Molecular identity of cyanobionts and mycobionts in *Peltigera membranacea*

Vanessa Iglesias Fernández

Supervisor: Prof. Ólafur S. Andrésson

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Faculty of Life and Environmental Sciences

Department of Biology

University of Iceland

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Special thanks for Britto,for your quality instructions and for explaining me so carefully all the most obviously things. Thank you for your patient with my mistakes and for being not only an instructor but also a person who worries about our work and takes care of us. You have introduced me a little bite more in the scientific world, thank you.

Thank you also to Sheeba Santhimi Manoharan, for helping me with everything in the laboratory and for being so nice with me.

Thanks a lot!!

Abstract

Cyanobacteria of the genus *Nostoc* are widespread in nature and occur both in a free-living state as well as in symbiotic associations, most prominently with fungi in lichens and leguminous plants [(1)].

A molecular phylogenetic approach was used to investigate whether specific types of *Nostoc* associate with different samples of lichenizing *Peltigera* fungi or whether fungal spores from different lichens species can associate with one or several widespread strains of symbiotic *Nostoc.*

The phylogenetic analysis was conducted on 16S ribosomal RNA genes from symbiotic strains of *Nostoc* and the Internal Transcribed Spacer (ITS) sequences of fungal rDNA.

Samples of the lichen *Peltigera membranacea* were identified and collected from different places in Iceland, mainly around Reykjavik between 2008- 2009.

The prokaryotic 16S ribosomal RNA gene and the eukaryotic Internal Transcribed Spacer rDNA region fragments were amplified by the polymerase chain reaction from DNA extracted directly from the collected lichens, and sequenced directly.
DNA extracted from lichen apothecia was also used for the ITS study.

The results confirmed that 16S ribosomal RNA gene sequences were identical between lichen specimens in Iceland but not in other geographical areas such as Canada and U.S.A.

The initial results from ITS sequences revealed heterogeneity in the mycobiont population of *Peltigera membranacea* in Iceland. But, still it needs more thorough ITS analysis to confirm this.

Table of Contents

1. Introduction……4

1. 1. Lichen……4

1.1.1- What is a lichen? Definition and main characteristics……...4

1.1.2- Types of lichens……5

1.1.3- Genus Peltigera……..6

1.1.4- Apothecia……...6

1.2. Cyanobacteria……..7

1.2.1 Nostoc……..7

1.3. Markers……8

2. Aims……9

3. Materials and methods……10

3.1. Lichen samples: Collection and processing……..10

3.2. DNA extraction from lichens……...11

3.3. PCR: Polymerase Chain Reaction…… ..11

3.4. Direct Sequencing……12

4. Results……..13

4.1. DNA extraction……13

4.2. Molecular Analysis of 16SrDNA gene sequences……...13

4.3. Molecular Analysis of 18S-26S ITS rDNA sequences……...25

4.4. Phylogenetics analysis……..33

5. Conclusions and discussion……35

References……..37

Appendix……38

1. Introduction

1.1.Lichen

1.1.1. What is a lichen? Definition and main characteristics

'A *lichen* is a stable self-supporting symbiotic association between a fungus (mycobiont) and an algae or/and a cyanobacteria (photobionts)' [(2)].

In this association, the photobiont provides the fungus with food (carbohydrate) and the fungus provides the photobiont with a suitable living environment for growth.

They are a successful life form, which is able to colonize a manifold of different habitats and substrates [(3)].

Components:

- The fungal component

  The main component of the lichen is the fungus, called mycobiont, which is generally responsable for shaping the vegetative body of the symbiotic lichen, known as 'thallus', and forming also the lichen secondary metabolites [(4)].

  The distinctive colors of many lichens result from the massive accumulation of these diverse secondary compounds, called 'lichen substances'.

  Lichen symbioses represent a major way of life among the Fungi [(5)].

- Algal and cyanobacterial components

  The algae and/or cyanobacteria are the photosyntetic constituents of the lichen, which may be distributed evenly throughout the thallus but, more often, are limited to a layer just below the upper surface.

  A few lichens regularly contain two photosynthetic partners (photobionts), a green algae and a cyanobacterium.

  Their biological importance is that the cyanobacteria are thought to help with the nitrogen metabolism of the lichens by fixing molecular nitrogen, and both types of photobiont cooperate in the process of the photosynthesis [(2)].

All lichens with cyanobacterial symbionts are called 'cyanolichens'[(5)].

1.1.2. Types of Lichens

There are two divisions according to:

A) Morphology: they have traditionally been divided into three main categories: crustose, foliose and fruticose lichens.

B) Composition: most cyanolichens can be divided into two main groups:

(1) bipartite cyanolichens: they are formed by one type of lichen-forming fungus and one type of cyanobacterial photobiont.

In most of them, the cyanobiont is the only photosynthetic component forming a photobiont layer below the upper cortex of the thallus [(6)].

(2) tripartite cyanolichens: they contain both green algal and cyanobacterial symbionts in addition to the lichen- forming fungus.

In most tripartite lichens the green algal is the primary photobiont, which occupies much of the thallus distributed in a primary layer under the upper cortex and produces most of the photosynthate. The cyanobacterium cells are confined to specialized structures called cephalodia, which form pink, grey or brown warts situated on the upper or lower surface of the thallus or even 'packets' inside it [(6)], and their main function is N_2 fixation.

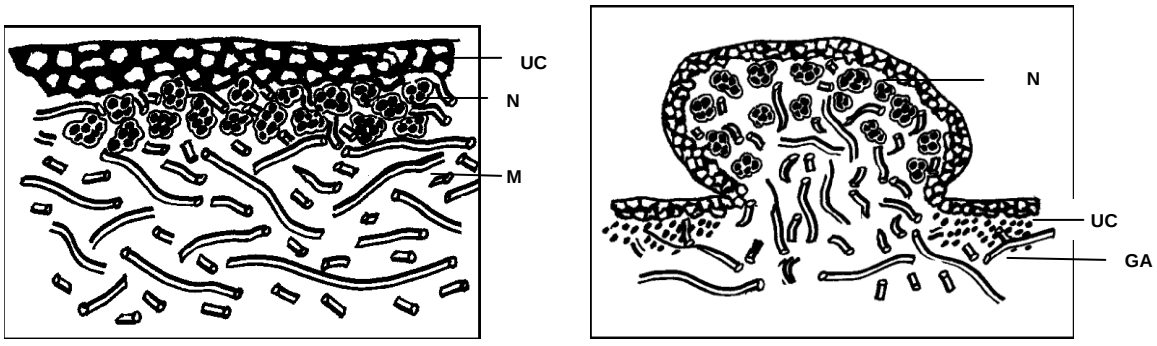


Figure 1: (A) Bipartite Peltigera. The mycobiont is responsible for the structure of the thallus with an upper cortex (UC), photobiont layer to which the *Nostoc* (N) cells are restricted, and a medulla (M).
(B) Tripartite Peltigera. A green alga (GA) is located in a layer underneath the upper cortex much like the *Nostoc* cells in the bipartite *Peltigera*. In this type, the *Nostoc* cells are confined to specialized structures, cephalodia, located on the upper surface of the lichen.
Picture taken from 'The Nostoc Symbiont of Lichens' [(6)]

1.1.3. Genus *Peltigera*

The genus Peltigera comprises ascomycota fungi which belongs to order Lecanorales, family Peltigeraceae.
Peltigeralean lichen-forming fungi are generally characterized by foliose, subfruticose or granular thalli with the presence of rhizines on the lower side [(7)].
This genus contains both bipartite and tripartite lichens.

1.1.4. Apothecia

The apothecia are structures along the edge of the thallus of different colors dependent on species and conditions.
They are the fruiting bodies from the fungal portion of the lichen that eventually release spores. However, spore release is thought to be a poor way for lichens to reproduce. When the spore reaches its new environment, the fungal component of the lichen begins to grow, but it must somehow find suitable algal and/or cyanobacterial partner.

Humans have exploited lichens for diverse purposes. Lichen substances exhibit a great diversity of biological effects, including antimicrobial, anti-inflammatory, analgesic, antipyretic, and antiproliferative and cytotoxic activities, and there has been a long standing interest in the pharmaceutical properties of compounds derived from lichens[(4)].

Definitely, lichens are complex organisms: they are multicellular, macroscopic and eukaryote. In fact, they can be considered as miniature ecosystems.

1.2. Cyanobacteria

Cyanobacteria include a large morphologically heterogeneous group of gram-negative photoautotrophic eubacteria [6].

These prokaryotic organisms form symbiotic associations with a wide range of plant and fungal hosts, almost exclusively with Ascomycota.

Identification of cyanobacterial photobionts in the intact lichen thallus is often impossible since the morphology of the photobiont is changed by the influence of the fungal partner.
However, using molecular techniques, at least a determination on the genus level is possible directly from the lichen thallus, using specific primers for cyanobacterial 16SrDNA [8].

1.2.1. Genus *Nostoc*

The genus Nostoc belongs to order Nostocales, family Nostocaceae by traditional clasification and subsection IV by bacteriologic classification [9].
This genus participates in a wide range of symbiotic associations with hosts from different groups of organisms [6].

The genus *Nostoc* is by far the most common cyanobiont in lichens, serving as a source of fixed carbon and nitrogen, as in bipartite cyanolichens.
The N_2-fixation takes place in structurally and physiologically differentiated cells where oxygen tension is kept on a low level, termed heterocysts.

Nostoc is an ecologically successful genus which contains both free- living and symbiotic species distributed in a wide range of terrestrial and aquatic ecosystems from the tropics to the arctic [9]

In most members of the order Peltigerales, Nostoc is thought to be the sole photobiont and also occurs as a secondary photobiont in many green algal lichens [1].

1.3. Molecular Markers

Molecular markers are strong tools for research on cyanobacterial diversity, identification and evolution. The success of most of them in cyanobacteria is due to the design of cyanobacteria- specific primers.
They play a central role in establishing species identity and reconstructing phylogenetic relations for Nostoc [(9)].

In this study, I evaluated 16S rRNA gene and 18S-26S rDNA internal transcribed spacer (ITS), used as phylogenetic markers at the intrageneric level.

- 16S rRNA gene

  The small subunit ribosomal RNA (16S rRNA) gene is the most popular molecular marker in reconstructing phylogenetic relations among cyanobacteria, but it is not sufficient for studies at the subgeneric level because it is highly conserved among closely related species and strains [(9)].

- 18S- 26S Internal Transcribed Spacer (ITS)

  It is a region of non- functional RNA situated between sequences 18S and 26S in the eukaryotic rRNA genes.

  This region exhibits a great deal of length and sequence variation; therefore, it has been used to provide insight into phylogenetic relationships within genera[(9)].

2. Aims

The purpose of this project is to study the molecular identity of *Nostoc* and the mycobiont in the lichen *Peltigera membranacea*.

The established questions are the following:

- Is there one or are there several different strains of *Nostoc* in the population of *Peltigera membranacea* in Iceland?
- Are these *Nostoc* symbionts the same as in other different geographical areas?
- Is there any species variation among thallus and apothecia in the population of the lichen *Peltigera membranacea*?

In order to find some answers to the questions described above, the research will focus on the genetic study with molecular markers, conducted in two different parts:

(i) the diversity of *Nostoc* symbionts in the lichen *Peltigera membranacea* in Iceland and the comparison of these strains with other symbiotic cyanobacteria

(ii) the variation within the population of fungus *Peltigera* among different parts of the lichen: thallus and apothecium.

3. Materials and Methods

1.1. Lichen Samples: Collection and Processing

Peltigera membranacea thalli were directly collected from the field at different times in different places.

For the first part of this project, the 16S rRNA gene analysis, four thalli were processed (see Table 1).

