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Characterization of Unusual Gymnamoebae Isolated from the Marine Environment

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Characterization of Unusual Gymnamoebae Isolated from the Marine Environment

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by

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ABSTRACT

Characterization of Unusual Gymnamoebae Isolated from the Marine Environment

Amoebae are important heterotrophic protists (protozoa) and this study focuses on three unusual forms from the marine environment. Amoebae are ubiquitous and play an important role as consumers in microbial communities. A common coastal ctenophore (*Mnemiopsis sp.*) is known to harbor an undescribed naked amoeba on the comb plate surface. The nature of the symbiotic association is unknown although electron microscopy suggests these micrograzers are degrading comb plates (Moss et al., 2001). A second amoeba isolate from mangrove waters is new to science by virtue of its distinctive trophic form that does not conform to any described species. A third isolate, *Acanthamoeba* spp. is unusual because it was isolated from offshore marine waters despite the fact it is normally found in soil. On rare occasions acanthamoebae invade the cornea and cause Amoebic Keratitis (AK). Characterization of these amoebae involved characterizing three important diagnostic features including morphology (size, form and locomotion), physiology (salinity tolerance) and molecular analysis. Salinity tolerance experiments (0g/l to 50g/l) were conducted to better understand the origin of the isolates while the molecular analysis was conducted with an aim to determine phylogeny of the isolates. The *Acanthamoeba* marine isolate was compared to non-marine strains isolated from other 'unusual' environments including chlorinated tap water, acidic soil (pH4) and marine fish scale mucus. These were included to determine their genotypes and to explore a suggested possible link between survivability in extreme habitats and pathogenicity (Booton et al., 2004). Characterizing the ctenophore amoeba included all three diagnostic features while studies on the second amoeba isolate involved physiological studies (salinity tolerance) and molecular analysis (ribotyping) based on small subunit (SSU) ribosomal RNA gene (Medlin et al., 1988; Smirnov et al., 2007). Studies on acanthamoebae included physiological studies and genotyping based on the 18S small subunit ribosomal RNA gene (Booton et al., 2004). The ctenophore amoeba had an optimum generation time at 10g/l salt (7.9 hours) suggesting that this is an estuarine amoeba although cells survived up to 50g/l. The amoeba from mangrove waters had its optimum generation time at 20g/l (34 hours) and amoebae survived up to 50g/l. The "marine" *Acanthamoeba* grew best at 15g/l salt suggesting that this may be an unusual strain with a unique genotype. Phylogenetic analyses showed that the marine strain was a T3 designate while tap water strains and acid tolerant acanthamoebae were T4 strains. The acid tolerant *Acanthamoeba* strain closely resembled *A. castellanii* previously associated with AK infections (98% bootstrap value; 0.2% dissimilarity). *Acanthamoeba* strain from fish mucus closely resembled T5 designates (78% bootstrap value; 5% dissimilarity). There was no notable trend seen with acanthamoebae genotypes and association with "extreme" environments. However, a T3 *Acanthamoeba* designate has previously been reported to tolerate salinity as high as 32g/l (Sawyer 1970, 1971). Interestingly, all three unusual amoebae isolates showed optimum growth between 10g/l and 20g/l indicating that amoebae, in general, may prefer this low salinity to save on energetic costs involved in expelling water via their contractile vacuoles.

Dedication

I would like to dedicate this to my family for all their support, prayers and encouragement: my father Solomon Njuguna Mbugua, my mother Grace Njoki Mbugua, my sisters Monica Waringa, Violet Wanja and Rose Wambui, and my brother Alan Mbugua.

Acknowledgements

I would like to recognize those who have helped me along the way: Dr. Andrew Rogerson, Dr. Wendy Trzyna, Dr. Philippe Georgel, Dr. Victor Fet, Dr. Dan Evans, David Neff (MIBC), Adam Short and Kyle Johnson. I also acknowledge support from Marshall University Undergraduate Research Grant, National Science Foundation for funding on the ctenophore amoeba study, and other collaborators on the ctenophore amoeba study: Dr. Rebecca Gast (Woods Hole Oceanographic Institute) and Dr. Anthony G. Moss (Auburn University), and all those who have been gracious enough to help me through my journey towards this scholarly achievement.

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Abbreviations

AK	Amoebic Keratitis
AS	Amoeba Saline
DNA	Deoxyribonucleic Acid
EM	Electron Microscopy
FOV	Field of View
HMDS	Hexamethyldisilazane
KV	Kilovolts
M/Y	Malt/Yeast
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscope
SSU rRNA	Small subunit ribosomal RNA
TEM	Transmission Electron Microscopy

Chapter I: General Introduction

1.1 Gymnamoebae

The term gymnamoebae is used to describe all naked or atestate amoebae with lobose pseudopodia. The term excludes all amoeboid protists with thin filopodia. The naked forms are those amoebae without an obvious cell covering external to the plasma membrane. On the other hand, the testate forms have a well defined shell around the cell with an aperture through which pseudopodia emerge for locomotion and feeding. The gymnamoebae are all non-spore forming amoebae (Page, 1983). These can take on many forms ranging from thin and finger-like (e.g. the genus *Vexillifera*) to polypodial with many radiating broad pseudopodia (e.g. the genus *Amoeba*). Some amoebae even have a single broad pseudopodium as found in the common vannellids. These 'fan-shaped' amoebae are frequently within the morphologically similar genera *Vannella* or *Platyamoeba*.

Other important features used to delineate gymnamoebae include; the form of locomotion (i.e. smooth and steady or eruptive), the presence or absence of a flagellate stage, the ultrastructural appearance of mitochondria (branched or tubular cristae), cytoplasmic inclusions, the appearance of the nucleus and nucleolus, and the floating form (with or without radiating pseudopodia) (Page, 1988; Rogerson & Patterson, 2000). But perhaps the most useful diagnostic feature, at least within the last 15 years, has been the ultrastructure of the thin covering (glycocalyx) found external to the cell membrane in the 'naked' amoebae. This can only be viewed by transmission electron microscopy (TEM) and takes on many forms that have been used to characterize genera. Even though the gycocalyx is important for identification not all amoebae can be fully distinguished in this way since some amoebae have a very thin glycocalyx with little diagnostic significance. However, others (e.g. *Mayorella*) have a thickened layer termed a cuticle or even differentiated structures such as the bundles of filaments (glycostyles) found in the genus *Vannella* or even the elegant organic scales characteristic of *Dactylamoeba and Korotnevella* (Page, 1983 & 1988; Smirnov and Brown, http://amoeba.ifmo.ru/guide.htm).

With the recent popularity of molecular approaches, morphological features have been shown to be less robust than once believed for discriminating between amoebal genera. For this reason, molecular methods (often based on ribosomal RNA genes) are now predominately used in identification and classification (Adl et al., 2005; Cavalier-Smith, 2004) although as shown below, they have not solved the problems inherent in protistan taxonomy.

1.2 Classification

Page (1988) based his classification of gymnamoebae on the convenient classical scheme of Butschli (1880-1889) who divided protozoa into four groups; Sarcodina (amoeboid organisms), Sporozoa (a parasitic group), Mastigophora (flagellated species) and Infusoria (ciliates). Here gymnamoebae were grouped within the phylum Rhizopoda (Page, 1988) (Fig. 1). Ultrastructural features discernible at the TEM level such as cell surface structures like the glycocalyx (Page, 1988; Rogerson & Patterson, 2000) resulted in a much improved classification scheme (Levine et al., 1980) delineating protozoa into seven phyla; Sarcomatigophora, Labyrinthomorpha, Apicomplexa, Microspora, Ascetospora, Myxospora and Ciliophora. The classification scheme by Levine et al. (1980) housed the subclass Gymnamoebia within the superclass Rhizopoda (Fig. 2) encompassing lobose protists.

Since then, there have been many changes leading to rearrangement of groups rendering some groups invalid (Hausmann, Hülsmann & Radek, 2003). The most recent classification scheme adopted by the International Society of Protistologists is based not only on morphological and ultrastructural features but also on molecular phylogenetics (Adl et al., 2005). The current scheme (Fig. 3) proposes six clusters of eukaryotic molecular phylogenies. Traditional taxonomy based on morphological characters identified naked amoebae as a monophyletic group sharing a common line of descent. The new classification scheme (Adl et al., 2005) suggests a monophyletic origin between some naked amoebae and testate (Testacealobosia) amoebae. The current scheme suggests that some naked amoebae groups like the Tubulina (e.g. *Amoeba, Entamoeba, and Saccamoeba*) (Levine et al., 1980) may share common ancestry with Testacealobosia. The classification of protists is in a state of flux and not all contributors agree on all points in the current scheme proposed by the International Society of Protistologists (Adl et al., 2005). Consequently the scheme is subject to modification as new information becomes available. In view of the paucity of information on amoebae in general, many researchers shy away from the group. Until their classification and identification problems can be resolved, many amoebae encountered in the field will be deemed ‘unusual’ and deserving of study as is the case for the three amoebae featured in this thesis.

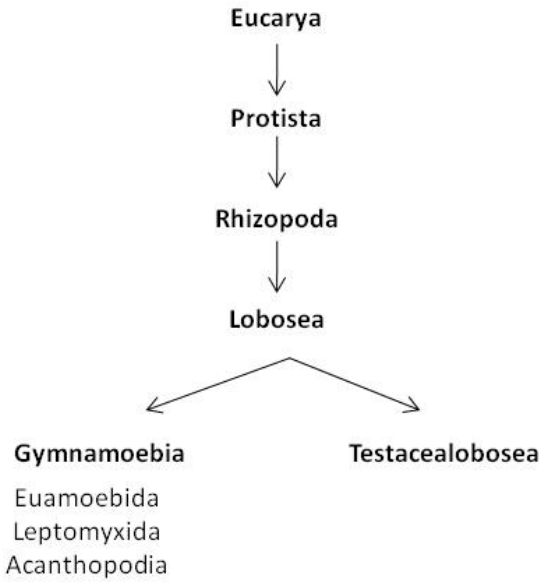


Figure 1: Classification of gymnamoebae according to Page (1988). This classification was based mainly on morphological features and comprises three main orders within the subclass gymnameobia.

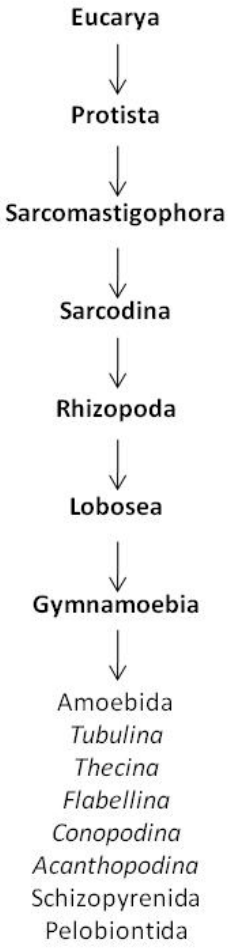


Figure 2: Classification of gymnamoebae based on morphological and ultrastructural features showing three main orders and suborders within Amoebida (Levine et al., 1980).

Figure 3: The current classification scheme proposed by the International Society of Protistologists showing six clusters of eukaryotes and assigned first ranks. The rank Tubulinea (within Amoebozoa) comprises both testate (Testacealobosia) and atestate amoebae (e.g. Tubulinida) (Adapted from Adl et al., 2005).

1.3 Diagnostic Features Evident at the Light Microscopical Level

Because of the aforementioned problems of unambiguously identifying naked amoebae, many researchers rely on using morphological features to characterize 'morphotypes' or 'morphospecies' of amoebae rather than risk mis-identifying an isolate to species. Only features discernable with a light microscope are given here since microscopy was the main tool used to distinguish amoebae isolated in the course of the current study. While there are obvious limitations with this approach, there are currently no routine methods available for unambiguously identifying species of gymnamoebae. As noted above, molecular approaches are gaining popularity, but the sequence data is not extensive enough to be used as the standard diagnostic approach. To date, the value of molecular methods has largely been to show the inadequacies of some of the traditional diagnostic features. For example, it has always been assumed that the ultrastructure of the glycocalyx was a rigid diagnostic feature. Up until a few years ago, two common genera, Vannella and Platyamoeba, were distinguished by their glycocalyx. This was either composed of long single filaments or bundles of filaments formed into structures termed glycostyles. However, recent molecular data suggests that these features are not reliable and may be artifactual depending upon the preparative methods used to prepare specimens for TEM (Sims et al., 1999; Peglar et al., 2003). In fact the data is so strong that all species may someday be fused into one genus. The following features were used in the present study:

Size. Length and breadth measurements are useful for characterizing amoebae moving under conditions favoring normal and sustained locomotion. Size can be useful for distinguishing species within a genus but it should be noted that there are also wide variations within a species or even a single strain. These differences may be caused by culture conditions (media formulations) and age of cultures. In older laboratory cultures, cells stop dividing but continue to build biomass. The size of the cyst (in the case of cyst-forming amoebae) and nuclear diameters are considered to be more reliable features and are less prone to the effects of culture conditions (Page, 1988).

Locomotive form and behavior. Observing the mode of locomotion of amoebae moving on a substratum under favorable conditions often permits identification to the level of the family or even genus (Page, 1988). Locomotive forms of amoebae can be cylindrical (limax) or

compressed or flattened. The cylindrical form may be branched (polypodial) as in *Amoeba proteus* or unbranched (monopodial) as in *Hartmannella* (Page, 1988).

Uroid. The posterior of the locomotive cell may have a feature termed the uroid. The form of the uroid (if present) can be used to help define different morphotypes. Both Page (1988) and Rogerson and Patterson (2000) showed that uroidal structures could be classified into eight types (Fig. 4). Examples of amoebae that can be identified by uroidal features include amoebae in the family Flabellulidae since all these amoebae exhibit trailing uroidal filaments (Page, 1983). The genus *Vexillifera* is unique with its fasciculate uroid. These swollen projections resemble remnant pseudopodia or subpseudopodia (Fig. 4c). Usually, adhesive uroidal filaments are produced by amoebae with a very thin or no discernible glycocalyx as in flabellulate amoebae (Page, 1988). The other uroidal types (Fig. 4a-f) are commonly seen on amoebae with thick surface coats such as found in the family Amoebidae (Page, 1988). Many differentiations are of non-adhesive origin and are products of internal processes associated with locomotion (Page, 1988).

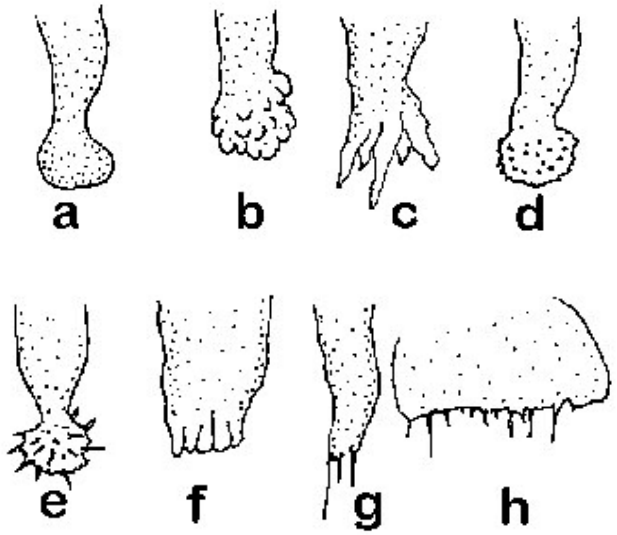


Figure 4: Typical uroidal structures in amoebae. A-bulbous; B- morulate; C-fasciculate; D-spineolate; E-villous-bulbous; F-plicate; G,H-adhesive uroidal filaments. (Adapted from Smirnov and Brown, http://amoeba.ifmo.ru/guide.htm).

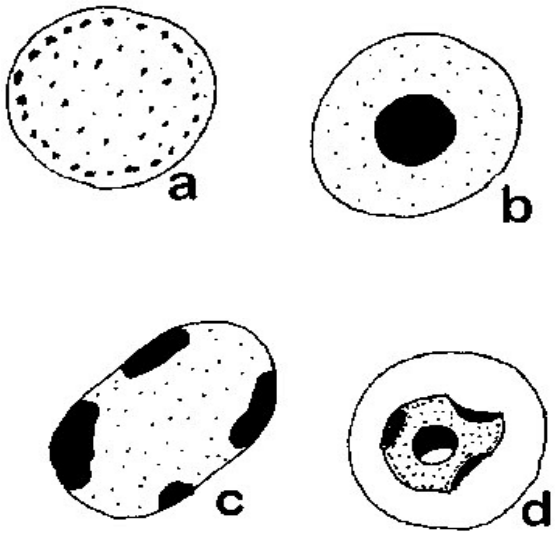
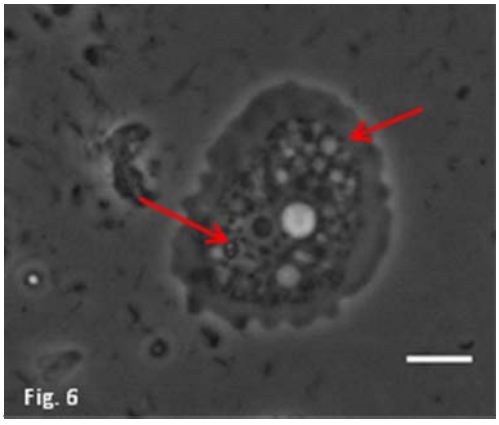


Figure 5: Basic types of nuclear structure in amoebae. A-granular nucleus; B-vesicular nucleus; C-nucleus with peripheral nucleoli; D-nucleus with complex nucleolus. (Adapted from Smirnov and Brown, http://amoeba.ifmo.ru/guide.htm)

Nuclear structure and number. The vast majority of amoebae have a single nucleus and are termed uninucleate. The few amoebae with more than one nucleus are either binucleate or multinucleate. The nucleus of the cell can be observed by phase contrast light microscopy or by epifluorescence microscopy incorporating DNA-specific fluorochomes such as DAPI. Fluorescence microscopy can also reveal the nucleolus although this structure is best studied by TEM. Most amoebae have a prominent central nucleolus as illustrated in Fig. 5b but some have characteristic nucleoli that can help identify the cell. *Amoeba proteus*, for example, has a granular nucleus with many scattered nucleolar fragments (Fig. 5a). Other nucleoli are discrete but not central in the nucleus. *Thecamoeba striata* has three or four parietal nucleoli (Fig. 5c) located on the periphery of the nucleus (Page, 1988) and *Vannella devonica* has a single parietal nucleolus (not illustrated) (Page, 1983). Many thecate amoebae display various complex nucleoli as shown in Fig. 5d.

Cytoplasmic inclusions. Crystals of various shapes, usually discernible by light microscopy, are the most obvious cellular inclusions in some naked amoebae. The function of these crystals is unknown although they are thought to be from waste excretions. Regardless of their origin or function, some species consistently have crystals of specific shapes. Some amoebae also have highly refractile cytoplasmic inclusions thought to be lipid globules. Again, the presence of these is consistent within species and they can be useful in distinguishing between genera (Smirnov & Brown, http://amoeba.ifmo.ru/guide.htm). Examples of families with cytoplasmic crystals include the Amoebidae and Hartmanellidae. Both families typically have truncated bipyramidal crystals. The absence of crystals is also useful. Crystals have never been found in thecate amoebae and only one species within the Vannellidae possesses crystals (Page, 1988). The genus with numerous conspicuous cytoplasmic crystals is *Cochliopodium* (Fig. 6, 7) (Page, 1988).



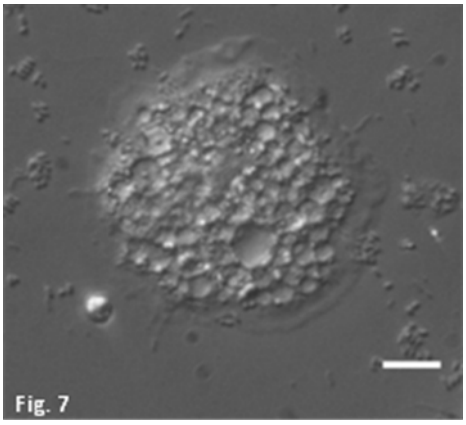


Figure 6, 7: Cytoplasmic crystals (see arrows) visible in fresh water naked amoebae (Fig. 6: Phase contrast light micrograph. Fig. 7: Integrated modulation contrast) of the genus *Cochliopodium*. Scale bar:10µm.

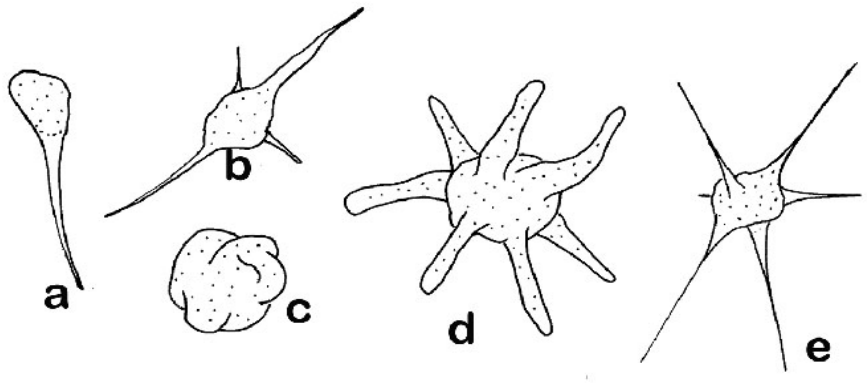


Figure 8: Typical floating forms of amoebae: a, b, c and e with radiating pseudopodia of different types; c-without defined pseudopodia. (Adapted from Smirnov and Brown, http://amoeba.ifmo.ru/guide.htm)

Floating forms. When cells are not attached to the substratum during locomotion and feeding they are free, floating in the water column. In such a state different genera adopt different floating forms. Some have long, tapering or radiate pseudopodia, for example in 'fan-shaped' morphotypes as in (Fig. 8a, 8b, 8e). Within this grouping are two common genera that, in some cases, can be distinguished by their floating form. *Vannella* tend to have long radiating pseudopodia with sharp tips while *Platyamoeba* usually have long pseudopodia with blunt tips. Other radiating pseudopodia can be short, or even absent, for example the hartmanellids as shown in Figs. 8c and 8d.

Hyaline Cap. A hyaline cap or zone, also termed the hyaloplasm (Fig. 9), is the clear cytoplasmic region that does not contain any optically visible inclusions in the advancing front of a locomoting amoeba (Page, 1988). This is especially obvious in the fan-shaped amoebae, the vannellids. In flabellulid amoebae the hyaline zone often has a wavy edge while in the genus *Thecamoeba* the hyaloplasm may appear distinctly wrinkled (Page, 1988). Some limax (tube-shaped) amoebae have different degrees of zone from a cap occupying half the length of the shell to a zone that is so small it is indistinct.

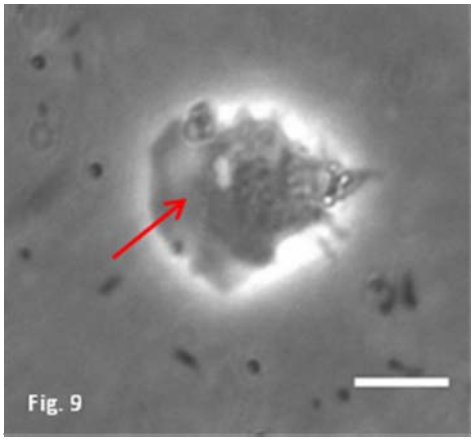


Figure 9: Photomicrograph of flabellulid amoeba showing hyaline cap (see arrow) obtained from Culture Collection of Algae and Protozoa (CCAP). Scale bar:5µm.

1.4 Physiological Features: Salinity Tolerance

Physiological characters (Page, 1988) have been used to help distınguish amoebae although the usefulness of these features is not always clear. Amoebae inhabiting deep muds are often anaerobes or microaerophiles and can only be grown under conditions of low or no oxygen. Thus, this feature is clearly important for the identification of amoebae from such habitats. Many studies have examined tolerance to temperature but often these ranges are related to time in culture since long term maintenance at a set temperature is a selective pressure that narrows their tolerance range. Salinity tolerance would also seem relevant and useful for distinguishing between 'freshwater/soil' amoebae and 'marine' amoebae, however, unpublished data (Rogerson, pers. comm.) showed that many soil amoebae can grow at 32g/l salt and marine amoebae can grow in freshwater media. These experiments did not even involve any acclimatization to new conditions. More comprehensive published studies show that naked amoebae show different responses to salinity although most seem to grow over wide ranges. Hauer et al. (2001) isolated naked amoebae from different sites ranging from 0g/l to 160g/l salt. One particularly resilient species was *Platyamoeba pseudovannellida*, from the Salton Sea, California (salinity ca. 48g/l) which was found to have a wide range of tolerance and grew well within the range 0g/l to 138g/l. In a different study, Hauer and Rogerson (2005) isolated two species of amoebae from the intertidal zone of a Florida beach and found that they grew within the salinity range of 2g/l to120 g/l.

In the present study, salinity tolerance was tested because it was relevant to at least two of the three amoebal types studied. One of the isolates featured in this thesis was a species of *Acanthamoeba.* What made this isolate unusual was its location when isolated, several miles offshore in Florida. This amoeba is a classic 'soil amoeba' and is rarely isolated in seawater. Sawyer (1970, 1971) was the first to document a species of *Acanthamoeba* from marine waters although this was collected from a sewage dumping ground off New York and the isolate may have been from a deposited cyst. He did show in the laboratory that *A. griffini,* isolated from salinity range 24g/l to 28g/l, had a tolerance range of 0g/l to 32g/l. In the study by Hauer and Rogerson (2005), *Acanthamoeba polyphaga*, grew over a salinity range of 0g/l to 12g/l salt. *Acanthamoeba* were frequently isolated from a sandy beach in Florida both from the intertidal zone and the upper beach sand (Booton et al., 2004). However, in this study, *Acanthamoeba* was not found in seawater adjacent to the beach.

The second amoeba in the present study was isolated from the surface of coastal ctenophores. Again, salinity tolerance was a relevant parameter to document since ctenophores are euryhaline and, depending upon currents, can be moved from full salinity waters to brackish estuarine waters. Clearly, if the attached amoebae are always present on the ctenophore they must also be capable of tolerating these salinity extremes.

The third amoeba featured in this thesis research was a morphologically unusual amoeba isolated from mangrove waters in Florida. Again, given the influxes of freshwater after tropical downpours and the elevated salinities due to evaporation events, the tolerance of this isolate to salinity was an important parameter to consider.

1.5 Ultrastructural Features: Electron Microscopy

Although specifically not a part of this present study, it is useful to note the main ultrastructural features that have been used, mainly by Dr. F. Page, to describe amoebae. The nature of the cell coat (glycocalyx, Fig. 10) and the structure of mitochondrial cristae (tubular or discoid, Fig. 12) are the two most important diagnostic characters revealed by TEM (Page, 1983). Ultrastructural features have been especially important for distinguishing amoebae that appear morphologically similar at the light microscope level. In some amoebae, TEM is helpful in determining the nature of the nucleus and nucleolus (Fig. 11) although as noted earlier, this information can often be obtained easier by epifluorescence microscopy.

Cell preparation for TEM is an expensive and time consuming process that involves chemical fixation of cells, dehydration through an alcohol series and embedding in a resin. After polymerization of the resin, ultrathin sections (ca. 10nm) are cut on a microtome. Before viewing in the microscope, material is stained with heavy metal stains (often uranyl acetate and lead citrate). The preparation methods can lead to artifacts of preparation and this is one of the main limitations of the method.

The cell surface structure (glycocalyx) is the most useful character (Page, 1988). However, inadequate electron microscopy fixation may complicate the appearance of the structure if it is damaged during preparation. This can lead to artifactual imaging, a serious concern if the glycocalyx is the sole distinguishing characteristic. For example in vannellids (fan-shaped amoebae) it is virtually impossible to distinguish the genera *Vannella* and *Platyamoeba* unless the nature of the glycocalyx is determined. In *Vannella* the surface consists of 'pentagonal glycostyles that are slightly more than 100nm in length (Page, 1988) while *Platyamoeba* has a "fuzzy" glycocalyx, with hexagonal arrangements of filamentous material (not glycostyles) discernible in favorable sections (Page, 1988) (Fig. 10). In practice, obtaining sections that unambiguously yield these two forms of glycocalyx is not straightforward (Rogerson, pers. comm.) and, as noted in section 1.2, recent molecular sequence data does not support these two groupings. Moreover, there are several other fan-shaped amoebae that have never been examined by TEM such as *Unda*. However, in some genera the ultrastructure of the glycocalyx is more robust and less prone to specimen preparation artifacts. The mayorellids have cuticles that preserve well and *Korotnovella* has distinctive surface scales that are also evident in TEM sections (Fig. 10).

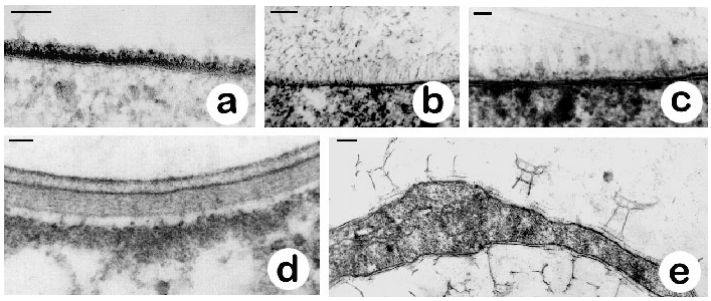


Figure 10

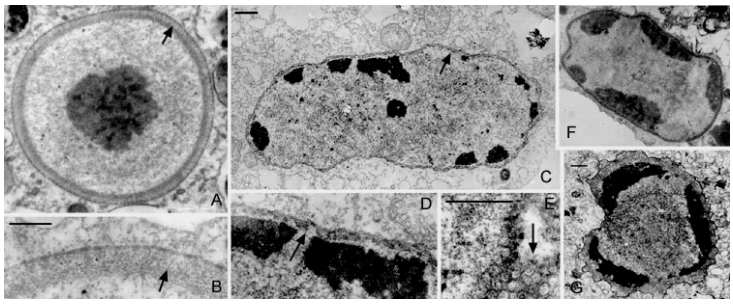


Figure 11

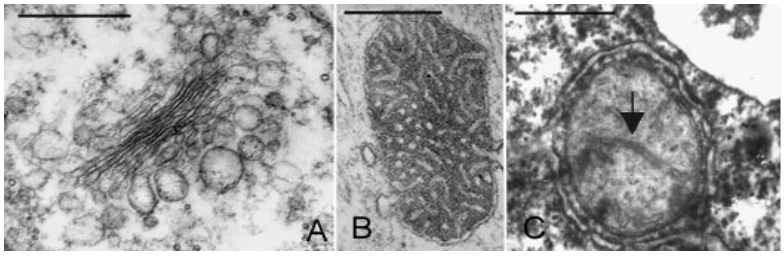


Figure 12

Figure 10: Cell coat of some amoebae species. A - amorphous cell coat of *Chaos glabrum*; B - filamentous cell coat of *Polychaos annulatum*; C-glycostyles of *Vannella*; D-thick, multilayered cell coat called "cuticle" of *Mayorella*; E-scales of *Korotnevella bulla*. Scale bar:100 nm.
Figure 11: Nuclei of some amoebae species. A-vesicular nucleus of *Saccamoeba limax* with fibrous nuclear lamina (arrowed in A and B); C-granular nucleus of *Chaos glabrum* with honeycomb nuclear lamina (arrowed in C; D-cross-section; E-tangential section of the lamina); F-nucleus of *Thecamoeba striata* with peripheral nucleoli; G-complex nucleus of *Polychaos annulatum*. Scale bar:500 nm.
Figure 12: Mitochondria of some amoebae species. A-dyctyosome of lobose amoeba *Chaos glabrum*; B-mitochondria of lobose amoeba *Thecamoeba striata* with tubular cristae; C-mitochondria of heterolobosean *Euhyperamoeba fallax* with flattened cristae (arrowed). Scale bar:500 nm. (Micrographs adapted from Smirnov and Brown, http://amoeba.ifmo.ru/guide.htm)

1.6 Identifying Amoebae Using Nucleotide Sequence Analysis

Molecular sequence data is increasingly useful for identification of amoebae to help validate amoeboid lineages based only on morphological and ultrastructural features. Low resolution of morphological, physiological and ultrastructural taxonomy has necessitated the use of molecular sequence data to elucidate the phylogeny of amoebae and other protists (Bolivar et al., 2001; Tekle et al., 2008).

Phylogenetic studies using small-subunit ribosomal RNA (SSU rRNA) gene sequences provide insights into phylogenetic relationships between amoebal groups. According to Sims et al. (2002) and Rogerson et al. (2002), phylogenetic analysis based on the SSU rRNA gene indicates that the common morphologically similar fan-shaped *Vannella* and *Platyamoeba* isolates have substantial genetic variability. Likewise, Smirnov et al. (2007) using phylogenetic analysis based on 18S SSU rRNA genes concluded that the presence or absence of a cell surface coat in *Vannella* and *Platyamoeba* respectively is an unreliable feature for delineating the two genera.

