

# **Morphological Characters and Molecular Analysis of Some Endolithic Green Algae**

Fidelia Okosisi ALIKA

Biology 533, Marine Algae, Summer 2012.

Contact Information:

Fidelia Okosisi ALIKA

Department of Plant Biology and Biotechnology,

Faculty of Life Sciences, University of Benin

P. M. B. 1154, Benin City, Nigeria.

[figwodo@yahoo.com](mailto:figwodo@yahoo.com)

Key words: Endoliths, Green algae, Strains, Morphology, Molecular.

# Morphological Characters and Molecular Analysis of Some Endolithic Green Algae

Fidelia Okosisi ALIKA

## Abstract

Morphological and molecular (ITS and TufA nuclear DNA) analysis was carried out on some endolithic green algal strains to examine their relationship and boring activity. Using the BLAST, the closest relative to most of the studied strains was the *Acrochaete* (= *Ulvella*) sp. Phylogenetic tree, maximum likelihood was used to compare study strains and some unpublished sequences obtained from O'Kelly Laboratory. It was observed from the maximum likelihood tree, that F442 and F446 strains have same evolutionary relationship which was supported by their similar morphological descriptions. The resulting clade of five (F190, F601, F646, F442/F446, F388/F408) sequences was distant from all other *Ulvella* sequences, suggesting a previously-unrecognized species group within *Ulvella*. Boring activity was not determined as the Scanning Electron Microscope (SEM) broke down during the study. Further research using several primers and phylogenetic trees analysis to confirm the appropriate identity of strains is recommended.

**Key words:** Endoliths, Green algae, Strains, Morphology, Molecular.

## Introduction

Endolithic organisms are diverse and comprised of a variety of fungi, cyanobacteria and eukaryotic macroalgae (green algae or Chlorophyta, red algae or Rhodophyta). They are found on/in a variety of calcium carbonated substrates, such as mollusk shells, limestone, loose carbonate sediment grains, glasses, calcareous algae and marine invertebrates, where they colonize fissures (chasmoendoliths), dwell in pores of substrates (cryptoendoliths), or actively bore and penetrate carbonate substrates (euendoliths) (Tribollet, 2008).

Euendoliths are distinguished from the cryptoendoliths and chasmoendoliths as the latter two dwell in their substrates with no dissolution action (Chacon *et al.*, 2006). The endoliths have various adaptative features, which allow them to live in shaded habitats; for example, endolithic algae in live corals absorbs far-red light effectively and have high

catalase activity which protects them from reactive oxygen species (Tribollet *et al.*, 2006).

Endoliths have far reaching ecological and environmental importance. First, they play a vital role in reef bioerosion where their boring activity dissolves the carbonate of the coral reefs, making them susceptible to abrasion and collapse (Tribollet, 2008). The collapse of such ecosystem affects other aquatic life that derives their nourishment, shelter and protection from them and the general aquatic ecosystem diversity (Gutierrez *et al.*, 2003). Second, euendoliths are relevant in paleontological studies, where they are used as proxies for differentiating photic and aphotic paleozones (Chacon *et al.*, 2006). Fossilized phototrophic eukaryotic algae and cyanobacteria are most common in the upper photic zone while the fungi communities dominated as light decreased and depth increased (Jeff, unpublished, 2011). In addition, they are food source for parrotfish and urchins (Tribollet, 2008). They are often pioneers in colonizing newly-exposed surfaces (Gutierrez *et al.*, 2003). Furthermore, they are used as bioindicators in assessing water quality (Al-Thuakir, 2002).

Species composition of euendoliths may be substrate specific and light dependant (Tribollet, 2008). Several works have been carried out on endolithic algae. Deng *et al.*, (2012) report that temperature variation had a remarkable influence in the thallus organization of *Acrochaete leptochaete* (Huber) Nielsen (Chaetophoraceae, Chlorophyta). In his study, Nielsen (1987) documents the taxonomy of some euendoliths among the blue green, green and red algae, and discovered three new green algal species, *Acrochaete endostraca* sp., nov., *Eugomonta stelligera* sp. nov., and *Ovillaria catenata*

gen. et sp. nov. Komarek and Anagnostidis (2005) publish a determination manual on endolithic cyanobacteria.

However, many of such works are largely based on morphological descriptions.

Nevertheless, in recent years, new species are being discovered with emergence of molecular tools. Molecular data has revolutionized taxonomy; showing convergence and divergence in genus and species. O' Kelly (per. comm., 2012) argues that molecular data is preferable to morphological characters as they may show similarity in gene relationship among species that may be morphologically plastic. Studies conducted for many years shown that tubular, *Enteromorpha* and blade, *Ulva* belong to the same genus, *Ulva* (Hayden et al. 2003). Lewis and McCourt (2004) claim that morphological and ultrastructural studies will still have a role in systematics in the future despite molecular tools as many morphological characters are yet to be discovered.

