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DETERMINATION OF CYANOBACTERIA DIVERSITY FROM HOT SPRINGS AROUND AYDIN PROVINCE BY CULTURE INDEPENDENT METHODS

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MASTER'S THESIS

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Ruth Maseko PHIRI

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LIST OF SYMBOLS AND ABBREVAITIONS

AXI **:** Growth media containing Ampicillin

BHI **:** Brain Heart Infusion

CO_2 **:** Carbon dioxide

DNA **:** Deoxyribonucleic Acid

E. coli **:** *Escherichia coli*

EDTA **:** Ethylenediaminetetraacetic acid

Fe (II) **:** Iron 2

Fe (III) **:** Iron 3

$Fe(OH)^+$ **:** Iron Hydroxide

HGT **:** Horizontal Gene Transfer

HSPs **:** Heat Shock Proteins

ICSB **:** International Committee on Systematic Bacteriology

ICSP **:** International Committee on Systematic of Prokaryotes

IPTG **:** Isopropyl β-D-1-thiogalactopyranoside

LSU **:** Large Subunit

MAAs **:** Mycosporine-like Amino Acids

PCR **:** Polymerase Chain Reaction

rRNA **:** Ribosomal Ribonucleic Acid

SDS **:** Sodium Dodecyl Sulfate

SSU **:** Small Subunit

Tem ºC **:** Temperature

TES **:** N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid

TSA **:** Tryptic Soy Agar

TSB **:** Tryptic Soy Brooth

UV **:** Ultra-violet

X-gal **:** 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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ÖZET

KÜLTÜRDEN BAĞIMSIZ METOTLARLA AYDIN İLİ VE ÇEVRESİNDEKİ SICAK SU KAYNAKLARINDA SİYANOBAKTERİ ÇEŞİTLİLİĞİN BELİRLENMESİ.

Phiri R M, Aydın Adnan Menderes Üniversitesi, Fen Bilimleri Enstitüsü, Biyoloji Programı, Yüksek Lisans Tezi, Aydın, 2021.

Amaç: Bu tezin amacı, Aydın ili ve çevresindeki kaplıcalardan kültürden bağımsız yöntemlerle siyanobakteri çeşitliliğini belirlemektir.

Materyal ve Metodlar: Toplam genomik DNA'lar su, çamur veya toprak örneklerinden izole edildi ve ekstrakte edilmiş çevresel DNA'lardan 16S rDNA genleri PCR yöntemi ile amplifiye edildi. Amplikonlar daha sonra *E.coli* hücrelerine klonlandı, mavi-beyaz koloniler arasından transforme olmuş beyaz koloniler seçildi ve M13 primerleri kullanılarak PCR reaksiyonları yapıldı. M13 PCR ile elde edilen amplikonlar dizilendi ve GenBank veri tabanında BLAST analizine göre diğer bakteriyal gruplar ile olan homolojiler belirlendi.

Bulgular: Klonlama sonucu elde edilen fragmanların toplam sayısı 298 olup, yapılan sekans analizi sonucunda 201 tanesinin siyanobakteriler ile, 97 tanesinin ise diğer bakteriyal filumlar ile en yüksek homolojileri gösterdikleri belirlenmiştir. BLAST analizi sonucu bulunan yüzde özdeşlik %78 ila %100 aralığındadır. En sık tespit edilen taksonlar, *Planktothricoides raciborskii, Trichocoleus desertorum, Spirulina subsalsa, Gleobacter violaceus, Leptolyngbya laminosa, Synechococcus* sp. ve *Alkalima pantanalense olarak* belirlenmiştir.

Sonuç: Çalışmamız, Türkiye'de Aydın İli ve çevresindeki termal ortamlardan termofilik siyanobakterilerin belirlenmesinde kültürden bağımsız bir yaklaşım içermesi açısından önemlidir ve ekstrem habitatlardaki siyanobakteri araştırmalarına yeni bakış açıları sağlayabilir.

Anahtar Kelimeler: Siyanobakteriler, Kültürden Bağımsız Yöntem, Kaplıcalar ve 16S rDNA.

ABSTRACT

DETERMINATION OF CYANOBACTERIA DIVERSITY FROM HOT SPRINGS AROUND AYDIN PROVINCE BY CULTURE INDEPENDENT METHODS

Ruth Maseko Phiri, Aydın Adnan Menderes University, Graduate School of Natural and Applied Sciences, Biology Program, Master Thesis, Aydın, 2021.

