

Phylogenetic analysis of members of the *Crenarchaeota* in association with marine invertebrates from Woods Hole, MA

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Abstract

Once thought to be a limited kingdom within the *Archaea*, mesophilic members of the *Crenarchaeota* have been shown to be a ubiquitous group in marine, freshwater and terrestrial environments. A few members have been found in association with marine animals, including a sponge, a deep-sea cucumber and two species of fish. This study explored the phylogenetic diversity of the *Crenarchaeota* among several marine invertebrates common to Woods Hole, MA using 16S rRNA gene sequence analysis. Genomic DNA was isolated from gut contents of a mussel (*Modiolus demissus*) and a tunicate (*Styela clava*). DNA was also extracted from the accessory nidamental gland (ANG) of the squid *Loligo peali*. This gland is known to have a dense extracellular bacterial consortium. A ~ 750 bp fragment was PCR-amplified using primers specific for crenarchaeotes in the ANG and mussel gut, but no product was observed using the tunicate gut DNA. The products were cloned, transformed into *E. coli*, and analyzed by RFLP. Nine different clones were successfully sequenced, 2 from the ANG and 7 from mussel gut. Four clones (1 from the ANG and 3 from mussel gut) grouped within the mesophilic *Crenarchaeota*. Five clones (1 from the ANG and 4 from mussel gut) grouped within the mesophilic *Euryarchaeota*. These data add to the growing body of studies that demonstrate the wide distribution and diversity of *Archaea* in the environment and more specifically, in association with animal tissue.

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Introduction

The domain *Archaea* is comprised of two kingdoms, the *Euryarchaeota* and *Crenarchaeota*. Once thought to be only extreme thermophiles, studies have found mesophilic crenarchaeotes in temperate and polar marine waters where they may contribute up to 30% of the prokaryotic biomass (4). This group is now known to contain mesophilic members that are found in almost every niche that has been examined by analysis of 16S rDNA sequences, including terrestrial and freshwater environments (2,8). The range of this as yet uncultivated group extends into associations with marine animals. To date, nonthermophilic crenarchaeotes have been found as a symbiont of a marine sponge (*Axinella mexicana*), in the digestive system of a deep-sea holothurian, and in the intestinal tracts of two species of marine fish (6,7,9). This study investigated the diversity of *Crenarchaeota* among various local marine invertebrates of Woods Hole, MA using phylogenetic analysis of the 16S rRNA gene of isolated crenarchaeal clones.

Materials and Methods

Animal collection and organ harvesting

Adult, female squid (*Loligo peali*) were obtained from the Marine Resource Center at the Marine Biological Laboratory (MBL). Squid were anesthetized on ice followed by rapid decapitation. Ventral dissections were performed and the accessory nidamental glands (ANG) were removed. Squid ANGs were rinsed three times in filtered sterilized sea water (FSSW) and homogenized in 100 ul of FSSW on ice. Homogenized samples were either prepared for microscopy or DNA extraction (see below). Mussels (*Modiolus demissus*) were collected from Sippewisset salt marsh and brought back to the MBL where they were dissected. The gut tract (MG) was removed and rinsed in FSSW followed by preparation for microscopy and DNA extraction. Tunicates (*Styela clava*) obtained from the MBL dock were also dissected. The gut tract (TG) was removed and treated in a similar fashion as the mussel gut tract.

DNA extraction

Genomic DNA from the ANG, MG, and TG were isolated using a *UltraClean* Soil DNA kit (*MoBio*, Solana Beach, CA). See *MoBio UltraClean Soil DNA Isolation Kit* handout, MBL Microbial Diversity, 1999. Approximately 100 mg of homogenized tissue from each of the three sources was used. Isolated DNA was separated on a 1% agarose gel using gel electrophoresis and visualized with GelStar nucleic acid stain and UV illumination.

PCR amplification of 16S rDNA with crenarchaeal primers

PCR conditions included a master mix consisting of 2.5 mM MgCl₂, all four dNTPS (each at 1.25 mM), 15 pmol each of crenarchaeal 89 forward 5'-ACGGCTCAGTAACRC-3' and archaeal 915 reverse 5'-GTGCTCCCCGCCAATTCCT-3' primers, 5U / ul of Taq DNA polymerase and 0.01% BSA. Master mix was added to template DNA and sterile dH₂O to a final reaction volume of 25 ul. A temperature profile of 95 °C for 1 min, 61 °C for 1 min and 72

°C for 1 min was used for 25 cycles. A PCR reaction without any template DNA served as a negative control.

Cloning, Transformation, RFLP analysis and Sequence Analysis

Fresh PCR product was cloned with the Invitrogen TOPO TA cloning kit using the cloning and transformation protocols for Microbial Diversity, 1999. PCR product was cloned into a plasmid vector (pCR2.1) containing *lacZ* and ampicillin resistance genes. The cloned vectors were used to transform *E. coli* TOP10 competent cells supplied with the kit.

