Mar. Biotechnol. 5, 450–457, 2003
DOI: 10.1007/s10126-002-0110-1

MARINE BIOTECHNOLOGY

Grouping Newly Isolated Docosahexaenoic Acid-Producing Thraustochytrids Based on Their Polyunsaturated Fatty Acid Profiles and Comparative Analysis of 18S rRNA Genes

Jianzhong Huang,[1] Tsunehiro Aki,[1,*] Toshihiro Yokochi,[2] Toro Nakahara,[2] Daiske Honda,[3] Seiji Kawamoto,[1] Seiko Shigeta,[1] Kazuhisa Ono,[1] and Osamu Suzuki[1,†]

[1]*Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan*
[2]*Biological Resources Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Ibaraki 305-8566, Japan*
[3]*Department of Biology, Faculty of Science and Engineering, Konan University, Kobe 658-8501, Japan*

Abstract: Seven strains of marine microbes producing a significant amount of docosahexaenoic acid (DHA; C22:6, n-3) were screened from seawater collected in coastal areas of Japan and Fiji. They accumulate their respective intermediate fatty acids in addition to DHA. There are 5 kinds of polyunsaturated fatty acid (PUFA) profiles which can be described as (1) DHA/docosapentaenoic acid (DPA; C22:5, n-6), (2) DHA/DPA/eicosapentaenoic acid (EPA; C20:5, n-3), (3) DHA/EPA, (4) DHA/DPA/EPA/arachidonic acid (AA; C20:4, n-6), and (5) DHA/DPA/EPA/AA/docosatetraenoic acid (C22:4, n-6). These isolates are proved to be new thraustochytrids by their specific insertion sequences in the 18S rRNA genes. The phylogenetic tree constructed by molecular analysis of 18S rRNA genes from the isolates and typical thraustochytrids shows that strains with the same PUFA profile form each monophyletic cluster. These results suggest that the C20-22 PUFA profile may be applicable as an effective characteristic for grouping thraustochytrids.

Key words: 18S rRNA gene, docosahexaenoic acid, molecular phylogeny, polyunsaturated fatty acids, *Schizochytrium, Thraustochytrium.*

INTRODUCTION

Docosahexaenoic acid (DHA, C22:6, n-3), a typical long chain polyunsaturated fatty acid (PUFA), is an important component of cell membranes, especially in brains and retinas of mammals (Connor et al., 1992). Clinical studies have shown that DHA is essential for the growth and development of the brain in infants, and for maintenance of normal brain function in adults (Martinez, 1992). In addition, DHA has many positive effects on diseases such as hypertension, arthritis, artherosclerosis, depression, thrombosis, and cancers (Horrocks and Yeo, 1999).

As the importance of DHA becomes better understood, the demand for DHA as a dietary additive has been increased. However, conventional fish oils may not be suitable to meet the increasing market for DHA owing to their

Received June 13, 2002; accepted October 25, 2002.
†*Current address:* Ikeura Patent Office, Tokyo, Japan.
**Corresponding author: telephone +81-824-24-7755; fax +81-824-24-7754; e-mail aki@hiroshima-u.ac.jp*

limited supply, lower content of DHA, and peculiar taste and odor. Thus, the search for oleaginous microorganisms rich in DHA as commercial sources is becoming more urgent. Single cell oils from marine thraustochytrids, which are widely distributed in the oceans around the world, are a satisfactory alternative to fish oil as a DHA source (Bowles et al., 1999; Lewis et al., 1999). Although about 15 strains of thraustochytrids including *Thraustochytrium aureum, T. roseum, T. aggregatum, Schizochytrium limacinum*, and *S. aggregatum* have been reported to produce significant amounts of DHA (20% to 40% of the total fatty acids), there are many potential strains yet to be explored (Lewis et al., 1999; Huang et al., 2001).

Thraustochytrids have been characterized by the presence of a sagenogenetosome, an ectoplasmic net, a cell wall with noncellulosic scales, and a life cycle consisting of vegetative cells, zoosporangium, and zoospores (Anthony et al., 1988; Moss, 1991). In their classification history, the taxonomic criteria were mainly based on morphologic characteristics. However, their evolutionary relationships (Anthony et al., 1988) and taxonomy (Bahnweg and Jäckle, 1986) were not well understood until molecular analysis of their 18S ribosomal RNA genes showed conclusively that thraustochytrids were deeply divergent from oomycetes and close to labyrinthulids (Cavalier-Smith et al., 1994; Leipe et al., 1994). In particular, findings described by Honda et al. (1999) revealed that the morphologic characteristics of the thraustochytrids currently used as taxonomic criteria should be reevaluated. Thus, it is essential to recognize these microorganisms by other criteria in addition to the conventional morphological characteristics. In this study, we isolated several unreported DHA-rich thraustochytrids and grouped them according to their 18S rRNA genes and PUFA profiles.