Sample ID	DNA ID	Place	Date
A	1	Keldur	23/09/08
B	34	Akureyri	16/06/09
D	VBB02	SKK-N	05/09/09
E	VBB01	Klukkn.Au	06/09/09

Table 1: Places and dates of lichen samples collected for the 16SrRNA Analysis

In the second part of the project, the ITS analysis, the same specimens were used as well as one additional thallus.

Apothecia samples from lichens were also cut and collected in plastic tubes (see Table 2).

Sample ID	DNA ID	Place	Date	Part of the lichen
A	1	Keldur	23/09/08	Thallus
B	34	Akureyri	16/06/09	Thallus
D	VBB02	SKK-N	05/09/09	Thallus
E	VBB01	Klukkn.Au	06/09/09	Thallus
18	18	Keldur	11/07/08	Thallus
C	32	Keldur	14/04/09	Apothecium
36	36	Keldur	15/10/09	Apothecium

Table 2: Places and dates of lichen samples collected for the 18S-26S rDNA ITS Analysis

Freshly collected thallus samples were transported in plastic boxes to the laboratory where they were processed. The processing consisted of several washes with running water and a final rinse with deionized water. All the small impurities from the field and insects were removed under a stereoscope using forceps. Then, each sample was left to air dry.

One portion of each specimen was deposited in an envelope in the lichen collection of the laboratory as a voucher specimen.

The rest of the samples of interest were stored at 4ºC for further analyses.

1.2. DNA Extraction from Lichens

Approximately 2 grams of air dried cleaned lichen fragments from each locality were ground separately in a cold mortar with liquid nitrogen. DNA was extracted from the resulting lichen powder (see protocol in Appendix).

1.3. Polymerase Chain Reaction (PCR) - Amplification

16SrRNA Analysis

Four *Nostoc* strains were used in this study.
The small subunit ribosomal RNA gene was amplified using oligonucleotide primers F9 and Cyar 1444 (see Table 3).
F9 primer is an universal primer whereas Cyar 1444 is specific for Nostoc.

PCR reactions were performed in 50 µl mixture containing:

- 36 µl distilled water
- 0.6 µl deoxyribonucleoside triphosphate (2 mM each dNTP)
- 5 µl Taq buffer
- 1.5µl of each primer forward and reverse (20mM each)
- 0.3 µl Taq polymerase (5u/ µl)
- 5µl DNA solution (template DNA)

E. coli was used as positive control and water as negative.

The amplification conditions were: an initial denaturation of 95ºC for 2 minutes, followed by 33cycles of 94ºC for 20 seconds, 55ºC for 20s, and 72ºC for 1.30 min, with a final extension of 72ºC for 10 minutes.

The PCR products were separated on 1.2% agarose gel electrophoresis and the bands were then visualized by UV light.

18S- 26S Internal Transcribed Spacer Analysis

Each DNA sample was amplified using both pairs of fungal specific primers ITS5- ITS2 and ITS 1- ITS 4 (see Table 4).
The same conditions that described previously were applied in the amplification of the ITS region of the samples.

1.4. Direct Sequencing

16SrRNA gene and Internal Transcribed Spacer Analysis

PCR products were directly sequenced (see protocol in Appendix).

The different primers used for sequencing of both analyses are shown in Table 3 and 4.

Primer	Nucleotide Sequences (5'-3')
F9	5'- GAGTTTGATCCTGGCTCAG- 3'
515F	5'- GTGCCAGCAGCCGCGGTAAATAC- 3'
515R	5'- GTATTTACCGCGGCTGCTGGCAC- 3'
850F	5'- AAACTCAAAGGAATTGACGG- 3'
850R	5'- CCGTCAATTCCTTTGAGTTT- 3'
1195F	5'- GAGGAGGGTGGGGATGACGTC- 3'
Cyar1444	5'- AAAGGAGGTGATCCAGCCAC -3'

Table 3: Primers used for amplification and sequencing of 16S ribosomal RNA gene.
The name of the primers refers to the position on the *E.coli* 16SrDNA sequence and the direction forward (F) or reverse (R).

Sequences were assembled with Codon Code Aligner and DNA Baser programs.

The final alignment was analyzed manually cheking the peaks of the bases on the trace chromatogram. The assembled and inspected sequences were then aligned with ClustalW Multiple Alignment using the bioinformatic program CLC Genomics Workbench 3 and results were confirmed using BLAST on the NCBI GenBank Database.

This sequence information was used to construct a phylogenetic tree with neighbor-joinig method and for the comparison of the evolutionary patterns of the regions analyzed.

Primer	Nucleotide Sequences (5'- 3')
ITS 1 (F)	5'- TCCGTAGGTGAACCTGCGG – 3'
ITS 2 (R)	5'- GCTGCGTTCTTCATCGATGC -3'
ITS 4 (R)	5'- TCCTCCGCTTATTGATATGC -3'
ITS 5 (F)	5'- GGAAGTAAAAGTCGTAACAAGG -3'

Table 4: Primers used for amplification and sequencing of 18S ribosomal RNA gene. (F) Forward (R) Reverse

4. Results

4.1. DNA Extraction from Lichens

Genomic DNA extracted from the lichen includes a mixture of mycobiont (eukaryotic) and photobiont (prokaryotic) genomes [10].

The presence of DNA was confirmed on 0.6% agarose gel electrophoresis (see fig. 2).

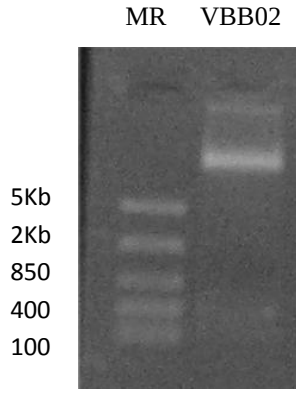
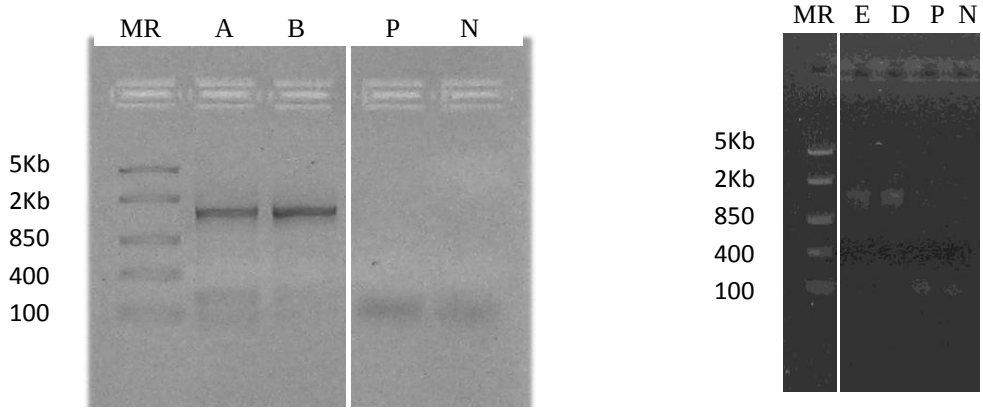


Figure 2: Photography of 0.6 % agarose gel electrophoresis of extracted lichen DNA.

4.2. Molecular analysis of 16S rDNA sequences

For examination of the cyanobacterial genomes, primers F9 and Cyar 1444 were used (Table 3). These primers allowed amplification of a product of the expected size, about 1.5 kb from the *Peltigera* specimens [10].



A – DNA 1
B – DNA 34
D – DNA VBB02
E - DNA VBB01
P – positive control (E. coli)
N – negative control (water)
MR – Middle Range (Moleculerl size marker)

Figure 3: Agarose gel electrophoresis of DNA fragments generated by amplification of 4 *P. membranacea* DNAs using 16S rDNA primers F9 (universal primer) and Cyar1444 (specific for Nostoc).

The resulting PCR products were sequenced using the primers of the table 3.

The 16S rDNA sequences obtained from the 4 samples of P. membranacea, approximately all of the same length, were aligned and blasted against GenBank records.

According to the GenBank Database results, all the sequences corresponded to the same species of Nostoc except sample A which gave other specie of Nostoc. This difference in BLAST results is only due to using query sequences of different lengths (see Table 5).

Sample ID	Contig Length (bp)	Blast Results	Accession nº	Query %	Max.Ident %
A	1378	Nostoc sp. Lukesova 1/86 partial 16SrRNA gene	AM711545.1	100	99
B	1425	Nostoc sp. SKSL2 16S rRNAgene	EU022727.1	100	99
D	1456	Nostoc sp. SKSL2 16S rRNAgene	EU022727.1	100	99
E	1464	Nostoc sp. SKSL2 16S rRNAgene	EU022727.1	100	99

Table 5: Sequences results according to NCBI GenBank Database.

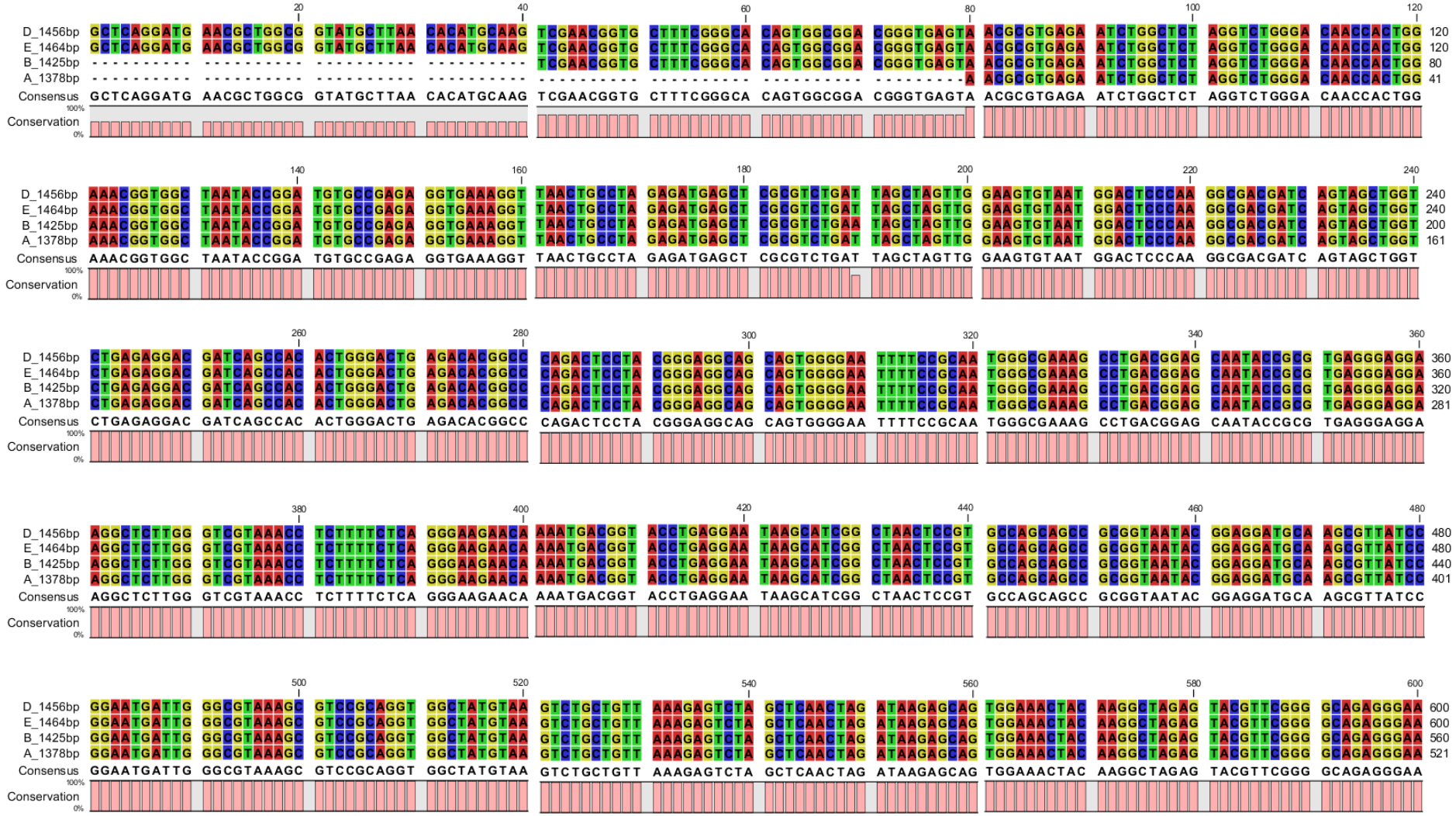
The sequences were checked manually in the chromatogram, base by base, and any real single nucleotide difference was found (see alignment in Figure 4).

