Diversity of Endophytes in Various Plants from Woods Hole, MA

By

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Introduction

Various plants from different environments around Woods Hole, MA were assessed for biodiversity endophytic symbionts. Endophytes are microorganisms (mainly fungi and bacteria) that inhabit tissues of plants and cause asymptomatic infections (Wilson, 1995). In a recent review, it has been summarized that endophytes can promote plant growth by a number of different mechanisms. These organisms are capable of solubilizing phosphate, producing indole acetic acid, vitamins, and nitrogen metabolism (Ryan et al., 2008).

The main interest in this study was to observe the endophytic biodiversity and if endophytic communities of plants differ according to the environments where the hosts are located. It is hypothesized that different endophytic organisms will be found when comparing the plant hosts from different environments.

The grass *Spartina alterniflora* was studied from the Little Sippewissett salt marsh (Fig. 1). This species of grass is the dominant vegetation of salt marsh sites along the east coast of North America and most abundant on the east of the United States. They can also be found along marshes in Western Europe. In Massachusetts alone, this grass covers approximately 7,940 acres (Teal, 1986).

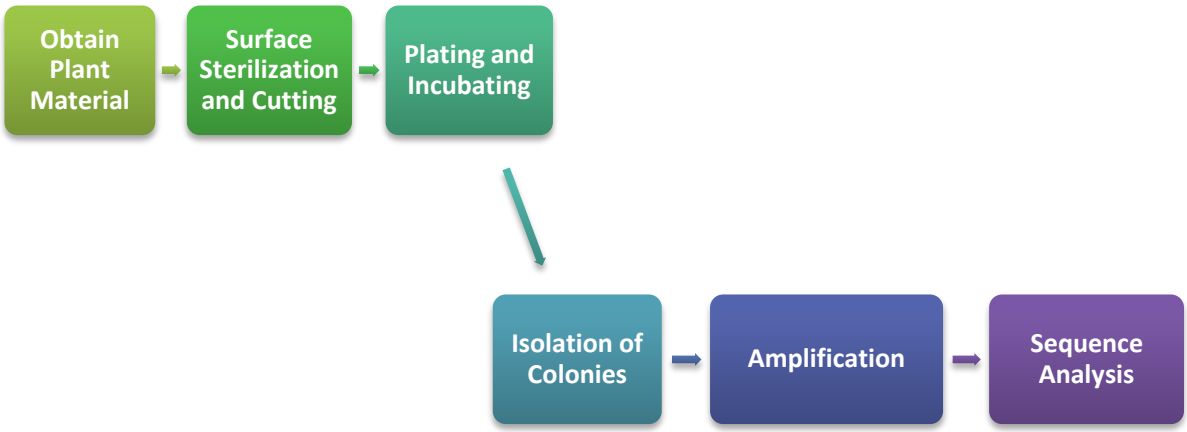
The sea algae *Ulva lactuca* was sampled from Woods Neck Beach. According to the World Register of Marine Species, this alga is widely distributed around the world. It can be found in the Northeast, Northwest, and around Long Island Sound in the United States. It can also be found in Europe and Africa. This alga grows the open coast attached to hard surfaces such as rocks or in sandy muddy areas if there is not much tidal movement.

Fig. 1 *Spartina alterniflora* grass.

Ulva lactuca

Materials and Methods

A diagram for the general procedure for the culture dependent approach is shown below.



A culture independent approach was also attempted with the leaves of *Ulva lactuca* and *Spartina alterniflora.* A diagram for the general procedure is shown below.

Sampling. Three different plants were sampled for this project. One leaf from each of three plants from the same species was studied from each site. The specimens were observed to be healthy and mature plants. Various grass samples of *Spartina alterniflora* were obtained from superficial pools where purple sulfur bacteria are found at Little Sippewisset Salt Marsh. These grass samples were sampled along with their roots. The plants with the most intact roots were chosen for the study. Another plant that was studied for the presence of endophytes was the bright green seaweed *Ulva lactuca* which was sampled from rocks at the bottom of Woods Neck Beach. A third sampling of leaves was performed on the Maple tree located next to the Swope Building. Seaweed samples were transported in ziplock bags containing seawater. The other samples from *Spartina alterniflora* and Maple were also stored individually in ziplock bags. In the case that they could not be immediately processed, the leaves were placed at 4˚C in order to stop decaying of the leaf which would cause saprophytes to be isolated. All samples were processed within 48 hours of being sampled.