Transformants were grown on Luria-Bertani media containing 100 ug/ml of ampicillin and 40 ug/ml of X-gal at 37 °C overnight. Fourteen ANG and 20 MG transformants were randomly selected for restriction fragment length polymorphism (RFLP) analysis. Whole colony PCR was used to amplify the plasmid-borne 16S rDNA from the 34 transformants. For this step, procedure was followed according to course protocols except an annealing temperature of 57 °C was used. PCR product was digested with the restriction enzymes *HinP1* (10U/ul) and *Msp1* (20U/ul). Digests were separated on a 2.0% Metaphor agarose gel. Eight unique ANG patterns and 16 unique MG patterns were observed. The amplified PCR products from each of these transformants were subsequently sequenced and nine resulting sequences were analyzed using ARB sequence data software. A neighbor joining tree was generated.

Fluorescent in situ hybridization (FISH)

Samples from the ANG were prepared for FISH using the course protocol provided by Scott Dawson. Two crenarchaeal specific probes, a universal probe, and an archaeal probe were used. Probe was omitted from one sample to serve as a negative control. Samples were counter-stained with DAPI and viewed under UV fluorescence microscopy using filters for DAPI and rhodamine.

Results and Discussion

Microscopy

Microscopy revealed that each of the three environments analyzed were comprised of extensive and diverse microbial communities (Fig. 1-7). Previous studies with the squid ANG have shown that this organ houses a diverse array of bacteria with different morphologies (1, 3, 5). This study also observed similar cell morphologies (Fig. 2-3). In addition, groups of rods forming star shaped clusters were noted (Fig. 4). The identity of these cells remains unknown and may be worthy of further study. The mussel and tunicate guts yielded a number of bacterial and eukaryotic cell types (Fig. 5-7). This is not unexpected since these animals are filter feeders, and their digestive tracts may be areas where large numbers of undigested microorganisms are concentrated.

FISH

To understand the archaeal diversity in the ANG, fixed samples labeled with archaeal and crenarchaeal primers were examined by fluorescence microscopy. Problems occurred with excessive autofluorescence in all samples in one trial and autofluorescence in the negative control in another experiment. This excessive autofluorescence may be due to degraded paraformaldehyde or residual squid tissue which tends to fluoresce when excited by UV light (SVN, personal observation). Further optimization of hybridization and washing conditions and design of mesophilic crenarchaeal primers with broader specificity will most likely alleviate these problems.

Molecular Biology

Genomic DNA extraction from all three tissues was successful as judged by nucleic acids labeled with GelStar after gel electrophoresis (Fig 8). The MoBio Soil extraction kit proved very useful for extracting bacterial and archaeal DNA from animal tissue and may provide an alternative to the more traditional phenol extraction methods that have been used with eukaryotic samples. The crenarchaeal primers amplified an approximate 750-800 base-pair (bp)

fragment in samples from the ANG and the MG (Fig. 9). No amplified product was observed from the tunicate gut. This was surprising since *S. clava* is a filter feeder and would likely contain a representative portion of the microorganisms in the water column of Eel Pond. The mesophilic *Crenarchaeota* have been described as a cosmopolitan group from almost every marine environment that has been analyzed (4). However, the conditions in which these organisms will grow is unknown. Perhaps they can not survive in the tunicate gut.

Environmental DNA from Eel Pond should also have been extracted and used as a template to amplify *Crenarchaeota* 16S rDNA. PCR product from this source would serve as an indicator of the free-living diversity of crenarchaeal species in Eel Pond.

RFLP analysis yielded 8 unique digest patterns from the ANG and 16 patterns from the MG (Fig. 10). Nine out of these 24 clones were successfully sequenced. Sequence analysis using ARB software revealed that four of these clones grouped within the *Crenarchaeota* (m102, m13, and m11 from MG; m14 from ANG) while the other five grouped within the *Euryarchaeota* (m3, m5, m6, and m8 from MG; m15 from ANG) (Fig. 11). Clone m102 clustered with other marine mesophilic crenarchaeotes. Clones m11, m13 and m14 clustered with terrestrial mesophiles. However, a close unidentified crenarchaeote, EnvJTB173 was isolated from deep-sea sediment (L. Li, Deep Star Project, unpublished data). Clones m3, m5, m6, m8, and m15 grouped within a group of euryarchaeotes which include *Thermoplasma spp.*. This group, like the mesophilic crenarchaeotes, has been shown to be another widely distributed cosmopolitan group in marine environments (4). Therefore, the presence of these marine clones within the *Euryarchaeota* may be expected. Future work should include complete sequencing of the rRNA gene to better understand phylogenetic relationships between these clones.

Overall, these data support the hypothesis that members of the *Archaea* are found in association with marine invertebrates from Woods Hole, MA. In the case of the mussel, whether these archaea are symbionts or transients passing through the digestive system remains unknown.