In the current study, identification of the mangrove amoeba was primarily based on molecular sequence analysis. Methods including primer design, PCR conditions and sequencing were developed from those applied in earlier studies by Medlin et al., 1988; Weekers et al., 1994; Brown et al., 2007; and Smirnov et al., 2007. Medlin et al. (1988) established conserved sequences from comparisons of 45 eukaryotic 16S-like rRNA sequences which could be used as starting points in PCR experiments. Amplification primer sequences used in the study by Medlin (1988) were considered in this study since identification of the mangrove amoeba was not possible at the light microscope level since this amoeba is new to science. Another set of eukaryotic primer pair sequences (Weekers et al., 1994) was considered in the current study since these primers (570C forward primer; 1200 reverse primer) have been used successfully in amplifying rDNA from amoebae (*Hartmannella* sp.). It is important to note that this is a first attempt to isolate and amplify DNA from the mangrove amoeba so the choice of primers was not straightforward, and as noted by Dr. R. Gast (pers. comm.) about 40% of amoebae cannot be amplified by conventional primers. Primer sequences used to amplify rDNA from amoebae in Smirnov's (2007) and Brown's (2007) study were the same one's used by Medlin et. al. (1988) as discussed above. The expectation in this study was that the primer sets used here would yield

nucleotide sequences that would lead to identification at least to the familial and genus level if at all not to the species level.

Another genus that relied heavily on morphological features to delineate species (this time at the light microscope level) was cyst appearance in *Acanthamoeba.* This feature alone was used to distinguish the approximate 21 species of named acanthamoebae. However, several studies based on molecular sequence data have demonstrated inconsistencies in using cyst morphology to identifying species of *Acanthamoeba*. Sawyer (1971) showed that salinity, or change in ionic strength of the growth media, can alter cyst morphology. Consequently, a study by Gast et al. (1996) based on complete sequences analysis of nuclear small ribosomal unit RNA genes from 18 strains revealed inconsistencies with cyst morphology as the main identification feature. In the same study, four sequence types were identified and three were obtained from single strains; Type 1 (T1) from *Acanthamoeba castellanii*, Type 2 (T2) from *Acanthamoeba palestinensis*, and Type 3 (T3) from *Acanthamoeba griffini*. The fourth sequence type (T4) included 15 isolates originally classified as *A. castellanii*, *A. polyphaga*, *A. rhysodes* or *Acanthamoeba* spp.. These genotypes have been supported by more recent analyses using the mitochondrial small subunit ribosomal RNA gene (Ledee et al., 1996). In a more recent study, classification based on 18S SSU rRNA sequences suggests 15 *Acanthamoeba* genotypes (groups T1-T15) (Shuster and Visvesvara, 2004; Booton et al. 2004). An acceptable benchmark for delineation of genotypes has been suggested to be 6-12% for differences among genotypes (Gast et al., 1996). Because of the clinical importance of this genus, there have been many studies on *Acanthamoeba* especially on identification of strains from the environment. An earlier study (Gast et al., 1996) showed that sequence variation was localized in 12 highly variable regions. Of the 12 highly variable regions, Booton et al. (2002) and Schroeder et al. (2001) established the use of 3 highly variable regions that could produce phylogenetic trees as robust as those based on the entire gene. These were designated diagnostic fragments 1, 2 and 3 (DF1, DF2, DF3). Of the three diagnostic fragments, a single variable and highly informative region (DF3) was named as the most useful in rapidly identifying genotypes (Booton et al., 2004). In the course of time, primers that are specific to the genus (referred to as JDP1 and JDP2 forward and reverse primers, respectively, for PCR and 892 and 892C forward and reverse primers, respectively, for sequence analysis) were defined and these were used in the current study to identify acanthamoebae. Some of the strain designates (T1, T2, T3, T4, T6, T10, T11 and12) have been associated with human

diseases, i.e. Amoebic Keratitis (AK) and granulomatous encephalitis. Recently, strains most commonly associated with AK include strains T2, T3, T4, T6, T11 while strains most commonly associated with granulomatous encephalitis include T1, T4, T10, T12 (Shuster and Visvesvara, 2004). Strain T4 is the most common environmental strain (Booton et al., 2004) and has been associated with both human diseases and a high number of AK infections, although it must be noted that its prevalence in AK infections might be a reflection of its abundance in nature. Whether genotypes T1 to T15 represent 15 species or variants within species is unknown at this time. In the current study, salinity tolerance experiments were coupled with genotype identification to investigate a hypothesis that strain designation may be related to tolerance to extreme environments. In light of this, additional acanthamoebae from extreme environments (acid environment [pH4], chlorinated tap water and marine fish mucus) were included to compare genotypes. This is an important step toward investigating links between pathogenicity and strain designations.

1.7 Ecology of Gymnamoebae

Most amoebae are bacteriovorous although larger amoebae will also feed on eukaryotic organisms such as flagellates, ciliates, diatoms, other microalgae and cyanobacteria (Page, 1988). Amoebae, like most protozoa, feed by pinocytocis (dissolved material) or phagocytosis (particulate material). They reproduce asexually by binary fission (Lee and Capriulo, 1990). Probable sexual reproduction is reported in the genus *Sappinia* (Page, 1988) although this is the only report of sex in amoebae and its relevance to the group is in question. Amoebae have been found in almost all aquatic habitats or sites with a film of water (i.e. soil). Page (1983) stated that he never failed to isolate amoebae from seawater samples, suggesting that they are numerically important. Since then there have been numerous studies detailing their abundance in a range of habitats.

As shown in Table 1, amoebae are common in terrestrial and aquatic habitats, are particularly numerous in soil, and are also abundant in freshwater and marine water habitats (Anderson & Rogerson, 1995; Rogerson & Gwaltney, 2000). Despite their numerical abundance, the ecological role of amoebae remains to be fully described. However, based on their densities and voracious feeding rates (Rogerson & Hauer, 2002; Butler & Rogerson, 1997)

they are presumed to be important consumers of bacteria and to play an important role in the microbial food web by recycling carbon and mineral nutrients (Butler & Rogerson, 1995).

Table 1: Comparison of numbers of naked amoebae (l-1) from different marine or brackish planktonic sites

Location	**Mean Numbers l^{-1}**	**Range**	**Reference**
Open ocean	nd	1-10	Davis et al., 1978
Open ocean	nd	4-37	Caron et al., 1986
Open ocean (aggregates)	nd	1900-53,200	Caron et al., 1986
Hudson estuary	2869	Up to 8000	Anderson & Rogerson, 1995
Clyde estuary	nd	800-15600	Anderson & Rogerson, 1995
Clyde estuary	nd	0-43000	Rogerson & Laybourn-Parry, 1992
Clyde estuary	nd	Up to 19000	Amstrong et al., 2000
Black sea	nd	0-380000	Murzov & Caron, 1996
Bermuda	75 000	nd	Anderson, 1988
Pacific, Antarctica (160m)	nd	up to 68000	Kopylov & Sashin, 1988
Pacific, Antarctica (surface)	nd	up to 30000	Kopylov & Sashin, 1988
Coastal Antarctica	2048	nd	Mayes et al., 1998
Mangroves	19430	2000-104000	Rogerson & Gwaltney, 2000
Salton Sea	117312	14560-237120	Rogerson & Hauer, 2002
Isle of Cumbrae, (Beach Sand)	2604	nd	Rogerson et al., 2000
Dania Beach (Beach Sand)	4236	nd	Rogerson et al., 2000
Mangroves	94640	nd	Rogerson et al., 2003

1.8 Project overview

The present study involved the characterization of three unusual isolates of naked amoebae (gymnamoebae) from the marine environment. Characterization was at the light microscope level and, where appropriate at the molecular level. Supplemental information on their behavior and tolerance to salinity is also given since this helps define their ecological role and function within the marine environment. Not surprisingly, the project leaves many unanswered questions but it does draw attention to three enigmatic amoebae that are worthy of further study.

Ctenophore Amoeba

This ctenophore amoeba is considered "unusual" because it is one of two common protistan epibionts on the comb plate surface of a common coastal ctenophore, *Mnemiopsis* sp. (Fig. 13) (Moss et al., 2001). Ctenophores (phylum Ctenophora), also known as comb jellies, are gelatinous predators of the marine environment. Ctenophores play an important ecological role acting as key predators in the coastal marine food web (Moss, 2001; Edmiston, 1979). They have a broad food spectrum which includes fish eggs and larvae, different kinds of smaller planktonic animals, and pelagic larvae of different benthic invertebrates (Hansson, 2006). *Mnemiopsis* is an invasive species in the Black Sea believed to have been introduced by ballast ship water (Oliviera, 2007) and this invasion demonstrates the capacity of ctenophores to disrupt the trophic food web in the absence of natural predators in the black sea where zooplankton were grazed and phytoplankton production increased (Oliviera, 2007). Ctenophores can live and reproduce in a wide salinity and temperature range (Oliviera, 2007).

Moss et al. (2001) reported protistan epibionts on the surface of ctenophores. These included the ciliate *Trichodina ctenophorii* and a small *Flabellula*-like gymnamoeba living amid the *Mnemiopsis* comb plates. Individual amoebae on the comb plate surface were approximately 15μm in width (Moss et al., 2001). Not surprisingly, given the paucity of information on the ecology of free-living amoebae, little information exists on the nature, or significance, of these surface associated protists.

Transmission electron microscopy revealed degradation of the comb plate cilia possibly by this amoeba. If true, this would impact the general health of the ctenophore (Moss et al., 2001) (Fig. 14). It is a previously undescribed amoeba and consequently new to science.

Moreover, understanding the nature of the association between the amoeba and the ctenophore may have implications on the ecological impact of ctenophore population densities.

The ctenophore amoeba had already been isolated from a comb plate surface of the ctenophore *Mnemiopsis* by Ms. Connie Versteeg (Nova Southeastern University, Florida). Ms. Versteeg demonstrated (by direct observation) that the frequency of occurrence on randomly captured ctenophores from around Florida was 85% (Versteeg, MS. Thesis, 2007). However, despite its abundance the isolate could only be cultivated from ctenophore tissue in ca. 2% of the ctenophores sampled. Scanning electron microscopy showed high numbers of the amoebae when they were encountered on comb plate surfaces with an average of 313 amoebae mm^{-2}. Extremely high surface densities (up to ~5000 mm^{-2}) were sometimes encountered by Moss (2001).

Neither Moss nor Versteeg were able to identify the ctenophore amoeba although some observations on its growth in culture were provided by Versteeg (M.S thesis, 2007). The aim of this section was to extend Versteeg's work by attempting to re-isolate amoebae from ctenophores and further characterization of this "unusual" amoeba using stock cultures from Ms. Versteeg.

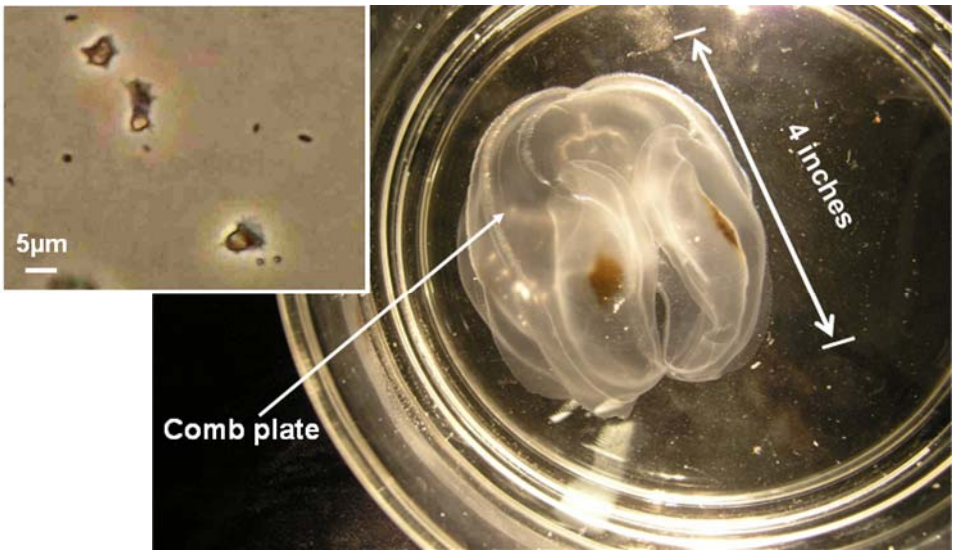


Figure 13: Common coastal ctenophore with arrow showing ciliated comb plate. Inset shows a clonal culture of novel amoeba isolated from comb plate surface of ctenophores, growing in sea salt media seeded with prey bacterium *E. coli*. Photo and Micrograph by A. Rogerson.

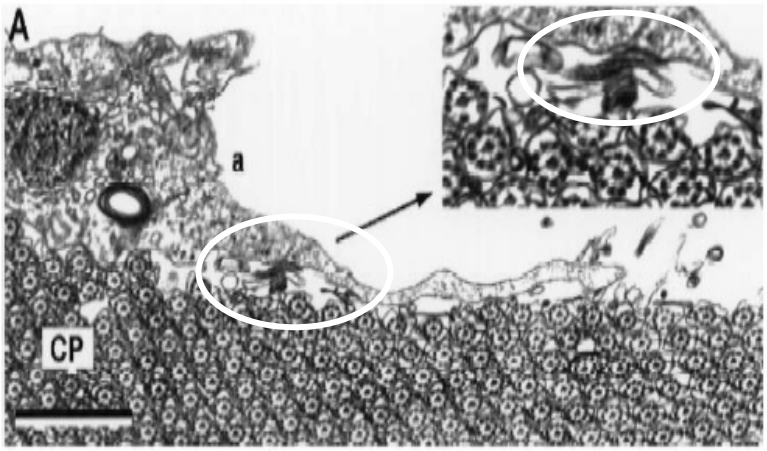


Figure 14: Electron micrograph showing amoeba crawling on ctenophore comb plate (cp). Inset shows loss of comb plate cilia on the advancing front of the pseudopodia (Image from Moss et.al., 2001); Scale bar:1µm.

Mangrove Amoeba

The mangrove amoeba (Fig. 15) was isolated by Ms. Tina Gwaltney from mangrove water from John U. Lloyd State Park, Dania Beach, Florida. The same strain was later routinely found in water samples taken from Port Everglades. The amoeba is unusual in terms of its morphology and slow rate of motion. Consequently it is easily overlooked unless the investigator is aware of this type. In short, the relative ease with which it can be found, at least in South Florida waters, suggests that it may be common.

The amoeba has an unusual trophic form quite unlike any named genera. Morphological features discernible by light microscopy suggest that the amoeba may belong to the family Thecamoebidae (Rogerson, pers. comm.). However, this is a highly speculative conclusion solely based on the fact that the surface coat appears to be thickened and wrinkled, similar to the coat of thecate amoebae. The main aim of this part of the research was to highlight the features of this unusual morphotype. Since the amoeba bears little resemblance to named species, the study will also use molecular methods to attempt to better understand its relationship to other naked amoebae.

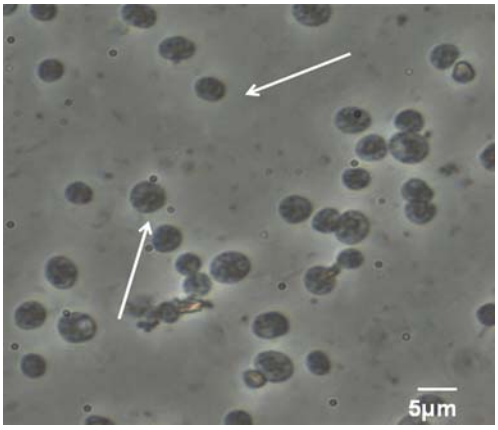


Figure 15: Phase contrast photomicrograph of a clonal culture of a novel amoeba strain (herein referred to as mangrove amoeba) obtained from mangrove water in Dania beach, Florida. Micrograph shows cells cultured *in situ* and growing optimally in 20g/l sea salt media seeded with bacterial prey (*E. coli*).

Marine *Acanthamoeba*

The third amoeba isolate featured in this thesis is a free-living amoeba of the genus *Acanthamoeba.* The isolate was unique because it was isolated from the marine environment (by divers three miles off-shore, Fort Lauderdale beach, Florida). This genus is not known to live in marine conditions although a few strains have been isolated previously, possibly from cysts (Sawyer, 1971) since acanthamoebae encyst when conditions become unfavorable. Acanthamoebae are common free-living soil/freshwater amoebae that are ubiquitous. However, there is increasing interest in this amoeba since some members of the genus are opportunistic pathogens known to cause a rare eye infection termed Amoebic Keratitis (AK). The strain most commonly isolated from AK patients is the genotype T4 (Booton et al., 2004).

This marine isolate (Fig. 16) provided the opportunity to identify a strain (by molecular genotyping) from an extreme environment; the salinity of the source water was ~32g/l, considerably higher than the salinity of soil pore water (ca. 1g/l). Tolerance to extreme environments, such as natural sea water, may be linked to pathogenicity since invasion of the cornea (as occurs in AK infections) presumably represents an 'extreme environment' for invading cells. The eye is bathed in enzymes (lysozyme) and has an elevated salinity (ca. 10g/l–5g/l). Moreover, osmotic challenges from lysed cells and changes in oxygen tension as the eye is invaded are unknown but presumably challenge amoebae that penetrate and reproduce within the corneal layers. With this in mind, the study included *Acanthamoeba* isolates from other extreme environments. Thus strains from chlorinated tap water, from the mucus of a marine fish and from an acidic environment of pH4 (Berkley Pit, Montana) were included in this study to determine whether genotype designation suggested a possible association between tolerance to extreme environments and pathogenicity. At least one study has correlated with extreme habitats (Booton et al., 2002).

Comparative *Acanthamoeba* strains from chlorinated tap water were obtained from Huntington's (WV) domestic water supply. The tap water in Huntington has a high degree of chlorination and amoebae surviving the water treatment system may have unusual properties (i.e. high tolerance) making them more likely to be opportunistically pathogenic. Moreover, there is a general belief that most infections result from rinsing contact lenses in tap water, a practice that can introduce amoebae onto the lens surface thereby promoting infection onto the eye. Searching for acanthamoebae in Huntington water constitutes the first study of its kind in WV and only the

second study to document the occurrence of these amoebae in U.S. water distribution System. The resistance of acanthamoebae cysts to chlorination was tested in this study.

The *Acanthamoeba* genotyping study also included all these "extreme" strains. One strain of *Acanthamoeba* was found living in the mucus layer on the scale surface of a marine fish (Fig. 18). This is an extreme environment by virtue of the high salinity of the marine environment. The other strain of *Acanthamoeba* from a hostile location was one isolated from amid moss in the vicinity of the Berkeley Pit, Montana (Fig. 17). This is an EPA superfund site since the runoff from mine tailings has flooded the pit and produced a flooded quarry with water at a pH of around 2.0. The moss close to the pit has a pH of ca. 4.0 (Dr. G. Mitman, pers. comm.). *Acanthamoeba* Neff strain is a laboratory strain maintained in Dr. Trzyna's laboratory (Biology Department, Marshall University) and is included here as a control in the *Acanthamoeba* study.

In summary, the comparative *Acanthamoeba* isolates were included in the study to determine whether their genotypes are unusual (new T genotype designation) or whether they were T4, the type most often found in corneal infections. Regardless, the data will provide a baseline for future studies if indeed tolerance to 'extreme environments' is associated with pathogenicity.

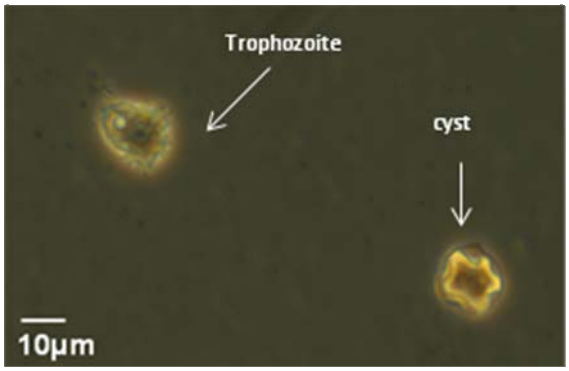


Figure 16: Phase contrast photomicrograph of "marine" *Acanthamoeba* trophozoite and cyst isolated from Fort Lauderdale beach, Florida. Scale:10µm.

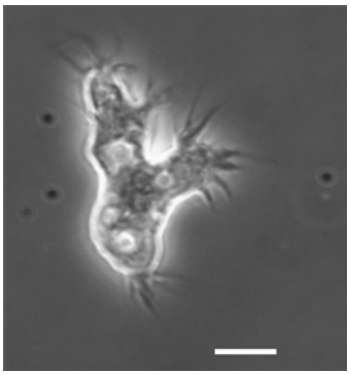


Figure 17: Phase contrast photomicrograph of acid tolerant *Acanthamoeba* strain (BP) isolated from Berkeley Pit, Montana. Scale:5µm

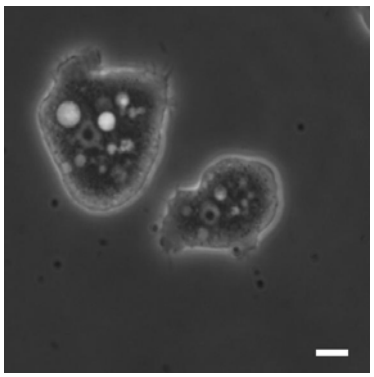


Figure 18: Phase contrast photomicrograph of *Acanthamoeba* strain (FH) isolated from the mucus layer on the scale surface of a marine fish. Scale:5µm

1.9 Project Aims

The research focuses on three "unusual" strains of naked amoebae that were all isolated from the marine environment.

a. Strain 1, the ctenophore amoeba.

b. Strain 2, the mangrove amoeba.

c. Strain 3, a 'marine' *Acanthamoeba*.

Aim 1: Isolate the ctenophore amoeba, or other naked amoebae, from the surface of ctenophores.

Aim 2: Illustrate the characteristics of the ctenophore amoeba and the mangrove amoeba using features discernible at the light microscope level.

Aim 3: Describe the physiological features of the ctenophore amoeba, the mangrove amoeba and 'marine' *Acanthamoeba* strains in terms of salinity tolerance and optimum growth responses.

Aim 4: Use molecular methods to obtain sequence data for the mangrove amoeba.

Aim 5: Survey Huntington (WV) tap water for strains of acanthamoebae.

Aim 6: Genotype 'marine' *Acanthamoeba* and other selected strains of acanthamoebae from 'extreme environments' (chlorinated tap water, fish mucus and acidic environment).

Chapter II: Materials and Methods

2.1 General Methods of Isolation and Culture of Amoebae in the Laboratory

2.1.1 Isolation of Amoebae

Due to their ubiquity, amoebae can be cultivated from different environments using enrichment cultivation methods (Page, 1983, 1988). As discussed earlier in chapter one the current study focused on amoebae isolated from a ctenophore comb plate surface (ctenophore amoeba), a mangrove habitat (mangrove amoeba) and an amoeba from water off-shore Fort Lauderdale Beach, FL (*Acanthamoeba* sp.). Also included in the study were amoebae isolates from fresh water (i.e. from domestic tap water) from Huntington, WV.

2.1.1.1 Isolation of Amoebae from Ctenophore (Mnemiopsis sp.) Comb Plates

Live coastal ctenophores (*Mnemiopsis sp*.) were obtained from Gulf Specimen Marine Laboratories Inc., Panacea, Florida on four occasions in the months of June (5^{th}, 28^{th}), September (10^{th}) and November (11^{th}) in 2007. On arrival, healthy ctenophores (Fig. 13) were first washed in three fresh rinses of sterile artificial sea water (32g sea salt in 1L filtered H_2O) to remove any unattached amoebae (i.e. contaminants). Fresh comb plate tissue visible with the naked eye was cut into small pieces (10mm x 10mm) and inoculated into culture media.

To optimize isolation of amoebae from the comb plates, tissue was inoculated into three different media formulations (Appendix I). Each of the media formulations was prepared at two concentrations, 28g/l and 10g/l. Media formulations were as follows; sterile soil extract in artificial sea water (28g/l and 10g/l), sterile artificial sea water (28g/l and 10g/l) seeded with *E. coli* and, sterile artificial Sea water (28g/l and 10g/l) enriched with 50µl Malt/Yeast agar block (see Appendix I). Each media type (at each concentration) was replicated six times, totaling 36 experimental plates. Cultures were examined by phase contrast inverted microscopy (at x630 total magnification) for the presence of amoebae at least once every week for six weeks. Whole ctenophores were also preserved in 5% gluteraldehyde for imaging on the SEM.

2.1.1.2 Isolation of Amoebae (Acanthamoeba sp.) from domestic tap water

Over an eight month study period, tap water samples were collected from four homes within a five mile radius of Huntington's (WV) water treatment plant. Volunteer collectors were instructed to let the cold water run for 60 seconds before collecting samples. On each sampling event, three 1L samples were collected from the water faucet and returned to the laboratory within one hour.

In the laboratory, each 1L sample was filtered through a 2µm pore size filter (Fig. 20) to collect any amoebae (trophs or cysts) in the sample. Prior to filtration chlorine levels and turbidity of water samples was measured using digital turbidity and chlorine meters respectively (Mettler Toledo, Utrecht, Nederland). To avoid damaging cells, gentle filtration using a suction hand pump was used (Fig. 19). After filtering, the still moist filter was transferred to a Petri dish containing non-nutrient AS agar streaked with the prey bacterium *E. coli.* Under such conditions amoebae migrate in the thin water film on top of the agar and consume the bacteria. Thus, even a single cell on the filter can be detected since amoebae multiply and soon form a dense population visible by low power microscopy (Fig. 21). It should be noted that this method does not reveal how many amoebae (whether a single cell or more than one amoeba) occurred on the filter prior to inoculation on agar plates. But the method does score the presence or absence of amoebae, although it does not distinguish between cysts and trophs in the water sample. Agar plates were observed 7 days after inoculation using a dissecting microscope. Positive plates with amoebae/protists were washed with amoeba saline (AS) and a few drops of suspension were added to a Petri dish containing liquid AS media. Cells were observed by phase contrast microscopy at high magnification (x630) to enable amoebae or other protists to be identified.

2.1.1.3 Clonal Isolation of Acanthamoeba from tap water

After establishing that the morphology of tap water amoebae isolates resembled *Acanthamoeba* (i.e. cells had spiny pseudopodia), cells in liquid AS media were agitated using a sterile transfer pipette and 50µl of the cell suspension was added to a fresh AS agar plate streaked with *E. coli.* As cells began to divide and migrate along the *E. coli* streak (Fig. 22), blocks of AS agar containing an individual cell were cut out and inoculated onto new AS agar plates streaked with *E. coli*. Once Clonal cultures grew, they were maintained by routinely sub-culturing onto fresh AS plates every few weeks.

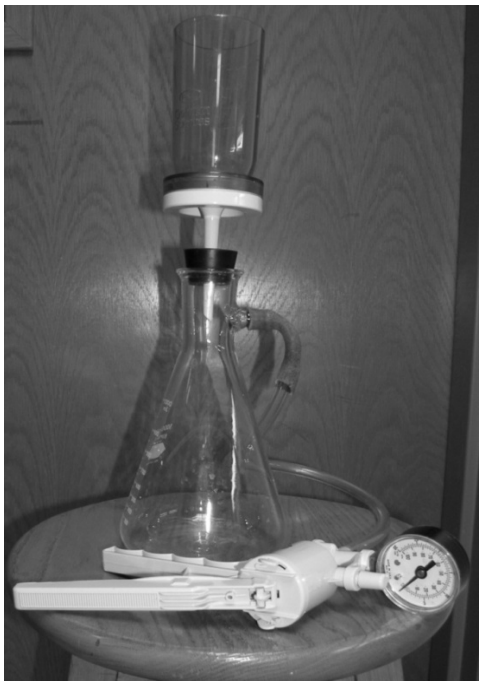


Figure 19: Filter apparatus used to process samples. One-liter samples were passed through 3µm filters. The hand-pump provided gentle filtration catering to possible trophic forms of amoebae or resistant cyst forms.

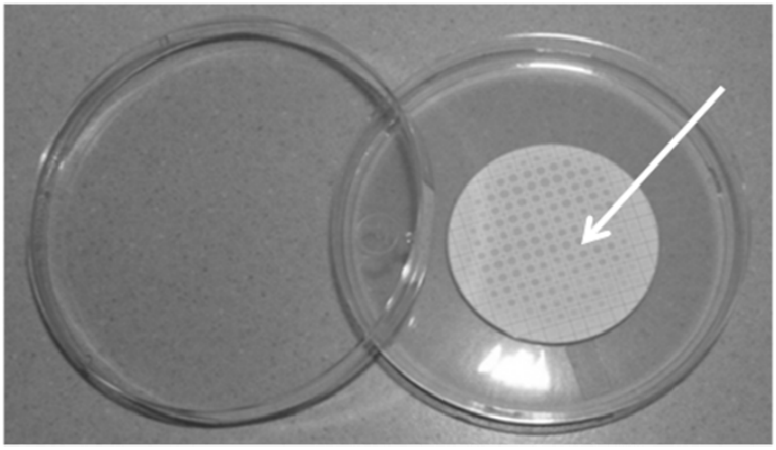


Figure 20: After water was passed through the filter (see arrow), it was placed on an agar plate seeded with the prey bacterium *E. coli*.

2.1.2 Culturing Amoebae

For the purposes of this study, and for long term preservation, it was necessary to maintain cultures in the laboratory. In this regard, isolates were maintained in their respective media promoting optimal growth and best approximating conditions in the natural environment.

2.1.2.1 Maintenance of Stock Cultures

Ctenophore amoebae isolated from live ctenophores by Connie Versteeg (Nova Southeastern University, 2007), were sub-cultured every 7 days and maintained in 10g/l artificial sea water media supplemented with prey bacterium *E. coli*.

The mangrove amoeba isolate from Ms. T. Gwaltney (Nova Southeastern University, 2007) was maintained in 20g/l artificial sea water media also supplemented with prey bacterium *E. coli*.

Marine acanthamoebae isolated from off-shore Fort Lauderdale Beach, FL and tap water acanthamoebae isolated from domestic tap water in Huntington, WV were maintained on AS agar plates (see Appendix I) streaked with *E. coli* prey. The following axenic cultures were maintained in proteose peptone media (Appendix I); *Acanthamoeba castellanii* (Neff strain), acid tolerant *Acanthamoeba* isolate (BP), *Acanthamoeba* isolate from fish scales (FH) and tap water *Acanthamoeba* isolate (A2).

Other tap water isolates included in the genotyping study (isolates coded A3, A4, A5) could not be axenicised successfully and were routinely maintained on AS agar plates streaked with *E. coli* prey.

2.1.2.2 Axenicisation of selected acanthamoebae

Axenic cultures of the acid tolerant isolate (BP), the fish isolate (FH) and tap water isolate (A2) were achieved by carefully cutting out a block of agar (3mm x 3mm) with at least one individual cell that had migrated away from the prey bacterium *E. coli* (confirmed using a dissecting microscope) on a stock culture plate. Excised blocks were inoculated into sterile culture flasks containing 25ml proteose peptone media supplemented with Penicillin/Streptomycin (Biowhittaker™, Cat. No. 17-602E). The Penicillin/Streptomycin (ratio 1:100) was added to kill of any bacteria that were on the inoculated agar block. In order to achieve fully axenic cultures, each culture was sub-cultured at least three times every five days by transferring 5ml of culture into sterile culture flasks containing 25ml of fresh sterile proteose peptone media. Axenic cultures of *Acanthamoeba* isolate (BP) and fish isolate (FH) were achieved in three weeks. An axenic culture of tap water *Acanthamoeba* isolate (A2) was achieved after a period of five weeks.

2.1.2.3 Long term preservation of Samples

For long term preservation of *Acanthamoeba,* isolates were inoculated onto AS (Appendix I) agar slants streaked with prey bacterium *E. coli* in capped Pyrex culture tubes (Cat. No. 11354-16). Frozen stocks of purified cloned DNA were prepared for preservation for all constructs by adding 500µl of cultures used in the miniprep procedure to 500µl of sterile glycerol (65%) and stored frozen at –80°C.

2.1.3 Enumeration of Amoebae in Liquid Culture

Ctenophore amoebae were cultured in liquid media in 50ml BD falcon cell culture flasks (growth area 2500mm^2) and in 55mm plastic plates with a growth area of 2375mm^2. Mangrove amoebae were also cultured in liquid media in 90 mm plastic plates with a calculated growth area of 6359 mm^2.

Cells were enumerated by averaging 10 random fields of view (FOV) using a Leica DMI 4000B phase contrast inverted light microscope and a 63X long working distance objective, total magnification x630. The total number of cells per culture vessel was estimated from the ratio of the area of the FOV and the growth area of the culture vessel. The diameter of FOV was determined using a stage micrometer at x630. The area of the FOV was calculated to be 2.29 x 10^5 µm^2.

