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FULL PAPER

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Taxonomic rearrangement of the genus *Ulkenia* sensu lato based on morphology, chemotaxonomical characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for *Ulkenia* and erection of *Botryochytrium*, *Parietichytrium*, and *Sicyoidochytrium* gen. nov.

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Abstract The genus *Ulkenia* is characterized by the naked protoplast stage within its life cycle. However, the 18S rRNA gene tree clearly shows that this genus is not a natural taxon, because our own isolates and reported strains separately form four well-supported monophyletic groups. These four groups are clearly distinguishable by their profiles of polyunsaturated fatty acids and carotenoid pigments and cell and colony morphology, e.g., persistence of sporangial wall, manner of the cell cleavage at the zoospore formation, and development of the ectoplasmic nets. Therefore, the four groups are assigned to four genera including three new genera, i.e., *Ulkenia* sensu stricto, *Botryochytrium*, *Parietichytrium*, and *Sicyoidochytrium* gen. nov.

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

Introduction

The class Labyrinthulomycetes is a phylogenetic group in the stramenopile organisms, which consists of two well-accepted families, i.e., Labyrinthulaceae and Thraustochytriaceae (see Honda et al. 1999; Leipe et al. 1994; Porter 1989). The family Thraustochytriaceae was established by Sparrow (1943, 1960) for chytrid-like, eucarpic, and epi- and endobiotic marine fungi attached to substrate by an ectoplasmic net and producing biflagellate zoospores. Porter (1989) reviewed this family as being composed of the following seven genera at that time: *Thraustochytrium* Sparrow emend. Johnson, *Schizochytrium* S. Goldst. et Belsky emend. Booth et Miller, *Ulkenia* A. Gaertn., Labyrinthuloides F.O. Perkins, *Japonochytrium* Kobayasi et M. Ookubo, *Aplanochytrium* Bahnweg et Sparrow, and *Althornia* E.B.G. Jones et Alderman. These genera had been distinguished by morphological features at various stages of their life cycle. For example, the genus *Schizochytrium* was characterized by successive binary division of vegetative cells. *Ulkenia* was characterized by producing naked protoplasts. However, more recently Honda et al. (1999) clearly showed that *Thraustochytrium*, *Schizochytrium*, and *Ulkenia* did not form a monophyletic group in the molecular phylogenetic tree of 18S rRNA genes. On the other hand, Leander and Porter (2000, 2001) and Leander et al. (2004) revealed that *Aplanochytrium kerguelense* Bahnweg et Sparrow and two *Labyrinthuloides* species, i.e., *L. minuta* (S.W. Watson et Raper) F.O. Perkins and *L. yorkensis* F.O. Perkins, formed a monophyletic group with comparatively compact distances among species of these genera in the 18S rRNA gene tree. Leander and Porter (2000) judged that *Aplanochytrium* and *Labyrinthuloides* are synonymous, based on the combination of molecular phylogenetic data and morphological features, and consequently emended the definition of the preferential genus *Aplanochytrium*, which included all species of *Labyrinthuloides* and one species of *Labyrinthula*, *L. thais* B.A. Cox et Mackin. Recently, Yokoyama and Honda (2007) clearly showed that their own isolates and reported strains that were identified as *Schizochytrium* spp. formed three separate well-supported monophyletic groups in the 18S rRNA gene tree. These groups can be distinguished by the combination of morphological characteristics, profiles of polyunsaturated fatty acids, and carotenoid pigments. Therefore, we proposed that species of the genus *Schizochytrium* were separated into three distinct genera, that is, *Schizochytrium* sensu stricto and two new genera, *Aurantiochytrium* R. Yokoy. et D. Honda and *Oblongichytrium* R. Yokoy. et D. Honda.

The genus *Ulkenia* was established by Gaertner (1977), but the 18S rRNA gene tree suggested that this genus is also not a natural taxon. Two *Ulkenia* species, *U. visurgensis*

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(Ulken) A. Gaertn. and *U. amoeboidea* (Bahnweg et Sparrow) A. Gaertn., were originally described as species of *Thraustochytrium*. So far, four more species have been described: *U. sarkariana* A. Gaertn., *U. profunda* A. Gaertn., *U. radiata* A. Gaertn., and *U. minuta* Raghuk. However, three *Ulkenia* species (*U. visurgensis*, *U. profunda*, and *U. radiata*) separated into two separate monophyletic groups in the 18S rRNA gene tree (Honda et al. 1999), which means that the definitive feature of this genus, i.e., the release of amoeboid cells before the formation of zoospores, was gained in each lineage. Moreover, amoeboid cells have been also observed in at least following four species of genera other than *Ulkenia*: *Schizochytrium mangrovei* Raghuk., *Schizochytrium limacinum* D. Honda et Yokochi (Honda et al. 1998), *Thraustochytrium striatum* Joa. Schneid. (Raghukumar 1992), and *Thraustochytrium gaertnerium* Bongiorni (Bongiorni et al. 2005). According to Gaertner (1972) and Raghukumar (1988), these organisms can be identified only if observed under standardized conditions such as when pollen-baited seawater is used. Hence, it is possible that the genus *Ulkenia* is not a natural taxonomic group.

In the present study, we investigated the taxonomic validity of the genus *Ulkenia* by examining our own isolates and reported strains based on morphological features, composition of polyunsaturated fatty acids (PUFAs) and carotenoid pigments, and molecular phylogenetic relationships.

Materials and methods

Samples and cultivation

Our own isolates are shown by SEK prefix (Table 1); these were isolated from seawater and sand collected in each location throughout Japan and Malaysia by the pine-pollen baiting method (Gaertner 1968). Cells were cultured at 25°C in medium-H (Honda et al. 1998).

Observations

Cells of each stage in the life cycle were cultured in both medium-H and seawater/pine-pollen cultures, as proposed by Raghukumar (1988). Light microscopic observations were made by a BX60 (Olympus, Tokyo, Japan) fitted with a Nomarski interference differential contrast objective. 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-bottom culture dish (Meridian Instruments Far East, Tokyo, Japan) filled with flesh 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 one frame every 2 s by Moto DV (Digital Origin, Los Angeles, CA, USA) on Power Mac G4 (Apple Computer, Cupertino, CA, USA).

Extraction of DNA

Cells were harvested after 4–7 days of culture. Total genomic DNA was extracted using the suggested method by 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 (Murray and Thompson 1980). To obtain almost complete 18S rRNA gene sequences, we used a polymerase chain reaction (PCR) protocol with a thermostable DNA polymerase (Ex Taq DNA Polymerase; Takara, Ohtsu, Japan) and amplification primers SR1 and SR12 (Nakayama et al. 1996). The best annealing temperature (45°–55°C) and reactive frequency (40–50 times) of PCR were tested and selected for each samples. We removed the amplified band of about 1800 bases in 0.5% TBE-agarose gel and subsequently extracted it, using Gene Clean Turbo (Q-Bio Gene, Carlsbad, CA, 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)

Table 1. List of newly isolated strains with the sources, collection localities and date

Taxon	Strain	Source	Locality	Date
Genus *Ulkenia*				
Ulkenia amoeboidea	SEK 214 (RT0167)	Seawater	Hiroshima, Japan	22 May, 2001
Genus *Sicyoidochytrium*				
Sicyoidochytrium minutum	SEK 354 (RT0305)	Seawater and sand	Langkawi Is., Malaysia	20 July, 2003
Genus *Botryochytrium*				
Botryochytrium radiatum	SEK 353 (RT0304)	Seawater and sand	Langkawi Is., Malaysia	20 July, 2003
Genus *Parietichytrium*				
Parietichytrium sarkarianum	SEK 351 (RT0301)	Seawater and sand	Penang Is., Malaysia	17 July, 2003
Parietichytrium sarkarianum	SEK 364 (RT0049)	Seawater	Iriomote Is., Japan	31 July, 2000

and optically aligned. Other strain sequences were downloaded from the GenBank constructed by the National Center of Biotechnology Information (NCBI) (accession numbers shown in Table 2). 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

