UNIVERSITY OF CALIFORNIA, SAN DIEGO

Phylogeny of Tropical Marine *Oscillatoria* and Correlation with Biologically Active Secondary Metabolites

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2011

The Thesis of Tracy Chan is approved and is acceptable in quality and form for publication on microfilm and electronically:

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University of San Diego, California

2011

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. William H. Gerwick for his support as my principal investigator and the chair of my committee. He has provided invaluable critique on my papers as well as insight on the direction of my project.

I would also like to acknowledge Niclas Engene, Ph.D, my mentor and inspiration for this project. Besides teaching me all the basic skills necessary for this project, he has also helped me with basic skill for life (such as time management, for example). I am very grateful that Niclas has taken the time to help me with my thesis despite being occupied with writing his own dissertation. In addition to Niclas, I would like to thank everyone at the Gerwick Lab especially the chemists, who have taught me almost everything I know about the chemical analysis part of my research.

I would like to thank Hyukjae Choi, Ph.D, for permitting me to use data regarding dolastatin 12, which is referenced in the abstract, introduction, and results and discussion sections of this paper. Isolation and structure characterization of dolastatin 12 from cyanobacteria from Curacao is unpublished data, and the date of publication has not been determined. Without these data, I would have had a hard time finding a starting point for chemical profiling and this project would have taken much longer. Hyukjae has also been a great teacher while I was learning how to do LCMS analysis.

I would also like to thank the governments of Curacao, Panama, and Papua New Guinea, for allowing the collection crew to gather environmental samples, as well as the collection crew for collecting them.

Lastly, I would like to acknowledge any other parties that were not mentioned but directly or indirectly involved in making this project possible.

ABSTRACT OF THE THESIS

Phylogeny of Tropical Marine *Oscillatoria* and Correlation with Biologically Active Secondary Metabolites

by

Tracy Chan

Master of Science in Biology

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Professor William H. Gerwick, Chair
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The goal of this study was to test the effectiveness of phylogenetics as a method of predicting secondary metabolite production. A phylogeny of 27 strains of tropical

marine cyanobacteria from 5 geographic locations was constructed based on the SSU (16S) rRNA gene. Evolutionary relationships among the strains were used to predict their production of several known natural products. Two strains from Curaçao were successfully predicted to produce dolastatin 12 and tumonoic acid B based on secondary metabolite production by related strains, while a morphologically similar but evolutionarily more distant strain from the same geographic location was correctly predicted to not produce these compounds. However, a strain from Panama that was closely related to the dolastatin 12 and tumonoic acid producers did *not* produce these compounds. This suggests that while evolutionary relatedness can be a useful tool for predicting secondary metabolite production, it is possible that environmental factors affect production of specific compounds. With this in mind, a phylogeny-guided approach is still an effective method of finding existing bioactive compounds of interest, and possibly new natural analogs of those compounds.

Introduction

Cyanobacteria, also known as blue-green algae, are a ubiquitous group of photosynthetic bacteria found in almost every imaginable environment on earth (Rothschild & Mancinelli 2001). Most likely the first organisms to possess the ability to perform photosynthesis, they are often credited with converting the early oxygen-poor atmosphere into an oxygen-rich one, allowing biodiversity to flourish (Kasting & Siefert 2002). Cyanobacteria are also primarily responsible for fixing relatively inert atmospheric nitrogen, making it available to other organisms (Kasting & Siefert 2002). In short, cyanobacteria were, and still are, an essential component in shaping the earth into what it is today.

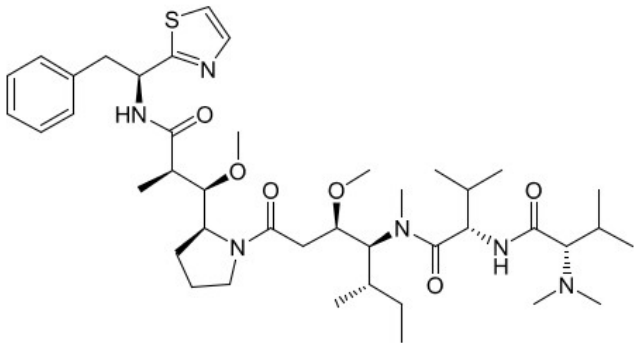
Humans have found multiple uses for cyanobacteria. Among these uses is cyanobacteria as a source of nutrition. *Spirulina* has been popularized as a health food because it is a good source of proteins, vitamins, and essential fatty acids (Belay *et al*., 1993). Harvested *Spirulina* can be found in health stores and supermarkets in the form of blended drinks or dry tablets. Besides providing nutrition, cyanobacteria may contribute to environmental conservation efforts. Cyanobacteria are capable of synthesizing long-chain fatty acids, which may be used as an alternative to the hydrocarbons found in diesel and other fossil fuels in the future (Hu *et al*., 2008). Some cyanobacteria are also capable of degrading harmful organic pollutants such as pesticides, and may be a less expensive and more natural way to deal with these chemical waste products (Kuritz & Wolk, 1995).

Cyanobacteria are also known to cause harmful algal blooms (Sharp *et al*., 2009). Toxins produced by the overabundance of certain cyanobacteria during these

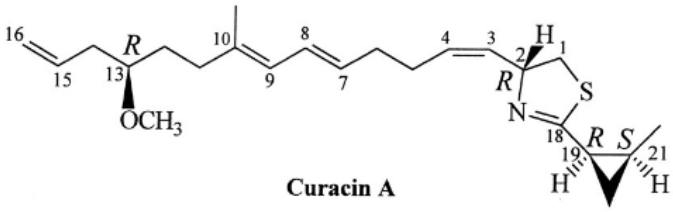
blooms are dangerous to human health as well as devastating for marine wildlife. These toxins are secondary metabolites produced by the cyanobacteria, presumably as a self-defense mechanism against predators (Nagle & Paul 1999; Ramaswamy *et al*., 2006). In order to predict and possibly prevent or reduce the occurrence of future harmful algal blooms, it is important to gain a better understanding of cyanobacteria and their secondary metabolite production.

Despite the destructiveness and negativity associated with cyanobacterial toxins, there are beneficial applications for these natural products. For example, the toxins could be used as a pest control agent. Cyanolide A (Figure 1.1), a potent molluscicide produced by the tropical marine cyanobacterium *Lyngbya bouillonii*, may be used to kill snails that are involved in the spread of schistosomiasis to humans (Pereira *et al*., 2010). Other toxins have medicinal uses. Curacin A, for example, which was extracted from another tropical marine cyanobacterium *Lyngbya majuscula*, has antimitotic and antiproliferative activities (Gerwick *et al*., 1994).

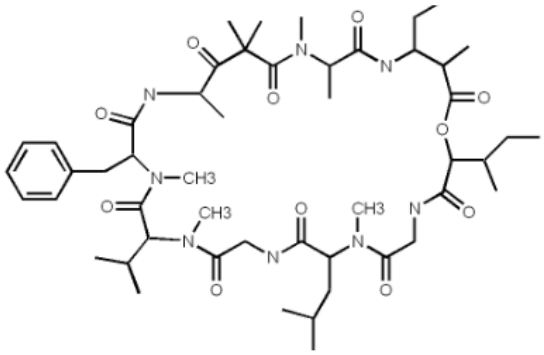
In addition to the natural products isolated directly from marine cyanobacteria, there are many natural products isolated from other marine organisms that are actually produced by symbiotic cyanobacteria (Proksch *et al.*, 2002). Marine sponges (Phylum Porifera) have received increasing attention in recent years due to the number of bioactive compounds isolated from them (Munro *et al*., 1999), but many of these compounds are actually produced by symbiotic microorganisms, including cyanobacteria. *Dysidea herbacea*, for example, hosts the symbiotic cyanobacterium *Oscillatoria spongeliae*, which produces multiple unusual polychlorinated and polybrominated compounds, some of which have antibiotic properties (Flatt *et al*.,



Dolastatin 10



Curacin A



Dolastatin 12

Figure 1.1: Dolastatin 10, curacin A, and dolastatin 12

2005). It is important to identify the true producer of bioactive compounds because harvesting these compounds directly from the source instead of the macroorganism will produce higher yields (Harrigan *et al*., 1998b). Another benefit of finding the source of natural products is that the biosynthetic pathway can be observed and syntheses of analogs can be modeled off of these pathways (Harrigan *et al*., 1998b).