2.2 Physiological Characterization of Amoebae

2.2.1 Salinity Tolerance

Cultures in liquid media

Ctenophore amoebae and mangrove amoebae were harvested from a dense exponentially growing culture by dislodging cells from the bottom of the stock dish using a cell scraper. Suspended cells were agitated using a transfer pipette to evenly suspend the amoebae. Five drops of amoebal suspension were added to the experimental plates containing fresh media and the prey bacterium *E.coli*. The prey bacterium suspension was prepared by adding a loopful of *E. coli* to 10ml milli-Q filtered water. The prey bacterium suspension was shaken vigorously and a dense drop was added to each plate. This ensured that there was a comparable abundance of bacterial prey in all experimental dishes. Varying concentrations of sterile artificial sea water media were prepared by dissolving sea salts (Sigma Scientific) in 1L of sterile filtered milli-Q water. Aliquots (8ml) of media at each salinity (AS, 5g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l) were added to plastic Petri plates (55mm). Amoeba Saline (AS) media was included as a control to simulate the fresh water environment (Salinity <1.0g/l). Culture dishes were observed using phase contrast microscopy at x630 total magnification and cell counts were averaged from at least 3 fields of view on each culture dish for each of the replicate dishes and media types. Experimental plates were incubated at ambient temperature (~25˚C) for the duration of the experiment.

Cultures on agar plates

Agar plates were specifically used for all *Acanthamoeba*-related experiments. Experimental media comprised 1.5% non-nutrient agar in AS media (see Appendix I) and dissolved sea salts were added at varying concentrations to provide a range of salt amended plates (AS, 5g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l). The plates were streaked with *E. coli* prey along the diameter of a 90mm plate using a sterile loop (Fig. 22). Agar regions containing between 50 to 100 cysts (Fig. 21) were identified on stock plates using a dissecting microscope. Blocks of the agar (ca. $5mm^2$) were removed with a sterile blade and inoculated, amoebae face down, onto fresh experimental plates at the end of an *E. coli* streak (Fig. 22). As the amoeba

divided and increased in density the distance between the inoculated agar block and the advancing front of amoebae along the *E. coli* streak increased with time (Fig. 21).

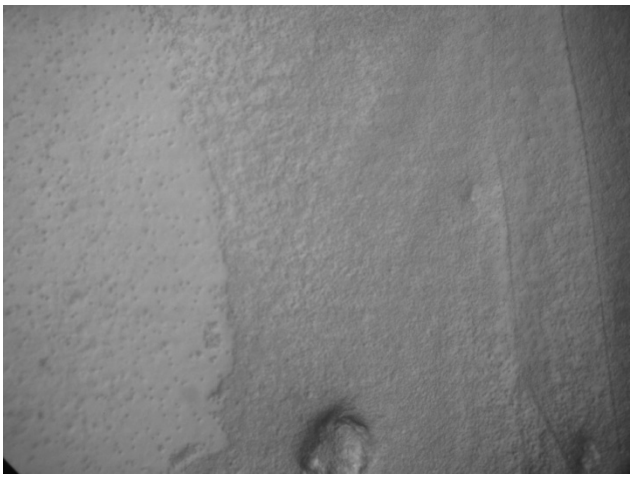


Figure 21: Photo showing thousands of amoebae migrating across the surface of the agar. Acanthamoebae consumed the bacteria added to the plate and replicated.

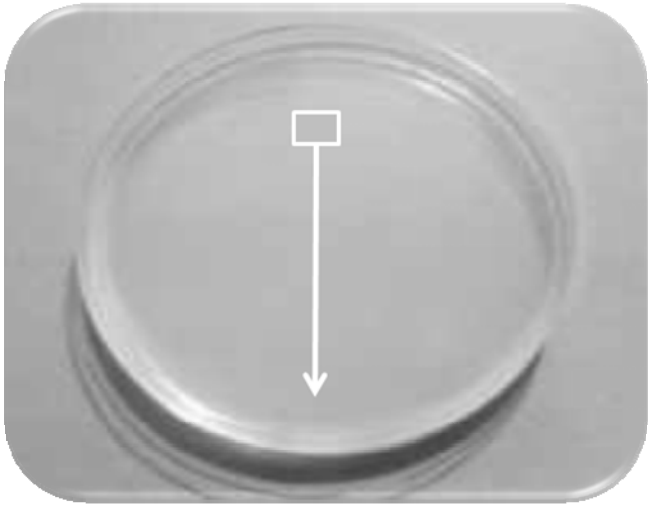


Figure 22: Photo of culture dish with red arrow showing direction of amoebal growth along prey bacterium originating from inoculated agar block.

Determination of Growth Rates/Migration Rates and Generation Times

Numbers of amoebae (ctenophore and mangrove amoebae) in three random fields of view (in each of the replicate plates) at the different salinities were counted every 12 hours (ctenophore amoebae) or 24 hours (mangrove amoebae) until cultures entered stationary phase. Average counts of viable active cells (those attached to the substratum and moving noticeably by pseudopodia) from three replicates at respective salinities were converted to numbers of cells per plate (see section 2.1.3) and growth curves were drawn and regressions calculated for the exponential phase of growth.

For acanthamoebae isolates growing on agar plates, the growth rate was indirectly determined by scoring migration rates (mm/hr). The advancing front of advancing amoebae moved along the bacterial streak as prey were consumed and amoebae replicated. The "marine" *Acanthamoeba* isolate (A1) rapidly migrated down the length of the *E. coli* streak. The distance moved by "marine" *Acanthamoeba* along the *E. coli* streak was observed and recorded every 12 hours for a total of 60 hours. The distance moved by the tap water *Acanthamoeba* isolate (A2) from inoculated agar block was observed and recorded every 24 hours for up to 72 hours.

The maximum rates of migration were determined from slopes at each of the different salinities. Likewise, cell counts over time yielded slopes for the ctenophore and mangrove amoebae. The slopes (determined by regression analysis) were used to compute the growth rate constant (K) according the formula by Stanier et al. (1976): $\text{Log}_{10}N_t - \text{Log}_{10} N_o / 0.301t$; where N_t is the final number of cells, and N_o is the initial number of cells, and t is time in hours. The generation time (g) was calculated as 1/ K.

Locomotive Rate for Ctenophore amoeba

Locomotive rates are useful in helping to identify amoebae. The locomotive rate was determined by computing the average distance moved per second by ten randomly selected amoebae in culture at different salinities. Although the mangrove amoeba like the ctenophore amoeba is new to science, identification of the mangrove amoeba was based primarily on molecular sequencing data (nucleotide sequences). Thus it was not necessary to explore locomotive rates of the mangrove amoeba isolate. Members of the genus *Acanthamoeba* are easily identified by their unique morphology and strains are usually identified by genotyping

(Schroeder et al., 2001; Booton et al., 2004). Thus exploring locomotive rates of *Acanthamoeba* isolates was also not necessary in this study.

2.2.2 Chlorine Tolerance of Amoebae

Two randomly selected amoebae [tap water *Acanthamoeba* isolate (A2) and *Vannella* sp.] from Huntington, WV's domestic water supply were treated to different levels of chlorine (Cl). Only cysts were used since it was assumed that the cyst stage was most likely to be found within the water distribution system. Cysts were immersed in water containing Cl as HOCl in bleach (0mg/L to 200mg/L) for 24 hours. After treatment they were rinsed by carefully dipping the agar block once, with cysts side up, in sterile AS contained in multi well tissue culture plates. Their viability was tested by plating agar blocks on agar plates with prey bacterium *E. coli*. Surviving cysts excysted, amoebae reproduced and formed dense populations on the plates.

2.3 Characterization of Ctenophore Amoeba Using Microscopical Methods

2.3.1 Scanning Electron Microscopy

Ctenophore Comb Plate Surface

The presence of amoebae on the comb plate surface of ctenophores was determined from micrographs obtained using a scanning electron microscope (SEM JEOL 5310). Ctenophores preserved in 5% gluteraldehyde were dissected to remove thin sections of comb plate tissue (approximately 5mm x 5mm) for post-fixation. The fixation procedure was modified from Dr. Moss' Jellyfish Fixation Protocol[1] (see Appendix I). The reagents were prepared as shown in Table 2.

Table 2: Scanning electron microscopy preparation reagents

Stock needed	Volume in final mix (ml)	Final concentration
Part A		
1.5M NaCl (32g/l sea water)	2	0.075-0.15M
0.8M NaCacodylate (pH 7.5)	5	0.2M
0.1M $CaCl_2$	2	0.01M
Sterile Milli-Q H_2O	6	
Part B		
4% OsO_4	5	1%

Osmium Post-Fixation

Pieces of ctenophore comb plate tissue (5mm x 5mm) were placed on glass coverslips. Reagents in part A and part B (Table 2) were mixed in the ratio 3:1 respectively in an ice bath since osmium tetroxide (OsO_4) is highly toxic and sublimes readily at room temperature. For initial fixation in 1% OsO_4, the cold "mix" was immediately added to the sample and left to stand on ice for 20 minutes until a port wine precipitate was distinctly visible. Upon seeing this precipitate the sample was washed in ice cold buffer consisting of equal volumes of 0.2M Na Cacodylate (pH 7.5) and 0.3M NaCl.

[1] Dr. Anthony G. Moss (Auburn University, Alabama) - Project collaborator (*Protistan epibionts of the ctenophore Mnemiopsis mccradyi Mayer*).

Samples were again fixed with 1% osmium tetroxide in 0.1M Na Cacodylate and 0.38M NaCl on ice for another 20 minutes. The sample, still kept on ice, was rinsed five times for 5 minutes each rinse with sterile filtered milli-Q water.

Dehydration

Coverslips holding the sample were slotted into a compartmentalized Coplin jar where the samples were dehydrated through an ethanol series [30%, 50%, 70%, 90% (x2), 95% (x3), and 100% (x 3)] in ten minute intervals. The sample was dehydrated to completion in hexamethyldisilazane (HMDS) in a 1:1 HMDS: EtOH mix followed by two dehydration steps in 100% HMDS for ten minutes each. In the final dehydration, just enough HMDS concentrate was left to cover the sample in the Coplin jar. The jar was left in a tilted position overnight in a safety hood to air dry. The samples on the coverslips were sputter-coated with a 10nm thick layer of gold/palladium and viewed on the SEM with an acceleration voltage of 20KV.

Ctenophore Amoebae

A 50 µl drop of amoebal cell culture was pipetted onto a clean glass coverslip and left overnight in a moist chamber to allow cells to adhere firmly to the glass surface. For primary fixative, a drop of 2.5% gluteraldehyde in 0.05M Cacodylate buffer (pH ~ 7.2), was added. After 30 minutes the samples were rinsed 2 times for 30 seconds each in 0.05M buffer by gently dipping the coverslips in small staining jars (Coplin jars) containing the buffer. For postfixation, 2% osmium tetroxide in 0.05M Cacodylate buffer was pipetted onto the samples and left for 1 hour. After fixation, the samples were gently rinsed in distilled water several times (30 seconds each) and dehydrated through an alcohol series 30%, 50%, 70%, 85%, 100% (x2) for 15 minutes each. Further dehydration was achieved through a HMDS dehydration series (1:1; HMDS: EtOH and 100% HMDS for 10 minutes each) and the samples were left to air dry. The fixed and dried amoebae samples were sputter coated with a 10nm thick gold/palladium layer and viewed on the SEM with an acceleration voltage of 20KV.

2.3.2 Phase contrast microscopy

Cell Size and Form of Ctenophore Amoeba

To determine cell form, active/locomoting and stationary/floating forms of amoebae were observed. The cell size was estimated using length and breadth measurements of 10 randomly selected active amoebae growing in different salinities and were measured from micrographs obtained using a digital camera attached to a Leica inverted phase contrast microscope using a 63X long distance working objective, total magnification, x630. Measurements were made from the anterior tip of the hyaline cap, distinctly visible in active amoebae, to the posterior end.

2.3.3 Epifluorescence Microscopy

Nuclear Position, Size and Number. For resolution of the nucleus of the ctenophore amoeba, DAPI (4'-6-Diamidino-2-phenylindole) staining was used with conventional florescence microscopy. DAPI is a DNA-specific fluorochrome that binds to dsDNA. Cells in a 5 ml suspension were fixed with 1% gluteraldehyde and stained with 0.1% DAPI (5 drops) for 30 minutes in the dark. After staining, cells were captured on a 0.2µm pore size black membrane (Nuclepore) and viewed under UV light by epifluorescence microscopy at 630X magnification.

2.4 Molecular Characterization

2.4.1 Genomic DNA Isolation

Genomic DNA isolations from amoebae were performed using either DNA STAT-60^{TM} reagent (Tel-Test Inc., Friendswood, Texas, Cat. No. TL-4420) for the mangrove amoeba or Qiagen's DNeasy Blood and Tissue Kit Cat. No. 69504 for acanthamoebae.

Acanthamoebae Genomic DNA Isolation

DNA was isolated from triplicate plates (90mm AS agar plates seeded with *E. coli)* for *Acanthamoeba* strains. Cells were scrapped off agar surfaces using a sterile cell scrapper and washed off using 1ml AS into sterile 90mm plastic plates containing 10ml AS. Acanthamoebae

(now in liquid AS media) were left to sit for 15minutes at ambient temperature to allow for cells to attach to the bottom of the plate.

A ratio of at least 1:1 (cyst: trophozoite) population was important to achieve since mature cysts do not break open to yield DNA. Schroeder et al. (2001) also alludes to the difficulty of obtaining PCR products from cultures consisting of only *Acanthamoeba* cysts. Therefore, a favorable population for the purposes of DNA isolation was harvested from 2-3 day old cultures when cells occupy a large portion of the plate surface area and before total encystment.

Presence of trophozoites (active form) in the fresh 10ml liquid AS media was confirmed using phase contrast microscopy. Since acanthamoebae adhere to the bottom of plates, the liquid AS media was decanted off into a waste beaker to remove excess prey bacterium *E. coli* and floating cysts. Thereafter, cells were dislodged using a cell scraper and agitated once more using a transfer pipette before transferring the cell suspension into 1.5ml microcentrifuge tubes. The cell suspension was centrifuged at 8000rpm for 10 minutes at 4°C and the supernatant was carefully poured off. The visible pellet was vortexed to form a slurry.

Mangrove Amoeba DNA Isolation

Mangrove amoebae were harvested from at least twenty 90mm culture dishes by first pouring off the cell growth media and blotting out the residual media. The mangrove amoebae adhered strongly to the bottom of the culture flask or plates after pouring off the media (this was confirmed using phase contrast microscopy). A 2ml aliquot of DNA STAT-60^{TM} reagent was added to the vessel containing the cells and was spread evenly through the growth surface by a gentle swirling motion for approximately 5 seconds. The homogenate was re-used for one more vessel before obtaining a fresh 2ml aliquot of reagent for subsequent washes of each experimental vessel. Residual homogenate was scrapped off the culture flasks or plates using a cell scrapper (this ensured that no cells or lysate was left adhering to the bottom of the flask, again confirmed visually using phase contrast microscopy) and the homogenate containing the lysed cells was poured into 30ml Cortex glass tubes. DNA precipitation using isopropanol solution was conducted overnight at 4°C. Because the precipitate was clear to almost invisible the supernatant was carefully decanted off before washing the pellet in 75% ethanol.

2.4.2 Primers

Mangrove Amoeba

Currently there are few molecular studies on gymnamoebae and since the mangrove amoeba is new to science, five sets of eukaryotic primers (Table 3) were tried in the mangrove amoeba study in an attempt to amplify ribosomal RNA genes (Brown et al., 2007; Smirnov et al., 2007; Medlin et al., 1988). These primers have been used separately in previous studies involving gymnamoebae for three different genera, *Hartmannella* (Weekers et al., 1994), *Vannella* (Smirnov et al., 2007) and *Sappinia* (Brown et al., 2007). The primers are herein referred to as: Med-F/Med-R, Silb-F/Silb-R (from Medlin, 1988 and Brown et al., 2007), Rib-F/F-R, S12.2-F/S20-R (from Smirnov, 2007), and 570C-F/1200R (from Weekers, 1994) (Table 3).

Primers used for direct sequencing were the primers used for respective PCR reactions. Primers used to sequence cloned PCR products in plasmid DNA were selected from promoter regions of TA plasmid vector used in the study (www.invitrogen.com).

***Acanthamoeba* spp.**

Molecular analysis was conducted on the "marine" *Acanthamoeba* strain (A1) and on comparative *Acanthamoeba* strains from varying 'extreme' environments [acidic environment (BP), fish mucous (FH), and chlorinated tap water (A2-A5)].

Acanthamoeba-specific primers JDP1 and JDP2 (see Table 3) targeting the informative region referred to as DF3 (Diagnostic Fragment No. 3) on the 18S nuclear ribosomal RNA gene were used (Booton et al., 2004; Schroeder et al., 2001). These primers were expected to yield 400bp to 500bp amplicons. This amplicon is reliably used to identify genotypes rapidly (Schroeder, 2001).

Primers used for direct sequencing were *Acanthamoeba*-specific 892 forward and 892C reverse primers (Booton et al., 2004). Primers used to sequence cloned PCR products in plasmid DNA were selected from promoter regions of TA plasmid vector used in the study (www.invitrogen.com).

Table 3: Primer sources and sequences for DNA amplification

Set No.	Primer	Primer Sequence (5' to 3')	Source
1	MedF	aac ctg gtt gat cct gcc agt	Medlin et al., 1988
	MedR	gat cct tct gca ggt tca cct ac	
2	SilbF	aac ctg gtt gat cct gcc agt	Brown et al., 2007
	SilbR	gat cct tct gca ggt tca cta c	Medlin et al., 1988
3	RibF	ctg gtt gat cct gcc agt	Smirnov et al., 2007
	RibR	gat cct tct gca ggt tca cta c	
4	S12.2F	gat cag ata ccg tcg tag tc	Smirnov et al., 2007
	S20R	gac ggg cgg tgt gta caa	
5	570cF	gta att cca gct cca ata gc	Weekers et al., 1994
	1200R	ggg cat cac aga cct g	

2.4.3 Polymerase Chain Reaction (PCR)

Amplification of Mangrove Amoeba

PCR parameters applied in the current study were modified from Brown et al. (2007) and were as follows: 5 minutes initial denaturation at 94°C followed by 30 cycles of 25s denaturation at 94°C, 60s annealing at 55°C, extension for 90s at 72°C. Final elongation was done for 3 minutes at 72°C. Annealing temperatures were adjusted depending on melting temperatures of primer sets used in the reaction as presented in Table 4 with an upper limit of -1°C to -2°C. PCR reagent amounts used per PCR reaction (total volume 25µl for all PCR reactions) are presented in Table 5. Amounts of DNA template were adjusted accordingly. Theoretically, one molecule of DNA is sufficient per PCR reaction however at least 50ng DNA template is recommended per reaction.

In order to eliminate "non-specific binding" of primers to DNA template, various factors were considered (see Table 6). These factors included primer selection (see also Tables 3 and 4), use of Q-solution in the PCR reaction mix, varying Mg^{2+} ion concentrations and varying annealing temperatures.

Q-solution is a reagent that is supplied in Qiagen kits whose purpose is to reduce the non-specific binding and effects of secondary structure of rRNA. The current study applied the use of Q solution at 1µl for a final PCR reaction volume of 25µl.

DNA isolated from mangrove amoebae at separate times was considered a variable though amoebae were a clonal culture grown under identical conditions. Mangrove amoebae

used in subsequent DNA isolations were a sub-culture of the cells used in previous isolations. After several DNA isolation attempts only two isolations were successful and this DNA was stored in individual 1.5ml Eppendorf tubes. This was carried out to provide enough material (DNA template) for PCR reactions.

Amplification of Acanthamoeba Isolates

PCR conditions were modified from Booton et al. (2004) and were as follows: 7 minutes initial denaturation at 95°C followed by 30 cycles of 60s denaturation at 95°C, 60s annealing at 55°C, extension for 120s at 72°C. This was followed by a final extension for 15 minutes at 72°C. DNA template volumes in the PCR reaction mix were adjusted according to the concentration DNA template yields from genomic DNA extraction procedures presented in results section (Chapter III).

Table 4: Melting temperatures of primers used in this study

Primer Source	**Primer ID**	**Primer Sequence (5 ‘ to 3’ end)**	**No. of Nucleotides**	**Melting Temp.°C**
Medlin et al., 1988	Med-F	aac ctg gtt gat cct gcc agt	21	63
	Med-R	gat cct tct gca ggt tca cct ac	23	58
Brown et al., 2007	Silb-F	aac ctg gtt gat cct gcc agt	21	63
	Silb-R	gat cct tct gca ggt tca cta c	22	58
Smirnov et al., 2007	Rib-F	ctg gtt gat cct gcc agt	18	62
	F-R	gat cct tct gca ggt tca cta c	22	58
Smirnov et al., 2007	S12.2-F	gat cag ata ccg tcg tag tc	20	52
	S20-R	gac ggg cgg tgt gta caa	18	61
Weekers et al., 1994	570c-F	gta att cca gct cca ata gc	20	54
	1200-R	ggg cat cac aga cct g	16	54
Booton et al. 2004; Schroeder et al. 2001	JDP1-F[¥]	ggc cca gat cgt tta ccg tgaa	21	68
	JDP2-R[¥]	tct cac aag ctg cta ggg gag tca	24	70

[¥]JDP1andJDP2 are primers specific to the genus *Acanthamoeba* and have been used in this study to specifically amplify *Acanthamoeba* DNA.

Table 5: PCR reaction mix

	Reaction volume (µl)	Final concentration
Template	-*	5-50ng
Forward primer (10µM)	2.5	1µM
Reverse primer (10µM)	2.5	1µM
Nucleotides	4	200µM each
Buffer$^{\epsilon}$ w/$MgCl_2$ (10x)	2.5	2mM ($MgCl_2$)
Taq polymerase$^{¥}$	0.25	1 unit
H_20	-	-
Total	25	

*Template volumes were adjusted according to DNA yield from cells. $^{\epsilon}$ Biolabs Cat. No. M0267S (5,000 U/ml)
$^{¥}$Thermpol Buffer with $MgCl_2$, Biolabs, Cat. No. B9004S (10X).

Optimizing PCR on Mangrove Amoeba Isolate

Table 6: Summary table of PCR reaction variables applied

Variables			**Primers**		
	Silb-f/Silb-r	**Rib-f/Fr**	**12.2f/S20r**	**Med-F/MedR***	**570C/1200R**
Annealing Temperature (°C)	42,45, 48, 55,56, 58	42	48, 50	48	48, 52
Q-Solution[¥]	applied	applied	n/a	n/a	n/a
DNA Template[¥]	applied	applied	n/a	n/a	n/a
Mg^{2+} ion concentration (mM)[¥]	2, 2.5, 3, 3.5, 4	n/a	n/a	n/a	n/a
Primer concentration* (2µM and 10µM	n/a	n/a	n/a	n/a	n/a

[¥]Experiments were run at 55°C annealing temperature which was determined to be the optimal annealing temperature. **n/a**: These experiments were not carried out while primer sets Silb-F/Silb-R and Rib-F/F-R were explored. *This variable was only applied to *Acanthamoeba castellanii* (Neff strain) a laboratory strain used in this study as a positive control. Appling the primer concentration factor on this strain was to help determine validity of the primers for subsequent PCR runs on mangrove amoeba isolate.

2.4.4 Gel Electrophoresis

2.4.4.1 Determination of Integrity of Genomic DNA Extracts

The quantity and quality of DNA extract was determined using a nanodrop (NanoDrop ND-1000 Spectrophotometer). The spectrophotometer was used to measure the DNA concentration (ng/µl) and optical density (OD) ratio. DNA quality measurement is based on OD at 260 nm being twice than at 280 nm if the solution contains pure DNA. If there is a contaminant, the OD ratio between 260 and 280 nm is decreased. Clean DNA has an OD260/OD280 between 1.8 and 2.0.

2.4.4.2 PCR Products

PCR products were analyzed on PCR grade agarose gel (FisherBiotech CAS 9012-36-6) prepared in 0.5X TBE (Appendix I). The band sizes were determined by comparing to a standard 1Kbp ladder (100bp – 12,000bp) after post-staining the gel with 1% ethidium bromide (EtBr) solution (Fisher BioReagents, Cat. No. BP1302-10) added to buffer in the ratio 1:10 and left to stand for 20 minutes on a shaking platform.

2.4.4.3 PCR Product Purification and DNA Extraction from Agarose Gels

Gel extraction was the preferred method of purification of DNA for the purposes of direct sequencing of amplified DNA fragments. This method was particularly applicable where templates yielded multiple bands in which case the DNA band of interest was cut out of the gel and purified for sequencing or cloning. Agarose gel stained with 1% EtBr solution was placed over UV lighting to illuminate DNA fragments of interest which were excised from the agarose gel and then purified using Qiagen's Gel Purification Kit (Qiagen, Cat. No. 28704). The clearly visible bands were excised from the agarose gel with a sharp sterile surgical blade and placed in pre-weighed 15ml tubes. The tubes containing the pieces of excised gel were weighed again. The difference in weight was the measurement used to determine the amount of buffer QG to be used. Subsequent steps in the procedure were followed according to the manufacturer's (Qiagen) specifications.

2.4.4.4 Analysis of Restriction Digested DNA Clones

For gel analysis of restriction digested samples 0.5ml tubes containing the sample were first spun briefly to collect all condensation. For each tube containing digested sample 2µl of 6X loading dye was added. Corresponding undigested samples of plasmids were also prepared for gel analysis (control experiments). Here 2µl of undigested sample was added to 8µl sterile milli-Q water and 2µl of 6X loading dye. The total volume of 12µl in each tube was carefully mixed by pipetting up and down, vortexing briefly before loading onto the agarose gel for electrophoresis.

2.4.5 TA Cloning of Gel Purified PCR products

2.4.5.1 Ligation Using TA pCRII Vector

The purified PCR product was ligated into a TA plasmid vector (pCRII TA Vector Dual Promoter cloning kit, Cat. No. 45-0007). TA plasmid vectors bear thymine bases on EcoRI restricted sites which bind to overhanging adenine bases on 5'-3' ends of the PCR product. Adenine bases on either end of the PCR product result from using particularly efficient Taq polymerase enzyme in the PCR reaction. The minimum volume of PCR product required for ligation into 50ng pCRII Vector to achieve at least 1:1 vector: insert ratio was determined from Invitrogen's protocol using the formula:

$$\text{Xng PCR product} = \frac{(\text{Ybp PCR product})(50\text{ng pCRII vector})}{\text{size of pCRII vector}}$$

NB:

Xng PCR product: The concentration of purified PCR product varied with template (mangrove amoeba and acanthamoeba isolates both ranged from 1.5ng/µl to 11ng/µl).

Ybp PCR product: PCR products of mangrove amoeba ranged from 500bp to 850bp while PCR products of acanthamoebae amplified with genus-specific primers (JDP1andJDP2) ranged between 400bp-500bp and acanthamoebae amplified with eukaryotic primers 570/1200R (Weekers et al., 1994) yielded a 1300bp fragment. Size of pCRII vector: 3900bp (vector map; www.invitrogen.com).

Yields obtained from gel purification ranged from 1.5ng/µl to 11ng/µl. At least 2µl to 3µl (3ng to 6.5ng) of PCR product was used for ligation reactions. Purifying the PCR product reduced the

possibility of salts in the PCR sample that would inhibit the action of the T4 DNA ligase in the reaction mixture (table 7).

Table 7: Typical ligation reaction mix

	1:1 (vector: insert) µl	1:3 (vector:insert) µl
Purified PCR product	1 (1.5-11ng)	3 (4.5-33ng)
10X ligation buffer	1	1
pCRII vector (2ng/µl)	2	2
Sterile dH_2O	5	3
T_4 DNA ligase	1	1
Total	10	10

The sample was incubated overnight at 14°C.

2.4.5.2 Transformation

The ligate was transformed into 'competent' cells (NEB Turbo Competent *E. coli* – High Efficiency kit. Cat. No. C2984H) rendered to uptake plasmid DNA containing ampicillin-resistant gene (pAMP). Transformations are detected by their antibiotic-resistant phenotype of blue/white colonies. The enzyme beta-galactosidase breaks down lactose to glucose and galactose. Bacteria without a plasmid turns blue, while bacteria with a plasmid forms white colonies. This method is a convenient selection mechanism to determine if the DNA fragment of interest has been inserted into the plasmid.

Two tubes containing 50µl of competent cells were thawed on ice for 10 minutes. For one of the tubes containing 50µl of competent cells, 5µl of the ligation reaction was added. To the other tube of competent cells, 5µl of pUC19 control DNA (1pg to 100ng plasmid DNA) was added. The tube containing pUC19 DNA served as a control to monitor transformation efficiency. The tubes were carefully flicked 4 to 5 times to mix DNA and cells (no vortexing was used in this procedure). To shock the cells, the mixture was placed on ice for 30 minutes followed by heat at precisely 42°C for exactly 30 seconds. Shocking creates a thermal imbalance on either side of the cell membrane creating a draft that sweeps plasmids into the cell. The mixture was placed on ice for 5 more minutes before pipetting the mixture into a 15ml cell

culture tube containing 950µl of SOC media at room temperature (media provided in the transformation kit with competent cells from NEB Cat. No. C2984H). This was placed in a shaking incubator (200rpm) at 37°C for 60 minutes to allow cells to recover and begin to express the antibiotic-resistance gene. After the one-hour incubation, cells were mixed thoroughly by flicking the tube and inverting before plating onto LB-Amp plates (see Appendix I).

The LB-Amp plates were coated with 100µl 'TruBlu' solution to promote expression of antibiotic resistant bacterial clones. SOC media was used to prepare serial dilutions of transformed cells. For pUC19 control DNA transformations, three serial dilutions were prepared 90%, 99% and 100%. Experimental ligation transformation dilutions were also prepared as follows: 90%, 100% and a pellet of cells (obtained after centrifugation) re-suspended in 100µl of SOC media. The total amount of transformation mix added onto each LB-Amp plate was 100µl. The plates were incubated overnight at 30°C and white colonies were re-plated on fresh LB-Amp plates and incubated overnight at 30°C to serve as back-up.

2.4.5.3 Plasmid Minipreps

In order to screen positive clones for the presence of an insert of expected size, plasmid minipreps were carried out. Positive clones (white colonies) were inoculated into 3mls LB-Amp broth and incubated overnight at 37°C on a shaking platform at 220 rpm. The same clones were duplicated by re-plating on LB-Amp plates and incubating overnight at 37°C. This was achieved using QIAprep Spin Miniprep Kit (Cat. No. 27104).

2.4.5.4 Restriction digestion of plasmid DNA from minipreps

Plasmids were subjected to restriction digests using EcoRI restriction enzyme to confirm the presence of an insert of expected size. Table 8 shows amounts used per restriction digest reaction mix.

Table 8: Restriction digest reaction mix

Solution	Amount (µl)
Plasmid DNA	3
Restriction Enzyme buffer (10X)	1
BSA (10X)	1
Sterile milli-Q water	4.5
Restriction enzyme (EcoRI)	0.5
Final Volume	10

The 'mix' was incubated at 37°C for one hour on a heat block.

2.5 Sequencing, Alignment and Phylogenetic Analysis

Purified PCR products and/or purified plasmid DNA containing PCR product were sent to sequencing facilities (Marshall University's Genomic Core Facility and/or Davis Sequencing, Davis, CA) for analysis. *Acanthamoeba* PCR products from *Acanthamoeba* isolates A1, A3, A4, A5, A6, FH were directly sequenced using 892/892C primers (useful in determining the DNA sequence of the informative DF3 region) (Schroeder et al., 2001) and subsequently concatenated for pairwise alignments. For plasmid DNA (*Acanthamoeba* PCR products from A2, A6, BP) TA plasmid vector sequences were removed prior to conducting pairwise alignments. *Acanthamoeba* spp. ***g***enotype determinations were done by phylogenetic analysis of sequence variation of DF3 region (Booton et al., 2004). Unresolved bases on experimental *Acanthamoeba* sequences were resolved by eye by examining primary data chromatograms and by comparing repeat sequences from respective plasmid clones. The 8 *Acanthamoeba* DNA sequences obtained in this study (See Appendix II) were aligned to each other and to previously determined *Acanthamoeba* sequences from GenBank (GenBank references: *Acanthamoeba castellanii* Castellani, U07413; *A. castellanii* Ma, U07414; *A. castellanii* Neff, U07416; *Acanthamoeba comandoni* AF019066; *Acanthamoeba culbertsoni* Al, AFOl9067; *Acanthamoeba griffini* Panola Mt.,AF019052; *A. griffini* Sawyer, AF019053; *A. griffini* TIOH37, S81337; *Acanthamoeba hatchetti* BH2, AF019068; *Acanthamoeba healyi* V013, AF019070; *Acanthamoeba lenticulata* JCI, U94739; *A. lenticulata* 7327, U9473 1 ; *A. lenticulata* 7212,U94732; *Acanthamoeba. Lugdunensis* Garcia, U07407; *Acanthamoeba palestinensis* Reich, U07411; *Acanthamoeba polyphaga* HC2, AF019056; *A. polyphaga* JacIS2, U07415; *Acanthamoeba* sp. Czech 4339, AF140711; *Acanthamoeba* sp. Czech 4706, AF140712; *Acanthamoeba* sp. Czech 43337, AF140713; *Acanthamoeba* sp. Czech 4528, AF140715; *Acanthamoeba* sp. Czech 4178, AF140716; *Acanthamoeba* sp. Czech 3668, AF140717; *Acanthamoeba stevensonii,* AF019069) using ClustalX 1.83 program.