Materials and Methods

Microorganisms and Culture Condition

Table 1 summarizes the sources and related information of the new isolates and typical strains used in this study. New thraustochytrid strains were isolated from seawater samples collected at several coastal areas in Hiroshima (strains H1-14 and H6-16), Ibaragi (N1-27 and N4-103), Miyagi (M4-103), and Nagasaki (A5-20) in Japan, and Fiji (F3-1), by using pine pollen as a bait. The collected zoospores were cultivated on a GPY-positive agar medium (20 g/L glucose, 10 g/L polypeptone, 5 g/L yeast extract, 15 g/L agar) and salinity equivalent to 50% of that of seawater (20 g/L sea salts; Sigma) until nonmycelial, yeast-like colonies appeared. *Thraustochytrium* sp. KK17-3 (Huang et al., 2001), *Schizochytrium limacinum* SR21 (Honda et al., 1998), and strain KH105 (T. Aki, manuscript in preparation) were directly activated from the reserve culture in our laboratory. The strain KH105 was tentatively allocated to the genus *Schizochytrium* because of its successive binary division of vegetative cells. A medium composed of 2% glucose and 1% yeast extract at salinity equivalent to 50% of that of seawater was used for the liquid cultivation unless otherwise indicated. The culture was shaken on a rotary shaker at 160 rpm in a 300-ml baffled flask containing 50 ml of the medium at 28°C for 4 days.

Fatty Acid Composition Analysis

At stationary growth phase, the cultured cells were harvested by centrifugation at 6000*g* for 20 minutes and then washed twice with deionized water. A portion of the wet cells were dried at 105°C for 12 to 18 hours, then their weights were gravimetrically measured for biomass determination. The other wet cells were used directly for extraction of total lipids by a chloroform-methanol (2:1, vol/vol) mixture in the presence of glass beads, as described previously (Aki et al., 1998). The extraction process was repeated 2 or 3 times to collect as much of the total lipid mass as possible. The total lipids were analyzed as fatty acid methyl esters, which were prepared by treatment with 10% methanolic HCl with the addition of eicosanoic acid (C20:0) as an internal standard. The samples were applied to a gas-liquid chromatograph (GC-17A; Shimazu) equipped with a flame ionization detector and a split injector on a TC-70 capillary column (GL Science) with temperature programming (190° to 220°C at 2°C/min). Fatty acid methyl ester peaks were identified and calibrated with the corresponding standard fatty acid methyl esters. Data were provided on the averages of 2 or 3 determinations.

Isolation and Sequencing of 18S rRNA Gene

The cultured cells harvested by centrifugation were ground into powder by treatment with liquid nitrogen and then lysed with TNE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.0). The genomic DNA was extracted by

Table 1. Strains Used in This Study With Molar% GC and Database Accession Numbers of Their 18S rRNA Genes

Taxon	Strain[a]	Mol% GC	Accession number
Stramenopiles, Labyrinthulida			
Aplanochytrium haliotidis[b]	n/a	44.4	U21338
Aplanochytrium kerguelense	KMPB N-BA-107	45.1	AB022103
Aplanochytrium minuta[b]	n/a	43.8	L27634
Japonochytrium sp.	ATCC 28207	45.0	AB022104
Labyrinthula sp.	AN-1565 (Nakagiri)	45.0	AB022105
Schizochytrium aggregatum	ATCC 28209	50.4	AB022106
Schizochytrium limacinum	NIBH SR21	45.1	AB022107
Schizochytrium minutum	KMPB N-BA-77	44.1	AB022108
Thraustochytrium aggregatum	KMPB N-BA-110	43.8	AB022109
Thraustochytrium aureum	ATCC 34304	47.4	AB022110
Thraustochytrium kinnei	KMPB 1694d	46.4	L34668
Thraustochytrium multirudimentale	KMPB N-BA-113	44.3	AB022111
Thraustochytrium pachydermum	KMPB N-BA-114	40.4	AB022113
Thraustochytrium striatum	ATCC 24473	44.4	AB022112
Ulkenia profunda	#29 (Raghukumar)	44.2	AB022114
Ulkenia profunda	KMPB N 3077 a	44.6	L34054
Ulkenia radiata	#16 (Raghukumar)	44.2	AB022115
Ulkenia visurgensis	ATCC 28208	45.1	AB022116
Isolates	A5-20	45.9	AB073303[d]
	F3-1[c]	45.1	AB073304[d]
	H1-14	45.1	AB073305[d]
	H6-16	50.2	AB073306[d]
	KH105[c]	49.3	AB052555
	KK17-3[c]	50.8	AB052556
	M4-103	42.8	AB073307[d]
	N1-27[c]	48.5	AB073308[d]
	N4-103	51.8	AB073309[d]
Stramenopiles, Bicosoecida			
Cafeteria roenbergensis	n/a	46.9	L27633
Alveolata, Dinoflagellata			
Prorocentrum micans	CAC LB113614	46.0	M14649