Bioactive compounds isolated from cyanobacteria often inspire synthetic compounds that can be made to have improved biological properties over those of the natural product. One successful example is that of dolastatin 10 (Figure 1.1). This natural product was originally isolated from the marine mollusk *Dolabella auricularia*, but the source of the compound was traced back to the marine cyanobacterium *Symploca* sp. upon which the mollusc feeds (Luesch *et al*., 2001;Ramaswamy *et al*., 2006). Dolastatin 10's potent antitumor activity against leukemia cells inspired efforts to synthesize an analog known as Soblidotin, or TZT-1027, which has been used to treat lung and renal carcinoma in clinical trials (Ramaswamy *et al*., 2006). In recent trials, TZT-1027 was linked by modified peptides to monoclonal antibodies in order to target cancer cells (Mayer *et al*., 2010).

Filamentous tropical marine cyanobacteria produce bioactive secondary metabolites with many other potential applications, as mentioned earlier, including anti-cancer and/or anti-inflammatory treatments (Gerwick *et al,* 2008; Tan, 2009). When one strain of cyanobacteria with such properties is found, it may be of interest to identify and study related strains in order to find the same or similar compounds that result from the same or related biosynthetic pathways. One reason for doing so would be to find a common strain that produces the same compound as a rarer strain, so that it would be

easier to collect and study as well as less harmful to cyanobacterial biodiversity. Another scenario in which finding related strains would be helpful is if the strain producing the compound of interest was hard to culture – finding a culturable cyanobacteria that continues to produce the secondary metabolites of interest would provide a sustainable source of these natural products. Yet another benefit of being able to identify related strains is that they may produce variants or analogs of the molecule of interest, which may be more potent or have additional bioactive properties.

Unfortunately, identification of related strains is often difficult due to incorrect taxonomic identifications and nomenclature associated with these organisms. The traditional classification system of marine cyanobacteria is based on very few characteristics, such as cell width, cell length, and growth morphology, which are sometimes unreliable as they may change under different growth conditions (Willmotte *et al*., 1991). The traditional system often does not reveal their evolutionary origin because phenotypical similarities may arise from environmental factors and/or convergent evolution (Engene *et al*., 2010). In fact, the variability in morphology often results in the misidentification of many *Oscillatoria* as *Lyngbya*, giving the illusion that the group *Lyngbya* is richer in secondary metabolites than it really is (Engene *et al*., 2011). This thesis will attempt to show that not only is correct nomenclature important in a general sense, such as understanding the origins and evolutions of natural product pathways in cyanobacteria, but it may also serve as a tool for locating bioactive compounds of interest.

Previous efforts have been made to group cyanobacteria based on chemotaxonomic information. Much of this work has focused on characterization of

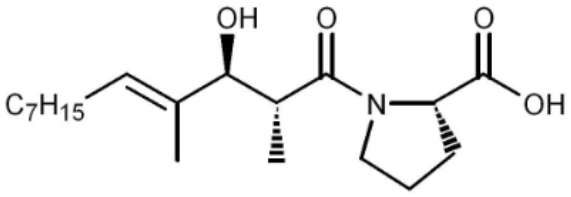
lipids and carotenoids, but recent work has been done to construct chemotypes based on secondary metabolites. In a study on *Lyngbya* spp. and *Symploca* spp., a "phylogenetic" tree based on chemical characteristics was constructed and compared to a tree based on the 16S gene sequence (Thacker & Paul, 2004). The tree based on chemotypes was not as high resolution, and there was a basal polytomy in the *Lyngbya* clade. However, there was a distinct separation between the *Symploca* and *Lynbgya* groups. This shows that chemotaxonomy may be a useful tool when used in combination with morphology and phylogeny, but a chemical profile alone is not sufficient to distinguish between closely-related strains. Although it might not be possible to use chemotaxonomy to predict evolutionary relationships among strains, it might be possible to do the reverse, since the 16S tree yielded a higher resolution phylogeny than the tree based on chemotypes. This thesis will test this hypothesis.

In order for phylogeny-guided secondary metabolite prediction to be effective, a very straightforward approach must be used in order to maximize ease and efficiency of the method. The purpose of this study was to show that given a previously described secondary metabolite and the cyanobacterium that produces it, it is possible to use phylogenetics to identify related strains that produce the same or similar compounds., In this study, a simple phylogenetic tree based on the 16S rRNA gene was used to determine relationships among tropical marine cyanobacteria. Then liquid chromatography-mass spectrometry (LCMS) was used to screen for previously described secondary metabolites. Complete chemical profiles of all the strains were not constructed, as that would have been difficult and time-consuming, and the point of this project was not to identify every secondary metabolite produced but to demonstrate the

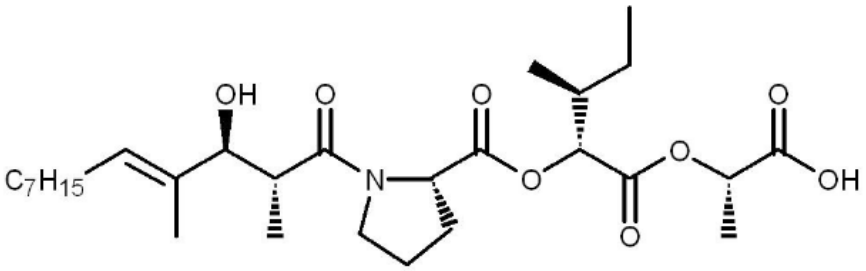
efficient identification of a single compound of interest – that is, a compound whose structure and mass are known, and whose biosynthetic origin of production (i.e. strain of the producing cyanobacterium) has been confirmed by other techniques.

Previous research in the Gerwick laboratory had revealed that a group of 4 strains of *Oscillatoria margaritifera* from Curaçao produces dolastatin 12 and the tumonoic acids A-C (Figure 1.2a) as well as E-G (Figure 1.2b) (Choi, 2011; Engene *et al.* 2010). Tumonoic acids were first discovered in an assemblage of *Lyngbya majuscula* and *Schizothrix calcicola* collected from Tumon Bay in Guam (Harrigan *et* al., 1999). Tumonoic acids A and D-I showed no activity in anticancer, antimalarial, anti-Chagas, antileishmania, or antimicrobial assays, except for tumonoic acid I, which had moderate antimalarial activity (Clark *et al.*, 2008). However, derivatives of these tumonoic acids may have bioactivity. For example, ethyl tumonoate A, a derivative of tumonoic acid A produced by cf. *Oscillatoria margaritifera* from Curaçao, displays anti-inflammatory activity and inhibitory activity of calcium oscillations in neocortical neurons (Engene *et al*., 2011).