To answer this, probes specific to the mussel clones could be made and used with FISH to visualize in what context these cells are in association with the animal tissue. Although the extent to which the squid ANG is open to the outside environment is unclear, it appears to be more of a closed system when compared to the mussel gut. The crenarchaeote and euryarchaeote clones may indicate that specific archaeal symbionts reside within the squid ANG. While the list of bacterial symbionts within the ANG is quite large (1, 4, 5), the extent to which the *Archaea* contribute to this symbiotic consortium is poorly understood. Using FISH, previous work from the Microbial Diversity course suggests that members of the *Archaea* are detectable within the ANG (1). This work represents the first phylogenetic analysis using 16S rDNA of putative archaeal symbionts within this organ.

Acknowledgments

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Literature Cited

1. Barbieri, E. and J. Gullledge. 1996. The accessory nidamental gland of *Loligo peali* and diversity of symbiotic bacteria. *Microbial Diversity*, MBL.
2. Buckley, D. H., J. R. Grabier, and T. M. Schmidt. 1998. Phylogenetic analysis of nonthermophilic members of the kingdom *Crenarchaeota* and their diversity and abundance in soils. *Appl. Environ. Microbiol.* 64: 4333-4339.
3. Chien, C. C. 1995. A study of microbiota in the accessory nidamental gland of the squid *Loligo peali*. *Microbial Diversity*, MBL.
4. DeLong, E. F., C. Schleper, R. Feldman and R. V. Swanson. 1999. Application of genomics for understanding the evolution of hyperthermophilic and nonthermophilic *Crenarchaeota*. *Biol. Bull.* 196: 363-366.
5. Ludek, Z. and D. Hughes. 1997. Microbial community of the egg capsule and accessory nidamental gland (ANG) of the squid *Loligo peali*. *Microbial Diversity*, MBL.
6. van der Maarel, M. J. E. C., R. R. E. Artz, R. Haanstra, and L. J. Forney. 1998. Association of marine Archaea with the digestive tracts of two marine fish species. *Appl. Environ. Microbiol.* 64: 2894-2898.
7. McInerney, J. O., M. Wilkinson, J. W. Patching, T. M. Embley and R. Powell. 1995. Recovery and phylogenetic analysis of novel archaeal rRNA sequences from a deep-sea deposit feeder. *Appl. Environ. Microbiol.* 61: 1646-1648.
8. Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science* 276: 734-740.
9. Preston, C. M., K. Y. Wu, T. F. Molinski and E. F. DeLong. 1996. A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. *Proc. Natl. Acad. Sci. USA* 93: 6241-6246.

Figure Legends

Figure 1. The accessory nidamental gland of a female squid (*Loligo peali*). The colored pigments are due to a consortium of bacterial symbionts.

Figure 2. The bacterial consortium of the ANG as viewed under phase-contrast microscopy.

Figure 3. Higher magnification phase-contrast light micrograph showing a wide range of cell morphologies among the symbionts found in the ANG.

Figure 4. Phase-contrast light micrograph showing rod-shaped bacteria forming star shaped clusters within the ANG.

Figure 5. Phase-contrast light micrograph showing the microorganisms comprising the gut of the tunicate *Styela clava*.

Figure 6. Light micrograph showing microorganisms within the gut of the mussel *Modiolus demissus*. Note the spirochete near the center of the figure.

Figure 7. Light micrographs of mussel gut contents showing both eukaryotic and prokaryotic components.

Figure 8. Agarose gel showing genomic DNA isolated from the squid ANG and TG (lanes 7 and 8 respectively). MG yielded similar results (data not shown). A 1 Kb ladder is shown in lane 12.

Figure 9. Agarose gel showing PCR product (approx. 800 bp fragment) using crenarchaeal primers. Lane 1 = 1 Kb ladder; lane 3 = negative control; lanes 4-7 contain 1:1000, 1:100, 1:10, and 1 ul respectively of TG template DNA; lanes 8-11 contain 1:1000, 1:100, 1:10 and 1 ul respectively of ANG template DNA; and lanes 12-15 contain 1:1000, 1:100, 1:10 and 1 ul respectively of MG template DNA.

Figure 10. RFLP analysis of 34 clone containing vectors of transformed *E. coli*. The nine clones that were successfully sequenced are indicated in red.

Figure 11. Phylogenetic tree based on partial archaeal 16S rDNA sequences obtained from the ANG and MG. The nine clones group within the *Crenarchaeota* and the *Euryarchaeota*.