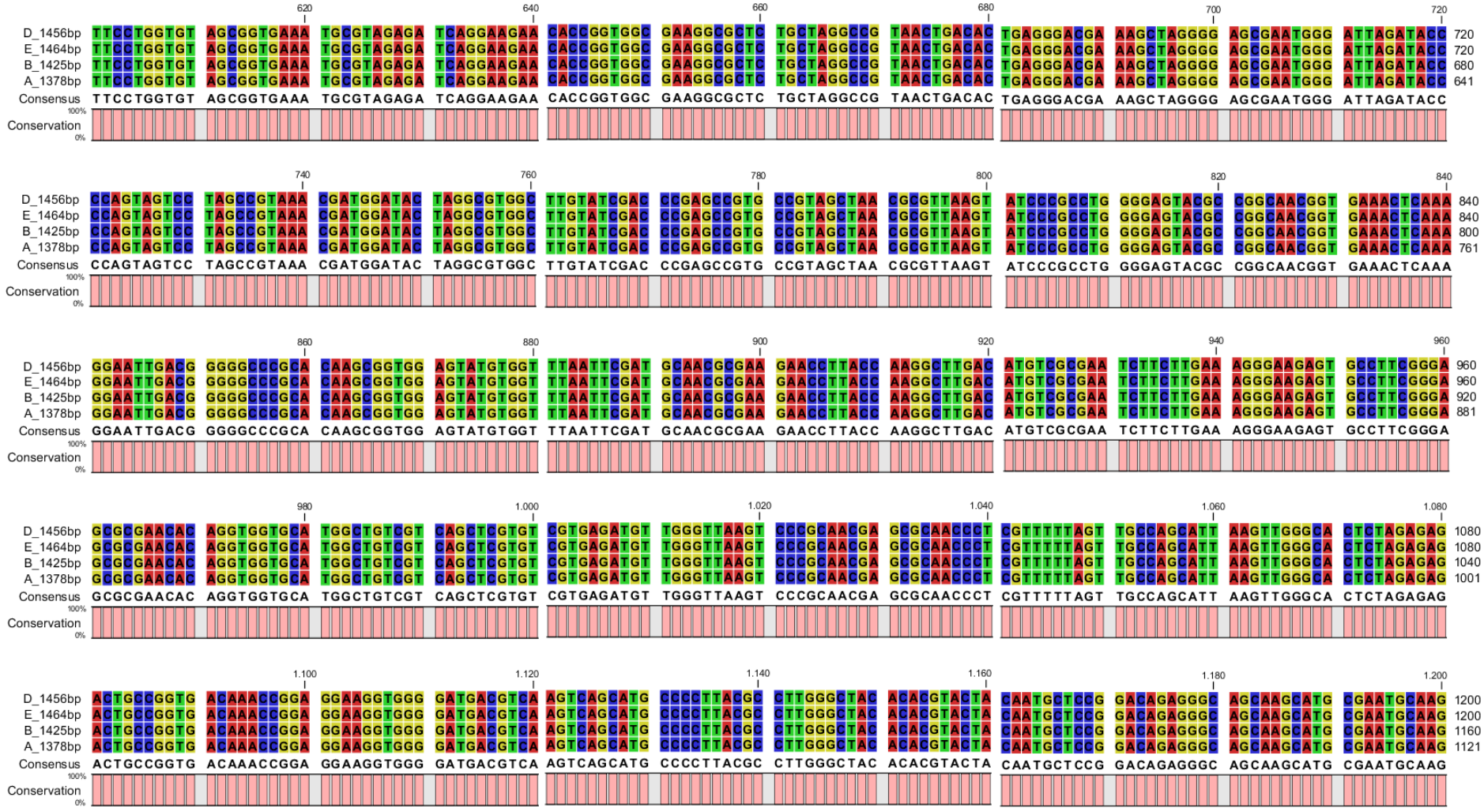
Due to the homology of these four 16S rDNA sequences, sample E was selected to make a comparison for being the longest.

Sample E was compared with sequences C01, C02, C03, C04 and C05 from the work of Celia, conducted in Iceland last semester.

When these sequences were compared by alignment, no difference was found.

All strains obtained in Iceland therefore share identical 16S rDNA sequences (see Figure 5).


D_1456bp
E_1464bp
B_1425bp
A_1378bp
Consensus
Conservation
100%
0%


620
640
660
680
700
720
D_1456bp TTCCTGGTGT AGCGGTGAAA TGCGTAGAGA TCAGGAAGAA CACCGGTGGC GAAGGCGCTC TGCTAGGCCG TAACTGACAC TGAGGGACGA AAGCTAGGGG AGCGAATGGG ATTAGATACC 720
E_1464bp TTCCTGGTGT AGCGGTGAAA TGCGTAGAGA TCAGGAAGAA CACCGGTGGC GAAGGCGCTC TGCTAGGCCG TAACTGACAC TGAGGGACGA AAGCTAGGGG AGCGAATGGG ATTAGATACC 720
B_1425bp TTCCTGGTGT AGCGGTGAAA TGCGTAGAGA TCAGGAAGAA CACCGGTGGC GAAGGCGCTC TGCTAGGCCG TAACTGACAC TGAGGGACGA AAGCTAGGGG AGCGAATGGG ATTAGATACC 680
A_1378bp TTCCTGGTGT AGCGGTGAAA TGCGTAGAGA TCAGGAAGAA CACCGGTGGC GAAGGCGCTC TGCTAGGCCG TAACTGACAC TGAGGGACGA AAGCTAGGGG AGCGAATGGG ATTAGATACC 641
Consensus TTCCTGGTGT AGCGGTGAAA TGCGTAGAGA TCAGGAAGAA CACCGGTGGC GAAGGCGCTC TGCTAGGCCG TAACTGACAC TGAGGGACGA AAGCTAGGGG AGCGAATGGG ATTAGATACC
100%
Conservation
0%
740
760
780
800
820
840
D_1456bp CCAGTAGTCC TAGCCGTAAA CGATGGATAC TAGGCGTGGC TTGTATCGAC CCGAGCCGTG CCGTAGCTAA CGCGTTAAGT ATCCCGCCTG GGGAGTACGC CGGCAACGGT GAAACTCAAA 840
E_1464bp CCAGTAGTCC TAGCCGTAAA CGATGGATAC TAGGCGTGGC TTGTATCGAC CCGAGCCGTG CCGTAGCTAA CGCGTTAAGT ATCCCGCCTG GGGAGTACGC CGGCAACGGT GAAACTCAAA 840
B_1425bp CCAGTAGTCC TAGCCGTAAA CGATGGATAC TAGGCGTGGC TTGTATCGAC CCGAGCCGTG CCGTAGCTAA CGCGTTAAGT ATCCCGCCTG GGGAGTACGC CGGCAACGGT GAAACTCAAA 800
A_1378bp CCAGTAGTCC TAGCCGTAAA CGATGGATAC TAGGCGTGGC TTGTATCGAC CCGAGCCGTG CCGTAGCTAA CGCGTTAAGT ATCCCGCCTG GGGAGTACGC CGGCAACGGT GAAACTCAAA 761
Consensus CCAGTAGTCC TAGCCGTAAA CGATGGATAC TAGGCGTGGC TTGTATCGAC CCGAGCCGTG CCGTAGCTAA CGCGTTAAGT ATCCCGCCTG GGGAGTACGC CGGCAACGGT GAAACTCAAA
100%
Conservation
0%
860
880
900
920
940
960
D_1456bp GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGTATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC AAGGCTTGAC ATGTCGCGAA TCTTCTTGAA AGGGAAGAGT GCCTTCGGGA 960
E_1464bp GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGTATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC AAGGCTTGAC ATGTCGCGAA TCTTCTTGAA AGGGAAGAGT GCCTTCGGGA 960
B_1425bp GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGTATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC AAGGCTTGAC ATGTCGCGAA TCTTCTTGAA AGGGAAGAGT GCCTTCGGGA 920
A_1378bp GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGTATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC AAGGCTTGAC ATGTCGCGAA TCTTCTTGAA AGGGAAGAGT GCCTTCGGGA 881
Consensus GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGTATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC AAGGCTTGAC ATGTCGCGAA TCTTCTTGAA AGGGAAGAGT GCCTTCGGGA
100%
Conservation
0%
980
1.000
1.020
1.040
1.060
1.080
D_1456bp GCGCGAACAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT CGTTTTTAGT TGCCAGCATT AAGTTGGGCA CTCTAGAGAG 1080
E_1464bp GCGCGAACAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT CGTTTTTAGT TGCCAGCATT AAGTTGGGCA CTCTAGAGAG 1080
B_1425bp GCGCGAACAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT CGTTTTTAGT TGCCAGCATT AAGTTGGGCA CTCTAGAGAG 1040
A_1378bp GCGCGAACAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT CGTTTTTAGT TGCCAGCATT AAGTTGGGCA CTCTAGAGAG 1001
Consensus GCGCGAACAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT CGTTTTTAGT TGCCAGCATT AAGTTGGGCA CTCTAGAGAG
100%
Conservation
0%
1.100
1.120
1.140
1.160
1.180
1.200
D_1456bp ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AGTCAGCATG CCCCTTACGC CTTGGGCTAC ACACGTACTA CAATGCTCCG GACAGAGGGC AGCAAGCATG CGAATGCAAG 1200
E_1464bp ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AGTCAGCATG CCCCTTACGC CTTGGGCTAC ACACGTACTA CAATGCTCCG GACAGAGGGC AGCAAGCATG CGAATGCAAG 1200
B_1425bp ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AGTCAGCATG CCCCTTACGC CTTGGGCTAC ACACGTACTA CAATGCTCCG GACAGAGGGC AGCAAGCATG CGAATGCAAG 1160
A_1378bp ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AGTCAGCATG CCCCTTACGC CTTGGGCTAC ACACGTACTA CAATGCTCCG GACAGAGGGC AGCAAGCATG CGAATGCAAG 1121
Consensus ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AGTCAGCATG CCCCTTACGC CTTGGGCTAC ACACGTACTA CAATGCTCCG GACAGAGGGC AGCAAGCATG CGAATGCAAG
100%
Conservation
0%