The aim of this study is to investigate the morphological characters and molecular data of some endolithic green macroalgae with the specific objectives to examine species similarities/variations and to observe the microboring activity of some species under controlled laboratory conditions.

## **2. Materials and methods**

### **2.1 Microscopy**

#### **2.1.1 Light microscope**

The general morphology (filament type, cell shape and size) of some specimens laboratory cultured (F190, F442, F446, F408, F188, F388, F514, F601a, F646, F550) was

observed, photographed and quantified under the light microscope, Nikon Eclipse E600 that is attached to a QImaging Micropublisher 5.0 RTV camera and computer.

### **2.1.2 Electron microscope: Shell Casting**

Oyster shells containing the cultured algal specimens were resin cast with a modification on the method developed by Golubric *et al.* (1970). Shell fragments were fixed in 1 % formalin solution for 15 minutes. Then, dehydrated in a graduated series of acetone solutions: 25 %, 50 %, 75 % and 100 %. Shell fragments were left in each concentration for at least 30 minutes. Thereafter, they were transferred to 50 % acetone and 50 % resin (EMBed 812) without hardener and left for 24 hours. Then these were placed in 100 % resin without hardener and allowed to stand for 24 hours. This allows the resin to infiltrate the microscopic boreholes. Fragments were embedded into 100 % resin with hardener and cure at 60<sup>0</sup>C for 24 hours.

Square sections were made with a drummel. These were transferred into concentrated hydrochloric acid (HCL), transferred into distilled water and then acetone. Sections were removed from acetone and allowed to dry and the inner surfaces of shells were glued to metal stubs. They were taken for scanning electron microscopy examination.

Unfortunately the machine broke down and this section of study was discontinued.

## **2.2 DNA extraction of the samples**

36 fresh specimens/strains (F187, F188, F189, F190, F191, F192, F193, F388, F408, F442, F446, F447, F471, F541, F550, F556, F581, F582, F593, F594, F597, F598, F599, F601, F604, F646, F648, F657, F674, F463, F464, F474, F522, F539, F540, F679) were randomly chosen from cultured samples, which were initially collected from

Massachusetts, Hawaii, California and Washington (See details in Table 1 in appendix).

The DNAs of strains were extracted using a commercial kit (UltraClean Plant DNA Isolation Kit, MO BIO Laboratories, Inc., Solana Beach, California, United States of America) following the manufacturer's instructions.

### 2.3 Amplification of DNA: PCR reaction

All DNA reactions were amplified using primer combinations (ITS-142R and 1762F; ITS1-265R and 1762R, TufA-1062R and 201F. See Table 1) (Fama *et al.*, 2002; Hayden *et al.* 2003; O'Kelly *et al.*, 2010; Saunders and Kucera, 2010). Each PCR tube contained the following: 25.0 IL TopTaq Mater mix (Bioline, Taunton, MA, USA), 21.0 IL Nuclease free water, 2.0 IL each primer (0.4 mM), and 2.0 IL extracted/template DNA.

	Quantity	ITS	ITS1	TufA
Primer 2	1 µl	142R	265R	1062R
Primer 1	1 µl	1762F	1762R	201F

Source: modified from Fama *et al.* (2002) and Hayden *et al.* (2003).

Primer solutions (1762F, 142R, 265R, 201F) and template (extracted DNA) were thaw to room temperature and mixed well before use. 25 µl tag, 21 µl ddH<sub>2</sub>O, 1 µl primer 1, 1 µl primer 2 were mixed. Vortex briefly and transferred to labeled PCR tubes. Then 2 µl of each extracted DNA was put into appropriated PCR tubes. Centrifuge briefly for 30 sec at 10 × 1000 g. PCR tubes were placed into the PCR machine, Whatman Biometra T3 thermocycler and operated according to manufacturer's instruction with a modification made in annealing temperature (47°C) for 30 cycles.

## 2. 4 Agarose gel electrophoresis

Agarose gel electrophoresis was employed for size separation of the PCR products. The size of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contain DNA fragments of known size run on the gel alongside the PCR products.

1.5 % (0.525 g) of agarose was weighed and placed into a conical flask and mixed with 35 ml of  $1 \times$  TBE buffer (this was prepared by adding 10 ml AccuGene  $10 \times$  TBE buffer and 90 ml water). The conical flask with content was microwave for a few seconds (60-120 sec.) until the solution was clear. Allow to cool to a level that one does not need a towel to hold the flask. The content in the flask was poured into a gel casting mold with gel combs placed (one at the middle and the other at the left edge) and allowed to solidify.

