DESIGNING DNA PRIMERS FOR THE NiFe HoxH HYDROGENASE GENE IN CYANOBACTERIA

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Abstract:

Cyanobacteria, a group of prokaryotic photoautotrophs, are considered one of the largest and most important groups of bacteria. Cyanobacteria can be found in almost any ecological niche including salt water, freshwater, terrestrial and extreme environments. Not only can cyanobacteria thrive in extreme environments, but these organisms possess the ability to produce hydrogen via hydrogenases. In previous studies conducted on cyanobacteria, the Nickel-Iron (NiFe) hydrogenases have become a focal point of research because these enzymes give the organism the capability to produce hydrogen gas. With increasing demands on fossil fuels and decreased availability, alternative fuel sources, such as hydrogen energy, have become a focal point of a multitude of studies.

In this study we designed general hydrogenase gene primers to identify several species of cyanobacteria. We collected the HoxH and 16sRNA DNA sequences for selected cyanobacteria in GenBank at the Nation Center for Biotechnology Information (NCBI). The sequences were aligned and examined by the CLUSTAL X 2.0 and SEQUENCHER informatics computer programs. Once several potential primers were created, a Basic Local Alignment Search Tool (BLAST) was used to identify the most promising HoxH primers. The main objectives achieved in this study were, 1) the design of several HoxH primers with the overall capability to identify unique HoxH gene, and 2) gathering supporting evidence that the conservation of the HoxH gene is fairly limited.

Introduction and Literature Review:

In the dynamic area of energy production, fossil fuels are the main resource used to produce energy. In today's world, virtually all aspects of people's lives require the use of energy in order to maintain the basic day-to-day activities such as transportation and communication. Although the use of fossil fuels has been a crucial component in the development of technology, in the past few decades concern of fossil fuel availability has become a primary issue throughout the world (Dresselhaus, 2001).

One area of science that has increasingly gained awareness, due to the decreased availability of fossil fuels, is the field of alternative energy technology. This discipline of science consists of a variety of possible sources and methods, with the main focus on yielding a useable and renewable energy source. A few specific alternative energy sources currently being researched include solar energy, nuclear energy, chemical energy and hydrogen energy. Hydrogen as an alternative resource is promising, yet there are a few obstacles that need further investigation.

Hydrogen is an attractive fuel source because its oxidation product is water, which is environmentally friendly, and it's highly abundant (Tamagnini *et al*., 2007 Dresselhaus, 2001). Conversely, two major obstacles with hydrogen energy are the means of storing the hydrogen, and the production of sole hydrogen gas (Schlapbach and Züttel, 2001). Currently, there are two main methods in which hydrogen gas can be produced, through steam reforming of hydrocarbons and by heating coal to 1,000^{o}C in the absence of oxygen (Grätzel, 2001). However, the reaction with coal makes a mixture of H_2 with CO, CO_2 and other gases, which have to be separated from hydrogen. Ultimately, to avoid the production of CO_2, this process requires energy, in form of nuclear, hydroelectric, wind or solar. Currently, two potential methods of hydrogen production, which require less energy, are being investigated, 1) photochemical production of hydrogen using sunlight to dissociate water and 2) microbial production of hydrogen (Grätzel, 2001; Schlapbach and Züttel, 2001).

One group of microorganisms that have been the subject of several recent reviews, due to their capability to produce hydrogen as a byproduct, are cyanobacteria (Levin et al., 2004; Sakurai & Masukawa, 2007; Tamagnini, 2002; Tamagnini *et al*., 2007). Cyanobacteria represent one group of diazotrophic or nitrogen

fixing, prokaryotes. This particular group of organisms is relatively diverse, with a size and GC content ranging from 0.5-40 µm and 35-72%, respectively. Furthermore, cyanobacteria display a relatively wide range of morphological diversity, including unicellular, filamentous and colonial forms (Tamagnini *et al*., 2007). Structurally, these photosynthetic organisms are Gram negative and lack flagella. Cyanobacteria are also capable of performing photosynthesis with most species containing only chlorophyll a, instead of both chlorophyll a and b, or just b (Swingley et al., 2008). As research continues on a variety of environments, including marine, freshwater and terrestrial, biological information concerning cyanobacteria continues to increase, further revealing the diversity of cyanobacteria (Tamagnini *et al*., 2007).

The main advantages of cyanobacterial H_2 production are cyanobacteria can use sunlight as an energy source, water as an electron source and air as a carbon and nitrogen source. Therefore, cultivation of cyanobacteria is relatively simple and inexpensive (Tamagnini *et al*., 2007). Even though there are promising benefits to H_2 production through cyanobacteria, there are a few general challenges being addressed and researched, one of which is manipulating the hydrogen producing and consuming genes to produce high quantities of H_2(Levin et al., 2004; Sakurai & Masukawa, 2007; Tamagnini, 2002; Tamagnini *et al*., 2007).

In general, cyanobacteria are one of the largest and most important groups of bacteria; they are able to perform oxygenic photosynthesis with the use of water as an electron donor, and have the capability of utilizing light energy to yield cellular biomass through CO_2 fixation (Tamagnini *et al*., 2007). Additionally, several species have the ability to convert nitrogen gas (N_2) to ammonia (NH_3). This process is called nitrogen fixation, a crucial component of the nitrogen cycle. In the process, several enzymes are utilized to yield ammonia and hydrogen from nitrogen, hydrogen and electrons. Ultimately, the hydrogen produced by nitrogen fixation is consumed by hydrogenases, which assist in maintaining an anaerobic environment, as well as producing H_2.

