g–l** Transmission electron micrographs of the electron-dense body and the para-nuclear body (PA) observed in vegetative cells of each strain. (**a, d** *Aurantiochytrium limacinum* NIBC SR21; **b, e, g, j, l** *Aurantiochytrium* sp. SEK 209; **c, f, h, i, k** *Aurantiochytrium* sp. SEK 217). *EDB*, electron-dense body; *G*, Golgi body; *Mt*, mitochondria; *N*, nucleus; *V*, vacuole. *Bars* **a–c** 30 µm; **d–f** 5 µm; **g–k** 0.2 µm; **l** 0.5 µm

Table 3. Profiles of carotenoid pigments of *Aurantiochytrium*, *Oblongichytrium*, and *Schizochytrium* sensu stricto strains

Strains	Astaxanthin	Phoenicoxanthin	Canthaxanthin	Echinenone	β-Carotene
Genus *Aurantiochytrium*					
Aurantiochytrium limacinum NIBH SR21	+	+	+	+	+
Aurantiochytrium sp. SEK217	+	+	+	+	+
Aurantiochytrium sp. SEK218	+	+	+	+	+
Aurantiochytrium sp. SEK209	+	+	+	+	+
Aurantiochytrium sp. N1–27	+	+	+	+	+
Aurantiochytrium sp. KH105	+	+	+	+	+
Genus *Schizochytrium*					
Schizochytrium aggregatum ATCC 28209	–	–	–	–	+
Schizochytrium sp. SEK210	–	–	–	–	+
Schizochytrium sp. SEK345	–	–	–	–	+
Schizochytrium sp. SEK346	–	–	–	–	+
Genus *Oblongichytrium*					
Oblongichytrium sp. SEK347	–	–	+	+	+
Other strains					
Japonochytrium sp. ATCC 28207	+	–	–	–	+
Thraustochytrium striatum ATCC 24473	+	+	+	+	+

several taxonomic problems for this genus and species have been pointed out. Booth and Miller (1969) reported that the strains had several features different from the original description, but they concluded that both strains should be identified as the same species, that is, *Schizochytrium aggregatum*, and emended the definition of the genus *Schizochytrium* sensu lato. Alderman et al. (1974) noted that *S. aggregatum* "seems most probably to be a complex of related organisms which will require considerable effort to separate." Honda et al. (1999) clearly showed that the