Table 2. Strains used in molecular phylogenetic analyses with sequence accession numbers

Taxon	Strain	Accession number	Reference
Genus *Ulkenia*			
Ulkenia amoeboidea	SEK 214 (RT0167)	AB290355	Takao et al. 2007
Ulkenia profunda	KMPB N 3077^T	L34054	Honda et al. 1999
Ulkenia profunda	BUTRBG 111	DQ023615	–
Ulkenia visurgensis	ATCC 28208^T	AB022116	Honda et al. 1999
Ulkenia visurgensis	BURAAA 141	DQ100296	–
Ulkenia sp. (*Japonocytrium* sp.)	ATCC 28207	AB022104	Honda et al. 1999
Genus *Sicyoidochytrium*			
Sicyoidochytrium minutum	NBRC 102975	AB290585	–
Sicyoidochytrium sp.	NBRC 102979	AB183659	–
Sicyoidochytrium minutum	SEK 354	AB355412	This study
Genus *Botryochytrium*			
Botryochytrium radiatum	SEK 353 (RT0304)	AB355410	This study
Botryochytrium radiatum	#16 (Raghukumar)	AB022115	Honda et al. 1999
Botryochytrium sp.	BUTRBC 143	DQ023614	–
Botryochytrium sp.	#29 (Raghukumar)	AB022114	Honda et al. 1999
Genus *Parietichytrium*			
Parietichytrium sarkarianum	SEK 351 (RT0301)	AB355411	This study
Parietichytrium sarkarianum	SEK 364 (RT0049)	AB244715	–
Parietichytrium sp.	F3-1	AB073304	Huang et al. 2003
Parietichytrium sp.	H1-14	AB073305	Huang et al. 2003
Parietichytrium sp.	NBRC 102984	AB290580	–
Other strains			
Aplanochytrium kerguelense	KMPB N-BA-107	AB022103	Honda et al. 1999
Aplanochytrium minutum	n/a	L27634	Leipe et al. 1994
Aplanochytrium stocchinoi	n/a	AJ519935	Moro et al. 2003
Aplanochytrium sp.	PR24-1	AF348519	Leander et al. 2004
Aurantiochytrium limacinum	NIBH SR21^T	AB022107	Honda et al. 1999
Aurantiochytrium mangrovei	RCC893	DQ367049	–
Aurantiochytrium sp.	KH105	AB052555	Huang et al. 2003
Aurantiochytrium sp.	N1-27	AB073308	Huang et al. 2003
Aurantiochytrium sp.	SEK 209 (NBRC 102614)	AB290574	Yokoyama and Honda 2007
Aurantiochytrium sp.	SEK 217 (RT0100P1)	AB290572	Yokoyama and Honda 2007
Aurantiochytrium sp.	SEK 218 (RT0040)	AB290573	Yokoyama and Honda 2007
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	Yokoyama and Honda 2007
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.	N8	AB246794	–
Labyrinthula sp.	N12	AB246795	–
Schizochytrium aggregatum	ATCC 28209	AB022106	Honda et al. 1999
Schizochytrium sp.	KK17-3	AB052556	Huang et al. 2003
Schizochytrium sp.	SEK 210 (NBRC 102615)	AB290576	Yokoyama and Honda 2007
Schizochytrium sp.	SEK 345 (NBRC 102616)	AB290577	Yokoyama and Honda 2007
Schizochytrium sp.	SEK 346 (NBRC 102617)	AB290578	Yokoyama and Honda 2007
Thraustochytrium aggregatum	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	Honda et al. 1999
Thraustochytrium pachydermum	KMPB N-BA-114	AB022113	Honda et al. 1999
Thraustochytrium striatum	ATCC 24473^T	AB022112	Honda et al. 1999
	BURABG 162	DQ100295	–
	C9G	AF474172	Anderson et al. 2003
	NBRC 102990	AB183664	–
	QPX	AY052644	Stokes et al. 2002
Outgroup			
Bacillaria paxillifer	n/a	M87325	–
Ochromonas danica	n/a	M32704, J02950	–

ATCC, American Type Culture Collection; 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); T, ex-type strain; n/a, not available

the ML method analysis, the transition/transversion (ti/tv) ratio of the HKY85 model (Hasegawa et al. 1985) was estimated by maximizing the likelihood value for neighbor-joining (NJ) topology. The best tree was found by the heuristic search method. Bootstrap values (Felsenstein 1985) were obtained from 100 resamplings. In the ME method analysis, 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. 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 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 Marine Art SF-1 (Tomita Pharmaceutical, Tokushima, Japan), and then incubated for 7–10 days at 25°C. 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°C to 220°C with 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 the internal standard.

Carotenoid composition analyses

Each strain was inoculated into a 500-ml conical flask containing 200 ml medium-H and subsequently 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 frozen in liquid nitrogen and dried by an oil rotary vacuum pump (GVD-050A; Ulvac, Kanagawa, Japan). The endopigments were extracted by suspending cells into 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 according to peaks of the standard sample and data of previous studies (Carmona et al. 2003).

Taxonomy

According to the comparison of all the data sets shown later, one genus is recharacterized and three new genera are established here. The strains of *Ulkenia* sensu lato form four well-supported monophyletic groups, which are distinguished by their profiles of polyunsaturated fatty acids and carotenoid pigments and cell and colony morphology, e.g., persistence of sporangial wall, manner of the cell cleavage at the zoospore formation, and development of the ectoplasmic nets. Therefore, these four groups are assigned to form genera including three new genera: i.e., *Ulkenia* sensu stricto, *Sicyoidochytrium*, *Botryochytrium*, and *Parietichytrium* gen. nov.

Ulkenia A. Gaertn. emend. R. Yokoyama, B. Salleh et D. Honda

Thallus epi- and endobiotic, interbiotic, thin-walled during active growth, with a rhizoidal system (syn. ectoplasmatic net) endobiotic or interbiotic. Thallus globose, subglobose, or pear shaped, very variable in diameter with varying and differing cell content during development. Colonies small. Ectoplasmic nets not well developed. At maturity, cell wall disappears totally, and the protoplast becomes fully naked or creeps like an amoeba out of the sporangium wall by a small opening at the apical part of the sporangium. After settlement, the protoplast becomes a zoosporangium, which released heterokont zoospores. Cells possess astaxanthin, phenicoxanthin, echinenon, and β-carotene.

Type species: *Ulkenia visurgensis* (Ulken) A. Gaertn.

Other species: *Ulkenia amoeboidea* (Bahnweg et Sparrow) A. Gaertn. (It was spelled as "*U. amoeboida*" in the original description), and *Ulkenia profunda* A. Gaertn.

Botryochytrium R. Yokoyama, B. Salleh et D. Honda, gen. nov.

Coloniae aliquantum magnae. Reticulum ectoplasmaticum aliquantum bene evolvens. Maturitate paries cellularum omnino evanescens et protoplastum denudatus. Protoplastus botruoideus per divisionem centripetalem, dein stelliformis ante formationem zoosporarum. Cellulae cum pigmentis "canthaxanthin," "echinenon," "β-carotene," et acido pingui "*n*-6 docosapentaenoic acid" aliquantum abundanto includens.

Colonies comparatively large. Ectoplasmic nets comparatively well developed. At maturity, cell wall deliquescing completely and protoplast becomes totally naked. Protoplast becoming botryose by a centripetal division, then becoming star-shaped before zoospore formation. Cells possessing canthaxanthin, echinenon, β-carotene, and comparatively abundant *n*-6 docosapentaenoic acid.