Hydrogen production process

In cyanobacteria, there are two pathways for H_2 production, 1) nitrogen fixation 2) bidirectional hydrogenase (Roeselers et al., 2007; Tamagnini, 2002; Tamagnini *et al*., 2007). As briefly discussed previously, one method in which H_2 is produced in cyanobacteria is through the process of nitrogen-fixation. The reduction

of atmospheric nitrogen (N_2) to ammonia (NH_3) is a result of a nitrogenase complex, which is illustrated by the generic reaction:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

The enzymes involved in this process can only function in anaerobic conditions, which require the formation of heterocysts and vegetative cells. Heterocysts are vital differentiated cells, specialized in maintaining an anaerobic environment for nitrogenase (Tamagnini, 2002; Tamagnini *et al*., 2007). Ultimately the transfer of electrons from the reduction of N_2 to NH_3 is a highly endothermic reaction, requiring a substantial amount of energy, in the form of ATP. Accompanied with this reaction is the reduction of protons to hydrogen, H_2 (Tamagnini 2002).

The hydrogen produced by the nitrogenase reaction is readily consumed by an uptake hydrogenase, which has been found in almost all examined nitrogen fixing cyanobacteria (Ludwig et al., 2006). The uptake hydrogenase plays a role in the transfer of electrons. Additionally, another hydrogenase, called bidirectional, has the capability to both take up and produce hydrogen. This particular enzyme has been found in both nitrogen-fixing and non-nitrogen-fixing cyanobacteria (Tamagnini, 2007; Tamagnini *et al*., 2007). In general, bidirectional hydrogenases are the predominant hydrogen producing enzymes in bacteria, and are activated under both N_2-fixing and non-N_2-fixing conditions, with the capability to produce significant amounts of H_2 (Vignais & Colbeau, 2004; Zhang *et al*., 2005).

Currently, it is widely accepted that the bidirectional hydrogenase is composed of five subunits, HoxE, HoxF, HoxU, HoxY, and HoxH. The three primary structures of the bidirectional hydrogenase are the large and small subunit and the diaphorase subunit (Zhang *et al*., 2005). The large subunit, HoxH, harbors the bimetallic active site containing nickel and iron, and has a molecular mass ranging from 50.0 to 56.0 kDa. The small subunit, HoxY, has a molecular mass ranging form 17.0 to 22.0 kDa, and is involved in containing and coordination of the FeS clusters (Zhang *et al*., 2005). The clusters function as the electron transfer domain between the electron donor (NADH) and acceptor (NAD^+), as well as the catalytic center of the enzyme. The diaphorase, which is encoded by HoxEFU, also aids in the function of electron transfer between the electron donor and acceptor (Tamagnini, 2002; Tamagnini *et al*., 2007; Zhang *et al*., 2005)

Recently phylogenetic analyse of the up-take hydrogenase (Hup) has become an area of interest, due to several studies manipulating the Hup genes to reduce the up-take of hydrogen gas, in order to increase the yield of H_2 gas (Tamagnini *et al*., 2007). Furthermore, other studies have proven a high degree of DNA sequence similarity of the Hup genes in cyanobacteria. Therefore, aligning DNA and protein sequences of the Hup genes of one cyan bacterium to another is more simplified. Contrary to the Hup DNA sequence, the bidirectional hydrogenase (Hox) is complicated by high levels of variation between species, and greater length variation that leads to uncertain alignment for many proteins. Although analysis of Hox genes is complicated, research in this particular area continues today (Tamagnini *et al*., 2007; Zhang *et al*., 2005).

The objective of this study is to design general primers with the capability to identify the HoxH hydrogenase gene in a variety of cyanobacteria, whether isolated or from environmental samples. In the most recent research on the HoxH gene, studies have created primers for specific species and small groups of similar cyanobacteria, but there are currently no specific primers available to examine a wide variety of cyanobacteria. The approaches being used in this particular study consist of: (1) informatic techniques for designing primers to detect HoxH hydrogenase gene in cyanobacteria and (2) phylogenetics of HoxH genes. These results should yield broad data on cyanobacteria containing the gene. Overall, the information obtained in this study will help to better understand and identify microorganisms that contain the HoxH hydrogenase gene.

Material and Methods:

Primer design

Amplification primers were designed on the basis of comparisons of available HoxH sequences for cyanobacteria in GenBank at the Nation Center for Biotechnology Information (NCBI). The DNA sequences as well as their protein translations were aligned in CLUSTALX 2.0 and Sequencher (Chenna et al., 2003; Roeselers et al., 2007). The alignments were used to identify regions of moderate to high conservation among the cyanobacterial sequences. Once potential DNA primer areas were selected, Basic Local Alignment Search Tool (BLAST) was used to aide in determining the most promising primers.

The primer selection process began after the alignment results were analyzed. Once areas of high nucleotide conservation were located, potential promising primers, with a optimal length of 16-24nucleotides, were identified and subject to BLAST analysis. The numerical values most examined in the BLAST results were maximum identity, query coverage, and E-value. These values were the main factor analyzed in order to propose a list of the most promising HoxH primers.

Results:

CLUSTAL X 2.0.11 and SEQUENCHER 4.0.11 analysis of 16S rRNA gene fragments

The 16S rRNA gene alignment from CLUSTAL X indicated two distinct clusters (Figure 1). The first alignment results, which included all available 16S rRNA of cyanobacteria known to posses the HoxH gene, showed very limited gene conservation, which is evident in the lack of black asterisks. Through further analysis, two major 16S rRNA gene groupings were developed (figures 2 and 3) that contained a higher degree of DNA conservation in each individual group. Similar results were revealed for the 16S rRNA gene in SEQUENCHER, except one grouping was formulated instead of two (figure 4). The 16S rRNA information gathered in CLUSTAL X and SEQUENCHER was used as a basic comparison for all cyanobacteria DNA collected.

CLUSTAL X 2.0.11 and SEQUENCHER 4.0.11 analysis of Hox H gene fragments

The HoxH DNA alignment results for CLUSTAL X indicated low nucleotide-to-nucleotide conservation, therefore all available cyanobacteria HoxH protein sequences were aligned in CLUSTAL X. The protein alignment showed one major grouping, which is evident in figure 5 by the light blue shaded area. A subgroup was identified in figure 5, indicated by the white shaded area, but very little amino acid conservation could be found. After refining the alignment, which can be seen in figure 6, the degree of amino acid conservation of the HoxH protein increased drastically.

