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Development of a PCR strategy for thraustochytrid identification based on 18S rDNA sequence

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Abstract The identification and characterization of the thraustochytrids, an emerging economically and biotechnologically important group of marine heterotrophic protists, is usually based on morphological characters. In this research we used molecular markers to identify thraustochytrids. We designed three sets of primers based on the 18S rDNA sequence alignment of known strains and employed a PCR (polymerase chain reaction) strategy to identify unknown thraustochytrid isolates. DNA from 26 thraustochytrids (three isolated from primary cell cultures of the tunicate *Botryllus schlosseri* and 23 from a coral holding aquarium) were amplified by these primers, revealing 21 isolates with three bands each, which were assigned to two groups according to PCR fragment sizes. Taxonomic characterizations were deduced by comparing with GenBank data. Four isolates were further studied by sequencing their 18S rDNA. Sequence alignments and phylogenetic analysis revealed that isolates from the coral aquarium (7-5 and 8-7) were highly similar to each other and 95.0–97.0% similar to *Thraustochytrium multirudimentale* and *Schizochytrium minutum*. Isolates from the tunicate primary cell cultures (BS1 and BS2) were also closely related to each other and 84.3–86.0% similar to labyrinthulid quahog parasite and *Thraustochytrium pachydermum*. AFLP (amplified fragment-length polymorphism) analysis revealed 2.5–3.6% differences within the genomic DNA of each group, showing that each isolate is different, although isolates within each group may belong to the same species, in spite of differences found in the general morphology.

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Introduction

The thraustochytrids, a common group of marine heterotrophic protists, are known as pathogens of edible invertebrates (Bower 1987; Mass et al. 1999), as predominant contaminants of marine invertebrate cell cultures (Rinkevich 1999, and literature therein), and as potential sources for valuable bioactive compounds (Lewis et al. 1998; Bowels et al. 1999). This last characteristic has been further illuminated by our results on primary cell cultures of sponges and tunicates (Rinkevich and Rabinowitz 1993; Ilan et al. 1996), revealing that thraustochytrid-infected cultures resist bacterial contamination better than freshly prepared cultures. An area of research we are currently pursuing is the isolation of thraustochytrids that produce novel bioactive compounds. A major obstacle, however, is the identification and proper systematic classification of different isolates.

Thraustochytrids belong to the phylum Labyrinthulomycota (Porter 1990) and were studied in the past mainly by mycologists (Barr 1992). Cells form naked globose or colonial structures, associated with thin or more-developed, scaled cell walls. Many of them develop ectoplasmic networks generated by a unique organelle termed the sagenogen (or sagenogenetosome). Some growing cells exhibit a gliding mobility associated with the ectoplasmic networks. Their reproduction activities involve the formation of heterokont, biflagellate zoospores (Porter 1990).

The phylum Labyrinthulomycota contains a single class, Labyrinthulea, and a single order, the Labyrinthulida, with two families, the Labyrinthulidae and the Thraustochytriidae (Olive 1975). Eight genera have been described in the family Thraustochytriidae through the use of morphological characterizations: *Althornia*, *Aplanochytrium*, *Corallochytrium*, *Japonochytrium*, *Labyrinthuloides*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia*, comprising more than 30 species (Raghukumar 1987; Porter 1990). However, *Corallochytrium* was later characterized by its 18S rRNA gene sequence and

phylogenetic analysis as a choanoflagellate protozoan, belonging to the Corallochytriidae family (Cavalier-Smith and Allsopp 1996). Half of all species reported in the Thraustochytriidae family (15 species) belong to the genus *Thraustochytrium*. Thraustochytrids were usually characterized by their morphological and developmental characteristics, such as the sorus development, the ectoplasmic net, the form of zoospores, the thickness of the sorus wall, the pigmentation of the thalli, the cleavage planes of the developing spores, and whether the zoospores are immediately motile or quiescent for a time after release (Porter 1990). According to Gaertner (1972), these organisms could be identified only if observed under standardized conditions, such as when pollen-baited seawater was used. Typically morphological phenotypes were unstable because of different nutritional conditions.