Objectives: The thesis's objectives were to determine the cyanobacterial diversity from hot springs around Aydın province and surrounding using culture independent methods.

Material and Methods: Total genomic DNAs were isolated from water, mud or soil samples and 16S rDNA genes were amplified from extracted environmental DNAs. Amplicons were then cloned into *E.coli* cells, transformed cells were chosen and PCR reactions were done using M13 primers. Amplicons obtained by M13 PCR, were sequenced and according to BLAST analyses homologies were determined in GenBank database.

Results: In this study we highlighted the Cyanobacterial diversity of hot springs in Aydın and its surroundings using culture independent methods as well as cloning of PCR amplified fragments of 16S rRNA genes. The total number of cloned colonies were 298 of which 201 belonged to Cyanobacteria phylum while 97 other phylum and the percentage identity found were in the range of 78% to 100%. Most abundant species detected were, *Planktothricoides raciborskii, Trichocoleus desertorum, Spirulina subsalsa, Gleobacter violaceus, Leptolyngbya laminosa, Synechococcus sp.* and *Alkalinema pantanalense.*

Conclusion: Our study is important for being first culture- independent approach to determine thermophilic cyanobacteria from thermal environments in Aydın Province and surrounding area, Turkey and may provide new insights into the Cyanobacteria researches from extreme habitats.

Keywords: Cyanobacteria, Culture Independent Method, Hot Springs and 16S rDNA.

1. INTRODUCTION

In microbiological research, hot springs are isolated aquatic habitats with extreme temperatures, distinct chemical compositions and unique microbial communities (Strunecky et al., 2018). The microbial organisms identified in hot springs in several parts of the world are mostly Bacteria or Archaea. They play important roles by maintaining several ecosystem functions like nutrient cycling, primary production, litter decomposition and also climate regulations. These microorganisms are of huge interest in scientific areas such as in biotechnology.

The lineage Bacteria has been classified into many classes and one of them is Cyanophyceae which has a phylum called Cyanobacteria. This aquatic group constitutes Gram-negative bacteria that can perform oxygenic photosynthesis. Cyanobacteria contain phycobiliproteins and Chlorophyll *a* as their primary photosynthetic pigments (Waterbury, 2006). They are highly adaptive photosynthetic organisms that live in soil, marine and fresh water, including those with extreme conditions. Life in thermophilic and psychrophilic conditions leads to varying cyanobacterial morphology (Yılmaz and Arık, 2016). With the development of molecular techniques, identification of microorganism's diversity has been made easy thereby assisting researchers obtain results within a short period of time. One of the molecular techniques that has been used in microbiology laboratories is the culture independent method.

This technique allows the direct examination of the DNA or RNA in samples without growing the microorganisms within, allowing researchers to study the total diversity from the bulk extract in a single step. Moreover, classical physiological and biochemical tests are substandard in rapidly analyzing and identifying the bacteria from microbial communities, because the bacterial populations involved are fastidious and usually similar nutritional requirements and environmental conditions cannot be replicated in the laboratory (Su et al., 2012). To add to that, it is important to determine the microbiological diversity because it provides us with the data about the variety of microbial species present at the selected study site.

This study aimed to assess the Cyanobacterial diversity in hot springs of Aydin province and its surrounding areas using Culture-independent method, 16S rDNA

sequencing. To our knowledge, this study is the first to use the aforementioned method to investigate Cyanobacteria diversity in hot springs in the selected area.

2. LITERATURE REVIEW

2.1. Extremophiles

In the early 1970's, some environments like saturated salt brines, cold arctic water, hot springs, pressurized abyssal waters, and acidic and alkaline water considered as too extreme to mankind where found to support microbial life. These microorganisms that thrive within such extreme environments are named as hyperthermophiles, barophiles, psychrophiles, thermophiles, alcaliphiles, acidophiles, and halophiles according to their optimal growth conditions (Stetter, 1999).

Hyperthermophiles grow at an optimum temperature above 80^{o}C. They are found in environments of active volcanism, hot springs as well as geothermally heated subterranean rocks. Mostly these microorganisms belong to the Bacteria and Archaea domains (Stetter, 2013). Thermophiles: are microorganisms capable of growing and reproducing at optimum temperatures between 45^{o}C and 80^{o}C. These organisms have thermostable and proteolytic resistant cell membranes and proteins that remain intact at such elevated temperatures (Aror and Panosyan, 2019).