Phylogenetic trees were built using Maximum Parsimony (MP) and Neighbor Joining (NJ) algorithms in PAUP* 4.10b (Swofford, 2000). These methods were based on the assumptions that transversions and transitions are equally probable and gaps were excluded from distance calculations. The reliability of internal branches in the NJ tree were assessed using bootstrap method in PAUP program with 100 replicates. Difference in nucleotide bases were calculated as a percentage in pairwise comparisons of sequences in distance matrices using

PAUP program. Trees were rooted with *Balamuthia mandrillaris* (GenBank reference number V039) sequence.

Mangrove amoeba nucleotide sequences were also edited based on primary data chromatogram and by comparing sequences yielded using respective forward and reverse primers. For cloned DNA plasmid, unresolved bases were compared with repeat sequencing of clones. Plasmid vector sequences were removed prior to performing a search in GenBank for similar amoebae sequences (National Centre for Biotechnology Information) database (www.ncbi.nlm.nih.gov). Sequences obtained in this study are in Appendix II.

Chapter III: Results

3.1 Prevalence of Amoebae in Diverse Environments

Amoebae are ubiquitous and occur virtually everywhere including aquatic and terrestrial habitats. As detailed in chapter one the current study focused on three amoebae isolated from the marine environment. In addition to the three marine isolates (ctenophore amoeba, mangrove amoeba, and a 'marine' *Acanthamoeba*), acanthamoebae from diverse 'extreme' environments including chlorinated tap water, fish mucus and an isolate from Berkely Pit, Montana (with an acidic environment of ~pH4) were included for comparisons. These additional acanthamoebae isolates were included since, like the 'marine' *Acanthamoeba,* these strains were isolated from 'extreme' environments.

3.1.1 Amoebae on Ctenophore (*Mnemiopsis sp.*) Comb Plates

Isolation of amoebae from the ctenophore surface resembling the presumed symbiont (ctenophore amoeba, see Fig. 23) were conducted on four occasions between June and November, 2007 using twenty-four animals purchased from Gulf Specimen Marine Laboratories Inc., Panacea, Florida. A total of 144 ctenophore comb plate samples (six samples per ctenophore) were processed (in multiple media formulations) and only one type of gymnamoeba (morphotype 1, see Fig. 24) was found on the surface of four of the 144 samples. The term 'morphotype' or 'morphospecies' is used here because of difficulties in identifying species at the light microscope level. This categorization clearly showed that the *Vexillifera*-like amoeba was smaller (~4µm) than the ctenophore amoeba (5-7µm). Moreover, the finger-like subpseudopodia radiating from the advancing edge of this isolate were quite different from the wavy-like advancing edge of the ctenophore amoeba (Fig. 23, 24). Indeed, it is the characteristic sub-pseudopodia that justify assigning morphotype 1 to the genus *Vexillifera* although no species have been described in this size range. The smallest named species is *V. minutissima* but these cells average 10µm in length (Page, 1983). Although the ctenophore amoeba was not isolated in any of the attempts, the one different amoeba encountered in this study was similar to one of the amoebae found on the ctenophore comb plates in a similar study by Versteeg (2007).

During the attempts to isolate amoebae, three different experimental media formulations at two salinity concentrations each (SE28, SE10, SWMY28, SWMY10, SW28, SW10; see

Appendix I) were used for enrichment cultivation of the amoebae. None of the media yielded the ctenophore amoeba over the course of this study although all will sustain the ctenophore amoeba in the laboratory. Media SW10 yielded morphotype 1 (*Vexillifera*-like amoeba) and media SW28 and SWMY10 yielded a small heterotrophic flagellate identified by its anterior proboscis as *Rhynchomonas* sp. (Fig. 25). Ciliated protozoa (Fig. 26a, b) were abundant in all media types.

The inability to isolate the Ctenophore amoeba in media formulations used for its routine cultivation (original strains from Versteeg) was surprising since the majority of ctenophores from Florida were shown to harbor amoebae using direct observation methods ((Versteeg, MS. Thesis, 2007). In the present study, the surfaces of animals were not observed before processing since they had been transported overnight and there was a need to set up cultures rapidly. Some material was, however, fixed for scanning electron microscopy. Observation of 9 tissue samples (10mm x 10mm pieces of comb plate tissue from three randomly selected ctenophores) revealed that one surface sample was rich in amoebae, assumed to be the ctenophore amoeba based on the morphology (Fig. 27). In this sample, the number of amoebae (Ctenophore amoeba) occurring on a randomly selected area on a 10mm x 10mm area of comb plate was estimated to be 784 amoebae mm^{-1}.

All the amoebae counted had a crescent-like form as described by Moss et al. (2001). These investigators were the first to note the presence of a presumed symbiotic amoeba on the ctenophore surface. While amoebae were expected to be ~15µm in size (Moss, 2001), amoebae in electron micrographs from this study were found to be between 11-13µm. The difference in size may have been due to differences in SEM sample preparation techniques. Interestingly, amoebae in culture were much smaller in size (usually around 5-7µm, see Fig. 23) although the size range increased in older cultures since amoebae had the ability to fuse and form much larger multinucleate cells (see Fig. 34).

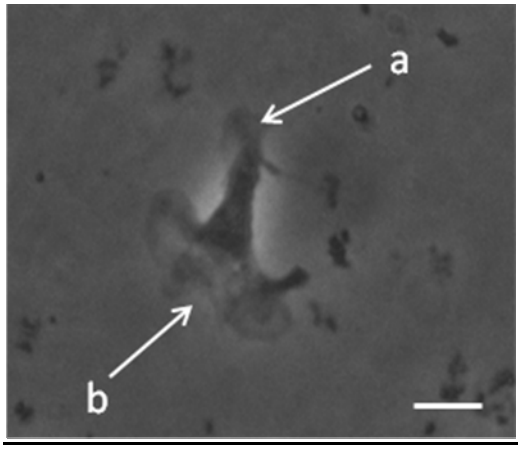


Figure 23: Phase contrast micrograph of ctenophore amoeba showing hyaline cap in the advancing front and trailing uroidal fillaments at the posterior end, features characteristic of *Flabellula*-like group. (Background: Bacterial prey). a) trailing uroidal fillaments b) Transparent hyaline zone in the advancing front. Scale bar:2µm.

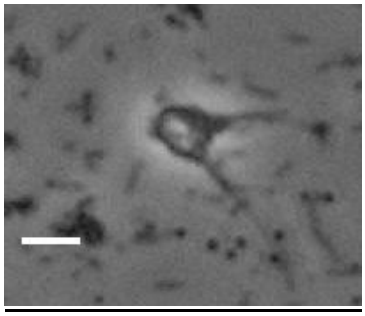


Figure 24: Phase contrast photomicrograph of morphotype 1 (*Vexillifera*-like) amoeba. Scale bar:5µm.

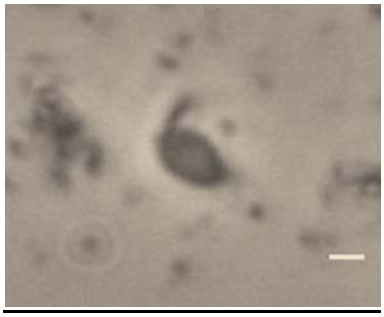


Figure 25: Phase contrast photomicrograph of flagellate (Rhynchomonas). Scale bar:2µm.

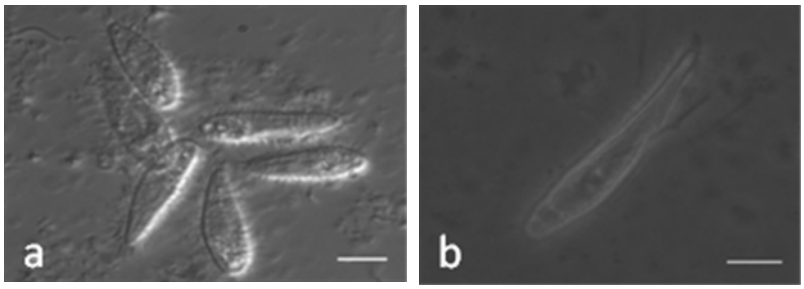


Figure 26a, b: Integrated Modulation Contrast (a) and Phase contrast (b) photomicrographs of ciliates from ctenophore comb plates. Scale bar (Fig. a):5µm. Scale bar (Fig. b):10µm.

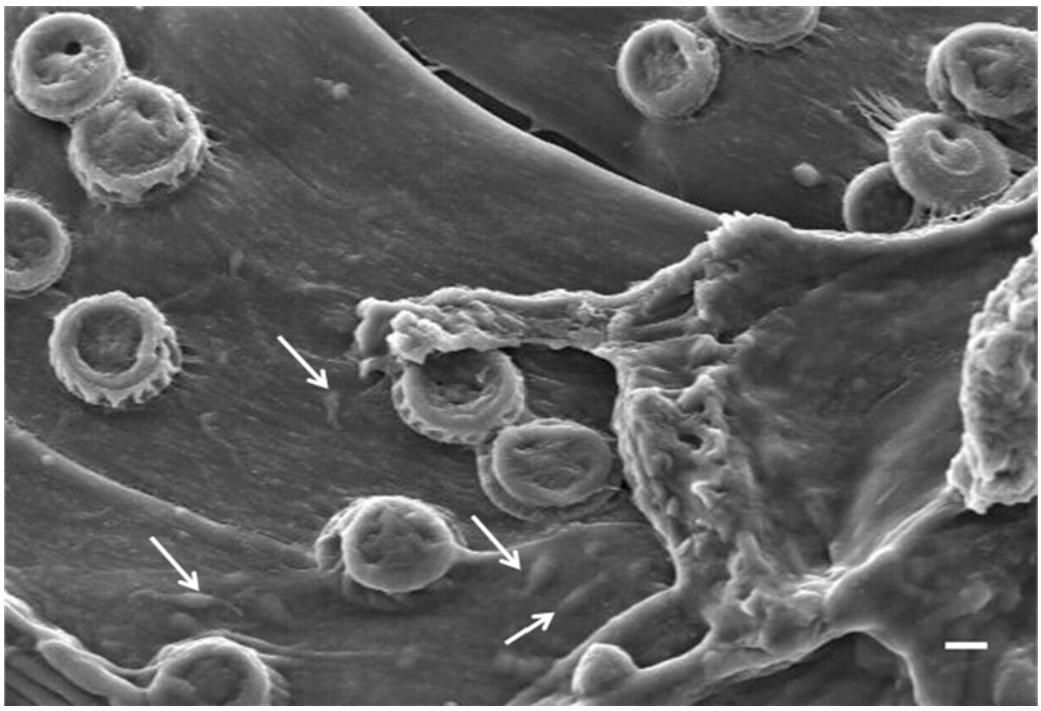


Figure 27: Scanning electron photomicrograph of ctenophore comb plate surface infested with the ciliate, *T. ctenophorii*. Arrows indicate ctenophore amoebae. The amoebae had a crescent-like shape comparable to the morphology of this amoeba detailed in a previous study by Moss et al. (2001). Scale bar:10μm.

3.1.2 Amoebae in domestic tap water (WV)

Some 15 years ago a link between tap water and *Acanthamoeba* eye infections was proposed, at least as it relates to contact lens wearers (Booton et al., 2004). Although non-lens wearers can suffer from amoebic keratitis (AK), contact lens wearers are more susceptible (Joslin C. E. et al., 2007). Amoebae invade the cornea, possibly through an existing lesion or abraded region, and migrate along nerve fibers. As they multiply and migrate they cause cellular damage and the painful, and sight-threatening disease, AK (Fig. 28). The belief then, and now, is that if contact lenses are rinsed in tap water, they can become contaminated with a few amoebae. During storage, the amoebae multiply on the lens surface which is often coated in bacteria. This provides the means of transferring a large infective dose of amoebae to the eye surface.

This pathway assumes that acanthamoebae are present in tap water and can withstand the rigors of water treatment plants (filtration and chlorination). Only one previous study has been conducted to examine US water supplies (Shoff et al., 2007). In this study by Shoff et al., (2007), it was found that 2.8% of all water samples in Florida contained *Acanthamoeba*. Almost 20% of the samples contained other types of amoebae not linked to pathogenicity.

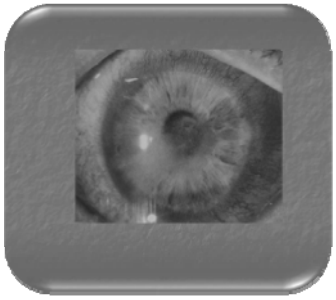


Figure 28: Eye infected with *Acanthamoeba*, the causative organisms in AK infections. The cloudy patch on the cornea is due to thousands of invading amoebae. Image courtesy of Dr. David Seal.

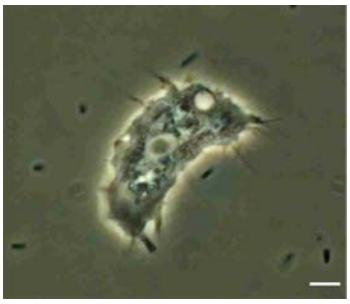


Figure 29: Light micrograph of *Acanthamoeba*. This common soil amoeba is around 20µm in length and easily recognizable by its spiny pseudopodia that radiate from the cell surface. Scale bar:5µm.

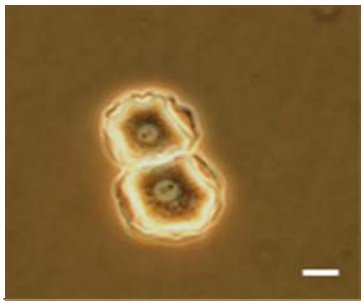


Figure 30: Resistant cysts of *Acanthamoeba.* When conditions become hostile (such as food depletion or desiccation) amoebae often form cyst stages. Cysts are about 12µm in diameter and can pass through sand filters commonly used in water treatment plants. Scale bar:5µm.

A recent outbreak of AK in the Chicago area (M. Shoff, pers. comm.) has renewed interest in tap water and amoebae. The present study attempted to find culturable acanthamoebae and other naked amoebae in the domestic water supply of Huntington, WV. Amoebae are common in soil and freshwater and frequently exist as both a trophic (i.e. feeding) form and a resting (i.e. cysts) form. The trophic form of *Acanthamoeba* is distinctive (Fig. 29) since the pseudopodial extensions, referred to as acanthopodia, are spiny in appearance. *Acanthamoeba* readily form resistant cysts (Fig. 30) that allow cells to survive the water treatment process. Cyst-forming amoebae other than acanthamoebae were also encountered in the water supply including *Vannella*-like and limax amoebae (Fig. 31, 32) perhaps also able to survive the high chlorine levels since they are able to form cysts. Of turbidity measurements obtained, levels were high on two occasions (0.33ntu and 2.03ntu). Chlorine levels were within EPA recommendations (up to 4mg/l) and were <1 mg/l in the months of July to October while levels >1mg/l were seen in months of June and July (Table 9). This is only the second study documenting the prevalence of amoebae in U.S. tap water supplies and the first for West Virginia.

Table 9: Water samples in five different locations on different days. Out of 173 liters sampled over 8 months, 31 one-liter samples were positive for amoebae out of which 20 were acanthamoebae. A wider range of amoebal types was seen in instances where the chlorine level tested was low.

Date	Location #	Isolate	Turbidity (ntu)	Chlorine (mg/l)
04/17/07	1	Acanthamoeba	-	-
05/17/07	1	Acanthamoeba	0.22	-
05/22/07	2	Acanthamoeba	0.33	-
05/24/07	1	Acanthamoeba	0.30	-
05/24/07	1	Acanthamoeba	0.08	-
05/31/07	1	Acanthamoeba	0.11	-
05/31/07	1	Other*	0.11	-
05/31/07	1	Acanthamoeba	0.11	-
05/31/07	1	Acanthamoeba	0.11	-
06/19/07	1	Acanthamoeba	0.71	1.76
06/26/07	1	Acanthamoeba	1.46	1.75
06/26/07	2	Acanthamoeba	2.03	1.46
07/10/07	1	Acanthamoeba	-	1.92
07/16/07	1	Other*	-	1.78
07/16/07	1	Acanthamoeba	-	1.78
07/26/07	1	Acanthamoeba	-	1.88
07/26/07	1	Acanthamoeba	-	1.88
08/24/07	5	Acanthamoeba, limax, Vannella	-	0.03
08/24/07	5	Acanthamoeba, limax, Vannella	-	0.03
08/24/07	5	Acanthamoeba, limax, Vannella	-	0.03
08/24/07	5	Acanthamoeba, Vannella	-	0.03
08/24/07	5	Acanthamoeba, Vannella	-	0.03
10/12/07	3	limax	-	0.86
10/12/07	3	limax	-	0.86
10/12/07	3	limax	-	0.86
10/12/07	4	limax	-	0.96
10/12/07	4	limax	-	0.96

Date	Location #	Isolate	Turbidity (ntu)	Chlorine (mg/l)
10/12/07	4	limax	-	0.96
11/09/07	4	limax	-	1.39
11/09/07	4	limax	-	1.39
11/09/07	4	limax	-	1.39

Locations #1 to #4 = Huntington area; Location #5 = Hurricane area (* unidentified protist cysts)

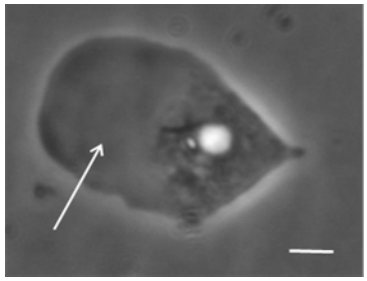


Figure 31: Micrograph (phase contrast) of vannellid amoeba isolated from tap water (Huntington, WV) cultured in AS media seeded with prey bacterium *E. coli*. Arrow shows broad spatulate hyaline zone characteristic of the genus *Vannella*. Scale bar:10µm.

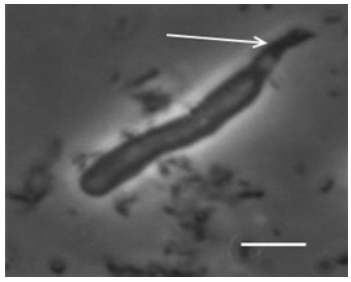


Figure 32: Micrograph (phase contrast) of limax amoeba isolated from tap water (Huntington, WV) cultured in AS media seeded with prey bacterium *E. coli*. Arrow shows conspicuous uroids in the posterior end of the amoeba. Scale bar:10µm.

3.2 Characterization of Ctenophore Amoeba

As discussed earlier in chapter one, efforts to characterize the ctenophore amoeba were predominantly based on morphological and physiological studies. Morphology (and behavior) of the ctenophore amoeba was determined using light microscopy, fluorescence microscopy and electron microscopy (SEM). Transmission electron microscopy was not explored in the current study since it is being conducted by collaborators in the overall project (see details in chapter one, section 1.8).

Cell Form. Ctenophore amoeba cells in culture were small (ca. 6 μm in length) with a very faint (thin) anterior hyaline zone (Fig. 23). In moving cells, this zone changed shape rapidly. The appearance of the hyaline zone and its ability to markedly change shape are unusual features in amoebae (not described previously) and suggest that this is an undescribed species, and possibly a new genus. Cells had occasional trailing filaments from the posterior uroid. The morphology and behavior of these amoebae were investigated in this study as an initial step towards describing a new species.

An interesting feature of this amoeba was that after 48h in culture, cells often began to fuse and form a network of cells (Fig. 33, 34). Amoebae would remain in this state for several days (ca. 96 h) before separating and reverting to single uninucleate amoebae. At that time, the numbers of cells would decrease as cultures entered stationary and death phases. Rapid population crashes were typical of this isolate and stock cultures rarely survived beyond 168 h. To maintain stocks, it was important to sub-culture the amoebae regularly, certainly within 7 days.

The morphology of the cell was studied by SEM (Fig. 36). Most attempts to prepare samples of amoeba on cover-glasses were unsuccessful because cells became dislodged during preparation. But in the few cells observed, images showed parts of the hyaline zone and a raised cell body. The cell appeared to be covered by short projections (pseudopodia) that might, in part, reflect the rapidly changing appearance of cells observed by light microscopy. However, it should be noted that the appearance of cells in light microscopy and electron microscopy differed, notably in the extent of the hyaline zone. It is postulated that fixation and preparative methods may cause cells to shrink and round up. Such shrinkage is a common problem with electron microscopy preparative methods. This would also account for the loss of samples from the glass surface.

Nucleus number, size and structure. In order to determine the nature and size of the nucleus, a DNA-specific fluorochrome (DAPI) was used for staining. A single nucleus, about 2μm in size, was evident confirming that the amoeba was uninucleate (Fig. 35), although when cells fused they become temporarily multinucleate. The single nucleus was characteristically amoeboid with a prominent (unstained) central nucleolus.

Rate of locomotion. Least cellular activity was observed at the lowest and highest salinities (0g/l, 5g/l, 40g/l and 50g/l). In fact, motion was so slow at these salinities that rates were not calculated. Locomotion was greatest at 10g/l which was also the salinity that promoted maximum growth. Here, cells travelled at 0.59 μm sec^{-1} (= 35.4 μm min^{-1}). This translates to some 6 cell lengths per minute. At 30g/l, the salinity closest to coastal waters, the rate of locomotion was half the rate at 10g/l (0.3 μm sec^{-1}) (Table 10).

Size. Measurements were made only on locomoting amoebae that were attached to the substratum for cells cultured at 10g/l, 20g/l and 30g/l sea salt. For amoebae growing at 0g/l, 5g/l, 40g/l and 50g/l measurements were made on both attached and floating forms since many of the amoebae were rounded floating forms. Results showed that length and breadth measurements varied considerably (as evidenced by magnitude of SE) and that even across the range of salinities promoting maximum growth and attachment (10g/l–30g/l salt) mean dimensions showed no clear trends. This underscores the variability of sizes within a species. Mean length of amoebae across these optimum salinities was 5.9 μm. When all the data were considered, the length across all salinities averaged 5.2 μm (Table 10). This slight decrease in mean size was probably due to the fact that floating cells were rounded and had smaller length dimensions. The mean cell lengths at salinity (10g/l) and at a salinity close to natural sea water (30g/l) were not significantly different ($p=0.95$; $\alpha=0.05$) while the differences in the mean cell breadths at 10g/l and at 30g/l were significant ($p=0.01$; $\alpha=0.05$). Conversely, the difference in mean cell lengths at 30g/l (close to natural sea water) and 50g/l (highest salinity) was significant (t-test: p-value= 0.04; $\alpha=0.05$) while the mean cell breadths at the same salinities (30g/l and 50g/l) was not significant ($p=0.15$; $\alpha=0.05$). Overall, the ANOVA test showed that the difference in mean cell lengths at all salinities tested was not significant ($p=0.15$; $\alpha=0.05$), while the difference in mean cell breadths at all tested salinities was significant ($p=0.01$: $\alpha=0.05$). Rounded floating forms of amoebae were seen at salinities 0g/l and 5g/l and at higher salinities 40g/l and 50g/l.

Table 10: Average length dimensions and average locomotion rate of ctenophore amoeba at different salinities (n=10)

Salinity (g/l)	Mean Length(µm)	SE	Mean Breadth(µm)	SE	Velocity (µm/sec)	SE
0	4.8	0.21	2.3	0.18	nd	nd
5	4.1	0.45	2.5	0.21	nd	nd
10	6.4	1.24	2.4	0.16	0.59	0.1
20	4.8	0.33	2.7	0.33	0.43	0.03
30	6.5	1.00	3.3	0.26	0.3	0.04
40	5.7	0.40	2.8	0.39	nd	nd
50	4.2	0.20	3.8	0.20	nd	nd

nd= no data

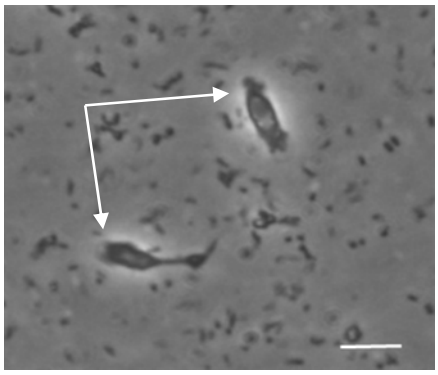


Figure 33: 12-hour culture of individual amoebae (ctenophore amoeba) in 32g/l salinity. Arrows show amoebae. Growth media also consists of prey bacterium *E. coli* visible in the background. Scale bar:5µm.

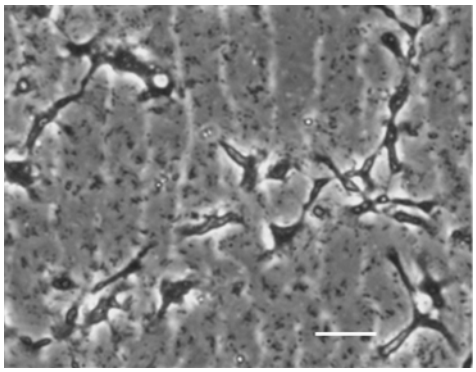


Figure 34: 48-hour culture with now fused amoebae (ctenophore amoeba) in the same media, 32g/l salinity. Scale bar:7µm.

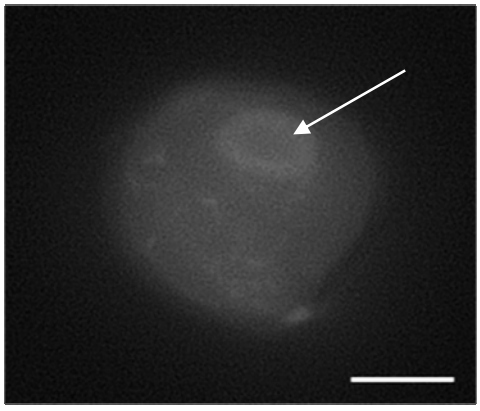


Figure 35: Amoeba cell fixed with 1% gluteraldehyde and stained with DNA-specific fluorochrome (DAPI). Image depicts an unstained nucleolus visible within a stained nucleus (see arrow). Scale bar:2µm.

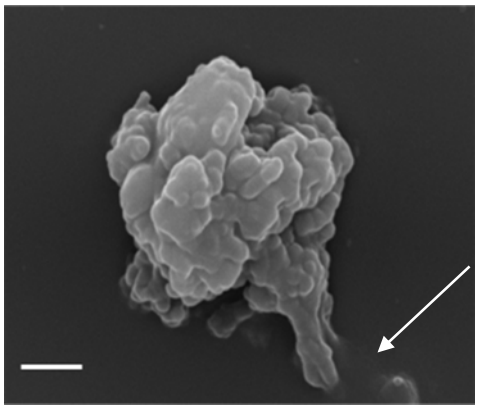


Figure 36: SEM image showing typical morphology of naked amoeba. Cells round up (after fixation with gluteraldehyde) and have extending pseudopodia (arrow) attached to the substratum. Definitive identification will require further work with the use of TEM. Scale bar:2µm.

3.3 Physiological Characterization of Amoebae

Factors useful in physiological characterization of amoebae include determination of growth rate, generation time and locomotive rate and are usually useful in distinguishing between species of amoebae (Page, 1988). All three marine isolates in the study (ctenophore amoeba, mangrove amoeba and 'marine' *Acanthamoeba*) have been subjected to the three above named factors for characterization. One more isolate, *Acanthamoeba* tap water isolate no. A1, is subjected to the same factors for comparison with the 'marine' *Acanthamoeba* isolate.

In addition, two amoebae isolates from tap water isolations (vannellid and *Acanthamoeba*) were subjected to varying chlorine levels to determine tolerance to this disinfectant. The vannellid isolate was used as a control because, like members of the genus *Acanthamoeba*, forms resistant cysts when conditions are unfavorable and would therefore form cysts in the presence of chlorine.

3.3.1 Salinity Tolerance

For all salinity tolerance experiments, growth rates were determined over a range of salinities (at ambient temperature 25°C). Log_{10} mean number of cells (n = 3, for ctenophore amoeba isolate and n=5, for mangrove amoeba isolate) were plotted against time (h) for the period when amoebae were in the exponential phase of growth (at least three data points spanning the exponential growth phase were included). This yielded linear relationships for which regression equations were calculated. Generation times were calculated from the slopes of the exponential phases of growth after transforming the densities to Log_{10} mean number of cells.

Ctenophore amoeba

In all cases (0g/l, 5g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l), the fit of the regression lines was significant ($p<0.05$) [Figs. 39 a(ii)-g(ii)], and high R^2 values reflecting the fit of the slope were obtained (0.64 – 0.97). The maximum mean number of cells recorded per Petri dish was 624,038 amoebae at 20g/l [Figs. 39 a(i)-g(i)]. This density represents the mean yield of cells per dish (n=3). Lowest yields were obtained at the extreme salinities (both high and low) (Table 11).

Surprisingly, the fastest growth was found at 10g/l salt (doubling time of 7.9h) (Fig. 41). This was shorter than the generation time at 30g/l (the salinity of coastal water) where amoebae divided every 29.9 h. A paired sample t-test comparison between generation times at 10g/l and

30g/l yielded a p-value of 0.0058 indicating that these growth rates were significantly different ($\alpha = 0.05$). Amoebae grew at 0g/l salt (i.e. freshwater conditions), however, the generation time was found to be 57.1h reflecting slower growth rate. The paired t-test suggests that the difference in generation times between salinities 0g/l and 10g/l was not significant (p-value=0.24; $\alpha = 0.05$).

As noted earlier, amoebae did not survive well in culture beyond 168 h. The sudden decrease in amoebal numbers after this time hints at a possible fastidious requirement of these amoebae as does the difficulty of establishing cultures from ctenophore tissue known to be rich in amoebae. Many other free-living amoebae remain viable well into stationary phase, however ctenophore amoeba rapidly died perhaps in response to lack of essential nutrients, reduced prey concentrations or a sensitivity to the build up of metabolites. The precise reasons are unknown but it was important to sub-culture amoebae at least every 6 days.

Table 11: Generation times and regression equations of ctenophore amoebae at each salinity based on exponential growth for up to 168 h. (n=3)

Salinity (g/l)	Generation time (h)	SE between replicates	Maximum number of cells recorded	R -square	Regression Equation	p-value
0	57.1	5.0	582906	0.64	y = 0.009x + 3.800	0.000141
5	30.3	13.7	69338	0.89	y = 0.006x + 4.171	1.02E-05
10	7.9	0.5	416026	0.96	y = 0.030x + 3.594	6.43E-07
20	25.2	1.0	624038	0.94	y = 0.027x + 4.013	9.9E-07
30	29.9	4.3	452457	0.88	y = 0.022x + 4.248	1.33E-06
40	27.6	2.6	391346	0.97	y = 0.024x + 3.900	1.3E-07
50	35.0	3.6	192735	0.88	y = 0.018x + 4.100	6.07E-07

Mangrove Amoeba

The salinity tolerance of the mangrove amoeba isolate was investigated by subjecting the amoebae to varying salinities (0g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l). Survival was observed at all salinities. The maximum number of cells per Petri dish (n=5) recorded was 303,205 at 20g/l (Table 12). Growth rates were computed from semi-log plots of cell density against time. Regression equations [Fig. 42 a(ii)-g(ii)] were generated for the exponential phase of growth and in all cases the fit of the regression lines was significant ($p<0.05$).

The fastest growth was found at 20g/l salt (doubling time of 34.0 h). This was shorter than the generation time at 30g/l (the salinity of coastal water) where amoebae divided every 53.2h. A paired sample t-test showed that the variation in generation times at 20g/l and 30g/l was significant with a p-value of 0.043687 ($\alpha=0.05$). Amoebae grew at 0g/l salt (i.e. freshwater conditions) however the generation time was found to be higher (55.3 h) than at optimum salinity (20g/l). A paired sample t-test showed that the difference in generation times at 0g/l and 20g/l was not significant ($P=0.14$; $\alpha=0.05$). Cells took longest to divide at 10g/l where the generation time was 202.3h. However, in light of the division rates at the other salinities, this result appears anomalously high, especially since the generation time at 0g/l (AS) was some four times faster. Overall, there was considerable variation in the division rate data and an ANOVA analysis showed that the generation times at all tested salinities (0g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l) had no significant variation ($p=0.5$; $\alpha=0.05$). The reason for this variation is unknown, but this amoeba did tend to be very slow moving such that cells would divide and remain in patches. The counting method employing random fields of view was prone to recording large differences between adjacent counts. In hindsight, an improved counting method should have been developed.