To overcome identification and classification difficulties, the gene encoding for the small subunit ribosomal RNA (SSU rDNA or 18S rDNA) has been widely used for molecular characterization and for phylogenetic analysis; the gene's ubiquity and low evolutionary rate are particularly advantageous in this respect (Cavalier-Smith 1993; Margulis et al. 1996; Van de Peer et al. 2000). Some thraustochytrids have already been studied on the basis of their 18S rDNA sequence (Cavalier-Smith et al. 1994; Leipe et al. 1994, 1996; Honda et al. 1999; Mass et al. 1999; Ragan et al. 2000) and through phylogenic analysis.

In the present study we aligned all the known 18S rDNA sequences from the phylum Labyrinthulomycota. Primers were designed accordingly and were used to identify thraustochytrids isolated from the seawater of a coral holding aquarium and from primary cell cultures of the tunicate *Botryllus schlosseri*. Further information was obtained by sequencing the 18S rDNA of four thraustochytrid isolates and by the construction of phylogenetic trees. An AFLP (amplified fragment-length polymorphism) molecular marker was also employed to compare different thraustochytrid isolates.

Materials and methods

Isolation of thraustochytrids

Colonies of *Stylophora pistillata*, a branching coral species, were collected in Eilat, Red Sea (29°33′83″N; 34°57′83″E <6 m depth) and, in Haifa, were maintained in aquaria with a running seawater system. The pollen-baiting technique (Raghukumar 1992; Bremer and Talbot 1995) was employed for thraustochytrid isolation. Round, open, plastic tubes, in which sterilized pine pollen was placed, were wrapped with 30 μm mesh size nylon filters at both ends and put into the coral holding aquarium for 7 days. Thereafter, the pollen was collected and streaked on petri-dish plates containing selective growth medium M [yeast extract (2 g l^{-1}), peptone (2 g l^{-1}), glucose (5 g l^{-1}) and agar (10 g l^{-1}) prepared in 50% seawater]. Streptomycin, penicillin G (250 mg l^{-1}, each) and germanium dioxide solutions (3 mg l^{-1}) were added to medium M to prevent bacterial contamination during isolation. Thraustochytrid cultures were maintained in a 25°C incubator. Observations were performed weekly under an inverted microscope.

Thraustochytrid cells were transferred from contaminated *Botryllus schlosseri* primary cell cultures (methodologies in Rinkevich and Rabinowitz 1993, 1994) to the selective growth liquid medium M (as above), in 24-well plates (Costar, USA). Serial dilutions of thraustochytrid cell suspensions were performed in order to establish single cell cultures. To support the growth of thraustochytrids, 10 ml MEM vitamin solution (100×: NaCl, 8.5 g l^{-1}; D-calcium pantothenate, 100 mg l^{-1}; choline chloride, 100 mg l^{-1}; folic acid, 100 mg l^{-1}; I-inositol, 200 mg l^{-1}; nicotinic acid, 100 mg l^{-1}; pyridoxin HCl, 100 mg l^{-1}; riboflavin, 10 mg l^{-1}; thiamine HCl, 100 mg l^{-1}) was added to 1,000 ml liquid medium M.

DNA extraction

Cultures originating from single cells were maintained in 24-well plates (Costar) in selective growth liquid medium M and subcultured to fresh medium M once a month. Subcultures were grown in 10-ml tubes with 3 ml of selective growth medium M (as above), incubated at 25°C in an orbital shaker at 150 rpm for 3 days.

Cell suspensions were centrifuged at 8,000 rpm (1.5 ml tubes) for 5 min at 4°C. The cell pellet was re-suspended with 200 μl lysis buffer (0.25 M Tris-Cl, 0.1 M Na_2-EDTA, 2% w/v SDS and 0.1 M NaCl, pH 8.2), and then homogenized. DNA was extracted by the phenol/chloroform/isoamyl alcohol–extraction and ethanol-precipitation protocol (Sambrook et al. 1989). Purified DNA was dissolved with 40 μl double-distilled water and kept at –20°C.

