Thraustochytrid-like isolates from marine bivalve mollusks

Maille Lyons, Christopher Dungan, Steven Roberts

INTRODUCTION

Interest in thraustochytrid biology, ecology, and systematics has resurfaced in recent years due to their elevated production of omega-3 fatty acids (Miller et al. 2007, Jain et al. 2007, Fan et al. 2007), their importance in marine microbial communities (Ramaiah et al. 2005, Bongiorni et al. 2005, Damare et al. 2008), and their role as pathogens of aquatic invertebrates (Anderson et al. 2003, Kvingedal et al. 2006, Lyons et al. 2007, Scharer et al. 2007). As a group, thraustochytrids are unicellular, eukaryotic, fungal-like protists whose classification is not clear. They may be classified in either the kingdom Straminipila (if following Dick 2001 and Ragukumar 2002) or Chromista (if following Cavalier-Smith 1993 and Cavalier-Smith et al. 1994). The corresponding phylum, class, order, and family designations are also dissimilar between the two classification systems. There are at least five genera of thraustochytrids (*Thraustochytrium, Schizochytrium, Ulkenia, Althornia,* and *Japonochytrium*) and possibly six (previous five plus *Labrinthuloides* and *Aplanochytrium,* now considered synonymous as *Aplanochytrium;* Leander and Porter 2000) if there are two lineages within the Labyrinthulomycetes (Honda et al., 1999), but not if there are three lineages (i.e. thraustochytrids, labyrinthulids, and aplanochytrids; Leander and Porter 2001). There may be up to nine genera if disputed groups (*Diplophrys*, *Elina,* and *Corallochytrium*) are also included. Regardless, all of these genera are distinguished by morphology, cell wall characteristics, and life history traits such as the method of zoosporulation, even though pioneering (culture-based) research suggested that these behavioral and morphological characteristics would not be sufficient to distinguish among species (Goldstein 1962, Goldstein and Belsky 1964, Booth and Miller 1968, Gaertner 1972).

The lack of proper systematic classification continues to be an obstacle in thraustochytrid research (Mo et al. 2002), but culture-independent molecular tools have been developed to characterize isolates suspected of belonging to the thraustochytrid assemblage. Using 18S rDNA molecular phylogeny, Honda et al. (1999) demonstrated that many of the thraustochytrid genera listed above do not form natural taxa. Subsequently, Leander and Porter (2001) also demonstrated that results based on molecular analyses did not support the descriptive taxonomy. Both groups of authors concluded that more sequences were necessary to determine the degree to which groupings of thraustochytrids were monophyletic. The need for more sequence data is further supported by recent observations using molecular techniques that have revealed the existence of novel thraustochytrids in laboratory cultures of invertebrates and invertebrate cell cultures (Mo et al. 2002, Rabinowitz et al. 2006, Scharer et al. 2007) and throughout the environment (Massana et al. 2004, Bongiorni et al. 2005). A primary objective of this paper is to describe 27 thraustochytrid-like isolates, including comparisons of 18S rDNA sequences, in order to better describe this relatively understudied group of organisms.

In the environment, thraustochytrids have been documented in coastal (Naganuma et al. 1998), oceanic (Raghukumar et al. 1990) and benthic (Santangelo et al. 2000) habitats, living on both plant (Sharma et al. 1994) and animal (Perkins 1973) substrates. Although generally considered saprophytic (Raghukumar 2002), several parasitic associations have been described for thraustochytrids and mollusks including octopuses (Polglase 1980), squid (Jones and O'Dor 1983), nudibranches (McLean and Porter 1982), abalone (Bower 1987a, 1987b, 1987c), and bivalves (Polglase 1986, Ragan et al. 2000). Non-parasitic associations of thraustochytrids and animals are poorly understood and rarely documented (Raghukumar 2002). A secondary objective of this paper is to document the association of thraustochytrids and mollusks that are not necessarily parasitic because all of our isolates were obtained from apparently healthy suspension-feeding bivalve mollusks from several locations over many years.