Etymology: *botryo-* = cluster, *chytrion* = pot, referring the shape of cells at the zoospore formation.

Table 3. Morphological features of examined strains

Organism	*Ulkenia amoeboidea*	*Ulkenia* sp.	*Sicyoidochytrium minutum*		*Botryochytrium radiatum*	*Parietichytrium sarkarianum*	
Strain	SEK 214	ATCC 28207	SEK 354	NBRC 102975	SEK 353	SEK351	SEK 363
Colony	small	small	small	small	large	large	large
Ectoplasmic nets	un-developed	un-developed	un-developed	un-developed	well-developed	well-developed	well-developed
Size of a zoospore (um)	2.5–3.5 × 3.5–5.0	2.0–4.0 × 3.5–5.5	2.0–2.5 × 3.0–4.0	1.5–2.5 × 2.5–4.2	2.5–3.5 × 3.5–4.2	3.0–4.0 × 4.5–6.0	3.0–4.0 × 4.0–6.5
Persistence of cell wall after releasing protoplast	+	+	–	–	–	+	+
Amoeboid activity	+	++	++	++	++	+	+

Type species: ***Botryochytrium radiatum*** (A. Gaertn.) R. Yokoyama, B. Salleh et D. Honda, comb. nov.

Basionym: *Ulkenia radiata* A. Gaertn., Veröff. Inst. Meeresforsch. Bremerh. 16: 150, 1977.

Parietichytrium R. Yokoyama, B. Salleh et D. Honda, gen. nov.

Coloniae aliquantum magnae. Reticulum ectoplasmaticum aliquantum bene evolvens. Paries cellurarum persistens post liberatum protoplastum. Protoplastus botryoideus per divisionem centripetalem, dein stelliformis ante formationem zoosporarum. Cellulae cum pigmentis "β-carotene" et acido pingui "docosatetraenoic acid" aliquantum abundanto includens.

Colonies comparatively large. Ectoplasmic nets comparatively well developed. Cell wall persistent after releasing protoplast. Protoplast becoming botryose by a centripetal division, then becoming star-shaped before zoospore formation. Cells possessing β-carotene and comparatively abundant docosatetraenoic acid.

Etymology: *paries* = wall, *chytrion* = pot, referring cell wall persistence after releasing amoeboid cell.

Type species: ***Parietichytrium sarkarianum*** (A. Gaertn.) R. Yokoyama, B. Salleh et D. Honda, comb. nov.

Basionym: *Ulkenia sarkariana* A. Gaertn., Veröff. Inst. Meeresforsch. Bremerh. 16:144, 1977.

Sicyoidochytrium R. Yokoyama, B. Salleh et D. Honda, gen. nov.

Cellulae vegetativae aliquantum parvae. Coloniae aliquantum parvae. Reticulum ectoplasmaticum non aliquantum evolvens. Maturitate paries cellularum omnino evanescens et protoplastus denudatus. Protoplastus compluriens dividens. Ad divisionem ultimam zoosporae constrictae elongatae formantae. Cellulae cum pigmentis "canthaxanthin," "echinenon," "β-carotene" includens.

Vegetative cells somewhat small. Colonies somewhat small. Ectoplasmic nets not well developed. At maturity, cell wall deliquescing completely and protoplast becomes totally naked. Protoplast dividing several times. At final division, zoospores forming by meaning of pinching and pulling. Cells possessing canthaxanthin, echinenon, and β-carotene.

Etymology: *sicyoideus* = gourd-shaped, *chytrion* = pot, referring the cell shape at the final division of zoospore formation, which is similar to the shape of Japanese gourd.

Type species: ***Sicyoidochytrium minutum*** (Raghuk.) R. Yokoyama, B. Salleh et D. Honda, comb. nov.

Basionym: *Ulkenia minuta* Raghuk., Veröff. Inst. Meeresforsch. Bremerh. 16:160, 1977.

Results

Morphological characteristics

Morphological features are summarized in Table 3. Colonies of the vegetative cells of *Ulkenia amoeboidea* SEK 214

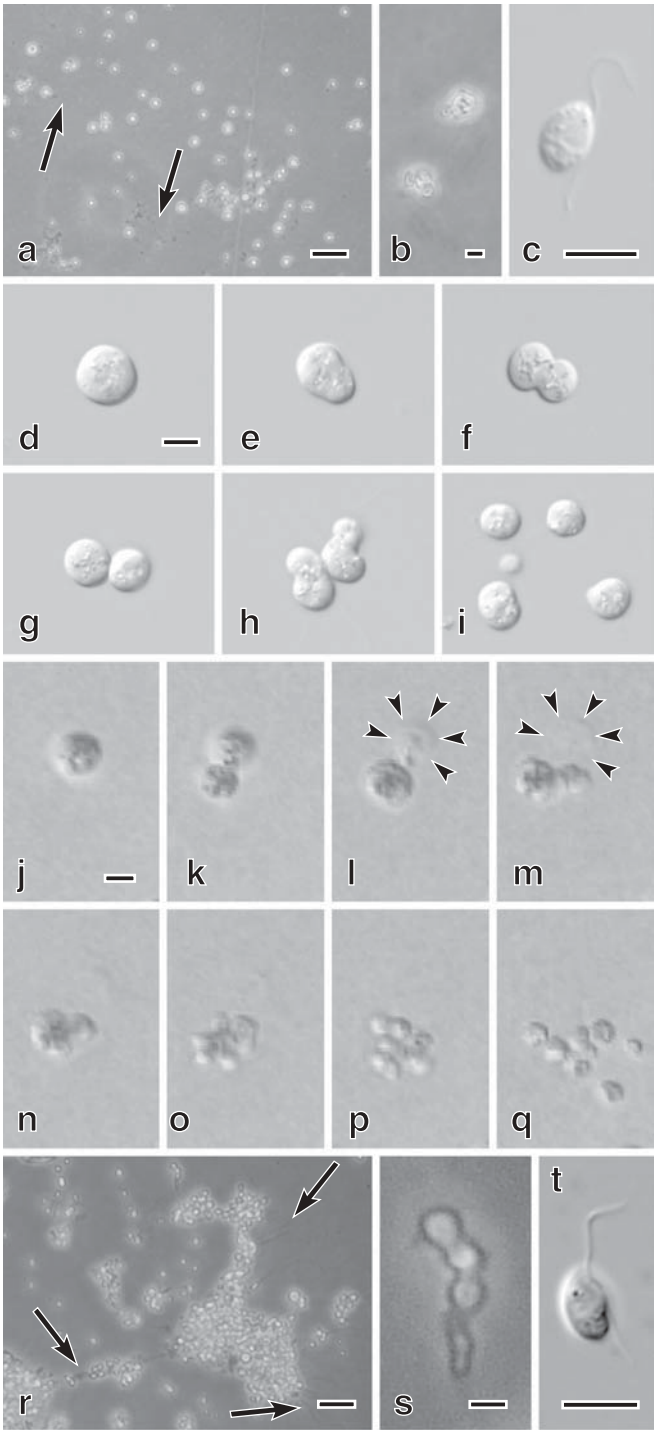


Fig. 1. Light micrographs of *Ulkenia* clade strains [**a–r** *U. amoeboidea* SEK 214; **s–u** *Ulkenia* sp. (deposited as *Japanochytrium* sp.) ATCC 28207]. **a** Cells with many fine ectoplasmic nets (*arrows*) in seawater/pine pollen cultures. **b** Amoeboid cells. **c** A zoospore. **d–i** Light micrographs showing division of amoeboid cells (**d** spherical shape cell; **e** free shape cell; **f** binary cell division; **g** two daughter cells; **h** binary cell divisions; **i** four daughter cells). **j–q** Continuous observation of amoeboid cell and zoospore formation [**j** vegetative cell; **k, l** protoplast that is out from the cell wall (*arrowheads*); **m** amoeboid cell changing from naked protoplast; **n** sessile amoeboid cell; **o** cell division; **p** eight daughter cells; **q** releasing zoospores]. **r** Gathering cells with many fine ectoplasmic nets (*arrows*) in seawater/pine pollen cultures. **s** Amoeboid cells. **t** A zoospore. *Bars* **a, r** 30 μm; **b, c, e, j, r–t** 5 μm