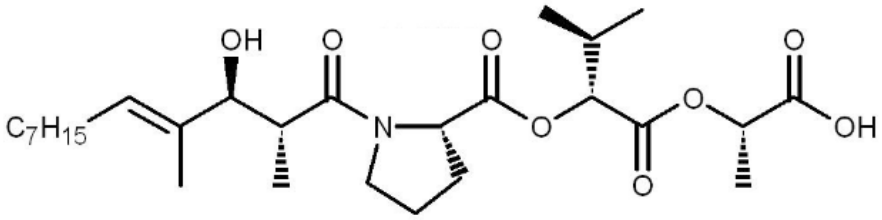
The dolastatins are a family of peptides isolated from the sea hare *Dolabella auricularia*. They inhibit cell proliferation and induce apoptosis in tumor and cancer cells by interfering with tubulin formation during mitosis of malignant cells. The most potent of the currently known dolastatins are dolastatin 10 and 15 (Amador *et al*., 2003), both produced by species of *Symploca* (Luesch *et al*., 2001). These are extremely cytotoxic molecules, showing exceptional anticancer and antitumor activity (Ramaswamy *et al*., 2006; Bai *et al*., 1992). As expected, natural and synthetic analogs and derivatives of these dolastatins have also shown high levels of cytotoxicity



Tumonoic acid A

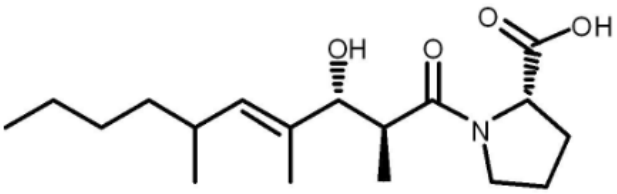


Tumonoic acid B

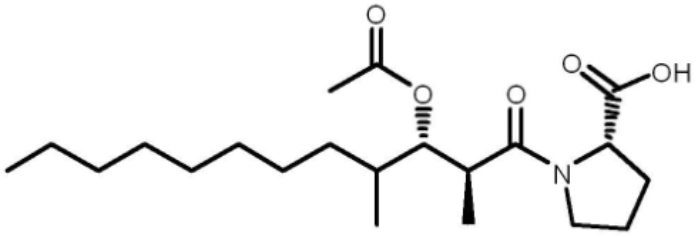


Tumonoic acid C

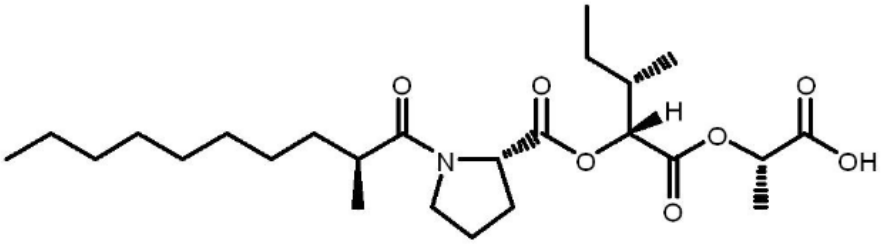
Figure 1.2a: Tumonoic acids A-C



Tumonoic acid E



Tumonoic acid F



Tumonoic acid G

Figure 1.2b: Tumonoic acids E-G

(Ramaswamy *et al*., 2006; Luesch *et al*., 2001). Dolastatin 12 (Figure 1.1), which was isolated from an assemblage of *Lyngbya majuscula* and *Schizothrix calcicola* (the same assemblage which yielded the tumonoic acids discussed above), is a potent disrupter of microfilament networks and has moderate antitumor activity (Harrigan *et al*., 1998a).

Dolastatin 12 and the tumonoic acids described above and their known producers were used as a starting point for this project. The central hypothesis of this study was that strains which were closely related to these *O. margaritifera* would also produce dolastatin 12 and tumonoic acids, while strains that were more distant would not.

Isolation and structural characterization of dolastatin 12 from *O. margaritifera* was performed by Hyukjae Choi, Ph.D, and referenced here with his permission.

Results and Discussion

The purpose of this study was to see if a simple 16S tree could be used to predict which strains would produce compounds similar to those in sister taxa. A clade of strains from Curaçao (cf. *Oscillatoria margaritifera*), as well as one closely-related strain from Papua New Guinea, were known to produce dolastatin 12 and tumonoic acids A-C and E-G (Choi, 2011; Engene *et al.* 2010). In addition, PAB-21-JUN-06-1 was known to produce tumonoic acids A and D-I (Clark, *et al*. 2008). Strains that were in the same clade as the known producers were predicted to produce the same or similar compounds while the rest of the strains were predicted not to produce them. With the exception of two strains, there was a direct correlation between phylogeny and natural product production. Figure 2.1 shows the compounds detected by LCMS and the strains that produced them.

Of the 27 strains analyzed, only 17 of them had sufficient sized samples to be analyzed for chemical constituents, and the rest of the samples were either lost or used up in other assays. Of these 17 samples, only 11 had enough material to perform LCMS analysis on VLC fractions, and thus only the crude extracts of the remaining 6 were analyzed. Table 2.1 shows which strains were analyzed by LCMS.

Of all the known compounds potentially of interest in this study, only two were detected successfully: tumonoic acid B and dolastatin 12. Although PNG-21-MAY-05-4 and the four strains of cf. *O. margaritifera* were confirmed to produce at least 6 tumonoic acids in a previous study (Engene *et al*. 2010), only tumonoic acid B was detected in the LCMS traces in this study. Tumonoic acid F was detected in NAC-20-DEC-08-7 which was one of the original confirmed producers. However, because

Figure 2.1: Compounds detected by LCMS and strains that produced them. This is the same phylogenetic tree shown in Figure 2.1. Strains found to produce tumonoic acid B are indicated by green squares next to their collection codes. Strains found to produce dolastatin 12 are indicated by red squares. Strains analyzed by LCMS but not found to produce either are indicated by blue squares. Unmarked strains were not analyzed by LCMS in this study.