Table 12: Generation times and regression equations of the mangrove amoeba at each salinity based on exponential growth for up to 168 h. (n=5)

Salinity (g/l)	Generation time (h)	SE between replicates	Maximum number of cells recorded	r -square	Regression equation	p-value
0	55.3	9.8	94487	0.70	y = 0.007x + 4.020	2.35E-05
10	202.3	30.7	62756	0.343	y = 0.002x + 4.354	1.47E-06
20	34.0	5.6	303205	0.468	y = 0.003x + 4.271	3.17E-05
30	53.2	4.2	116346	0.347	y = 0.002x + 4.263	8.55E-06
40	68.8	14.3	88846	0.042	y = 0.000x + 4.347	1.11E-05
50	88.5	130.0	69808	0.189	y = -0.001x + 4.342	3.16E-05

Acanthamoeba Isolates

In the case of acanthamoebae, a group of amoebae that grow well on agar seeded with the prey bacterium *E. coli,* migration rate can be used as an index of growth since migration (distance travelled along a streak of bacteria) is a reflection of how fast a culture divides. A rapidly growing strain advances rapidly and shows high density of cells at the advancing front (Fig. 37).

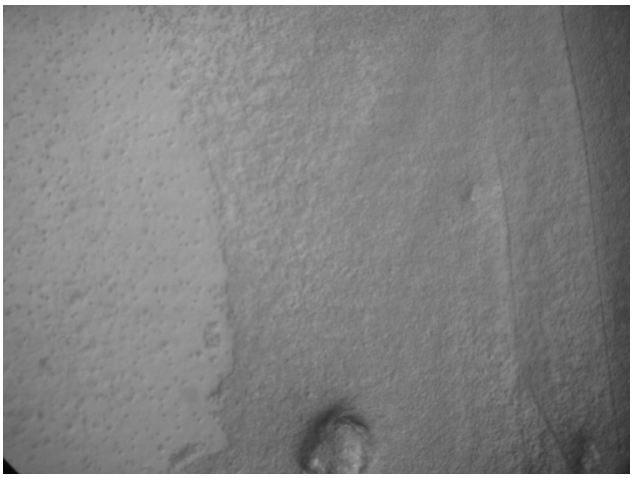


Figure 37: Photo showing advancing trophic *Acanthamoeba* moving along a streak of the bacterium *E. coli*. Typically these amoebae only moved a few microns as they fed. Distance travelled in millimeters is due to replication.

While the tap water strain A2 showed very little growth at extreme salinities and no growth at 45g/l, both *Acanthamoeba* isolates (tap water and marine) grew at 0g/l, 5g/l, 10g/l, 15g/l, 20g/l, 25g/l, 30g/l, 35g/l and 40g/l (Fig. 38). [Overall, the tap water isolate showed lower migration rates except at 5g/l and 10g/l where mean migration rate were higher (5.11, SE±0.5 and 5.96, SE±0.4 respectively) than marine isolate (4.58, SE±0.8 and 5.21, SE±1.1 respectively)]. At these salinities (5g/l and 10g/l), the variation in mean migration rates according to the t-test for the marine isolate and the tap water isolate was insignificant since separate t-tests at each salinity yielded a p-value of 0.6 (α=0.05). The best (highest) mean migration rate for the marine isolate was found at 15g/l (5.49, SE±0.3) while the tap water isolate performed best at 10g/l (5.21, SE±0.4). These optimum salinities (10g/l and 15g/l) are close to the salinity/osmotic conditions found in the human eye (Dr. D. Seal, pers. comm.) No significant variation (p-value=0.51) was detected between the two treatments (ANOVA $p<0.05$) for both

isolates. Overall, the marine isolate performed better than the tap water isolate since migration was evident even at the higher salinities (40g/l and 45g/l) while the tap water isolate did not migrate at all at 45g/l and advanced minimally at 40g/l (0.09mm/hr). Significant variation in mean migration rates (t-test; $p<0.05$) between the two isolates was only seen at higher salinities i.e. 30g/l, 35g/l, 40g/l and 45g/l ($\alpha=0.05$) suggesting that their physiological properties may be different. In conclusion, both isolates survived well over a wide salt range (surprising for the tap water isolate). However, the marine isolate grew better at higher extremes (40g/l-45g/l).

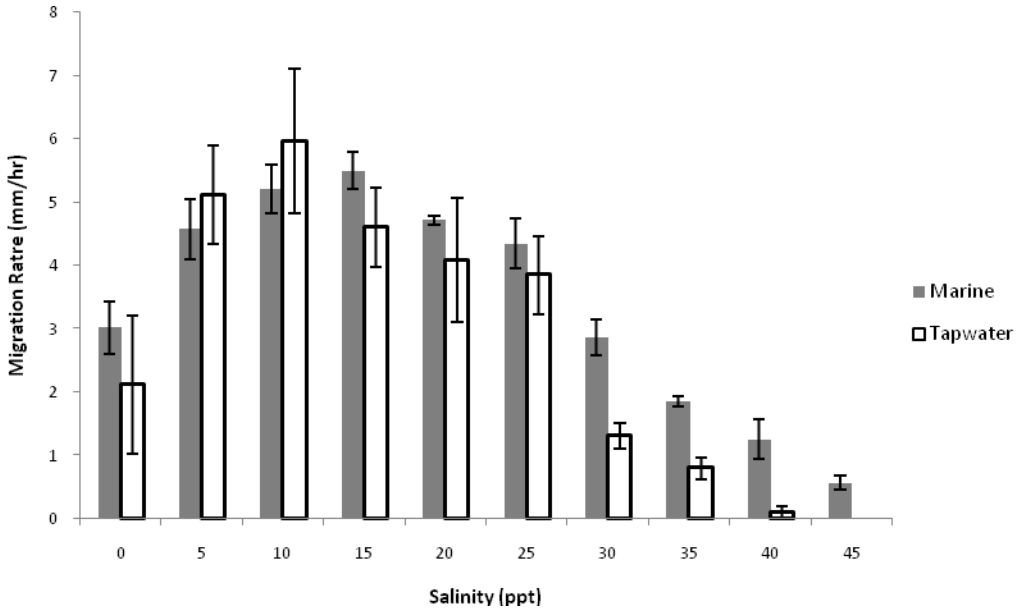


Figure 38: Comparison of migration rates as a measure of growth rates (Booton et al., 2004), of a marine (A1) and tap water strain (A2) grown at different salinities (0g/l, 5g/l, 10g/l, 15g/l, 20g/l, 25g/l, 30g/l, 35g/l and 40g/l, 45g/l). n=3

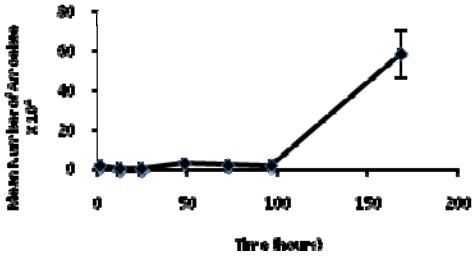


Figure 39(a) (i)

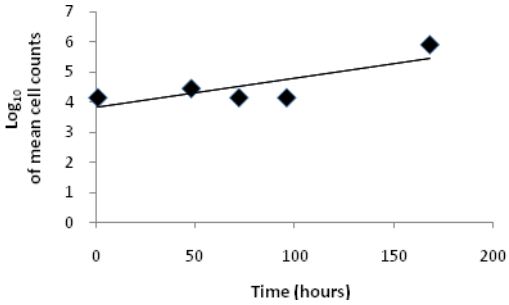


Figure 39(a) (ii)

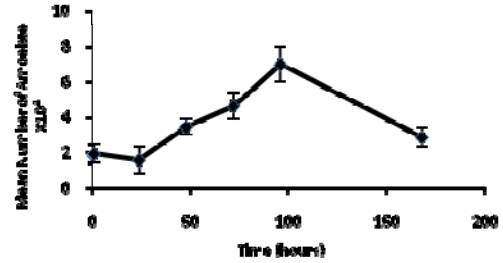


Figure 39 (b) (i)

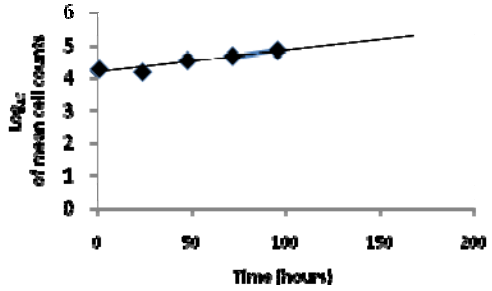


Figure 39(b) (ii)

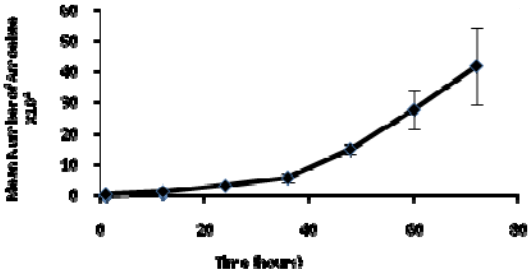


Figure 39 (c) (i)

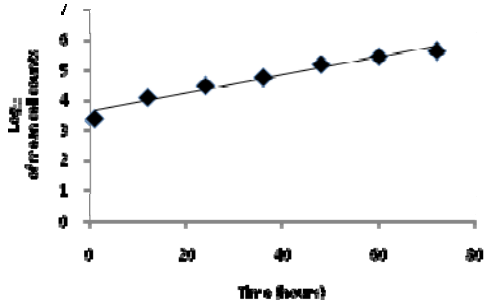


Figure 39(c) (ii)

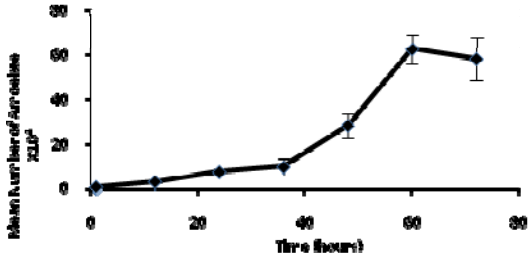


Figure 39 (d) (i)

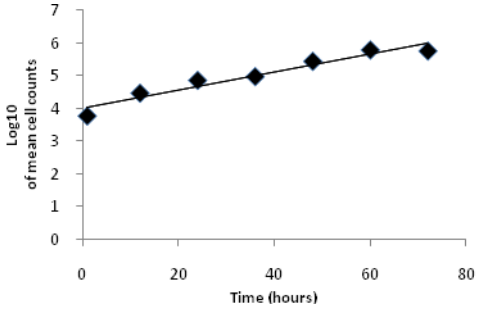


Figure 39 (d) (ii)

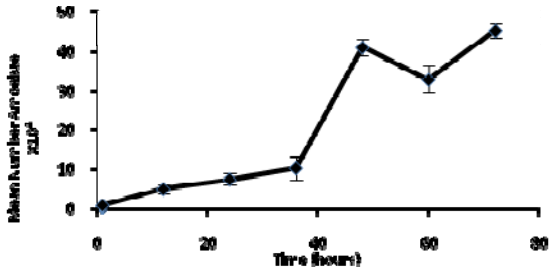


Figure 39 (e) (i)

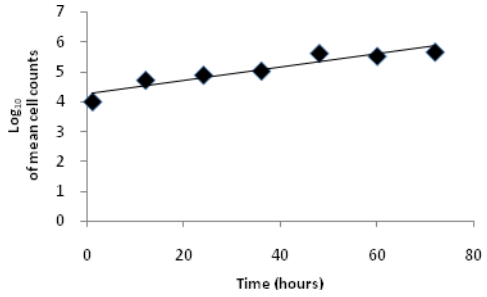
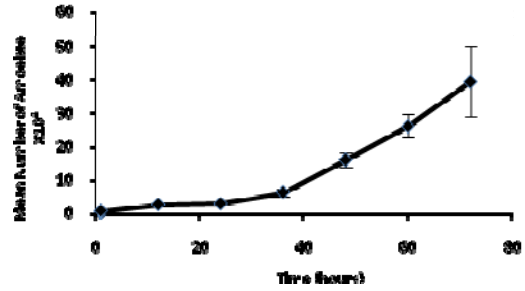


Figure 39 (e) (ii)



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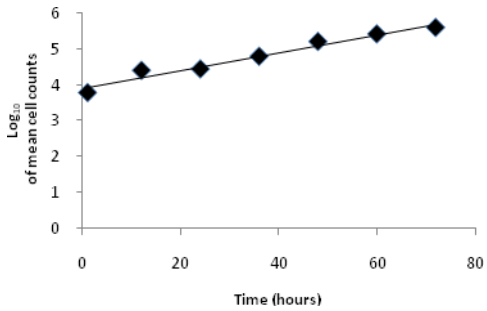


Figure 39 (e) (ii)

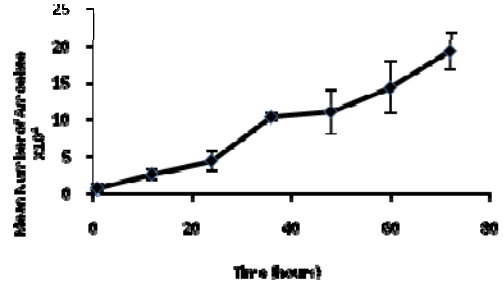


Figure 39 (g) (i)

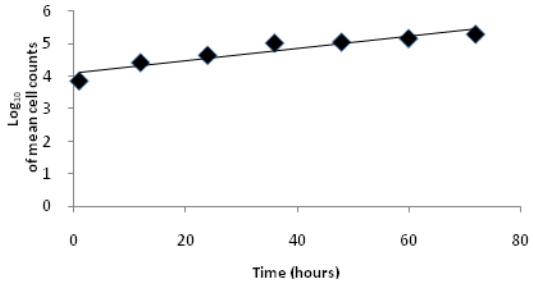


Figure 39 (g) (ii)

Figure 39 a(i)-g(i): Growth curves of ctenophore amoebae over time (up to 168h) at salinities 0g/l, 5g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50 g/l. n=3.

Figure 39 a(ii)-g(ii):: Growth curves for ctenophore amoeba based on Log_{10} of mean cell counts per plate against time (h) at salinities 0g/l, 5g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50 g/l. n=3.

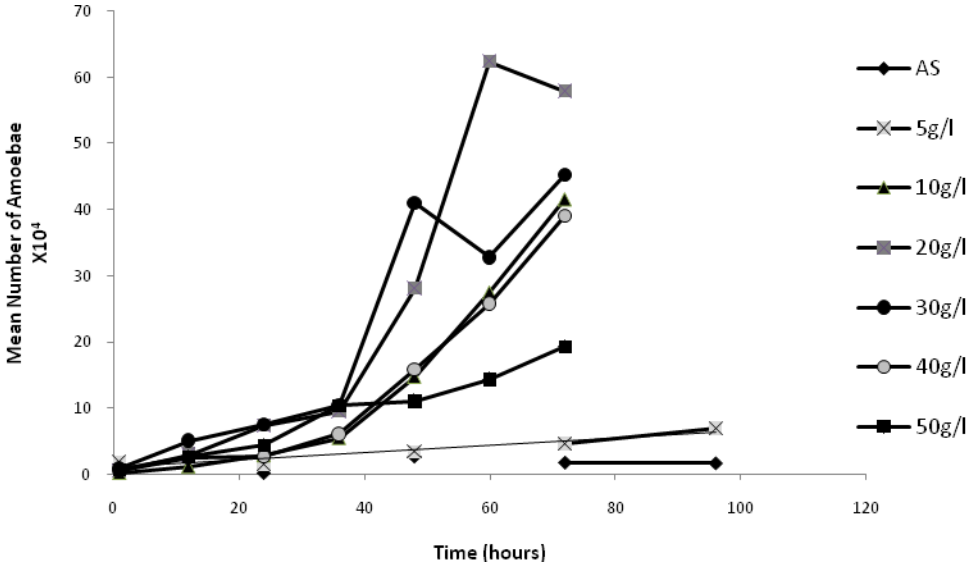


Figure 40: Summary of growth curves for ctenophore amoebae at different salinities (0g/l, 5g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50 g/l)

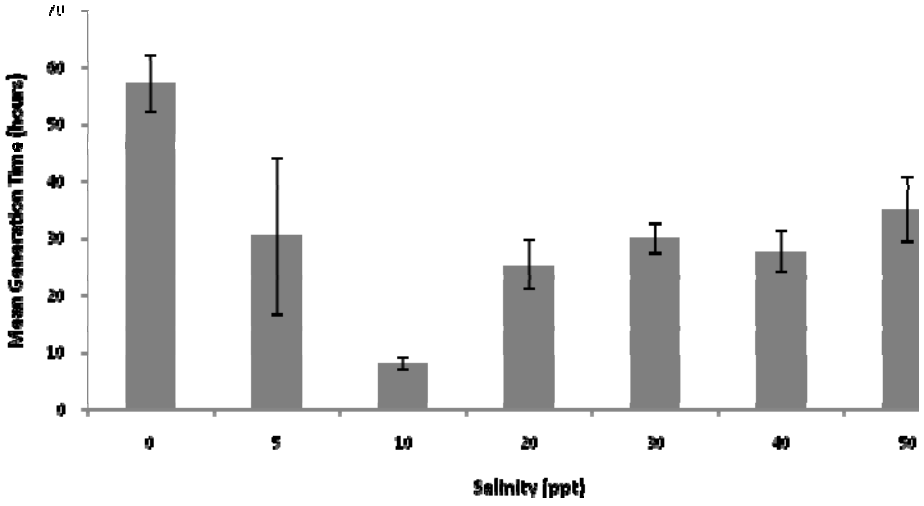


Figure 41: Mean generation time for ctenophore amoebae in h at salinities 0, 5, 10, 20, 30, 40, 50 (n=3).

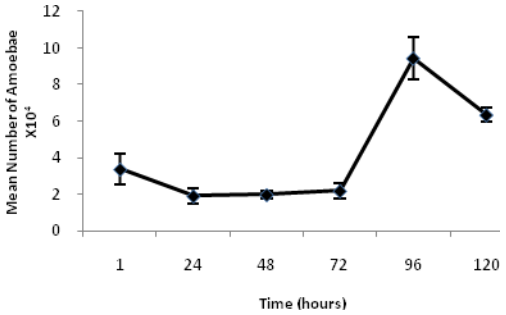


Figure 42 (a) (i)

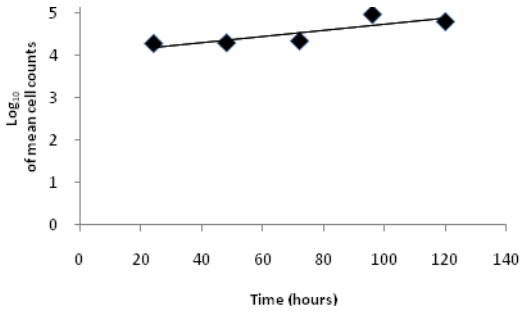


Figure 42 (a) (ii)

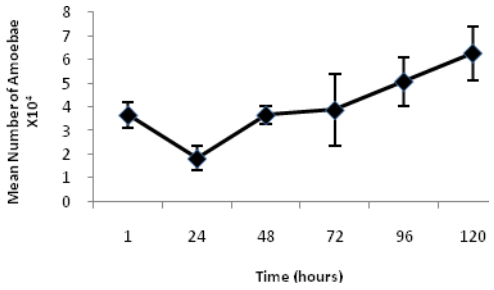


Figure 42 (b) (i)

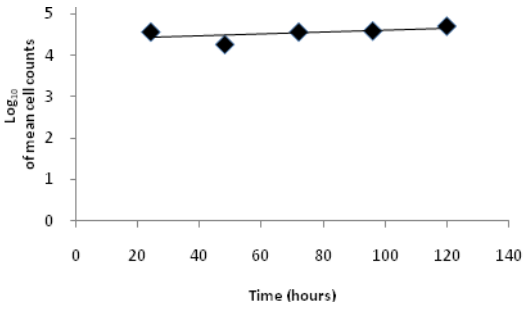


Figure 42 (b) (ii)

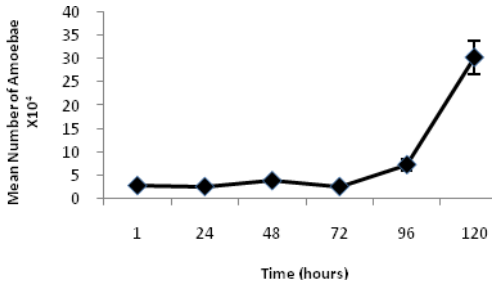


Figure 42 (c) (i)

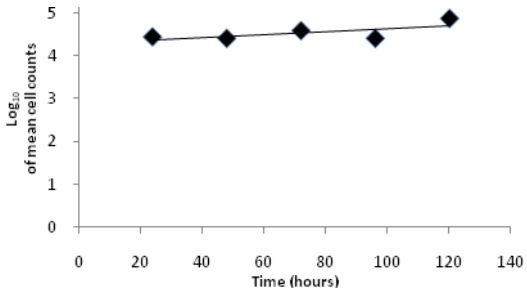


Figure 42 (c) (ii)

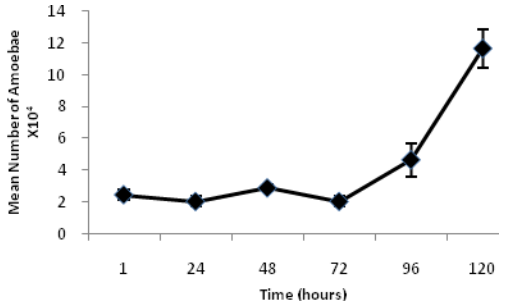
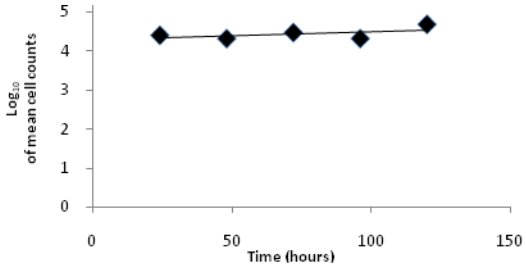


Figure 42 (d) (i)



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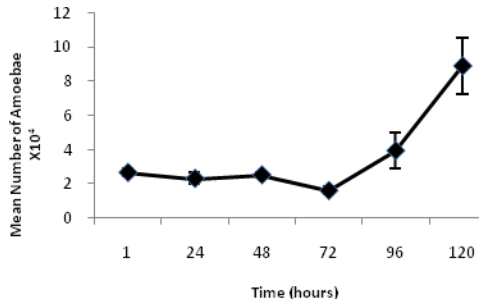
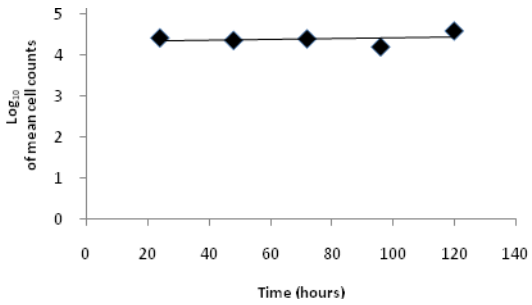


Figure 42 (e) (i)



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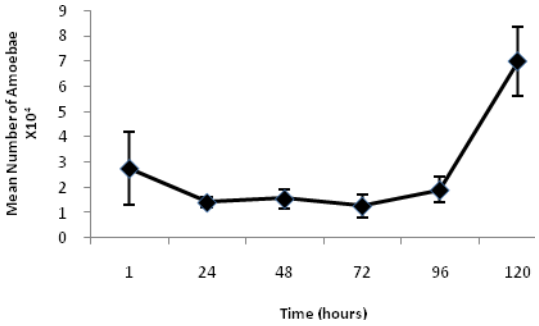

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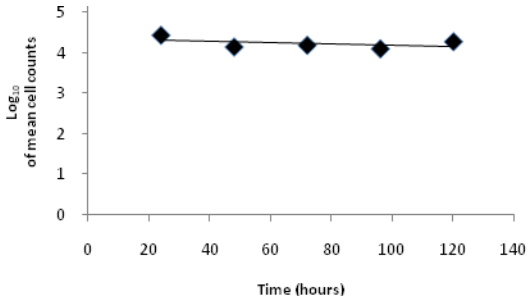


Figure 42 (f) (ii)

Figure 42 a(i)-f(i): Growth curves for the mangrove amoeba at different salinities (0g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l). n=5.

Figure 42 a(ii)-f(ii): Growth curves for the mangrove amoeba based on Log_{10} of mean cell counts per plate against time (h) at salinities 0g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l. n=5.

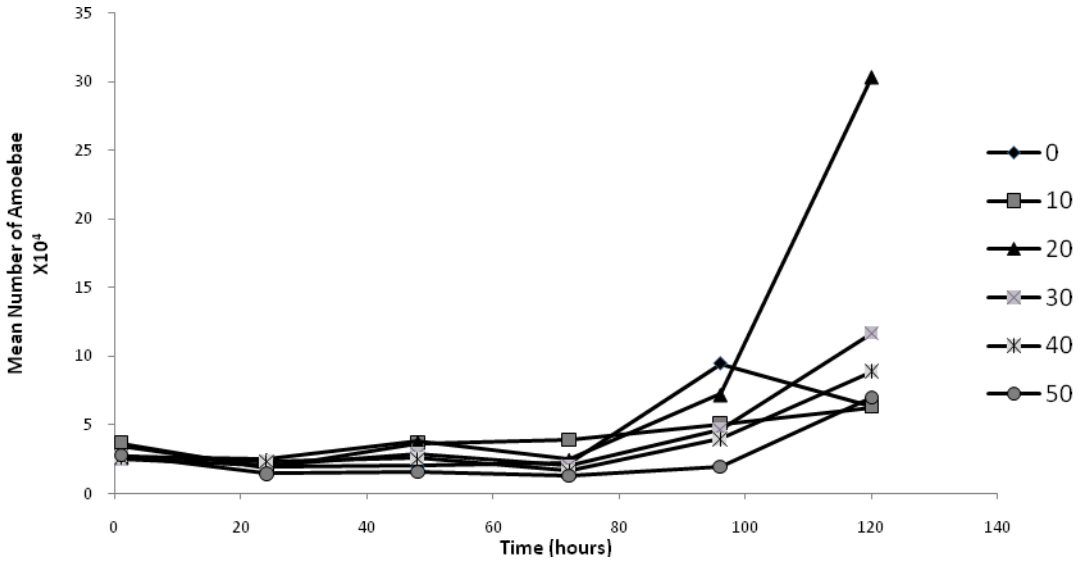


Figure 43: Summary of growth curves for the mangrove amoeba at different salinities (0g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l).

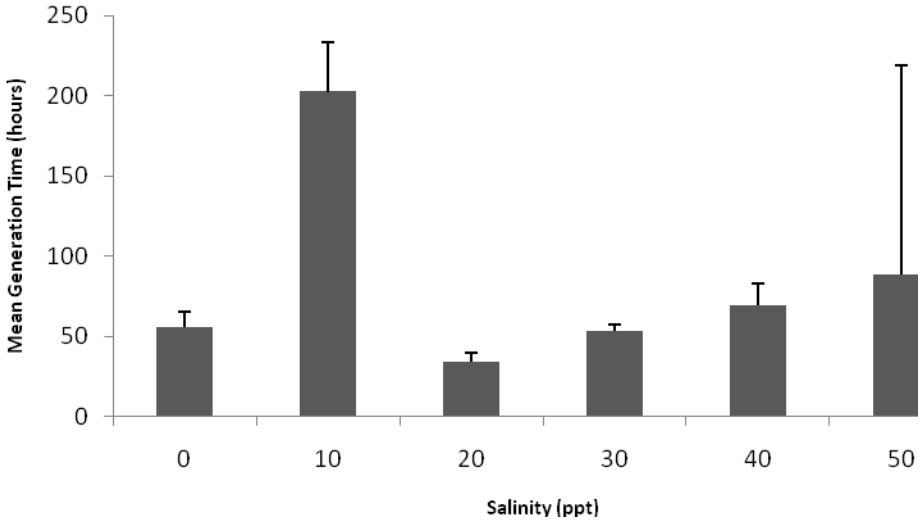


Figure 44: Mean generation time of mangrove amoeba in h at salinities 0g/l, 10g/l, 20g/l, 30g/l, 40g/l, 50g/l. n=5

3.3.2 Chlorine Tolerance of Amoebae

Table 13: Comparing survivability of *Acanthamoeba* cysts (Tap water strain A2) (A2a – A2f) versus the cyst-forming vannellid amoeba (S7a-S7f) at varying chlorine concentrations (2 – 200 mg/l). Inoculations were replicated 6 times (a-f) on non-nutrient AS plates streaked with *E. coli* as a food source for each chlorine level. Survival of cysts was scored after one week of inoculation

Chlorine Conc. (mg/l)												
	A2a	A2b	A2c	A2d	A2e	A2f	S7a	S7b	S7c	S7d	S7e	S7f
AS	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	¥	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+
20	-	-	-	-	-	-	+	+	+	+	+	+
25	-	-	-	-	-	-	+	+	+	+	+	+
30	-	-	-	-	-	-	+	+	+	+	+	+
35	-	-	-	-	-	-	+	+	+	+	+	+
40	-	-	-	-	+	-	+	+	+	+	+	+
45	-	+	-	-	-	-	+	+	+	+	+	+
50	-	-	+	-	-	-	+	+	+	+	+	+
200	-	-	-	-	-	-	-	-	-	-	-	-

AS = Amoeba Saline, +/- = Survival; No survival respectively
¥No inoculation

Approximately 18% of the tap water samples (n = 173) were positive for amoebae with the majority (12%) positive for the amoeba of interest, *Acanthamoeba* (Table 14).
The method assumes at least one cell was present on the filter (i.e. from the 1 L sample), however, it should be noted that some filters probably held more than one cell, so amoebae may be more prevalent in tap water than reflected in this 18% value.

The dominance by *Acanthamoeba* was unexpected. The only comparable study for the U.S. found amoebae in 19.4% of samples but acanthamoebae were only present in 2.8% of samples. Nineteen different types of amoebae were found in the Florida study but only 3 types were found in Huntington samples. In addition to acanthamoebae, *Vannella* (Fig. 31) and an unidentified elongate limax amoeba (Fig. 32) were present.

Water treatment in Huntington relies on filtration and chlorination. While it is possible that acanthamoebae survive in the distribution system as living amoebae (trophozoites) within

the biofilm layer coating pipes, it is most likely they were present as cysts. Consequently, a series of experiments examined the ability of amoebal cysts to tolerate chlorine. In all the trials with *Acanthamoeba* and *Vannella,* cysts survived up to 14 mg/l chlorine for 24h (Table 13) and *Vannella* showed enhanced tolerance surviving up to 50 mg/l chlorine. These levels are far in excess of permissible levels used by treatment plants. The U.S. Environmental Protection Agency (EPA) sets an upper limit of 4 mg/l at distribution. Typically, chlorine levels fall markedly during delivery. Measured levels in the samples taken from faucets ranged from 0.86 to 1.92 mg/l chlorine.

3.4 Molecular Characterization

In the current study molecular characterization was based on ribosomal RNA (rRNA) gene analysis. Ribosomal RNA genes are universally distributed and are functionally equivalent in all known organisms. They code for RNAs that can be found in nuclear or mitochondrial genomes (Mindell and Honeycutt, 1990). Hence, the rRNA gene is a reliable molecular chronometer most commonly used for defining evolutionary relationships in phylogenetic studies (Sogin, 1985) whereby nucleotide sequence differences in hypervariable regions on rRNA genes reflect strain variations.

To carry out molecular characterization it was necessary to isolate DNA from the mangrove amoeba and from acanthamoebae isolates. Molecular characterization involved use of primers based on 18S rRNA gene for amplification of probable rDNA fragments with informative regions on amoebae. The nucleotide base sequences on amplicons were determined either by direct sequencing or after cloning DNA from PCR products.

3.4.1 DNA Isolation

This study was the first attempt to isolate DNA from the mangrove amoeba isolate. For acanthamoebae growing axenically (Neff strain, acid tolerant isolate, isolate from fish mucus and one tap water isolate) it was not difficult to isolate DNA for PCR. However for acanthamoebae growing on agar plates it was important to monitor growth using a dissecting microscope for a comparatively higher population of trophs than cysts so that so that enough cells (trophs) were available for DNA isolation since cysts do not break open easily.

DNA isolation procedures employed in this study (Qiagen's DNeasy Blood and Tissue kit and DNA Stat reagent) were non-exclusive to eukaryotic DNA isolation but yielded DNA from bacterial prey present in the media as well as from amoebae.

DNA isolations were conducted on the mangrove amoeba using DNA Stat reagent (see section 2.4.1) while *Acanthamoeba* DNA isolation was carried out using either Qiagen's DNeasy kit (section 2.4.1) or DNA Stat reagent. For higher yields, DNA from axenic *Acanthamoeba* cultures was obtained using Qiagen's Kit while DNA from acanthamoebae growing on agar plates streaked with *E. coli* was isolated using DNA Stat reagent. The amount of DNA yields from isolation procedures are summarized in Table 15.

3.4.2 Determination of DNA quality

In the current study, the purity of genomic DNA (based on 260/280 OD ratio) and nucleic acid concentration (ng/µl) was determined using a NanoDrop ND-1000 Spectrophotometer (see table 15 for values). This was done to ensure that high quality DNA was being used for PCR.