Thraustochytrid 18S rDNA primer design

All 18S rDNA sequences belonging to the phylum Labyrinthulomycota were obtained from GenBank (Table 1), and were aligned by GCG sequence analysis software packages (Genetics Computer Group, Madison, Wis., USA). Primers were designed along the sequences (an example is given for the *Thraustochytrium aureum* 18S rDNA sequence: Fig. 1; Table 2). Six primer combinations (FA1RA1, FA1RA2, FA1RA3, FA2RA2, FA2RA3 and FA3RA3) were employed for the PCR (polymerase chain reaction) amplification, in order to analyze thraustochytrid 18S rDNA sequences. The deduced PCR fragment sizes for primer sets FA1RA1, FA2RA2 and FA3RA3 were between 520 and 650 base pairs (bp), covering almost the whole length of the 18S rDNA sequence, with each fragment overlapping ~70 bp. Primers F and R (Table 2) were designed later on, in accordance with 18S rDNA sequences of four thraustochytrid isolates, BS1, BS2, 7-5 and 8-7 (see "Results"), for the purpose of re-checking our sequences. Both these primers were also located on the *T. aureum* 18S rDNA sequence (Fig. 1; Table 2).

PCR assay

A 2 μl thraustochytrid DNA (~100 ng) sample was added to 20 μl PCR reaction solution containing 5 mM deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP) solution, 0.5 U of supertherm DNA polymerase and 1.25 μM of each primer in the reaction buffer. PCR amplifications were performed in a PTC-100 programmable thermal controller (MJ Research, USA) with the following cycling program: denaturation for 3 min at 95°C, 35 amplification cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, with a 10 min extended elongation step. PCR products were visualized on an ethidium bromide–stained 2% agarose gel.

Sequencing of PCR products and phylogenetic analysis

PCR products of four thraustochytrid samples (12 DNA fragments altogether) were purified from agarose gels using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany), cloned into pGEM-T Easy Vector according to the manufacturer's instructions (Promega, USA), and sequenced. Three sequences of each sample were combined together to form the 18S rDNA sequence. Obtained 18S rDNA sequences were compared with each

Table 1. GenBank database availability list for 18S rDNA sequences of thraustochytrids (*T*) and its sister group labyrinthulids (*L*)

Species and strain identification	Order	Accession no.	Reference
Aplanochytrium kerguelense	T	AB022103	Honda et al. (1999)
Japonochytrium sp. ATCC 28207	T	AB022104	Honda et al. (1999)
Labyrinthula sp. AN-1565	L	AB022105	Honda et al. (1999)
Labyrinthulid quahog parasite	T	AF155209	Mass et al. (1999)
	T	AF261664	Ragan et al. (2000)
Labyrinthuloides haliotides	T	U21338	Leipe et al. (1996)
Labyrinthuloides minuta	T	L27634	Leipe et al. (1994)
Schizochytrium aggregatum	T	AB022106	Honda et al. (1999)
Schizochytrium limacinum	T	AB022107	Honda et al. (1999)
Schizochytrium minutum	T	AB022108	Honda et al. (1999)
Thraustochytrium aggregatum	T	AB022109	Honda et al. (1999)
Thraustochytrium aureum	T	AB022110	Honda et al. (1999)
Thraustochytrium kinnei	T	L34668	Cavalier-Smith et al. (1994)
Thraustochytrium multirudimentale	T	AB022111	Honda et al. (1999)
Thraustochytrium striatum	T	AB022112	Honda et al. (1999)
Thraustochytrium pachydermum	T	AB022113	Honda et al. (1999)
Ulkenia profunda	T	L34054	Cavalier-Smith et al. (1994)
Ulkenia profunda no. 29	T	AB022114	Honda et al. (1999)
Ulkenia radiata	T	AB022115	Honda et al. (1999)
Ulkenia visurgensis	T	AB022116	Honda et al. (1999)