MATERIALS AND METHODS

Obtaining isolates: Twenty seven thraustochytrid-like isolates were obtained from suspension-feeding bivalve mollusks including oysters (*Crassostrea virginica*, *Crassostrea ariakensis*), soft shell clams (*Mya arenaria*) and northern quahogs (*Mercenaria mercenaria*) from Hog Island, Virginia, Chesapeake Bay, Behai, China or Yaquina Bay, Oregon from 1991 to 2005. Isolates were stored cryopreserved (Dungan and Hamilton, 1995) by pelleting axenic cultures, re-suspending pellets in a freezing medium (5-10% v/v DMSO in culture medium), and slowly freezing (1°C per minute) in foam blocks in liquid nitrogen.

Imaging: In order to photograph specimens, isolates were aseptically revived from frozen pellets and grown in 10 ml of one of two culture media (DME/F12-3 or KMEM-10) in 25 ml, vent-capped, tissue culture flasks in a 27°C incubator. Cultures were monitored and photographed with an inverted microscope.

DNA Sequencing: Cells were revived from a cryopreserved state, as described above and harvested after 3 days of growth. Genomic DNA was extracted using QIAamp DNA Tissue Kits, following the manufacturer's protocol with one modification. The elution step (200 µl of QIAamp's AE® buffer) was increased from 1 to 5 minutes as recommended in Audemard et al. (2004). In order to characterize differences among isolates at the molecular level, a fragment of the 18s rDNA was amplified and sequenced from isolates. Specifically, general labyrinthulomycete primers, previously described by Stokes et al. (2002), were used to amplify a 450 bp PCR fragment. Products were sequenced in both the 5' and 3' direction. All sequences were trimmed to produce a 320 bp overlapping region. Using BLAST, sequences were compared to the NCBI nr database to identify sequences with high homology (Altschul et al 1997). Twelve sequences were identified and include *Thraustochytrium pachydermum* (GenBank Accession #AB022113), QPX (GenBank Accession #AF155209), QPX isolate NY0313808CC1 (GenBank Accession #DQ641204), *Labyrinthuloides haliotidis* (GenBank Accession #U21338), Thraustochytriidae sp (GenBank Accession #AF257314), *Thraustochytrium caudivorum* (GenBank Accession #EF114355), *Thraustochytrium aureum* (GenBank Accession #AB022110), *Thraustochytrium kinnei* (GenBank Accession #DQ367053), *Thraustochytrium striatum* (GenBank Accession #AB022112), *Thraustochytrium multirudimente* (GenBank Accession #AB022111), *Oblongospora* sp. (GenBank Accession #AB290575), and *Thraustochytrium* sp. (GenBank Accession #AF257316). The combined sequences, in addition to the corresponding region from *Batrachochytrium* sp (GenBank Accession #AF051932) were aligned using ClustalW. A guide tree was generated based on the alignment (MacVector 7.2.3).

RESULTS

Thraustochytrid isolates were examined using light microscopy. Distinguishing morphological characteristics are difficult to identify given their simple morphology (Figure 1). In general they ranged in size from 4 to 100 um, some of which have an easily identified cell wall (Figure 1A), while others consistently produce endoplasmic net structures (Figure 1B).

A region of 18s rDNA was sequenced in all thraustochytrid-like isolates. Of the 27 isolates four where identical to other sequences deposited in NCBI's GenBank. These included three isolates with 100% sequence similarity with *T. aureum* (DQ890366, DQ890367, DQ890368). These three isolates were obtained from bivalves collected at locations on both the East and West Coast of the United States and from three different host species. The only other isolate that possessed 100% sequence identity with a described organism was DQ890378. This isolate was from a hard clam, *M. mercenaria* and was most similar to the hard clam pathogen known as Quahog Parasite X (QPX; Whyte et al. 1995).