SEQUENCHER was used to align the HoxH DNA sequences, which resulted in more promising results. Unlike the CLUSTAL X results, several moderately conserved cyanobacterial groupings were identified in SEQUENCHER and labeled as contig, which can be viewed in figure 7. Based on the contigs created, several potential HoxH primers were designed (Table 1). The primers were then examined by BLAST to give the maximum identity and E values for all available organisms in GenBank, which can also be seen in Table 1.

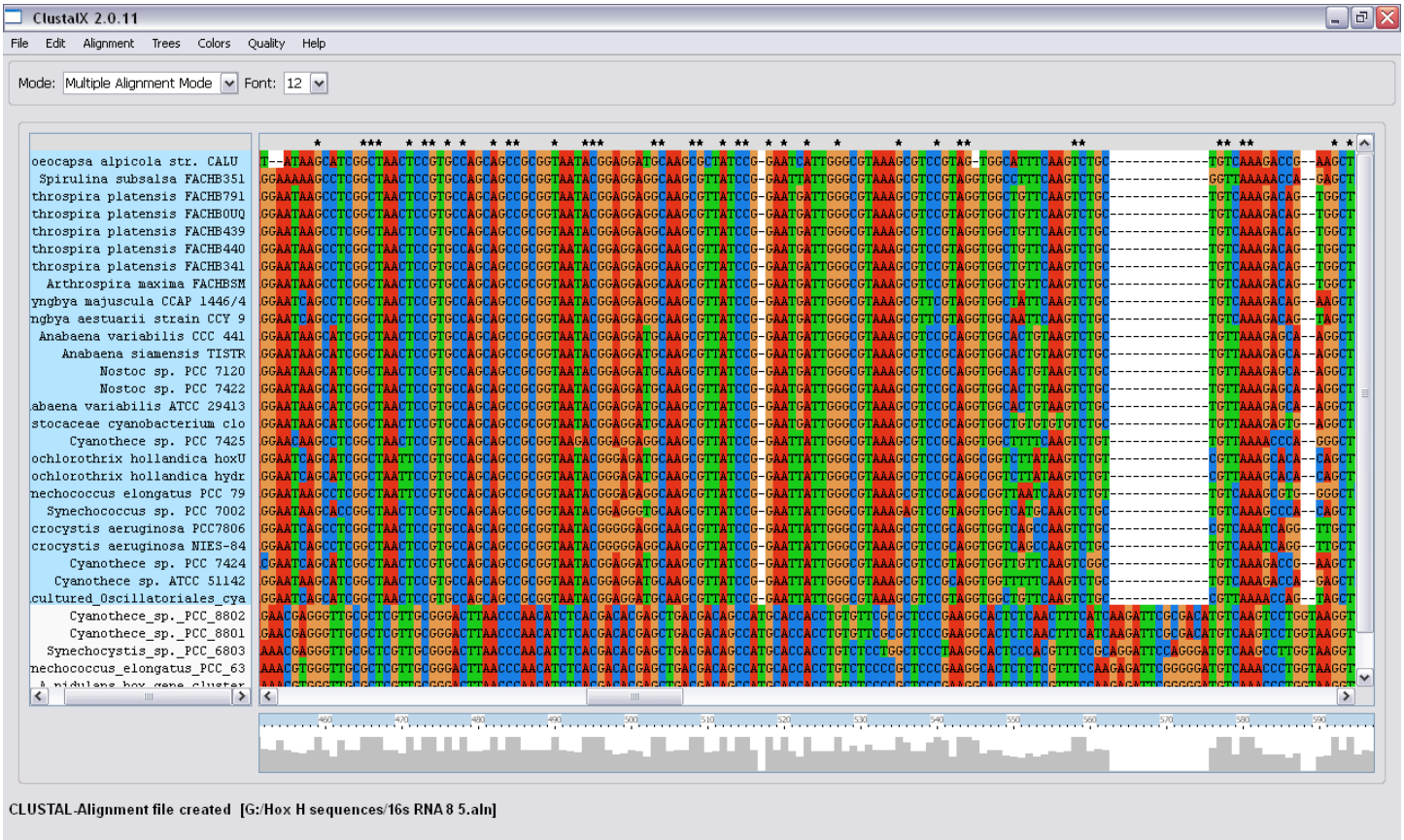

Fig. 1. The 16S rRNA alignment results for CLUSTAL X. The column to the far left gives the name of the specific cyanobacteria, and to the right of each name is the specific 16S rRNA sequence. Each nucleotide has its own distinct color (Thymine- green, Adenine-

Red, Cytosine- Blue, Guanine- Orange). The blue and white shaded areas classify the cyanobacteria into the two different groupings. Columns of full conservation are given a black asterisk *.