Surface Sterilization and Tissue Processing. Leaves and roots were washed with distilled water to remove excess debris. Once washed they were surface sterilized to eliminate epiphytes. Leaves were immersed in 70% ethanol for 1 minute, 10% NaOCl for 3 minutes, and 70% ethanol for 30 seconds. Finally, the leaves were washed three times with sterile water to wash away the remaining alcohol and NaOCl. Negative controls were prepared using the water from the last wash in order to determine if the surface sterilization technique was successful. The plant material was then placed on a sterile Petri dish where pieces were cut using a scalpel following proper aseptic techniques. Leaves were cut into pieces that were approximately 3mm x 3mm in size. Roots fragments were approximately 3mm in length.

Endophyte Cultures. Surface sterilized leaf fragments and root fragments of *Spartina alterniflora* were inoculated from each site into nitrogen-free media (NFM) and 50% tryptic soy agar (TSA) plates. The samples that came from *Ulva lactuca* were inoculated onto Marine Agar (MA). Marine Agar has been previously used in other successful studies for the isolation of endophytes of a seagrass (Couto-Rodríguez, 2009). The leaf fragments from Maple were inoculated onto 50% TSA and NFM. The plates were incubated at 30˚C. Once endophytes colonies were observed growing from the edges of the leaf fragments these were isolated to a pure culture. Those endophytes which grew on NFM were isolated again on NFM in order to make sure that they were capable of fixing nitrogen. Negative controls were prepared by placing 100µL of the water used in the last wash on media. 50% Potato dextrose agar media (PDA) was prepared in order to isolate and grow fungal colonies.

Community Analysis. A community analysis of the bacterial endophytes present in some samples was attempted. DNA was extracted directly from leaf pieces that had been previously surface sterilized following the instructions of the MOBIO PowerSoil® DNA Isolation Kit. Bacterial 16S rRNA genes were amplified using the 8F and 1492R primers. Once the appropriate sized band was observed on a 1% agarose gel, it was excised and DNA was recovered using the Millipore Gel DNA Extraction Kit.

DNA Extractions from Isolates. DNA extraction from each isolate was performed. These were carried out by taking a single colony and placing it in a PCR tube with 75-100µl of sterile water. They were placed in the thermal cycler where they were boiled for 10-15 minutes.

DNA Amplification. Isolates were identified based on their sequences for the 16S rRNA gene. The primers 8F and 1492R were used for the amplification. In order to amplify fungal colonies, the primers ITS1/ITS4 were used (White et al., 1990). Finally the PCR products were visualized in 1% agarose gels.