belonging to *Ulkenia* sensu stricto tended to disperse and did not form comparatively large colonies (Fig. 1a). Amoeboid cells (Fig. 1b) and biflagellate zoospores were observed (Fig. 1c). Figure 1d–i showed that amorphous cells repeated binary division. During continuous observation, naked protoplasts escaped from the opening formed by a partial melting of the cell wall, and moved with active amoeboid motility (Fig. 1j–l). The remains of the cell wall after the release of the protoplast were also observed (Fig. 1m). The naked protoplasts were rounded, and divided to form spores. About 30 min after forming nonflagellate spores, spores obtain flagella and are released as zoospores (Fig. 1n–q). *Ulkenia* sp. (deposited as *Japonochytrium* sp.) ATCC 28207, also belonging to the *Ulkenia* clade, tends to form colonies with more cells gathering formed than SEK 214 (Fig. 1r). Amoeboid cells (Fig. 1s) and biflagellate zoospores are formed (Fig. 1t). During continuous observation of this strain, the release of naked protoplasts, similar to that by SEK 214, was observed (data not shown).

The cells of *Sicyoidochytrium minutum* SEK 354 and NBRC 102975 did not form large colonies of cells. The cells or small colonies extended simple unbranched ectoplasmic net elements to the pine pollen (Fig. 2a,k). Amoeboid cells with active motility (Fig. 2b,l) and heterokont zoospores (Fig. 2c,m) were observed. After two or three successive divisions of the protoplast, the cell became mature and was ready to form zoospores (Fig. 2g,h,r–t). After about 5 min, each cell synchronously divided once, and 8 or 16 small cells were formed as a result (Fig. 2g,h,r–t). Each small cell became a zoospore with heterokont flagella and swam away (Fig. 2i,u). During the final synchronous division stage, cells were characteristically shaped like dumbbells by a process of pinching and pulling (Fig. 2j,v). After the release of zoospores, no remains of the cell wall were observed. Sometimes comparatively larger cell(s) did not form zoospores, which is possibly a proliferous body (Fig. 2u).

The cells of *Botryochytrium radiatum* SEK 353 form comparatively large colonies of ca. 100 μm in the diameter with well-developed ectoplasmic nets. The ectoplasmic net elements merged and connected to each other, so the cells form a large cluster (Fig. 3a). Amoeboid cells with active motility (Fig. 3b) and heterokont zoospores (Fig. 3c) were observed. The cell walls of the matured vegetative cell disappear (Fig. 3d), and an amoeboid protoplast is released (Fig. 3e–g). After ca. 10 min, the protoplasts settle and zoospore formation occurs, and ca. 16 heterokont zoospores swim away (Fig. 3h–j). After the zoospores are released, there are no proliferous bodies nor remains of the cell wall of the sporangium. During the detailed observation of the zoospore formation, the protoplast changed its shape into a botryose form by synchronous multipolar budding and formed small cells (Fig. 3k–l). These small cells are produced by the pinching and pulling of cell division, and finally the prematured zoospore cells are formed (Fig. 3m,n).

The colonies of *Parietichytrium sarkarianum* strains SEK 351 and 364 are also large but smaller than *Botryochytrium radiatum* SEK 353, although their ectoplasmic nets are comparatively well developed (Fig. 4a,m). Amoeboid cells with active motility (Fig. 4b,n) and heterokont zoospores

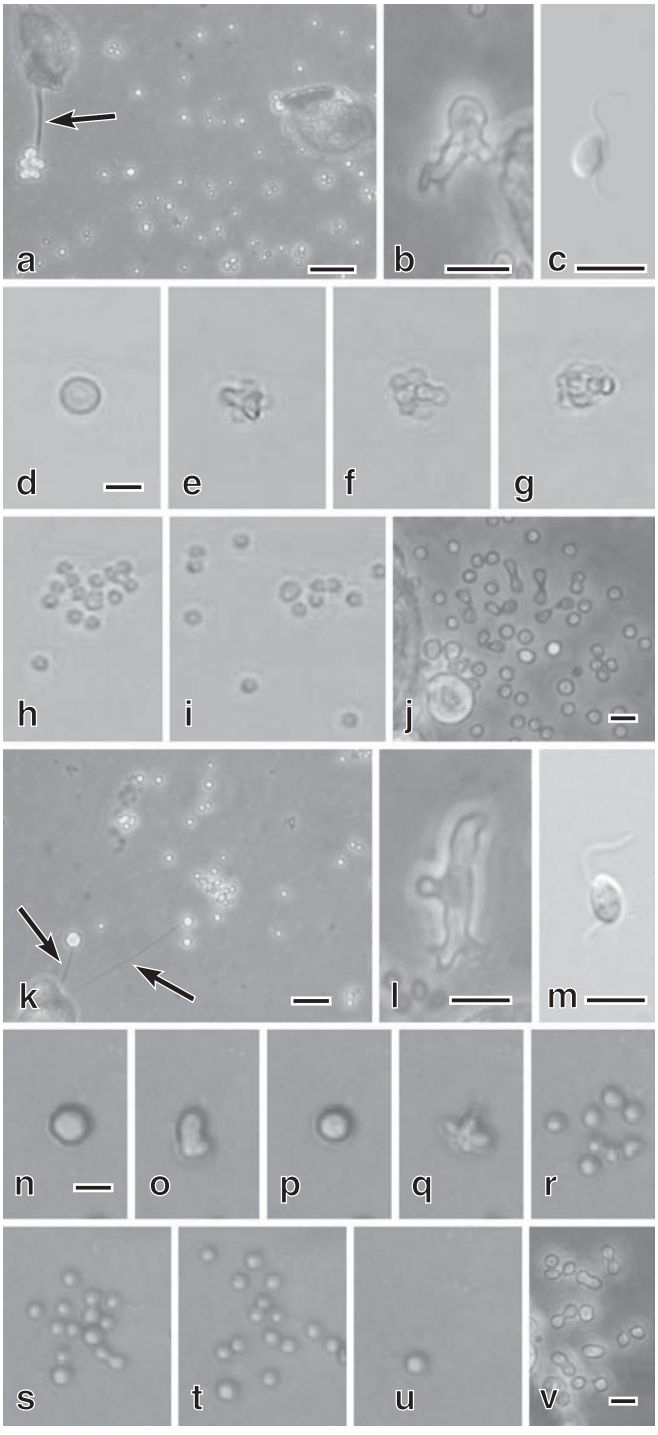


Fig. 2. Light micrographs of the *Sicyoidochytrium* clade strains (**a–j** *S. minutum* SEK 354; **k–v** *S. minutum* NBRC 102975). **a, k** Cells with simple unbranched ectoplasmic nets (*arrows*) in seawater/pine pollen cultures. **b, l** Amoeboid cell. **c, m** Zoospore. **d–i, n–u** Continuous observation of zoospore formation through cell division [**d** mature sporangium; **e** cell division; **f** eight daughter cells; **g** synchronous, dumbbell-like binary cell division; **h** sixteen spores without motility; **i** zoospores obtaining two flagella and releasing; **n** vegetative cell; **o** amoeboid cell; **p** rounded and matured sporangium; **q** cell division; **r** eight daughter cells; **s** the synchronous, dumbbell-like binary cell division; **t** sixteen spores without motility; **u** zoospores with two flagella releasing and remaining proliferous body (*arrowhead*)]. **j, v** The dumbbell-like binary cell division. *Bars* **a, k** 30 μm; **b–d, j, l–n, v** 5 μm