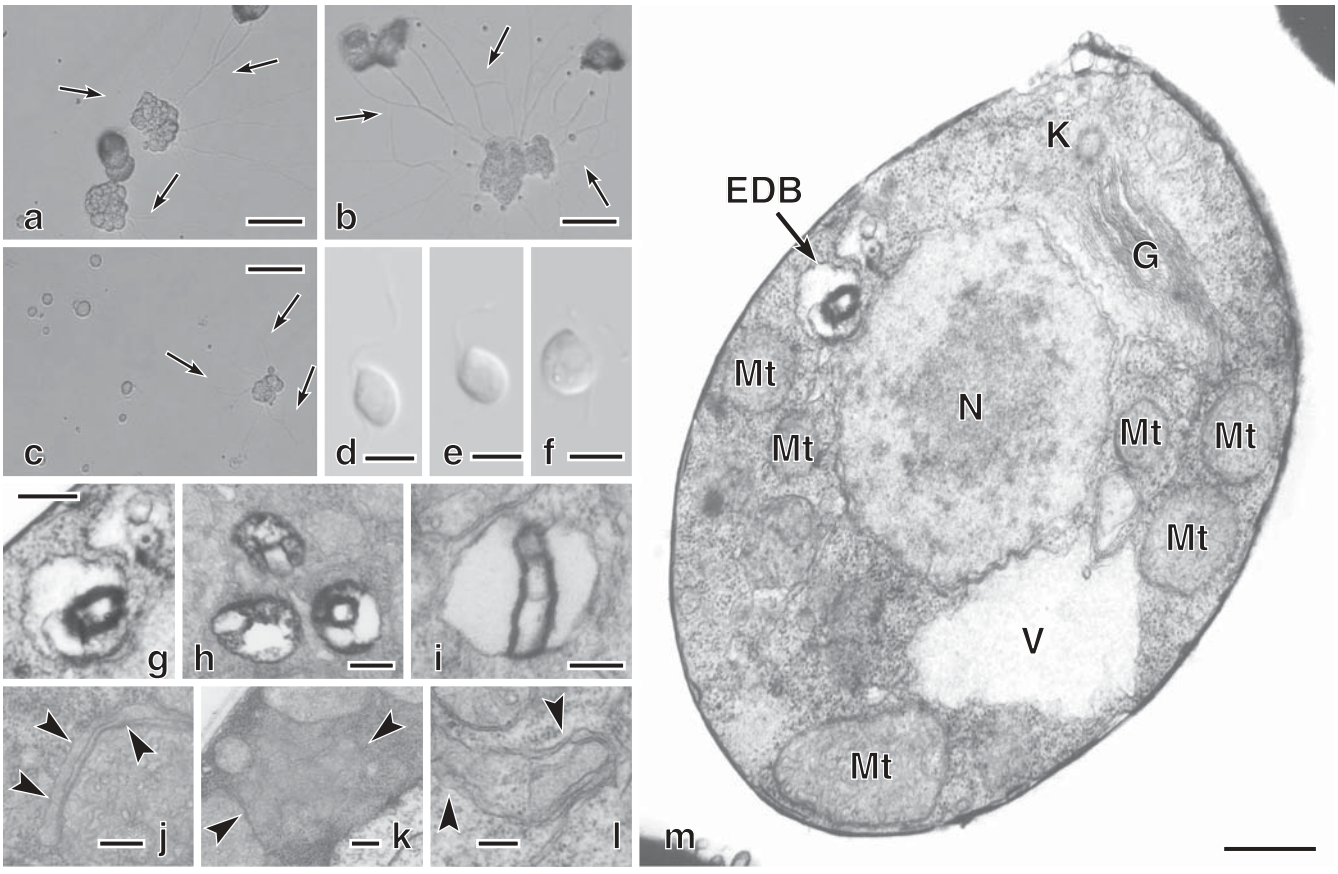

Fig. 4. Micrographs of the genus *Schizochytrium* sensu stricto. **a–c** Light micrographs showing a large cluster of cells with well-developed ectoplasmic nets (*arrows*) in seawater/pine pollen cultures. **d–f** Light micrographs of the ovoid-shaped zoospore. **g–m** Transmission electron micrographs of the electron-dense body and the para-nuclear body (*arrowheads*) observed in vegetative cells of each strain. (**a, d, g, j, m** *Schizochytrium aggregatum* ATCC 28209; **b, e, h**, **k** *Schizochytrium* sp. SEK 210; **c, f, i, l** *Schizochytrium* sp. SEK 346). *EDB*, electron-dense body; *G*, Golgi body; *K*, kinetosome; *Mt*, mitochondria; *N*, nucleus; *V*, vacuole. *Bars* **a–c** 30 µm; **d–f** 5 µm; **g–l** 0.2 µm; **m** 0.5 µm

strains identified as species of *Schizochytrium* sensu lato appeared at different lineages in the 18S rRNA gene phylogenetic tree; therefore, this molecular phylogeny strongly suggested that the currently applied taxonomic identification of the genus *Schizochytrium* sensu lato might require reconsideration.

The first report of the polyphyly of *Schizochytrium* sensu lato already showed that members of this genus appeared in three distinct lineages (Honda et al. 1999). However, each of the lineages was composed of only a single strain, so that it was difficult to judge the taxonomic treatment because of the small data set. In the present study, the molecular phylogenetic trees show that each of the lineages is composed of 5 to 11 strains with reasonable genetic variation, which is indicated by the genetic distances (i.e., branch lengths) among the strains in each group. The well-supported monophyletic genera *Aplanochytrium* and *Labyrinthula* are composed of 6 and 4 strains in our trees whose genetic variation corresponds to those of three phylogenetic groups of *Schizochytrium* sensu lato. It is reasonable to treat each phylogenetic group not as the lower (i.e., species) or higher (i.e., family) taxonomic rank but as genus rank. Moreover, we revealed that these three groups could clearly be distinguished by combining phenotypic characteristics obtained by light and electron microscopy, and in profiles of the PUFAs and carotenoid pigments, although it is impossible to distinguish each group using only a single feature. Therefore, these three groups were considered to be three different genera; that is, the genus *Schizochytrium* sensu stricto, after emendation of the definition, and the two new genera, *Aurantiochytrium* and *Oblongichytrium*.