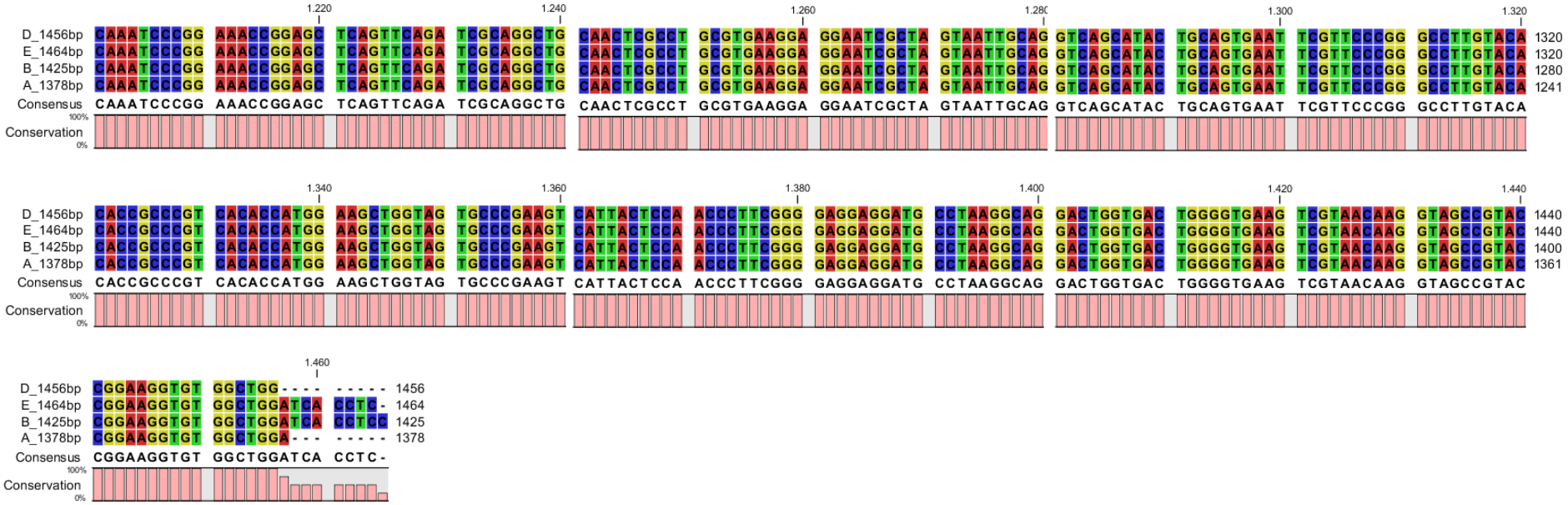
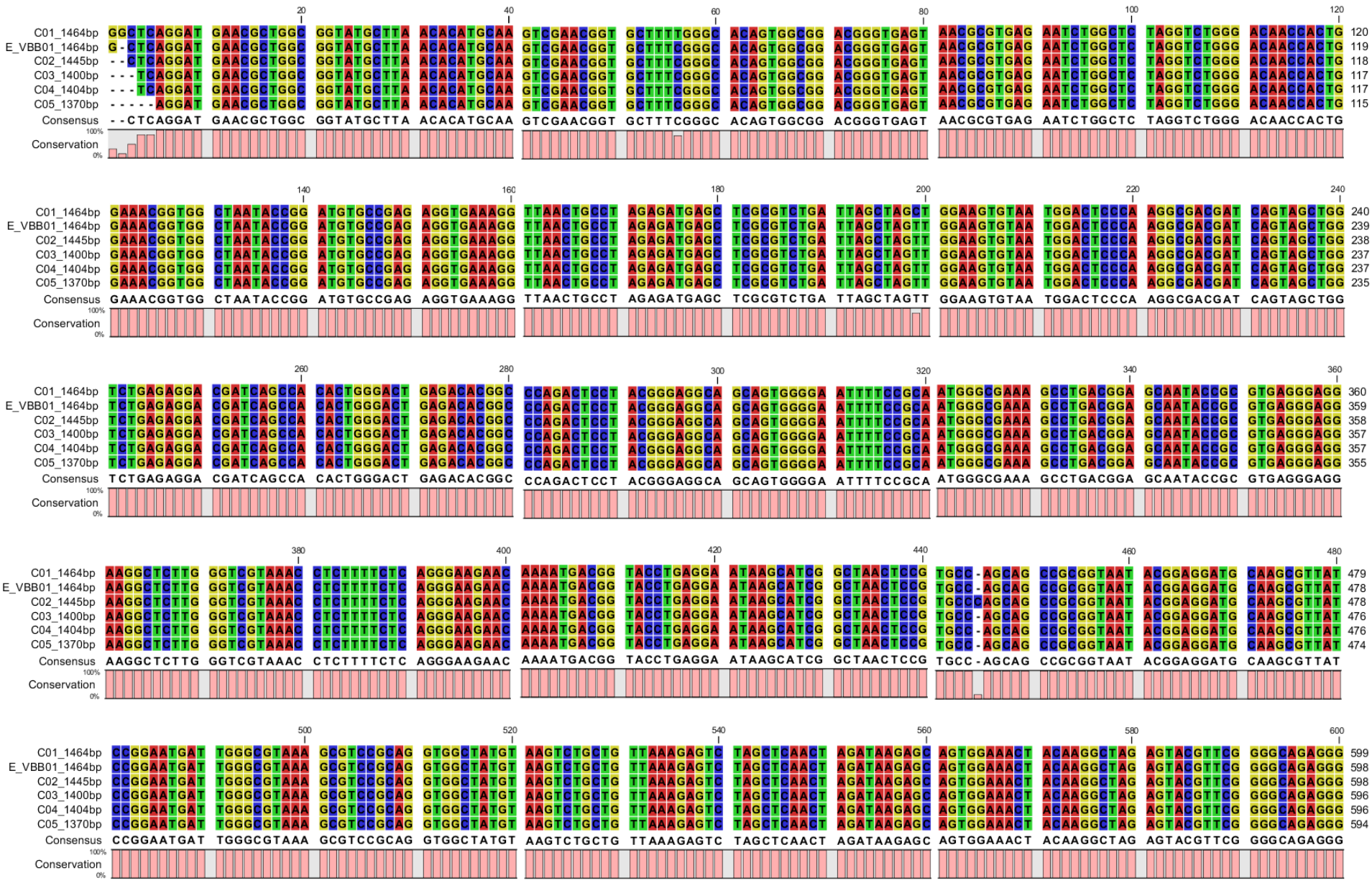


Figure 4: Alignment of 16S rDNA sequences from *P. membranacea*


C01_1464bp
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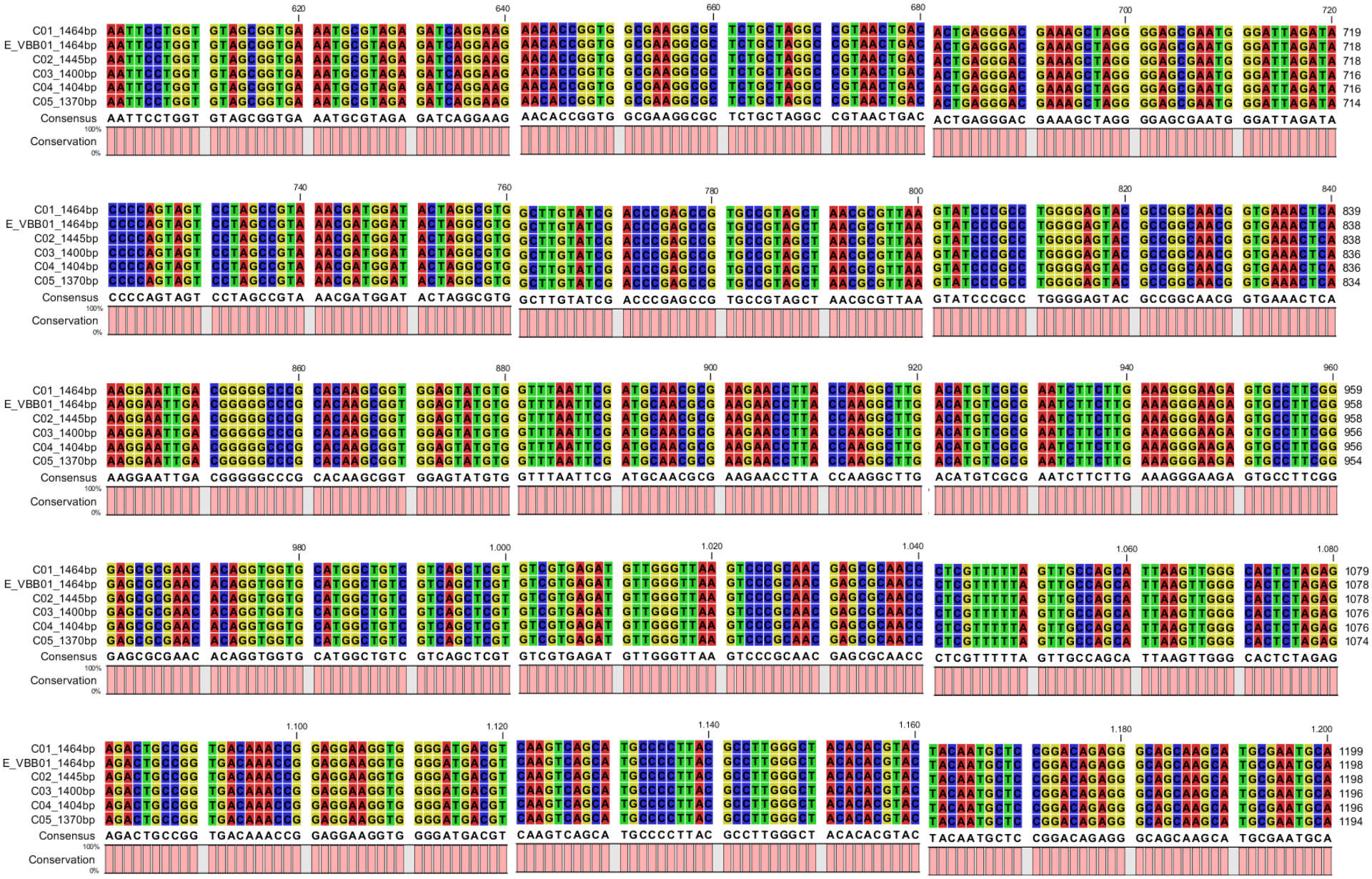
C02_1445bp
C03_1400bp
C04_1404bp
C05_1370bp
Consensus
Conservation


C01_1464bp
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C02_1445bp
C03_1400bp
C04_1404bp
C05_1370bp
Consensus
Conservation
100%
0%

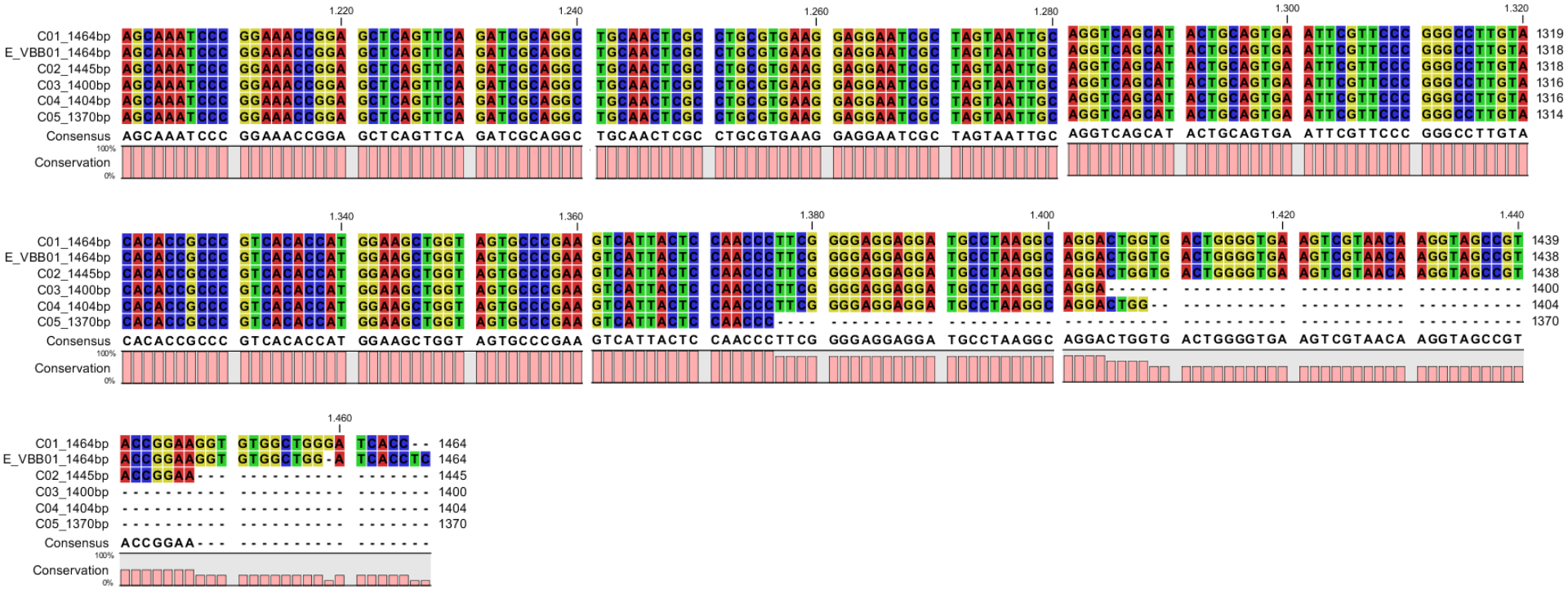


Figure 5: aligment of the comparison with sample E and samples from work of Celia.