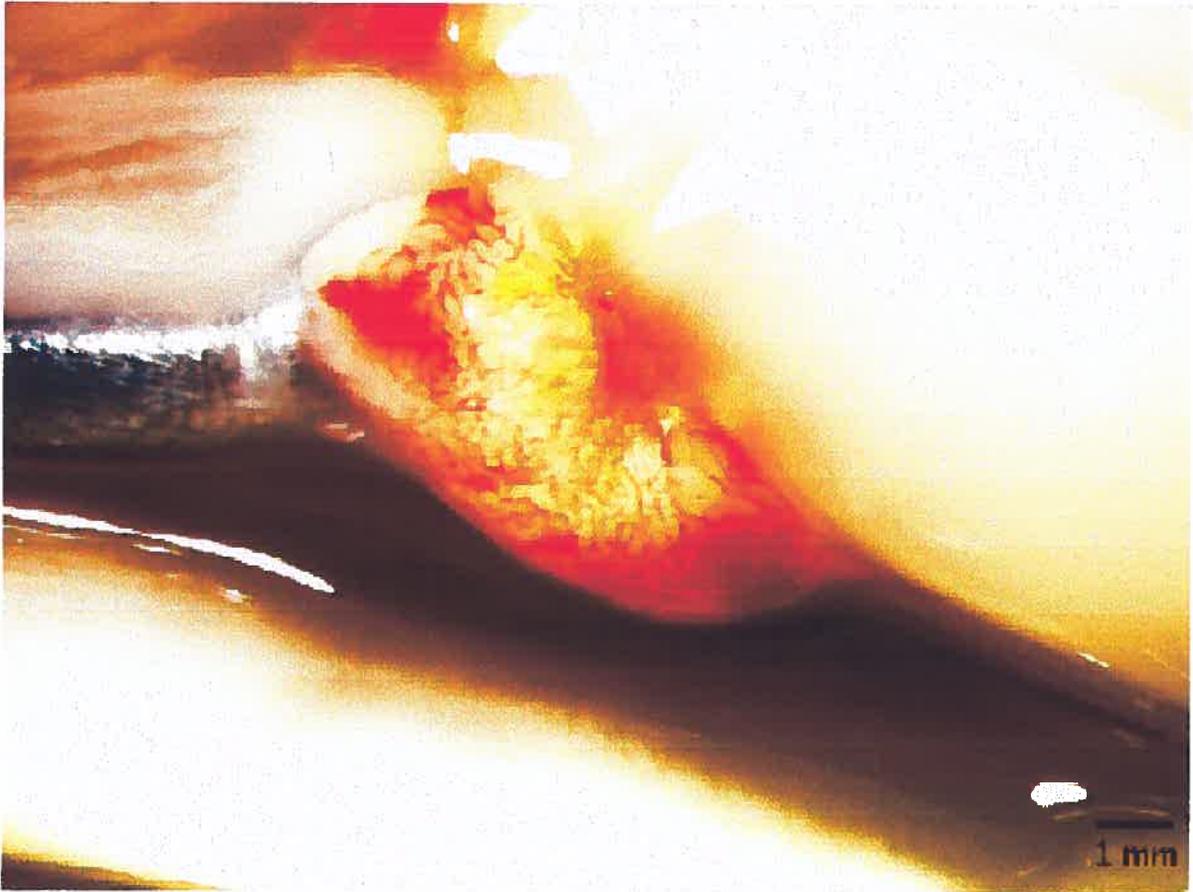


Figure 1

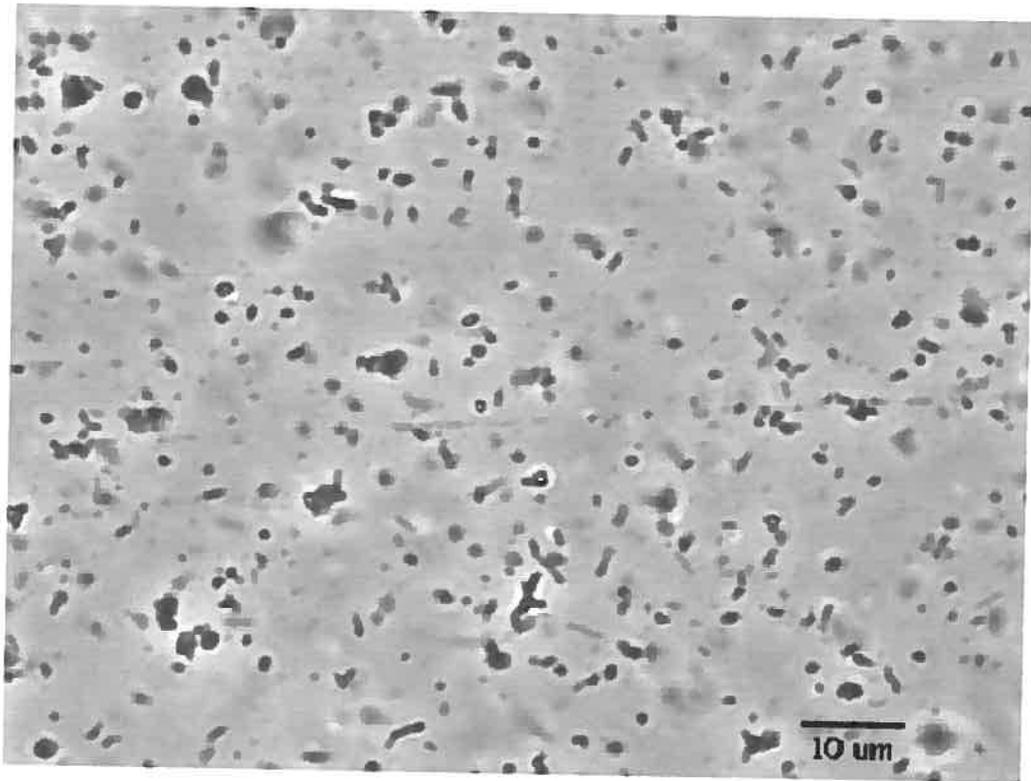


Figure 2