Thermophilic photosynthetic microorganisms have been reported worldwide, such as from Kamchatka, the Indian subcontinent, the Balkan Peninsula, North Africa, as well as the Yellowstone National Park in Wyoming, USA (Brock, 1995). Hot springs in the Yellowstone National Park are the largest and best studied; they have phototrophic microbial mats flourishing at approximately 74 °C (Castenholz, 1984; Mehta and Satyanarayana, 2013). Psychrophilic microorganisms: can grow at an optimum temperature of about 15 ^{o}C or even less but cannot grow above 20 ^{o} C. The other kind of microorganisms that can exhibit activity at temperatures close to the freezing point of water and also are able to grow at 20 - 30 ^{o}C are psychrotolerants (Fendrihan et al.,2003).

Acidophiles are organisms that are capable of growing and reproducing under highly acidic conditions with pH levels less than or equal to 2.0.

Alkaliphilic microorganisms thrive in conditions with high pH levels of around 9.0 and above.

Halophiles: are microorganisms that can thrive in high concentrations of salts such as 0.2 M – 0.5 M (1- 30 %). These organisms have higher concentrations of compatible solutes and inorganic ions in their cytoplasm enabling them to inhabit hypersaline environments which are widely distributed in different geographical areas on Earth, like salt pans, saline lakes, saline soils and salt marshes (Amoozeger et al., 2019).

Barophiles: are microorganisms that are able to grow and reproduce at high pressure conditions of more than 40 MPa (Kato and Bartlett., 1997).

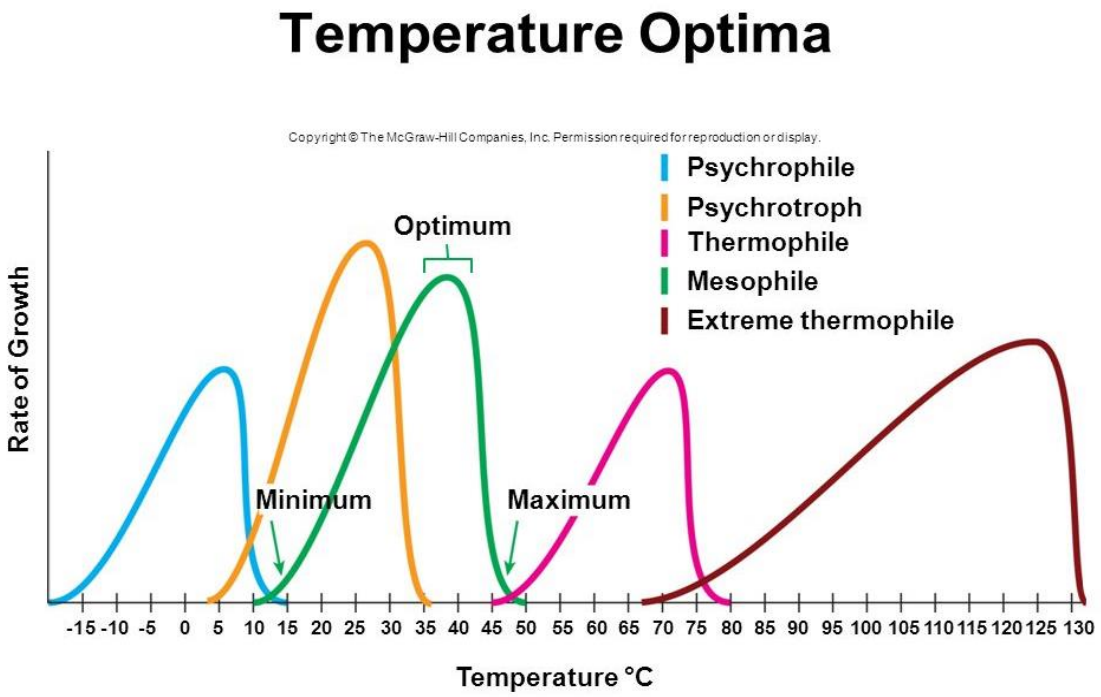


Figure 2.1. Showing the relationship between the growth rate and temperatures of psychrophile, psychrotroph, thermophile, mesophile and extreme thermophile (https://slideplayer.com/slide/8195290/.)