[a]Abbreviations of the culture collections: ATCC, American Type Culture Collection; CAC, Cambridge Algae Collection (U.K.); KMPB, Kulturensammlung Mariner Pilze Bremerhaven, Alfred-Wegener-Institut für Polar und Meeresforschung (Germany): NIBH, National Institute of Bioscience and Human-Technology (Japan). n/a, not available.

[b]*A. haliotidis* and *A. minuta* had been considered to belong to the genus *Labyrinthuloides* (Leander and Porter, 2000).

[c]Strains F3-1 (Lali et al., 2001) and KK17-3 (Huang et al., 2001) most likely belong to the genus *Thraustochytrium,* and strains KH105 and N1-27 (Lali et al., 2001) to the genus *Schizochytrium.*

[d]Determined in this study.

a standard phenol and chloroform–isoamyl alcohol protocol (Murray and Thompson, 1980). To obtain an almost complete 18S rRNA gene, we used a polymerase chain reaction (PCR) protocol with amplification primers 18S1, 5′-TACCTGGTTGATCCTGCCAG-3′, and 18S12, 5′-CCTTCCGCAGGTTCACCTAC-3′ (Honda et al., 1999). A 50-μl PCR reaction mixture contained 5 μl of 10× PCR buffer, 20 mM each of deoxyribonucleotide triphosphates, 0.5 μM of each primer, 2 U of *Taq* polymerase (Takara Shuzo, and 1 μg of the genomic DNA. After initial denaturing at 95°C for 5 minutes, PCR amplification was performed in the thermal cycler (Gene Amp PCR System 9600; Applied Biosystems Inc.), using a program of 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C for 30 cycles, followed by a 10-minute extension at 72°C. The PCR product was purified by CONCERT Rapid PCR Clean-up System

(Life Technologies, Inc.) and then directly used or cloned into pGEM-T Easy Vector (Promega) for sequence analysis with a capillary DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems Inc.) according to the manufacturer's instruction. Sequences were determined for both strands of 18S rRNA gene using primers 18S1, 18S12, and other primers that correspond to the internal region of the DNA template (Honda et al., 1999).

Molecular Phylogenetic Analysis

The nucleotide sequences of 18S rRNA genes from the related microorganisms were obtained from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome.html). Sequences to be examined were aligned through a profile alignment process by CLUSTAL W for Power PC, Version 1.74 (Thompson et al., 1994). The positions with gaps and undetermined or ambiguous bases were removed for further analysis. The sequence alignment file (alignment number: ALIGN_000240) was available from the European Bioinformatics Institute FTP server (ftp://FTP.EBI.AC.UK/pub/databases/embl/align/) by anonymous FTP. Phylogenetic trees were generated using the neighbor-joining (NJ) method (Saitou and Nei, 1987) and the maximum-likelihood (ML) method (Felsenstein, 1981) with taxons *Prorocentrum micans* and *Cafeteria roenbergensis* as an outgroup. The NJ analysis was performed using PAUP* Version 4.0b10 (Swofford, 1998), and distances were estimated by the ML method with Felsenstein's (F84) model (Felsenstein, 1984). A tree with a maximum log-likelihood value was obtained when the transition-to-transversion ratio was 0.81. The statistical significance of the tree branches was assessed by 1000 bootstrap resamplings (Felsenstein, 1985).

Results

PUFA Profiles Produced by Thraustochytrid Isolates

In liquid cultures, all of the isolates except H1-14 and F3-1 produced a significant amount of DHA comprising 22% to 53% of total intracellular fatty acids (Figure 1). Their DHA contents were equivalent to those produced by previously isolated strains such as *S. limacinum* SR21 (34.1%), *Schizochytrium* sp. KH105 (46.9%), and *Thraustochytrium* sp. KK17-3 (52.1%).