$1 \times$  TBE buffer was poured into gel rack or electrophoresis chamber until it was half filled. Gel with wells is transferred into the gel rack. 1 % (1  $\mu$ l of dye, SybrSafe was mixed in 99  $\mu$ l of distilled water. This is done in the dark as the dye is light sensitive) of dye, SybrSafe was placed on a parafilm and mixed with 2  $\mu$ l loading buffer/dye (bromophenol blue (Sigma, B8026), sucrose and water). The loading gel adds colour to the sample and because they are negatively charged, they move in the same direction as DNA during electrophoresis and makes it easy to monitor progress of the gel.

9  $\mu$ l of DNA ladder (Hi-low DNA marker) was mixed with 1  $\mu$ l SybrSafe and put in the first wells of gel on both sides. 5  $\mu$ l of each extracted DNA is mixed with the Sybrsafe containing the loading gel and transferred to subsequent wells. These were electrophoresed

at 110V and allowed to run for 30 mins. The gel was removed and analysed in a Kodak EDAS 290 Transilluminator UVP attached to a camera with imaging software, Kodak Gel Camera Imaging to view the separation and weight of bands.

## **2.5 DNA Sequencing and construction of phylogenetic tree**

DNA products and pictures of gels were sent to a company, GENEWIZ for sequencing. Results obtained were assembled using the software, Sequencer, aligned (using Cluster Muscle software) and edited. Sequences were then submitted to BLAST search engine (National Center for Biotechnology Information, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) for initial phylogenetic matching. Some sequences obtained by O'Kelly on his ongoing research were also used to match them and phylogenetic tree was constructed using maximum likelihood routine of MEGA5.

### 3. Results

#### 3.1 Morphological Analysis

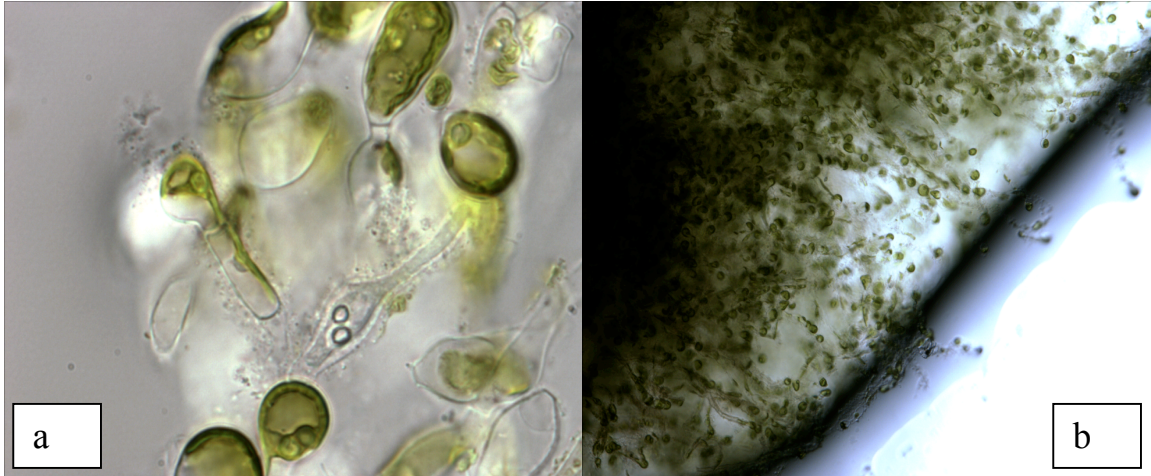


Fig. 1: F189 strains (a) Cell shape (b) filaments

Description: Cells are more spherical than cylindrical and contain big pyrenoids (1 or 2) in each cell. Cultured at 23 °C. Cell dimension: Length (8.09 – 14.98  $\mu\text{m}$ ), Width = 1.91 – 9.86  $\mu\text{m}$ . Mag.  $\times 10 \times 100$  (oil immersion).