2.2. Background of Thermophilic Cyanobacteria

Cyanobacteria are photosynthetic bacteria naturally found in all environments on Earth. They are usually unicellular, but often times they can grow in colonies. These important bacterial group have been around for more than 3.5 billion years. Cyanobacteria are believed to be responsible for the oxygenation of the oceans and atmosphere of the early earth during the Great Oxidation Event 2.4 billion years ago. Before the 20th century, they

used to be described as algae or rather called Cyanophyta or blue green algae because they are photosynthetic and aquatic (Demoulin et al., 2019). Iron is a key metal of the universe that played an important part in the evolution of Cyanobacteria and other life. The liquid iron of the earth's core created a magnetic field that made survival of life on our planet possible and the ferredoxins (iron sulfur protein) are believed to have played a key part in the growth of fermentative bacteria (Mandal and Rath, 2015).

The ancestors of Cyanobacteria and Proteobacteria are thought to have employed Fe $(OH)^{+}$ as a primary electron donor for CO_2 fixation because the last common ancestor of some archaea and bacteria are able to reduce Fe (III) to Fe (II) and by doing so, they conserve energy which supports growth. It is also believed that before oxygen production from splitting water, Cyanobacteria probably used Fe $(OH)^{+}$ as the electron donor for CO_2 fixation unlike anoxygenic phototrophs (Mandal and Rath, 2015). According to the endosymbiotic theory, the chloroplasts found in eukaryotic algae and plants originated from cyanobacterial ancestors by the process of endosymbiosis and the phylogenetic estimates constructed with plastids, bacteria, and eukaryotic genomes also indicating that plastids are most closely related to Cyanobacteria (Mandal and Rath, 2015). Thermophilic Cyanobacteria can develop over 45°C and are less distributed compared to mesophilic ones (Whitton and Potts, 2000). The upper limit of temperature for Cyanobacteria species is believed to be approximately 73^0C hot water and *Synechococcus sp* were reportedly to be inhabitants. (Strunecký et al., 2018).