However, it was noted that the occurrence of PUFAs other than DHA greatly varied among the new isolates and

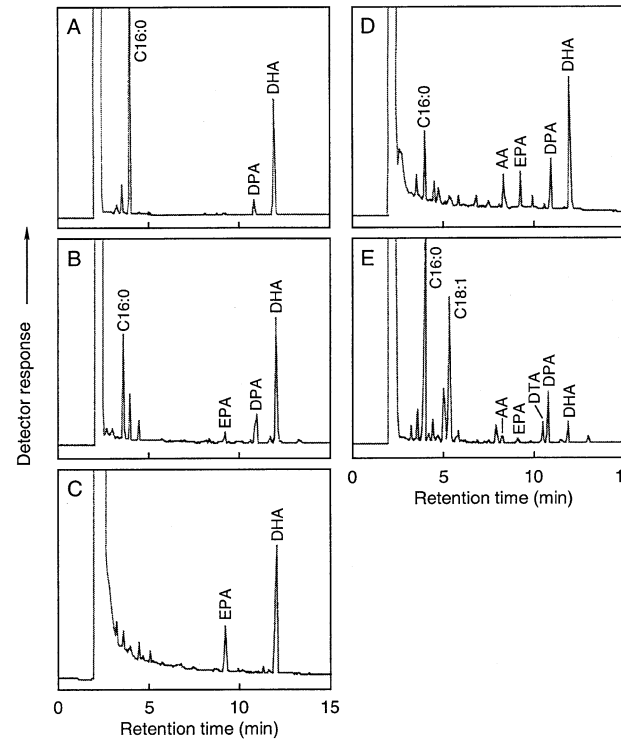

Figure 1. Typical gas chromatograms of total fatty acid from the isolated strains A5-20 (**A**), N1-27 (**B**), M4-103 (**C**), N4-103 (**D**), and H1-14 (**E**). C16:0, palmitic acid; C18:1, oleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DTA, docosatetraenoic acid; DPA, n-6 docosapentaenoic acid; DHA, docosahexaenoic acid.

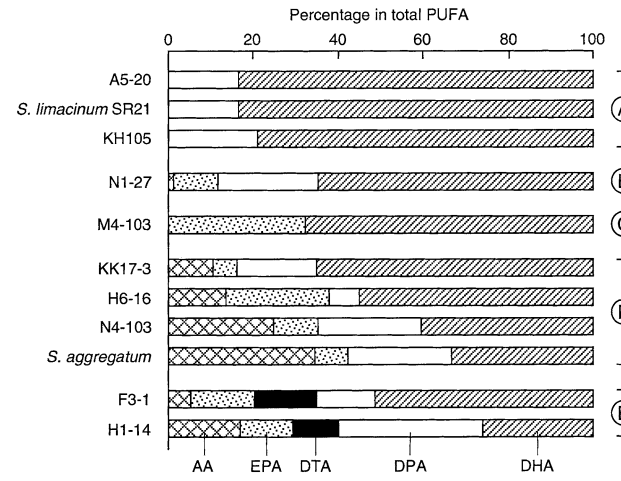

Figure 2. Grouping of the isolates based on their C20 and C22 PUFA profiles. Abbreviations are in the legend to Figure 1.

preserved strains. In light of the presence of respective PUFAs, they could be categorized into the following 5 groups. Strains A5-20 (Figure 1, A) and KH105 as well as *S. limacinum* SR21 accumulating only n-6 docosapentaenoic acid (DPA, C22:5, n-6) at 12.4%, 7.7%, and 6.7% of total

Table 2. Pairwise Similarity and Distance Matrix of 18S rRNA Sequences of Thraustochytrids and *Prorocentrum micans* (out group)[a]