The new genus *Aurantiochytrium* is erected for *Aurantiochytrium limacinum*, *A. mangrovei*, and nine strains of unidentified species. The characteristic morphological features of this genus are that the cells in the growth phase tend not to form large colonies regardless of media and not to develop ectoplasmic net elements, and these features can be recognized as a critical difference from *Schizochytrium* sensu stricto and *Oblongichytrium*. Under cultivation with liquid nutrient media, small colonies of *Aurantiochytrium* look like fine grains on the bottom of the flask, whereas the two other genera form large colonies that develop into small balls in the media. The PUFA composition shows that an arachidonic acid level less than 5% was not seen in the other two genera (see Fig. 6). Moreover, the accumulation of astaxanthin is a unique feature among the three groups.

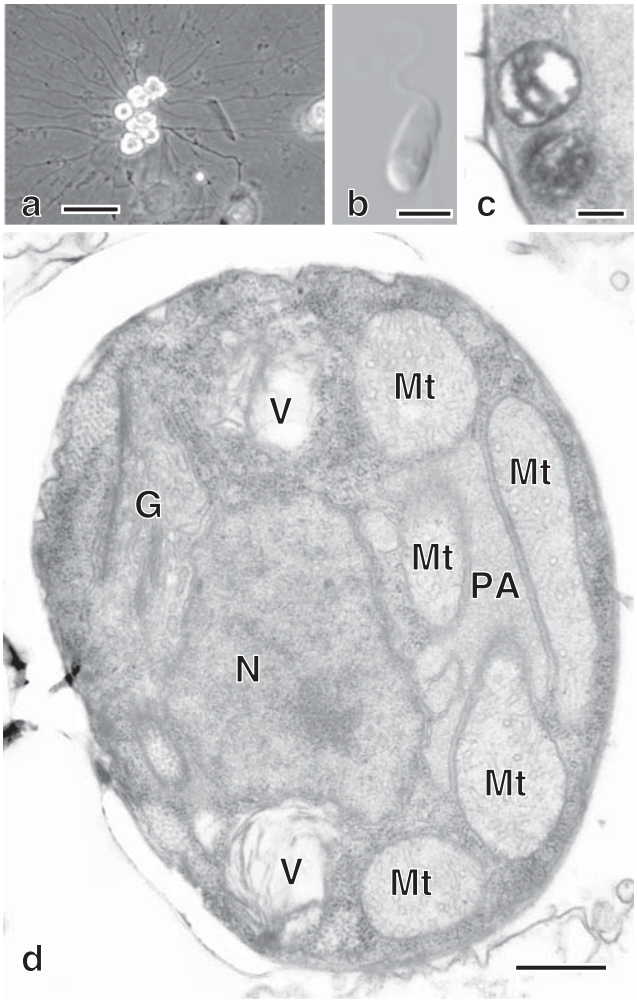

Fig. 5. Micrographs of *Oblongichytrium* sp. SEK 347. **a** Light micrograph shows a large cluster of cells with well-developed ectoplasmic nets in seawater/pine pollen culture. **b** Light micrograph of narrow elliptical zoospore. **c** Transmission electron micrograph of the electron-dense body. **d** Thin section of a young vegetative cell with para-nuclear body. *G*, Golgi body; *Mt*, mitochondria; *N*, nucleus; *PA*, para-nuclear body; *V*, vacuole. *Bars* **a** 30 µm; **b** 5 µm; **c** 0.2 µm; **d** 0.5 µm