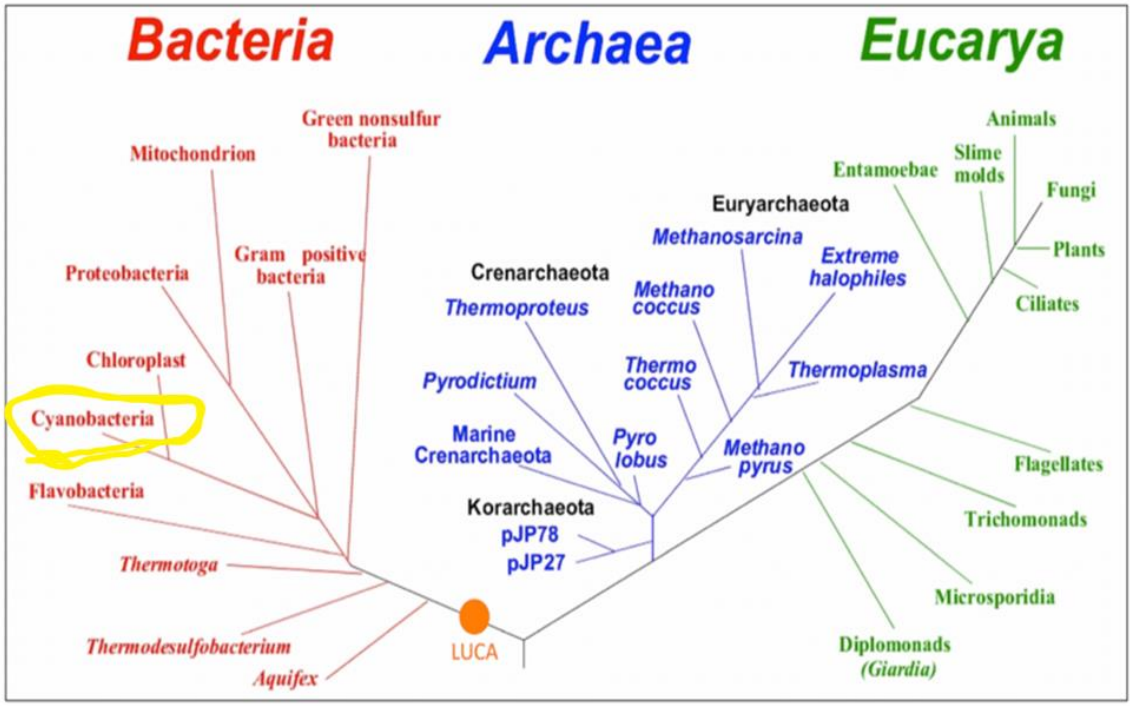


Figure 2.2. The universal phylogenetic tree with the three domains of Life (Bacteria, Archaea and Eukarya) constructed using comparative rRNA gene sequencing (Madigan et al., 2012).

2.3. Cyanobacteria Taxonomy/Classification

Stanier et al. (1978) proposed the inclusion of Cyanobacteria in Bacterial taxonomic schemes but through time there was a conflict between these bacterial taxonomic names with botanical nomenclature (Oren 2004; Oren and Garrity, 2014). Then after two decades, a note to General Consideration 5 (1999) for Cyanobacteria was developed under the rules of the International Committee on Systematic Bacteriology (ICSB)/ and published by International Committee on Systematic of Prokaryotes (ICSP) (De Vos and Trüper, 2000; Oren and Garrity, 2014). The phylum Cyanobacteria is made up of the classes Gloeobacteria and Hormogoneae; six orders: Chroococcales, Oscillatoriales, Nostocales and Stigonematales and recently discovered, Gloeobacterales, and Pleurocapsales; two families Prochlorotrichaceae and Prochloraceae; and the genera *Planktothricoides, Halospirulina, Prochlorococcus, Prochlorothrix,* and *Prochloron* (Mandal, 2015).

Chroococcales:

Chroococcales accounts for 10% of all isolated secondary metabolites from marine environments. There are 70 species of non-heterocystous Cyanobacteria (Chroococcales) in 55 genera and 8 families. The families included in Chroococcales are *Aphanothecaceae*, *Microcystaceae*, *Chroococcaceae*, *Cyanobacteriaceae*, *Cyanothrichaceae*, *Entophysalidaceae*, *Gomphosphaerşaceae* and *Stichosiphoraceae.* The organism responsible for harmful algal bloom is *Microcystis aeruginosa*. It belongs to the Microcystaceae family but members of Chroococcales may not be a clade according to the indications of molecular data (Waterbury, 2006).

Oscillatoriales:

This order has cylindrical, coin-like, or rather barrel-shaped cells linked together end-to-end to form long, unbranched filaments, and all the cells in the filament are almost similar or the filament might be partially different mostly at either one the ends. Oscillatoriales has many different species of Cyanobacteria even though the ones associated with planktonic Cyanobacteria blooms are few. The following are the examples of the families in the order Oscillatoriales, (Matthews, 2016; Komárek, et al., 2014).

- *Cyanothecaceae*
- *Borziaceae*
- *Coleofasciculaceae*
- *Microcoleaceae*
- *Homoeotrichaceae*
- *Oscillatoriaceae*
- *Gomontiellaceae*

Nostocales:

Members of this order have special prominent cells that are heterocystes and akinets. They represent an enormous and monophyletic cluster of filamentous cyanobacterial species with varied thallus. Nostocales contains isopolar unbranched, falsely or true branched types and the filaments of some families have heteropolar structure (Komárek J, et al., 2014).

- *Godleyaceae*
- *Scytonemataceae*
- *Chlorogloeopsidaceae*
- *Rivulariaceae*
- *Symphyonemataceae*
- *Tolypothrichaceae*
- *Nostocaceae*
- *Aphanizomenonaceae*
- *Gloeotrichiaceae*
- *Capsosiraceae*
- *Stigonemataceae*

Pleurocapsales:

Members of the Pleurocapsales order reproduce by multiple fission. However, their ability to divide by binary, as well as by multiple, fission differentiates them from endospore-forming Chamaesiphonales members, like *Dermocarpa*. Pleurocapsales has the following families (Komárek J, et al., 2014):

- *Hydrococcaceae*
- *Dermocarpellaceae*
- *Xenococcaceae*
- *Pleurocapsaceae*

Gloeobacterales:

This order has one monophyletic family (*Gloeobacteraceae)* and one genus (*Gloeobacter).* This genus clearly located at the base of all Cyanobacteria as an independent taxonomical position and is the only genus without thylakoids (Rippka et al., 1974; Mareš et al., 2013; Komárek et al., 2014)

Synechococcales:

Synechococcales has over 70 genera with both unicellular and filamentous types, even though most taxa do not have sequence data. The following are the families placed in Synechococcales order (Komárek et al., 2014).