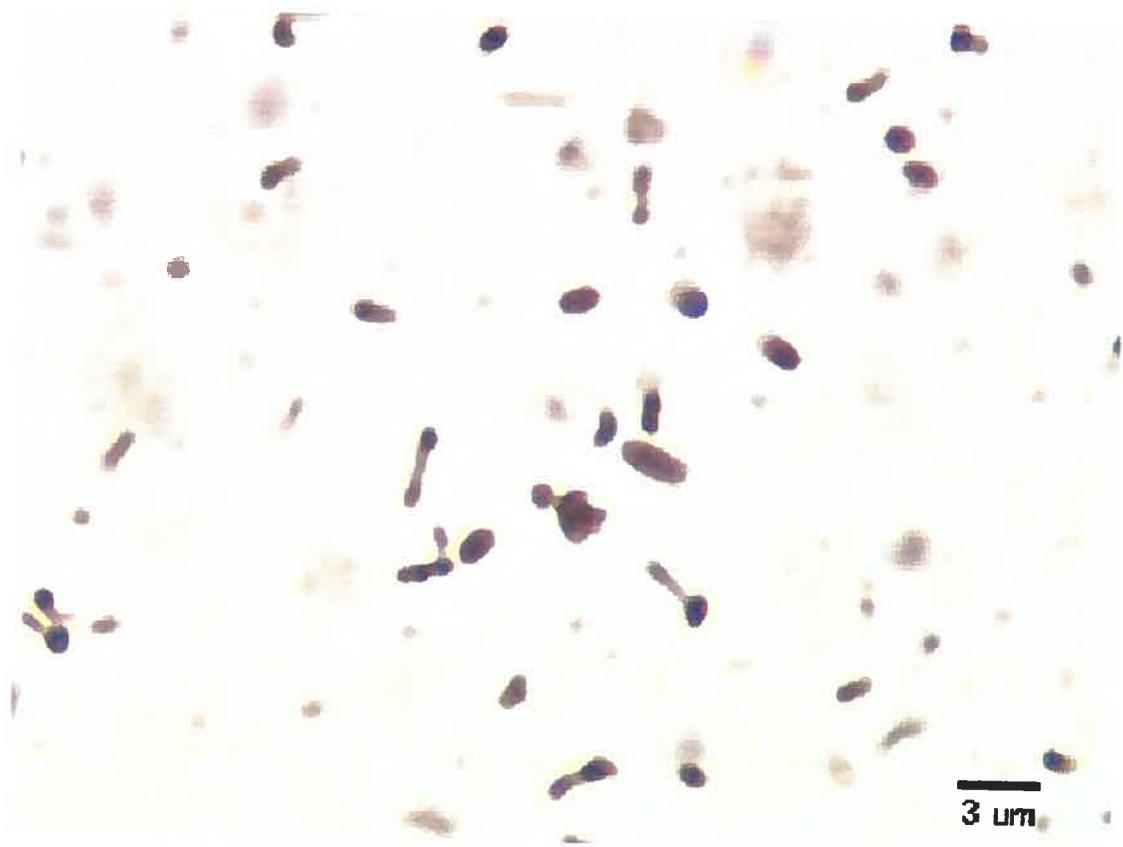


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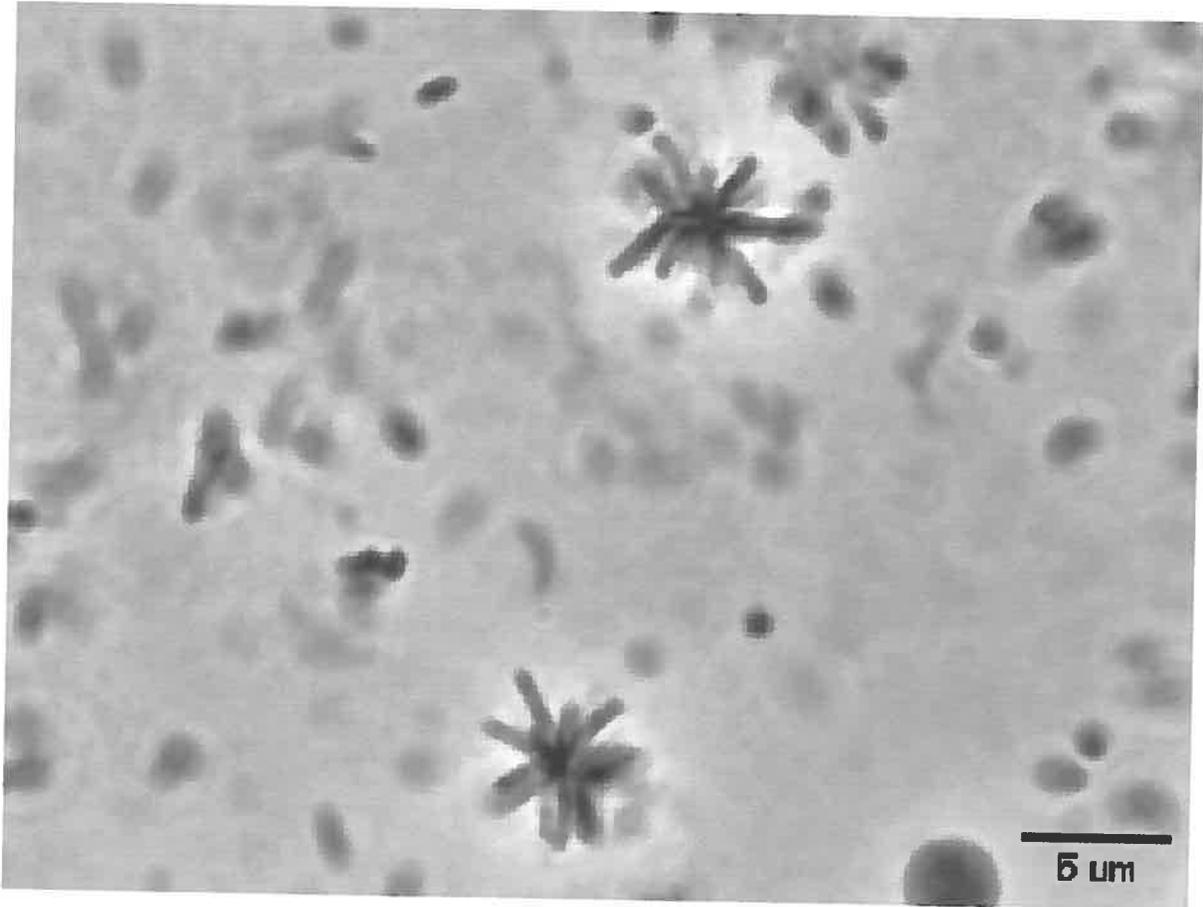