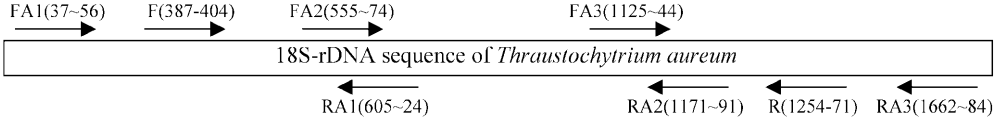

Fig. 1. *Thraustochytrium aureum*. Location of the designed primers on the 18S rDNA sequence of *T. aureum* (GenBank accession number AB022110)

other and with GenBank sequences by using the FASTA search program. Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei using the program CLUSTAL W (version 1.81, Thompson 1994) with 10,000 bootstrap trails.

AFLP reaction and analysis

DNA samples of the four above-mentioned thraustochytrid isolates were examined by AFLP analysis. Analysis was carried out using the AFLP Starter Primer Kit (GibcoBRL, Life Technologies, Paisley, UK) based on the protocol described in the products manual and by Barki et al. (2000). Digestion with *Mse*I and *EcoR*I restriction enzymes was performed in a 20 µl volume for 4 h at 37°C. The digested genomic DNA fragments were then ligated to the *Mse*I and *EcoR*I adapter to generate template DNA for amplification. PCR was performed in two consecutive steps: the pre-amplification step and the selective amplification step. The primers used for the selective amplification were as follows: *EcoR*I primer: 5′-AGACTGCGTACCAATTCACAACA-3′ and *Mse*I primer: 5′-GATGAGTCCTGAGTAACAG-3′; E-ACA and M-CAC; E-ACG and M-CTG; E-ACG and M-CTC; E-ACG and M-CAC. The *EcoR*I selective primers E-ACA and E-ACG were ^{32}P-labeled before amplification. Selective amplification products were separated on a 7.5% denaturing polyacrylamide (sequencing) gel. PCR band fingerprinting was visualized by autoradiography and analyzed by scoring the alleles.

Results

General morphological description of thraustochytrids

Thraustochytrid cells first appeared attached to the pine pollen after 3 days in culture. Within 5–7 days after plating, they developed on the selective medium M plates as colonies of a single or a few layers of cells, ranging from 1 to 5 mm in colony diameter. The colony surface area was usually rough, and the shape was irregular. Thraustochytrid colonies had orange, bright orange, reddish orange or white color morphs. The peripheral cells of a thraustochytrid colony were easy to observe, ranging from 2 to 12 µm in cell diameter. Ectoplasmic networks commonly radiated away from the thraustochytrid colonies. From time to time actively swimming zoospores appeared for short periods (~2 h) in the liquid film surrounding colonies.

Thraustochytrids isolated from primary cell cultures of the tunicate *Botryllus schlosseri* did not grow on medium M plates. They were selected by serial dilution and grown in liquid medium M. The cells were usually of a rounded shape and 3–40 µm in diameter, depending on their developmental stage (young sori or thalli).

Table 2. Designed PCR primers and their specific locations (in base pairs) on the *Thraustochytrium aureum* ATCC 34307 18S rDNA sequence (GenBank accession number AB022110)

Primer	Sequence (5′–3′)	Location
FA1	AAAGATTAAGCCATGCATGT	37–56
F	GGGAGCCTGAGAGACGGC	387–404
RA1	AGCTTTTTAACTGCAACAAC	605–624
FA2	GTCTGGTGCCAGCAGCCGCG	555–574
RA2	CCCGTGTTGAGTCAAATTAAG	1171–1191
FA3	CTTAAAGGAATTGACGGAAG	1125–1144
R	GGCCATGCACCACCACCC	1254–1271
RA3	CAATCGGTAGGTGCGACGGGCGG	1662–1684

Table 3. Deduced PCR product sizes (in base pairs) of known thraustochytrid and labyrinthilid 18S rDNA sequences from GenBank data, evaluated by the designed primers. Note: *Schizochytrium limacinum* and *Thraustochytrium aggregatum* 18S rDNA deduced PCR products were omitted because their sequences were not complete. The FA2RA2 primer set could not be used to deduce product size for the 18S rDNA sequences of *Schizochytrium aggregatum* and *Ulkenia profunda*

