STUDENT PROJECT REPORT TO THE UNIVERSITY OF HAWAIʻI MARINE OPTION PROGRAM

Systematics of the Trentepohliales (Ulvophyceae, Chlorophyta) on the windward coast of Oʻahu

Pauleen Fredrick

Marine Science Department
University of Hawai'i at Hilo

Advisors
Dr. Alison Sherwood
Internship Supervisor
Department of Botany
University of Hawai'i at Mānoa

Emily Johnston
Internship Mentor
Department of Botany
University of Hawai'i at Mānoa

Dr. Karla McDermid
Faculty Advisor
Marine Science Department
University of Hawaii at Hilo

Dr. Steven Colbert
MOP Instructor
Marine Science Department
University of Hawaii at Hilo

December 10, 2014

Abstract:

The molecular systematics of Hawaiian representatives of the order Trentepohliales, specifically the genera *Trentepohlia* and *Printzina*, was studied by the examination of cultured specimens collected from Waimanalo, Kailua, La‘ie, Kahana, O‘ahu, and other specimens collected directly from sites along Pali Highway and Malaekahana. Samples were grown at different salinity levels. Thirteen Trentepohliales specimens were sampled from culturing plates for both molecular and morphological analyses. Morphological data were examined in culture and five different species of *Trentepohlia* were identified In contrast, phylogenetic analysis revealed four species of *Trentepohlia* and *Printzina* using the 18S rRNA marker and two species of *Printzina* using the chloroplast marker (rbcL). Growth and species diversity were also surveyed in culturing plates and results showed an increase in species diversity under conditions where salinity was low and very few occurring at conditions where salinity was high. Results showed that morphological and molecular species identification do not always correspond to each other and that salinity is an important factor in determining morphology and growth in these species.

Introduction:

Subaerial algal communities are very rich and widespread in tropical and temperate regions (Rindi *et al.* 2008). These algae play important roles as environmental pollution indicators (Freystein *et al.* 2008), commercial producers of carotenoids (i.e., β-carotene), and potential biofuels (Lopez-Bautista *et al.*2006a). Communities of these microorganisms are recognizable by the pigmented layer they produce on stable, exposed surfaces above soil, including both natural and artificial substrata (Lopez-Bautista *et al.* 2006b). Currently, limited information on the systematics of tropical terrestrial microalgae is known due to the fact that these organisms are relatively understudied compared to the terrestrial algae of temperate regions (Lòpez–Bautista *et al.* 2007; Sherwood 2004).

Green algae (Chlorophyta) of the order Trentepohliales are generally reported as the most diverse and widespread subaerial algae in tropical and subtropical regions (Rindi & Lopez-Bautista 2007). The order Trentepohliales consists of a single family, the Trentepohliaceae,

which includes the following genera: *Cephaleuros, Phycopeltis Physolinum, Stomatochroon,* and *Trentepohlia* (Lopez-Bautista *et al.* 2002). These organisms grow on humid natural and artificial substrata (rocks, stems, bark, cement, soil, etc.) in a community that includes other subaerial organisms such as lichens or fungi (Lopez–Bautista *et al.* 2002). Rindi *et al.* (2006) documented several species of subaerial algae (of the genera *Trentepohlia,* and Printzina; Trentepohliales) and discovered a previously undescribed species, *Spongiochrysis hawaiiensis,* growing in a bright golden-yellow biofilm on bark of *Casuarina* trees in coastal areas of the windward side of Oʻahu (Rindi *et al.*2006).

Based on preliminary observations of subaerial algal communities in the Hawaiian archipelago, the Trentepohliales appear to be very diverse and abundant, occurring on a variety of substrata in numerous locations. These algae have unique morphological features (i.e. branching, filamentous growth, habit of thallus, shape and size of vegetative cells, arrangement of reproductive structures, presence of hair like cells, and type of substratum colonized) that distinguish species from each other (Rindi and Guiry 2002). Contemporary studies, focusing on morphometric characters of the Trentepohliales, have shown that morphological features can vary at different spatial and temporal scales in different species. This variation makes identification of species based solely on morphology difficult, if not impossible, and highlights the need for genetic sequencing to determine species boundaries (Rindi and Guiry 2002). In this study we will concentrate our efforts on the genetic sequencing and molecular systematics of the Trentepohliales to better understand species delimitation and distribution on the windward side of Oʻahu. Furthermore, we want to take into consideration the effect of salinity on communities of the order Trentepohliales.