Results

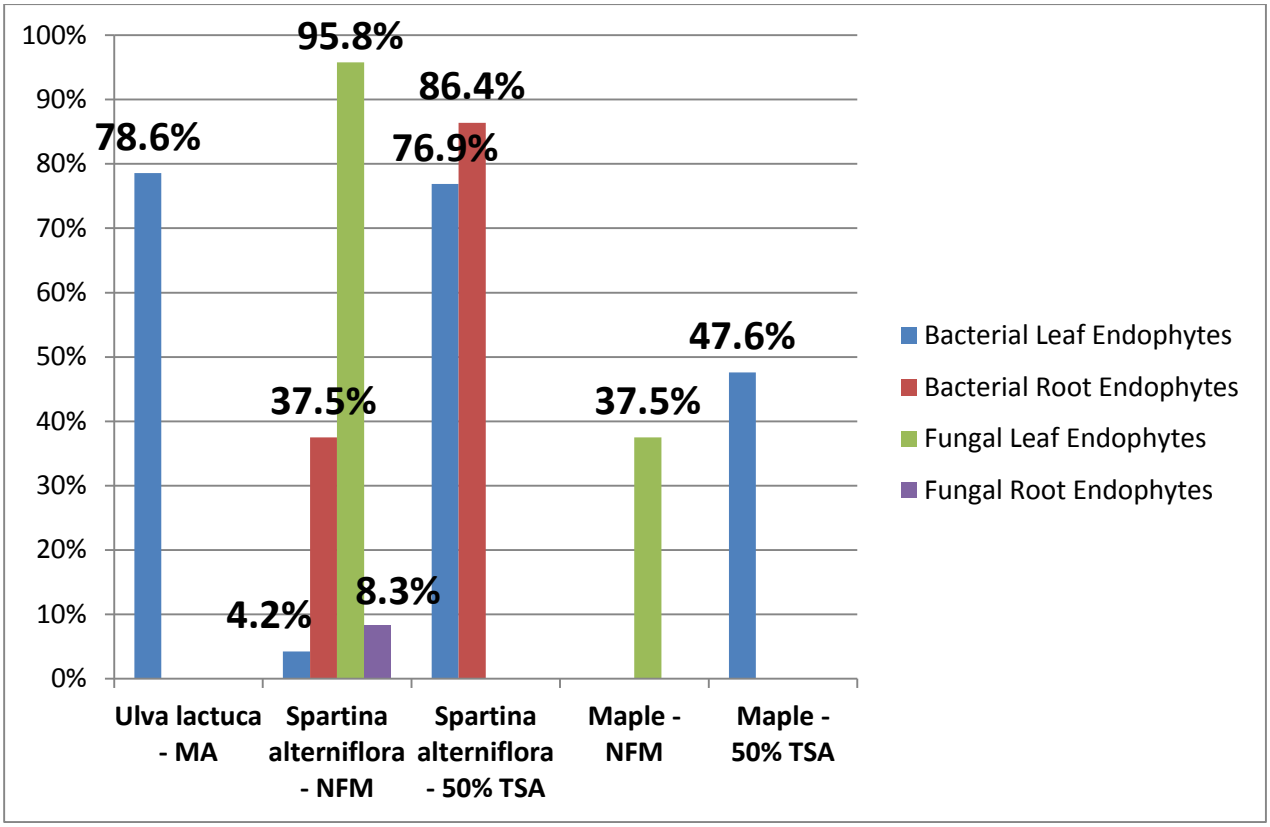
Culture Dependent Methods

The results of how many colonies grew from the total amount of leaf or root fragments sampled is shown on the table below.

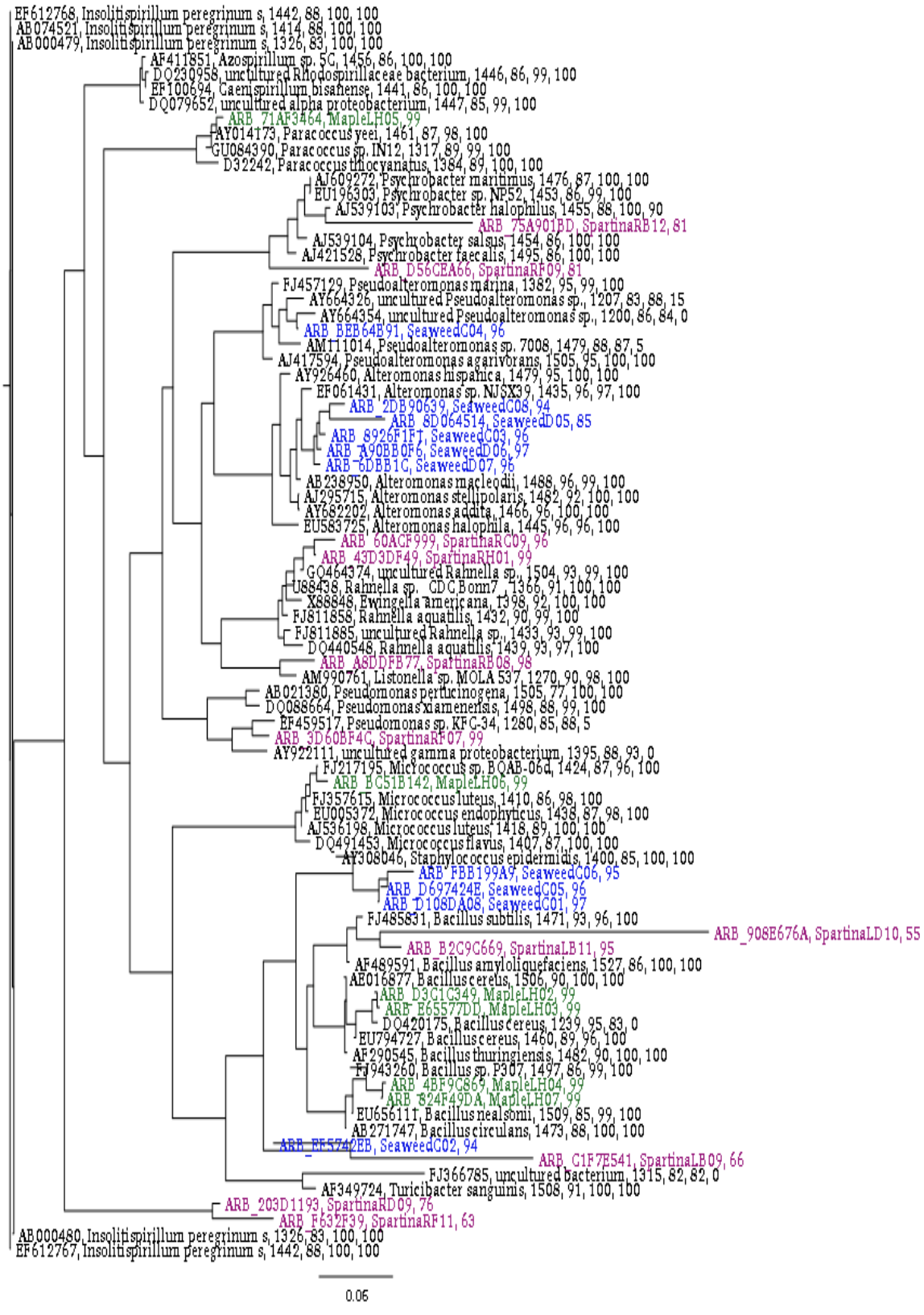
	Seaweed – Marine Agar	*Spartina alterniflora* – Nitrogen Free Media	*Spartina alterniflora* – 50% Tryptic Soy Agar	Maple – Nitrogen Free Media	Maple – 50% Tryptic Soy Agar
Bacterial Leaf Endophytes	22/28	1/24	20/26	0/24	10/21
Bacterial Root Endophytes	0/0*	9/24	19/22	0/0*	0/0
Fungal Leaf Endophytes	0/28	23/24	0/26	9/24	0/21
Fungal Root Endophytes	0/0*	2/24	0/22	0/0*	0/0