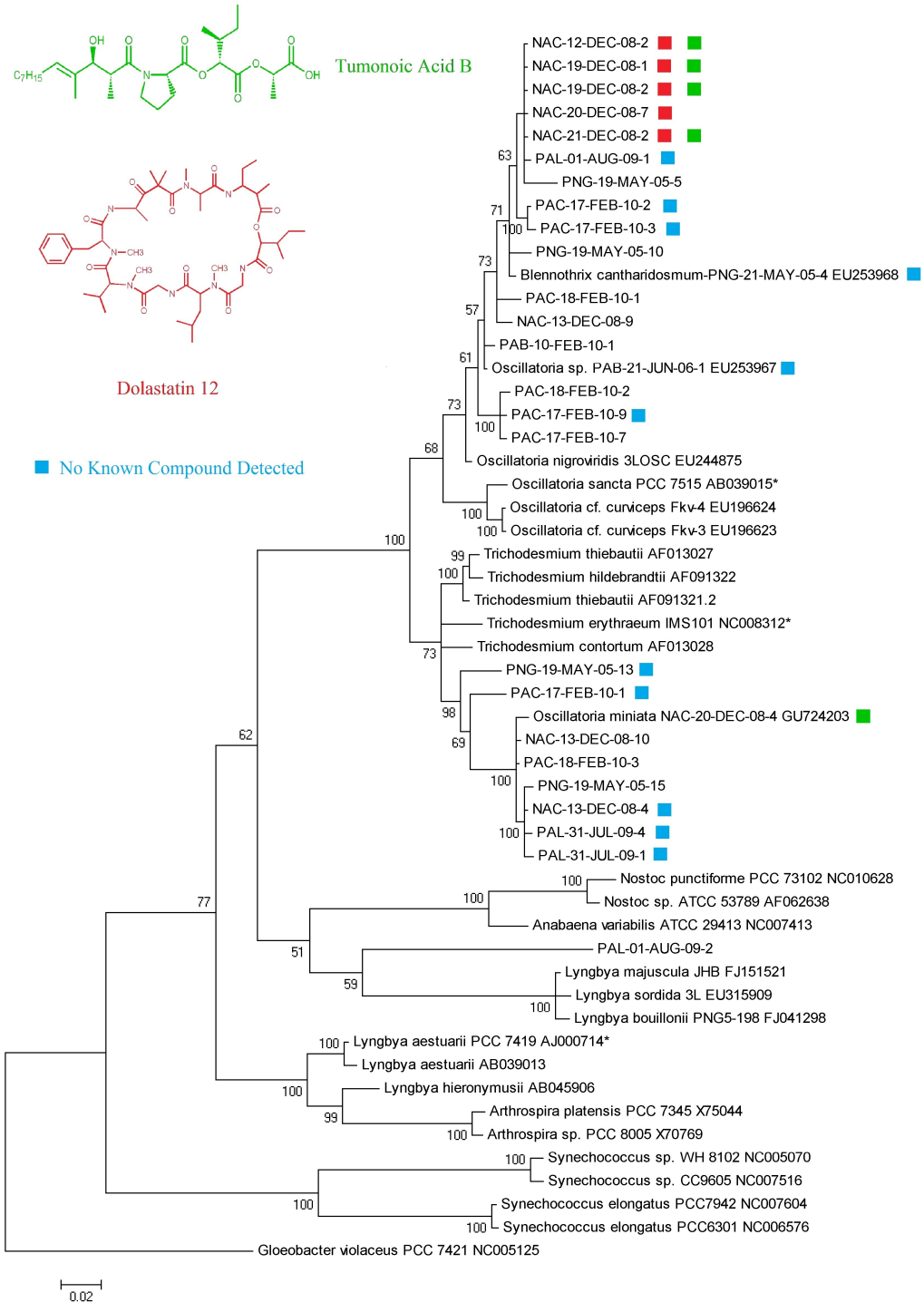

Tumonoic Acid B
Dolastatin 12
No Known Compound Detected
NAC-12-DEC-08-2
NAC-19-DEC-08-1
NAC-19-DEC-08-2
NAC-20-DEC-08-7
NAC-21-DEC-08-2
PAL-01-AUG-09-1
PNG-19-MAY-05-5
PAC-17-FEB-10-2
PAC-17-FEB-10-3
PNG-19-MAY-05-10
Blennothrix cantharidosmum-PNG-21-MAY-05-4 EU253968
PAC-18-FEB-10-1
NAC-13-DEC-08-9
PAB-10-FEB-10-1
Oscillatoria sp. PAB-21-JUN-06-1 EU253967
PAC-18-FEB-10-2
PAC-17-FEB-10-9
PAC-17-FEB-10-7
Oscillatoria nigroviridis 3LOSC EU244875
Oscillatoria sancta PCC 7515 AB039015*
Oscillatoria cf. curviceps Fkv-4 EU196624
Oscillatoria cf. curviceps Fkv-3 EU196623
Trichodesmium thiebautii AF013027
Trichodesmium hildebrandtii AF091322
Trichodesmium thiebautii AF091321.2
Trichodesmium erythraeum IMS101 NC008312*
Trichodesmium contortum AF013028
PNG-19-MAY-05-13
PAC-17-FEB-10-1
Oscillatoria miniata NAC-20-DEC-08-4 GU724203
NAC-13-DEC-08-10
PAC-18-FEB-10-3
PNG-19-MAY-05-15
NAC-13-DEC-08-4
PAL-31-JUL-09-4
PAL-31-JUL-09-1
Nostoc punctiforme PCC 73102 NC010628
Nostoc sp. ATCC 53789 AF062638
Anabaena variabilis ATCC 29413 NC007413
PAL-01-AUG-09-2
Lyngbya majuscula JHB FJ151521
Lyngbya sordida 3L EU315909
Lyngbya bouillonii PNG5-198 FJ041298
Lyngbya aestuarii PCC 7419 AJ000714*
Lyngbya aestuarii AB039013
Lyngbya hieronymusii AB045906
Arthrospira platensis PCC 7345 X75044
Arthrospira sp. PCC 8005 X70769
Synechococcus sp. WH 8102 NC005070
Synechococcus sp. CC9605 NC007516
Synechococcus elongatus PCC7942 NC007604
Synechococcus elongatus PCC6301 NC006576
Gloeobacter violaceus PCC 7421 NC005125
0.02

Table 2.1: LCMS analysis of extracts used in this study.

Collection Code	Crude Extract	VLC Fractions
NAC-12-DEC-08-2	YES	YES
NAC-13-DEC-08-4	YES	YES
NAC-13-DEC-08-9	NO	NO
NAC-13-DEC-08-10	NO	NO
NAC-19-DEC-08-1	YES	NO
NAC-19-DEC-08-2	YES	YES
NAC-20-DEC-08-4	YES	NO
NAC-20-DEC-08-7	YES	YES
NAC-21-DEC-08-2	YES	NO
PAB-21-JUN-06-1	YES	NO
PAC-17-FEB-10-1	NO	YES
PAC-17-FEB-10-2	YES	YES
PAC-17-FEB-10-3	YES	YES
PAC-17-FEB-10-7	NO	NO
PAC-17-FEB-10-9	YES	YES
PAC-18-FEB-10-1	NO	NO
PAC-18-FEB-10-2	NO	NO
PAC-18-FEB-10-3	NO	NO
PAL-01-AUG-09-1	YES	YES
PAL-01-AUG-09-2	NO	NO
PAL-31-JUL-09-1	YES	YES
PAL-31-JUL-09-4	YES	YES
PNG-19-MAY-05-5	NO	NO
PNG-19-MAY-05-10	NO	NO
PNG-19-MAY-05-13	YES	NO
PNG-19-MAY-05-15	YES	NO
PNG-21-MAY-05-4	YES	NO

tumonoic acid F was not detected in any of the other strains, these data were not included in the Figure 2.1 for the purpose of clarity. All but tumonoic acid B evaded detection (except for tumonoic acid F as described below), so it was the only one used as a "known" compound in this project.

Detection of secondary metabolites depended on several factors including the amount of compound present, the ability of the molecule to be ionized, and the solubility of the compound in solvents used to extract and dissolve it. Lack of detection of a compound does not confirm its absence. Only tumonoic acid B was detected in samples from strains known to produce several tumonoic acids, so it is possible that these compounds were produced but undetected in the other strains analyzed in this study as well. Tumonoic acid B was the most abundant secondary metabolite in the strains in which was discovered (Harrigan *et al*., 1999), so the successful detection of tumonoic acid B was probably due to its relative abundance in the strains from this current study. Tumonoic acids were not detected in the oldest sample analyzed in this study, PNG-21-MAY-05-4, a known producer of several tumonoic acids (Clark *et al*., 2008), which suggests that these bioactive compounds (including tumonoic acid B) may have decayed over time. It is also possible that the tumonoic acids (and/or dolastatin 12) evaded detection because they were present as derivatives such as ethyl tumonoates (Engene *et al*. 2011).

All 27 strains used in this study were selected to be of the *Oscillatoria* morphotype described in *Bergey's Manual* (Castenholtz *et al.*, 2001). The measurements of the 27 strains are presented in Table 2.2 Measurements that are listed as "NA" ("not available") were from strains that were preserved badly or too damaged to take accurate

Table 2.2: Morphological characterization of 27 strains of cf. *Oscillatoria*. Filament widths were taken from three separate filaments and averaged. Cell measurements were taken from 10 adjacent cells from three different filaments. Fields marked "NA" indicate that the specimens were not in good condition and could not be measured accurately.