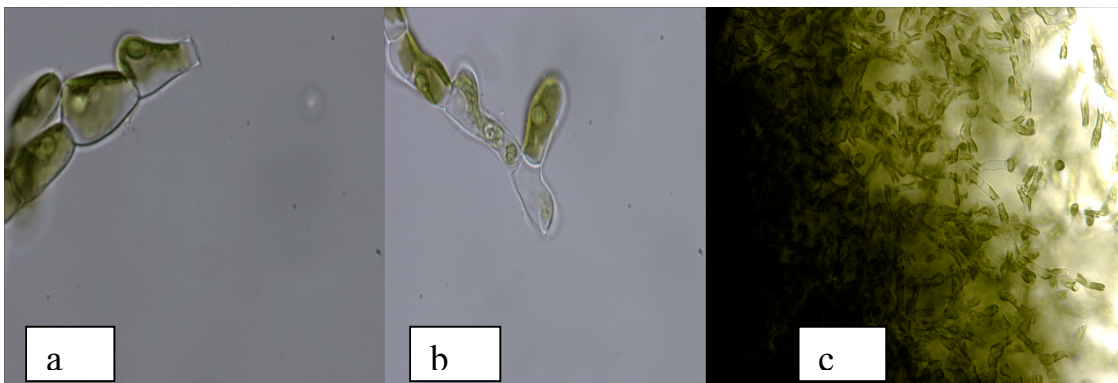


Fig. 2: F190 strains (a) and (b) Cell shape (c) filaments

Description: The cells are bigger and elongated/cylindrical with 1 big pyrenoid in each cell. Cultivated at 23°C. Cell dimension: Length (7.38- 29.99  $\mu\text{m}$ ), Width = 2.997 – 11.67  $\mu\text{m}$ . Mag. x 10 x 100.



Fig. 3: F442 strains (a) and (b) Cell shape (c) filaments

Description: Elongated/Cylindrical cells with big pyrenoid. Grown at 15 °C (16:8 light-dark) at constant light intensity of 20  $\mu\text{Em}^{-2}\text{s}^{-1}$ . Cell dimension: Length (9.58 – 12.74  $\mu\text{m}$ ), Width (2.76 – 3.44  $\mu\text{m}$ ). Mag. x 10 x 100.

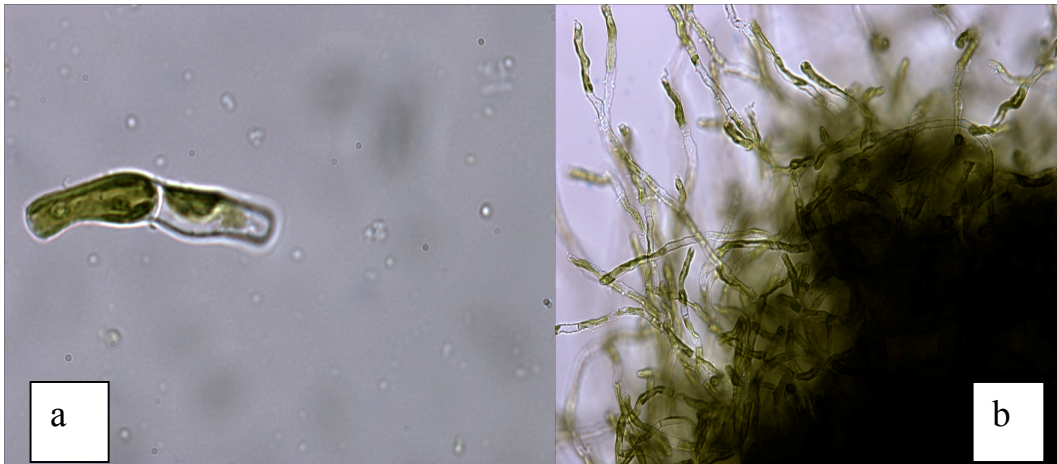


Fig. 4: F446 strains (a) cell shape (c) filaments

Description: Elongated/Cylindrical cells with big pyrenoid. Grown at 15 °C (16:8 light-dark) at constant light intensity of  $20 \text{ uEm}^{-2}\text{s}^{-1}$ . Cell dimension: Length (8.43 – 17.26  $\mu\text{m}$ ), Width (2.93 – 3.62  $\mu\text{m}$ ). Mag. x 10 x 100.