**Ulva lactuca* does not form roots therefore none could be sampled. No roots were sampled for the Maple tree either, due to the difficulty of obtaining a representative sample.

The percent of colonization of bacterial and fungal endophytes for each of the plants and media used are represented in the following bar graph.



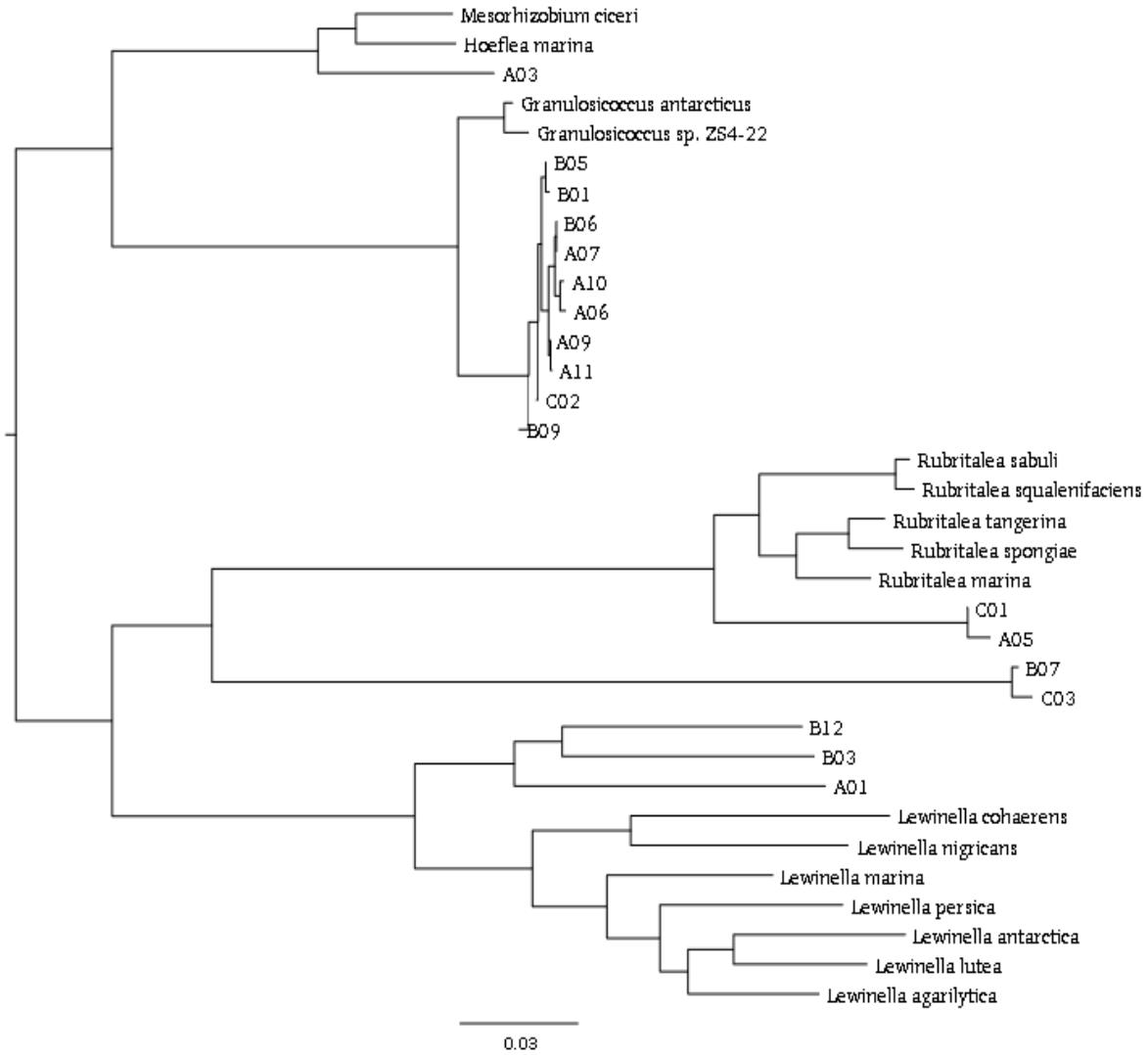
In total, 53 isolates from the different plants and treatments were attempted to be sequenced after obtaining a positive PCR reaction. All PCR reactions that were submitted for sequencing were performed along with a negative control. From the 53 PCR reactions that were submitted, only 26 came back with good enough sequences in order to do any subsequent analyses. The sequences that were good enough, were placed aligned using the SINA Web aligner from the SILVA ribosomal rRNA database (http://www.arb-silva.de/aligner/). Once the sequences were aligned they were uploaded into ARB software for their analysis (http://www.arb-home.de/). The tree on the following page represents the sequences that were obtained which were good enough for analysis. The neighbor joining tree that is shown, was created using ARB but was exported into FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).