Collection Code	Filament Width (µm)	Cell Length (µm)	Cell Width (µm)	Cell width/length ratio (µm)	Cell Wall Constriction	Apical Cells
NAC-12-DEC-08-2	20	4.0	19.0	4.75	6.33%	rounded
NAC-13-DEC-08-4	24	3.0	22.0	7.33	4.00%	rounded
NAC-13-DEC-08-9	23	3.0	22.0	7.33	2.50%	rounded
NAC-13-DEC-08-10	25	4.0	22.0	5.50	4.66%	rounded
NAC-19-DEC-08-1	21	2.3	20.0	8.70	6.66%	rounded
NAC-19-DEC-08-2	21	4.0	20.0	5.00	4.00%	rounded
NAC-20-DEC-08-4	22	3.0	21.0	7.00	2.66%	rounded
NAC-20-DEC-08-7	20	2.6	15.0	5.77	1.00%	rounded
NAC-21-DEC-08-2	38	3.0	35.0	11.67	<0.5%	rounded
PAB-21-JUN-06-1	NA	NA	NA	NA	NA	rounded
PAC-17-FEB-10-1	22	NA	NA	NA	NA	rounded
PAC-17-FEB-10-2	20	2.7	18.0	6.67	<0.5%	rounded
PAC-17-FEB-10-3	21	3.3	20.0	6.06	<0.5%	rounded
PAC-17-FEB-10-7	NA	NA	NA	NA	<0.5%	rounded
PAC-17-FEB-10-9	25	NA	NA	NA	<0.5%	rounded
PAC-18-FEB-10-1	25	5.5	25.5	4.64	<0.5%	rounded
PAC-18-FEB-10-2	28	4.1	27.0	6.59	<0.5%	rounded
PAC-18-FEB-10-3	21	6.4	19.5	3.05	<0.5%	rounded
PAL-01-AUG-09-1	36	3.2	30.2	9.44	<0.5%	rounded
PAL-01-AUG-09-2	NA	NA	NA	NA	NA	rounded
PAL-31-JUL-09-1	41	3.3	31.5	9.55	4.70%	rounded
PAL-31-JUL-09-4	43	5.3	33.0	6.23	4.60%	rounded
PNG-19-MAY-05-5	NA	NA	NA	NA	NA	NA
PNG-19-MAY-05-10	NA	NA	NA	NA	NA	NA
PNG-19-MAY-05-13	19	2.2	17.0	7.73	NA	rounded
PNG-19-MAY-05-15	NA	NA	NA	NA	NA	NA
PNG-21-MAY-05-4	NA	NA	NA	NA	NA	rounded

measurements.

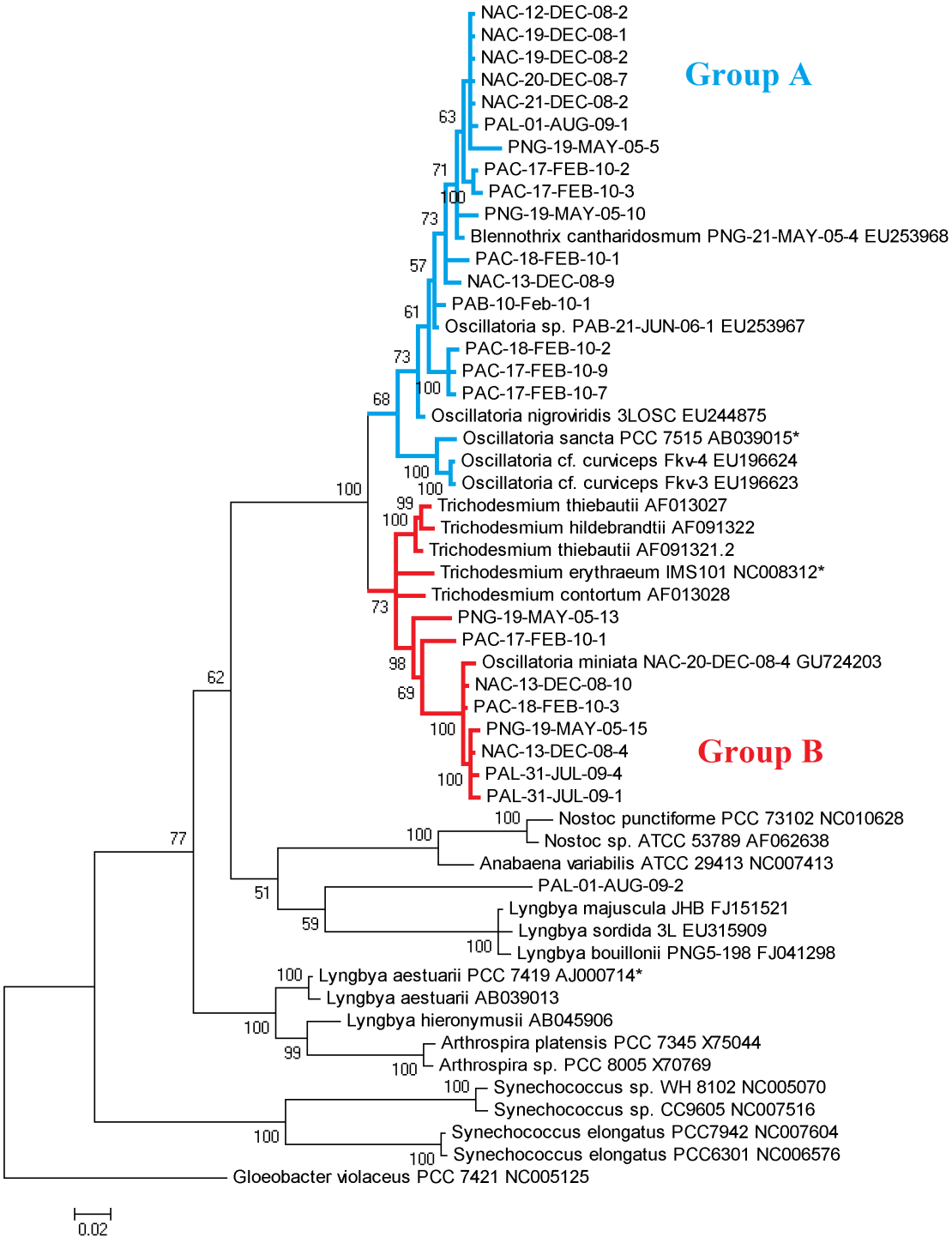
According to the 16S rRNA tree, the 27 strains split into 2 main groups (see Figure 2.2). The first group (which will be referred to as "Group A") is closely related to the true *Oscillatoria* clade (type-strain *Oscillatoria sancta* PCC 7515 GenBank acc. Nr. AB039015) while the second group (which will be referred to as "Groups B") is more closely related to the *Trichodesmium* group (type-strain *Trichodesmium erythraeum* IMS101 GenBank acc. Nr. NC008312). This means that the *Oscillatoria* morphotype is polyphyletic and should be revised. Group A may be still be considered *Oscillatoria* because strains in that group are in the same clade as the type-strain. As for Group B, it could either be renamed as *Trichodesmium,* or established as a new group.

There does not appear to be any significant morphological characteristic distinguishing Group A from Group B strains. There also does not to be any significant similarities among strains from the same geographic location. The fact that strains from Group A could not be distinguished from those from Group B illustrates the inadequacy of morphology-based taxonomic systems to identify new strains.

According to the LCMS data, NAC-12-DEC-08-2 produces dolastatin 12 as predicted by its phylogeny. However, neither of the Panamanian strains (PAC-17-FEB-10-2 and PAC-17-FEB-103) produced it. None of the other analyzed strains were found to produce dolastatin 12, which was also predicted, but it is also possible that it was undetected because the compound may exist as a closely related analog or derivative, or the compound is produced in levels too low to be detected by the equipment employed.

Tumonoic acid B was detected in NAC-12-DEC-08-2 as expected, but it was also detected in evolutionarily distant NAC-20-DEC-08-4. Another surprising finding

Figure 2.2: Maximum-likelihood (PhyML) phylogenetic inference of 27 strains of cf. *Oscillatoria* based on the SSU (16S) rRNA gene. The clade highlighted in blue is Group A and the clade highlighted in red is Group B. The evolutionarily distant *Gloebacter violaceus* PCC 7421 was used as an outgroup. Bootstrap values displayed are from 1,000 replicates. The scale bar is 0.02 substitutions per nucleotide position.