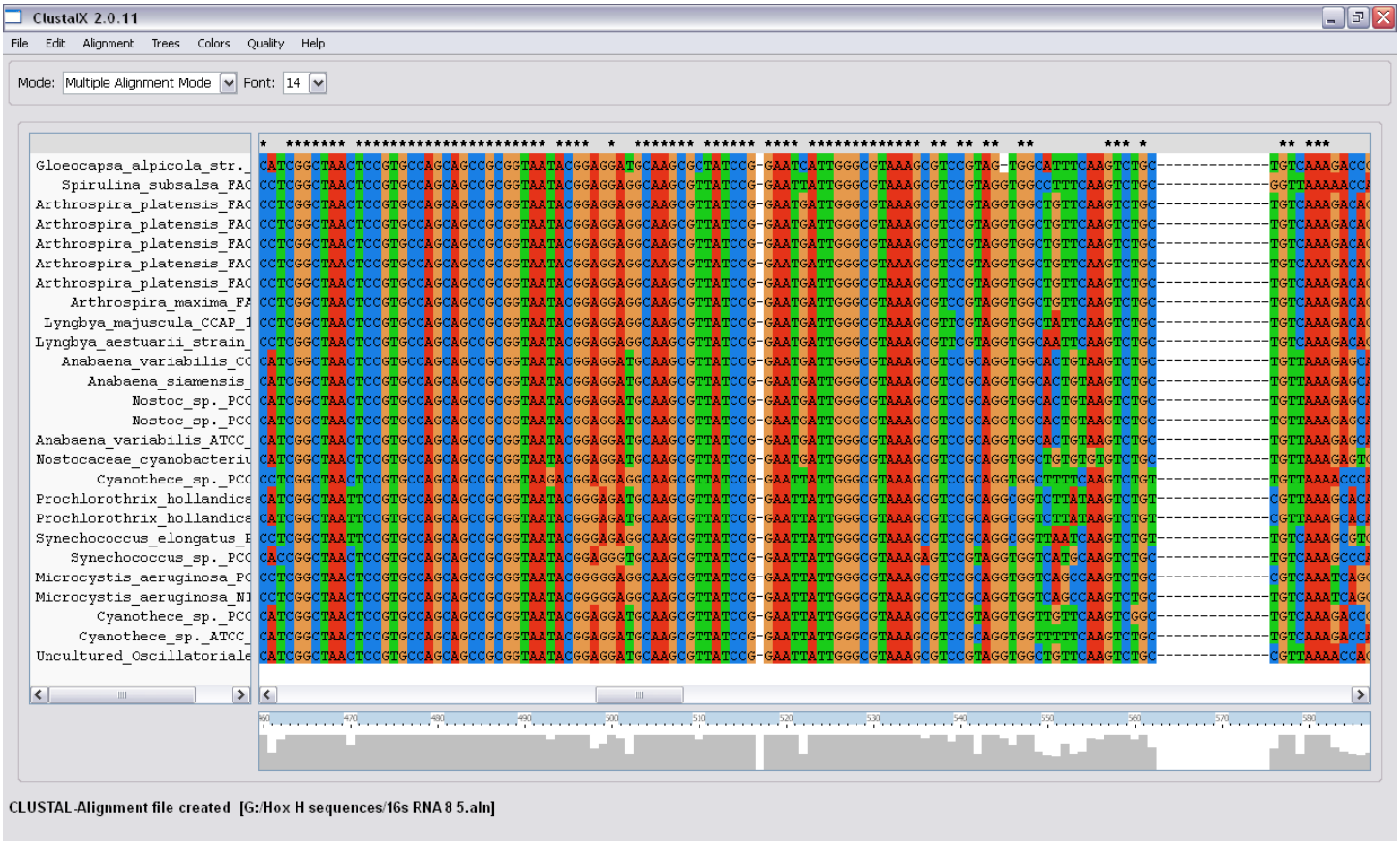

Fig. 2. Adjusted 16S rRNA alignment results for CLUSTAL X. (Refer to figure 3)