After the results obtained with the samples from Iceland, another comparison was conducted in order to see some possible geographical differences.

Several cyanobacterial 16S rDNA gene sequences from other geographic areas available in GenBank Database were used together with the sample E of the present study.

Additionally, a 16S Nostoc genome sequence from recent work at the University of Iceland was included in the comparison (see Table 6).

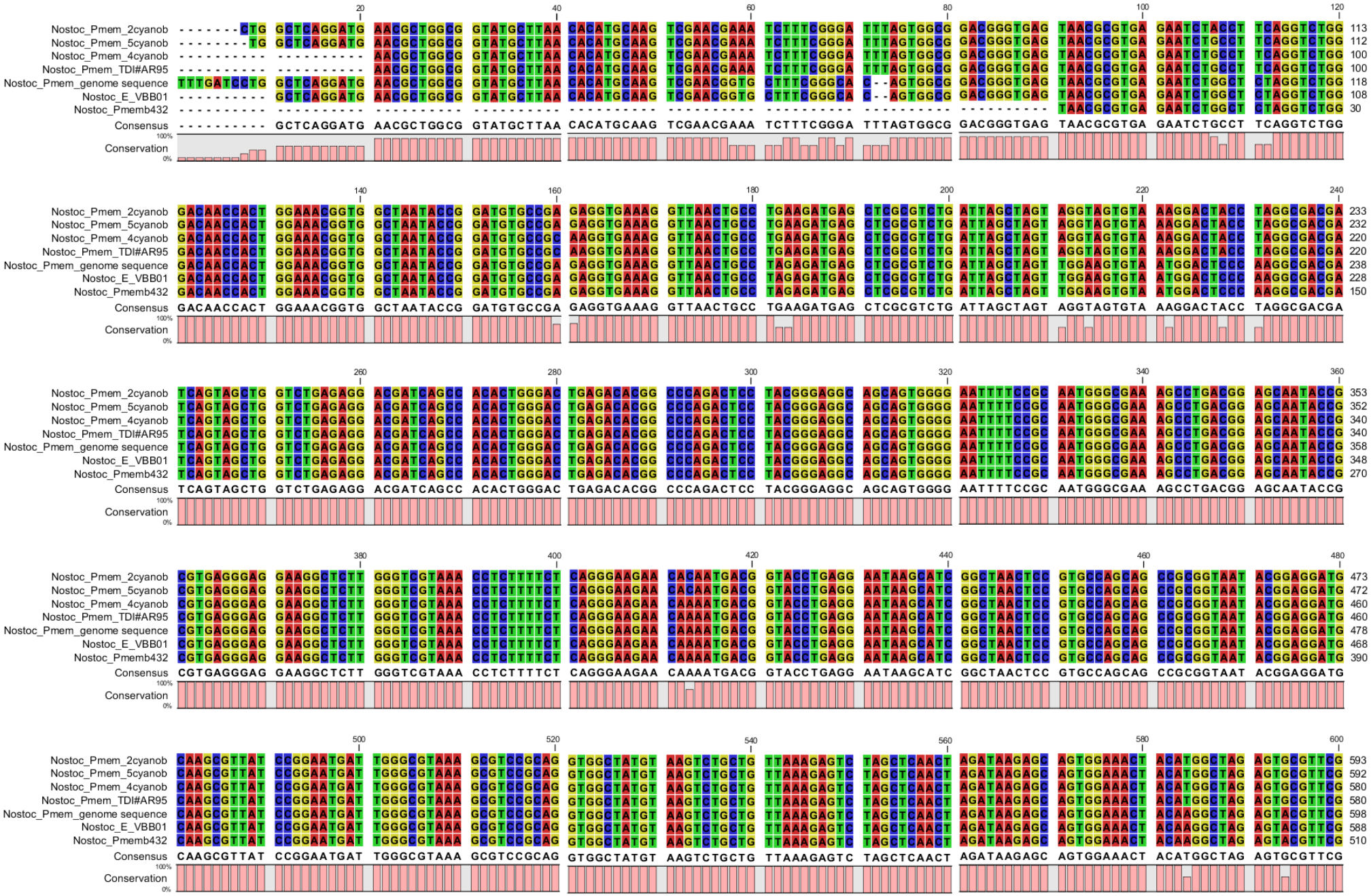
	Accession nº	**Strain**	**Place**	**Isolation source**	**Reference**	**Length (bp)**
1	DQ185227.1	*Nostoc sp. Pmem* 2cyanob.	Durham, USA	*P.membranacea* lichen specimen voucher	BLAST	1443
2	DQ185248.1	*Nostoc sp. Pmem* 4cyanob.	Durham, USA	*P.membranacea* lichen specimen voucher	BLAST	1423
3	DQ185247.1	*Nostoc sp. Pmem* 5cyanob.	Durham, USA	*P.membranacea* lichen specimen voucher	BLAST	1451
4		*Nostoc sp*. TDI#AR95	Vancouver, Canada	*P.membranacea* lichen- field sample	Unpublished (thesis)	1397
5	EF102282.1	*Pmemb* 432	Botanical museum, Helsinki	*P.membranacea* lichen- field sample	BLAST	640
6		*Pmem* Genome sequence	Keldur- Iceland	*P.membranacea* lichen- field sample	Recent Research	1478
7		*Nostoc sp.* VBB01	Iceland	*P.membranacea* lichen- field sample	Current Research	1464

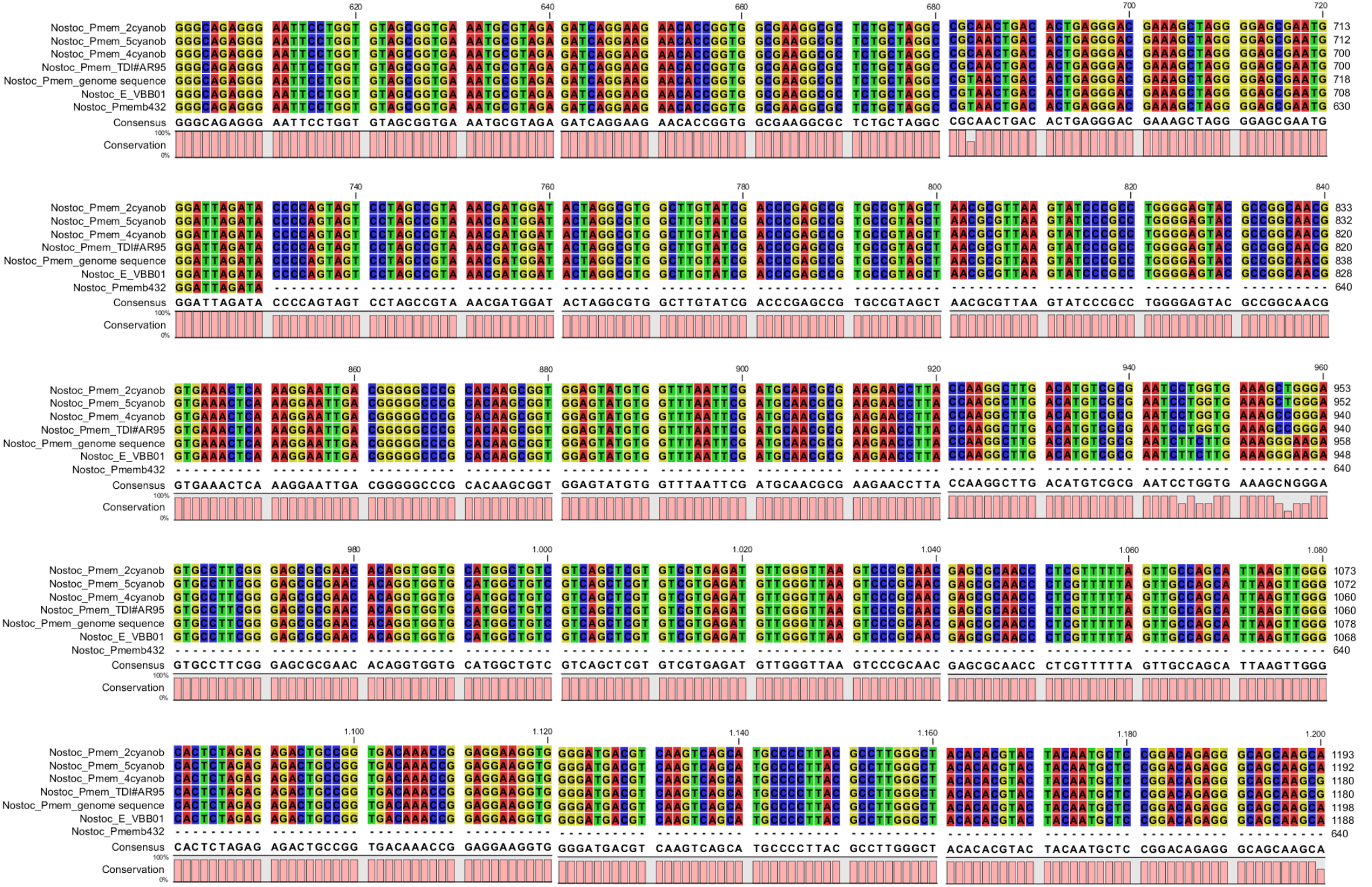
Table 6: Sequence data used for geographical comparison.
1,2,3 : O'Brien H., Miadlikowska J., Lutzoni, F. (2005). Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera*
4: Miao, V.P.W, Rabenau, A., Lee, A., (1997).Cultural and Molecular Characterization of Photobionts of *Peltigera membranacea*
5: Myllys, L., Stenroos, S., Thell, A., Kuusinen, M. (2006). High cyanobiont selectivity of epiphytic lichens in old growth boreal forest of Finland.

Nucleotide variations were analyzed by alignment with the CLC Genomics program.

The results revealed that sequences P.mem 4 cyanob. and TD#AR95 showed exactly the same sequence and the pair of sequences P.mem 2 cyanob and P.mem 5cyanob. differed in only one nucleotide.

With regards to the general comparison, several differences were found between both strains from Iceland and the rest of the strains. Specifically, in the 640 bases stretch the Icelandic strains are identical to the Finnish strain and both have 15 differences from the American strains including the Canadian strain (see Figure 6).