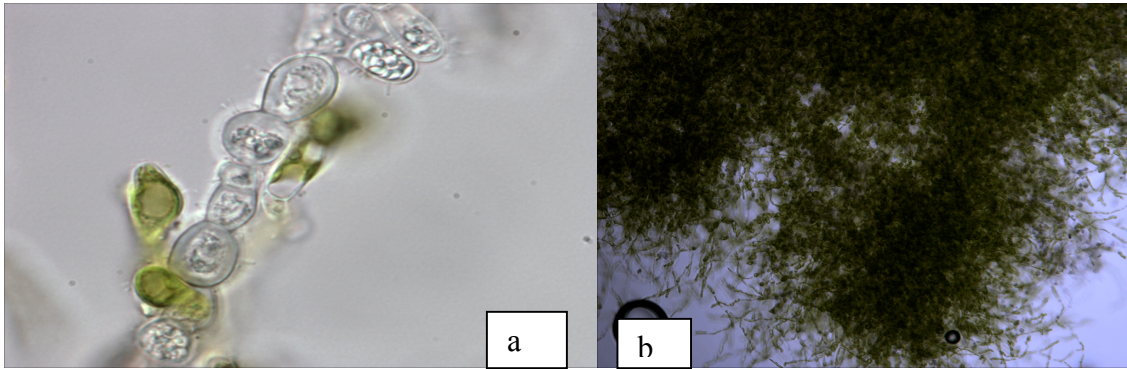


Fig. 5: F514 strains (a) cell shape (c) filaments

Description: Has both elongated/Cylindrical cells and nearly isodiametrical spherical cells with clusters of gypsum crystals. Grown at 23 °C (12:12 light-dark) at constant light intensity of  $20 \text{ uEm}^{-2}\text{s}^{-1}$ . Cell dimension: Length (3.96 – 11.21  $\mu\text{m}$ ), Width (5.01 – 9.74  $\mu\text{m}$ ). Mag. x 10 x 100.

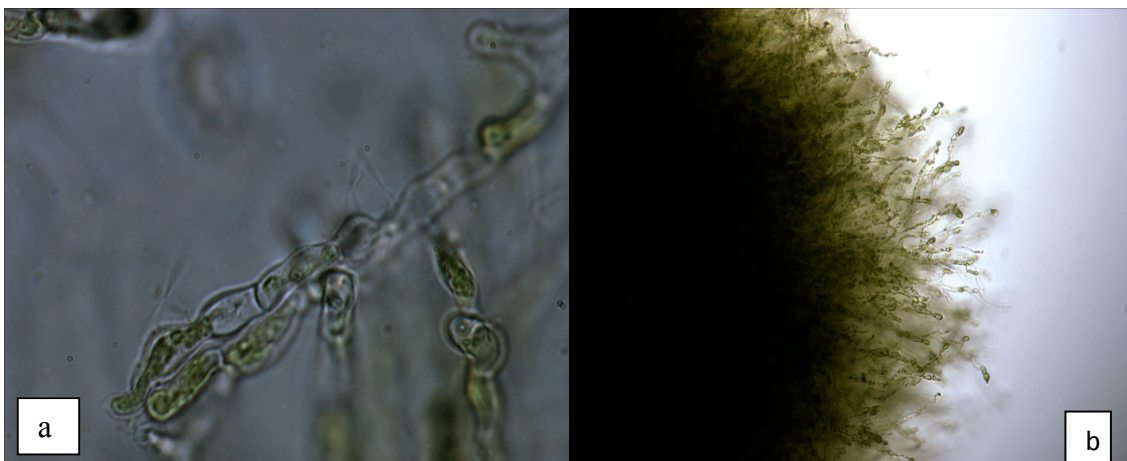


Fig. 5: F550 strains (a) cell shape (c) filaments

Description: Has both elongated/Cylindrical cells and spherical cells with pyrenoids.

Grown at 23 °C (12:12 light-dark) at constant light intensity of 20  $\mu\text{Em}^{-2}\text{s}^{-1}$ . Cell

dimension: Length (5.80 – 15.04  $\mu\text{m}$ ), Width (3.34 – 9.11  $\mu\text{m}$ ). Mag. x 10 x 100.