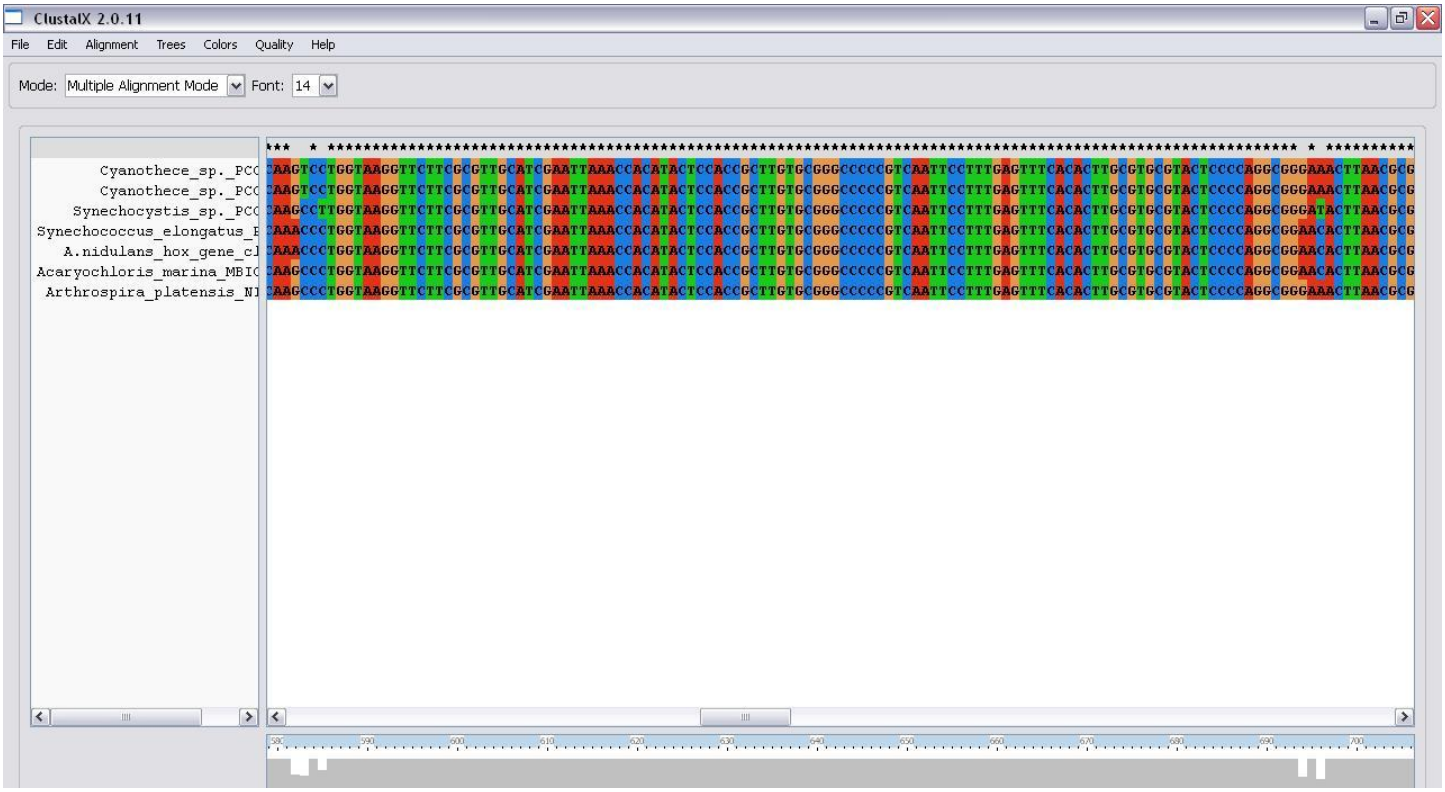

Fig. 3.

Fig. 2 and 3. The refined 16S rRNA alignment results for CLUSTAL X. After adjusting the alignment into two groups, the degree of DNA conservation increased in both groupings, which is evident in the increased appearance of black asterisks.

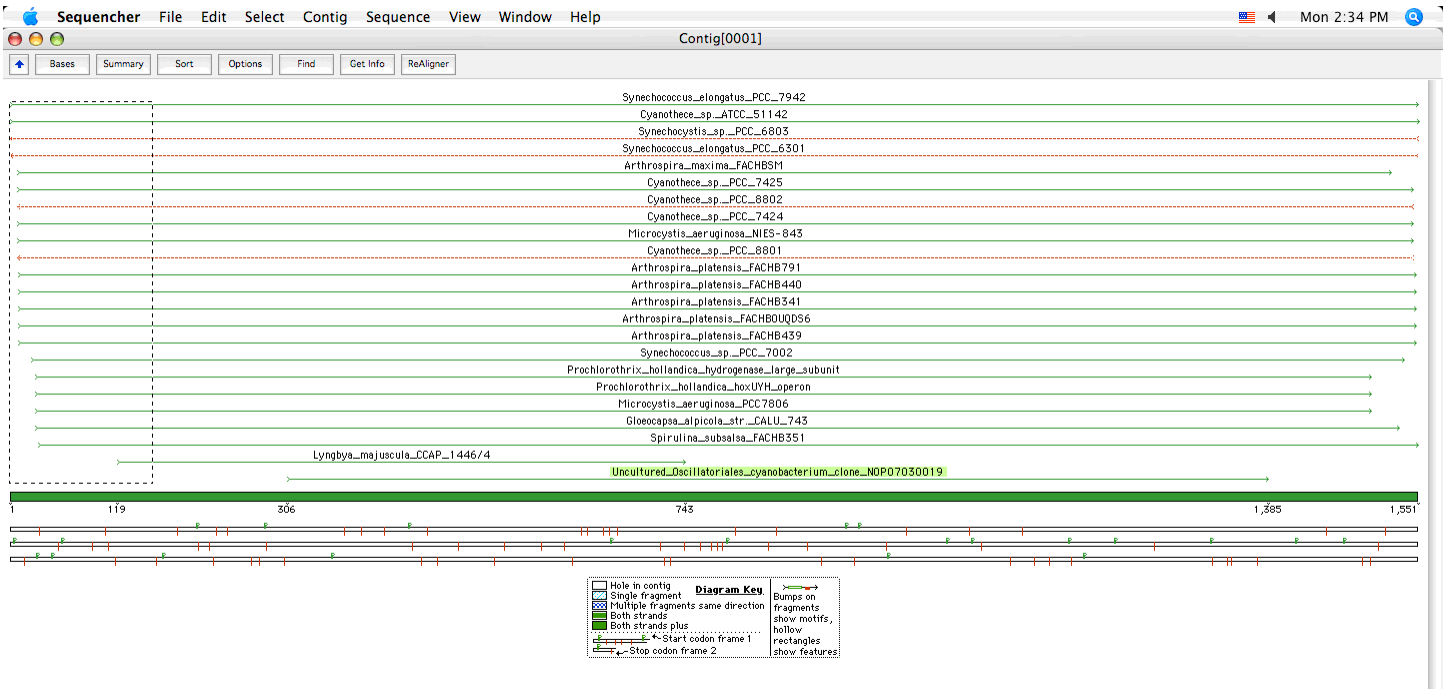

Fig. 4. The 16S rRNA alignment results for SEQUENCHER. One single 16S rRNA grouping was formulated. The lines indicate the 16S rRNA sequences of the cyanobacteria. Green lines represent DNA sequences starting from left to right, while red lines represent reverse sequences starting from right to left.