Strains	Product size (bp) of fragments deduced by primer sets:		
	FA1RA1	FA2RA2	FA3RA3
Aplanochytrium kerguelense	589	644	525
Japonochytrium sp. ATCC 28207	588	639	541
Labyrinthula sp. AN-1565	554	628	521
Labyrinthulid quahog parasite	583	628	539
Labyrinthuloides haliotidis	587	633	540
Labyrinthuloides minuta	581	648	527
Schizochytrium aggregatum	593	–	543
Schizochytrium minutum	590	647	528
Thraustochytrium aureum	587	636	556
Thraustochytrium kinnei	596	634	550
Thraustochytrium multirudimentale	590	655	527
Thraustochytrium striatum	587	631	549
Thraustochytrium pachydermum	594	632	546
Ulkenia profunda	588	–	552
Ulkenia profunda no. 29	577	625	550
Ulkenia radiata	577	611	548
Ulkenia visurgensis	586	645	551

Deduced PCR products of thraustochytrid and labyrinthulid sequences

Deduced PCR products for the thraustochytrid and labyrinthulid sequences based on GenBank data (Table 3) indicated a gel signature of typically two to three bands for each sequence (Fig. 2). These bands revealed high polymorphism between the different species studied, and distinguished groups according to band sizes.

PCR analysis of new thraustochytrid isolates

DNA samples from 26 thraustochytrids (23 from the coral aquarium, 3 from *B. schlosseri* primary cell cultures) were subjected to PCR amplifications with the primer sets FA1RA1, FA2RA2 and FA3RA3. All 26 DNA samples were amplified by primer set FA3RA3, 25 were amplified by FA1RA1 (96.2%) and 23 were amplified by the primer set FA2RA2 (88.5%). In total, 21 (80.8%) DNA isolates each produced three bands. The PCR amplification products for these 21 isolates could be assigned to two groups. Three thraustochytrids isolated from *B. schlosseri* primary cell cultures (BS1, BS2 and BS3) gave typically 585, 625 and 540 bp fragment sizes, while 18 of the thraustochytrids isolated from the coral aquarium by pollen baiting (Fig. 3) gave 593, 652 and 527 bp fragment sizes. These PCR outcomes were compared with the deduced bands for known thraustochytrid species. The most closely related taxa are: labyrinthulid quahog parasite (Mass et al. 1999) and *Labyrinthuloides haliotidis* (Bower 1987) for thraustochytrids isolated from *B. schlosseri* primary cell cultures; *Thraustochytrium multirudimentale* and *Schizochytrium minutum* for thraustochytrids isolated by pollen baiting from the coral holding aquarium (Table 4).

Phylogenetic and 18S rDNA sequence analyses

Four thraustochytrid strains (BS1, BS2, 7-5 and 8-7) were selected for 18S rDNA gene cloning and sequencing (accession numbers AF257314, AF257315, AF257316 and AF257317, respectively). The GCG sequence analysis software packages (Genetics Computer Group, Madison, Wis., USA) were used to align the 18S rDNA sequences of all known thraustochytrids and the labyrinthulid (list in Table 3), and the four new isolates (BS1, BS2, 7-5 and 8-7). A neighbor-joining tree (Fig. 4) indicated that BS1 and BS2 (isolated from *B. schlosseri* primary cell cultures) formed a unique group, close to the labyrinthulid quahog parasite QPX and *Thraustochytrium pachydermum*. Thraustochytrids 7-5 and 8-7 isolated from coral holding aquarium were closely related to *T. multirudimentale* and *S. minutum* in this phylogenetic tree (Fig. 4).