### 3.2 Molecular Analysis

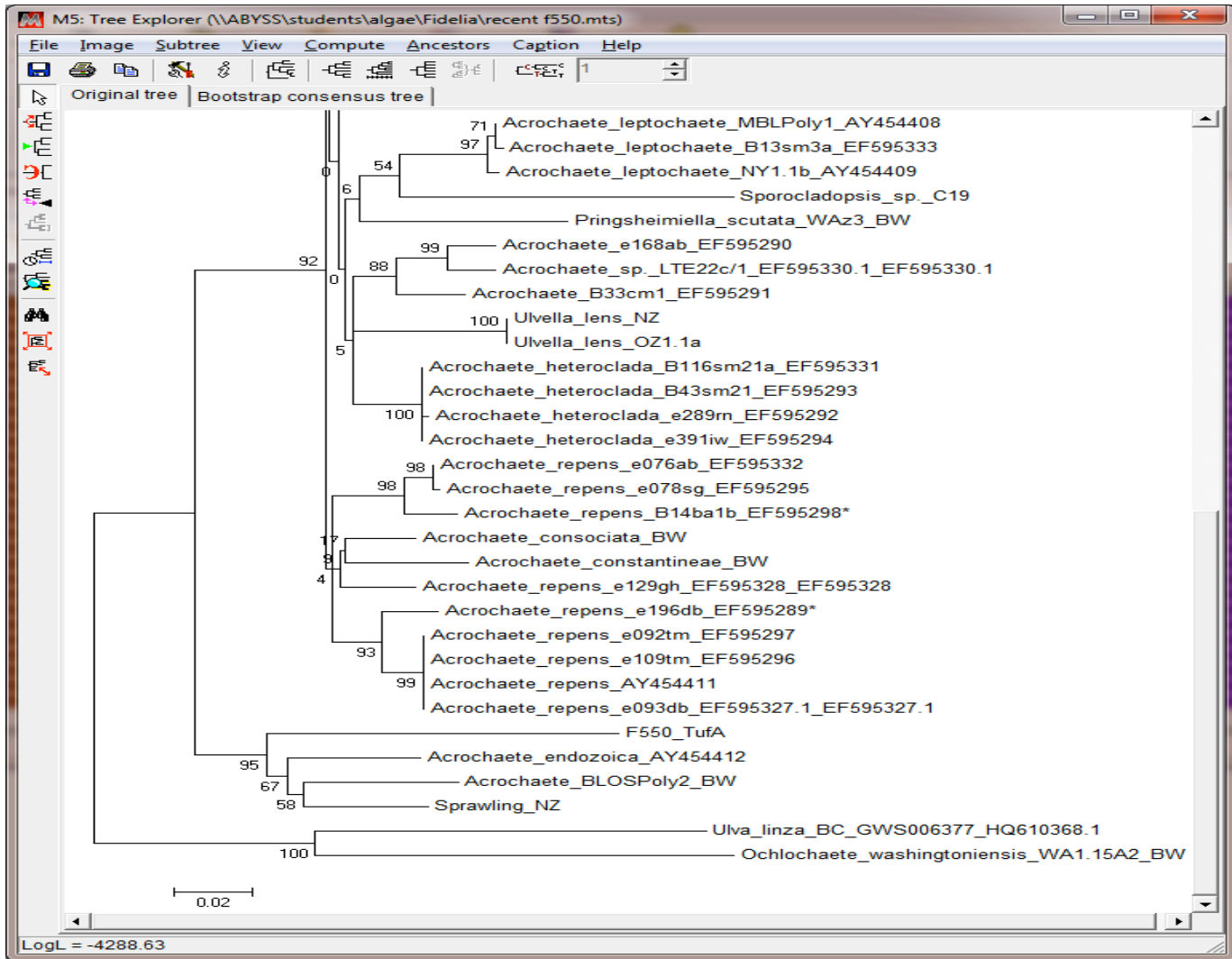


Fig. 6. Maximum-likelihood (ML) tree of F550 TufA sequences. (scale at bottom).