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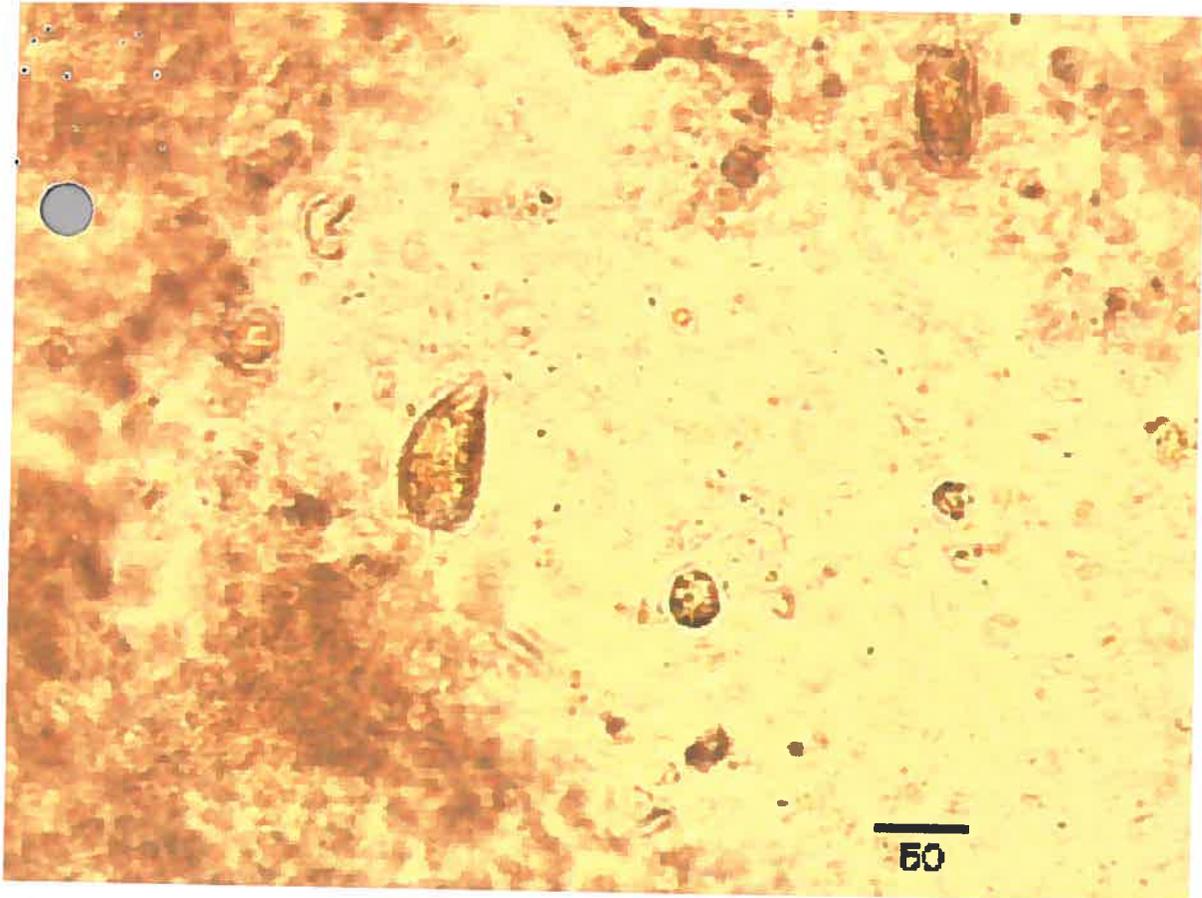