- *Acaryochloridaceae*
- *Prochloraceae*
- *Merismopediaceae*
- *Synechococcaceae*
- *Coelosphaeriaceae*
- *Chamaesiphonaceae*
- *Leptolyngbyaceae*
- *Romeriaceae*
- *Schizotrichaceae*
- *Pseudanabaenaceae*
- *Heteroleibleiniaceae*

Spirulinales:

Spirulinales members have a special phylogenetic position and are characterized by typical, regularly screw-like coiled trichomes without sheaths and also cytology and ecological characteristic. *Spirulinaceae* is the only family found in this order and *Filamentous cyanobacterium* ESFC-1 together with *Spirulina subsalsa* PCC9445 are some of the species that belong to this family (Komárek et al., 2014).

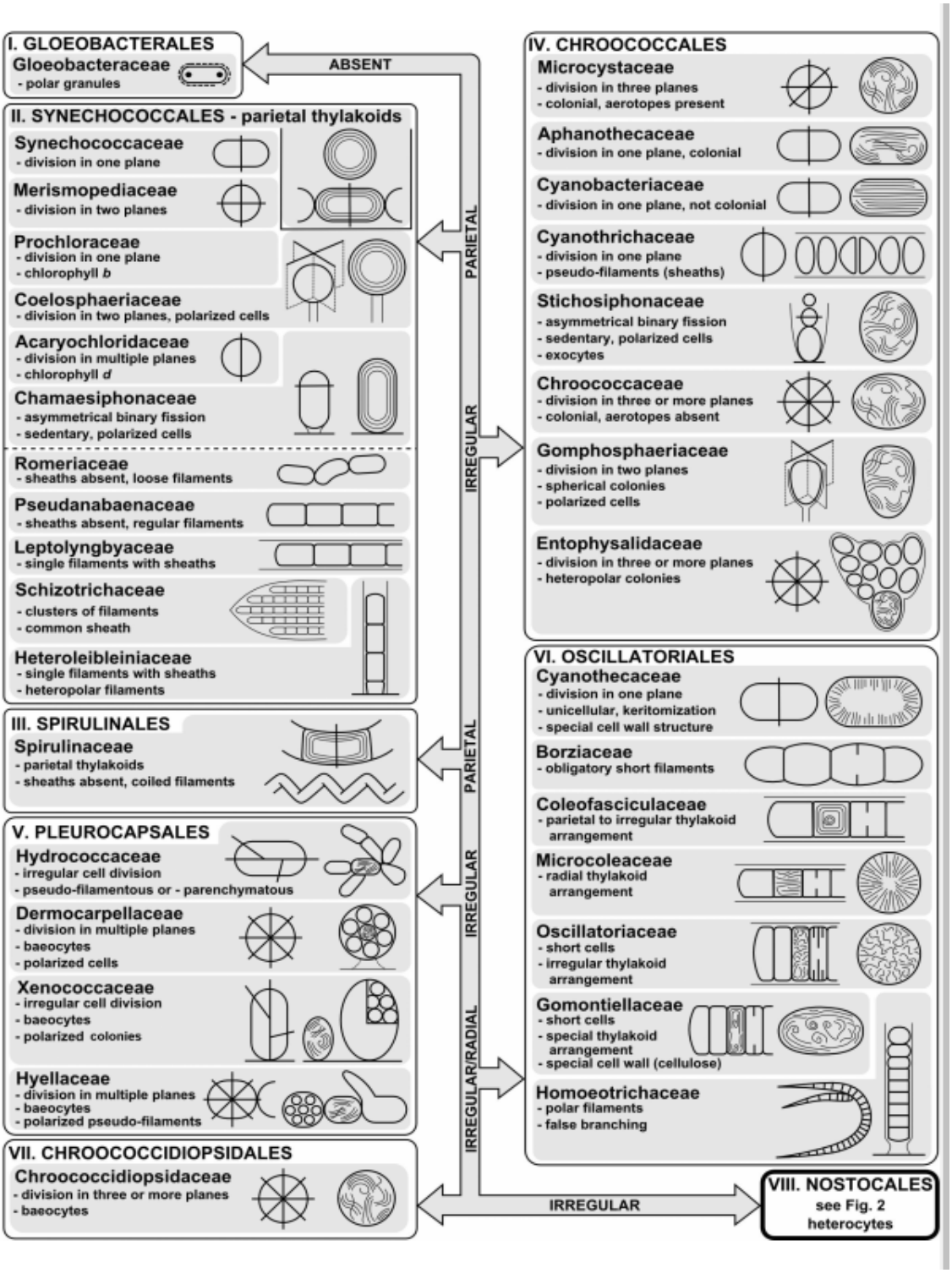


Figure 2.3. Illustrations of different cyanobacterial orders and families with the important taxonomic characters used to identify them (Komárek et al., 2014).