Materials and Methods:

Data sampling was obtained in two ways. In the first sampling data, three locations were selected along the windward coast of Oʻahu, representing most of the known range of *Spongiochrysis's:* Kailua Beach Park, Kahana Beach Park, and Malaekahana. These sites were chosen to match the sites in previous study (454 metabarcoding), except Malakahana was substituted for the nearby Laʻie Beach Park because of private property and restricted full access to inland *Spongiochrysis*-dominated biofilms. On April 5th, 2014, bark from randomly selected individual, single trees at three distances from the high-tide shore line, 0-5 m, 35-40m, 70-75 m was sampled. Biofilm material was removed from each tree separately with a new razor, combined in a petri dish, and shaken to randomize. The second set of samples, identified as *Trentepohlia* and *Printzina* in the field, was collected directly from tree bark, rocks and artificial substrata from sites along the Pali Hwy and at Malaekahana, generating an additional three specimens (Table 2).

This material was used to culture eight replicates of four different salinity treatments (0 ppt (parts per thousand), 12 ppt, 24 ppt and 36 ppt) using the following media: Alga-gro (Carolina Biological Supply , Burlington, North Carolina) diluted to regular strength in dH_2O and with 1 µL of Ampicillin (cGMP compliant facility, Grand Island, New York) per mL media. Salinity levels were generated with Instant Ocean Sea Salt (Marine land) by adding one gram salt for every part per thousand salinity per liter and checked with a Salinity Refractometer (Sinotech RHS 10 ATC). The media was then solidified with 1.5% Agar (Sigma Agar). The cultures were allowed to grow for two and a half months. Cultured microorganisms were examined with a Zeiss Discovery V12 Stereomicroscope and colonies representing each morphospecies on a plate, were sampled for both molecular and morphological vouchers. Microscopical analysis of the morphological vouchers was conducted using a Zeiss Axio Imager A1 (Carl Zeiss Ltd., United Kingdom) for species identification. A representative specimen for each *Trentepohlia* species found in culture was selected for further molecular analysis, for a total of 11 specimens (Table2).

For the molecular analysis (DNA extraction, Polymerase Chain Reaction (PCR), Gel Electrophoresis, Purification, and Sequencing) of the specimens from parts two and three above, the following protocol was observed: DNA was extracted using a Qiagen Dneasy Plant Mini Kit following the manufacturer's protocol. The 18S ribosomal marker (nuclear) for each of the 13

samples were then PCR amplified using the following protocol: For each 25 µL reaction 9.0 µL dH_2O (nanopure water), 5.0 µL reaction buffer, 1.5 µL 50mM $MgCl_2$ 1.5 µL of 1% biovine serum albumin solution (BSA), 1.0 µL of each 18S (SR1, SS11H, 18SC2,and SSU897) and rbcl markers (RH1and RT1134) (Table 1), 4.0 µLdNTPs, 1.0 µL of MangoTaq (polymerase enzyme; Bioline, Meridian Life Science Company), and 1.0 µL of total genomic DNA using the following cycle: an initial denaturation step at 94°C at one min. followed by 35 cycles of 30 seconds at 94°C, primer annealing at 58°C for one minute and extension at 72°C for five minutes, and a final extension phase of an additional five minutes in a Mastercycler ep gradient S (LabCommerce,Inc., Hauppauge , New York) PCR products were visualized with gel electrophoresis to verify the success of the reactions. Successful products were purified using Exosap (Affymetrix UK Ltd., Santa Clara,CA) (5µl of PCR products and 2 µl of Exosap) using the following cycle: 15 minutes at 37°C followed by an additional cycle of 15 min.at 80°C.Purified PCR products were sequenced at the ASGPB Facility.

Chromatograms and consensus sequences were assembled and aligned, in combination with Trentepohliales sequences downloaded from GenBank (Benson *et al.* 2001) using Geneious R7(Drummond et al, 2010) and the alignment was corrected by eye. To infer species-level relationships, phylogenetic inferences were conducted using Bayesian Inference (BI) analysis (MrBayes version 3.2; Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003, Ronquist *et al.* 2012) and Maximum Likelihood (ML) analysis (RAxML version 7.2.6, Stamatakis 2006). BI and ML analyses were run under a General Time Reversible (GTR) model with a gamma distribution. BI analyses were run for a minimum of 3,000,000 generations or longer, until the standard deviation of the split frequencies was less than 0.01 and the first 10% of sampled trees were discarded as burning. ML analyses were run for 1000 generations using a parsimony-inferred starting tree with 1000 bootstrap replicates using a random starting tree. Phylogenetic trees were outgroup rooted with sequences of *Halimeda*.