Nostoc_Pmem_2cyanob
Nostoc_Pmem_5cyanob
Nostoc_Pmem_4cyanob
Nostoc_Pmem_TDI#AR95
Nostoc_Pmem_genome sequence
Nostoc_E_VBB01
Nostoc_Pmemb432
Consensus
Conservation
100%
0%


620
640
660
680
700
720
Nostoc_Pmem_2cyanob GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGCAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 713
Nostoc_Pmem_5cyanob GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGCAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 712
Nostoc_Pmem_4cyanob GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGCAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 700
Nostoc_Pmem_TDI#AR95 GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGCAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 700
Nostoc_Pmem_genome sequence GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGTAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 718
Nostoc_E_VBB01 GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGTAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 708
Nostoc_Pmemb432 GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGTAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG 630
Consensus GGGCAGAGGG AATTCCTGGT GTAGCGGTGA AATGCGTAGA GATCAGGAAG AACACCGGTG GCGAAGGCGC TCTGCTAGGC CGCAACTGAC ACTGAGGGAC GAAAGCTAGG GGAGCGAATG
100%
Conservation
0%
740
760
780
800
820
840
Nostoc_Pmem_2cyanob GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG 833
Nostoc_Pmem_5cyanob GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG 832
Nostoc_Pmem_4cyanob GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG 820
Nostoc_Pmem_TDI#AR95 GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG 820
Nostoc_Pmem_genome sequence GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG 838
Nostoc_E_VBB01 GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG 828
Nostoc_Pmemb432 GGATTAGATA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 640
Consensus GGATTAGATA CCCCAGTAGT CCTAGCCGTA AACGATGGAT ACTAGGCGTG GCTTGTATCG ACCCGAGCCG TGCCGTAGCT AACGCGTTAA GTATCCCGCC TGGGGAGTAC GCCGGCAACG
100%
Conservation
0%
860
880
900
920
940
960
Nostoc_Pmem_2cyanob GTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGTATGTG GTTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG ACATGTCGCG AATCCTGGTG AAAGCTGGGA 953
Nostoc_Pmem_5cyanob GTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGTATGTG GTTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG ACATGTCGCG AATCCTGGTG AAAGCTGGGA 952
Nostoc_Pmem_4cyanob GTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGTATGTG GTTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG ACATGTCGCG AATCCTGGTG AAAGCCGGGA 940
Nostoc_Pmem_TDI#AR95 GTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGTATGTG GTTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG ACATGTCGCG AATCCTGGTG AAAGCCGGGA 940
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Nostoc_E_VBB01 GTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGTATGTG GTTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG ACATGTCGCG AATCTTCTTG AAAGGGAAGA 948
Nostoc_Pmemb432 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 640
Consensus GTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGTATGTG GTTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG ACATGTCGCG AATCCTGGTG AAAGCNGGGA
100%
Conservation
0%
980
1.000
1.020
1.040
1.060
1.080
Nostoc_Pmem_2cyanob GTGCCTTCGG GAGCGCGAAC ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCGTTTTTA GTTGCCAGCA TTAAGTTGGG 1073
Nostoc_Pmem_5cyanob GTGCCTTCGG GAGCGCGAAC ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCGTTTTTA GTTGCCAGCA TTAAGTTGGG 1072
Nostoc_Pmem_4cyanob GTGCCTTCGG GAGCGCGAAC ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCGTTTTTA GTTGCCAGCA TTAAGTTGGG 1060
Nostoc_Pmem_TDI#AR95 GTGCCTTCGG GAGCGCGAAC ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCGTTTTTA GTTGCCAGCA TTAAGTTGGG 1060
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Nostoc_E_VBB01 GTGCCTTCGG GAGCGCGAAC ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCGTTTTTA GTTGCCAGCA TTAAGTTGGG 1068
Nostoc_Pmemb432 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 640
Consensus GTGCCTTCGG GAGCGCGAAC ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCGTTTTTA GTTGCCAGCA TTAAGTTGGG
100%
Conservation
0%
1.100
1.120
1.140
1.160
1.180
1.200
Nostoc_Pmem_2cyanob CACTCTAGAG AGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCAGCA TGCCCCTTAC GCCTTGGGCT ACACACGTAC TACAATGCTC CGGACAGAGG GCAGCAAGCA 1193
Nostoc_Pmem_5cyanob CACTCTAGAG AGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCAGCA TGCCCCTTAC GCCTTGGGCT ACACACGTAC TACAATGCTC CGGACAGAGG GCAGCAAGCA 1192
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Nostoc_Pmem_TDI#AR95 CACTCTAGAG AGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCAGCA TGCCCCTTAC GCCTTGGGCT ACACACGTAC TACAATGCTC CGGACAGAGG GCAGCAAGCG 1180
Nostoc_Pmem_genome sequence CACTCTAGAG AGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCAGCA TGCCCCTTAC GCCTTGGGCT ACACACGTAC TACAATGCTC CGGACAGAGG GCAGCAAGCA 1198
Nostoc_E_VBB01 CACTCTAGAG AGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCAGCA TGCCCCTTAC GCCTTGGGCT ACACACGTAC TACAATGCTC CGGACAGAGG GCAGCAAGCA 1188
Nostoc_Pmemb432 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 640
Consensus CACTCTAGAG AGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCAGCA TGCCCCTTAC GCCTTGGGCT ACACACGTAC TACAATGCTC CGGACAGAGG GCAGCAAGCA
100%
Conservation
0%

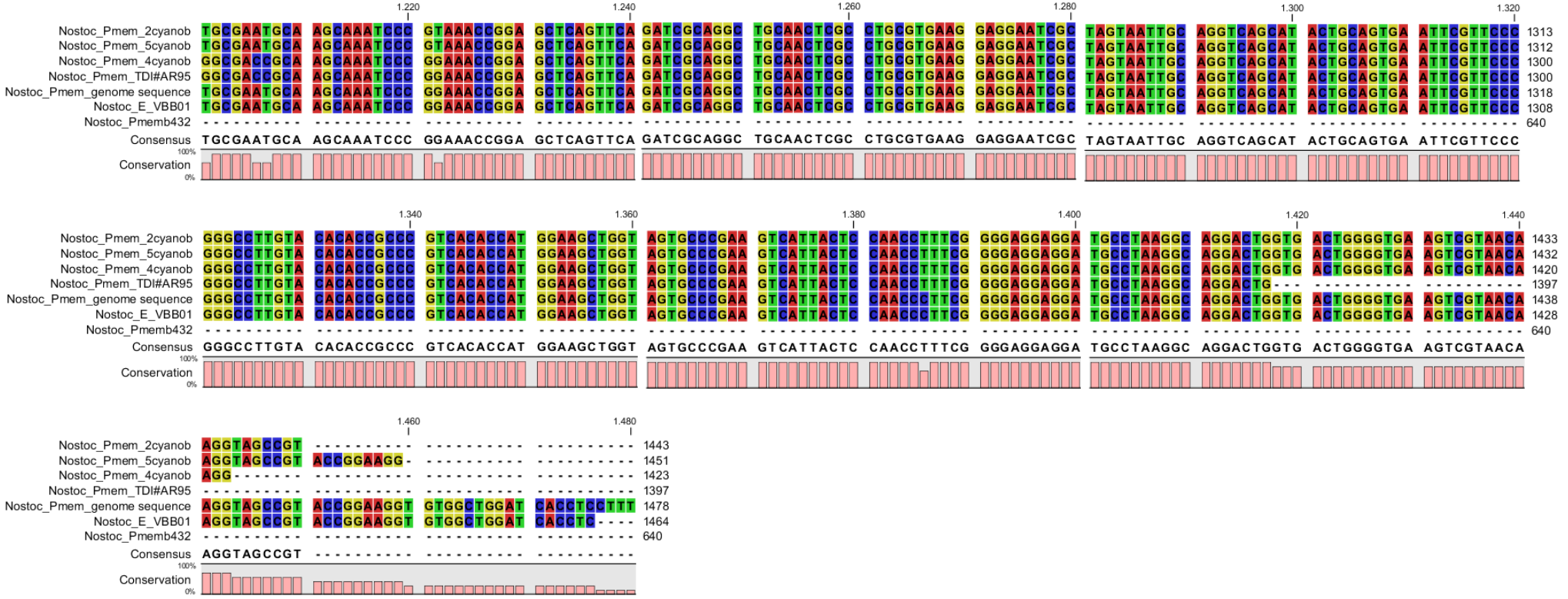
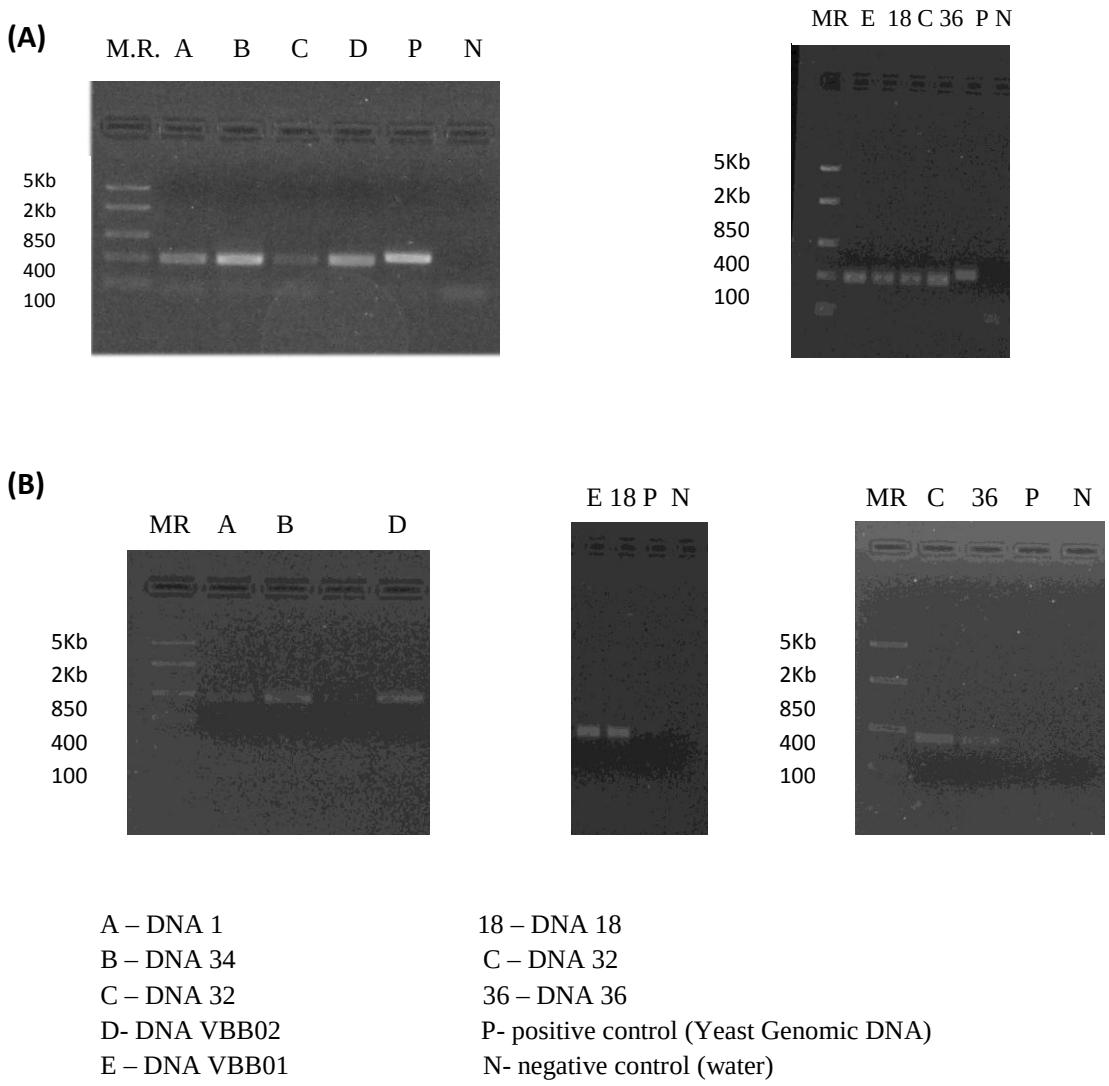


Figure 6: Alignment of the comparison with sample E of this project and other samples from GenBank Database

4.3. Molecular analysis of 18S-26S Internal Transcribed Spacer rDNA sequences

PCR was performed to amplify the 18S- 26S rRNA ITS region from fungal DNA extracted from thallus and apothecium. The fungal specific primers used are shown in Table 4.