Figure 5



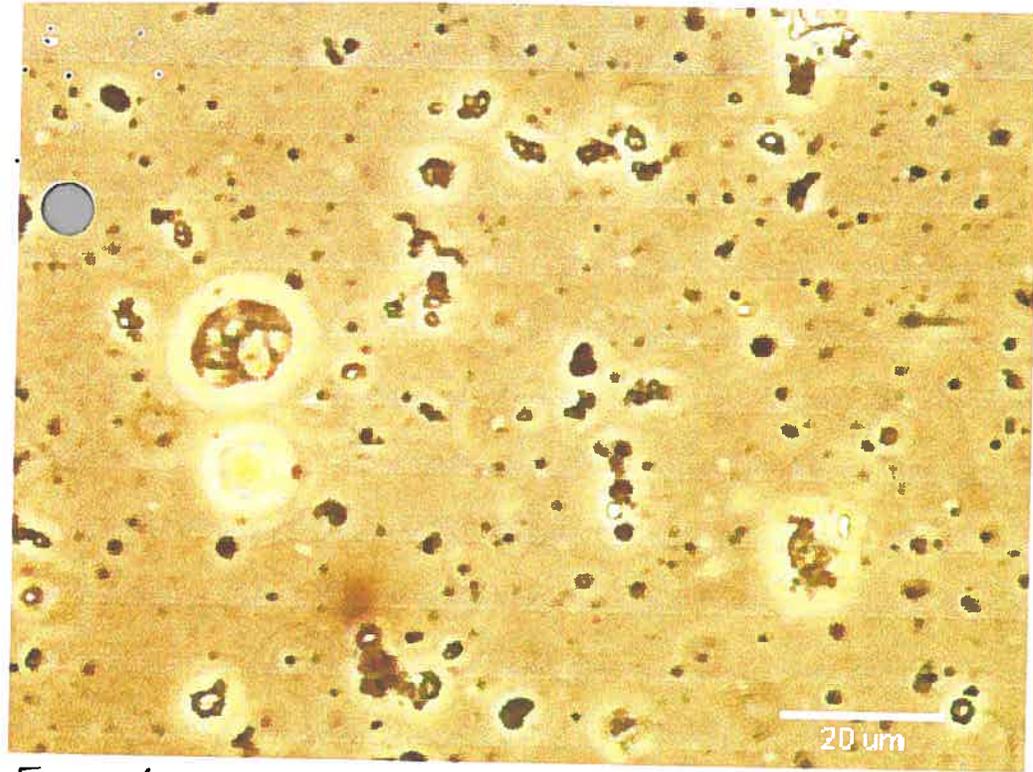


Figure 6



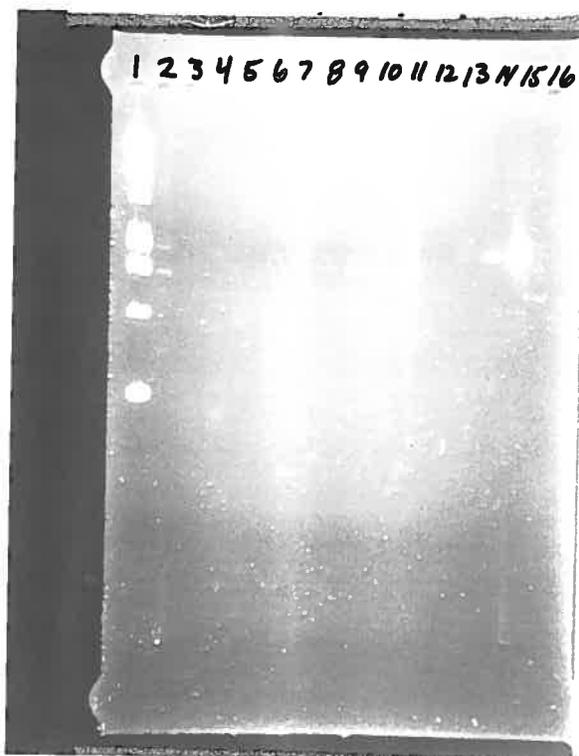


Figure 7



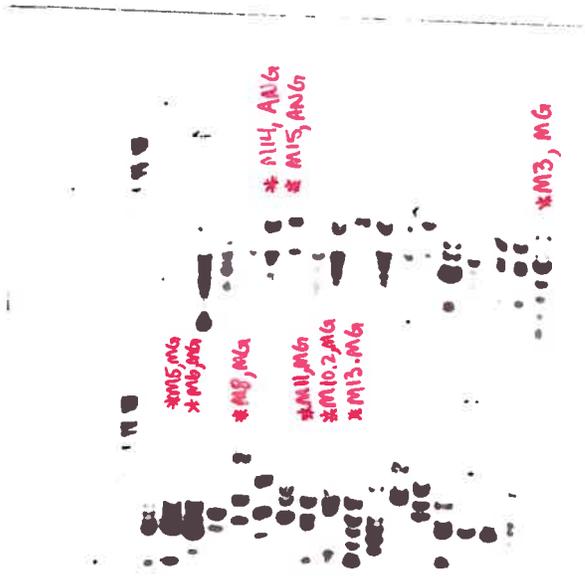
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Figure 8