Table 15: Summary table of cell counts, DNA yields and 260/280 OD ratio of DNA extracts from mangrove amoeba and acanthamoebae isolates in the study

Isolate ID	No. of Cells	Total amount of DNA (µg) *	260/280 ratio
Mangrove amoeba	3.3×10^{11}	22.2	2.35
Mangrove amoeba	5.2×10^{12}	0.97	2.15
Acanthamoeba "marine" isolate [¥] (A1)	3.73×10^{5}	0.45	2.10
Acanthamoeba tap water isolate (A2) [£]	5×10^{6}	30.33	2.10
Acanthamoeba tap water isolate (A3) [¥]	1.99×10^{5}	22	2.03
Acanthamoeba tap water Isolate (A4) [¥]	5.83×10^{6}	76	1.78
Acanthamoeba tap water Isolate (A5) [¥]	4.13×10^{6}	30	1.86
Acanthamoeba acid tolerant isolate (BP) [£]	5×10^{6}	65	1.83
Acanthamoeba sp. isolated from the mucus of a marine fish (FH) [£]	5×10^{6}	12.72	0.63
Acanthamoeba castellanii Neff strain (A6) [€£]	5×10^{6}	220.43	2.06

NB:

***** 260/280 OD ratio of approximately 2 is optimal for PCR reactions.

[¥]Axenicization attempts were unsuccessful and cells were cultured and maintained on agar plates streaked with prey bacterium *E. coli*. Cultures (trophozoites) were harvested for DNA extraction not more than 3 days after a new culture had been established. Several attempts were made to extract DNA from *Acanthamoeba* cysts but no DNA was isolated. [£]*Acanthamoeba* strains were successfully grown axenically. [€]*Acanthamoeba castellanii* (Neff strain ATCC no. 30010), commonly used lab strain maintained in the Dr. Wendy Trzyna's laboratory (Biology Department, Marshall University) included in the study to serve as a control. All tap water *Acanthamoeba* strains (A2, A3, A4, and A5) were obtained from Huntington's (WV) domestic tap water.

3.4.3 Polymerase Chain Reaction

3.4.3.1 Primer Design

For genotyping of *Acanthamoeba* isolates, the well established genus-specific primer pair, JDP1/JDP2, (Booton et al., 2004; Schroeder et al., 2001) was used here. PCR products for all *Acanthamoeba* isolates examined in this study were in the expected size range of 450bp to 550bp.

Because the mangrove amoeba had not been previously described and no molecular data was available for this isolate, various primer sets based on highly conserved sequences and used previously to amplify 18 rRNA gene sequences from other naked amoebae were selected for these studies.

Optimization of PCR employed variables such as varying the annealing temperatures, use of Q-solution, and altering Mg^{2+} concentration (the optimization variables are discussed in section 2.4.3.3).

Optimizing Primers and Annealing Temperatures. Smirnov (2007) reported that when he came across species of amoebae that were difficult to amplify "custom-made" primers were used to amplify the SSU rRNA gene in several fragments (Smirnov et al., 2007). The mangrove amoeba isolate in this study also proved difficult to amplify despite the application of different primer sets and numerous attempts to optimize various PCR reaction parameters.

The primer pair, Silb-F/Silb-R, was used previously to amplify an 18S SSU rRNA fragment from *Vannella anglica* (Smirnov et al., 2007). Using this pair, a series of PCR reactions were run using various annealing temperatures including ; 42°C, 45°C,48°C, 55°C, 56°C, 58°C, (melting temperatures for Silb-F/Silb-R are 63°C and 58°C respectively). PCR products obtained yielded multiple bands of different sizes (ranging from 200bp to 1800bp) at different annealing temperatures investigated with this primer set (Silb-F/Silb-R) as visualized by ethidium bromide staining on agarose gels as shown in Figs. 46–50 for 42°C, 45°C,48°C, 55°C. Overall, this primer set (Silb-F/Silb-R) did not yield highly specific products using the PCR conditions described in the methods section. However, a prominent band at approximately 800bp was obtained repeatedly at temperatures of 42°C, 45°C, and 55°C.

A similar trend was noted with primer sets Rib-F/F-R (melting temperatures 62°C and 58°C respectively) and Med-F/Med-R (melting temperatures 63°C and 58°C respectively) (Figs.

45, 48). Rib-F/F-R were also used previously by Smirnov et al. (2007) to amplify an 18S SSU rRNA fragment from *Vannella anglica.* In an attempt to reduce the presence of non-specific products, the Rib-F/F-R was remade and subjected to more rigorous purification procedures. When this highly purified Rib-F/F-R primer set was used (annealing temperature 55°C), the multiple bands previously observed with the non-purified form of these primers were eliminated and one distinct band (~800bp) was observed on ethidium bromide stained gels (Fig. 45b). This band was excised from gels and sequenced. At an annealing temperature of 42°C, however, this same Rib-F/F-R primer set yielded multiple PCR products, although the most prominent band was still observed at ~800bp. A similar outcome was observed with the Med-F/Med-R primer set at annealing temperature 48°C (Fig. 48). A PCR product of ~1800bp was expected for both of these primer sets (Rib-F/F-R and Med-F/Med-R). A faint band of this size is visible in Figs. 47a and 53. Additional experiments are needed to further optimize the PCR conditions in order to determine if this is the relevant band. When the Rib-F/F-R primer pair was used with *Acanthamoeba* Neff strain genomic DNA as template in PCR, (at an annealing temperature of 55°C), a single band of the expected size (~1800bp) was obtained (Fig. 45c).

From these experiments, the optimal annealing temperature for all three primer sets (Silb-F/Silb-R, Rib-F/F-R and Med-F/Med-R) was determined to be 55°C (-7°C and -3°C melting temperature, respectively). Consequently, this annealing temperature (55°C) was applied to PCR reactions designed to optimize other reaction parameters including Mg^{2+} concentration and the addition of Q-solution (Qiagen), described below.

A fourth primer set Primer set, 12.2f/S20r (melting temperatures 52°C and 61°C, respectively) used by Smirnov et al. (2007) to amplify a smaller rDNA fragment (~680 bp) from *Vannella anglica,* was also used. Results yielded multiple bands (~650bp and ~550bp) at annealing temperatures 48°C and 50°C respectively as visualized by ethidium bromide staining on the gel (Figs. 51, 52), although the most distinct band at both temperatures was observed at ~650bp.

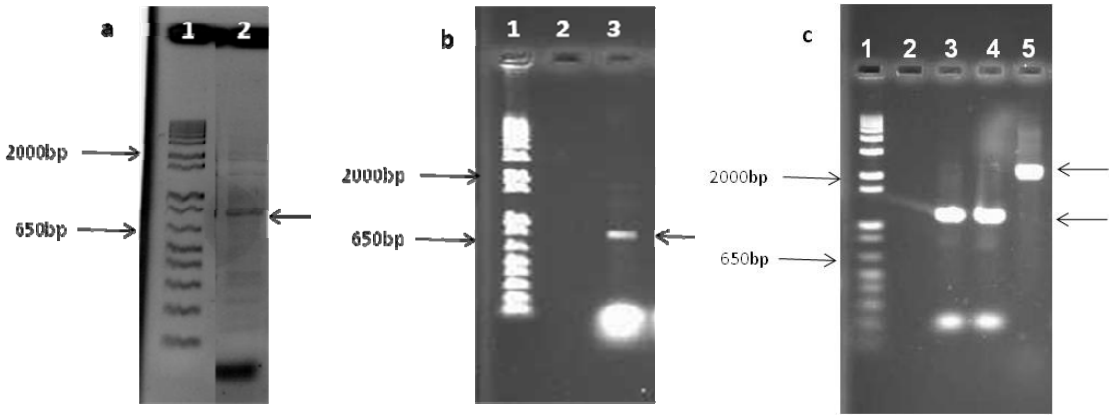
The final primer set considered in the mangrove amoeba study was 570C/1200R (melting temperatures 54°C and 54°C, respectively). Weekers et al. (1994) used this primer set to amplify SSU rRNA genes of *Hartmannella vermiformis* (a naked amoeba) and obtained a PCR product at ~900bp. In the current study amplification using this primer set (570C/1200R) was carried out at annealing temperatures 42°C and 48°C. This yielded a PCR product of ~350bp as determined by

agarose gel electrophoresis and a faint band ~1300bp was also seen on the ethidium bromide stained gel (Fig. 53). At higher annealing temperatures (50°C and 52°C) no amplified products were evident on the gel.

Mg $^{2+}$ Concentration. The presence of Mg^{2+} ions is required for the activity of Taq polymerase and the concentration of Mg^{2+} can affect the outcome of PCR reactions. Experimental PCR reactions were carried out using the Silb-F/Silb-R primer set. Even though varying magnesium concentrations were used (2.5mM, 3mM, 3.5mM and 4mM) no notable change in the resultant PCR products was detected. At all concentrations tested multiple bands were obtained on ethidium bromide stained gel although some slight differences in intensity of bands was observed (Fig. 54).

Q-Solution. Ribosomal RNAs contain considerable amounts of within-molecule base pairing to form specific secondary structures (Mindell and Honeycutt, 1990). The secondary structures of rRNA may result in weak non-specific binding in a PCR reaction.

In the current study, Q-solution, a reagent that is supplied in Qiagen kits to reduce the effect of secondary structure of rRNA was used. Primers used in this experiment were Rib-F /F-R. In this case even though amplification was expected, no amplification was achieved for PCR reactions with added Q-solution (1µl per 25µl total PCR reaction volume) as determined by ethidium bromide staining of agarose gel (data not shown). No further trials using Q-solution were conducted.



Figures 45 (Panel a, b): Agarose gels stained with ethidium bromide showing PCR products amplified from mangrove amoeba DNA using Rib-F/F-R primer set at annealing temparatures 42°C (panel a, lane 2) and 55°C (panel b, lane 3). Both lane 2 (panel a) and lane 3 (panel b) show a distinct band of size ~800bp. **Panel c** shows agarose gel stained with ethidium bromide of PCR product from *Acanthamoeba* Neff strain (positive control) where *Acanthamoeba* Neff strain DNA was amplified using Rib-F/F-R primer set (panel c, lane 5) at an annealing temperature of 55ºC and using 570C/1200R primer set (panel c, lanes 3,4) at annealing temperature of 50°C. Lane 5 (panel c) shows a band of size ~2000bp. Lane 1 in panels a, b, c show 1kbp ladder. Arrows to the right of the gels show bands of interest.

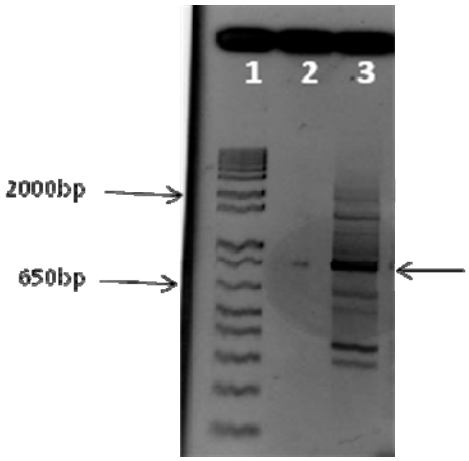


Figure 46

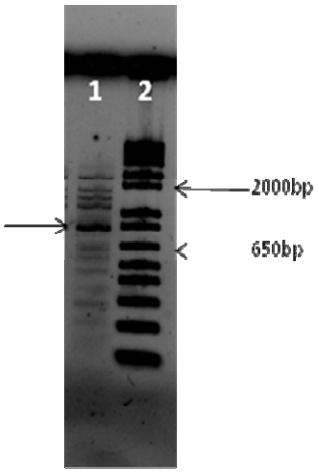


Figure 47

Figures 46, 47: Agarose gels stained with ethidium bromide showing PCR products amplified from mangrove amoeba DNA using Silb-F/Silb-R Primer set at annealing temparatures 42°C (Fig. 46. Lane 3) and 45°C (Fig. 47, lane 2). PCR yielded non-specific products. The expected band size using Silb-F/Silb-R primer set was ~1800bp. Lane 1 (Fig. 46) and lane 2 (Fig. 47) shows 1kbp ladder. Arrows to the right of the gel in Fig. 46 and to the left of Fig. 47 show bands of interest.

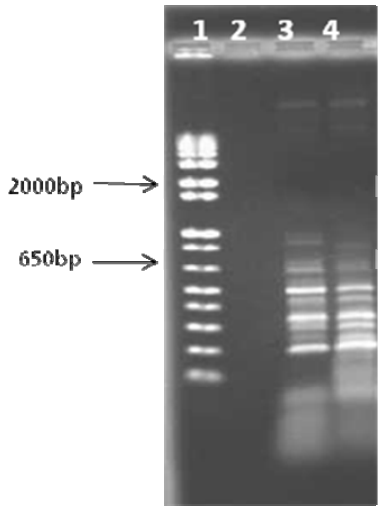


Figure 48

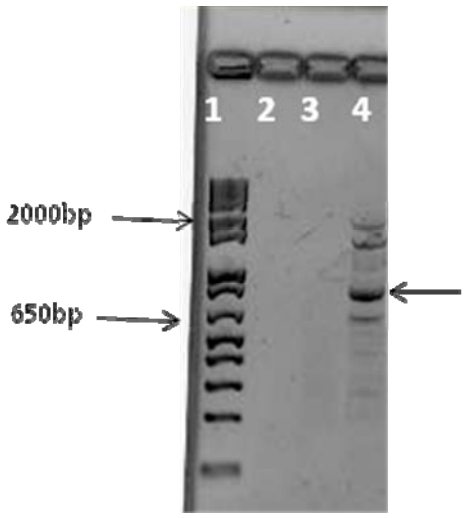


Figure 49

Figures 48, 49: Agarose gels stained with ethidium bromide showing PCR products amplified from mangrove amoeba DNA using Silb-F/Silb-R primer set at annealing temparature 48°C (Fig. 48, lane 3), Med-F/Med-R primer set at an annealing temparature of 48°C (Fig. 48, lane 4) and Silb-F/Silb-R primer set at annealing temparature 55°C (Fig. 49, lane 4). PCR yielded non-specific products. Lane 1 shows 1kbp ladder in both figures. Arrow to the right of the gel shows band of interest.

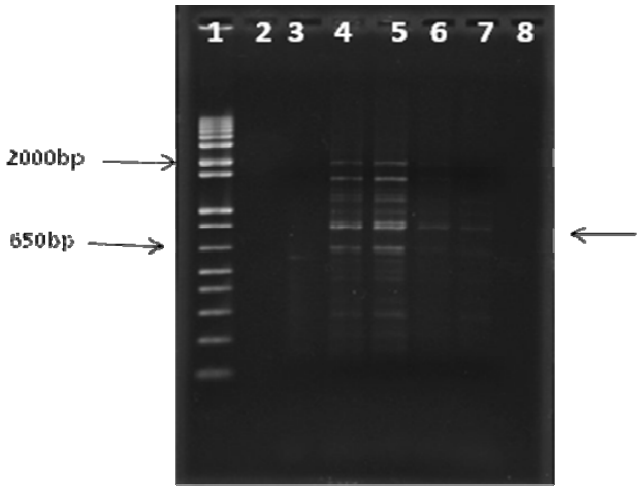


Figure 50

Figure 50: Agarose gel stained with ethidium bromide showing PCR products amplified from mangrove amoeba DNA using Silb-F/Silb-R Primer set at annealing temparatures 56°C (lanes 4, 5) and 58°C (lanes 6, 7). PCR yielded non-specific products. Lane 1 shows 1kbp ladder. Arrow to the right of the gel shows band of interest as previously seen in Fig. 45b of a distinct band ~800bp obtained using Rib-R/F-R primer set (see Fig. 45b).

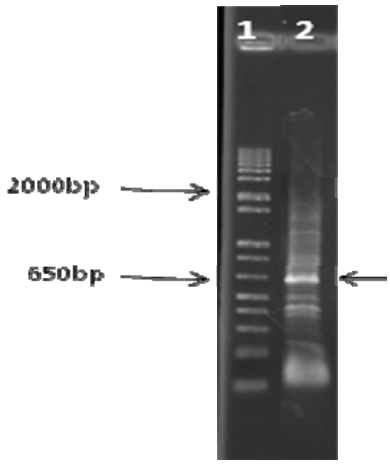


Figure 51

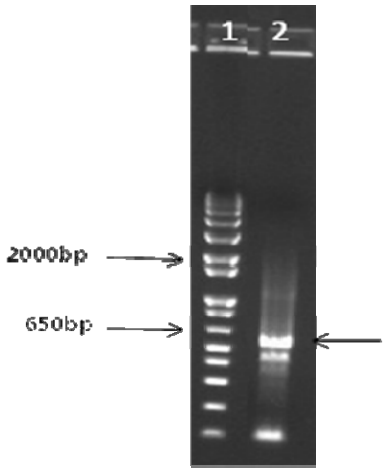


Figure 52

Figure 51, 52: Agarose gels stained with ethidium bromide showing PCR products amplified from mangrove amoeba DNA using 12.2f/S20r primer set at annealing temparatures 48°C (Fig. 51, lane 2) and 50°C (Fig. 52, lane 2). The expected band size using 12.2f/S20r primer size was ~680bp. Lane 1 shows 1kbp ladder in both figures. Arrows to the right of the gels show bands of interest.

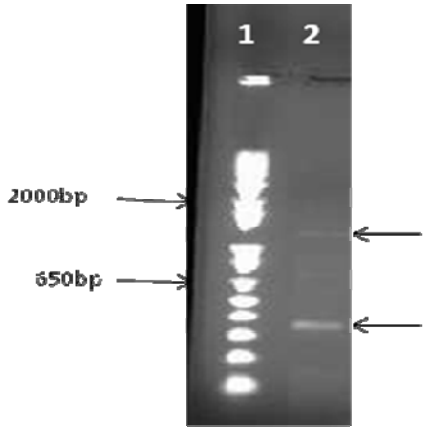


Figure 53

Figure 53: Agarose gel stained with ethidium bromide showing PCR products amplified from mangrove amoeba DNA using 570C/1200R primer set at an annealing temparature of 48°C. A band of size ~350bp band was obtained (lane 2). A faint band ~1300bp (lane 2) was also resolved on the gel. The expected band size with this primer set was ~900bp. Lane 1 shows 1kbp ladder. Arrows to the right of the gel show bands of interest.

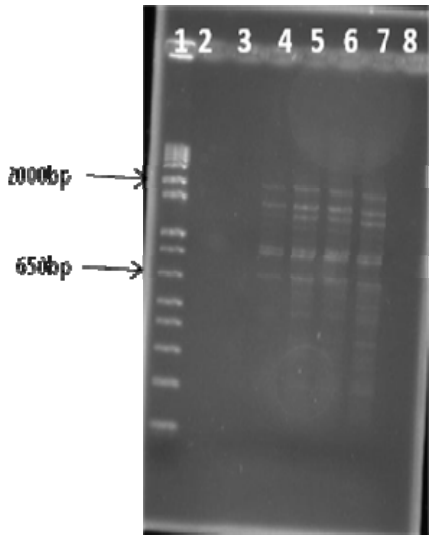


Figure 54

Figure 54: Agarose gel stained with ethidium bromide showing PCR products from mangrove amoeba DNA amplified using Silb-F/Silb-R primer set at different $MgCl_2$ concentrations. Annealing temperature for this reaction was set at 55°C. Lane 2 and 3: Empty; Lane 4: $MgCl_2$ 2.5 mM; Lane 5: $MgCl_2$ 3 mM; Lane 6: $MgCl_2$ 3.5 mM; Lane 7: $MgCl_2$ 4mM; Lane 8: empty. No visible effect was seen with change in $MgCl_2$ concentrations. Lane 1: 1kbp ladder.

3.4.4 Polymerase Chain Reaction on *Acanthamoeba* Isolates

Marine Acanthamoeba Isolate. The amount of DNA required for optimal amplification of the 'marine' *Acanthamoeba* isolate using *Acanthamoeba*-specific primers JDP1/JDP2 was 1ng per 25µl PCR reaction. Amplified DNA of the 'marine'*Acanthamoeba* isolate yielded a band at ~470 bp (see Fig. 55) defined by ethidium bromide staining. This band was excised and DNA was purified by gel purification prior to direct sequencing of the PCR product.

Tap water Isolates. Tap water *Acanthamoeba* isolates (A1, A2, A3, A4) required different amounts of DNA concentration for PCR. In order to amplify DNA from isolate no. A1, 50ng DNA template was used for a 25µl PCR reaction. Amplification of three (A2, A3, A4) of the four tap water isolates was not straightforward hence it was necessary to determine the optimum amount of DNA template required for each PCR reaction. Isolates no. A2 and A4 were difficult to amplify a second time. The initial amplification of isolates A2 and A4 were obtained during preliminary experiments in this study and DNA concentrations were not determined at that time. Nonetheless, PCR reactions were carried out for isolates A2 and A4 and PCR products were sequenced directly using primers 892/892C (*Acanthamoeba* sequencing primers). Amplification of isolate no. A3 required 5ng DNA template per 25µl PCR reaction. Bands of ~450bp were obtained (Figs. 56, 57) for each of the isolates (A2, A3, A4, A5). Bands were excised and gel purified (Qiagen) prior to sequencing.

Acid tolerant Acanthamoeba Isolate. Acanthamoeba-specific primers (JDP1/JDP2) were used to amplify DNA from the acid tolerant strain (BP). The PCR product showed positive results with a band size of ~490bp (Fig. 58).

Acanthamoeba Isolate from Fish Mucus. The *Acanthamoeba* isolate from fish mucus was amplified using *Acanthamoeba*-specific primers JDP1 and JDP2 and yielded a band of ~440bp (Fig. 59).

Acanthamoeba castellanii (Neff strain). The eukaryotic primer set, 570C/1200R, used to amplify *Acanthamoeba* spp. and *Hartmannella* spp. in previous studies (Weekers et al., 1994) was used for PCR reactions to amplify mangrove amoeba (mangrove amoeba) as shown in Fig. 45c and Fig. 61. In order to establish validity of the primers DNA from *Acanthamoeba* spp. (Neff strain) which was cultured axenically in Dr. Wendy Trzyna's laboratory (Biology Department, Marshall University) was amplified using this primer set (570C/1200R) and this

was done at two primer concentrations (2mM and 10mM) at annealing temperature 50°C. Both concentrations resolved a band at ~1300bp as seen on agarose gel stained with ethidium bromide (Fig. 61, lanes 2-5). The same strain (*Acanthamoeba* Neff strain) was amplified using *Acanthamoeba*-specific primers (JDP1/JDP2) and resolved a band of expected size of ~450bp (Fig. 60).

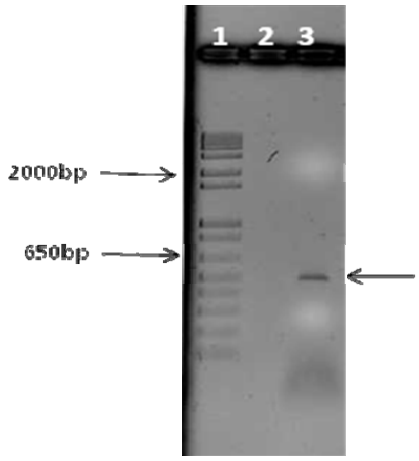


Figure 55

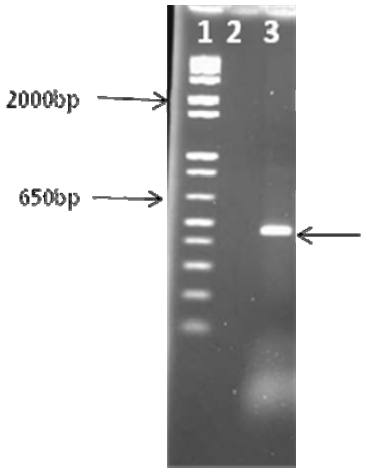


Figure 56

Figure 55, 56: Agarose gels stained with ethidium bromide showing PCR products amplified from 'marine' *Acanthamoeba* isolate (A1) (Fig. 55, lane 3) and tap water *Acanthamoeba* isolate (A2) (Fig. 56, lane 3). Amplification was done using *Acanthamoeba*-specific primers JDP1/JDP2 and yielded bands of expected size (450-500bp). Lane 1 shows 1kbp ladder in both figures. Arrows to the right of the gels show bands of interest.

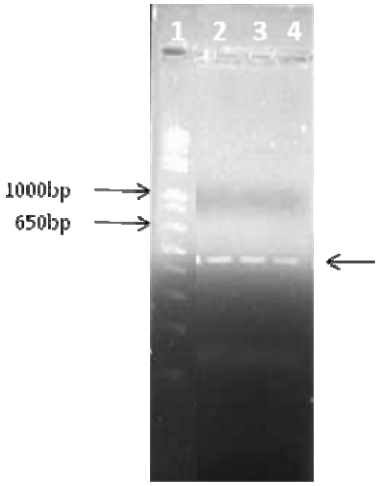


Figure 57

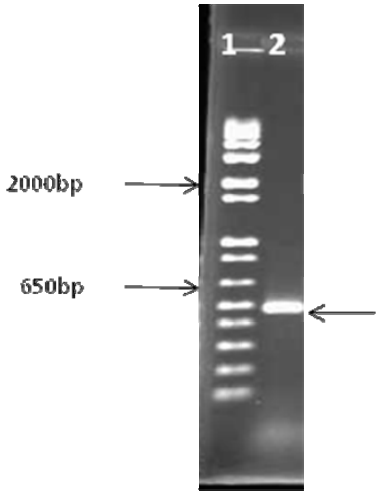


Figure 58

Figure 57, 58: Agarose gels stained with ethidium bromide showing PCR products amplified from tap water acanthamoebae A3, A4, A5 (Fig. 57 lanes 2, 3, 4, respectively) and acid tolerant *Acanthamoeba* strain (BP) (Fig.58, lane 2). Amplification was done using *Acanthamoeba*-specific primers JDP1/JDP2 and yielded bands of expected size (450-500bp). Lane 1 shows 1kbp ladder in both figures. Arrows to the right of the gels show bands of interest.

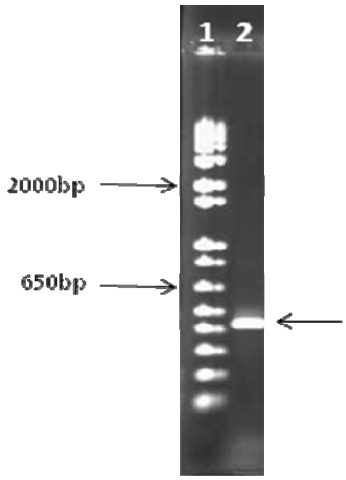


Figure 59

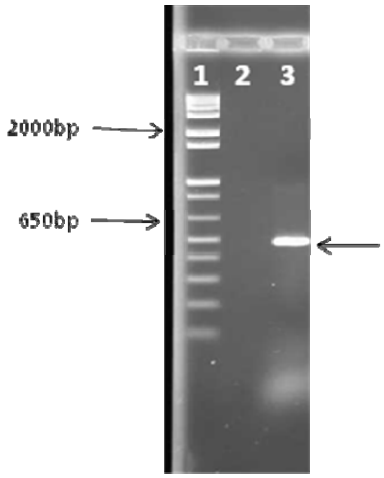


Figure 60

Figure 59, 60: Agarose gels stained with ethidium bromide showing PCR products amplified from *Acanthamoeba* isolate from fish mucus (FH) (Fig. 59, lane 2) and *Acanthamoeba* Neff strain (Fig. 60, lane 3). Amplification was done using *Acanthamoeba*-specific primers JDP1/JDP2 and yielded bands of expected size (450-500bp). Lane 1 shows 1kbp ladder in both figures. Arrows to the right of the gels show bands of interest.

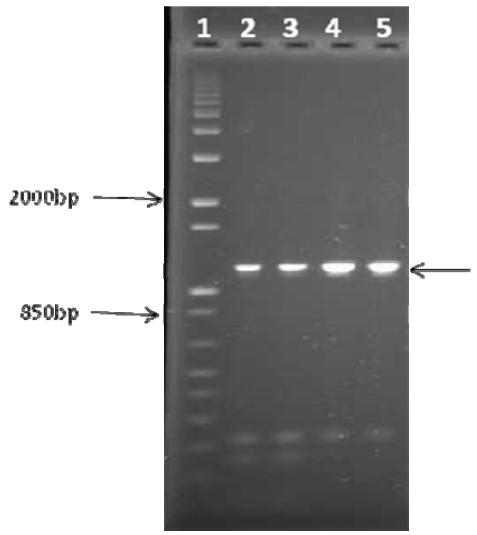


Figure 61

Figure 61: Agarose gel stained with ethidium bromide showing *Acanthamoeba* Neff strain PCR product amplified using eukaryotic primer set 570C/1200R. Lanes 2 and 3 show PCR product using this primer set (570C/1200R) at 2mM concentration while lanes 4 and 5 show PCR product using the same primer set (570C/1200R) at 10mM concentration. This PCR was done to establish optimal primer concentration for subsequent reactions with this primer set. Lane 1 shows 1kbp ladder. Arrow to the right of the gel shows bands of interest.

3.4.5 TA Cloning of Gel Purified PCR products

Mangrove Amoeba Isolate

PCR products were ligated into a TA vector and successfully transformed into competent *E. coli* cells. Positive colonies containing inserts (white colonies) were purified and screened by restriction digestion analysis prior to sequencing. Four bands (lane 1) were resolved. Band (a) ~3900bp (Fig. 62) corresponds to size of TA vector inserts (vector map; www.invitrogen.com) while band (b) ~800bp (Fig. 62) corresponds to size of the PCR product from the mangrove amoeba (ligate). Additional bands [band (c) ~450bp and Band (d) ~390bp (Fig. 62)] may represent cleavage products of the 800bp band, however these smaller bands were not analyzed further. Lane 3 (Fig. 62) is the undigested clone [band (e) ~4700bp (Fig. 62)] and corresponds to size of TA vector plasmid plus the ~800bp PCR product.

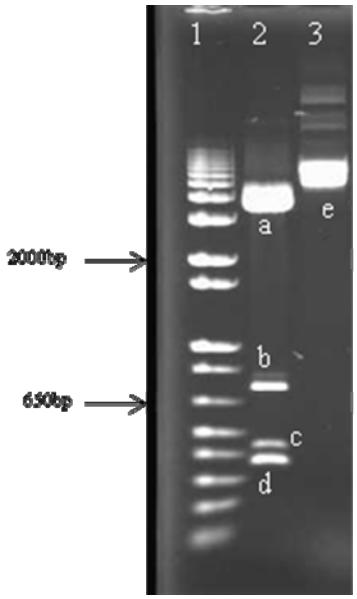


Figure 62

Figure 62: Agarose gel stained with ethidium bromide showing restriction digestion done using EcoRI restriction enzyme (lane 2) of purified PCR product of mangrove amoeba (800bp fragment). Four bands (lane 1) were resolved. Band (a) ~3900bp, band (b) ~800bp, band (c) ~450bp, band (d) ~390bp and band (e) ~4700bp.

Acanthamoeba Isolates

Tap water Isolates. Restriction digested cloned DNA of *Acanthamoeba* tap water strain A2 was analyzed on a 0.8% agarose gel, resolving two distinct bands; one being the expected size corresponding to the PCR product before cloning (~470bp) (Fig. 63 bands no. ii and iv) and the other fragment corresponding to the TA vector before ligation (~3900bp). This indicated that the plasmid had been fully digested (vector map; www.invitrogen.com). Control lanes of undigested recombined plasmids were also run on the same gel, resolving a distinct fragment of about 4390bp (Fig. 63 band no. iii) corresponding to a combined size of the PCR product and the TA vector (490bp + 3900bp).

Acid tolerant Acanthamoeba Isolate. PCR products amplified from *Acanthamoeba* strain (BP) genomic DNA using JDP1/JDP2 primer set were cloned into the TA vector. To confirm the presence of insert, plasmids were restriction digested with EcoRI and analyzed by agarose gel electrophoresis. This resolved two distinct bands; one being the expected band corresponding to the size of the PCR product before cloning (~490bp) (Fig. 64, band no. ii) and the other fragment corresponding to the TA vector without insert (~3900bp) (Fig. 64, band i). Control lanes of undigested plasmid were also run on the same gel, resolving a fragment of about 4390bp (Fig. 64, band no. iii), which correspond to the combined size of the PCR product and the TA vector (490bp + 3900bp). Restriction digestion of plasmids yielded two fragments (a, b).

Acanthamoeba castellanii (Neff strain). PCR products were purified and ligated into a TA vector, and transformed into competent *E. coli* cells. Restriction digested samples revealed band sizes corresponding to the PCR product size and linearised TA vector plasmid (Fig. 65; lanes 3 and 5) while undigested control samples of plasmid DNA resolved a larger band size, ~ 4300bp, consisting of the PCR product (~3900bp) (Fig. 65; lanes 4, 6).