NAC-12-DEC-08-2
NAC-19-DEC-08-1
NAC-19-DEC-08-2
NAC-20-DEC-08-7
NAC-21-DEC-08-2
PAL-01-AUG-09-1
PNG-19-MAY-05-5
PAC-17-FEB-10-2
PAC-17-FEB-10-3
PNG-19-MAY-05-10
Blennothrix cantharidosmum PNG-21-MAY-05-4 EU253968
PAC-18-FEB-10-1
NAC-13-DEC-08-9
PAB-10-Feb-10-1
Oscillatoria sp. PAB-21-JUN-06-1 EU253967
PAC-18-FEB-10-2
PAC-17-FEB-10-9
PAC-17-FEB-10-7
Oscillatoria nigroviridis 3LOSC EU244875
Oscillatoria sancta PCC 7515 AB039015*
Oscillatoria cf. curviceps Fkv-4 EU196624
Oscillatoria cf. curviceps Fkv-3 EU196623
Trichodesmium thiebautii AF013027
Trichodesmium hildebrandtii AF091322
Trichodesmium thiebautii AF091321.2
Trichodesmium erythraeum IMS101 NC008312*
Trichodesmium contortum AF013028
PNG-19-MAY-05-13
PAC-17-FEB-10-1
Oscillatoria miniata NAC-20-DEC-08-4 GU724203
NAC-13-DEC-08-10
PAC-18-FEB-10-3
PNG-19-MAY-05-15
NAC-13-DEC-08-4
PAL-31-JUL-09-4
PAL-31-JUL-09-1
Nostoc punctiforme PCC 73102 NC010628
Nostoc sp. ATCC 53789 AF062638
Anabaena variabilis ATCC 29413 NC007413
PAL-01-AUG-09-2
Lyngbya majuscula JHB FJ151521
Lyngbya sordida 3L EU315909
Lyngbya bouillonii PNG5-198 FJ041298
Lyngbya aestuarii PCC 7419 AJ000714*
Lyngbya aestuarii AB039013
Lyngbya hieronymusii AB045906
Arthrospira platensis PCC 7345 X75044
Arthrospira sp. PCC 8005 X70769
Synechococcus sp. WH 8102 NC005070
Synechococcus sp. CC9605 NC007516
Synechococcus elongatus PCC7942 NC007604
Synechococcus elongatus PCC6301 NC006576
Gloeobacter violaceus PCC 7421 NC005125
Group A
Group B
0.02

was that tumonoic acid was not detected in PAL-01-AUG-09-1. However, on the basis of LCMS data, it appears to produce tumonoic acid E. Unfortunately, tumonoic acid E wasn't detected in any of its confirmed producers, so there were no LCMS scans to use as a reference to confirm its presence in PAL-01-AUG-09-1. No other strains were found to produce tumonoic acid B. Again, as with dolastatin 12 or any other compound, it is possible that a strain may produce tumonoic acid B but in such low levels that this was undetected by the mass spectrometer.

The most interesting exception to the trends seen in the other strains (i.e. that closely-related strains produced similar compounds) was that NAC-20-DEC-08-4 produced tumonoic acid B despite its genetic distance from the rest of the tumonoic acid producers. NAC-20-DEC-08-4 was from Curaçao like 4 of the other known tumonoic acid producers and chemotype similarity among samples from the same location was also observed in another study on *Lyngbya majuscula* (Thacker & Paul, 2004), so this was not a surprise. The relationship between chemotype and geographic location may suggest that lateral gene transfer could be a mechanism by which it acquired this ability, but this is unlikely because tumonoic acids were found in cyanobacteria from Tumon Bay, Guam (Harrigan *et al*., 1999) and Bocas del Toro, Panama, which are geographically distant from Curaçao, making horizontal gene transfer improbable.

If horizontal transfer is not the mechanism, then the only other explanation (aside from sample contamination, which is unlikely) is that the pathway for tumonoic acid production is very ancient and that NAC-20-DEC-08-4 and the original known producers must have shared a common ancestor that possessed this pathway. One way to test this hypothesis would be to try to find one of the biosynthetic genes involved in

the pathway and see if it is present in all the descendents of that common ancestor. However, the focus of this study was to see if a quick and simple 16S rRNA phylogeny could be used to predict secondary metabolite production. A future study might involve analysis of the natural product pathways as well as a screening of the *Trichodesmium* and *Oscillatoria* type-strain clades for tumonoic acid production (since they would be descendents of the same common ancestor that produced the tumonoic acids).

Another possible explanation for the exception of NAC-20-DEC-08-4 is that it was growing in an assemblage of cyanobacteria but that was mostly comprised of NAC-20-DEC-08-4 so that the other strains, which in this case would presumably be making the tumonoic acids, were undetected by microscopy (in which only a small sample was examined). If there was only a small amount of this other strain, it 16S DNA would have been overshadowed by that of NAC-20-DEC-08-4 during competition for primers in the PCR step, thus evading detection during the sequencing step when multiple clones were submitted for proofreading.

Besides using phylogenetics to predict secondary metabolite production, it can also be used (perhaps in conjunction with chemotaxonomy) to establish unambiguous nomenclature. The need for a naming system that is not completely dependent on morphology can be seen in the results of this study. The *Blennothrix catharidosmum* in which the tumonoic acids were isolated (Clark *et al*., 2008) belongs to Group A, meaning that this "*Blennothrix*" is actually an *Oscillatoria*. Revising the current naming system will lead to a better understanding of true sources of natural products, which will guide research efforts to find new natural products and better understand the origins and evolution of these natural product pathways.

Another useful application of chemotaxonomy would be to determine secondary metabolite producers in assemblages of cyanobacteria. Many interesting compounds are isolated from assemblages of several cyanobacteria and it is difficult to determine which of the strains is producing the compound of interest. For example, dolastatin 12 was extracted from an assemblage of *Lyngbya majuscula* and *Schizothrix calcicola*. Neither of these strains was used in this study, but it is expected that the strain that is more closely related to the other dolastatin 12 producers is the likely true producer. This could be a useful tool when selecting individual strains for culture and harvest of their natural products.

This study has shown that there is little or no correlation between secondary metabolites produced and morphology. This finding is in agreement with a previous study on phylogeny of tropical marine cyanobacteria focusing on the *Lyngbya* morphotype (Engene *et al*., 2011). Also, as predicted, there was a correlation between phylogeny and secondary metabolites produced. Considering that 15 of the 17 analyzed strains were correctly predicted to produce or not produce tumonoic acid B and dolastatin 12, and that there were only 2 exceptions to this trend, this phylogeny-guided approach to natural products discovery remains an effective although not guaranteed method.

Isolation and structural characterization of dolastatin 12 from *O. margaritifera* was done by Hyukjae Choi, Ph.D, and referenced here with his permission.

Materials and Methods

Field Collection

Samples were collected by snorkeling or shallow-water SCUBA from Panama, Curaçao, Palmyra, and Papua New Guinea from 2005 to 2010 (see Table 3.1). Genetic samples were stored in RNA*later*® (Ambion Inc.) while environmental samples were stored in a 1:1 mixture of EtOH:H_2O. Both were kept frozen at -20°C.

Microscopy

Genetic samples were thawed and rinsed with SWBG-11 and visible debris and organisms were removed with sterile tweezers under an Olympus VMZ dissecting microscope.

An Olympus IX51 epifluorescent microscope (1000X) equipped with an Olympus U-CMAD3 camera was used to photograph and identify the samples. Samples were selected for this study if they were identified as *Oscillatoria* type according to *Bergey's Manual* (Castenholtz *et al.*, 2001). Some of the samples (particularly the older ones) were in poor condition and were not identifiable by microscopy, so they were selected for this study if they were reported to be *Oscillatoria* morphotypes according to field collection notes. However, if they were found to be genetically distant after sequencing, they were left out of this study.