Organism	1	2	3	4	5	6	7	8	9	10	11	12
1 A5-20	—	0.0203	0.0928	0.1024	0.1541	0.1946	0.1942	0.2013	0.1965	0.1819	0.1826	0.2052
2 *S. limacinum*	91.9	—	0.1182	0.1288	0.1761	0.2187	0.2255	0.2253	0.2220	0.2100	0.2093	0.2390
3 KH105	84.7	83.1	—	0.0743	0.1685	0.1792	0.1935	0.1870	0.1808	0.1838	0.1848	0.2045
4 N1-27	81.5	81.1	87.3	—	0.1683	0.1870	0.2017	0.1996	0.1966	0.1889	0.1888	0.2130
5 M4-103	74.4	75.1	76.3	75.6	—	0.1813	0.1780	0.1746	0.1747	0.1634	0.1563	0.2004
6 N4-103	72.8	73.5	77.3	77.7	74.8	—	0.0833	0.0650	0.0896	0.1845	0.1853	0.2167
7 KK17-3	74.0	74.1	77.9	75.1	75.3	85.5	—	0.0854	0.0642	0.1856	0.1830	0.2272
8 *S. aggregatum*	73.8	74.7	76.2	75.7	76.1	86.5	86.4	—	0.0847	0.1885	0.1854	0.2185
9 H6-16	73.5	74.1	77.2	75.8	75.8	84.6	89.9	85.5	—	0.1782	0.1881	0.2274
10 F3-1	73.0	73.6	75.4	74.6	75.6	74.9	75.8	75.4	76.6	—	0.1028	0.2174
11 H1-14	73.6	74.4	77.2	75.5	76.4	75.3	77.8	75.8	77.0	83.9	—	0.2164
12 *P. micans*	72.1	72.4	74.9	75.0	72.0	73.2	74.0	73.2	72.8	73.3	74.0	—

[a]The values on the *lower left* diagonal section are percentages of similarity between the sequences, and on the *upper right* the 18S rRNA evolutionary distances as estimated by the maximum-likelihood method with Felsenstein's model (Felsenstein, 1984).

fatty acids, respectively, in addition to DHA, formed group A. Strain N1-27 (Figure 1, B) accumulating DPA and eicosapentaenoic acid (EPA, C20:5, n-3) at 19.1% and 8.5%, respectively, could be assigned to group B. Strain M4-103 (Figure 1, C) alone made up group C because of the accumulation of EPA at 31.0% but not DPA. The other strains including N4-103 (Figure 1, D), H6-16, and KK17-3 could be allocated to group D owing to their production of arachidonic acid (AA, C20:4, n-6) at 13.6%, 7.0%, and 6.3%, respectively, besides DPA as well as EPA. The typical strain *S. aggregatum* ATCC 28209 was also examined and classified into group D. Strains H1-14 (Figure 1, E) and F3-1 could be discriminated from the other isolates by the presence of n-6 docosatetraenoic acid (DTA, C22:4, n-6; 11–15% of total PUFAs) in addition to the accumulation of oleic acid (C18:1, n-9) as a major unsaturated fatty acid (22% to 50%) with a lower DHA content (less than 10%). Thus, it was reasonable to assign the strains H1-14 and F3-1 to a separate group (group E).

The propriety of our categorization of the thraustochytrids into 5 groups by PUFA profile comparison is further highlighted by the composition of C20 and C22 PUFA as shown in Figure 2. Here, it should be pointed out that the C20 and C22 PUFA profiles of each isolate were not liable to change with different media consisting of either glucose (5 g/L), peptone (1 g/L), and yeast extract (1 g/L), or glucose (20 g/L) and corn steep liquor (5 g/L), although the occurrence of short-chain fatty acids (less than C18) varied slightly with the different culture media (data not shown). In addition, the cultivation temperature showed little effect on the PUFA profiles in the new isolates, although low temperature (15°C) could improve the total content of long-chain PUFAs in the fatty acid fraction compared with the normal temperature (28°C) (data not shown). Such stability of the C20 and C22 PUFA profile increased its validity as an effective characteristic for grouping these thraustochytrids.

Molecular Phylogenetic Analysis on 18S rRNA Gene Sequence

The 18S rRNA genes from the new isolates were sequenced, and 2088 nucleotides sites were examined for sequence comparison. The selected sites contained the relatively compact molar percentage of G + C (Table 1) ranging from 40.4% (*T. pachydermum*) to 51.8% (strains N4-103). This check could prevent bias on the proper phylogeny estimations (Hasegawa and Hashimoto, 1993).

All of the new isolates were found to possess an insertion sequence in the region between the nucleotide positions 1690 and 1718 (strain A5-20, TTAATTCTAATTTTTGGAA; F3-1, TTCAGTTTCTTTTGA; H1-14, TTTTTATCATTTCAT; M4-103, TTTTTGTTTCTTTCATTTTTTGATTGAAC; N1-27, TTTTC GCTGCTCG; N4-103, TGGGCGTTG; H6-16, TCTTTTCTTTGGGA), which was characteristic of the thraustochytrid phylogenetic group described by Honda et al. (1999). Although the sequences in the insertion region were not significantly homologous to each other, their occurrence completely warranted grouping, the 7 new isolates as thraustochytrids.