The majority of isolates (19) were most similar to *T. aureum*. The sequence similarity of these 19 isolates ranges from 97% - 100%. Of these 19 isolates, all hosts, tissue types of isolation, and source locations were represented. Five isolates described here are genetically similar to *T. caudivorum*. The source of host shellfish for these isolates includes Virginia and China.

Insert FIGURE

Figure 1. Representative photographs of the thraustochytrid-like isolates from a variety of suspension-feeding bivalves. Scale bars = 50 um.

Table 1. Thraustochytrid-like isolates described in this study. Information regarding host bivalve, GenBank Accession number of 18s rDNA sequence, and most similar Thraustochytrid (based on DNA sequence) provided. Isolate Accession numbers with the same superscript number indicate sequences are 100% identical. Double asterisk indicates DNA sequence of respective Thraustochytrid isolate is identical to sequence in GenBank.

Accession #	Bivalve host	Geographic Location	Tissue	Organism with most similar BLAST hit
DQ890350	*Crassostrea virginica*	Chesapeake Bay	hemolymph	*T. aureum*
DQ890351	*Crassostrea ariakensis*	China	visceral mass	*T. pachydermum*
DQ890354	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum*
DQ890355	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*

DQ890356	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*
DQ890357	*Crassostrea ariakensis*	China	visceral mass	*T. caudivorum*
DQ890358	*Crassostrea ariakensis*	China	visceral mass	*T. aureum*
DQ890359	*Mercenaria mercenaria*	Virginia	mantle	*T. caudivorum*
DQ890360	*Mercenaria mercenaria*	Virginia	mantle	*T. caudivorum*
DQ890361[1]	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*
DQ890362[2]	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum*
DQ890363	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum*
DQ890364[2]	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*
DQ890365	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*
DQ890366	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum* **
DQ890367	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum* **
DQ890368	*Crassostrea virginica*	Chesapeake Bay	hemolymph	*T. aureum* **
DQ890369[1]	*Crassostrea virginica*	Chesapeake Bay	hemolymph	*T. aureum*
DQ890370	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*
DQ890371	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum*
DQ890373	*Mercenaria mercenaria*	Virginia	mantle	*T. aureum*
DQ890374	*Mercenaria mercenaria*	Virginia	mantle	*L. haliotidis*
DQ890375	*Mercenaria mercenaria*	Virginia	mantle	*T. caudivorum*
DQ890376	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum*
DQ890377	*Mya arenaria*	Oregon	labial palps and gills	*T. aureum*
DQ890378	*Mercenaria mercenaria*	Virginia	mantle	QPX **
DQ890379	*Mercenaria mercenaria*	Virginia	mantle	*T. caudivorum*