EF612768, Insolitispirillum peregrinum s, 1442, 88, 100, 100
AB074521, Insolitispirillum peregrinum s, 1414, 88, 100, 100
AB000479, Insolitispirillum peregrinum s, 1326, 83, 100, 100
AF411851, Azospirillum sp. 5C, 1456, 86, 100, 100
DQ230958, uncultured Rhodospirillaceae bacterium, 1446, 86, 99, 100
EF100694, Caenispirillum bisanense, 1441, 86, 100, 100
DQ079652, uncultured alpha proteobacterium, 1447, 85, 99, 100
ARB_71AF3464, MapleLH05, 99
AY014173, Paracoccus yeei, 1461, 87, 98, 100
GU084390, Paracoccus sp. IN12, 1317, 89, 99, 100
D32242, Paracoccus thiocyanatus, 1384, 89, 100, 100
AJ609272, Psychrobacter maritimus, 1476, 87, 100, 100
EU196303, Psychrobacter sp. NP52, 1453, 86, 99, 100
AJ539103, Psychrobacter halophilus, 1455, 88, 100, 90
ARB_75A901BD, SpartinaRB12, 81
AJ539104, Psychrobacter salsus, 1454, 86, 100, 100
AJ421528, Psychrobacter faecalis, 1495, 86, 100, 100
ARB_D56CEA66, SpartinaRF09, 81
FJ457129, Pseudoalteromonas marina, 1382, 95, 99, 100
AY664326, uncultured Pseudoalteromonas sp., 1207, 83, 88, 15
AY664354, uncultured Pseudoalteromonas sp., 1200, 86, 84, 0
ARB_BEB64E91, SeaweedC04, 96
AM711014, Pseudoalteromonas sp. 7008, 1479, 88, 87, 5
AJ417594, Pseudoalteromonas agarivorans, 1505, 95, 100, 100
AY926460, Alteromonas hispanica, 1479, 95, 100, 100
EF061431, Alteromonas sp. NJSX39, 1435, 96, 97, 100
ARB_2DB90639, SeaweedC08, 94
ARB_8D064514, SeaweedD05, 85
ARB_8926F1F7, SeaweedC03, 96
ARB_A90BB0F6, SeaweedD06, 97
ARB_6DBB1C, SeaweedD07, 96
AB238950, Alteromonas macleodii, 1488, 96, 99, 100
AJ295715, Alteromonas stellipolaris, 1482, 92, 100, 100
AY682202, Alteromonas addita, 1466, 96, 100, 100
EU583725, Alteromonas halophila, 1445, 96, 96, 100
ARB_60ACF999, SpartinaRC09, 96
ARB_43D3DF49, SpartinaRH01, 99
GQ464374, uncultured Rahnella sp., 1504, 93, 99, 100
U88438, Rahnella sp. CDC Bonn7, 1366, 91, 100, 100
X88848, Ewingella americana, 1398, 92, 100, 100
FJ811858, Rahnella aquatilis, 1432, 90, 99, 100
FJ811885, uncultured Rahnella sp., 1433, 93, 99, 100
DQ440548, Rahnella aquatilis, 1439, 93, 97, 100
ARB_A8DDFB77, SpartinaRB08, 98
AM990761, Listonella sp. MOLA 537, 1270, 90, 98, 100
AB021380, Pseudomonas perfectinogena, 1505, 77, 100, 100
DQ088664, Pseudomonas xiamenensis, 1498, 88, 99, 100
EF459517, Pseudomonas sp. KFG-34, 1280, 85, 88, 5
ARB_3D60BF4C, SpartinaRF07, 99
AY922111, uncultured gamma proteobacterium, 1395, 88, 93, 0
FJ217195, Micrococcus sp. BQAB-064, 1424, 87, 96, 100
ARB_EC51B142, MapleLH06, 99
FJ357615, Micrococcus luteus, 1410, 86, 98, 100
EU005372, Micrococcus endophyticus, 1438, 87, 98, 100
AJ536198, Micrococcus luteus, 1418, 89, 100, 100
DQ491453, Micrococcus flavus, 1407, 87, 100, 100
AY308046, Staphylococcus epidermidis, 1400, 85, 100, 100
ARB_FBB199A9, SeaweedC06, 95
ARB_D697424E, SeaweedC05, 96
ARB_D108DA08, SeaweedC01, 97
FJ485831, Bacillus subtilis, 1471, 93, 96, 100
ARB_908E676A, SpartinaLD10, 55
ARB_B2C9C669, SpartinaLB11, 95
AF489591, Bacillus amyloliquefaciens, 1527, 86, 100, 100
AE016877, Bacillus cereus, 1506, 90, 100, 100
ARB_D3C1C349, MapleLH02, 99
ARB_E65577DD, MapleLH03, 99
DQ420175, Bacillus cereus, 1239, 95, 83, 0
EU794727, Bacillus cereus, 1460, 89, 96, 100
AF290545, Bacillus thuringiensis, 1482, 90, 100, 100
FJ943260, Bacillus sp. P307, 1497, 86, 99, 100
ARB_4BF9C869, MapleLH04, 99
ARB_824F49DA, MapleLH07, 99
EU656111, Bacillus nealsonii, 1509, 85, 99, 100
AB271747, Bacillus circulans, 1473, 88, 100, 100
ARB_EE5742EB, SeaweedC02, 94
ARB_C1F7E541, SpartinaLB09, 66
FJ366785, uncultured bacterium, 1315, 82, 82, 0
AF349724, Turicibacter sanguinis, 1508, 91, 100, 100
ARB_203D1193, SpartinaRD09, 76
ARB_F632F39, SpartinaRF11, 63
AB000480, Insolitispirillum peregrinum s, 1326, 83, 100, 100
EF612767, Insolitispirillum peregrinum s, 1442, 88, 100, 100
0.06