The 18S rDNA sequences of thraustochytrids BS1 and BS2 are 99.8% identical to each other. BS1 is 85.8% identical to the labyrinthulid quahog parasite QPX, a pathogen of *Mercenaria mercenaria* (Mass et al. 1999; Ragan et al. 2000), and 84.3% identical to *T. pachy-*

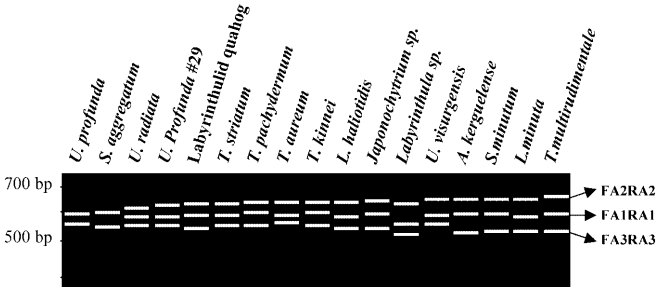

Fig. 2. Illustration of deduced PCR products for known thraustochytrid sequences, arranged according to the number of deduced PCR fragments per sequence and their sizes. FA1RA1, FA2RA2 and FA3RA3 indicate the PCR primer sets (see Table 2). For unabbreviated scientific names see Table 1

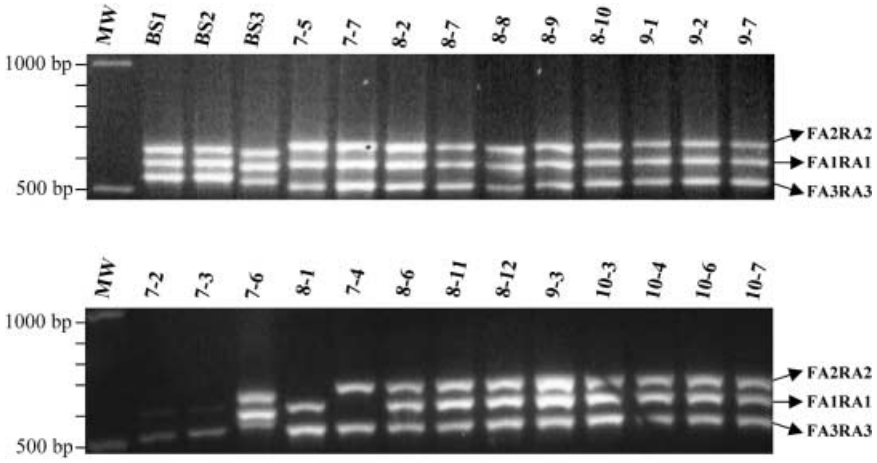

Fig. 3. PCR fragment-length analysis of 26 thraustochytrid isolates. (*MW* molecular weight marker; *FA1RA1*, *FA2RA2* and *FA3RA3* PCR products for the corresponding primer set). Isolate identification numbers are shown at the top of each lane

Table 4. Comparison of 18S rDNA PCR fragment lengths of the isolated thraustochytrid samples with known thraustochytrid 18S rDNA PCR products (deduced from GenBank data)

Samples	PCR fragment size (bp)	Most closely related taxon
BS1, BS2, BS3	585, 625, 540	Labyrinthulid quahog parasite (583, 628, 539 bp) *Labyrinthuloides haliotidis* (587, 633, 540 bp)
7-5, 7-7 to 9-7; 8-6, 8-11 to 10-7 (Fig. 3)	593, 652, 527	*Thraustochytrium multirudimentale* (590, 655, 527 bp) *Schizochytrium minutum* (590, 647, 528 bp)

dermum (Honda et al. 1999). BS2 has 86.0% identity to the labyrinthulid quahog parasite QPX and 84.3% identity to *T. pachydermum*. The 18S rDNA sequences of thraustochytrids 7-5 and 8-7 are 99.3% identical to each other. Thraustochytrid 7-5 has 97.0% identity to *T. multirudimentale* and 96.0% to *S. minutum*. Thraustochytrid 8-7 is 96.2% identical to *T. multirudimentale* and 95.0% to *S. minutum*.

Although very similar to each other in their 18S rDNA sequences (99.8% identity for BS1 and BS2, 99.3% identity for 7-5 and 8-7), each isolate showed different morphological characteristics. While in the same growth stage, BS2 cell size (up to 30–40 μm) was smaller than that of BS1 (up to 70–80 μm). Cultured on the solid medium M plate, isolate 7-5 colonies had a bright orange color morph as compared to the deep orange color of 8-7 colonies.