Table 1: Primers used for amplifying and sequencing *Trentepohlia* spp. genomic DNA

Species	**Primers (Markers)**	**Primer Sequence 5'-3'**	**Reference**
Trentepohlia spp.	SR1 (18S/SSU)	TACCTGGTGATCCTGCCAG	Boedeker *et al.* 2013
Trentepohlia spp.	SS11H (18S/SSU)	CCTTTAAGTTTCAGCCTTGCGACC	Leliart *et al.* 2007; Boedeker *et al.*2013
Trentepohlia spp.	18SC2 (18S/SSU)	TCCGCAGGTTCACCTACGGAG	Boedeker *et al.* 2013
Trentepohlia spp.	SSU897 (18S/SSU)	GGTGAAATTCTTGGATTTGCGAAAG ACG	Boedeker *et al.* 2013
Trentepohlia spp.	RH1 F(*rbc*L)	ATGTCACCACAAACAGAAACTAAAG C	Rindi *et al.* 2009
Trentepohlia spp.	RT1134 (*rbc*L)	CATGTGCCAAATGTGAATACC	Rindi *et al.*2008

Table 2: Thirteen *Trentepohlia* samples observed and used for molecular analysis with culture numbers and site collected.

#	**Sampled Species**	**Identification Number**	**Data obtained :**
1.	*Trentepohlia spp.*	00068-1	Culture plate
2.	*Trentepohlia spp.*	00001-3	Culture plate
3.	*Trentepohlia spp.*	00151-2	Culture plate
4.	*Trentepohlia spp.*	00128-2	Culture plate
5.	*Trentepohlia spp.*	00054-2	Culture plate
6.	*Trentepohlia spp.*	080714-2a	Malaekahana
7.	*Trentepohlia spp.*	080714-3b	Pali Look out
8.	*Trentepohlia spp.*	080714-4b	Pali Look out
9.	*Trentepohlia spp.*	00045-1	Culture plate
10.	*Trentepohlia spp.*	00025-1	Culture plate
11.	*Trentepohlia spp.*	00057-2	Culture plate
12.	*Trentepohlia spp.*	00127-4	Culture plate
13.	*Trentepohlia spp.*	00192-2	Culture plate

Results:

Morphological Analysis:

Five different Trentepohliales species were identified morphologically as Trent 1, 2, 3, 4, and 5 based on cell morphology from culturing plates (Table 3). Based on the morphological data from the culturing plates, species diversity (Trent 1 to 5) varied at locations along the

windward coast where specimens were collected (Figure 1). Species diversity also varied under different environmental conditions or salinity level ranging from 0 parts per thousand (ppt), 12 ppt, 15 ppt, 24 ppt, 35 ppt, and 36 ppt (Figure 2). In areas where salinity is high, species diversity is low and in areas where salinity is low species were more diverse.

Table 3: *Trentepohlia* species identified based on cell morphology.

Species	**Morphology**
Trent 1	Cells are circular; cells forming filaments
Trent 2	Cells are more rectangular with a rounded terminal (endpoint); filamentous
Trent 3	Cells are more elongated; cells forming filaments
Trent 4	Two cell shapes (circular & elongated cells); chain-like cells
Trent 5	Two cell shapes (elongated and rectangular)