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Figure 9

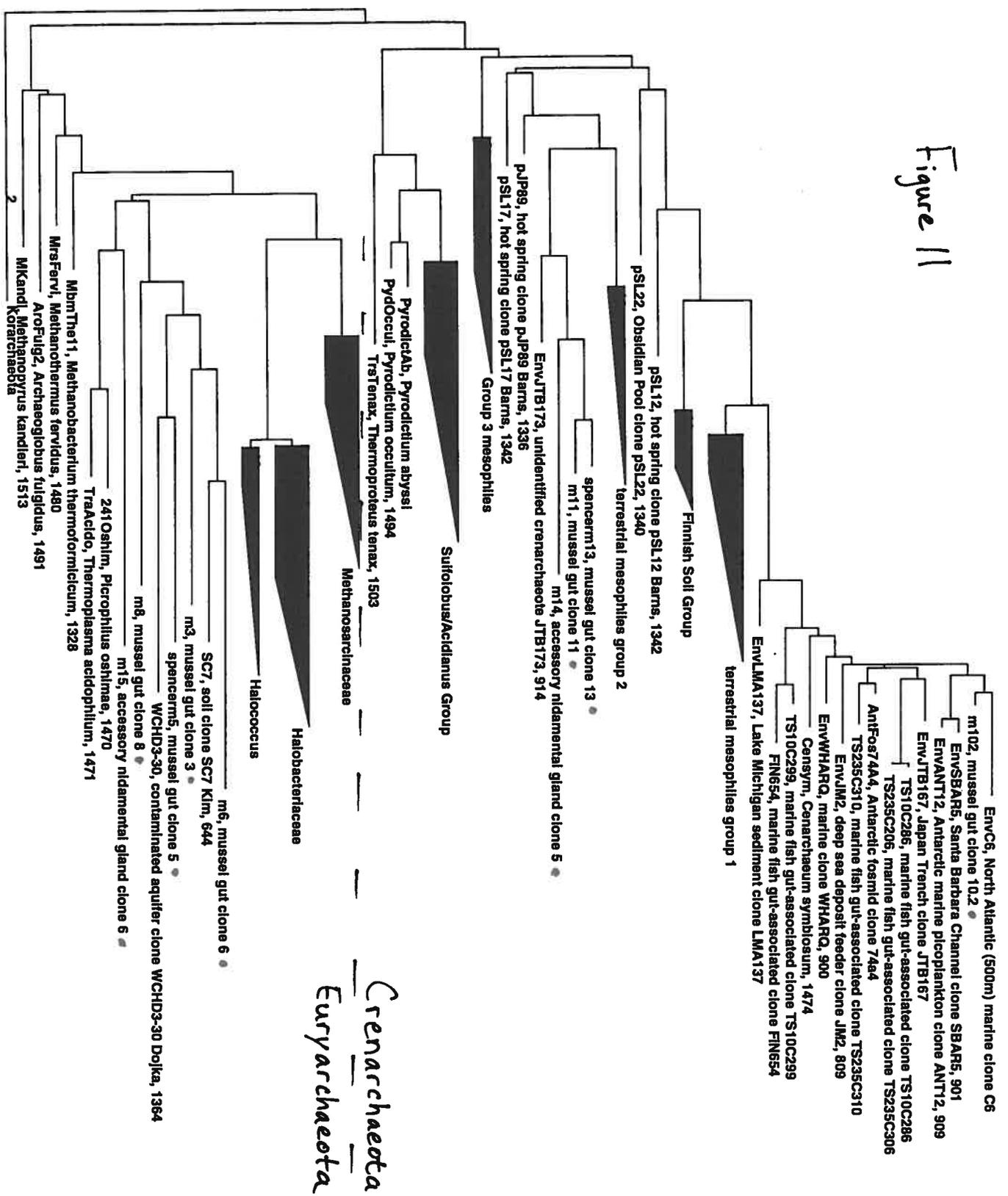


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Figure 10

Figure 11



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