Culture Independent Approach Results

Two clone libraries were attempted for a community analysis of *Ulva lactuca* and *Spartina alterniflora*. However, once the clones were obtained, they were placed on a single 96-well plate for sequencing. A positive PCR was obtained from amplifying the 16S rRNA gene of a leaf blade of *Spartina alterniflora* and of a leaf of *Ulva lactuca*. These were then ligated into a vector using the TOPO TA Cloning Kit and they were transformed into *E. coli* cells.

This method was only partially successful. Sequences were obtained from 95 out of the 96 wells. 28 sequences were obtained from *Ulva lactuca* while 68 were obtained from *Spartina alterniflora.* Of the 28 sequences obtained from *Ulva lactuca* 8 belonged to chloroplast sequences and were discarded from the analysis. All 68 sequences from *Spartina alterniflora* belonged to chloroplasts and therefore they could not be analyzed further. A phylogenetic tree was constructed and exported to FigTree. The neighbor joining tree shown below represents those clones that were obtained from *Ulva lactuca.*



Discussion

Culture Dependent Results

The percents of colonization varied among bacterial and fungal endophytes between leaves and roots of the various plants studied. The percent of colonization was greatest for fungal endophytes in leaves of *Spartina alterniflora* that were grown on nitrogen free media (95.8%). This however, should not be interpreted as fungi that fix nitrogen. These fungi are most likely feeding off the remainder of the nitrogen in the plant tissue where they inhabit. Only 1 colony of bacterial endophytes was obtained from *Spartina alterniflora* growing on Nitrogen Free Media. No fungi were isolated from portions of *Ulva lactuca* growing on Marine Agar.

From observing the data obtained for the grass *Spartina alterniflora* it is evident that nitrogen free media was the most efficient culture media used during this study to isolate fungal endophytes. The media that was most efficient for obtaining bacterial endophytes was 50% tryptic soy agar. In terms of those bacteria that grew on 50% tryptic soy agar, it was also evident that more were recovered from the root portions of the plant compared to the leaf portions (86.4% vs 76.9%). This is as expected since most bacterial endophytes should be encountered at the root portions of a plant where they are in close contact to the soil.