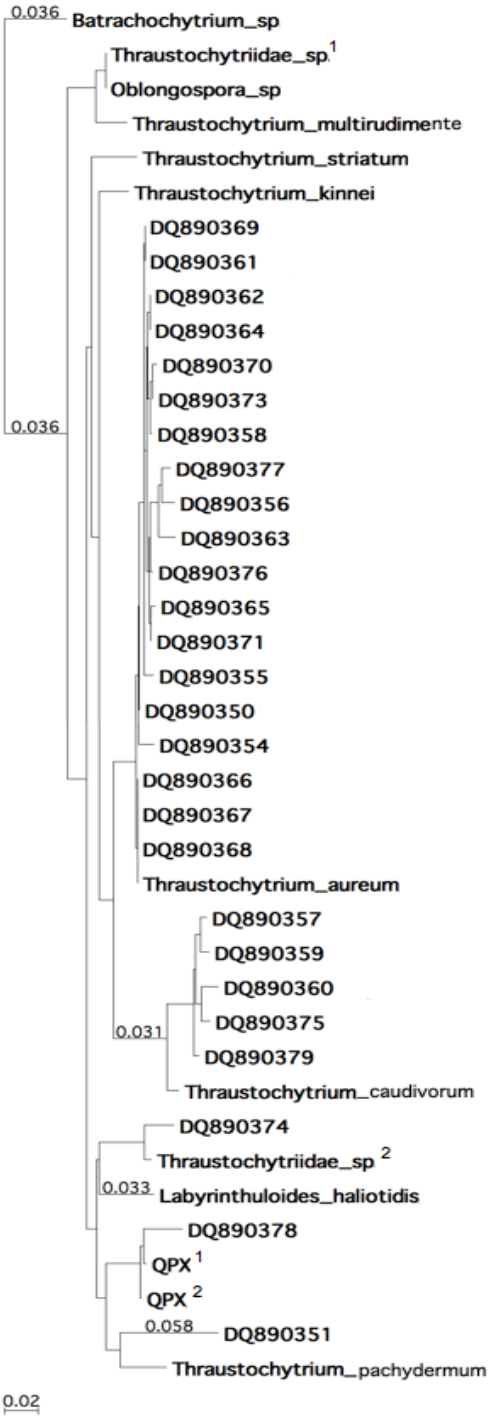

Figure 2. Sequence comparison tree based on CLUSTALW alignment of partial 18s rDNA sequence of Thraustochytrid-like isolates (denoted by accession numbers) and most similar sequences available in GenBank. Sequences include *Thraustochytrium pachydermum* (GenBank Accession #AB022113), QPX[1] (GenBank Accession #AF155209), QPX[2] isolate NY0313808CC1 (GenBank Accession #DQ641204), *Labyrinthuloides haliotidis* (GenBank Accession #U21338), Thraustochytriidae sp[1] (GenBank Accession #AF257314), *Thraustochytrium caudivorum* (GenBank Accession #EF114355), *Thraustochytrium aureum* (GenBank Accession #AB022110), *Thraustochytrium kinnei* (GenBank Accession #DQ367053), *Thraustochytrium striatum* (GenBank Accession #AB022112), *Thraustochytrium multirudimente* (GenBank Accession #AB022111), *Oblongospora* sp. (GenBank Accession #AB290575), *Thraustochytrium* sp[2] (GenBank Accession #AF257316), and *Batrachochytrium* sp (GenBank Accession #AF051932).

DISCUSSION

Research efforts concerning thraustochytrids are increasing, but there remains a lack of basic information necessary to clarify systematics of these taxa. This is particularly the case for thraustochytrids that are not parasites of invertebrate hosts such as bivalve mollusks. The results of our research augment morphological and molecular information regarding 27 thraustochytrid isolates, including 23 unique 18s rDNA sequences. These combined data will allow a better characterization of the thraustochytrid assemblage and facilitate an improved understanding of thraustochytrid distribution and ecology.

Nineteen of the isolates are genetically similar to *T. aureum*. *T. aureum* is an obligate aerobe first described by Goldstein (1963) after isolation from coastal waters around Woods Hole, Massachusetts. It was noted for its "striking capacity for morphological variation" (Goldstein 1963) and named *aureum* because cultures (especially older ones) had a golden color (Ulken 1990). Since then, *T. aureum* has also been isolated from the oceanic waters of Fladen Ground in the North Sea (Gaertner and Raghukumar 1980).

Of the isolates similar to *T. aureum*, three isolates had 18s rDNA sequence identical to *T. aureum* and thus we have classified the isolates as such. While it is possible the remaining 16 isolates are strains of *T. aureum*, it is beyond the scope of the work presented here to make such classifications. Nevertheless, based on the classification of the organisms most similar to these new isolates, no two species within a single genus were more similar than 96% in the region examined, while the sequence similarity of the 16 *T. aureum*-like isolates reported here ranged from 97-99%. Interestingly, there were some species that have been classified into different genera despite highly similar 18s rDNA sequences. For example, *Oblongospora* sp. and *T. multirudimentale* are 98% identical in the region examined.