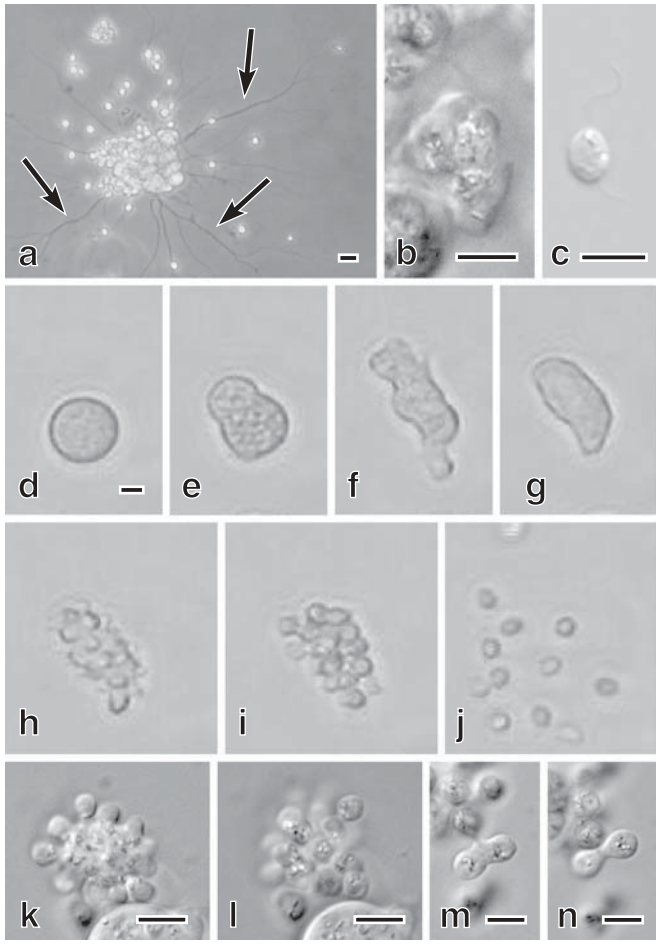
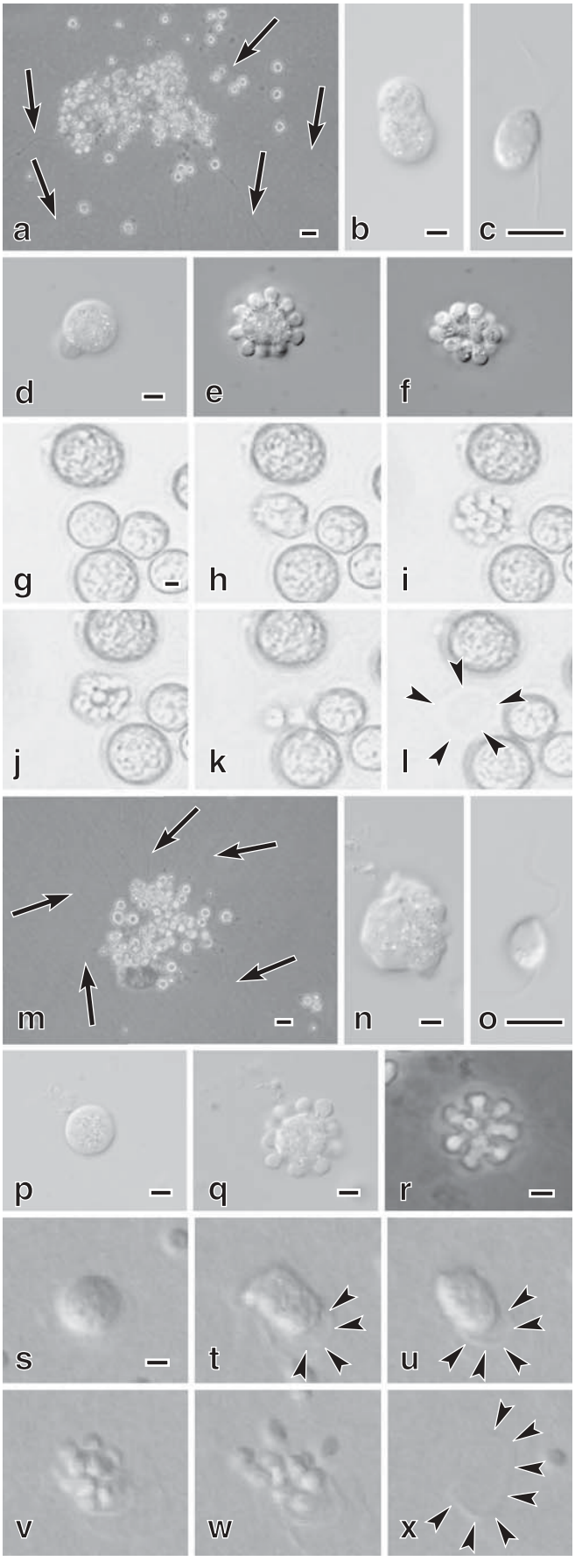


Fig. 3. Light micrographs of the *Botryochytrium* clade strains (**a–n** *B. radiatum* SEK 353). **a** Large clusters with well-developed ectoplasmic nets (*arrows*) in seawater/pine pollen cultures. **b** Amoeboid cell. **c** Zoospore. **d–j** Continuous observation of the zoospore formation (**d** mature sporangium; **e** protoplast escaping from thin cell wall; **f** amoeboid motion; **g** rounded and matured sporangium; **h** radiated cell division synchronous multipolar budding; **i** zoospores ready to be released; **j** zoospores released without persistent cell wall). **k, l** Radiated cell division synchronous multipolar budding. **m, n** Zoospore formation by pinching and pulling. *Bars* **a** 30 μm; **b–d, k–n** 5 μm

(Fig. 4c,o) are formed. Vegetative cells release protoplasts without remarkable motility (Fig. 4g,h,s,t), and after 1–2 min the released protoplast settles and changes shape into the botryose form by synchronous multipolar budding (Fig. 4d–f,i,p–r) and finally forms zoospores with heterokont flagella (Fig. 4j,k,v,w). After releasing the protoplast and zoospores, the cell walls persist (Figs. 4l,u,x).

Molecular phylogeny of 18S rRNA gene sequences

Almost the entire length of 18S rRNA gene sequences of five strains was determined and deposited in the DNA Data Bank of Japan (DDBJ) (see Table 2), and the alignment was deposited in TreeBASE (matrix accession number: M3464 at http://treebase.org/). We used 1242 sites for comparison among all operational taxonomic units without the gaps and ambiguous sites. The members of the genera *Labyrinthula*,



Aplanochytrium, *Schizochytrium*, *Aurantiochytrium*, and *Oblongichytrium* formed a monophyletic group supported by a high bootstrap value in both ML and ME analyses. The *Ulkenia* strains characterized in this study and recorded *Ulkenia* sensu lato strains were separated into four independent monophyletic groups with support of 100% bootstrap values in both ML and ME analyses (Fig. 5).