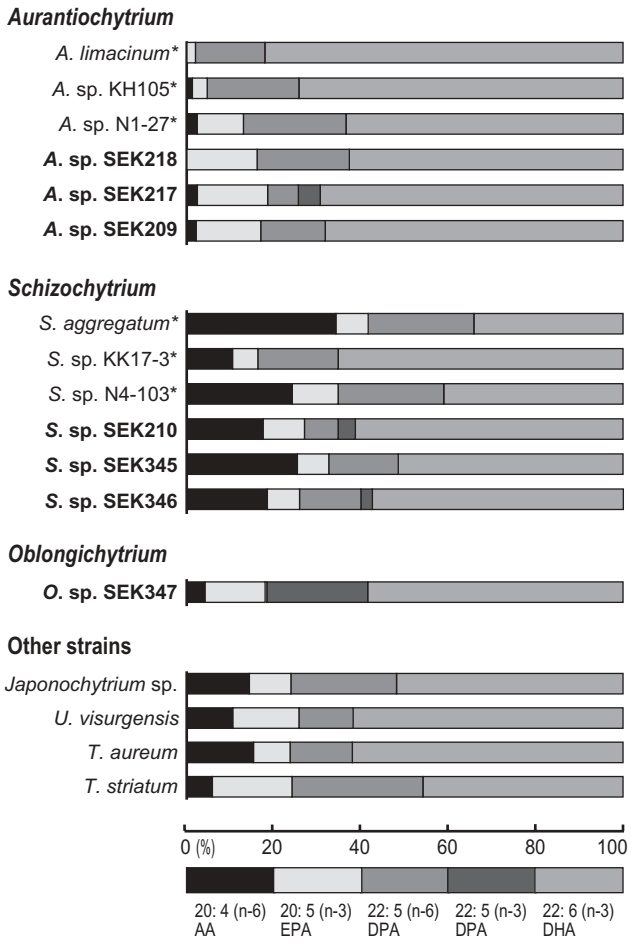

Fig. 6. Profiles of polyunsaturated fatty acids of thraustochytrid strains. *Bold characters* indicate the original isolates whose profiles were analyzed in this study. The genus *Aurantiochytrium* strains have a content of less than 5% of arachidonic acid and about 80% docosahexaenoic acid; the genus *Schizochytrium* sensu stricto strains have a content of about 20% arachidonic acids; and the genus *Oblongichytrium* sp. SEK 347 has a content of about 20% *n*-3 docosapentaenoic acid. Profiles of other strains are shown: *Japonochytrium* sp. ATCC 28207, *Ulkenia visurgensis* ATCC 28208, *Thraustochytrium aureum* ATCC 34304, *Thraustochytrium striatum* ATCC 24473. The data of the strains indicated by *asterisks* were reported by Huang et al. (2003)

Aurantiochytrium limacinum and *A. mangrovei* share the characteristic feature of releasing amoeboid cells in nutrient media. However, our original strains (i.e., *Aurantiochytrium* sp. SEK-217, -218, and -209) never released amoeboid cells (data not shown), so this feature is not a distinguishing characteristic for this genus. It is noted that *A. limacinum* NIBC SR21 is the ex-type strain, but *A. mangrovei* RCC893 is not, although it was collected from the mangrove area in Hong Kong (K.M. Tsui, personal communication). Unfortunately, the ex-type strain of *A. mangrovei* has been lost (S. Raghukumar, personal communication), so the phylogenetic position of this species will be revealed after obtaining data from the correctly identified strains collected from the type locality, Goa, in India.

The second new genus, *Oblongichytrium*, is composed of *O. minutum*, *O. multirudimentale*, and *Oblongichytrium* sp. SEK-347, 7-5, and 8-7. This genus is well characterized by the narrow ellipsoidal zoospore, ca. 20% docosapentaenoic acid in total PUFA, and accumulation of canthaxanthin and β-carotene, but no astaxanthin. Unfortunately, only *Oblongichytrium* sp. SEK-347 was examined for PUFA profile and carotenoid pigment in the present study, because the ex-type strain or any other living culture of *O. minutum* and *O. multirudimentale* were probably lost and not available from the culture collection. However, the original description of both species clearly showed the characteristic shape of the zoospores (fig. 4g–i in Gaertner 1981; figs. 12–13 in Goldstein 1963). We assume that members of this genus have similar profiles of PUFA and carotenoid pigments.

In addition, *O. multirudimentale* was originally described as a species of the genus *Thraustochytrium* whose characteristic features in the diagnosis are as follows: (1) 2 to 4 proliferous bodies (= rudiments) of the zoosporangium, and (2) "sub-fusiform or fusiform" ellipsoidal zoospores

(Goldstein 1963). It is noteworthy that the division of older sporangia on seawater agar medium was clearly shown (Goldstein 1963). This phenomenon may suggest this species possesses the ability of vegetative cell division. In fact, the dividing "sporangia" look like vegetative cells before zoospore formation in the original micrograph (fig. 18 in Goldstein 1963), because small cells (= immature zoospores) were not observed in the "sporangia." Therefore, this species should actually be classified as a species of the genus *Oblongichytrium*, but it is necessary to reexamine the foregoing observation carefully.