Although thraustochytrids (as a group) are considered cosmopolitan (Porter 1990), little is known about the environmental distribution of *T. aureum* or any other specific thraustochytrid species. This is because large-scale ecological studies (reviewed by Raghukumar 2002) enumerate thraustochytrids using either the pine pollen baiting method (Gaertner 1968) or an acriflavine direct detection technique (AfDD; Raghkumar and Schaumann 1993). This yields valuable information on the total number of thraustochytrids per volume of sediment or seawater, but neither of these methods is specific enough to distinguish thraustochytrids to a species (or even genus) level. On the contrary, our results have documented both *T. aureum* and *T. aureum*-like isolates collected from both the East (e.g., 12 isolates from Virginia and Chesapeake Bay) and West (e.g., 7 isolates from Newport, Oregon) Coasts of the United States. This suggests that not only do the thraustochytrids as a group have a cosmopolitan distribution, but an individual species of thraustochytrid (*T. aureum*) may also have a broad distribution.

Five of our isolates are genetically similar to *T. caudivorum*. This is a newly described thraustochytrid found to be pathogenic to the marine flatworm, *Macrostomum lignano* (Scharer et al. 2007). This small (1-1.5 mm) free-living flatworm generally lives between sand grains in the Northern Adriatic Sea, but the isolates describe by these authors were collected from lesions on worms grown in mass cultures in the laboratory. In contrast, four of the five isolates in our study were obtained from the mantle tissues of the hard clam, *Mercenaria mercenaria* held in flow-through aquarium tanks in Virginia. The fifth isolate was obtained from the visceral mass (i.e., internal organs) of the Asian oyster, *Crassostrea ariakensis*, originally from China, but held in similar seawater tanks in Virginia. None of our isolates caused visible lesions in the marine bivalves, further supporting the opportunistic nature of *T. caudivorum* suggested by Scharer et al. (2007). Interestingly, Scharer et al. (2007) also found *T. caudivorum* in long-term laboratory cultures of another flatworm, the acoel *Isodiametra pulchra* obtained from the Gulf of Maine. Although the authors report this was most likely due to accidental contamination within their laboratory, our results support their other explanation which suggested that *T. caudivorum* has a wide distribution and was collected during the original sampling efforts.

Overall, our results demonstrate that thraustochytrids were associated with a variety of tissues and organs (gills, palps, mantle, hemolymph, visceral mass) of several suspension-feeding bivalves (hard

clams, soft-shelled clams, and 2 species of oysters) and suggest an ecological relationship between thraustochytrids and bivalves that is not strictly parasitic. This relationship is potentially an artifact of the cosmopolitan distribution of thraustochytrids and the feeding behavior of bivalves, but if that were the case we would expect a similar distribution of the types of isolates from all four bivalves examined. A closer examination of Table 1 reveals all isolations from both the Eastern oyster (*C. virginica*) and the soft-shelled clam (*Mya arenaria*) yielded *T. aureum* or a *T. aureum*-like isolate. In comparison, isolations from the hard clam (*M. mercenaria*) resulted in 18s rDNA sequences similar to four different thraustochytrids including, *T. aureum*, *T. caudivorum*, a clam pathogen (QPX), and an abalone pathogen (*L. haliotidis*). Similarly, three isolations from the Asian oyster (*C. ariakensis*) yielded sequences similar to three different thraustochytrid species (i.e., *T. aureum*, *T. caudivorum*, and *T. pachydermum*).

The relationship between thraustochytrids and bivalves may be commensalistic, but it might also be symbiotic if the presence of thraustochytrids on the internal tissues of bivalves has a probiotic effect on water-borne pathogens (viral, bacterial, or protozoan) of the bivalve. This is supported by the observations of Mo et al. (2002) that report thraustochytrid-infected cell cultures of invertebrates resist bacterial contamination more so than thraustochytrid-free cell cultures and merits further research.