The *Ulkenia* clade included ex-type strains of *U. visurgensis* ATCC 28208 (the type species of the genus *Ulkenia*) and *U. profunda* KMPB N3077a, and the strains *Ulkenia amoeboidea* SEK 214 and *Ulkenia* sp. ATCC 28207. The *Botryochytrium* clade included the strains *Botryochytrium* sp. 29 and *B. radiatum* 16, isolated by Dr. Raghukumar, and our own isolate *B. radiatum S*EK 353. The *Parietichytrium* clade was composed of our own isolates, *P. sarkarianum* SEK 351 and *P. sarkarianum* SEK 364, and strain H1-14, F3-1 and NBRC 102984. The *Sicyoidochytrium* clade diverged with a long branch from the internal node and included our own isolate and strains: *S. minutum* SEK 354, *S. minutum* NBRC 102975, and NBRC 102979.

PUFA and carotenoid composition

PUFA profiles of arachidonic acid (AA, C20:4, *n*-6), eicosapentaenoic acid (EPA, C20:5, *n*-6), docosatetraenoic acid (DTA, C22:4), docosapentaenoic acid (DPA, C22:5, *n*-3 and *n*-6), and docosahexanoic acid (DHA, C22:6, *n*-3) were compared among the strains in the four genera and other strains including isolates reported by Huang et al. (2003). Figure 6 shows that *U. amoeboidea* SEK 214 and SEK 357 contained about 70% DHA whereas *Ulkenia* sp. ATCC 28207 contained about 15% arachidonic acid and about 50% DHA. All the *Sicyoidochytrium* clade strains contained less than 5% AA. *Botryochytrium radiatum* SEK 353 accumulated about 40% of *n*-6 DPA. All *Parietichytrium* clade strains contained about 10% DTA, which is unique among the thraustochytrid strains examined.

Ketocarotenoids profiles of β-carotene, echinenone, canthaxanthin, phoenicoxanthin, and astaxanthin were compared (Table 4). These pigments are intermediates of the synthetic pathway of astaxanthin from β-carotene. The strains of the *Ulkenia* clade possess all the pigments already mentioned except canthaxanthin, resulting in red-orange colonies. *Sicyoidochytrium* clade strains and *Botryochytrium* SEK 353 accumulate canthaxanthin and β-carotene, but not astaxanthin and phoenicoxanthin. Because most

Fig. 4. Light micrographs of the *Parietichytrium* clade strains (**a–l** *P. sarkarianum* SEK 351; **m–x** *P. sarkarianum* SEK 364). **a, m** Gathering cells with well-developed ectoplasmic nets (*arrows*) in seawater/pine pollen cultures. **b, n** Amoeboid cell. **c, o** Zoospore. **d, p** Mature sporangium, **e, f, q, r** Radiated cell division. **g–l, s–x** Continuous observation of zoospore formation [**g** mature sporangium; **h** amoeboid cell; **i** cell division; **j** spores without motility; **k** zoospores released; **l** remnant cell wall (*arrowheads*); **s** mature sporangium; **t** protoplast escaping from thick cell wall (*arrowheads*) with amoeboid motion; **u** rounded and matured sporangium; **v** cell divided into zoospores; **w** zoospores released; **x** remnant cell wall (*arrowheads*)]. *Bars* **a, m** 30 μm; **b–d, l, n–s** 5 μm

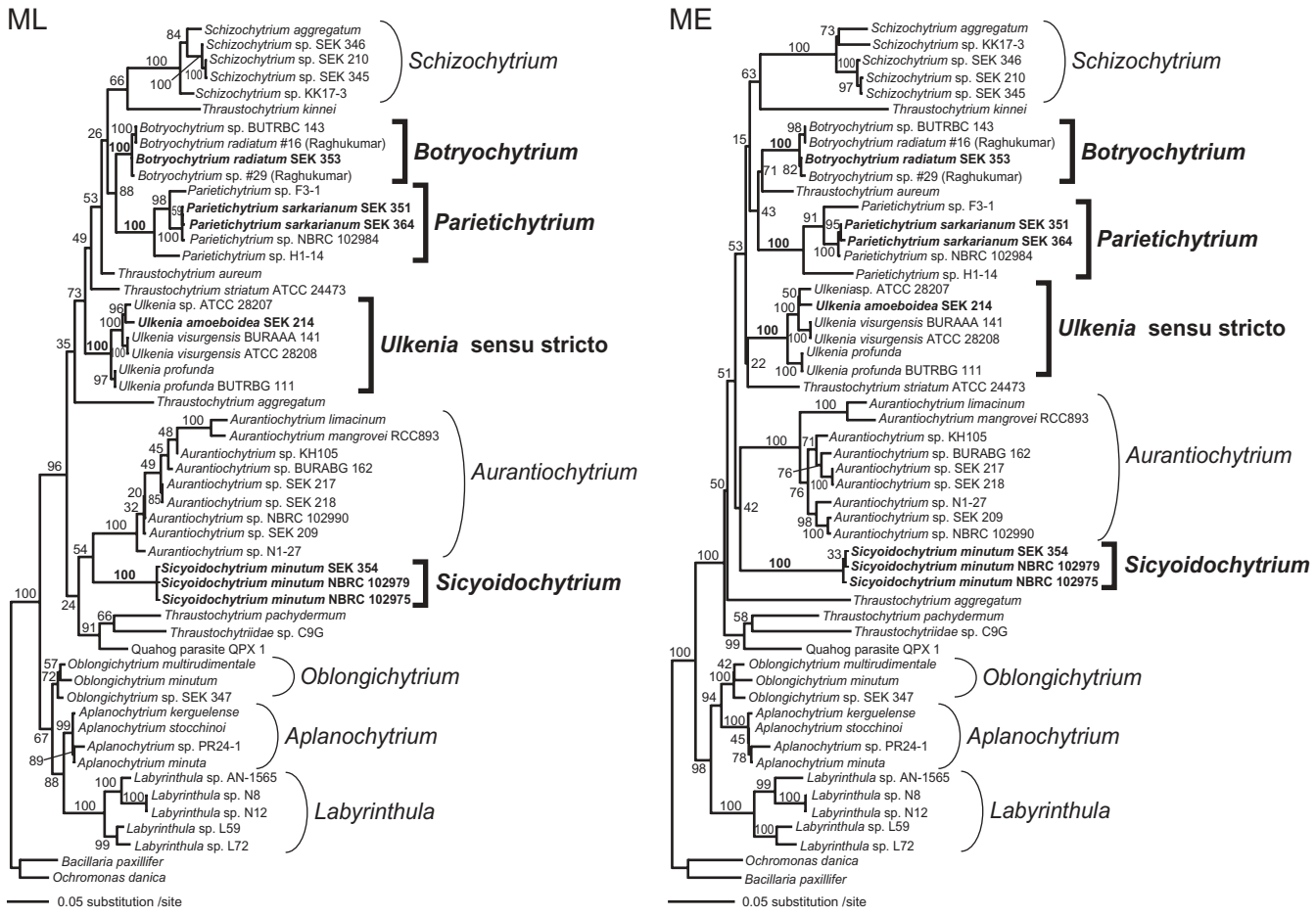


Fig. 5. Phylogenetic trees of Labyrinthulomycetes using 18S rRNA gene with *Bacillaria paxillifer* and *Ochromonas danica* as outgroup (53 operational taxonomic units; 1242 nucleotide sites). **a** Best maximum likelihood (ML) tree (log-likelihood = –12744.30779) on the HKY85 model (ts/tv ratio = 0.991175). **b** Best minimum evolution (ME) tree constructed from distances estimated by the ML method (log-likelihood = –12744.30779) with HKY85 model (ts/tv ratio = 0.991175). 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

Table 4. Comparison of carotenoids profiles among four clades: *Botryochytrium*, *Parietichytrium, Sicyoidochytrium,* and *Ulkenia* sensu stricto