Observing the phylogenetic tree constructed from the sequencing data obtained, it can be preliminarly determined that the most diverse bacterial endophytes came from the grass *Spartina alterniflora.* It is important to mention though, that a lot of the samples to be sequenced that were submitted did not make it back or were of poor quality and could therefore change the results that are being observed during this study.

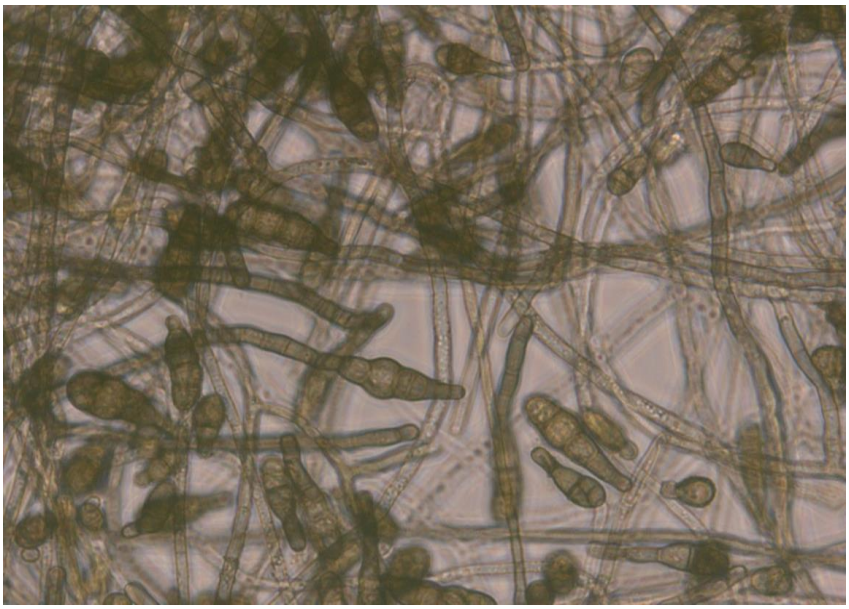
Most of the sequences that were recovered from *Spartina alterniflora* came from the root portion of the plant. Some of these bacteria were close to *Psychrobacter halophilus* and *Psychrobacter faecalis*. Species of *Psychrobacter* can be isolated from cold to warm and slightly to highly saline ecosystems. *Psychrobacter* belongs to the family Moraxellaceae and belongs to the Gammaproteobacteria (Bowman, 2006). Other sequences recovered from the roots of *Spartina alterniflora* grouped closely with *Rahnella*, specifically a member of an uncultured *Rahnella* species. Previously, *Rahnella aquatilis* have been found in the rhizosphere of soybean and has therefore been found to be somewhat in a relationship with plants (Yong Kim et al., 2006). Another sequence obtained grouped together with *Listonella* sp. This bacterium is particularly interesting because it has been found that *Listonella anguilarum* has been described as a diazotroph living in the rhizosphere of mangroves and could be contributing nitrogen to *Spartina alterniflora.* This was also one a colony that was able of growing on nitrogen free media. This can also help confirm that the sequence obtained belongs to a diazotrophic *Listonella.* Another root inhabiting endophyte grouped together with *Pseudomonas* sp. This is of no surprise since species of *Pseudomonas* sp. are constantly easily identified as endophytes in a variety of hosts. The bacteria that were isolated from the leaves of *Spartina alterniflora* clustered with

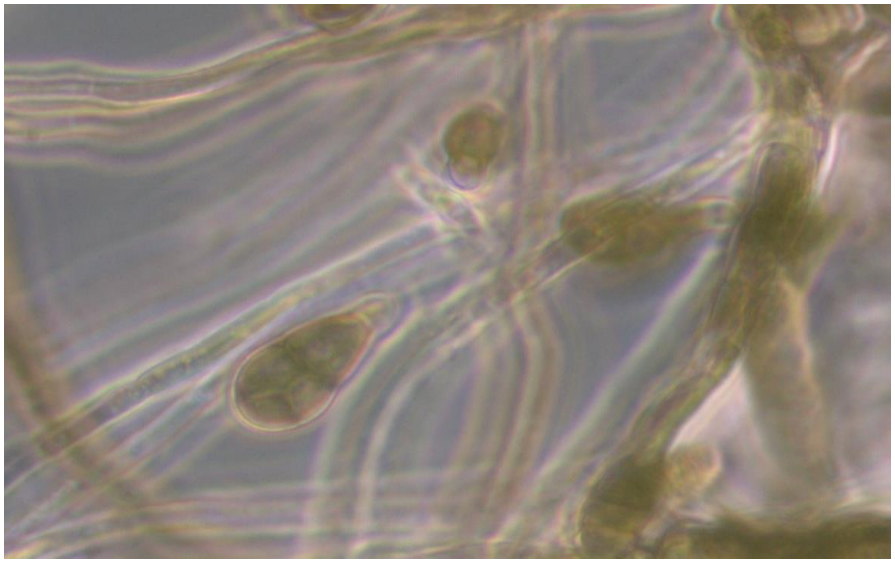
a different group of organisms than those that were isolated from the roots. Three of the sequences that were recovered from isolates from the leaves grouped together with different species of *Bacillus.* Two of the sequences were closely related to *Bacillus cereus* while another was more distantly related to *Bacillus circulans.*