Results were cheked using a 1.2% agarose gel.



A – DNA 1	18 – DNA 18
B – DNA 34	C – DNA 32
C – DNA 32	36 – DNA 36
D- DNA VBB02	P- positive control (Yeast Genomic DNA)
E – DNA VBB01	N- negative control (water)

Figure 7: Photograph of agarose gel electrophoresis of PCR products generated by amplification using ITS primers. (A) ITS 5- ITS 2 primers were used for amplification. (B) ITS 1- ITS4 primers were used for amplification.

Agarose gel electrophoresis analysis showed a single band from each sample, resulting in a product approximately 400bp for ITS 5-ITS 2 pair and 700 bases long for ITS 1- ITS 4 primers.

The nucleotide sequences of the resulting amplification products were aligned and blasted to the NCBI GenBank Database.

All samples were identified as *Peltigera membranacea* as expected.

Sample ID	Contig Length (bp)	Blast Results	Accession nº	Query %	Max.Ident %
A	683	*Peltigera membranacea* ITS 1,5.8SrRNA gene, ITS2	AY257907.1	96	95
B	684	*Peltigera membranacea* ITS 1,5.8SrRNA gene, ITS2	AY257907.1	96	95
D	684	*Peltigera membranacea* ITS 1,5.8SrRNA gene, ITS2	AY257907.1	96	94
E	677	*Peltigera membranacea* strain H0B020708-0-9-3 18S rRNA gene, ITS 1,5.8S rRNA gene, ITS 2	FJ709033.1	98	96
18	681	*Peltigera membranacea* strain H0B020708-0-9-3 18S rRNA gene, ITS 1,5.8S rRNA gene, ITS 2	FJ709033.1	98	95
C	697	*Peltigera membranacea* ITS 1,5.8SrRNA gene, ITS2	AY257907.1	94	87
36	676	*Peltigera membranacea* strain H0B020708-0-9-3 18S rRNA gene, ITS 1,5.8S rRNA gene, ITS 2	FJ709033.1	98	95

Table 7: Sequences results according to NCBI GenBank Database.

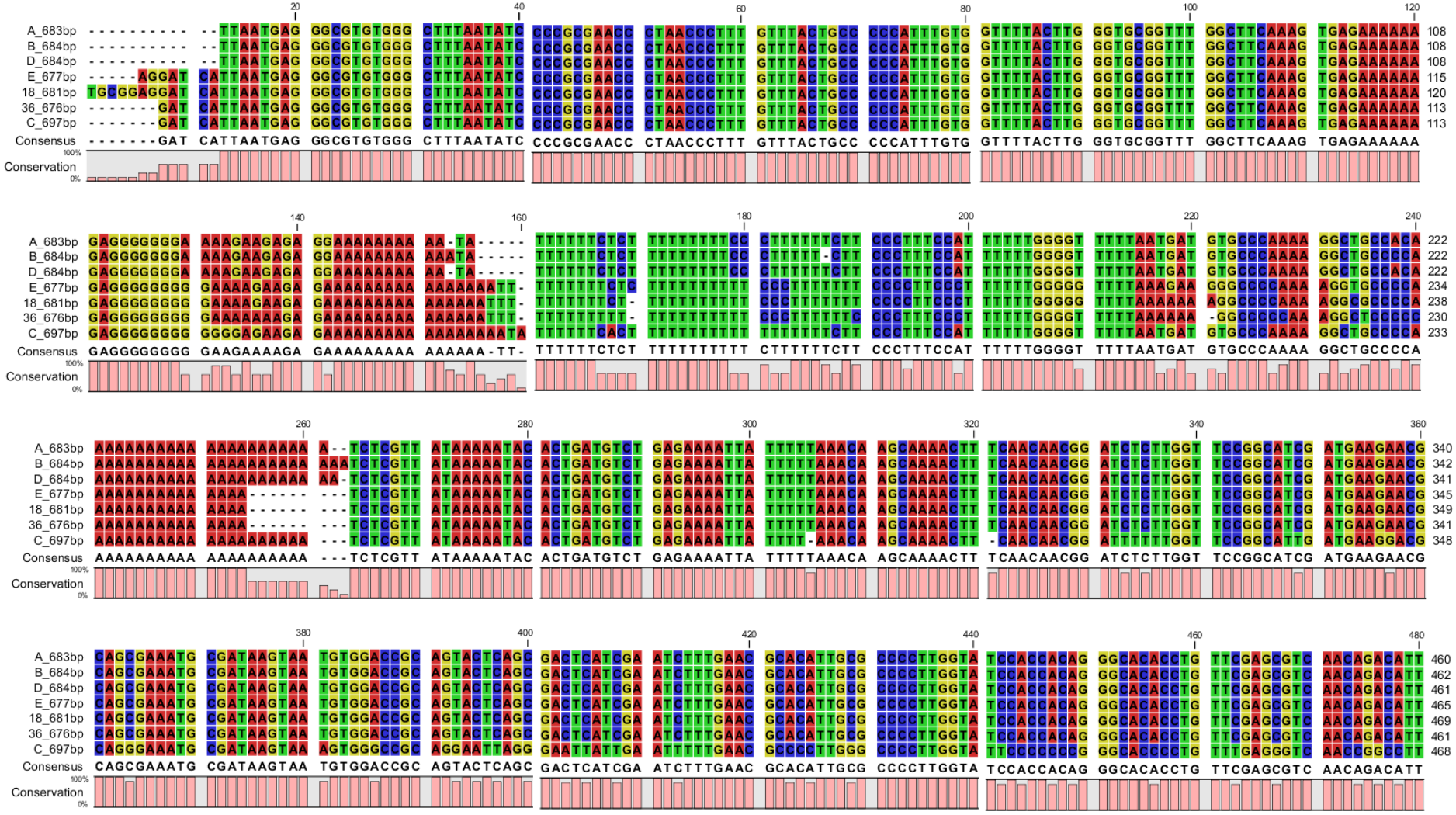
The alignment was cheked by eye, which revealed some variations between the sequences (see alignment in Fig.8).

Analyzing samples from the thallus, it was found that samples E and 18 had exactly the same sequence which differed in about 20 nucleotides from the sequence of the other samples A, B and D. In sequence D one nucleotide difference from samples A and B was detected in the position 662 (see black circle in fig. 8).

Between samples from the apothecium, some different nucleotides were visualized in the alignment but after reviewing the peaks of the bases no reliable difference was detected due to the low quality of the sequencing of the sample C.

In the region of the sequence C where the quality was quite good, which corresponded with the beginning and the end of the sequence, the alignment was perfect.

In the comparison among the samples from the thallus and the samples from the apothecium, sample 36 is very close to samples E and 18 from the thallus with only 8 nucleotides differences.


A_683bp
B_684bp
D_684bp
E_677bp
18_681bp
36_676bp
C_697bp
Consensus
Conservation

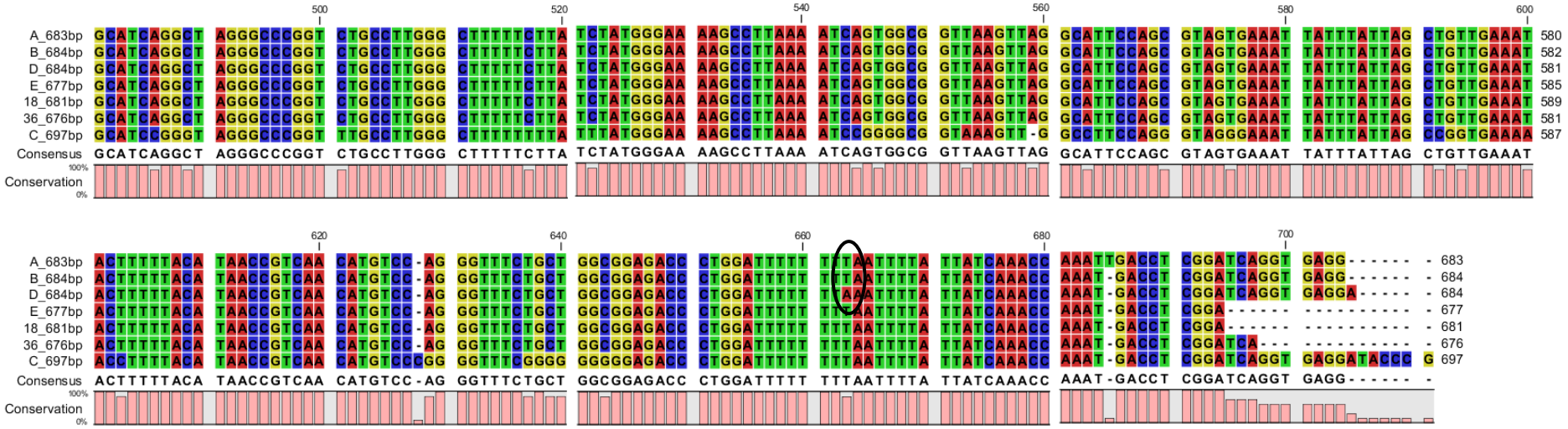


Figure8: Alignment of Internal Transcribed Spacer (ITS) region from *Peltigera membranacea*.

4.4. Phylogenetic Analysis

16S rRNA gene sequences

The phylogenetic tree was constructed with the program MEGA 4.1, using Bootstrap test 'Neighbor-Joining' (see phylogenetic tree in figure 9).

For that, the four sequences of the current project were taken along with sequences from Celia's work and sequences used in the geographical comparison. The sample from Helsinki wasn't used due to the shorter length.

Furthermore, an additional sequence from a free-living Nostoc strain was used in order to have a sequence with which to compare.