As shown in the lower left of Table 2, comparatively higher homologies were found within the strains with the same PUFA profile. For example, sequence homologies between strains inside group D (including strains N4-103, KK17-3, H6-16 and *S. aggregatum)* indicated comparatively higher values (84.6% to 89.9%), whereas their homologies to the other strains outside group D were less than 80%. This partially supported the grouping with respect to their PUFA patterns.

Based on the distance-matrix values given in Table 2, a phylogenetic tree was constructed by the NJ method as depicted in Figure 3. The allocations of the known thraustochytrids in our tree were essentially consistent with previous reports (Cavalier-Smith et al., 1994; Honda et al., 1999). As for the new isolates, strains A5-20 and KH105 formed the phylogenetic cluster A, together with *S. limacinum* SR21, which was supported by a high bootstrap value (100% on the NJ tree). The strain M4-103 formed a cluster with *Aplanochytrium haliotidis.* Four strains including N4-103, KK17-3, H6-16, and *S. aggregatum* constituted the monophyletic cluster D. Furthermore, strains H1-14 and F3–1 formed an independent cluster E. The last 2 groupings were apparently robust, supported by a bootstrap value of 100% on the NJ tree. A phylogenetic tree generated by the ML method also revealed similar configurations of each cluster and positioning of the isolates as grouped by PUFA profiles (data not shown).

It should be noted that the strain N1-27 (group B) is very close to the strains of group A, especially to KH105, in the phylogenetic tree. In fact, their 18S rRNA gene sequences were up to 87.3% homologous (Table 2), which partly accounted for their close relationship. This also could be reflected by their close similarity in PUFA profiles (Figure 2).

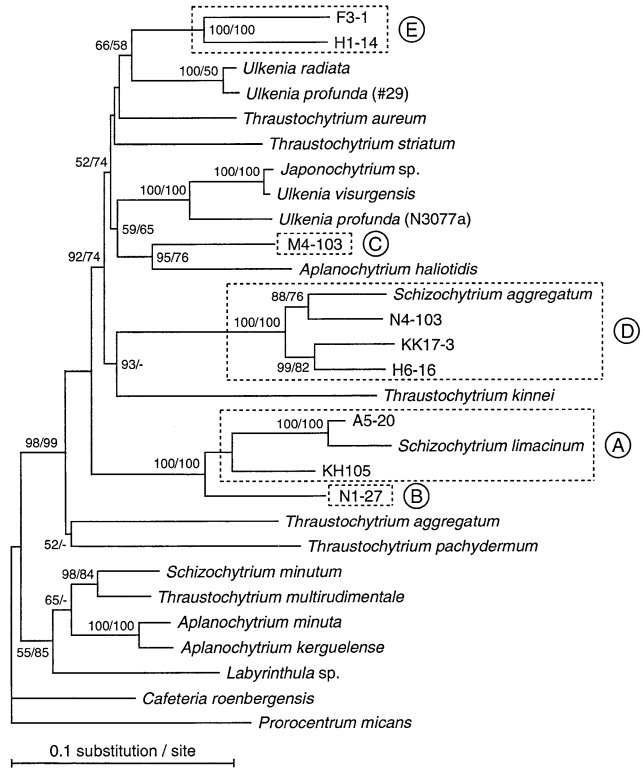

Figure 3. Phylogenetic tree based on 18S rRNA genes from thraustochytrids with *Prorocentrum micans* and *Cafeteria roenbergensis* as an outgroup. The neighbor-joining (NJ) tree was constructed from the distances estimated by the maximum likelihood (ML) method with F84 models. The numbers at each internal branch show bootstrap values (NJ/ML) only for the nodes supported by more than 50% of 1000 replicates.

Discussion

In our phylogenetic tree, none of the 3 typical genera including *Thraustochytrium, Schizochytrium,* and *Aplanochytrium* could form each monophylogenetic group. For example, *S. aggregatum* and *S. limacinum,* both exhibiting successive division of vegetative cells which was considered a key criterion for identification of the genus *Schizochytrium* (Goldstein and Belsky, 1964; Moss, 1986), scattered in separate nodes of the phylogenetic tree. In addition, such strains as *T. kinnei, T. aureum,* and *Ulkenia profunda,* which produced proliferation bodies, dispersed in different phylogenetic clusters (Figure 3). This clearly indicated that the morphologic characteristics could not accurately reflect the evolutionary relationships between thraustochytrids. On the contrary, our new and preserved isolates possessing the same PUFA profile formed each phylogenetic block (Figure 3). For example, strains A5-20, KH105, and *S. limacinum* SR21 having the DHA/DPA profile formed cluster A in the phylogenetic tree. Similarly, strains with profiles DHA/DPA/EPA, DHA/EPA, DHA/DPA/EPA/AA, or DHA/DPA/EPA/AA/DTA constituted their individual phylogenetic clusters. Moreover, PUFA profiles in the isolates were not liable to change with different culture media or temperatures. The results indicated that PUFA profiles could be an effective characteristic for grouping these new thraustochytrids.