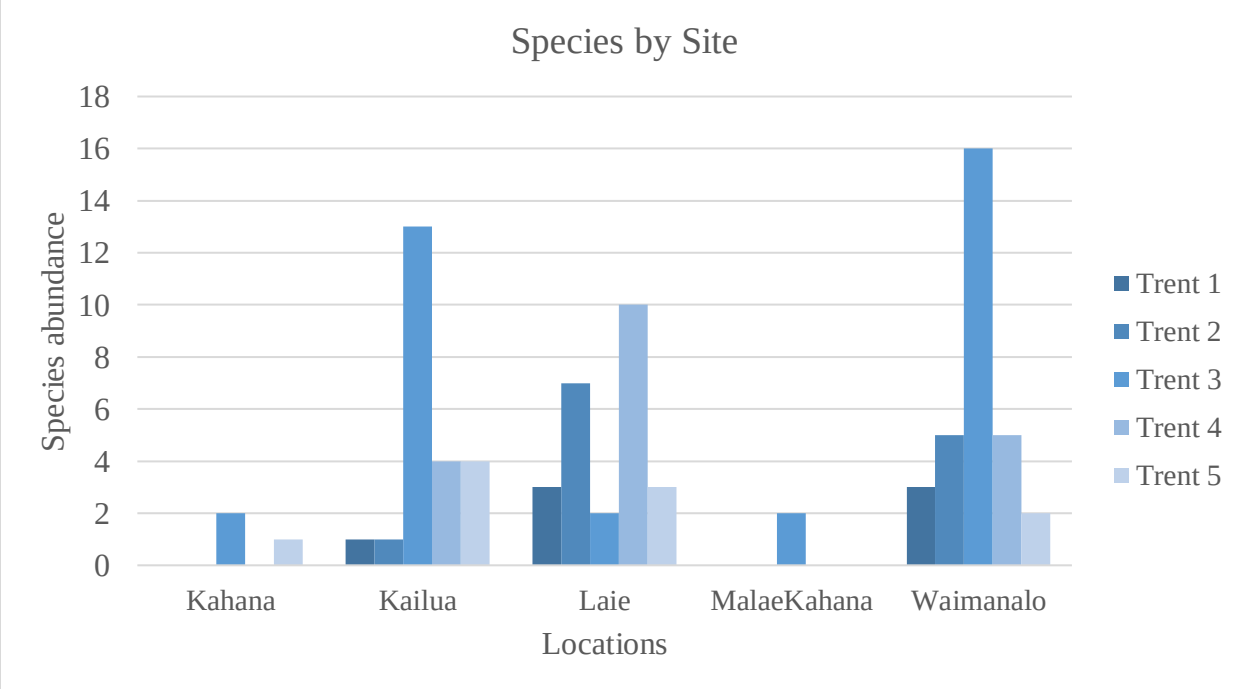

Figure 1: Species diversity along the windward coast of O‘ahu.

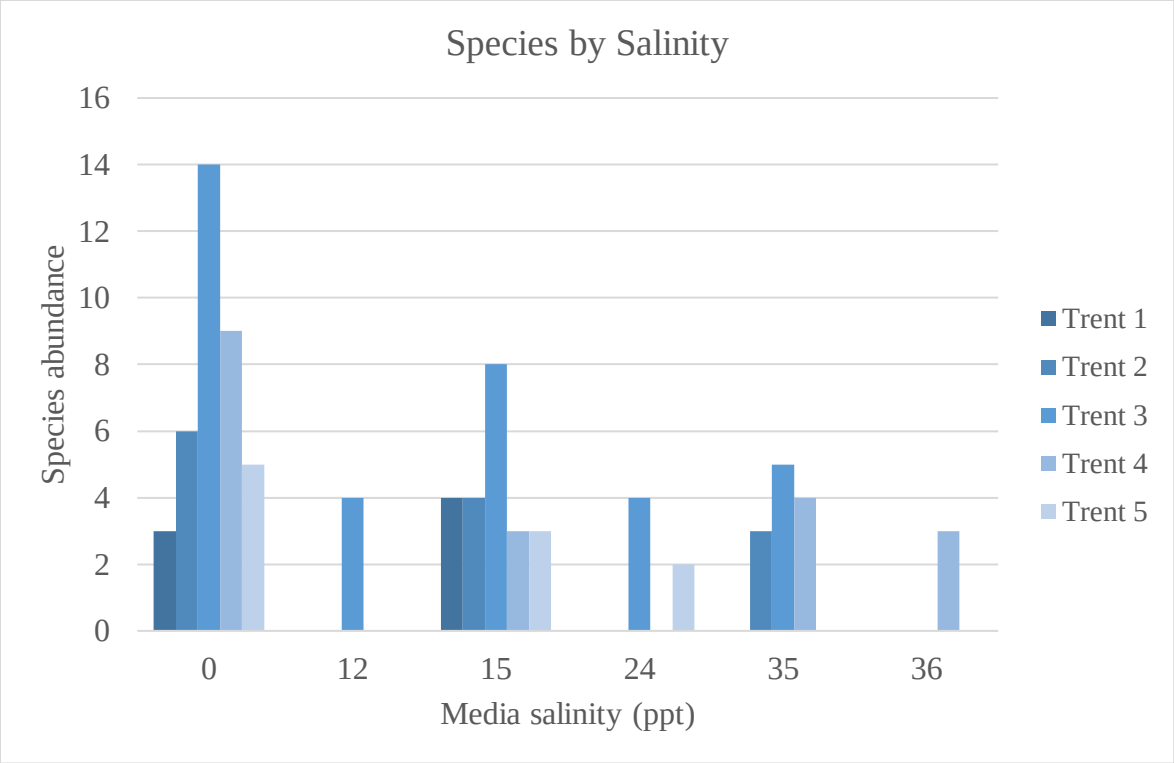

Figure 2: Species diversity by salinity levels.