The members of the third group should be classified in the genus *Schizochytrium* sensu stricto. This group includes the strain recorded as ATCC 28209 that is identified as *S. aggregatum*, but it should be noted that this strain is not the ex-type strain. Although the ex-type strain of *S. aggregatum* has been lost, ATCC 28209 has been distributed from ATCC to a number of researchers and used for investigation with several approaches (Raghukumar 1988b; Honda et al. 1999; Huang et al. 2003). In our observations, the morphology of ATCC 28209 fundamentally agrees with the original description (data not shown), so this strain can be treated as the standard strain of *S. aggregatum*. *Schizochytrium* sensu stricto was newly defined with the emendation by adding the three following characteristics: (1) accumulation of ca. 20% arachidonic acid in total PUFA, (2) accumulation of β-carotene without astaxanthin and canthaxanthin, and (3) 18S rRNA gene sequences distinct.

Leander and Porter (2001) used their isolate (T91-7) of *S. aggregatum*, which was located in the clade of the *Oblongichytrium* and formed a sister-group with *O. minutum* in the 18S rRNA phylogenetic tree (data not shown). Although the type of *S. aggregatum* was isolated from a seawater sample in New Haven, Connecticut, USA, strain T91-7 was isolated from the red alga *Polysiphonia* Grev. in Meridian, Georgia, USA, and strain ATCC 28209 was isolated from seawater in Germany. The correct identification and phylogenetic placement are clearly in need of further investigation for these strains and future isolates from the type locality. These strains are strongly expected to be examined on the morphology of the zoospore and analysis of the PUFA and carotenoid profiles.

As already mentioned, taxonomic positions were determined for four species except *Schizochytrium octosporum* in the genus *Schizochytrium* sensu lato. Presently there are no molecular phylogenetic data, PUFA, or carotenoid profiles of *S. octosporum*, but detailed morphological observation was reported in the original description (Raghukumar 1988b). This organism formed large colonies with developed ectoplasmic net elements and released relatively narrow-shaped zoospores (figs. 8 and 13 in Raghukumar 1988b). Hence, we judged that this organism should be classified in the genus *Oblongichytrium* following the aforementioned taxonomic criteria. It is, however, necessary to reexamine the isolates from the type locality, Rosfjord in Norway.

The species of the genera *Thraustochytrium* and *Ulkenia* appeared in five and two lineages, respectively, in our phylogenetic tree, and unidentified organisms also formed independent lineages (see Fig. 2). This observation probably means that further taxonomic rearrangement might be necessary for these lineages. Unfortunately, there are few morphological features reflecting the phylogenetic relationships and not influenced by culture conditions in thraustochytrids. However, the present study strongly suggests that the genus-level clades could be clearly distinguished by combining morphological and chemotaxonomic features, although it is hard to distinguish between groups on the basis of a single feature. Similar situations have occurred in the taxonomy of yeasts and related organisms, and their taxonomic systems have been based on molecular phylogeny (Kregervan Rij 1984; Kurtzman and Fell 1998). Careful analyses and comparisons of both phenotypic and molecular characteristics will establish a well-accepted taxonomic system for the Labyrinthulomycetes.

Acknowledgments We are grateful to Dr. T. Yokochi (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) for technical advice and kindly providing the data of the PUFA composition of *Japonochytrium* sp. ATCC 28209, *Thraustochytrium aureum* ATCC 34304, *Thraustochytrium striatum* ATCC 24473, and *Ulkenia visurgensis* ATCC 28208. We thank Drs. T. Nakahara and Y. Kumon (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) for technical advice and support concerning analysis by gas chromatography. We also express our thanks to Drs. Y. Yamaoka and M.L. Carmona (National Institute of Advanced Industrial Science and Technology, Hiroshima, Japan) for technical support in analysis by HPLC. We thank Dr. Y. Takao (National Research Institute of Fisheries and Environment of Inland Sea, Hiroshima, Japan) who kindly provided the thraustochytrids strain, SEK 209. We also thank Prof. T. Takaso (University of the Ryukyus, Okinawa, Japan), who kindly supported us in collecting samples in Iriomote Island. This study was supported in part by the Sasagawa Scientific Research Grant from The Japan Science Society, the Ministry of Education, Science, Sports, Culture and Technology, Japan (Grant No. 12740475), and the Hirao Taro Foundation of the Konan University Association for Academic Research.