Measurements of each strain were taken (presented in Table 2.2). Measurements of cell width, cell length, and cell wall constriction of three filaments of each strain were taken and averaged.

Table 3.1: Collection locations and depths. NAC codes indicate collections from Curaçao. PAB and PAC codes indicate collections from Panama in Bocas del Toro and Coiba, respectively. PAL codes indicate collections from Palmyra, and PNG codes indicated collections from Papua New Guinea. Depths are given in feet. "Snorkel" indicates depths of approximately 1-4 ft. "NA" indicates that the collection depth was not recorded.

Collection Code	Collection Site	Depth (ft.)
NAC-12-DEC-08-2	Curaçao – south of Carmabi Station	50
NAC-13-DEC-08-4	Curaçao – Playa Kalki	50-55
NAC-13-DEC-08-9	Curaçao – Playa Kalki	Snorkel
NAC-13-DEC-08-10	Curaçao – Grote Knip	Snorkel
NAC-19-DEC-08-1	Curaçao – Marie Pampoen	Snorkel
NAC-19-DEC-08-2	Curaçao – Caracas Bay	15-25
NAC-20-DEC-08-4	Curaçao – Caracas Bay	NA
NAC-20-DEC-08-7	Curaçao – Pierbaai Reef	20-25
NAC-21-DEC-08-2	Curaçao – Jan Theil Beach	9-10
PAB-21-JUN-06-1	Panama – Bocas del Toro	NA
PAC-17-FEB-10-1	Panama – Coiba – Isla Afuera Channel	Snorkel
PAC-17-FEB-10-2	Panama – Coiba – Isla Leones	Snorkel
PAC-17-FEB-10-3	Panama – Coiba – Isla Leones	Snorkel
PAC-17-FEB-10-7	Panama – Coiba – Isla Leones	Snorkel
PAC-17-FEB-10-9	Panama – Coiba – Isla Leones	Snorkel
PAC-18-FEB-10-1	Panama – Coiba – Isla Uvas	Snorkel
PAC-18-FEB-10-2	Panama – Coiba – Isla Uvas	Snorkel
PAC-18-FEB-10-3	Panama – Coiba – Isla Uvas	Snorkel
PAL-01-AUG-09-1	Palmyra – north beach of Cooper Island	Snorkel
PAL-01-AUG-09-2	Palmyra – north beach of Cooper Island	Snorkel
PAL-31-JUL-09-1	Palmyra – north beach of Cooper Island	Snorkel
PAL-31-JUL-09-4	Palmyra – north beach of Cooper Island	Snorkel
PNG-19-MAY-05-5	Papua New Guinea – Pigeon Island	10-30
PNG-19-MAY-05-10	Papua New Guinea – Credner Islands	25
PNG-19-MAY-05-13	Papua New Guinea – S4°14.105' E 152°25.605'	3-4
PNG-19-MAY-05-15	Papua New Guinea – S4°14.105' E 152°25.605'	3-4
PNG-21-MAY-05-4	Papua New Guinea – S4°07.270' E152°26.666'	30

DNA-isolation, PCR-amplification and Cloning

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Inc.) following the manufacturer's specifications. DNA concentration and purity were measured using a DU® 800 spectrophotometer (Beckman Coulter Inc.).

The PCR reaction volumes consisted of 1µL (~50ng) of DNA, 1µL of each primer (10µM), 12.5µL of Master Mix M750B (Promega Inc.), and 10.5µL dH_2O, for a total reaction volume of 25µL. The PCR reactions were performed in an Eppendorf® Mastercycler® gradient as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification: 20s at 95°C, 20s at 50°C, and 1.5 min at 72°C; and final elongation for 3 min at 72°C. PCR products were purified using a MinElute® PCR Purification Kit (Qiagen Inc.) before subcloning into competent *E. coli* using the pGEM®-T Easy Vector Systems PCR Cloning Kit (Invitrogen Inc.), according to the manufacturer's instructions. Plasmid DNA was isolated using a QIAprep® Spin Miniprep Kit (Qiagen Inc.) and sequenced bidirectionally with M13 vector primers. Plasmid DNA from two different colonies were submitted for sequencing and compared to ensure that there were no proofreading errors, and also to confirm that the 16S gene of a single strain was amplified (in cases the cyanobacteria grew in assemblages of more than one *Oscillatoria*).

Phylogenetic Inference

The 16S rRNA genes of the 27 selected strains, as well as 26 type-strains and reference strains selected from *Bergey's Manual* (Castenholz, 2001) were used in the phylogenetic analysis. The unicellular *Gloeobacter violaceus* PCC 7421 (GenBank acc.

Nr. NC005125) was included as an evoluntionarily distant out-group. The PCR products of the 27 tropical marine strains were about 1400bp, but due to the lack of complete 16S rRNA sequences from related species and type-strains in online databases (NCBI, GenBank), a 1080bp truncated region was used for the alignment. Gene sequences were aligned in MEGA5 using ClustalW with default parameters for gap opening and extension penalities. A maximum likelihood (PhyML) phylogenetic tree (Figure 2.2) was in TOPALi v2.5 using the GTR model. Bootstrap values were obtained from 1,000 replicates.

Chemistry Extraction

Environmental samples were extracted several times with a 2:1 mixture of dichloromethane:methanol (DCM:MeOH). A portion of each crude extract was separated into nine fractions by vacuum liquid chromatography (VLC) using progressively more polar mixtures of hexanes, ethyl acetate, and methanol.

Liquid Chromatography Mass Spectrometry (LCMS)

Volumes of crude extracts as well as VLC fractions were dried down and redissolved in acetonitrile (ACN) to a 1 mg/mL concentration. LCMS was performed with a Finnigan LCQ Advantage Max spectrometer with a Finnigan Surveyor HPLC system equipped with Thermo Finnigan Surveyor PDA plus detector. HPLC was performed using a Waters 515 pump and a Waters 996 photodiode array detector.

Analysis of LCMS Data

Usually without performing tandem mass spectrometry (MS-MS), it would be impossible to determine the presence of a particular compound, but since several strains were already confirmed by MS-MS to produce dolastatin 12 and tumonoic acids in previous studies (Clark *et al*., 2009; Engene *et al*., 2010; Choi, 2011), it was possible to determine the identity of compounds based solely on molecular weight and retention time. LCMS traces of known producers of dolastatin 12 and tumonoic acids were used as references to find other strains that produced the same compounds. An example of detection of dolastatin 12 in NAC-12-DEC-08-2 using known producer NAC-19-DEC-08-2 as a reference is shown in Figure 3.1.

Figure 3.1: Detection of dolastatin 12 in NAC-12-DEC-08-2 using NAC-19-DEC-08-2 as a reference.