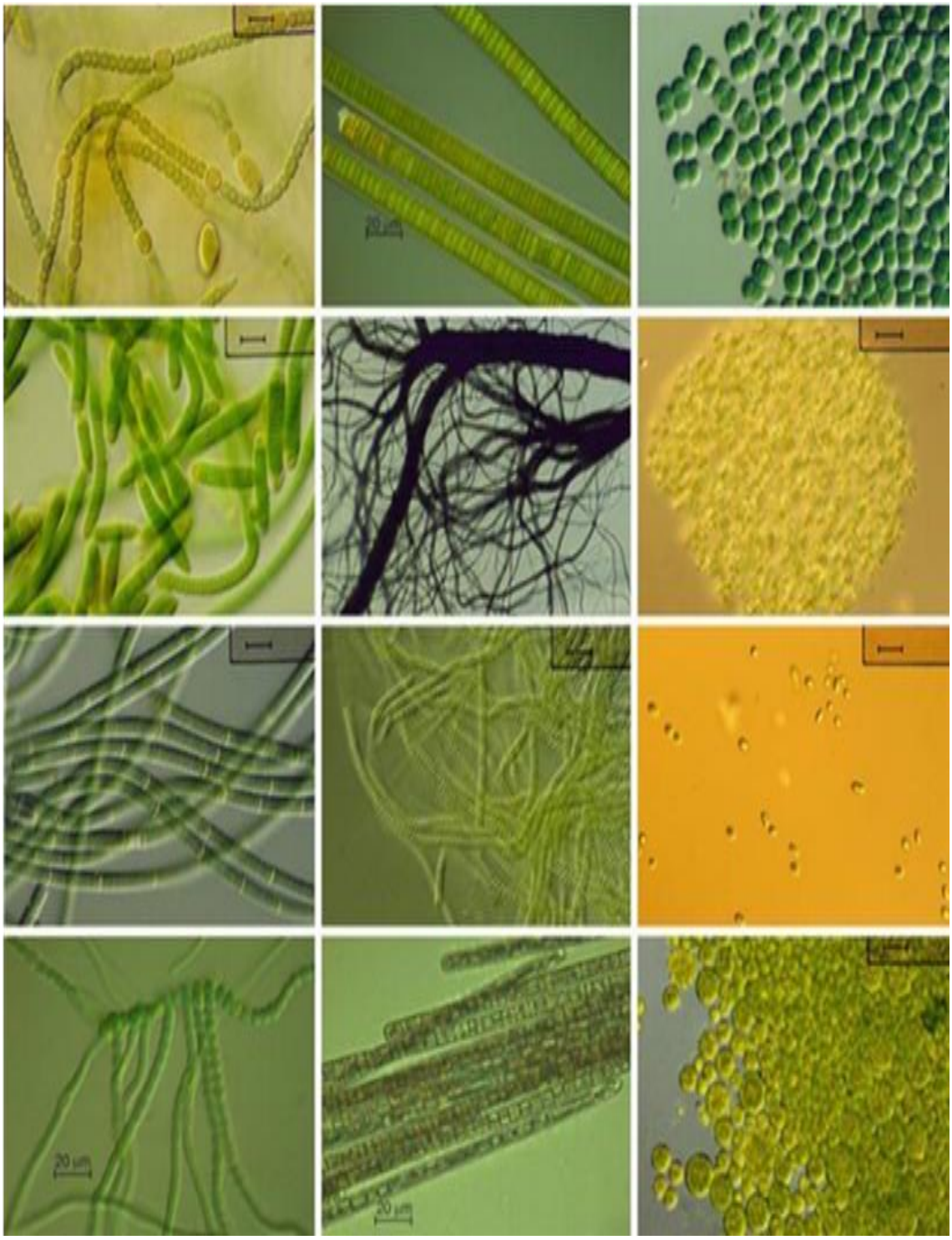


Figure 2.4. Images of different cyanobacteria species

2.4. The Mechanism of Adaptation in Thermophiles

Thermophilic Cyanobacteria are known to provide an efficient platform to validate above hypotheses relating to the evolution of their adaptability to varied environment because they establish themselves in diverse aquatic and terrestrial ecological niches with varied ranges of salinity, temperature, pH, water potential and radiation (Al-Haj et al., 2016, Kumar et al., 2019). Some Cyanobacteria species can be found in endolithic (interior of rocks) environments. This endolithic environment protects these organisms from environmental stresses such as extreme temperature, desiccation, UV radiation, and high photosynthetically active radiation (400 to 700 nm) (Olsson-Francis, et al., 2010).

Many research on the mechanism of adaptation in thermophiles show that, thermophilic bacteria are able to tolerate high temperatures due to genomic changes caused by horizontal gene transfer (HGT), a process that allows organisms of different species to exchange their DNA. HGT is important in the adaptation of bacteria and bacterial genome evolution. HGT occurs by either homologous sequences being replaced by the new sequence or by acquiring foreign sequence by gene integration through, conjugation, transformation and transduction. Gene mutations also contribute to the adaptation of thermophiles in extreme environments; for example, genomic examination of mutations in thermophiles, like *Sulfolobus acidocaldarius* and *Thermus thermophiles,* showed that thermophiles' base substitutions occur at a lower frequency compared to mesophiles (Kissling et al., 2013).

One other reason is that thermophilic genomes' base biases are structured in a way that supports stability in high temperatures. Research has shown that the guanine and cytosine contents in genomes are vital in maintaining the stability of DNA. The expression of temperature- sensitive genes in thermophiles also play critical roles in the thermal adaptation of thermophiles and heat shock proteins (HSPs) which are found in thermophiles are well known proteins that respond to heat stress as well as protecting against cellular damage that is induced by heat stress (Wang et al., 2015).