References

Aki T, Hachida K, Yoshinaga M, Katai Y, Yamasaki T, Kawamoto S, Kakizono T, Maoka T, Shigeta S, Suzuki O, Ono K (2003) Thraustochytrid as a potential source of carotenoids. J Am Oil Chem Soc 80:789–794

Alderman DJ, Harrison JL, Bremer GB, Jones EBG (1974) Taxonomic revisions in the marine biflagellate fungi. The ultrastructural evidence. Mar Biol 25:345–357

Amon JP, Perkins FO (1968) Structure of *Labyrinthula* sp. zoospores. J Protozool 15:543–546

Anderson RS, Kraus BS, McGladdery SE, Reece KS, Stokes NA (2003) A thraustochytrid protist isolated from *Mercenaria mercenaria*: molecular characterization and host defense responses. Fish Shellfish Immunol 15:183–194

Bahnweg G, Sparrow FK (1972) *Aplanochytrium kerguelensis* gen. nov. spec. nov., a new phycomycete from subantarctic marine waters. Arch Mikrobiol 81:45–49

Bongiorni L, Jain R, Raghukumar S, Aggarwal RK (2005) *Thraustochytrium gaertnerium* sp. nov.: a new thraustochytrid stramenopilan protist from mangroves of Goa, India. Protist 156:303–315

Booth T, Miller CE (1968) Comparative morphological and taxonomic studies in the genus *Thraustochytrium*. Mycologia 60:480–496

Booth T, Miller CE (1969) Morphological development of an isolate of *Schizochytrium aggregatum*. Can J Bot 47:2051–2054

Bowles RD, Hunt AE, Bremer GB, Duchars MG, Eaton RA (1999) Long-chain *n*-3 polyunsaturated fatty acid production by members of the marine protistan group the thraustochytrids: screening of