Molecular analyses:

Thirteen Trentepohliales samples in total were amplified and analyzed with different primers or markers (Table 1) to identify specimens to species level. Two molecular markers were used, a chloroplast marker (*rbc*L primers: RH1 and RT1134) and the 18S rRNA nuclear marker (18S primers: SSU897 & 18SC2, and SR1 & SS11H). Five out of 13 samples worked for rbcL marker were identified as Trent 1, 4, and 5 (Figure 3). Only nine samples worked for the 18S marker but because of additional specimens, our samples appeared in different clades (Figure 4).

All Bayesian Inference (BI) trees were used to examine the relatedness or phylogenetic relationship of collected species with additional sequences from Genbank (Figs 3 & 4). Most of the collected samples formed clades with *Printzina lagenifera*, *Trentepohlia arborum*, and *Trentepohlia annulata*. For the *rbc*L marker, our preliminary data of *Trentepohlia* species we collected are shown with few other sequences from Genbank (Figure 3). In the *rbc*L phylogenetic tree, shaded areas showed which samples formed clades with *Printzina* and *Trentepohlia*. Based on the 18S marker, a phylogenetic tree was generated and several sequences of the genera *Trentepohlia* and *Printzina* from Genbank formed clades with the collected

samples, as shown in the shaded areas. The specimens for which there were both *rbc*L and 18S data formed clades with the same species in each tree. However, 18S sequences were available for additional specimens, and these sequences formed clades with additional species (Figure 4).