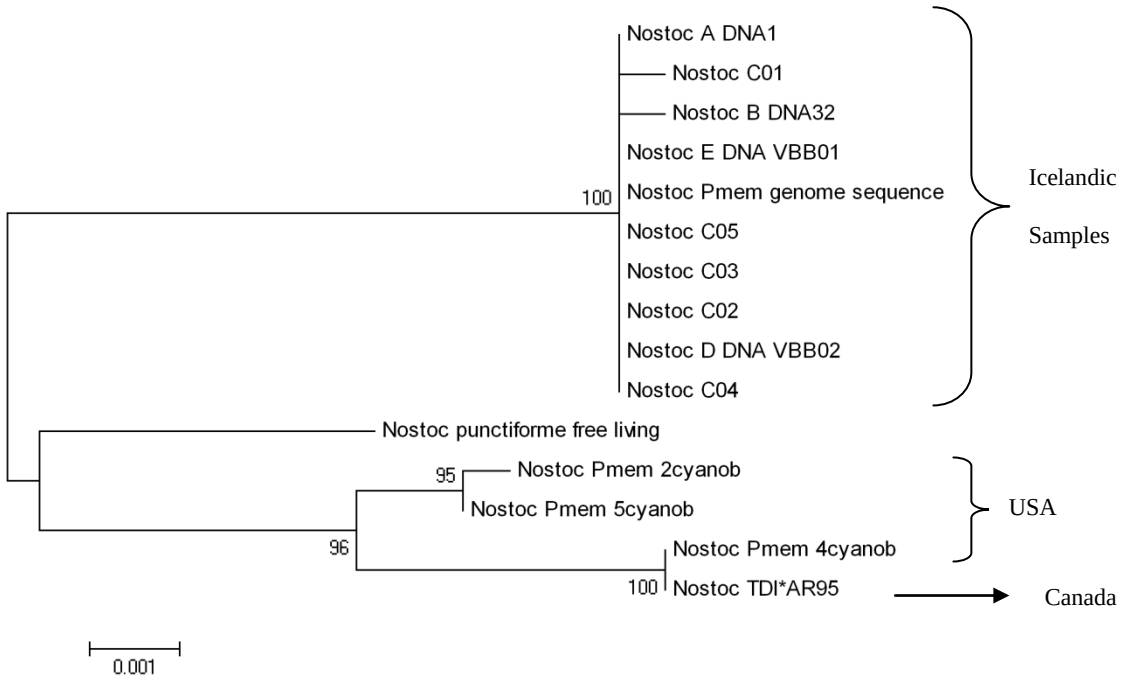


Figure 9: Phylogenetic tree of 16S rRNA gene made with the Bootstrap test 'Neighbor- joining' method.

The tree showed two categories as it was expected. One of the categories joined all Nostoc strains from Iceland whereas the other category grouped *Nostoc* strains from U.S.A. and Canada. *Nostoc punctiforme* presented a separate branch as expected.

The *Nostoc* from Helsinki, which was not used for making the tree, should be placed in the same category as the samples from Iceland.

The results suggest that all strains from Iceland and the strain from Helsinki are closely related as well as the pair of strains P.mem 4 cyanob and TD#AR95.

18S- 26S Internal Transcribed Spacer (ITS) rDNA sequences

For making the phylogenetic tree, the five thallus samples and two from apothecia were used together with another two sequences of *Peltigera membranacea* ITS region from Durham (USA) found in GenBank (see tree in figure 10).
All the samples have more or less the same length.

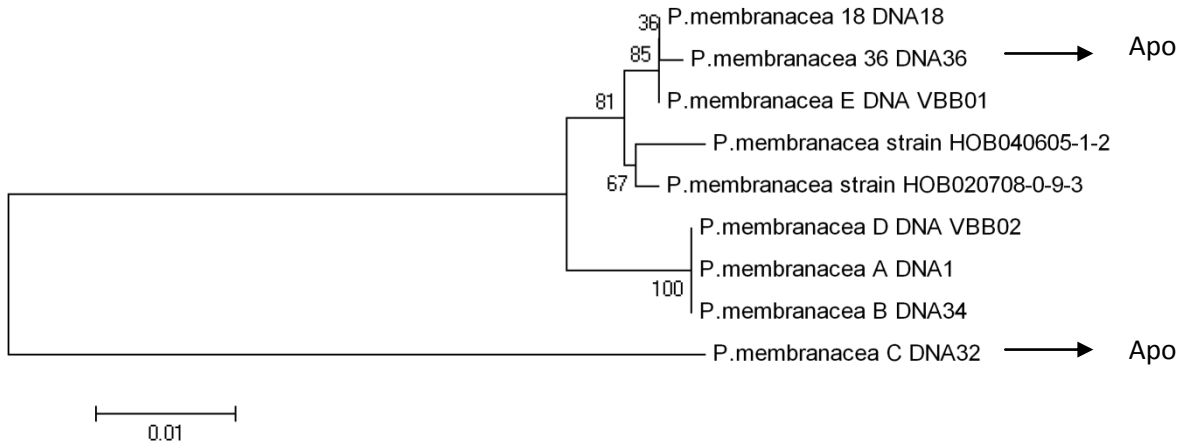


Figure 10: Phylogenetic tree of ITS region of *Peltigera membranacea* made with the Bootstrap test 'Neighbor- joining' method.

Three well differentiated groups were formed. The first group clustered samples E and 18 from thallus and sample 36 from apothecium was also placed in the same group.
The second group included samples A, B and D from thallus and the third widely diverging group was formed by sample C.

5. Conclusions and Discussion

Molecular analysis of 16S rDNA gene sequences

The same strain of *Nostoc* was found in association with the lichen *Peltigera membranacea* collected in the different places in Iceland.
16S rDNA gene data confirmed a completely homogeneity in the population of symbiotic *Nostoc* in Iceland.
The same homogeneity was observed in the comparison with Celia's Icelandic sequences but not in the comparison with sequences from other geographical areas, where considerable differences were found.

At the population level, it can be concluded that no cyanobiont diversity was found in Iceland. Each lichen thallus contains the same single strain of *Nostoc*.
On the other hand, on the geographical level, sequence variation in symbiotic *Nostoc* is considerable. The site where lichens grow seems to determine what strain of *Nostoc* appears in the *P. membranacea* symbiosis.

Molecular analysis of 18S-26S Internal Transcribed Spacer rDNA sequences

In the second part of this research, ITS sequences of different *Peltigera membranacea* were compared to check the species variation within thallus and apothecia and in the population.

There is no significant support for a real homogeneity between the *P. membranacea* samples. The reason for the heterogeneity might be

(i) DNA of the thallus and apothecium wasn't extracted from the same specimen of lichen sample.
(ii) The quality of the sequence was not completely reliable.

The phylogenetic tree showed small but significant differences placing sample C alone in a different group from the rest of the samples, which were placed in two other groups.
Samples A and 18 from thallus were not placed in the same group, in spite of belonging to the same place, probably because they were collected in different times and not at the exact same location.

Finally, it can be concluded that the level of homogeneity within the population of the fungus *Peltigera membranacea* in Iceland is not high.

From a genetic and evolutionary perspective, this study shows that associations between cyanobacteria and lichen- forming fungi can be very specific and stable.

- There is only a single *Nostoc* strain within the *Peltigera membranacea* population.
- The identity of the *Nostoc* symbiont is dependent on collection site for the species investigated.
- There is more variation in the *P. membranacea* fungal (mycobiont) 18S-26S ITS sequences than in the cyanobiont 16SrRNA sequences, and this may reflect a greater heterogeneity in the fungal mycobiont population than in the *Nostoc* cyanobiont population.

Regarding the ITS analysis, firm conclusion would require further investigation with the intention of going deeper into the mentioned study.

References

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Appendix

Protocol for Lichen DNA Isolation (*Peltigera membranacea*)

1. Sample of interest is collected from the field and washed running tap water for several times and finally it is rinsed with deionized water.
2. Sample is cleaned under microscope to remove the debris from the field.
3. Clean lichens (about 2 grams) are grounded with liquid nitrogen in a cold morter. The powder material is collected into pre- cooled 50 ml tubes.
4. Add 25 ml of Lysis buffer (50mM EDTA, 100 mM NaCl, 10mM Tris HCl pH 8.0, 1% SDS). Buffer temperature should be 65°C prior to use.
5. Tubes are kept in the 65 °C water bath for 20 minutes, during the incubation the tubes are shaken for many times.
6. After the incubation, the soup is collected from the tubes and distributed evenly in 2ml EP tubes.
7. Tubes are spinning it 4k for 4 minutes.
8. The pellet is discarded and supernatant will distribute in new 2 ml tubes, approximately 1.25 ml each. Then, add 535 µl of 10.5M of NH4Ac and mix well. Tubes are kept in the ice for 20 minutes.
9. Tubes are spinning it 4k for 4 minutes and the peller is discarded.The supernatant is collected in the new 2ml tubes for next step.
10. Add 800 µl of Isopropanol into supernatant and inver the tubes for several times. The tubes are kept in ice for 20 minutes.
11. Tubes are spinning it 14k for 4 minutes. The supernatant is discarded and the pellet is saved for the next step.
12. Add 100 µl of TE buffer with RNAase to the pellet of each tube (stock concentration of RNAase 10 mg/ml. It should be 4 µl for 1 ml of solution)
13. Then the tubes are kept in the thermomixer at 50° C for 20- 60 minutes until pellet get dissolved.
14. The tubes go for short spinning to bring down the condensed waters during the time of incubation.
15. The supernatant is pooled into 1.5 ml tubes (4 samples per tube)
16. Add equal volume of Phenol:Chloroform (1:1) to the tubes and mixed well
17. Spinning it for 14k for 4 minutes. The top phase of the solution is pooled out without disturbing the interphase.
18. The equal volume of saturated chloroform is added to the supernatant and mixed
19. Spinning it for 14k for 4 min. After spinning, top layer is pooled out or proceed with next steps (if needed it can be confirmed through 0.6% agarose gel for DNA)
20. Add (1:10) of 3M NaAc. (i.e.) If supernatant was 500 µl means add 50 µl
21. Add double amount of 100% ETOH to the solution and mixed well. Keep the tubes in ice for 3 minutes.
22. Spinning it for 14k for 4 minutes. Supernatant is discarded and pellet is preserved for next step.
23. Pellet is washed with 70% ETOH.Approximately 500 µl.
24. Spinning it 14K for 4 minutes, discard the supernatant and pellet goes for air dry to make sure ethanol gets evaporated.
25. Add 50 µl of TE buffer to the pellet and keep it in the thermomixer at 50ºC for 3 minutes to dissolve the pellet. Then the tubes go for short spinning.
26. Load 5 µl of the sample into a 0.6% of agarose gel to confirm the DNA bands.
27. Store the tubes in the freezer.

Protocol for Direct Sequencing Reaction- PCR products

1. Exo SAP Mixture (with 10% of extra amount required for reaction)

Double distilled water	16.28 µl
Exo I buffer/ Phosphatases	4.4 µl
Ant. Phosphatases	0.88 µl
Exo I	0.44 µl
DNA (PCR products)	5 µl (different samples for each tube)

2. The reaction tubes were incubated in the PCR machine at 38°C for 35 mins, heat inactivated at 80°C for 20 min and 12°C for forever (exo- sap program).
3. Sequence Reaction Mixture

Double distilled water	88.2 µl
Sequencing Buffer	46.2 µl
Big Dye	8.4 µl

4. In each tube
   11.5 µl of Sequence reaction mixture
   1.5 µl of primer forward or primer reverse (10 µM)
   2 µl of Exo Sap DNA solution
   Total each tube has 15 µl of sequence reaction mixture.
5. Sequencing reaction cycle(30 cycles): tubes are setting in the PCR machine with the program as follows, 96°C for 10 seconds, 96°C for 10 sec, 55°C for 5 sec, 60°C for 2 min, 12°C for 7 min and 12°C for forever.
6. Precipitation of DNA

   Precipitation Mix for 8 samples:

Milli Q water	450 µl
NaOAc 3 M	50 µl
Glycogen	2.5 µl

7. Add to each tube 50 µl of Precipitation Mix and 10 µl of sample and pipette mix gently
8. Add 125 µl of 96 % ice cold ETOH and pipette mix.
9. Centrifugate them 5500 r.p.m. for 30 minutes at 4°C.
10. Supernatant is discarded with air suction.
11. Add 250 µl of 70% ice cold ETOH.
12. Centrifugate them 10500 r.p.m. for 5 minutes at 4°C twice.
13. Supernatant is removed with air suction.
14. Samples are kept in a dark place for drying until total evaporation of ETOH.
15. After drying completely add 20 µl of HIDI and mix well (vortex and spin).
16. Heat the samples at 95°C for 2 minutes.
17. Chill on ice for 2 minutes.
18. Vortex and spin the samples.
19. Load samples in ABI plate and read the sequences in the sequencer.