isolates and optimization of docosahexaenoic acid production. J Biotechnol 70:193–202
Carmona ML, Naganuma T, Yamanoka Y (2003) Identification by HPLC-MS carotenoids of the *Thraustochytrium* CHN-1 strain isolated from the Seto Island Sea. Biosci Biotechnol Biochem 67:884–888
Cavalier-Smith T, Allsopp MTEP, Chao EE (1994) Thraustochytrids are chromists, not Fungi: 18S rRNA signatures of Heterokonta. Phil Trans R Soc Lond B 346:387–397
Cejp L (1959) Contribution to the knowledge of the karatinophilic Phycomycetes. Academia Republicii Populare Romanî: 129–138
Cienkowski L (1867) Über den Bau und die Entwickelung der Labyrinthuleen. Arch Mikrosk Anat 3:274–310
Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376
Gaertner VA (1968) Eine methode des quantitativen nechweises nieberer, mit pollen koderbarer pilze im meerwasser und im sediment. Veröff Inst Meeresforsch Bremerhav Suppl 3:75–92
Gaertner VA (1977) Revision of the Thraustochytriaceae (lower marine fungi) 1. *Ulkenia* nov. gen. with description of three new species. Veröff Inst Meeresforsch Bremerhav 16:169–157
Gaertner VA (1981) A new marine phycomycete, *Schizochytrium minutum*, sp. nov. (Thraustochytriaceae) from saline habitats. Veröff Inst Meeresforsch Bremerhav 19:61–69
Goldstein S (1963) Development and nutrition of new species of *Thraustochytrium*. Am J Bot 50:271–279
Goldstein S (1973) Zoosporic marine fungi (Thraustochytriaceae and Dermocystidiaceae). Annu Rev Microbiol 27:13–26
Haeckel E (1868) Monographie der moneren. Jena Z Med Naturwiss 4:64–137
Honda D, Yokochi T, Nakahara T, Erata M, Higaschihara T (1998) *Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean. Mycol Res 102:439–448
Honda D, Yokochi T, Nakahara T, Raghukumar S, Nakagiri A, Schaumann K, Higashihara T (1999) Molecular phylogeny of labyrinthulids and thraustochytrids based on the sequencing of 18S ribosomal RNA gene. J Eukaryot Microbiol 46:637–647
Huang J, Aki T, Yokochi T, Nakahara T, Honda D, Kawamoto S, Shigeta S, Ono K, Suzuki O (2003) Grouping the newly isolated docosahexanoic fatty acid profile and comparative analysis of 18S rRNA genes. Mar Biotechnol 5:450–457
Johnson TW, Sparrow FK (1961) Fungi in oceans and estuaries. Cramer, Weinheim, pp 330–333
Jones EBG, Alderman DJ (1971) *Althornia crouchii* gen. et sp. nov. A marine biflagellate fungus. Nova Hedwigia 21:381–399
Karling JS (1981) Predominantly holocarpic and eucarpic simple biflagellate Phycomycetes. Cramer, Vaduz
Kazama FY (1974) Ultrastructure of *Thraustochytrium* sp. zoospores. IV. External morphology with notes on the zoospores of *Schizochytrium* sp. Mycologia 66:272–280
Kazama FY, Zachary AL, Schornstein KL (1975) Observation on *Thraustochytrium* sp.: development and behavior in culture. Can J Bot 53:360–374
Kreger-van Rij (1984) The yeasts, a taxonomic study, 3rd ed. Elsevier, Amsterdam
Kurtzman CP, Fell JW (1996) The yeasts, a taxonomic study, 4th ed. Elsevier, Amsterdam
Kobayashi Y, Ookubo M (1953) Studies on the marine Phycomycetes. Bull Natl Sci Mus Tokyo 33:53–65
Kumon Y, Yokoyama R, Yokochi T, Honda D, Nakahara T (2003) A new labyrinthulid isolate, which solely produces *n*-6 docosapentaenoic acid. Appl Microbiol Biotechnol 63:22–28
Kumon Y, Yokoyama R, Haque Z, Yokochi T, Honda D, Nakahara T (2006) A new labyrinthulid isolate that produces only docosahexaenoic acid. Mar Biotechnol 8:170–177
Leander CA, Porter D (2000) Redefining the genus *Aplanochytrium* (phylum Labyrinthulomycota). Mycotaxon 76:439–444
Leander CA, Porter D (2001) The Labyrinthulomycota is comprised of three distinct lineages. Mycologia 93:459–464
Leander CA, Porter D, Leander BS (2004) Comparative morphology and molecular phylogeny of aplanochytrids (Labyrinthulomycota). Eur J Protistol 40:317–328
Leipe DD, Wainright PO, Gunderson JH, Porter D, Patterson DJ, Valois F, Himmerich S, Sogin ML (1994) The stramenopiles from a molecular perspective: 16S-like rRNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis*. Phycologia 33:369–377
Levine ND, Corliss JO (1963) Two new subclasses of sarcodines, Labyrinthulia subcl. nov. and Proteomyxidia subcl. nov. J Protozool 10(suppl):27
Levine ND, Corliss JO, Cox FEG, Deroux G, Grain J, Honigberg BM, Leedale GF, Loeblich AR, Lom J, Lynn DH, Merinfeld D, Page FC, Poljansky G, Sprague V, Vavra J, Wallace FG (1980) A newly revised classification of the Protozoa. J Protozool 27:37–58
Lewis TE, Nichols PD, McMeekin TA (1999) The biotechnological potential of thraustochytrids. Mar Biotechnol 1:580–587