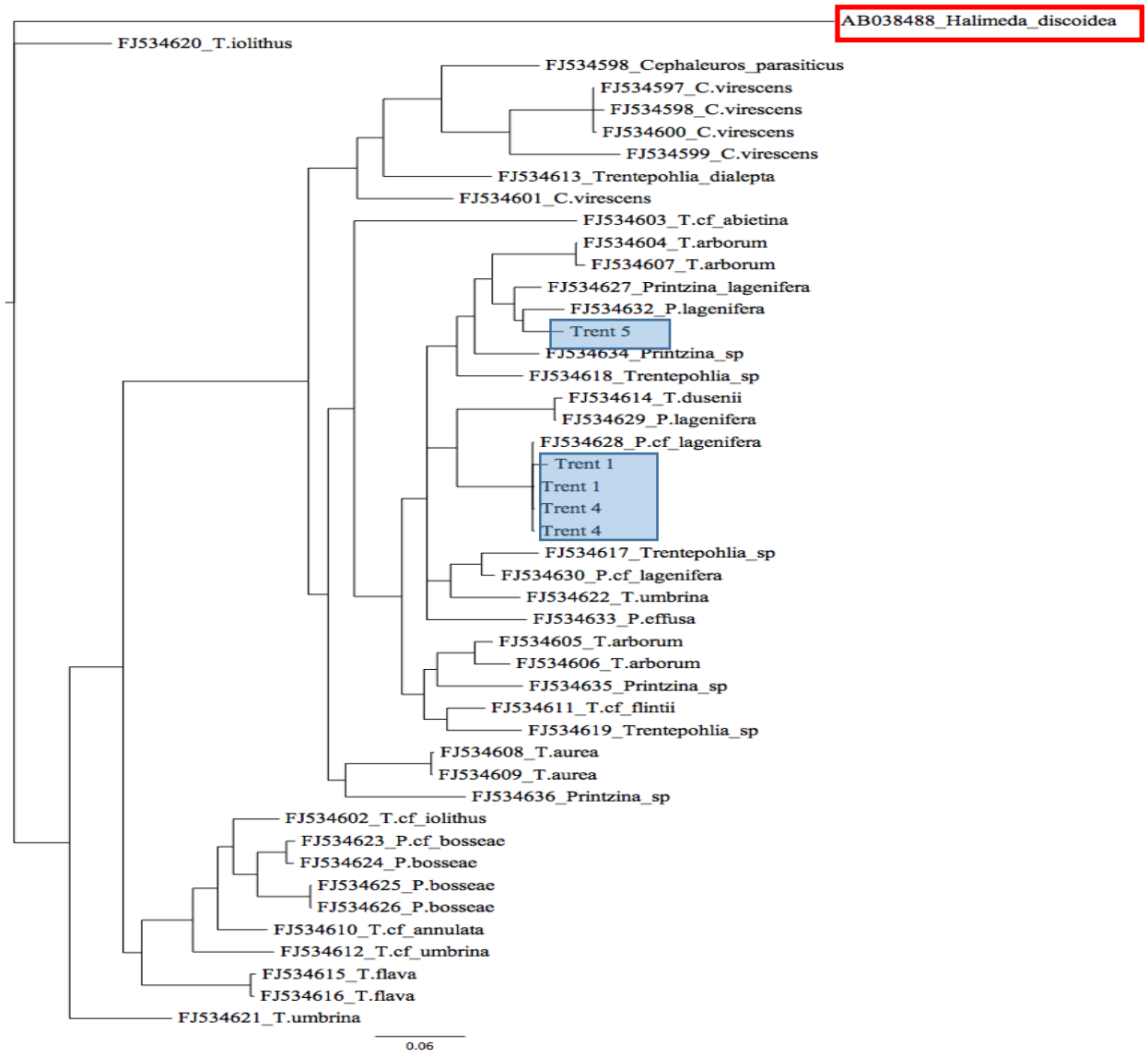

Figure 3: Phylogenetic tree for *Trentepohlia* species using the plastid encoded marker *rbc*L. The analysis included collected samples and additional sequences from GenBank. Shaded sequences show collected specimens. Open shapes (not shaded) shows outgroup species (*Halimeda*).