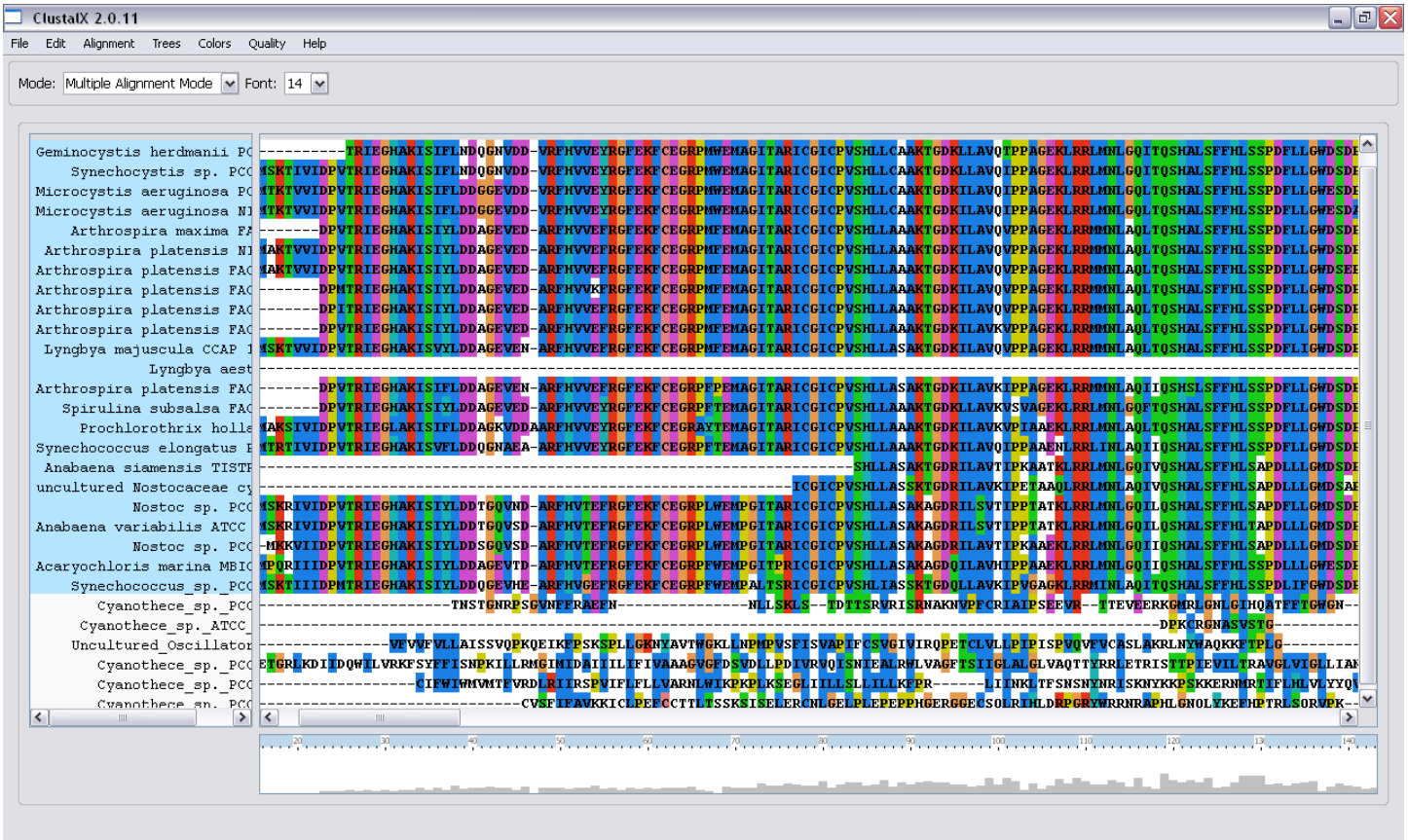

Fig. 5. The HoxH protein alignment results for CLUSTAL X. No areas of conservation were indicated, but two major groupings were distinguished based on the shaded areas of blue and white. In the white shaded area, very little consensus can be seen, unlike the blue shaded area where organisms have a high degree of conservation at the protein level.

Fig. 6. The refined HoxH protein alignment results for CLUSTAL X. After removing the previouse shaded white area in Figure 6, the number of asterisk increased which indicates a higher degree of conservation. The colons "**:**" and periods "**.**" placed above a column indicates inconsistencies with the protein alignment (Lyngbya aestuarii strain CCY 9616, Anabaena siamensis TISTR 8012 and Uncultured Nostocaceae cyanobacterium clone NOP07030021 were removed from the blue shaded group due to insufficient protein length)