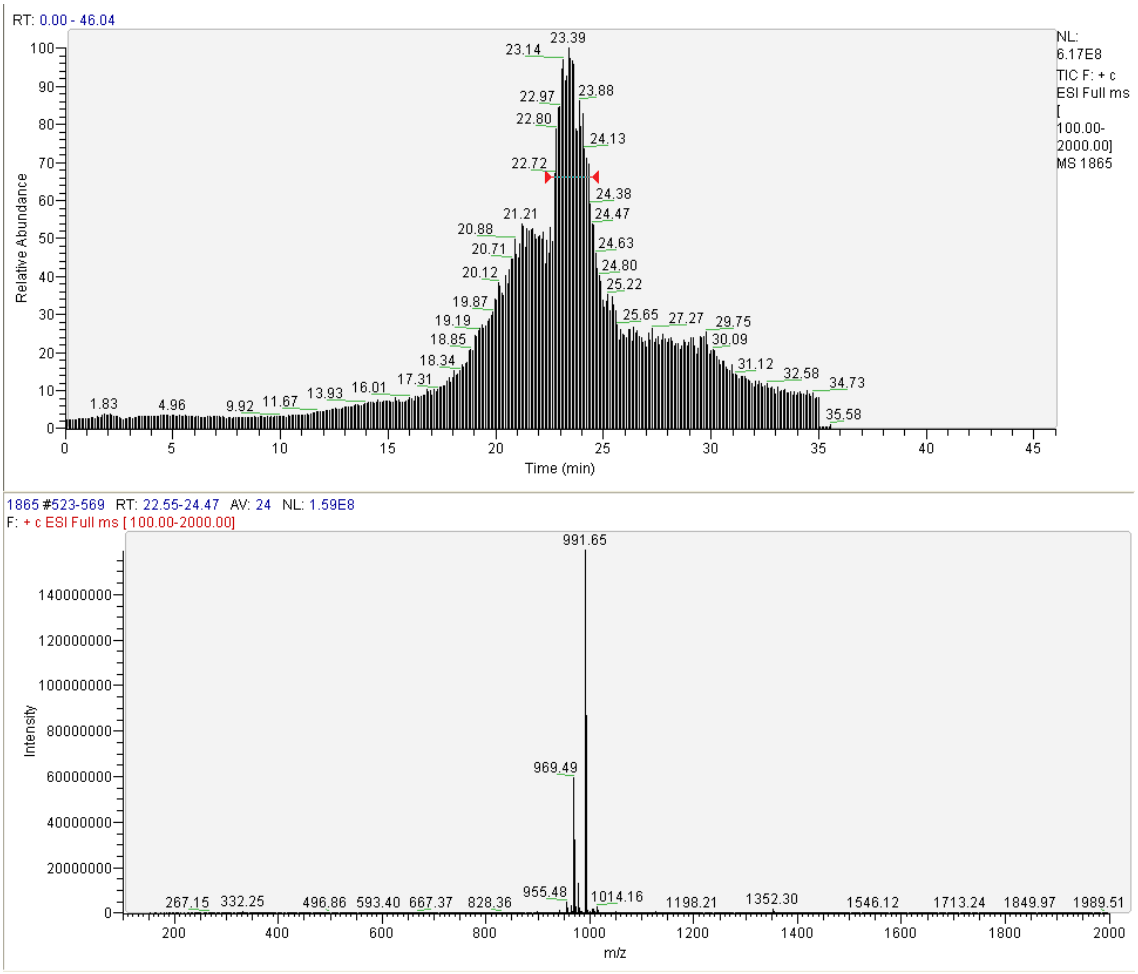
(A) and (B) are positive ion mode LCMS traces (so only molecules that can carry a positive charge are shown). The top frames of both (A) and (B) show the relative abundance of material in the column over time. The bottom frames show the molecular weights of the compounds present and the amounts of each compound present, at the time in which the most material accumulated in the column (i.e. the main peak), which is marked by red arrows in the top frames.

Dolastatin 12 (m/z = 968.59) is seen in both cases ionized as either the H+ adduct (m/z = 968.59 + 1) or Na+ adduct (m/z = 968.59 + 23). The similar retention times (approximately 22 to 25 minutes), indicate that these are most likely the same compound.

(A) NAC-19-DEC-08-2

(B) NAC-12-DEC-08-2

A.


RT: 0.00 - 46.04
Relative Abundance
Time (min)
NL: 6.17E8 TIC F: + c ESI Full ms [100.00-2000.00] MS 1865
1865 #523-569 RT: 22.55-24.47 AV: 24 NL: 1.59E8
F: + c ESI Full ms [100.00-2000.00]
Intensity
m/z
991.65
969.49
955.48
1014.16

B.

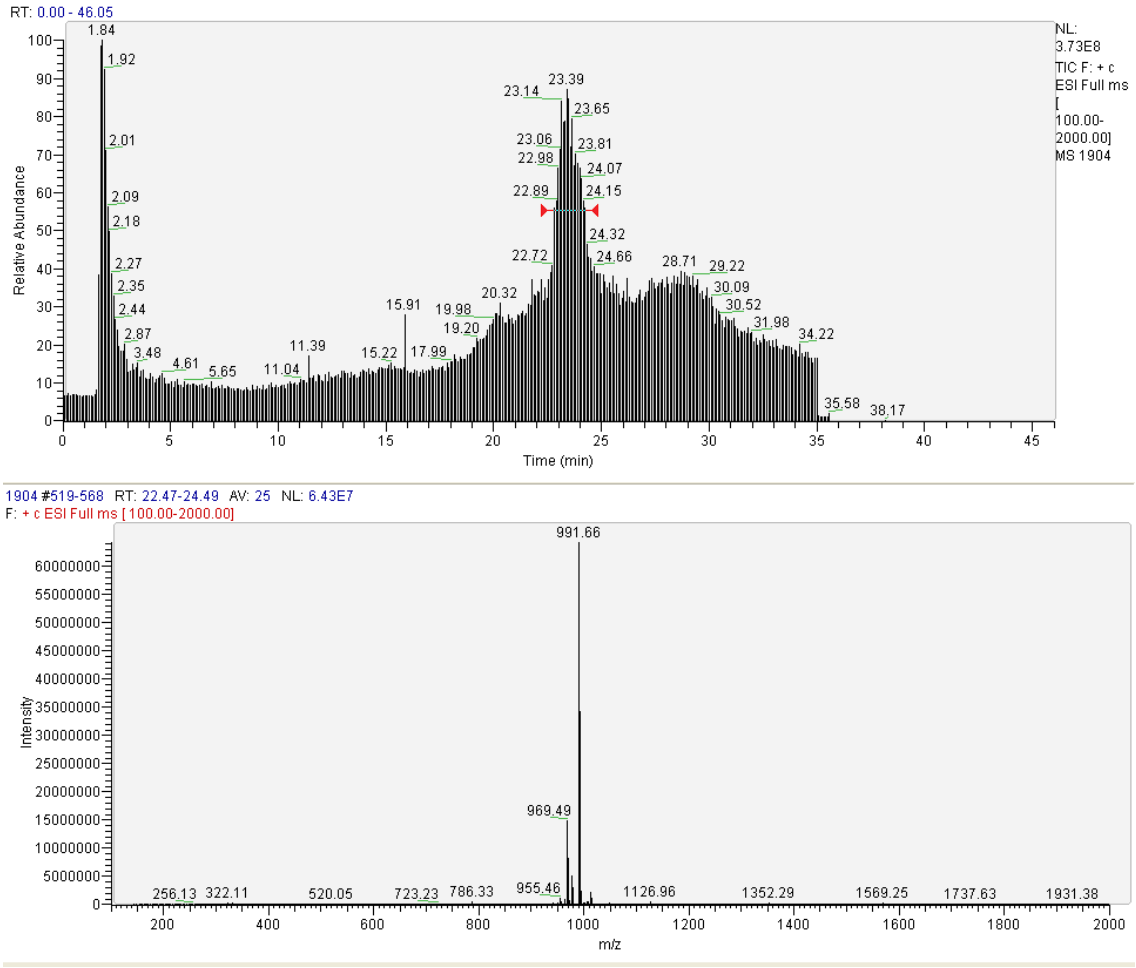


Figure 3.1. continued

Conclusion

There is a direct correlation between phylogeny and secondary metabolite production in the strains of cf. *Oscillatoria* studied in this project. This study has demonstrated the effectiveness of a phylogeny-guided approach to natural products discovery. The presence or absence of two known compounds was predicted correctly for most of the strains. As for the strains that were inconsistent with the predictions, possible explanations include but are not limited to environmental factors or horizontal gene transfer. However, since the data suggest that these instances are rare, hopefully phylogeny-guided search for natural products will continue to aid marine drug discovery research efforts.

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