AFLP analysis

AFLP band analysis revealed that isolates BS1 and BS2, as well as 7-5 and 8-7, were highly similar to each other (Fig. 5, partially presented). Calculating all scorable bands produced by the five sets of primers, BS1 and BS2 shared 294 out of 305 alleles (96.4%), and 7-5 and 8-7 shared 383 common alleles out of 393 (97.5%).

Discussion

Thraustochytrid species are usually characterized by their developmental modes, sorus form, and their spore type (Porter 1990). However, these traditional systematic approaches are not sufficient, since only a limited number of morphological characters are available for thraustochytrid identification.

Molecular markers can therefore be helpful as an additional, independent parameter for thraustochytrid identification. Among molecular markers, the small subunit ribosomal gene has proven to be an invaluable tool in molecular phylogenetic studies (Van de Peer et al. 2000), due to its ubiquity, size and low evolutionary rate. SSU rRNA molecular phylogeny has completely revolutionized the study of microbial taxonomy (Woese 1987).

Honda et al. (1999) have extensively studied the 18S rDNA sequences of major thraustochytrid groups and have constructed phylogenetic trees. Their work, as well as additional research on the 18S rDNA sequences of thraustochytrids and related species (Cavalier-Smith et al. 1994; Leipe et al. 1994, 1996; Mass et al. 1999; Ragan et al. 2000), provides us with the background needed to study new thraustochytrid isolates by 18S rDNA molecular markers.

In the present study, we employed three specially designed sets of primers, representing conserved regions of thraustochytrid 18S rDNA, in order to characterize different thraustochytrid strains. Twenty-six thraustochytrid isolates have been studied by this PCR strategy, revealing that not all thraustochytrids have the same profile of PCR bands. This further illuminates the high degree of variation characteristic of this group of organisms. Only 21 samples evidenced all three PCR bands; of these samples, PCR fragment-length signatures distinguished between two groups. Group 1 samples (isolated from *Botryllus schlosseri* primary cell cultures) were closely related to labyrinthulid quahog