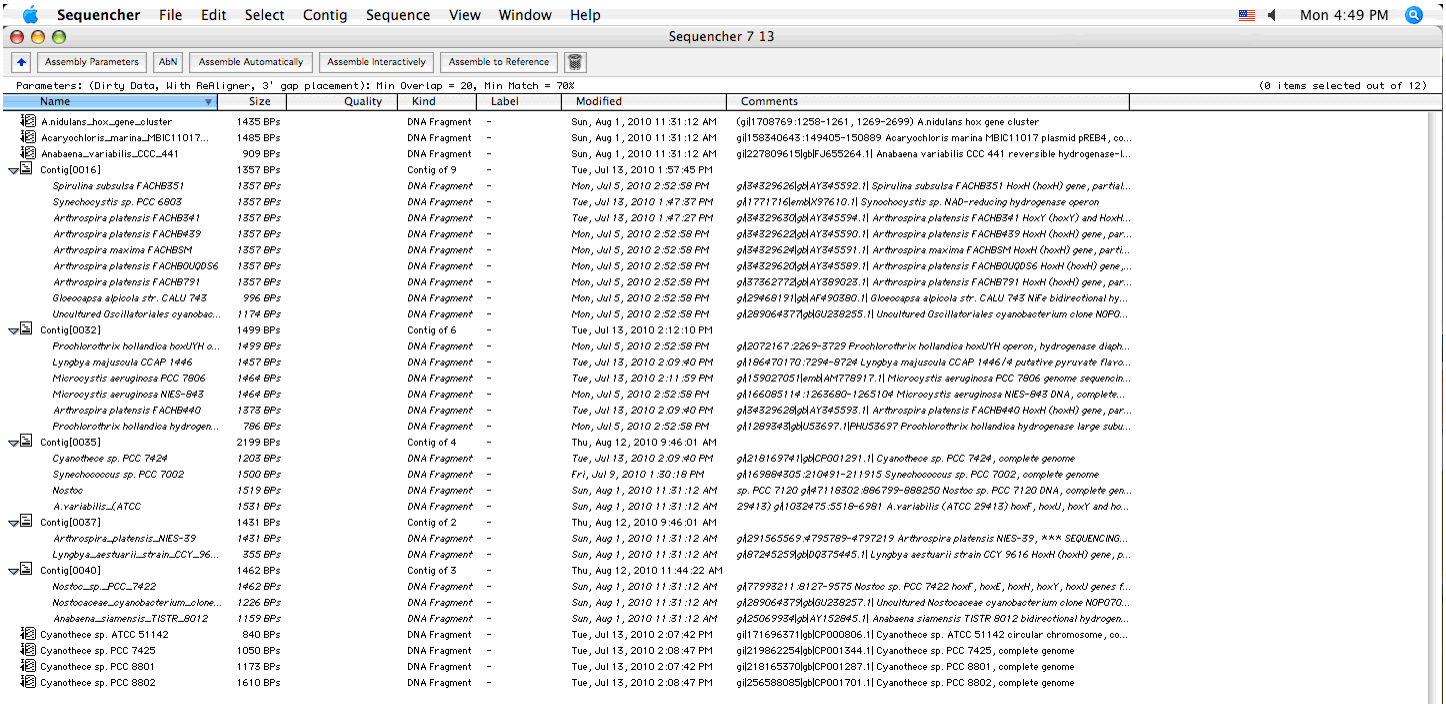

Fig. 7. The HoxH DNA alignment results for SEQUENCHER. 5 cyanobacterial groupings were formulated, which are indicated by the headings: Contig 0016, Contig 0032, Contig 0035, Contig 0037, and Contig 0040. Underneath each Contig are the cyanobacteria

with the most closely related HoxH gene sequence. Seven species of cyanobacteria were either unable to be placed in one of the Contigs, or were removed from a Contig due to low sequence similarity to other cyanobacteria within the Contig.

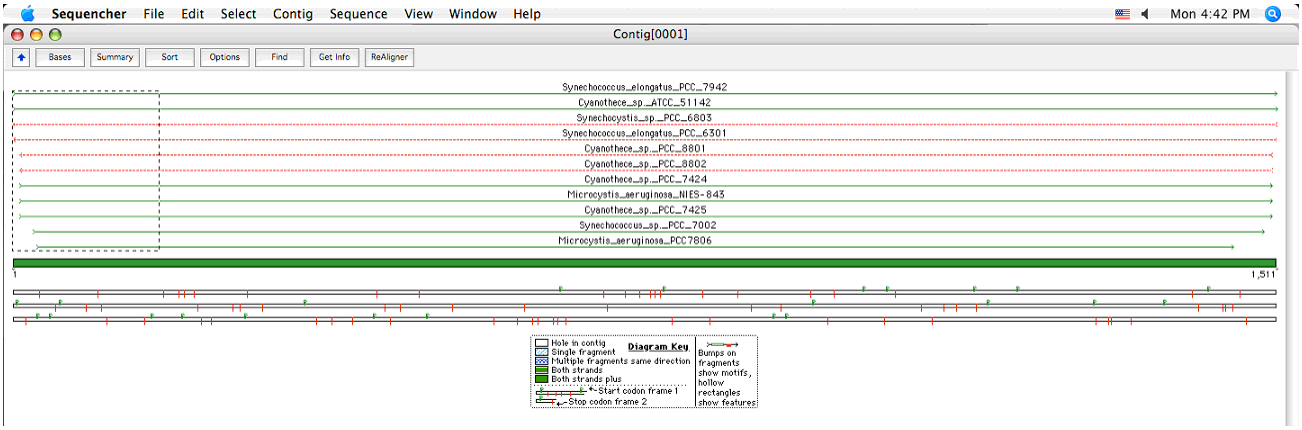

Fig. 8. Show the ability of SEQUENCHER to compare reversed DNA sequence. This technique can be seen via the red lines with arrows. Forward sequences are represented by the green lines with arrows. Figure 8 also displays the direction in which sequences are analyzed; the green lines represent sequences read from left to right, while the red lines read from right to left.