	Astaxanthin	Phenicoxanthin	Canthaxanthin	Echinenon	β-Carotene
Genus *Ulkenia*					
Ulkenia amoeboidea SEK 214	+	+	–	+	+
Ulkenia sp. (*Japonocytrium* sp.) ATCC28207	+	+	–	+	+
Genus *Sicyoidochytrium*					
Sicyoidochytrium minutum SEK 354	–	–	+	+	+
Sicyoidochytrium sp. SEK 361	–	–	+	+	+
Sicyoidochytrium minutum NBRC 102975	–	–	+	+	+
Genus *Botryochytrium*					
Botryochytrium radiatum SEK 353	–	–	+	+	+
Genus *Parietichytrium*					
Parietichytrium sp. SEK 211	–	–	+[a]	–	+
Parietichytrium sp. SEK 212	–	–	–	–	+
Parietichytrium sarkarianum SEK 351	–	–	+[a]	–	+
Parietichytrium sarkarianum SEK 364	–	–	+[a]	–	+
Parietichytrium sp. NBRC 102984	–	–	–	–	+

[a] Pigments were detected at 450 nm by HPLC

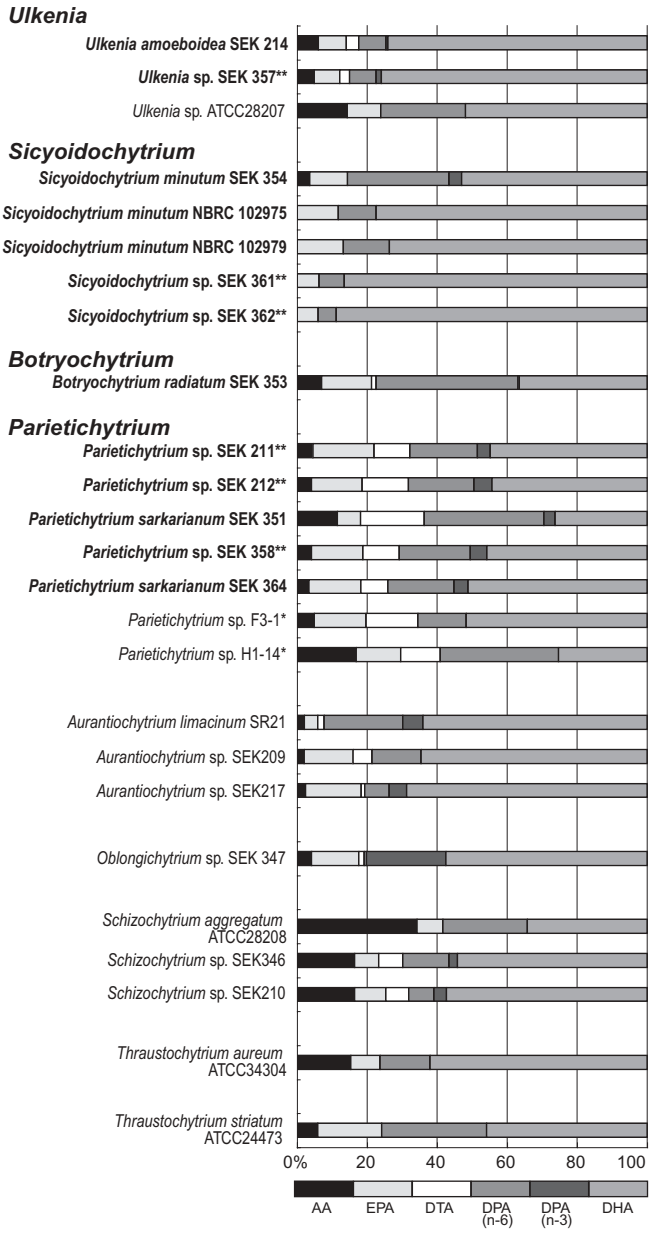


Fig. 6. Profiles of polyunsaturated fatty acids of thraustochytrid strains. *Bold characters* indicate the original isolates whose profiles were analyzed in this study. *Ulkenia amoeboidea* SEK 214 and SEK 357 each contain about 70% docosahexaenoic acid; *Ulkenia* sp. ATCC 28207 has about 15% arachidonic acid and about 50% docosahexaenoic acid; *Sicyoidochytrium* clade strains contain less than 5% arachidonic acid; *Botryochytrium radiatum* SEK 353 contains about 40% *n*-6 docosapentaenoic acid; *Parietichytrium* clade strains have a content of about 10% docosatetraenoic acid. Profiles of other strains are shown; data of the genus *Aurantiochytrium* strains, the genus *Oblongichytrium* strains, the genus *Schizochytrium*, *Thraustochytrium aureum* ATCC 34304, and *Thraustochytrium striatum* ATCC 24473 were previously reported in Yokoyama and Honda (2007). The data of the strains indicated by *single asterisks* were reported by Huang et al. (2003). Molecular phylogeny data of the strains indicated by *double asterisks* were not used in this study. *AA*, arachidonic acid (C20:4, *n*-6); *EPA*, eicosapentaenoic acid (C20:5, *n*-6); *DTA*, docosatetraenoic acid (C22:4); *DPA*, docosapentaenoic acid (C22:5, *n*-3 and *n*-6); *DHA*, docosahexanoic acid (C22:6, *n*-3)

strains of *Parietichytrium* clade only contain β-carotene, the colonies are light yellow.

Discussion

The genus *Ulkenia* was established by Gaertner (1977) by characterizing the naked protoplast stage within its life cycle. Two species, *U. visurgensis* and *U. amoeboidea*, were transferred from the genus *Thraustochytrium*, and four species were newly described (Gaertner 1977). However, more recently Honda et al. (1999) unequivocally showed that the *Ulkenia* species formed two distinct clades in the 18S rRNA gene tree. Sequence data of several strains identified as the genus *Ulkenia* have been recorded in the database (e.g., *Ulkenia visurgensis* strain BURAAA 141, *Ulkenia profunda* strain BUTRBG 111, and *Ulkenia* sp. strain BUTRBC 143, whose sequence data were recorded to GenBank by Jaritkhuan, Suanjit, and Manthachitra; see Table 2), and all of them belong to the two clades reported by Honda et al. (1999). The ex-type strains or identified strains of the genus *Ulkenia* sensu lato could not be obtained from culture collections at the present time. However, the molecular phylogenetic positions of the three species *U. visurgensis*, *U. profunda*, and *U. radiata* have been reported. Therefore, we carefully observed the morphology over the life history for our own isolates, and successfully determined strains identified as the following species, whose molecular phylogenetic data were not reported previously: *U. sarkariana*, *U. amoeboidea*, and *U. minuta*. Eventually, we revealed that all six *Ulkenia* species were located separately into four well-supported and distinct monophyletic groups in the 18S rRNA gene tree.

The first phylogenetic group includes *U. visurgensis*, *U. profunda*, and *U. amoeboidea*. *Ulkenia visurgensis* strain ATCC 28208 is the ex-type strain, and this species is the type species of the genus *Ulkenia*. *Ulkenia profunda* strain KMPB N 3077a is also the ex-type strain. These two species can be distinguished from each other by the presence or absence of proliferous bodies after releasing zoospores. Own isolate SEK-345 was identified as *U. amoeboidea* based on the following observations: (i) the protoplast is released from the pore of the cell wall, and (ii) the released protoplast is divided into four cells and transformed to zoospores. However, we have not yet found any characteristics that positively support the close relationships of these three species (see also Gaertner 1977; Raghukumar 1982a,b).