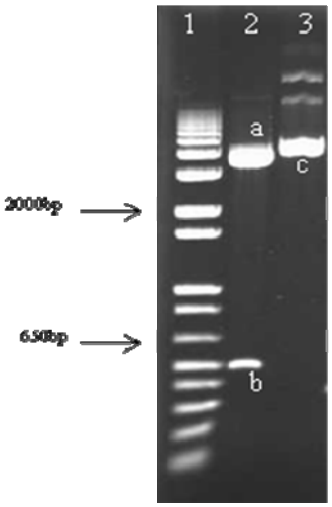


Figure 63

Figure 63: Agarose gel of restriction digests stained with ethidium bromide showing PCR product of tap water *Acanthamoeba* isolate (A2) in lane 2. Lane 3 shows the corresponding undigested clone of tap water *Acanthamoeba* isolate (A2). Digested plasmids yielded two fragments (bands a, b). Undigested plasmid reveals a band ~4390bp indicating no digestion in the absence of EcoRI restriction enzyme. Lane no. 1: 1kbp ladder.

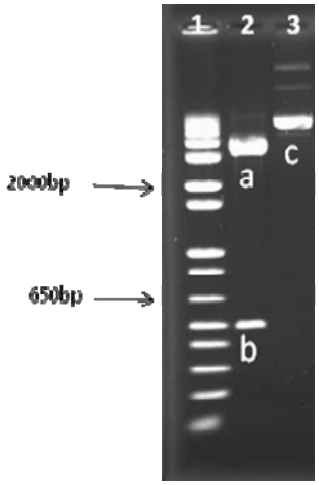


Figure 64

Figure 64: Agarose gel of restriction digests stained with ethidium bromide showing PCR product of acid-tolerant *Acanthamoeba* isolate (BP) in lane 2. Lane 3 shows corresponding undigested clone of acid-tolerant *Acanthamoeba* isolate (BP). Undigested plasmid reveals a band ~4390bp indicating no digestion in the absence of EcoRI restriction enzyme. Lane no. 1: 1kbp ladder.

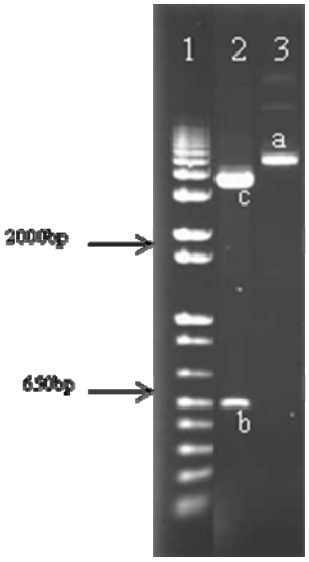


Figure 65

Figure 65: Agarose gel of restriction digests stained with ethidium bromide showing PCR product of *Acanthamoeba* Neff strain (A6) in lane 2. Undigested plasmid reveals a band ~4390bp indicating no digestion in the absence of EcoRI restriction enzyme. Lane 3 shows digested plasmid yielding a fragment with a size that corresponds to the PCR product size of *Acanthamoeba* Neff strain (A6) approximately 490bp. Control lanes with undigested plasmid in lane 3 show a band (c) ~4390bp indicating no digestion in the absence of EcoRI restriction enzyme. Lane no. 1 shows a ladder of size 1kbp.

3.5 Sequencing Analysis

PCR products generated throughout these studies were sequenced. PCR products were either direct sequenced or cloned prior to sequencing. For sequencing from clones, plasmid sequences were appropriately trimmed from either side of the insert to eliminate plasmid DNA sequences from the analysis.

Mangrove amoeba

For the mangrove amoeba, PCR products and clones (as described in previous sections) were sequenced. Sequencing results are included in Appendix II. Sequences were subjected to BLAST search analysis against all available sequences in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The most significant matches obtained were protozoan sequences including a marine flagellate *Telonema antarcticum* as well as two protists used in Medlin et al.'s study (1988); *Plasmodium falciparum* and *Dictyostelium discoideum* a soil-living amoeboid slime mould. No amoeba matches were obtained. However, since there has been relatively limited research on the identification of naked amoebae at the molecular level, and hence limited amoebal sequences available in GenBank, it is not surprising that a search for matches failed to yield matches to amoebal sequences. Sequence matches obtained were protozoan which is encouraging for further studies.

Acanthamoeba spp.

Acanthamoeba isolates were characterized based on 18S SSU rRNA gene using well established primers for diagnostic fragment 3 (DF3, approximately 100bp) (Booton et al., 2004). Even though the DF3 region is not as robust for phylogenetic tree building this region, used reliably in previous acanthamoebae genotyping studies (Booton et al., 2004) was used in this study to identify genotypes. After comparing nucleotide bases of the DF3 hypervariable region on experimental strains and *Acanthamoeba* strains available in GenBank, results showed that the marine *Acanthamoeba* strain matched T3 designates, particularly T3 designate referenced S81337 in GenBank (78%), as shown in Figure 66.

Two of the four tap water strains (strains A2, A3) were most similar to T4 designates referenced U07410 and AF140721 (69%) (Figs. 66, 67). The Neff strain (positive control) was a T4 designate as expected (100%) (Figs. 66, 67). The *Acanthamoeba* strain from fish mucus (FH)

matched T5 designates from GenBank (78%) (Figs. 66, 67). The acid tolerant strain from Berkley Pit, Montana (BP) was highly similar to pathogenic T4 designate *Acanthamoeba castellanii* (98%) (Figs. 66, 67). Strains A2 and A3 were closely related to each other and to members of T4 designates (Fig. 66). Tap water strains A4 and A5 were close to each other and to T9, T12 designate clade. (Figs. 66, 67). Figure 66 presents the resulting phylogenetic tree under maximum parsimony algorithms (tr: tv weighting 1:1) which exhibited the best statistical support for a maximal number of clades. Numbers under clades represent bootstrap support based on 100 replicates. Figure 67 presents genetic distance matrix (uncorrected "p") for 7 *Acanthamoeba* strains from the current study (*Acanthamoeba* Neff strain, A6, was used in this study as a positive control). Details of tree parameters are provided in the figure legends.

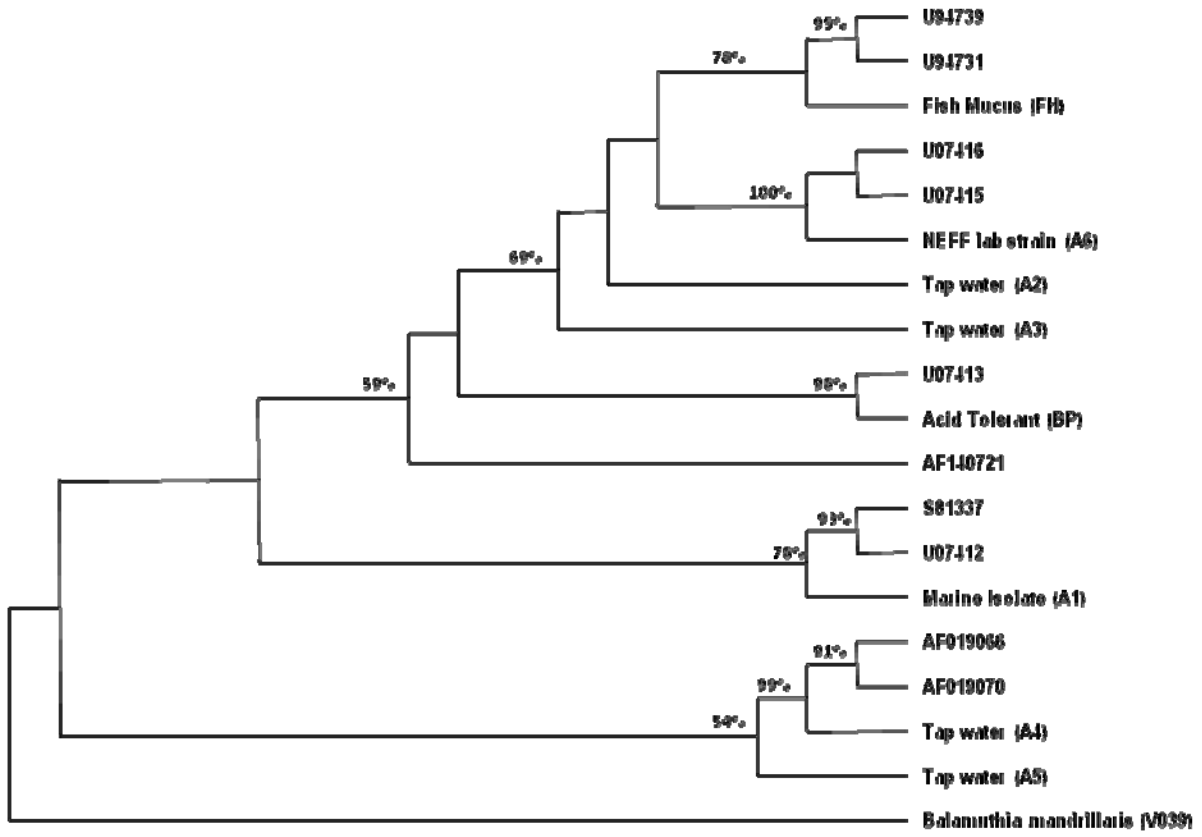


Figure 66: Maximum Parsimony (MP) tree; transition: transversion 1:1; Gaps are treated as "missing"; *Balamuthia mandrillaris* as outgroup. Of 749 total characters, 246 are constant, 226 are parsimony-informative. Bootstrap values > 50% (parsimony criterion) are indicated at nodes. KEY: A1=*Acanthamoeba* marine strain; A2, A3, A4, A5=*Acanthamoeba* tap water strains; A6=*Acanthamoeba* Neff strain. BP=*Acanthamoeba* acid tolerant strain; FH=*Acanthamoeba* strain from fish mucus. *Acanthamoeba* strains from GenBank used in alignments and represented in tree include *Acanthamoeba lenticulata* JCI (U94739), *A. lenticulata* 7327 (U94731), *A. polyphaga* JacIS2 (U07415), *A. castellanii* Neff (U07416), *Acanthamoeba castellanii* Castellani (U07413), *Acanthamoeba* sp. Czech 4436 (AF140721), *A. griffinii* TIOH37 (S81337), *A. griffinii* S7 (U07412), *Acanthamoeba comandoni* (AF019066) and *Acanthamoeba healyi* V013 (AF019070).

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 S81337	-												
2 U07412	0.00810	-											
3 U94739	0.09428	0.09239	-										
4 U94731	0.09433	0.09246	0.00447	-									
5 Fish mucus	0.11330	0.12283	0.05658	0.05639	-								
6 Balamuthia	0.14302	0.14572	0.15449	0.15428	0.23660	-							
7 U07416	0.06976	0.06593	0.09173	0.09183	0.11984	0.15127	-						
8 U07415	0.06976	0.06593	0.09173	0.09183	0.11984	0.15127	0.00000	-					
9 A6	0.05484	0.04444	0.07707	0.07709	0.06143	0.14386	0.00000	0.00000	-				
10 AF140721	0.06194	0.05264	0.09652	0.09892	0.09725	0.14561	0.03624	0.03624	0.03680	-			
11 U07413	0.07373	0.07230	0.09226	0.09479	0.13499	0.16875	0.05248	0.05248	0.03149	0.02169	-		
12 BP	0.05540	0.04716	0.07623	0.07866	0.07904	0.14913	0.03480	0.03480	0.03308	0.02409	0.00222	-	
13 A2	0.04429	0.04450	0.07483	0.07496	0.06124	0.14631	0.02222	0.02222	0.02194	0.00745	0.01328	0.01538	-
14 A3	0.05732	0.05739	0.09199	0.09245	0.06843	0.17882	0.02993	0.02993	0.02973	0.01461	0.02223	0.02240	0.00518
15 A1	0.06141	0.07078	0.12620	0.12632	0.15706	0.17717	0.10082	0.10082	0.07859	0.08992	0.11442	0.07998	0.06743
16 A5	0.11287	0.10009	0.13598	0.14127	0.63039	0.14358	0.06996	0.06996	0.07129	0.01988	0.05008	0.05518	0.03128
17 A4	0.37880	0.37850	0.42767	0.42750	0.58353	0.47454	0.36384	0.36384	0.35896	0.37185	0.35530	0.35875	0.33434
18 T9	0.63354	0.62997	0.61308	0.61285	0.59184	0.61726	0.63065	0.63065	0.62079	0.62146	0.63417	0.62925	0.61754
19 T12	0.67536	0.67378	0.69871	0.69639	0.68801	0.71853	0.68349	0.68349	0.67819	0.67671	0.68540	0.68656	0.67438

Uncorrected ("p") distance matrix (continued)

	16	17	18	19
16 A5	-			
17 A4	0.20141	-		
18 AF019066	0.64872	0.66653	-	
19 AF019070	0.66304	0.66864	0.58665	-

Figure 67: Distance matrix (uncorrected "p") showing dissimilarity values (% differences). KEY: A1=*Acanthamoeba* marine strain; A2, A3, A4, A5=*Acanthamoeba* tap water strains; A6=*Acanthamoeba* Neff strain (lab strain). BP=*Acanthamoeba* acid tolerant strain; FH=*Acanthamoeba* strain from fish mucus. *Acanthamoeba* strains from GenBank used in alignments and represented in tree include *Acanthamoeba lenticulata* JCI (U94739), *A. lenticulata* 7327 (U94731), *A. polyphaga* JacIS2 (U07415), *A. castellanii* Neff (U07416), *Acanthamoeba castellanii* Castellani (U07413), *Acanthamoeba* sp. Czech 4436 (AF140721), *A. griffinii* TIOH37 (S81337), *A. griffinii* S7 (U07412), *Acanthamoeba comandoni* (AF019066) and *Acanthamoeba healyi* V013 (AF019070).

Chapter IV: Discussion

Amoebae are ubiquitous in the marine environment and Page (1983) remarked that he had never failed to isolate amoebae from a sample of seawater. Accordingly, various studies have shown that naked amoebae are numerically important in marine habitats. For example, Rogerson and Laybourn-Parry (1992) reported up to 43,000 amoebae l^{-1} in the Clyde estuary water column in Scotland while Anderson and Rogerson (1995) found up to 15,600 amoebae at the same site in a follow up study. More recently, Rogerson and Gwaltney (2000) reported up to 104,000 amoebae l^{-1} in subtropical Florida in the water column of a mangrove site while Anderson (1998) counted 75,000 amoebae l^{-1} in a brackish pond in Bermuda. In the Florida mangrove study, 91.67% of all planktonic amoebae were attached to suspended flocs (Rogerson et al., 2003) probably derived from resuspended sediments. Since amoebae posses pseudopodia the high numbers of amoebae at these sites are thought to be predominantly floc-associated (Rogerson et al., 2003). In a previous study, Rogerson et al. (2000) found numbers of amoebae ranging from 438 to 13,035 cm^{-3} in beach sediments at Kames Bay, Isle of Cumbrae, Scotland, UK and Dania Beach, FL, USA. Likewise, Smirnov and Thar (2003) recovered 17 species of amoebae from $2mm^3$ sandy sediments from the brackish-water Nivå Bay (Baltic Sea, The Sound).

It is not atypical that the presumed symbiont, encountered on ctenophore comb plate surface, and the amoeba isolated from mangrove waters in Florida were new to science. In a study by Rogerson and Hauer (2002), 40% of amoebae isolated from the Salton Sea, California, were considered to be new, undescribed species. The same study illustrated an impressive diversity with 45 different taxa reported from a single body of water. Likewise, in mangrove waters, 43% were estimated to be new species (Rogerson and Gwaltney, 2000). Butler and Rogerson (2000) encountered several isolates that were probably new species and most were very small (<10µm) in size. The small size and lack of rigid morphological features on which to base identifications may be a major reason why few workers have chosen to study this group of protists and the many undescribed species. Studies by Anderson and Rogerson (1995), Butler and Rogerson (1995), and Rogerson and Gwaltney (2000) have all shown that about 60% of all amoebae isolated are less than 10µm in size and yet less than 10 species in this size range have ever been described in the literature.

As earlier noted, the specific aim of this study was to characterize three unusual amoebae (ctenophore amoeba, mangrove amoeba and *Acanthamoeba* spp.) using morphological and physiological features. Two of these species (ctenophore amoeba and mangrove amoeba) may be new to science and the third amoeba (*Acanthamoeba* spp.) may be a new genotype. In light of this, the study serves as an initial step towards identifying new species. It also serves to alert future workers in microbial ecology about these new isolates that could easily be overlooked. The most notable feature of the ctenophore amoebae was its unusual and distinctly wavy hyaline edge that changed shape rapidly. Unlike the hyaline zone of other amoebae (such as the vannellids), this zone was markedly transparent and could only be detected by careful observation (Fig. 23). Normally, the zone is dark in amoebae with a prominent hyaline cap when observed under phase contrast microscopy, however, the indisctict zone in the ctenophore amoeba indicates that it was thicker than normally found on other fan-shaped amoebae (thin objects in phase contrast are rendered dark). The nature of the hyaline zone, and the small size of the cell, suggests that the amoeba is undescribed and new to science.

Stock cultures for characterization were provided by Ms. C. Versteeg (Nova Southeastern University, Florida). However, part of the study was to use enrichment cultivation methods to attempt to isolate naked amoebae, including the ctenophore amoeba, from comb plates of ctenophores from the Gulf of Florida. Unfortunately, attempts to isolate this amoeba were unsuccessful and confirmed the results of Versteeg (MS, Thesis, 2007). Using slightly different media formulations in an attempt to improve isolations, the trials here yielded only ciliates (Fig. 26a, b), flagellates (Fig. 25) and a *Vexillifera*-like amoeba (Fig. 24). Comparable results were observed by Versteeg (MS, Thesis, 2007) in her earlier study although she did isolate 11 morphotypes of amoebae including the *Vexillifera*-like isolate encountered in the current study. Versteeg isolated the presumed symbiont on only a few occasions (i.e. in 2% of the 94 ctenophores processed) although direct observation showed that the surface of about 90% of all ctenophores harbored amoebae. In the present study, 28 ctenophores were processed but none yielded the presumed symbiont. Failure to isolate the ctenophore amoeba from comb plates in the current study may have been due to the smaller number of animals processed or due to the different media formulations that were used. One fact is clear, although these amoebae are common on the ctenophore surface, both Versteeg's results and the present data showed high densities on the surface, they are fastidious in growth requirements when attempting to isolate

them into cultures constituting bacterial prey. However, once established, the amoebae grow rapidly and demonstrate that this partnership is not an obligatory association between amoebae and the ctenophore comb plates.

The isolate does bear a resemblance to a described genus. The broad hyaline zone and the conspicuous trailing uroidal filaments observed in this amoeba are characteristic of members of the family Flabellulidae. Interestingly, a member of this family, *Flabellula calkinsi*, a free-living amoeba, has been reported to be associated with marine invertebrates (Page, 1983). The ctenophore amoeba is considerably smaller that this species but may belong to the genus. It should be noted that a very similar isolate was reported by Rogerson and Gwaltney (2000) from mangrove waters. Thus, the fact that they grow in culture and its presumed presence in sub-tropical regions suggest that this may be a free-living amoeba capable of thriving on the ctenophore surface; an opportunistic pathogen if it is indeed digesting comb plates. Baldock et al., 1980 presented a relationship between cell size (volume) of free-living amoebae and generation time. This regression of $\log_{10}$ generation time against $\log_{10}$ cell size (volume) was determined for free-living amoebae growing at various temperatures (10º, 15 º, 20 º, 25 º). This was based on a view expressed by Fenchel (1974) that there exists a general relationship between growth rate and body size for all microorganisms. The location of the amoebae on the curve was examined. Cell volume of the ctenophore amoeba was estimated from the relationship $\log_{10}$ cell volume (μm^3) = 0.882 + 3.117 $\log_{10}$ nuclear diameter (μm) (Rogerson, 1993). With a nuclear diameter of 2 um, the volume was estimated to be 66.06 μm^3. The optimum generation time of 7.9 hours at 10g/l showed that the ctenophore amoeba has a growth rate faster than expected when compared with other free-living amoebae growing under optimum conditions (Figs. 41, 68). Clearly, this amoeba in culture with prey bacteria grows very fast relative to other amoebae. This underscores the uniqueness of this strain and perhaps its fast growth helps it to become established on the ctenophore surface. Alternatively, the difficulty of establishing cultures may imply that only some individual cells can establish clonal cultures. These few clones may be atypical of the population in general. For now, this high growth potential remains unexplained and should be investigated in future studies.

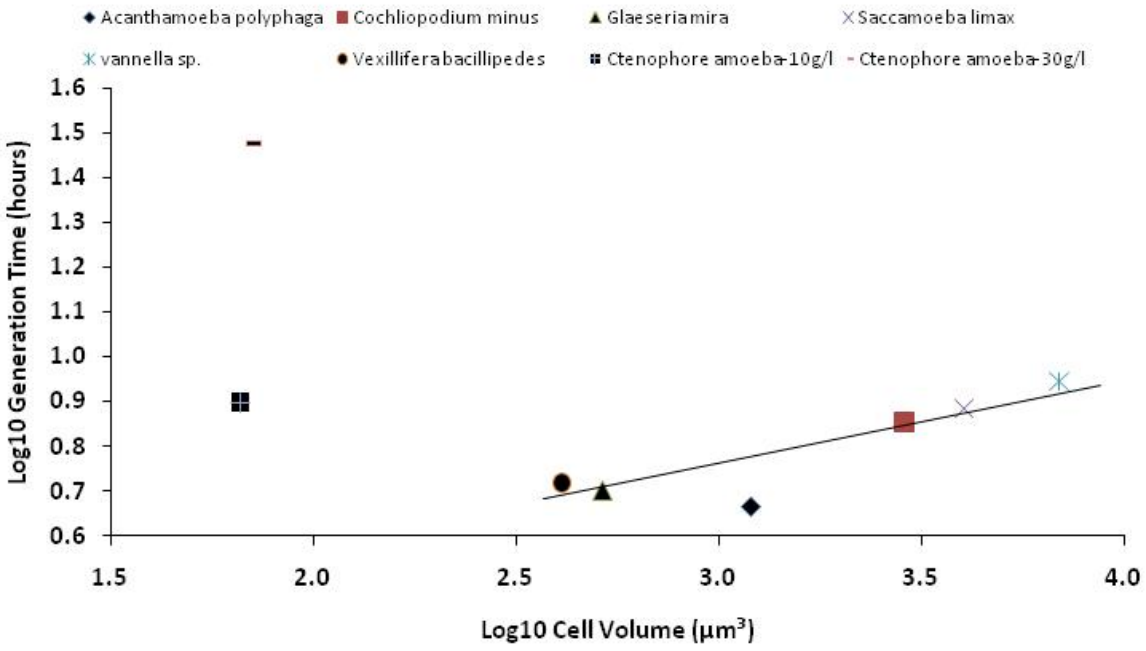


Figure 68: Regression of $\log_{10}$ generation time (hours) against $\log_{10}$ cell size of ctenophore amoeba growing at 10g/l (optimum conditions) and at 30g/l (~ sea water) and free-living amoebae growing at optimum temperatures (Baldock et al.'s study; 1980). R^2=0.799; P-value=0.016. See legend for key.

As noted earlier, Versteeg (MS, Thesis, 2007) recovered a total of 11 different morphotypes of naked amoebae from ctenophore comb plates while in the current study only one morphotype was recovered by enrichment cultivation. Media formulations used in the study by Versteeg (2007) were different from media used here since salinity trials indicated that faster growth was obtained at lower salinities. Thus media used to attempt to optimize isolations of the presumed symbiont incorporated low salinity formulations. Even though the ctenophore amoeba did not culture out, the presence of the amoeba on ctenophore tissue was confirmed by SEM (Fig. 14). Moss et al. (2001) reported that a morphotype, resembling the *Vexillifera*-like amoeba found in the current study, occurred on ctenophore comb plates at a much lower density than that of the presumed symbiont (~400 mm^{-2}). In the same study by Moss et al. (2001), SEM micrographs showed that the presumed symbiont occurred on the comb plate surface at population densities as high as 2,726±395mm^{-2}.

After 48 hours in culture the cells of the ctenophore amoeba began to fuse and remained in this state for up to 72 hours. In their fused state, cells formed multinucleate plasmodia, up to ~130µm in length. The reasons for this are unclear although it could be a form of sexual reproduction, which would be an interesting occurrence since there has only been one genus of

gymnamoeba (*Sappinia*) reported to undergo sexual reproduction (Page, 1988). The ctenophore cells remained in their fused or multinucleate state for up to 72 hours before they reverted back to individual cells. Thereafter, cultures become unstable and after 168 hours since established, cells started to die off and the population crashed. For this reason cultures were always sub-cultured into fresh media at 168 hours to maintain long term, vibrant, cultures. Alternatively, the fusion of cells may be an adaptation to help cells stay attached to the surface of the ctenophores or to enhance the ability of cells to consume large ciliary masses on the comb plates. This fusion followed by a reversion to single cells, however, might be a culture phenomenon in response to deteriorating conditions in cultures.

In culture, the presumed symbiont was usually about 6µm in length and approximately 8-9µm wide. This size was close to that reported by Versteeg (2007) where length was (7µm) and the width was up to 15µm, similar to that reported by Moss et al. (2001) from measurements taken from micrographs. The slightly smaller sizes found in the present study may reflect different culture conditions.

All comb plates examined contained ciliates (Fig. 26) and a large dinoflagellate symbiont (*T. ctenophorii*) reported by Moss et al. (2001). Abundance of ciliates on the ctenophore comb plate surface may have caused competition for bacteria with grazing amoebae again contributing to failure to isolate amoebae from the 28 animals tested. Certainly, the numbers on the surface were far fewer than reported by Moss et al. (2001). As noted earlier, they found the average number of *Flabellula*-like amoebae (presumed symbiont) on comb plates was 2,726±395 amoebae mm^{-2} while Versteeg (Thesis, 2007) reported a lower population density averaging 313 mm^{-2} on ctenophore surface (range 0 to 946 amoebae mm^{-2}). In the current study, an estimated density of 784 amoebae mm^{-2} was encountered. The lower numbers reported in the current study compared to Moss et al. (2001) may have been due to the ciliates outcompeting amoebae. Even so, densities of 300 amoebae or greater on a mm^2 surface are impressive and attest to the success of this amoeba in this biotic habitat. In all three studies (the current study; Versteeg, 2007; Moss et al., 2001), the ctenophore amoeba was clearly dominant over all other amoebae on the ctenophore comb plate surface. Other amoebae, including the 10 morphospecies isolated by enrichment cultivation by Versteeg (2007), were never observed by direct observation. However, the *Vexillifera*-like amoeba was common to all three studies.

Rogerson et al. (1991) encountered 27 species of amoebae on the surfaces of living macroalgae off the west coast of Scotland and demonstrated that the marine amoeba *Trichosphaerium sielboldi* was able to utilize 50% of the tissue biomass of macroalgae with ease within seven days in culture. This suggests that at least some free-living amoebae have the ability to effectively digest and degrade tissue of multicellular organisms. This supports the view that the ctenophore amoeba may be obtaining nutrients directly from digestion of the comb plate surface. Armstrong et al. (2000) found that amoebae on living algae numbered $20mm^{-2}$ (well short of the densities on ctenophores) and postulated that they were utilizing algal carbon directly. In the study by Rogerson et al. (1991), the amoeba *Trichosphaerium*, was capable of digesting several species of seaweeds and could even be maintained axenically solely in the presence of autoclaved seaweed tissue (Polne-Fuller et al, 1990). Amoebae have also been encountered on the surface of marine fish. Versteeg (M.S. Thesis, 2007) determined the presence of amoebae on the surface of coastal fish. Out of 36 scale samples examined, 12 marine fish proved positive for amoebae on 16 occasions (44%). Four morphotypes were found and all fish with positive results had more or less the same frequency of amoebae. This occurrence generally affirms that amoebae are more common on vertebrate and invertebrate surfaces than expected. It is probable that the presumed symbiont could be breaking down the ctenophore comb plate tissue and utilizing this for nourishment. It is, however, impossible to conclude that the ctenophore amoeba is an obligate symbiont because amoebae cultures (obtained from Ms. Connie Versteeg) have been maintained in the laboratory for some 3 years. The evidence suggests that they are opportunistically pathogenic although ctenophores with heavy infestations do not appear to be damaged. In fact, Versteeg tended to find more amoebae on 'healthy' looking ctenophores.

At the SEM level, unusual surface projections from individual amoebae were observed (Fig. 36). Individual cells fixed on glass cover slips appeared different from those living on surface tissue. This may be because amoebae *in situ* (on ctenophores) are 'down in the mucus' on the surface and fix better without shrinking (as seen on amoebae fixed on glass cover slips). This is an interesting observation especially since the same preparation technique was used for individual cell SEM preparation as comb plate surface SEM preparation. This may mean that this particular amoeba (presumed symbiont) prefers to attach itself to tissue rather than other surfaces such as glass and plastic.

Ctenophore amoebae and mangrove amoebae survived, and reproduced, at all salinities tested over the range 0g/l-50g/l salt while the marine *Acanthamoeba* spp. survived up to 45g/l although whereby in this case the highest salinities (35g/l-45g/l) yielded lowest migration rates (and presumably lowest growth rates). At the extreme salinities (high and low), the ctenophore and mangrove amoebae were frequently observed to be floating, rather than as attached, motile cells. Although it has never been demonstrated, it is generally believed that amoebae need to be attached to feed thus time spent suspended in the medium would impact consumption and account for reduced growth rates observed. This suggests that these extreme salinities (0g/l, 5g/l, 40g/l and 50g/l) were close to the survival limits for these amoebae although salinities with these extremes would rarely be encountered in coastal and mangrove waters. It is also worth noting that ctenophore bearing amoebae swept by currents into hypersaline waters may shed their amoebal symbionts if the floating behavior *in situ* mirrors that observed in laboratory culture. The lack of amoebae on the surface of a ctenophore (observed in SEM preparations here and in 2% of ctenophores observed by Versteeg) may have been a result of the ctenophore being swept by currents into low salinity environments ahead of collection.

Amoebae have also been isolated from extreme marine environments on a few previous occasions. Of note, Hauer et al. (2001) reported that at extreme salinities (low and high), cells of the marine amoeba *Platyamoeba pseudovannellida* isolated from the Salton Sea, California (salinity 44g/l), became rounded and generally less active. Even so, this amoeba (*Platyamoeba pseudovannellida*) could survive over the impressive salinity range tested, 0g/l-138g/l. Hauer and Rogerson (2005) isolated amoebae from hypersaline ponds (160g/l) around the perimeter of the Salton Sea. From these studies, it was concluded that marine gymnamoebae were euryhaline.

The ctenophore amoeba behaved in a similar manner with amoebae at extreme salinities (low and high) becoming rounded and less active. There was no discernible trend with size measurements (length and breadth) on the ctenophore amoeba over the range of salinities. Statistically, however, the breadth measurements of the ctenophore amoeba were significantly affected by the range of salinity treatments 10g/l-50g/l (ANOVA; p-value=0.009; α=0.05) and the average cell size (n=10) ranged between 2.4-3.8μm. The same test (ANOVA; α=0.05) showed that the variation reflected by the difference in mean lengths of cells across salinities 10g/l-50g/l was not significant (p-value=0.15). These results may reflect measurements on

'rounded' cells, or at least cells less active, that would be smaller than for cells moving fast under more ideal conditions.

Surprisingly, maximum growth of this supposed marine amoeba was at 10g/l rather than the salinity of seawater (32g/l). This suggests that the source of the amoeba might be brackish water or even from freshwater runoff. Although less activity was seen at the highest salinities, cells continued to reproduce. As discussed earlier, salinity did not affect the size or morphology of cells, however, amoebae were most active at the lowest salinity and the data is consistent with the view expressed above that marine amoebae are generally euryhaline.

None of the isolates showed optimum growth at 30g/l even though all three strains were obtained from the marine environment at around 32g/l salt. The optimum salinity for ctenophore amoeba was seen to be at 10g/l (generation time=7.9 h; SE=±0.5) while optimum salinity for mangrove amoeba was at 20g/l (generation time=34 h; SE±5.6) and optimum salinity for marine *Acanthamoeba* spp. was at 15g/l (migration rate=5.5 $mmhr^{-1}$; SE±0.3). Complementary molecular studies to identify the ctenophore amoeba at the molecular level are being undertaken by collaborators at Woods Hole Oceanographic Institute, Massachusetts and Auburn University, Alabama.

Multiple nucleotide sequences obtained for the mangrove amoebae using PCR primers based on 18S SSU ribosomal RNA gene yielded matches other than amoebae when sequences of PCR products were subjected to a BLAST search (www.ncbi.nlm.nih.gov). Important to note however, is that results from BLAST showed close matches to ciliate and flagellate nucleotide sequences also based on 18S SSU rRNA gene. Since there are not many studies at the molecular level on naked amoebae and because the mangrove amoeba and ctenophore amoeba are new to science, it is not entirely unusual that GenBank did not yield any matches to amoebae. According to Dr. R. Gast (pers. comm.) amoebae are generally difficult to amplify and attempts to identify the ctenophore amoeba using nucleotide sequences based on 18S SSU rRNA yielded ciliate and flagellate matches to ctenophore amoeba and these results are comparable to nucleotide sequence analysis of the mangrove amoeba in the current study.