Mo C, Douek J, Rinkevich B (2002) Development of a PCR strategy for thraustochytrid identification based on 18S rDNA sequence. Mar Biol 140:883–889
Moro I, Negrisolo E, Callegaro A, Andreoli C (2003) *Aplanochytrium stocchinoi*, a new Labyrinthulomycota from the Southern Ocean (Ross Sea, Antarctica). Protist 154:331–340
Moss ST (1980) Ultrastructure of the endomembrane-sagenogenetosome-ectoplasmic net complex in *Ulkenia visurgensis* (Thraustocytriales). Bot Mar 23:73–94
Moss ST (1985) An ultrastructural study of taxonomically significant characters of the Thraustochytriales and the Labyrinthulales. J Linn Soc (Bot) 91:329–357
Nakahara T, Yokochi T, Higashihara T, Tanaka S, Yaguchi T, Honda D (1996) Production of docosahexaenoic and docosapentaenoic acid by *Schizochytrium* sp. isolated from Yap Island. J Am Oil Chem Soc 73:1421–1426
Nakayama M, Watanabe S, Mitsui K, Uchida H, Inoue I (1996) The phylogenetic relationship between the Chlamydomnadales and Chlorococcales inferred from 18S rDNA sequences data. Phycol Res 44:47–55
Olive LS (1975) The Mycetozoans. Academic Press, New York, pp 215–292
Perkins FO (1972) The ultrastructure of hold-fasts, "rhizoids" and "slime track" in traustochytriaceous fungi and in *Labyrinthula* spp. Arch Microbiol 84:95–118
Perkins FO (1973) A new species of marine labyrinthulid *Labyrinthuloides yorkensis* gen. nov. spec. nov.: cytology and fine structure. Arch Microbiol 90:1–17
Perkins FO (1974) Phylogenetic consideration of the problematic thraustochytriaceous–labyrinthulid–*Dermocystidium* complex based on observation of fine structure. Veröff Inst Meeresforsch Bremerhav 5:45–63
Porter D (1972) Cell division in the marine slime mold, *Labyrinthula* sp. and the role of the bothrosome in extracellular membrane production. Protoplasma 74:427–448
Porter D (1974) Phylogenetic considerations of the Thraustochytriaceae and Labyrinthulaceae. Veröff Inst Meeresforsch Bremerhav 5:19–44
Porter D (1989) Phylum Labyrinthulomycota. In: Margulis L, Corlis JO, Melconian M, Chapman DJ (eds) Handbook of Protoctista. Jones and Bartlett, Boston, pp 388–398
Raghukumar S (1982a) Fine structure of the thraustochytrid *Ulkenia amoeboidea*. I. Vegetative thallus and formation of the amoeboid stage. Can J Bot 60:1092–1102
Raghukumar S (1982b) Fine structure of the thraustochytrid *Ulkenia amoeboidea*. II. The amoeboid stage and formation of zoospores. Can J Bot 60:1103–1114
Raghukumar S (1988a) *Schizochytrium mangrovei* sp. nov., a thraustochytrid from mangroves in India. Trans Br Mycol Soc 90:627–631
Raghukumar S (1988b) *Schizochytrium octosporum* sp. nov. and other thraustochytrids from the North Sea (Rosfjord, Norway). Trans Br Mycol Soc 90:273–278
Reynolds ES (1963) The use of lead citrate at high pH as an electronopaque strain in electron microscopy. J Cell Biol 17:208–212
Rzhetsky A, Nei M (1992) A simple method for estimating and testing minimum-evolution trees. Mol Biol Evol 9:945–967
Rzhetsky A, Nei M (1993) Theoretical foundation of the minimum-evolution method of phylogenetic inference. Mol Biol Evol 10:1073–1095
Shimizu S, Kawashima H, Shinmen Y, Akimoto K, Yamada H (1988) Production of eicosapentaenoic acid by *Mortierella* fungi. J Am Oil Chem Soc 65:1455–1459
Sparrow FK (1936) Biological observations on the marine fungi of Woods Hole waters. Biol Bull 70:236–263

Sparrow FK (1943) The aquatic Phycomycetes, exclusive of the Saprolegniaceae and *Pythium*. University of Michigan Press, Ann Arbor
Sparrow FK (1960) The aquatic Phycomycetes exclusive of the Saprolegniaceae and *Pythium*, 2nd edn. University of Michigan Press, Ann Arbor
Sparrow FK (1969) Zoosporic marine fungi from the Pacific Northwest (U.S.A.). Arch Mikrobiol 66:129–146
Stokes NA, Ragone Calvo LM, Reece KS, Burreson EM (2002) Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria*. Dis Aquat Org 52:233–247
Swofford DL (2003) PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0b 10. Sinauer Associates, Sunderland, MA
Thompson JD, Higgins DC, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position, specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
Valadon LRG (1976) Carotenoids as additional taxonomic characters in fungi: a review. Trans Br Mycol Soc 67:1–15
Yamaoka Y, Carmona ML, Oota S (2004) Growth and carotenoid production of *Thraustochytrium* sp. CHN-1 cultured under superbright red and blue light-emitting diodes. Biosci Biotechnol Biochem 68:1594–1597
Yokochi Y, Honda D, Higashihara T, Nakahara T (1998) Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. Appl Microbiol Biotechnol 49:72–76