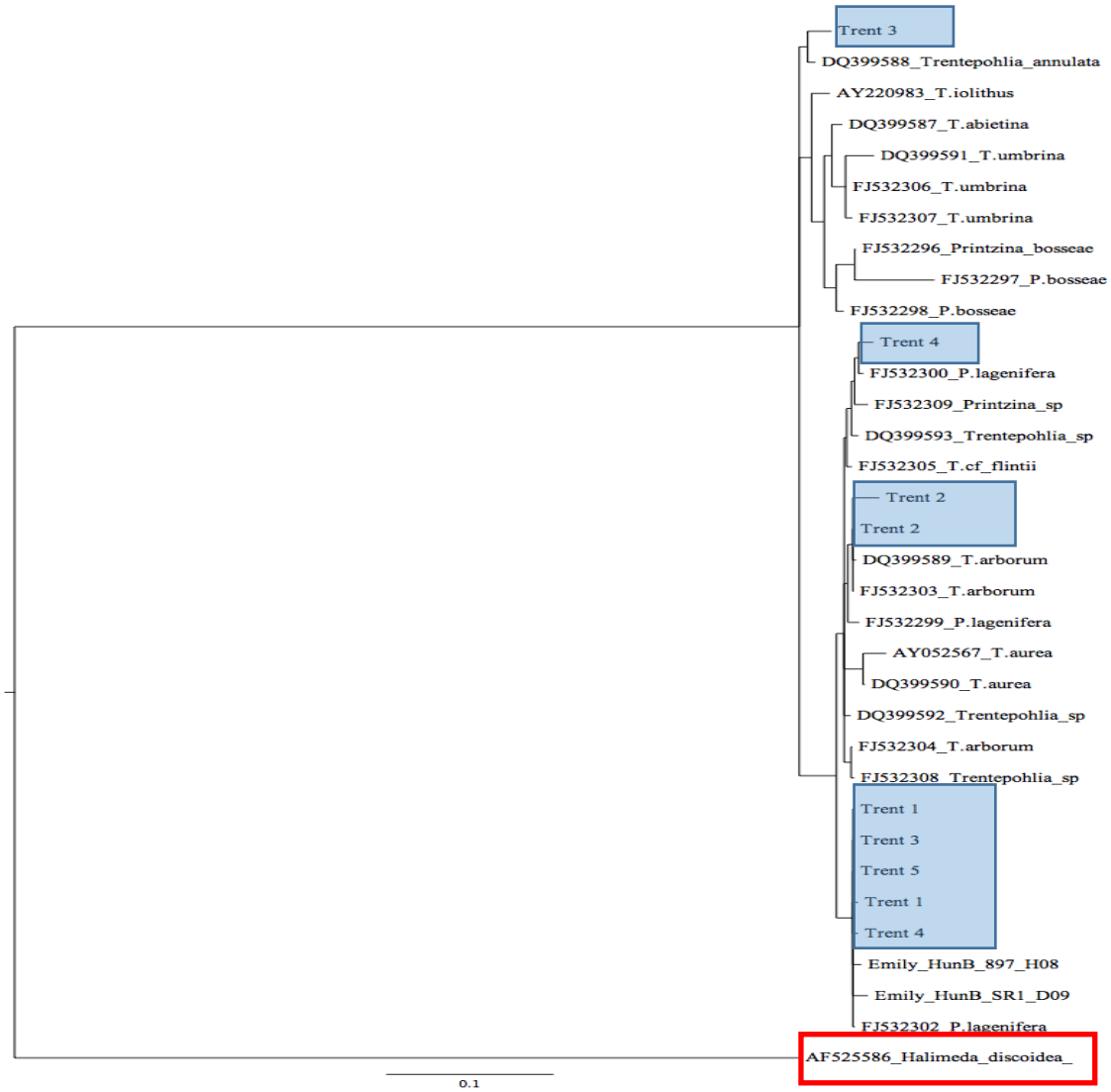

Figure 4: Phylogenetic tree for *Trentepohlia* species using the 18S rRNA marker. The analysis included collected samples and additional sequences from GenBank. Shaded sequences show collected specimens. Open shapes (not shaded) shows out group species (*Halimeda*).

Discussion:

The present study of the molecular systematics of *Trentepohlia*, led to the identification of 13 samples belonging to two genera, *Printzina* and *Trentepohlia*, of the order Trentepohliales. Eleven out of the 13 samples were *Printzina* species and only three samples were revealed as *Trentepohlia* species. Applying molecular techniques to samples collected (13) from culturing

plates and sites along the Pali Highway and Malaekahana, we were able to positively identify the majority of the species. Phylogenetic analyses of the 18S rRNA marker and the chloroplast rbcL datasets resulted in the same species identifications for our collected specimens. The *rbc*L datasets (Figure 3) revealed few of our samples being successful which is surprising since Rindi *et al.* (2009) previously reported that the *rbc*L gene has proved to be very useful for phylogenetic analysis of different taxonomic levels. Phylogenetic analysis of the 18S data sets showed that four species of *Printzina* and *Trentepohlia* seemed to match our collected samples supported by different nodes and clades on the tree (Figure 4). Genetic analysis of the same marker (18S) also revealed that two samples of Trent 2 (collected samples) were potentially two different species due to a high degree of genetic variation (based on long branch length) and formed a clade with *Trentepohlia arborum*.

Environmental salinity was also taken into consideration to assess species identification. Six levels of substrate salinity were present in culturing plates: 0 ppt, 12ppt, 15ppt, 24ppt, 35 ppt, and 36 ppt were examined to explain the importance of environmental salinity in communities where *Trentepohlia* species occur and how congruent the morphological data were with the molecular analyses. Substrate salinity is an important factor in resolving plant species distribution and their ability to tolerate different environmental conditions (Alpha *et al*.1996). According to the culturing study of subaerial algae, only Trentepohliales species forming tufts on agar were used for this study to classify species morphologically. Five different species of Trentepohliales were identified based on cell shape (circular, elongated, rectangular, or mixed). Figure 2 indicates that in areas where salinity was low, Trentepohliales species tended to have better growth rates and higher diversity and not surprisingly, in areas where salinity was high we saw limited growth and species diversity. Five locations (Waimanalo, Kailua, La‘ie, Malaekahana, and Kahana) were surveyed along the windward coast of O ‘ahu where *Trentepohlia* was previously seen within 150 m of the high tide shoreline, a range that experiences a high degree of salt loading from the wind (Alpha *et al*. 1996). Very diverse communities of *Trentepohlia* occurred at Waimanalo, La‘ie, and Kailua Beach Parks but more limited communities were present at Malaekahana and Kahana Beach Parks (Figure 1).

As previously reported in Rindi *et al.* (2009), morphological and phylogenetic analyses do not correspond well within the Trentepohliales and they vary greatly from species to species. For this order, morphological circumscription is insufficient to define species. Given the

morphological data revealed by our results, molecular analyses would be an ideal solution for species classification in the order Trentepohliales (Rindi *et al*. 2009), and is therefore highly applicable to this work.