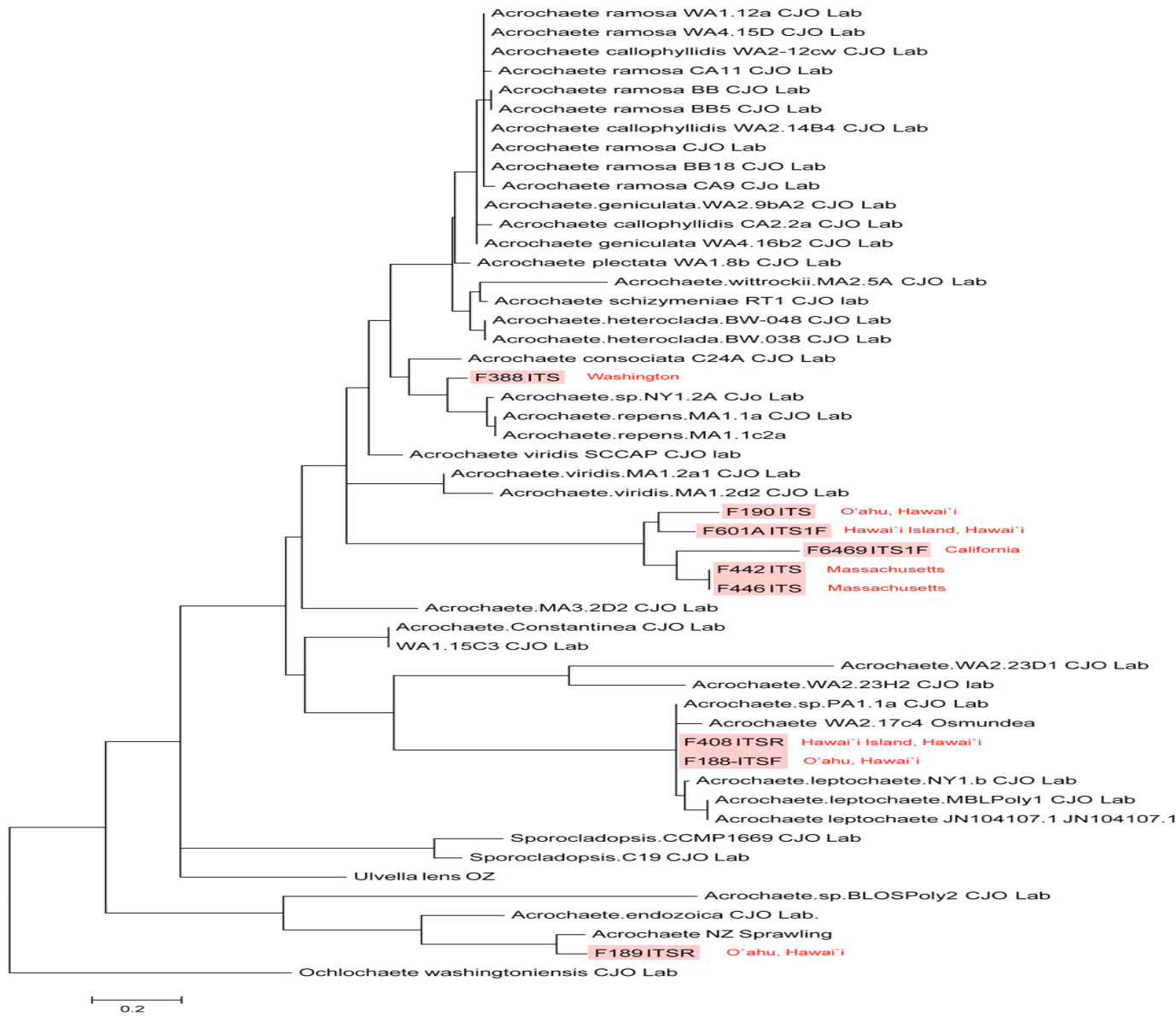


Fig.7. Maximum-likelihood (ML) tree of some strains internal transcribe spacers (ITS) sequences. (scale at bottom).

Using the nucleotide BLAST (GenBank), F190 sequence (>F190\_ITS

TATTAAACCATCAGCGCAAGAAACCAGCTACAAAAACAGAACTTATTCGAG  
CTGAAGCAGCCACATTCCCCGCAGCTGTCCGCAATAGGTTTACCCGGCCAGG  
AGGGATCATTGAAACCGATCAAACCGGACCTCCCGAGACCGAGGGCGCCCC  
GACTCTGGCTGAAAAAAGATGCGCTCCGACGCCTCAGCCAGTCGGATTATTT  
TTCCACCCACGACAAACCTCTGACCTGAACCTACTCTGCAGGCACCGCCTGC  
GGACTGAATCAGATAACTCTGAACAACGGATATCTTGGCTCTCGCAACGATG  
AAGAACGCAGCGAATGCGATAGTAGTGTGAATTGCAGAATTCCGTGAATCAT  
CGAATCCCGCCCAGGGAGCTGGACCTGGCCCCCKGYGGATCTYYMCGCC  
GGRCCGGCTGAAAGGCAGAGMTMCCGCACGGACCCAGAGCGGCCGCGGTTA  
GGTAGGTAGCTCGCTACTRCTAACC GGAGCCGRRCMCRGCRGRCASMYKAA  
ACRCTACCCGCTGAACTTAA) appeared to match with *Enteromorpha intestinalis*

only at a conserved region of the 5.8S gene.

The sequence of a 75 bp fragment of the ITS1 gene from *Ulva* strain F674 (which O'Kelly assembled and annotated was too short for phylogenetic analysis (>F674

AAACCGATCAAACCAATCACAGAGCACCTGCGGGCGCTCACCTCGCCCCTCA  
CGGGGCGGGGTGGGGCGGTCCGC), but in a BLAST search it is 99% similar to an undescribed *Ulva* species from Humboldt County, California, GenBank sequence number AY422520.

## Discussion

From the results, it appears there are some similarities between strains/specimens. F442 and F446 have elongated/cylindrical cells with big pyrenoids and both are grown at 15 °C. This is supported by the maximum likelihood tree that place them in the same clade and from the alignment, it appears F442 ITS is the same as F446 ITS.

The F190 sequence does not have any close matches to sequences in GenBank; the closest matches in BLAST for the entire sequence are to sequences assigned to *Acrochaete* (= *Ulvella*) or to sequences assigned to the red algal genus *Laurencia* which (O'Kelly unpublished) actually represent *Ulvella* endophytes within the *Laurencia* thalli.