In practice, the fatty acid composition of cellular lipids has proved to be a valuable and stable criterion to characterize, differentiate, and identify various organisms including bacteria (Guckert et al., 1991), fungi (Stahl and Klug,

1996), yeasts (Gunasekaran and Hughes, 1980), and higher plants (Wolff et al., 2001). The PUFA composition has also been a practical chemotaxonomic marker at the subgeneric level for fungus *Mortierella* that produces a significant amount of C18 and C20 PUFA (Amano et al., 1992). These data underlined the notion that the fatty acid compositions could offer valuable physiologic information that might not be available only from morphologic characteristics.

In general, PUFAs were synthesized via alternating steps of desaturation and elongation of precursor fatty acid. One pathway involved in the elongation of EPA and AA to C22:5n-3 and DTA, respectively, and the desaturation of the latter to form DHA and DPA by the final Δ4 desaturation, was recently confirmed in *Thraustochytrium* sp. (Qiu et al., 2001). Given that the Δ4 desaturation step is rate-limiting, DTA and C22:5n-3 are expected to accumulate as observed in our strains F3-1 and H1-14. Besides, the strain M4-103 was unusual because it lacked n-6 PUFA including DPA (n-6), which has been regarded as an identification marker for the order Labyrinthulales (Ellenbogen et al., 1969; Findlay et al., 1986). This may be because of a high activity on ω-3 (n-3) desaturation in M4-103, which is a key reaction for conversion of n-6 fatty acids to n-3 (Cook, 1996). The other pathway for PUFA biosynthesis involving fatty acid enzymes related to synthase and polyketide synthase, but not the above-mentioned enzymes (desaturase and elongase), has been proposed in *Schizochytrium* (Metz et al., 2001). Since the double bonds on the PUFA molecules were formed by dehydration and isomerization of keto groups in cycles of polyketide-forming chain elongation reaction, the C20 PUFA may not be the direct precursor of C22 PUFA in *Schizochytrium.* The related issue to be solved will be whether or not the thraustochytrids carrying Δ4 desaturase have such a distinct pathway for PUFA biosynthesis.

Briefly, the different PUFA profiles present in our new isolates probably resulted from the distinct enzymes and/or their regulations responsible for PUFA biosynthesis. These distinguishable PUFA profiles in the isolates could reflect their individual physiologic characteristics, which was supported by the coincidence in grouping them based on the analyses of their PUFA profile and 18S rRNA genes. In view of their high DHA contents and different PUFA profiles, these new isolates not only show potential as commercial DHA sources, but also provide excellent models for elucidating the mechanism of DHA biosynthesis in thraustochytrids by the genetic approach.

References

Aki, T., Matsumoto, Y., Morinaga, T., Kawamoto, S., Shigeta, S., Ono, K., and Suzuki, O. (1998). Lipid composition of newly isolated polyunsaturated fatty acid producing fungus, *Achlya* sp. ma-2801. *J Ferment Bioeng* 86:504–507.

Amano, N., Shinmen, Y., Akimoto, K., Kawashima, H., Amachi, T., Shimizu, S., and Yamada, H. (1992). Chemotaxonomic significance of fatty acid composition in the genus *Mortierella* (Zygomycetes, Mortierellaceae). *Mycotaxon* 45:257–265.

Anthony, H., Chamberlain, L., and Moss, S.T. (1988). The thraustochytrids: a protist group with mixed affinities. *Biosystems* 21:341–349.

Bahnweg, G., and Jäckle, I. (1986). A new approach to taxonomy of the thraustochytriales and labyrinthulales. In: *The Biology of Marine Fungi,* Moss, S.T. (ed). Cambridge, U.K.: Cambridge University Press, 131–140.

Bowles, R.D., Hunt, A.E., Bremer, G.B., Duchars, M.G., and Eaton, R.A. (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.

Cavalier-Smith, T., Allsopp, M.T.E.P., and Chao, E.E. (1994). Thraustochytrids are chromists, not fungi: 18S rRNA signatures of Heterokonta. *Phil Trans R Soc Lond B* 346:387–397.