Conclusion:

Morphological data sets revealed the effect of environmental salinity on species diversity across a certain transect along the windward coast and five species of Trentepohliales were identified based on cell morphology. Conversely, molecular data revealed only four species that appeared in clades of *Printzina* and *Trentepohlia*, indicating that morphology may more dependent on environmental salinity, rather than species identity. This study showed that molecular techniques for the identification of terrestrial green algae are very useful for species identification and bring up a number of interesting questions that should be pursued in further research, including species competition with other subaerial organisms at different salinity levels.

For future studies, additional samples of *Trentepohlia* should be collected from coastal areas around O‘ahu and other neighboring islands. Amplification of different markers used for the two genera might also be interesting in comparisons to better identify species. Conducting more salinity trials and culturing more *Trentepohlia* species is necessary to determine species ecological preferences.

Acknowledgements:

This work is supported by the NSF (National Science Foundation), the Pacific Internship Program for Exploring Science (PIPES) and the Highly Engaged Learning Placement program for Pacific islanders (HELP). Special thanks to the Sherwood Lab members especially my mentor (Dr. Alison Sherwood), project supervisor (Emily T. Johnston), Rachael Wade, Fisher Johnston (supervisor’s son), and Ernesto A. Olmos for their support in collecting samples and analyzing data. I also want to thank Ayesha Genz and Karla McDermid (UHH Faculty advisor) for the feed backs on my projects report. The support received from fellow interns (PIPES interns) is also thankfully acknowledged.

References:

Alpha, C.G, Drake D.R, and Goldstein, G. (1996). Morphological and physiological responses of Scaevola sericea (Goodeniacea) seedlings to salt, spray, and substrate salinity. American Journal of Botany, 83:86-92.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., Sayers, E. W. 2001. GenBank. *Nucleic Acids Research*, 39: D32-37.

Drummond, A.J. et al. (2010) Geneious v5.5. Available at http://www.geneious.com (last accessed 12 August 2014)

Freystein, Katharina, Salisch M, and Reisser W. (2008). Algal Biofilms on Tree Bark to Monitor Airborne Pollutants. Springer Link,

Huelsenbeck, J. P. and F. Ronquist. (2001). MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

López-Bautista, J.M., Debra A. Waters, D.A. & Chapman, R.L. (2002). The Trentepohliales revisited.Constancea 83: 1-23.

López-Bautista, J. M., Rindi, F., & Guiry, M. D. (2006a) Molecular systematics of the subaerial green algal order Trentepohliales: an assessment based on morphological and molecular data. *International Journal of Systematic and Evolutionary Microbiology* 56: 1709–15.

López-Bautista, J.M., Kapraun, D.F. & Chapman, R.L. (2006b). Nuclear DNA content estimates in the Trentepohliales (Chlorophyta): phylogenetic considerations .*Algological Studies* 120: 41-50.

Rindi F. & Guiry M.D.2002. Diversity, life history and ecology of *Trentepohlia* and *Printzina* (Trentepohlialies, Chlorophyta) in urban habitats in Western Ireland. *Journal of Phycology* 38:39-54.

Rindi, F., Guiry, M.D. & López-Bautista, J.M. (2006). New records of Trentepohliales (Ulvophyceae, Chlorophyta) from Africa. *Nova Hedwigia* 83: 431-449.

Rindi, F. & López-Bautista, J.M. (2007). New and interesting records of *Trentepohlia* (Trentepohliales, Chlorophyta) from French Guiana, including the description of two new species. *Phycologia* 46: 698-708.

Rindi, F. & López-Bautista, J.M. (2008). Diversity and ecology of Trentepohliales (Ulvophyceae, Chlorophyta) in French Guiana. *Cryptogamie, Algologie* 29(1): 13-43.

Rindi, F. Allali, H.A, Lam Daryl W., Lopez- Bautista, J.M (2009). An overvies of the biodiversity and biogeography of terrestrial green algae. Nova Sci.: 1-15.

Ronquist, F. and J. P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Ronquist, F., Teslenko, M. ,vander Mark P. ,Ayres, D.L., Darling A., Hohna S., Larget B., Liu L., Suchard ,M.A., Huelsenbeck, J.P. 2012. MRBAYES 3.2: efficient Bayesian phylogenetic and model choice across a large model space. Bioinformatics 61: 539-42.

Sherwood, A. R. (2004). Bibliographic checklist of the non-marine algae of the Hawaiian Islands.Bishop Mus Occ Pap Series 80: 1-23.

Stamatakis, A. 2006.RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688 – 2690.