The *Japonochytrium* sp. strain ATCC 28207 is also located in this phylogenetic group. The monotypic genus *Japonochytrium* is characterized by the presence of the apophysis (i.e., swelling part) at ectoplasmic nets, and was originally isolated from the surface of the seaweeds *Gracilaria confervoides* (L.) Greville (red alga) and *Cladophora japonica* Yamada (green alga) at seashores of the Pacific Ocean in Japan (Kobayashi and Ookubo 1953). However, this strain ATCC 28207 is not the ex-type strain, which was previously isolated from the surface of *Laminaria* (brown alga) in England (Alderman et al. 1974). During our observations, the apophysis-like structure was never observed in either

enriched or pine pollen–seawater media. On the other hand, this strain released amoeboid cells and showed two to four connected amorphous cells, which is similar to the characteristics of *U. amoeboidea* (Bahnweg and Sparrow 1974; Gaertner 1977). It is difficult to give any conclusion about the identification and taxonomy of the genus *Japonochytrium* from our data. However, it is reasonable to assume that strain ATCC 28207 should be recognized as an unidentified species of *Ulkenia*, attaching significance to the transformation of the amoeboid cells.

The PUFA composition varies among the strains of the first phylogenetic group, but *U. amoeboidea* SEK-345 and *Ulkenia* sp. (deposited as *Japonochytrium* sp.) ATCC 28207 have a similar composition of carotenoid pigments, in which both strains lack the accumulation of canthaxanthin (Table 4). This composition was also found in *Schizochytrium* sensu lato (Yokoyama and Honda 2007), but it is unique in thraustochytrids. Therefore, *U. visurgensis*, *U. profunda* and *U. amoeboidea* should be recognized as species of the genus *Ulkenia* sensu stricto, whose monophyly is supported by the molecular phylogeny and carotenoid pigment composition.

The second phylogenetic group includes strains previously identified as *U. radiata* and *U. profunda*. There are two strains of *U. profunda* (i.e., BUTRBC 143 and #29), which were identified by S. Jaritkhuan, S. Suanjit, and V. Manthachitra, and S. Raghukumar, respectively (see also Honda et al. 1999). As mentioned earlier, the ex-type strain of *U. profunda* is located in a different group recognized as *Ulkenia* sensu stricto in the 18S rRNA gene tree. It is reasonable to treat these two strains as *Ulkenia* sp., not *U. profunda*. On the other hand, the characteristics of our original strain SEK-353 correspond well with the original description of *U. radiata*; i.e., the sporangium releases a amoeboid cell without leaving a sloughed cell wall, and the released amoeboid cell transforms into a star-shaped cell. The PUFA and carotenoid pigment compositions were analyzed and compared for only a single strain, SEK-353, in this group. The PUFA composition is characterized by a comparatively high amount (>40%) of n-6 DPA. This strain accumulates canthaxanthin, echinenon, and β-carotene, and the same composition is also obtained for the genus *Oblongichytrium* (Yokoyama and Honda 2007). Yokoyama and Honda (2007) proposed that distinct monophyletic groups of thraustochytrids in 18S rRNA gene tree should be treated as distinct genera, and the delimitations were also supported by their morphological and chemotaxonomic data. Based on this opinion, this second phylogenetic group should be classified at the genus level, and therefore we propose a new genus, *Botryochytrium*. However, only a single strain SEK-353 was analyzed for morphology, PUFA, and carotenoid comparison, so it is possible that the definition of this genus will need to be amended after sufficient accumulation of data.

The third phylogenetic group includes unidentified strains that form an independent lineage characterized by molecular phylogeny and PUFA composition. Huang et al. (2003) suggested that strains of each 18S rRNA molecular phylogenetic group shared a similar PUFA composition. Their original strains, F3-1 and H1-14, were characterized by accumulation of comparatively high amounts of DTA, and formed a distinct monophyletic group in the 18S rRNA gene tree. Our own isolates, SEK-211, -212, -351, -358, and -364, and NBRC 102984, form a well-supported monophyletic group and had a similar PUFA composition as strains F3-1 and H1-14 (data not shown). These strains should be identified as *Ulkenia sarkariana* sensu Gaertner (1977) based on their morphological features, that is, the wall of the vegetative cell is deposited after releasing the amoeboid cell. On the other hand, amoeboid cells of these strains transform into star-shaped cells, which behavior was not mentioned in the original description (Gaertner 1977). So, it is necessary to examine the presence of this characteristic in the isolates from the type locality, Brinkamahof in Germany, for final judgment of the species identification. The uniformity of this group also appears in the composition of carotenoid pigment, which consists only of β-carotene, occasionally with a trace of canthaxanthin. Therefore, we propose a new genus, *Parietichytrium*.

The last phylogenetic group is a newly recognized monophyletic lineage in the thraustochytrid phylogeny, which is composed of our own isolate SEK 354 and the NBRC strains. There are few variations in 18S rRNA gene sequences among the strains of this group, and the strains share the same composition of carotenoid pigments. The vegetative cells are comparatively small, for example, 1.5–2.5 × 2.5–4.2, and the amoeboid cell divides by a process of pinching and pulling, after which it gives rise to the zoospores. These characteristic features justify identifying these strains as *Ulkenia minuta* sensu Raghukumar (1977). These strains accumulate canthaxanthin, echinenon, and β-carotene, which is similar to the genera *Oblongichytrium* and *Botryochytrium* (Yokoyama and Honda 2007; present study). We propose a new genus *Sicyoidochytrium*, especially allocating importance to the zoospore formation via the process of pinching and pulling, i.e., dumbbell-like binary cell division.

This study and that of Yokoyama and Honda (2007) clearly show that the genus-level phylogenetic groups in the Labyrinthulomycetes can be distinguished by combination of morphological and chemotaxonomic features such as the profiles of PUFAs and carotenoid pigments, although it is hard to distinguish each group based on a single feature. Based on the general consideration for the past taxonomic researches and our studies, we propose the following key to the families and genera of the single order Labyrinthulales in the class Labyrinthulomycetes (Fig. 7). Strains of the genus *Thraustochytrium* and unidentified species still locate at the several independent lineages in the molecular phylogenetic tree (Fig. 5). It is necessary to establish the taxonomy for these organisms based on careful analyses and comparisons of both phenotypic and molecular characteristics.

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1. Spindle-shaped vegetative cells that glide inside ectoplasmic nets..... Family Labyrinthulaceae
Single genus: *Labyrinthula*

1. Globose or subglobose vegetative cells not enrobed in ectoplasmic nets..... Family Thraustochytriaceae
2. Migration of vegetative cells observed in an extended period and aplanospores always formed..... *Aplanochytrium*
2. Migration of vegetative cell, when present, at early stage..... 3
3. Vegetative cells without ectoplasmic nets..... *Althornia*
3. Vegetative cells with basal rhizoid-like ectoplasmic nets..... 4
4. Vegetative cells with apophysis in ectoplasmic nets..... *Japonochytrium*
4. Vegetative cells without apophysis in ectoplasmic nets..... 5
5. Thallus occurring successive binary division of vegetative cell..... 6
5. Thallus developing into a single zoosporangium or amoeboid cell..... 8
6. Small colony, not well-developed ectoplasmic net..... *Aurantiochytrium*
6. Large colony and well-developed ectoplasmic net..... 7
7. Oblong zoospores and producing canthaxanthin and β-carotene..... *Oblongichytrium*
7. Ovoid zoospores and producing only β-carotene..... *Schizochytrium*
8. Thallus developing into a single zoosporangium..... *Thraustochytrium*
8. Thallus developing into an amoeboid cell..... 9
9. Small colony, not well-developed ectoplasmic net..... 10
9. Large colony, well-developed ectoplasmic net..... 11
10. Zoospores forming by meaning of pinching and pulling..... *Sicyoidochytrium*
10. Zoospore forming without pinching and pulling division..... *Ulkenia*
11. Persistence of cell wall after releasing an amoeboid cell..... *Parietichytrium*
11. Cell wall dissolved after releasing an amoeboid cell..... *Botryochytrium*

Fig. 7. Key to the families and genera of the single order Labyrinthulales in the class Labyrinthulomycetes

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