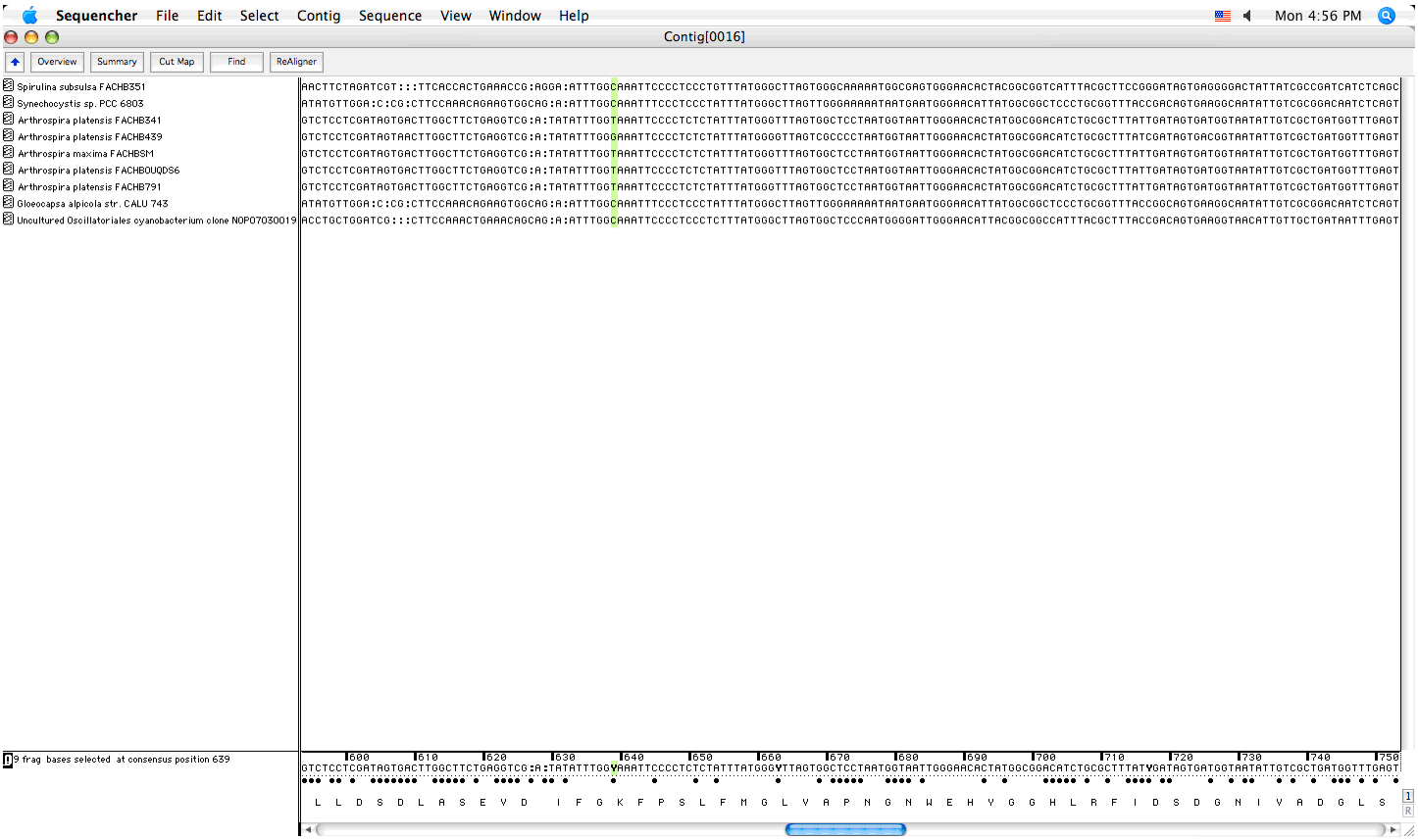

Fig. 9. Figure 9 displays the advancement of ambiguous terms in the SEQUENCHER program. Ambiguous terms are used to indicate aligned columns with equal occurrence of nucleotides. In this screen shot, an example of an ambiguous term is indicated by the bold **Y** at the bottom screen, highlighted in green. In this particular case, the **Y** represents the fairly equal occurrence of C, cytosine, and T, Thymine in this particular column. There are several ambiguity letters: R-guanine (G) or adenine (A), Y- Thymine (T) or cytosine (C), K- (G or T), M- (A or C), S- (G or C), W- (A or T), B- (G,T or C), D- (G,A or T), H- (A,C or T), V- (G,C or A).

Discussion:

This study describes a molecular approach to designing the most promising HoxH primers, with the capability to detect a variety of cyanobacteria, for future research of environmental samples. It was shown in previous studies that a PCR approach based on cyanobacterial 16S rRNA genes is not suitable for examining the diversity of cyanobacteria, due to high similarity of the gene from one group to another (Case et al., 2007). Supporting these finding are the CLUSTAL X 2.0.11 and SEQUENCHER 4.0.11 alignment results of the partial and full 16S rRNA cyanobacterial gene sequences obtained from GenBank. The cyanobacteria collected were formulated into one highly conserved group, which indicates a high similarity in these species (Fig. 4).

Furthermore, partial and full HoxH genes for the same cyanobacteria were obtained, aligned in CLUSTAL X 2.0.11 and SEQUENCHER 4.0.11. In previous studies, it had been shown that high levels of length variation and unavailability of HoxH sequences has led to uncertainty with concern to protein and DNA alignment (Tamagnini *et al*., 2007). Based on this previous information, the protein sequences of the cyanobacteria were first aligned in CLUSTAL X 2.0.11. The results in figures 5 and 6 indicate one major alignment grouping with moderate amino acid conservation, which supports the findings of Tamagnini *et al (*2007).

Following the protein alignment of the HoxH gene, the DNA alignment of the HoxH cyanobacterial genes were aligned in both CLUSTAL X 2.0.11 and SEQUENCHER 4.0.11. When the DNA sequences were aligned in CLUSTAL X 2.0.11, the results were inconclusive, showing very limited conservation throughout the sequences. These results were most likely due to the lack of advanced alignment techniques, such as reverse alignment, in the CLUSTAL X 2.0.11 program. Subsequently, the HoxH sequences were aligned in SEQUENCHER 4.0.11, which is a more powerful alignment program, with the capabilities to align sequences in reverse direction (Fig. 8) as well as insert ambiguity letters which can be seen in (Figure 9).

SEQUENCHER 4.0.11 was able to align the cyanobacteria sequences into 5 major groupings, contig 0016, contig 0032, contig 0035, contig 0037 and contig 0040 (Table 1). All 5 groups contained a moderate degree of nucleotide conservation. Subsequently, the 5 contigs were used to locate potentially promising primers, 16-24 bases long, with relatively medium to high conservation with no more than 2 ambiguous bases, with one exception in contig 0016 (Table 1). The potential primers were subjected to a BLAST search to identify the

DNA sequences of all organisms, available at the NCBI, matching and/or similar to the potential primer DNA sequence. The most promising potential primers, with the highest degree of cyanobacteria diversity, are identified in Table 1.

In recent studies on cyanobacteria hydrogenases, the up-take hydrogenase (Hup) has become another area of focus as well as Hox. After examining the alignment results from this study, the reasons for focusing on the Hup genes is more evident. Unlike the Hox genes, the Hup genes have been proven to have relatively high similarity at the DNA and protein levels; therefore primers have been designed to identify a variety of cyanobacteria in environmental samples (Roeselers et al., 2007). Although the Hox genes seems to be less conserved in cyanobacteria, when compared to the Hup genes, the Hox gene plays a more direct role in H^+ production and consumption, where as Hup genes only control H^+ up-take. Therefore, research on the Hox genes needs to continue. In the future, the primers designed is this study will be examined and tested, through polymerase chain reaction (PCR), on environmental soil-substrate samples collected on the Maxton Plains Alvar, a rare and extreme environment for organisms.

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