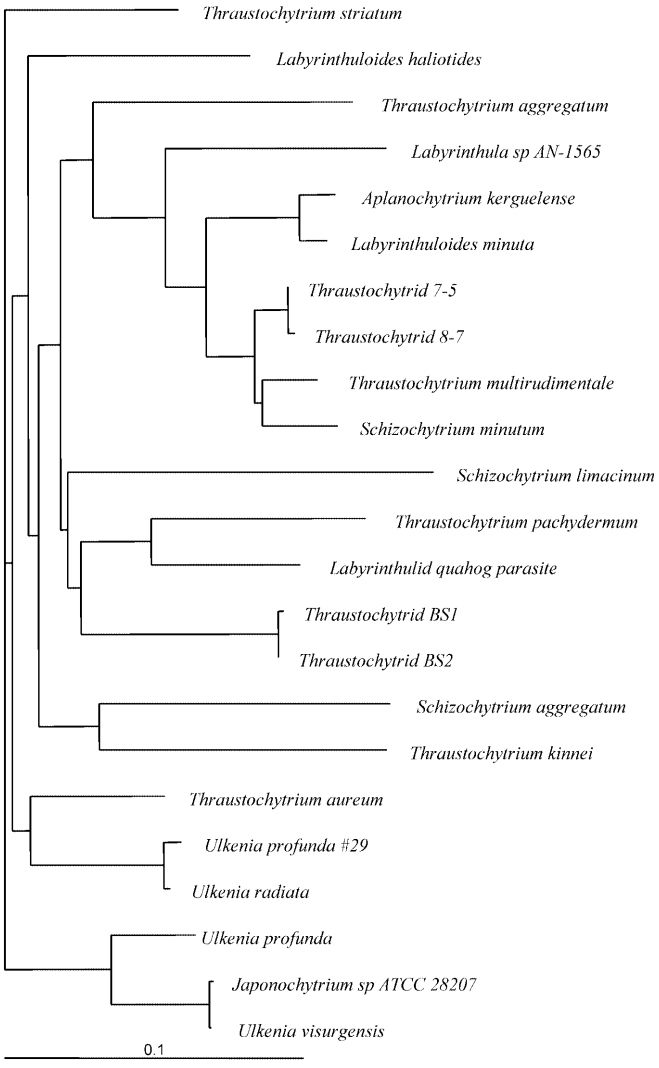

Fig. 4. Phylogenetic analysis of thraustochytrid isolates (BS1 and BS2, 7-5 and 8-7) based on 18S rDNA nucleotide sequence data. The phylogenetic tree was constructed by the neighbor-joining method

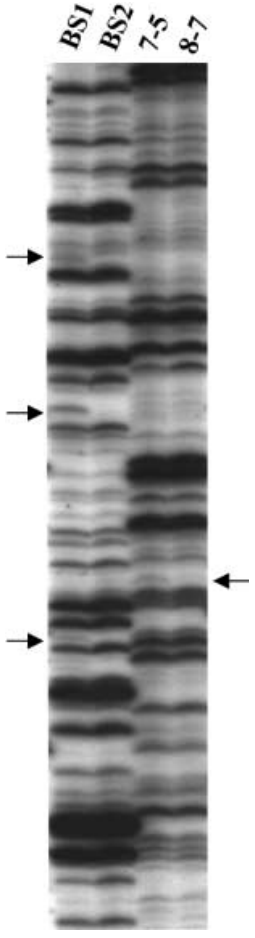

Fig. 5. E-ACG and M-CTG primer combination for AFLP analysis of thraustochytrid genomic DNA (a representative partial profile). Differences between BS1 and BS2 (*left arrows*) and between 7-5 and 8-7 (*right arrow*) are revealed by polymorphic bands

parasite and *Thraustochytrium pachydermum*. Group 2 samples (isolated from the coral aquarium) were closely related to *Thraustochytrium multirudimentale* and *Schizochytrium minutum*. These taxonomic relationships were further confirmed by comparing 18S rDNA sequences and by phylogenetic analysis, supporting the suggestion that our PCR strategy can be used as a sensitive tool for thraustochytrid identification. The phylogenetic analysis further revealed that the thraustochytrids BS1 and BS2 might be considered as a new group on the genus level.

A 5% difference between 18S rDNA sequences may be regarded as the threshold for distinguishing between species (J. Marcelo, Universidade de São Paulo, personal communication). Thus, we may consider the thraustochytrids BS1 and BS2 to be the same species; likewise, the thraustochytrids 7-5 and 8-7 can be considered as the same species.

It should be stated, however, that isolates BS1 and BS2 are clearly different from each other. The same holds for 7-5 versus 8-7. This conclusion stems from the second, independent test of AFLP band comparisons, an analysis done on the whole thraustochytrid genome. The 2.5–3.6% levels of non-shared alleles and morphological differences between the isolates of each group clearly indicate that each isolate is different; this may also be valid for most of the other 17 isolates which typically showed three PCR bands. It is, however, too early to draw conclusions on the species status in this group of organisms, although some findings have been published to support species separation. Two isolates of *Ulkenia profunda* (accession number L34054, Cavalier-Smith et al. 1994; accession number AB022114, Honda et al. 1999) exhibiting the same morphological characters shared only 86.1% identity to each other. These two isolates were therefore separated as putatively distinct species in phylogenic studies done by Honda et al. (1999); this separation is supported by our results (Fig. 4).

Isolates 7-2, 7-3 (revealing only a single band in the PCR procedure) and 7-6, 8-1 and 7-4 (characterized by two bands, each) probably represent different species or non-related systematic groups. These results represent the possible high diversity of taxa found in this group, not yet completely unveiled. Therefore, application of the above PCR strategy for identification based on the 18S rDNA gene sequence provides a novel, highly significant tool in the study of thraustochytrids.

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