The match to *U. intestinalis* is to a conserved region of the 5.8S gene, and consequently this match will be to most if not all *Ulva* species. That the BLAST program only selected the *U. intestinalis* sequence is one of the many artifacts inherent in BLAST.

Phylogentic trees are without problems; for example, depending on how the sequences are computed, different relationships may be obtained which may contradict morphological observations. Nevertheless, phylogenetic trees, which include unpublished sequences from the O'Kelly laboratory (they are not in GenBank), give a more robust view of the relationships of the F190 sequence. It clusters with four other new (F190, F601, F646, F442/F446,) *Ulvella* sequences in the tree. The resulting clades of five (F190, F601, F646, F442/F446, F388/F408) sequences is distant from all other *Ulvella* sequences thus far recorded; this result suggests that these five sequences, representing four strains, constitute a previously-unrecognized species group within *Ulvella*.

## **Recommendation**

For a conclusive argument to be made on phylogenetic relationship (similarities and differences), it is recommended that further research be carried out on these species using different primer types, a complete analysis (both ITS and TufA) and different phylogenetic trees (distance, maximum parsimony, maximum likelihood and neighbor joining) be conducted for all strains.

## **Acknowledgement**

I am indeed grateful to Friday Harbor Laboratories and the University of Washington for the fellowship granted to me to attend the summer 2012 Marine Algae course. Thanks are also due to my tutors, Bob Waaland, Tom Mumford and the teaching assistant, Kathy Dobkowski. My sincere gratitude goes out to my mentor, Charles O'Kelly and his research technologist, Geneva Mottet. I appreciate the hospitality expressed by my course mates, other colleagues and staff of Friday Harbor Laboratories during my stay. I will like to acknowledge the University of Benin, Nigeria for the study leave granted to me.

## References

- Al-Thuakir, A. A. (2002). Effect of oil pollution on euendolithic cyanobacteria of the Arabian Gulf. *Environmental Microbiology*, 4(2): 125-129.
- Chacon, E., Berrendero, E., Pichel, F. G. (2006). Biogeological signatures of microboring cyanobacterial communities in marine carbonates from Cabo Rojo, Puerto Rico. *Sedimentary Geology*, 185: 215-228.
- Deng, Y., Tang, X., Huang, B., Teng, L., Ding, L. (2012). Molecular identification and culture observation on *Acrochaete leptochaete* (Chaetophoraceae, Chlorophyta) from China.
- Famá, P., Wysor, B., Kooistra, W. H. C. F. & Zuccarello, G. C. 2002. Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tufA* gene. *J. Phycol.* 38:1040–50.
- Golubric, S., Brent, G. and Le Campion-Alsumard, T. (1970). Scanning electron microscopy of endolithic algae and fungi using a multipurpose casting embedding technique. *Lethaia*, 3: 203-209.
- Gutiérrez, J. L., Jones, C. G., Strayer, D. L., & Iribarne, O. O. (2003). Mollusks as Ecosystem Engineers: The Role of Shell Production in Aquatic Habitats. *Oikos*, 101(1), 79-90.
- Hayden, H.S., Blomster, J., Maggs, C.A. Silva, P.C. Stanhope, M.J. and Waaland, J. R. (2003). Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct evolutionary entities. *European Journal of Phycology*, 38: 277-294.
- Jeff, J. (2011). Survey of shell boring microorganisms across a depth gradient at Point Caution, on San Juan Island, WA. ZooBot Research Apprenticeship.

- Komárek, J., & Anagnostidis, K. (2005). *Cyanoprokaryota. 2.Teil: Oscillatoriales*.  
Munich, Germany, Elsevier GmbH, 759 pp.
- Lewis, L. A. and R. M. McCourt (2004). Green algae and the origin of land plants.  
*American Journal of Botany*, 91: 1535-1556.
- Nielsen, R. (1987). Marine algae within calcareous shells from New Zealand. *New Zealand Journal of Botany*, 25: 425-438.
- O'Kelly, C. Kurihara, A., Shipley, T.C. and Sherwood, A. (2010). Molecular Assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian Islands. *Journal of Phycology*, 46: 728-735.
- Tribollet, A., Langdon, C., Golubic, S., Atkinson, M. (2006). Endolithic microflora are the major primary producers in dead carbonate substrates of Hawaiian coral reefs. *Journal of Phycologia*, 42: 292-303.
- Tribollet, A. (2008). The boring microflora in modern coral reef ecosystems: a review of its roles. In: Wisshak M., Tapanila, L. (2007). *Current Developments in Bioerosion*, Springer-Verlag, Berlin.
- Saunders, G. A., Kucera, H. (2010). An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. *Cryptogamie Algologie* 31: 487-528.