Connor, W.E., Neuringer, M., and Reisbick, S. (1992). Essential fatty acids: the importance of n-3 fatty acids in the retina and brain. *Nutr Rev* 50:21–29.

Cook, H.W. (1996). Fatty acid desaturation and chain elongation. in eukaryotes In: *Biochemistry of Lipids, Lipoproteins and Membranes,* Vance, D.E., and Vance, J.E. (eds.). Amsterdam: Elsevier Science, 129–152.

Ellenbogen, B.B., Aaronson, S., Goldstein, S., and Belsky, M. (1969). Polyunsaturated fatty acids of aquatic fungi: possible phylogenetic significance. *Comp Biochem Physiol* 29:805–811.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 17:368–376.

Felsenstein, J. (1984). Distance methods for inferring phylogenies: justification. *Evolution* 38:16–24.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791.

Findlay, R.H., Fell, J.W., Coleman, N.K., and Vestal, J.R. (1986). In: *The Biology of Marine Fungi,* Moss, S.T. (ed). Cambridge, U.K.: Cambridge University Press, 91.

Goldstein, S., and Belsky, M. (1964). Axenic culture studies of a new marine phycomycete possessing an unusual type of asexual reproduction. *Am J Botany* 51:72–78.

Guckert, J.B., Ringelberg, D.B., White, D.C., Hanson, R.S., and Bratina, B.J. (1991). Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. *J Gen Microbiol* 137:2631–2641.

Gunasekaran, M., and Hughes, W.T. (1980). Gas-liquid chromatography: a rapid method for identification of different species of Candida. *Mycologia* 72:505–511.

Hasegawa, M., and Hashimoto, T. (1993). Ribosomal RNA trees misleading? *Nature* 361:23.

Honda, D., Yokochi, T., Nakahara, T., Erata, M., and Higashihara, 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., and Higashihara, T. (1999). Molecular phylogeny of labyrinthulids and thraustochytrids based on the sequencing of 18S ribosomal RNA gene. *J Eukaryot Microbiol* 46:637–647.

Horrocks, L.A., and Yeo, Y.K. (1999). Health benefits of docosahexaenoic acid (DHA). *Pharmacol Res* 40:211–225.

Huang, J., Aki, T., Hachida, K., Yokochi, T., Kawamoto, S., Shigeta, S., Ono, K., and Suzuki, O. (2001). Profile of polyunsaturated fatty acids produced by *Thraustochytrium* sp. KK17-3. *J Am Oil Chem Soc* 78:605–610.

Leipe, D.D., Wainright, P.O., Gunderson, J.H., Porter, D., Patterson, D.J., Valois, F., Himmerich, S., and Sogin, M.L. (1994). The stramenopiles from a molecular perspective: 16S-like rRNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis. Phycologia* 33:369–377.

Lewis, T.E., Nichols, P.D., and McMeekin, T.A. (1999). The biotechnological potential of thraustochytrids. *Mar Biotechnol* 1:580–587.

Martinez, M. (1992). Tissue levels of polyunsaturated fatty acids during early human development. *J Pediatr* 120:S129–138.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., and Browse, J. (2001). Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* 293:290–293.

Moss, S.T. (1986). Biology and phylogeny of the Labyrinthulales and Thraustochytriales. In: *The Biology of Marine Fungi,* Moss, S.T. (ed). New York, N.Y.: Cambridge University Press, 105–129.

Moss, S.T. (1991). Thraustochytrids and other zoosporic marine fungi. In: *The Biology of Free-living Heterotrophic Flagellates,* Patterson, D.J., Larsen, J. (eds). Oxford: Clarendon Press, 415–425.

Murray, M.G., and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8:4321–4325.

Qiu, X., Hong, H., and MacKenzie, S.L. (2001). Identification of a delta 4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea. J. Biol Chem* 276:31561–31566.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.

Stahl, P.D., and Klug, M.J. (1996). Characterization and differentiation of filamentous fungi based on fatty acid composition. *Appl Environ Microbiol* 62:4136–4146.

Swofford, D.L. (1998). PAUP*. Phylogenetic analysis using parsimony. Version 4. Sunderland, Mass.: Sinauer Associate.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (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.

Wolff, R.L., Lavialle, O., Pedrono, F., Pasquier, E., Deluc, L.G., Marpeau, A.M., and Aitzetmuller, K. (2001). Fatty acid composition of pinaceae as taxonomic markers. *Lipids* 36:439–451.