In terms of the endophytes obtained from Maple tree leaves, these were apparently less diverse than those obtained from the leaves and roots of *Spartina alterniflora*. However, it is important to mention that fewer sequences obtained from isolates that came from Maple trees were included for analysis and this could affect the amount of diversity being observed in the phylogenetic tree. The sequences that were found though were closely grouped in the *Bacillus* genus. Two of the sequences grouped with *Bacillus cereus* and the other to with *Bacillus* sp. Other two sequences of bacterial endophytes that came from the Maple tree were closely related to *Micrococcus* sp. and *Paracoccus yeei.* Both *Micrococcus* and *Paracoccus* have been previously identified as endophytes of banana plants (Thomas and Thyvalappil, 2009).

Finally, it appeared that the bacterial endophytes were least diverse in the sequences that were analyzed from *Ulva lactuca*. Most of these sequences grouped around only two groups. Three of the sequences grouped with *Staphylococcus epidermidis. S. epidermidis* was also found to be an endophyte of banana (Thomas and Thyvalappil, 2009). Three other sequences grouped with *Alteromonas* and *Pseudoalteromonas*. These are typical marine bacteria and have been previously described as endophytes of seagrass beds (Couto-Rodríguez, 2009).

Some PCR reactions from fungal isolates were submitted for sequencing. However, not all sequences were recovered. Luckily, one of the fungi that grew from the roots of *Spartina alterniflora* could be easily identified because of its distinct spore. The shape of the spore suggests strongly that this fungus belongs to the genus *Alternaria.* Below, are photographs taken of the hyphae and the spores of the fungus. Another one of the fungi that was obtained from a Nitrogen Free Media plate was described as been the oomycete *Halophytophthora* sp. However, the percent of query and of homology was very low so it is not a very reliable sequence.





Culture Independent Results

Around 20 sequences from the endophytic bacterial community of *Ulva lactuca* were able to be analyzed phylogenetically using the program ARB. The clone labeled A03 belonged to the Rhizobiales and the family Phyllobacteriaceae from the Alphaproteobacteria. Most of the clones sequenced belonged to the Chromatiales from the Gammaproteobacteria. These clones grouped closely together with the bacterium *Granulosicoccus antarcticus* and *Granulosicoccus* sp. Three other clones grouped closely together with bacteria from the genera *Lewinella* sp. Both *Granulosicoccus* and *Lewinella* have also been identified as epiphytes of an Ulvacean alga (Tujula et al., 2010). This definitely reinforces the fact that these organisms can be found in close association to this alga. Other clones grouped together

with *Rubritalea,* which belongs to the Verrucomicrobiales, and has been previously described living in association with a sea sponge (Scheuermayer et al., 2006). Two clones clustered together in the SR1 candidate division.

Conclusion

In conclusion, the endophytic community that was best described during this project belonged to the grass *Spartina alterniflora* which was sampled from the Little Sippewissett purple sulfur pools. These also had the highest percent of colonization of bacteria. However, it would have been more descriptive if more sequencing data would have been recovered. The endophytes that were described during this mini project by phylogenetic association have also been found in previous hosts as endophytes.

A distinct difference was observed between the sequences of the clones and the sequences of the organisms that were obtained by culture methods. A combination of both culturing methods and culture independent methods seems to be the most efficient way for describing the total endophytic community of a plant instead of just relying on one of the approaches alone. It was also apparent by the sequences of the isolates obtained that the endophytic communities differ among hosts sampled. Most of the endophytes recovered from *Ulva lactuca* represented marine species of organisms. Some organisms that were recovered from *Spartina alterniflora* belonged to halophilic, diazotrophs, and other common terrestrial endophyte species. Finally, no halophiles or marine bacteria were isolated from the Maple Tree at Swope. Further testing and more in depth analysis of the isolates that were not able to be sequenced would be necessary to prove this statement. However, what is being seen so far does suggest that the environment as well as the host tends to dictate what microbial communities are present in the interior of vegetable tissue.

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