A study by Booton et al. (2004) showed that acanthamoebae are commonly isolated from salt-water environments notably T4 strains commonly associated with the rare sight threatening disease, Amoebic Keratitis (Aitken et al., 1996; Dua et al., 1998; Kennedy et al., 1995). The eye disease is rare and unlikely to pose a significant risk to beach users even if clinically important

species were present (Booton et al., 2004). However, it is important to note that there have been recent outbreaks of the eye disease, one occurrence in Chicago as reported by the Center for Disease Control, CDC (2007) and another recent incident in Florida (Shoff et al. 2007). It is hypothesized that acanthamoebae invade the eye through microscopic abrasions on the cornea (caused by contact lenses, sand grains or dust particles). This allows the active form of the amoebae (trophozoite) to invade the eye aided by the thin, spiny pseudopodia that characterize the genus (Booton et al., 2004). The disease is so rare that it is estimated to infect only 1 in 10,000 contact lens wearers (Rogerson, pers. comm.). This estimate is comparable to an earlier estimate that infection occurs in 0.33 cases per 10,000 contact lens wearers (Lam et al. 2002). Frequent contact with disease-causing micro-organisms, including amoebae, leads to immunity and helps protect against infection. But physical stress (abrasion by contact lenses) is thought to lead to incidences of infection (Booton et al. 2004). Not surprisingly, AK has been attributed to inappropriate cleaning of contact lenses. In particular, rinsing in tap water containing cysts is thought to result in the attachment, and subsequent proliferation, of amoebae on the lens surface (Seal et al., 1995).

It is surprising that the *Acanthamoeba* spp. was isolated from a marine habitat since this is commonly known to be a soil amoeba. In a study by Booton et al. (2004), acanthamoebae were present in 38% of wet and dry sand samples obtained from beach sand although they were never isolated from marine water samples adjacent to the beach. In the same study it is postulated that the acanthamoebae were in cyst form concentrated in the sand although laboratory studies did confirm that they could reproduce from 0g/l-30g/l salt. This is not surprising given the study by Hauer et al. (2001) showing remarkable salinity tolerance of amoebae in general. However, this is the first report of salinity tolerance in a 'freshwater amoeba' and makes the distinction between marine and freshwater amoebae extremely gray.

Salinity tolerance of the marine A*canthamoeba* isolate was compared to an isolate from chlorinated tap water isolated in Huntington, WV. Data showed that acanthamoebae (regardless of habitat) tolerated a wide range of salt levels (0g/l up to 40g/l or 45g/l). Optimum growth at 15g/l and 10g/l (marine and chlorinated tap water isolates respectively) suggests that acanthamoebae prefer this concentration possibly because they expend less energy expelling water taken up by osmosis. It is interesting that these levels are close to salinity of the eye. It is difficult to establish whether the acanthamoebae isolates were in cyst or active form at the time

of isolation. Booton et al. (2004) postulated that high abundance of *Acanthamoeba* in sand relative to water (source of isolates in the study) and tolerance to high salinities suggests that acanthamoebae are active in sand, rather than surviving as cysts, probably because sand affords a protected habitat rich in prey bacteria.

In the current study, the prevalence of naked amoebae in Huntington's domestic water system was examined. Results showed an unexpected dominance of acanthamoebae. Of the 173 one-liter samples processed from water faucets in Huntington, approximately 18% of the samples were positive for amoebae and 12% were positive for *Acanthamoeba* (Table 2). It should be noted that this is probably an underestimate of abundance since there may have been more than one cell (or cyst) on the filter after processing the one-liter sample. Moreover, not all amoebae can be cultured in the thin water film on the surface of the agar used to enrich for amoebae on the filter. If a range of media formulations had been used, additional amoebae may have been detected. This is only the second study of amoebae in tap water in the U.S. The other study was conducted in Florida (Shoff et al., 2007). One major difference between the Huntington data and the Florida study was the high proportion of acanthamoebae in WV water samples (12%) relative to FL (2.8%). The reasons for this are unclear but it should be noted that in the Florida study, swabs were taken from water cisterns to sample amoebae in the biofilm. In Huntington, biofilms were absent (presumably because of high chlorination at source) so one-liter samples were filtered. Another obvious difference is in the source of the water. In Florida, water is taken from a shallow aquifer while in WV water is taken from the Ohio River which is presumably richer in amoebae, particularly after heavy rainfall events.

As mentioned above, the water in Huntington is chlorinated (typically 2mg/l) and presumably explains why fewer morphotypes were found in WV relative to FL. The survival of acanthamoebae was probably due to their ability to form resistant cysts that can withstand these levels of chlorination. As shown in Table 2, when chlorination levels were lower, more types of amoebae were found. The tolerance of the cysts of both *Acanthamoeba* and the vannellid amoeba (both from Huntington) were examined and these stages withstood up to 14 mg/l in the case of *Acanthamoeba* and up to 50 mg/l in the case of *Vannella*. These levels are far in excess of permissible levels demanded by the U.S. Environmental Protection Agency (EPA) [i.e. ca. 4 ppm chlorine].

The presence of free-living amoebae in drinking water is not regulated under current water quality regulations and protists generally do not cause health concerns. However, until the etiology of the AK condition is fully understood, it underscores the need for contact users not to store lenses in tap water and to use multi-purpose cleansing solutions instead. The work points the way to future research. Are there trophic amoebae living in the tap water system or are amoebae only present as cysts? And does the presence of amoebal cysts suggest a need to better treat domestic water? The inability of current practices to inactivate or remove these cysts suggests treatment would also fail to kill obligate pathogens such as *Cryptoporidium*, *Giardia*, and *Entamoeba*. These parasites do cause infections when ingested and perhaps the easily cultivated naked amoebae are useful surrogate organisms for use if future improved standards are demanded.

As mentioned earlier, this study aimed to determine the genotype of the marine *Acanthamoeba* spp.. Acanthamoebae from other extreme environments (fish mucus, acid environment; pH4 and chlorinated tap water) were included to determine whether genotype was a function of extreme environment. It was further reasoned that 'extreme acanthamoebae' may show a link to AK. Phylogenetic analysis showed that the marine *Acanthamoeba* isolate was genotype T3 (98% bootstrap value; 0.2% dissimilarity). Sawyer (1970, 1971) was the first to show that some *Acanthamoeba* are salt-tolerant. He showed that *A. griffini*, a T3 designate, could grow between 0g/l and 32g/l. He also demonstrated that *A. polyphaga*, a T4 designate could grow in both freshwater and marine conditions. In this regard, it is not unusual that the marine *Acanthamoeba* spp. isolate in this study resembled T3 designates in GenBank while the acid tolerant and tap water isolates resembled T4 designates.

The most commonly isolated genotype from AK patients is the genotype T4. But the T3 designate has also previously been associated with AK infections (Khan et al., 2002). However, it should be noted that the T4 strain is also the most commonly isolated strain from the environment and its predominance in AK patients may simply be a reflection of its abundance in nature (Booton et al., 2004; Shoff et al., 2007). Since the marine isolate was obtained from a habitat with a salinity of 32g/l and water isolates were obtained from tap water (corresponding to 0g/l salinity), slower growth at 32g/l for the marine isolate and slower growth at 0g/l for the tap water isolate may have been because amoebae were not acclimatized to the salt treatments. The isolate from the mucus of a marine fish was found to be a T5 designate closely resembling T5

ref. strain U94739 in from GenBank (78% bootstrap value; 5% dissimilarity). T5 designates have not been associated with disease but are commonly encountered in sewage (Booton et al., 2004) which could have been the source of these amoebae in coastal fish.

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Appendices

Appendix I: Media Formulations

Soil Extract (28ppt, 10ppt)

Prepare soil extract stock solution by adding tap water to a beaker containing fresh agricultural garden soil. The supernatant water should occupy approximately four-fifths of the depth. Autoclave the mixture for one hour at 250°F. Following sterilization, decant the liquid (soil extract) and store at 4°C. Soil Extract media used for amoebae cultures was prepared as follows:

Soil Extract (stock solution)	10ml
K_2HPO_4 (0.1% w/v)	2ml
$MgSO_4.7H_2O$ (0.1% w/v)	2ml
KNO_3 (1.0% w/v)	2ml
Sea Salts	28g /10g (depending on media concentration required)
Distilled water	84ml
Total Volume	100ml

Sea Water (32ppt, 28ppt, 10ppt) (SW32, SW28, SW10)

Sterile filtered distilled water	1L
Sea salts	32g or 28g or10g
E. coli[2] suspended SW28/10	500µl

Sea Water (20ppt) enriched with E. coli and supplemented with Malt/Yeast (SWMY20)

Sterile filtered distilled water	1L
Sea salts	28g/10g
Malt Extract	0.1g
Yeast Extract	0.1g
E. coli[1]	

Sea Water (28ppt, 10ppt) supplemented with Malt/Yeast agar block (SWMY28, SWMY10)

Sterile filtered distilled water	1L
Sea salts	28g/10g
Malt/Yeast agar block	100µl

[2] Comprises a loopful of *E. coli* suspended in respective culture media mix for consistency. For example, *E. coli* was suspended in SW28 liquid media prior to inoculation of experimental plates containing amoebae cultured in SW28 media.

Malt/Yeast Agar

Sterile filtered distilled water	1L
Bacteriological agar	15g
Malt Extract	0.1g
Yeast Extract	0.1g

Media was sterilized by autoclaving at 250°F for 15 minutes.

Amoeba Saline (AS)

NaCl	1.2g	*
$MgSO_4.7H_2O$	0.04g	
$CaCl_2.2\ H_2O$	0.04	
Na_2HPO_4	1.42g	
KH_2PO_4	1.36g	

*Stock solutions were prepared by adding solute to 100ml filtered distilled water
A final dilution was made by adding 10ml of each stock solution to filtered distilled water to make 1 litre. Media was sterilized by autoclaving at 250°F for 15 minutes.

Amoeba Saline Agar

Amoeba Saline	1L
Bacteriological Agar	15g

Media was sterilized by autoclaving at 250°F for 15 minutes.

10X TBE

Tris Base	108g
Boric Acid	55g
EDTA	7.45g
dH_2O	1L

0.5X TBE

Mix 10X TBE and distilled water in the ratio 1:20

LB-Amp Plates (500ml)

Add to a 1L sterile beaker 350ml milli-Q water and a magnetic stir bar and place on a hot plate (medium heat). Add to the water 5g Proteose Peptone, 2.5g Yeast extract and 5g NaCl. Bring pH to ~7.0 using 1N NaOH. Transfer mixture to sterile graduated cylinder and bring up the volume to 500ml using milli-Q water. Tranfer solution to a 1L autoclavable bottle and add 7.5g bacteriological agar and sterilize contents in autoclave. Prior to sterilization, weigh and label bottle and indicate the weight on the label. After sterilization allow the media to cool and weigh bottle again. If there is a difference in weight, this indicates evaporation of liquid during sterilization and this can be replaced by adding back water in the amount of the difference in weight (1g=1ml). After cooling, add 1000x ampicillin solution in the ratio 1:1000 (ampicillin:media), in this case add 500µl ampicillin to the 500ml of media. Pour warm temperature media in 90mm petridishes and leave in hood to cool and solidify before use.

Appendix II: Sequence Analysis

Key

*Sequences obtained from one or more clones of DNA from the amoeba.
Important Notes: The primers (TA vector promoter regions) used include SP6 Forward Primer, T7 reverse primer or M13 F/R forward and reverse primers respectively. Identity codes are arbitrarily assigned to the isolates. Reverse primer sequences were converted to the reverse complements for all pairwise alignments. Pairwise and multiple alignments were obtained using Biological Sequence Alignment Editor (BioEdit) program.

Mangrove amoeba Nucleotide Sequences M13 forward/reverse primers EcoRI Restriction site:

>Clone No. 1A (PCR primers: Silb-F/Silb-R) Sequencing primers M13 F/R
AATTCGGCTTAACCTGGTTGATCCTGCCAGTTTGTGCCGGGGCCTGCCAGTTCGCAGGTCGGGATGGGAATCGGCATGTCGCTCGGCGGATTTCCCG
GTGCATTTGCCGCATGGATCGGCT

>Clone No. 2B (PCR primers: Silb-F/Silb-R) Sequencing primers M13 F/R
CYYWWMWWWRGGGGCSAATTGGGGCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAACCTGGTTGATCCT
GCCAGTCGGTCGATCAAGACAGCCCAGAATATTCATCAGGGTCGATTTACCTGACCCGGAAGCGCCAATGATCGCAACCATTTCGCCGCGTTCAATC
GTCAGGTTGACGCCCTTTAGCACGGCAATGCGATCCTCGCCCGCCGGGTAGTGCCGCCACAGATCGCGGATTTCGATCAGCGGGGTTTTGTGAGTGT
TGCTCCCTATCTGGTTCATGATCAGAACATCCCCCCCGGGCCACGACGTATGGATTGGCTGGTGGCGACCACACCGCTGGACGCGCCGATAACAATG
TCGTCATTTTCGGAAATGCCACTGATGATCTGGGCGTTGATATTGGTGTTCATGCCGACCGTGACCGGCTTTTCAATCGGGTCACCTTTGTCGTTAA
GAACCTTAACGGCATATAGCCCGTCCTTGTCCGGACCACTCAGCGCGGCGGACGGAATGATCAACGCATTTTCAACTTCGTTTAGTGTGATGGTGAC
TTCGGCTGTCATCCAGACGCGCAGGCGGTGATCCGGATTGGCGATGTCAAACAGACCATTGTAATAAACGGCTTCGTCATCGGTATTGTCGTCGTCA
TCCTCGATTCCGGTCGGGGCGGGTTCGATGGCGCGAAGGGTGCTTTCATAGCGCGTGTCGGGATCGCCCAGAATGGTGAAGCTGACCGGTTGGGCCT
TCACGAACGCGGATGACGTCGCCTCGGATATTTCGGCCTCGACCGTCATGGTGTCGAGCTGTGCGAGTTTTTATGAWGGTC

>Clone No. 3B (PCR primers: Silb-F/Silb-R) Sequencing primers M13 F/R
GWYYWAAWWARGGCGATTGGGCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAACCTGGTTGATCCTGCC
AGTCCCGCACGCATTTTGGGCATATGCCGGATACGGTCCCAAAACGGCAATAATCCGATCACCAGAAGAAACGCGGGCAGGAAAATGGCGATCAGGG
CAATCATCGCCCCTGCGATCCCGCCTGTATCGGTTTGCATCACGGCACCCAGATAGGCAGCAAAGGTAAAAATCGGCCCCGGTACTGCCTGCGTTGC
GCCGTAACCGGCAAGGAACGTATCCGTATCGACCCAACCGGGCGCGACAACAGCTTCCTGCAGCAGTGGCAGCACCACATGGCCGCCACCAAAAACC
AGCGCCCCGCTGCGATAAAATCCGTCAAATACGGCAAGCCATGATGACGATGTGACCTGTACCAGTAACGGCAACCCGATCAGCAGTACACCAAAGA
TTGCCAACGCGAACACCGCCACCTTCGGGGAAATACCGGCAGCCAGCTTCCCGTCCAGCTTGACCAGATCAGCCTGCAGGAACAGCGTCCCGGCAAC
CAGCCCACCGACAATGACGATGGCCTGCATGCCCGCACCACTAAATGCGATCATCAGTGCGGCAGCAATTATGGCAACAACCGCACGCGGAATATCA
GGGCACAGGCTTCGCGCCATGCCCCAAAGAGCGTGTGCGACAACACCAACGGCAACCAGTTTGAGCCCCCATATCATGGGACCAGTGGCGGCGAGGT
TACCGCCAGCCAAACCAAGCCCAAGTGCGACAAGAACAATCGCCGATGGCATGGTAAAGCCGATCCATGCGGCAAATGCACCGGGGAAATCCGCCGA
GCGACAT GC

>Clone No. 3C (PCR primers: Silb-F/Silb-R) Sequencing primers M13 F/R
GMMMCAATWWRGGGCGATTGGGCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAACCTGGTTGATCCTGCC
AGTCCCGCACGCATTTTGGGCATATGCCGGATACGATCCCAAAACGGCAATAATCCGATCACCAGAAGAAACGCGGGCAGGAAAATGGCGATCAGGG
CAATCATCGCCCCTGCGATCCCGCCTGTATCGGTTTGCATCACGGCACCCAGATAGGCAGCAAAGGTAAAAATCGGCCCCGGTACTGCCTGCGTTGC
GCCGTAACCGGCAAGGAACGTATCCGTATCGACCCAACCGGGCGCGACAACAGCTTCCTGCAGCAGTGGCAGCACCACATGGCCGCCACCAAAAACC
AGCGCCCCGCTGCGATAAAATCCGTCAAATACGGCAAGCCATGATGACGATGTGACCTGTACCAGTAACGGCAACCCGATCAGCAGTACACCAAAGA
TTGCCAACGCGAACACCGCCACCTTCGGGGAAATACCGGCAGCCAGCTTCCCGTCCAGCTTGACCAGATCAGCCTGCAGGAACAGCGTCCCGGCAAC
CAGCCCACCGACAATGACGATGGCCTGCATGCCCGCACCACTAAATGCGATCATCAGTGCGGCAGCAATTATGGCAACAACCGCACGCGGAATATCA
GGGCACAGGCTTCGCGCCATGCCCCAAAGAGCGTGTGCGACAACACCAACGGCAACCAGTTTGAGCCCCCATATCATGGGACCAGTGGCGGCGAGGT
TACCGCCAGCCAAACCAAGCCCAAGTGCGACAAGAACAATCGCCGATGGGCATGGTAAAGCCGATCCATGCGGCAAATGCACCG

>mangrove (PCR primers: Silb-F/Silb-R); Sequencing primer Silb-F
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCNNNNNGNGNNNNNNNNTTNNNNAATGNNNGCCNTGNCTTCTTACCGCGACNAGNGGNNTTGTGGTC
NCCCACTGGATTACCAANTTGACCNCTTGCATCATNATACTCGTACTTGGNGTTGTACGTCNCGCTTANCGTTGTGGTGAATGAACCCTNNTTGTTG
GTGTCNTATGTCCATCNAGCGGTAGAGTCGAACAAGTCTACCCNTACTGTCGCTACNTTCTGAGATTGAGGTACCANNCNAACNACACNTTGAGTTN
ACCGATNTNNGTCACGCGANCATCACCCTGCGCTGCCAACCANGNATTGNCCNNTTCACNAGTCNNTGACCCNNGTGTTGGGTCNNAAGATGAACGA
TTTGAACCAATGGCTGCCAGNNATGAATCAAACTCTTCACCAACCACCTCTTCTGTNNCCNGCGAACNAATACGGTCTGTGTNCTCAATANACTGGT
NNTCCAAACTGATGCTCNNATCATTGACTGGNTCCCAGGTAAAACCNNCGNTCCANATATCGAACGTNNCANGNTTCANNCCNNGGTTACNNNANNG
NNANTTNNNNNNCGNTNANTCNCCNCCTGTGACAGCNNNNNCAACGANNANNATCTCACCACNNTTTTCNTTCGGATCNNANNNANNCNCATCANCT
TCANNGGNGNCANGAACNNNNNACANATTNNANNNCNNNNNNCCNATNTTGNNNNNNCTTNNCNNGCAGTNCN

>mangrove (PCR primers: Silb-F/Silb-R); Sequencing primer Silb-R
NNNNNNNNNNNNNNGNNNNNNNNNNCNNNNNGNGNNNNNNNTTNNNNANNGNNNGCCNTGCCTTCTTACCGCGACCAGNCGNNTTGTGNTCACCCA
CTGGATTACCAACTTGACAACTTGCATCGNNNTACTCGTACTTGGTGTTGTACGTCNCGCTNNNGTTGTGGTGAATGAACCCTCGTTGTTGGTGTCN
TATGTCCNTCGAGCGGTAGAGTCGAACAGGTCTACCCNTACTGTCGCTACNTTCTGAGATTGAGGTACCACACNAACNACACGTTGAGTTGACGGAT
CACGGGTCACGCNNNNATCACCCTGCGCTGCCAACCAGGNATTGNCCGNTTCACNAGTCGCTGACCCANGTGTTGGGTCNNAANATGATCGATTTCA
ACCAATNGCTGCCANCAAGGCATCAAACTCTNCANAANCCACGNCGTCAGTACNCANANTACNAATACGGTCTGTGTACTCAATANACTGGTANTCC
AAACTGANGCTCNNATCATTGACTGGTTCCNANGNAAAACCANNGTTCCANATATCGAACGNTTCANGNNNCNAGCNNGGNTTACNANATGAACANC
TNGNNNCNCCGTNCNNNNNNCCACCTGTGACNGGGNCATCANCNAANCAANATCTCACCACNNTNTTCGTTCNGATCNNACNGANTCGCNTCAACTG
CACNTGGTGNCATNNNNNNNCACCNNNTNNAGNNNNGNANCCNTNNNNGNNNNNCNNNCNCTCGNANTGN

>Clone No. C (PCR primers: 570C/1200; Sequencing primer, T7
AGCCTYTWRAWGMWGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTGGGCATCACAGACCTGTCACGGACGGGCAGCGTGTGGC
CGGTATATACCGGCGCTCCGTCATGCTCGCTAGCGGACGCTACGCCATGCTCGATGACGGTATGGGGTTCAGCCTAGTGCCGTGGCGGCCGATGATC
GAACAGCGACTAGGTCAGCAACTTACAGCGGTGGTGCGTGGTGATTTTGTATCATGGGACTTGGGCCGAGCACGAGGACCGGCAATCAGCTGATGCC
GATAGAACGTTAATGCTCAAGGTTAAGGGGGCTGTTCCCGGACTTCCTCTGAAGGCGCGGAGAAACGTAGCAACGGCATCTTTGGCCCAGTGATCGT
GCTGCGCGGCAAACGCTGGAATATGGGGATGCATCAAACACTGARTTCACCCCAGCACTTACCATATCACTTCAAAGGCGCGGCGATGGAGTCAAAT
CCAAGGCCTGCAAGGTCCAGTTCACCTTCATATACAGCTCTTTCTAGTCTTTCCGCCACGATACGTTTGATCTTGGCAGAGCCGGCAAGGTGAAAGC
GCGCAGCCTGAGCTCGGGAAACCTTGGGCTTCGGCCGCAATCATGCGATATAGCGCCAACCCGTCAGGCGGATGGCATTGGAGGACGTTCCGATGCT
AGAGCTGGCGGACATGTCGGTTACGTCTCTTTCTTCCTCTACTGTTGTTACGGATACGGAGCATGCCAGGTCTTGTGTTTTCGTACCGGCCAGATCG
GTTTTTTACCGATATTGACCACACGAGGAATTCGCCACATAATGCCATTTTTAGTAACGAACCGATCGGTTCGGTTTGGTTCWAGTCTGRAGTSYMG
CAYMCCTKAMGGCAYKGKTACTAGTYGGAWGCCGA

>Clone 570C (PCR primers: 570C/1200); Sequencing primer, SP6
GMMYWWRWRTYMAGSKTKGSTACGASCTCGGWCCACTAGTAMCSSCCGCCAGTGTGCTGGAATTCGGCTTGWAMYWCMMSCTCCMRWTCSGKKAGYC
CCTAAARGKGKGATWCCCCCGCSRWKTCCTCGTGATCGGYTCTCGSYAAARAATYGATYCKRCYTGACSTTTCCRAAMRACCTGTTATGCCSCRWAT
YGSKWCYWCCTGKARATKAAAAMTCMTGYGYWTCSWAMWTGTCSSCMTGCTTTASMTCCCGATTTCCMTCSARTGCCWTCCGCRGGTTGKGTTGGCG
STATWTCGSAYGATTGCRTGCGARYCYMARSGWYYCYMGAGCTMGGGYTGYGCGKTTWCMYYTYMCCRSCTCTGCCMWCAWCRCMCCYRYYRRGGYG
RWGWMACYMAMAAAARMTKWATWGGAASSKSCWMTGSAWYGTGYARGGSATGGATTTSACTCAMTCACCSCRCCTTTAMAGWGATRTGCKAWRYTMT
GARTGKGAACATGTGTTTGATSCCTCSCGWGRTYMCAKCGTTTGMCRMSCGATCTGTCWCTGGGAAAAAGATGSASSTGCTATGTTTCTCCKCSKCT
WCAGARGAAGWSGRRGAAMRASCCCYTTMCSATWTGKCCTYMACSTYCWATMKSSRAMMGTTSWYYGMCGGTMCTCGYGCKCGGSSYWAGYSCYATG
AYACATGATCGCCRCSCAMCMCCRCGGTTWCYTGATGACCTACTCGCTGCTTTCATCATSGSSGCTCTGCCASTACGATGAWMTCCATGACGTAATM
CWRAWGGAGTAKCTCTGMTRGYGAGCTGGACCGTAGCGSCSTWAWTYYGACYCATCGCCGCCSCTYTGGARCKGGWCTGGTGAKKGCWGGCCGAATT
CAGAGAATTGWMKTCAYMCCCTGKATGCCRSCTSTAGGCTGCGCTCASAAGRGACCTRGTCSCACGTATGCT

>Clone 570(B) (PCR primers: 570C/1200); Sequencing primer, T7
TCCCYCWKAAWGMATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTGTAATTCCAGCTCCAATAGCGTATATCAAAGTTGTTG
CAGTTAAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGTCGTCGGGTCAAACCGGCGACTGCGTTGGCGTTGCGGGCTCGGTC
CGTCGGTGGACCCTCGTGGTCTTAATCGGCGTGTCAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTGCTATTGAGTTAGTGGGGACGT
CACAGGGGGCTCATCGTCGTCATGCAAATGGCGGCGGTGGGTCCCTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGA
TCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTA
ATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAA
GGATGTTTTCATTAATCAAGGACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGA
GACGTTGAATACAAAACACCACCATCGGCGCGGTCGTCCTTGGCGTCTGTCCCTTTCAACGGGGGCAGGCGCGAGGGCGGTTTAGCCCGGTGGCACC
GGTGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAGCTGAACTTAAGGAATGACGGAAGGGCA
CACCAGAGTGGAGCCTGCGGCTTAATTTGACTCAACMCGGGGAAAC

>Clone 570(B) (PCR primers: 570C/1200); Sequencing primer, SP6
TMAMWARKSWYAGGKTTGCTMCGAYCTCGGATCCACTAGTAASGGCCGCCAGTGTGCTGGAATTCGGCTTGGSCATCACAGACCTGTTATTGCCTCA
AACTTCCGCTGGCTAGGSGCGCAGCASYCCCTCTAAGAAGCACGGACGCCGAACCCTACCGCCCACACGGACCCGGGCCCTGCCGGCGGGATGACGT
CSCCGCGAAGCGACMCCMCCCCGCAGCGGACCGCGKGAGCCACGCATGGGTTTTGAKGGACGGGTTAGCGCGGCATATTTAGCAGGTTARGGTCTCG
TTCGTTAACGGAATTAACCAGACAAATCACTCCACCAACTARGGACGGCCATGCMCCACCMCCCATAGAATCAARAAAGAGCTATCAATCTGTCAAT
CCTTACTATGTCSGGACCTGGTAAGTTTCCCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGT
TTCASCCTTGCGACCATACTCCCCCCGGAACCCAAAGACTTATGATTTCTCACAAGCTGCTAGGGGAGTCATTCACCGGTGCCACCGGGCTAAACCG
CCCTCGCGCCTGCCCCCGTTGAAAGGGACAGACGCCMAGGACGACCGCSCCGATGGTGGTGTTTTGTATTCAACGTCTCCTAATCGCTGGGYSGGCA
TCGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTCCTTGAWTAGWGAAAAMMWTCCTTGGGCAGATGCTTTCGC
AGAAGTTWAATCTTTCATAAATCCAGAATTTCACCTCCTGACAATTAATATTAATGCCCCCACTATCCTATTAATCATTACCCTAGTCCTCSCGCTG
CMCAAACAACTGAAAATARGGAGGGACAGGGTCCTA

Acanthamoeba Sequence Analysis

All sequences are concactenated to make the "full" length of each PCR product spanning the DF3 region (combination of 892 and 892C sequencing primers). The 892C primer sequence was useful in identifying the strains while both primer sequences (892/892C) were useful for phylogenetic analysis. *Only one primer sequence, 892C primer was recovered for tapwater strain A5. A1-A5 (tap water strains), A6 , BP and FH are arbitrary codes for acanthamoebae in the study.

>Marine *Acanthamoeba* isolate, A1; Sequencing primers 892/892C
STCCCAAGCTGCTAGGGAGTCATTCACCGGTGCCATGCGAGCTAAGCCGTCCCGATGATGCCGCCTTGTGAGCGACCCACCAAAGACGACCGCAATG
CGCATGGTGGTGTTTTTGTATTCAACGTCTCCTAATCSCTGGTYGGCATYGTTTAMGGTTAAGWGTTGGCCGGTATKTGWTAMTTTCYGATRKGCTA
ATWTTGGGTTWTTSAKAGGGAACACRTTAMMRAAGCATMCSTCAGGAGAAGTTAATSTRACCRARATTGGCCCAGATCGTTTACCGTGAAAAAATTA
GAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACAGTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTGC
CGCGAGGACCAGGGTAATGATGAATTGGCACAGTTMGGTTTTTTTYAWACTTCTGCGAAGCATCTGCCAGGATGTTTTCATTAATCAAGAACGAAAG
TTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCCCCATGCG
CATTGCGGTCGTCTTTGGTGTGTCGCTCACAAGGCGGCATCATCGGGACGGCTTAGCTCGCATGGCACCGGTGAATGACTCCCTAGCAGCTTGTGAG
AAAATWAAATATTAMTGCCCCCAAYTATCCCTATTAAWCRTTAACCTGGKCCTCGSRGMMAAYCAAYTGAAAATAGGAGGACAGGGTCCTATTCCAT
TATCCCATGCTAATGTATTCGGTGGCATAAAATTGRATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGC

> Tap water isolate, A2; Sequencing primer T7
GGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGA
CCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGT
GAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCG
GTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCTAGCAGCTTGTGAGAA

>Tap water isolate, A3; Sequencing primers 892/892C
TGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGG
ACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTGGGGCTTCTGCGAAGCATCTGCCAGGATG
TTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGT
TGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGGTGGCACCGGTGAATGACT
CCCTAGCAGCTTGTGAGAA

>Tap water isolate A4; Sequencing primers 892/892C
AWMCGKGRTTRGTCSCAAWTATGCTTRCCCTAGTCCTCGCGCTGCCAAAACCAACTGAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAA
TGYATTGGCTGGCAGAAAGTTGGATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGKWMCGATCTGGGCCATTTCAGGGCCGGCTAGGGGACGST
TTAGCCCGGGGGCKCGGTGAATGACTCCCTAAYTCATTGTGAGAAATAACGTATACAAGCCCAAACCGACCCATACTTAACCATAMCCKATGCCGAC
CASTGATTAAGASACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGGTTAGCCCGGT
GGCACCGGTGAATGACTCCCTARCAGCTTGTGAKAA

>Tap water isolate, A5; Sequencing primer 892C
CTSTTSMWTTYTKGSGGAASCTCTGCCAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAAC
CATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCSGTCTTTCSGGGCCGGCGCGGG
GACGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCTACAGCTTGTGAGAAAT

>Acid tolerant *Acanthamoeba*, BP; Sequencing primers T7
GGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGA
CCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGT
GAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTATTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGCGCGGTCGTCCTTGGCGTCG
GTCCTTCACGGGGCCGGCGCGAGGGCGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCTAGCAGCTTGTGAGAA

>*A. Castellanii castelani* Neff strain, A6; Sequencing primers T7
GGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGA
CCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGT
GAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGCGCGGTCGTCCTTGGCGTCT
GTCCCTTTCAACGGGGGCAGGCGCGAGGGCGGTTTAGCCCGGTGGCACCGGTGAATGACTCCCTAGCAGCTTGTGAGAA

>FH (*Acanthamoeba* from fish mucus); Sequencing primers 892/892C
TTCTCACAAGCTGCTAGGGAGTCATTCACGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCGGGCAGATATTTTTCCTGCCACCGAA
TACATTAGCATGGGATAATGGAATAGGACCCTGACCTCCTATTTTCAGTTGGTTTTGTTTACAGTGAGGTCATCAGGGTAATGATAATAGGGATAGT
GGTTMKKTTA

Curriculum Vitae

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Lab Instructor, Human Biology BSC 105, Marshall University, 2007
Lab Instructor, Human Biology BSC 105, Marshall University, 2008

Research Laboratory Experience

Research Assistant, Chemistry Department, Marshall University (Aug-Dec 2006)
Research Assistant, Biology Department, Marshall University (2007-2008)

Research Grants

Marshall University Graduate Advisory Board Thesis Grant (2007)
Louis Stokes Alliance Minority Participation (LSAMP) Research Grant (May – August 2007)
European Union research scholarship recipient (June 2005)

Posters

Mbugua, M. and A. Rogerson (2007). Characterization of Unusual Gymnamoebae from the Marine Environment. Marshall University, Sigmi-Xi Research Symposium.

Short, A., L. Bevins, M. W. Mbugua, W. Trzyna and A. Rogerson (2008). Prevalence of Acanthamoeba in Huntington Tap Water. Annual Undergraduate Research day at the Capitol, Charleston, WV.

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