Thermophilic Cyanobacteria, like other thermophiles, have thermostable and proteolytic resistant cell membranes and proteins that remain intact at elevated temperatures. For example, it was observed that the number of HSPs in protein complexes

increased as the temperature increased, indicating that HSPs further protected those interacting proteins undergoing higher temperatures (Wang et al., 2015).

2.5. Thermophilic Habitats

There are various hot springs in many regions around the world. Biologists have observed organisms living in thermal waters since the mid-19th century. Hot water resources can be found worldwide in countries such as West America, Central Africa, New Zealand, Iceland, Japan, Italy, Indonesia, etc. The most extensive studies on microorganisms living in thermal habitats, however, have been carried out in Yellow Stone National Park. This park is one of the most important places in the world with thermal properties**.**

Picture 2.1. Yellowstone National Park USA, showing Grand Prismatic Hot Spring. (https://www.yellowstonepark.com/page/about-us.)

Turkey is located in an earthquake prone zone. It is rich in hot water resources. It has been determined that the richest region in Turkey in terms of thermal resources is in the Aegean Region with 123 resources and among these, İzmir is the richest province with 31 resources. Thermal springs in Turkey have been used mostly in heating greenhouses and residences, for industrial activities like dry ice production and in the tourism sector as therapeutic spas. Turkey's geological characteristics, temperature and radioactivity were

found to melt mineral content and the accessibility with the total of 410 thermal source entering the literature. (Özşahin and Kaymaz, 2013).

Taking note of the determined biological wealth it is as important as determining the underground resources of the country (Özşahin and Kaymaz, 2013). Below is Turkey's map at 500 m and 100 m depth showing the underground temperatures and the distribution. It is seen that higher temperatures exist in Aegean region compared to other regions of the western region of the country. Figure 2.6. shows Turkey's 1000 m depth temperature distribution map with kriging interpolation.

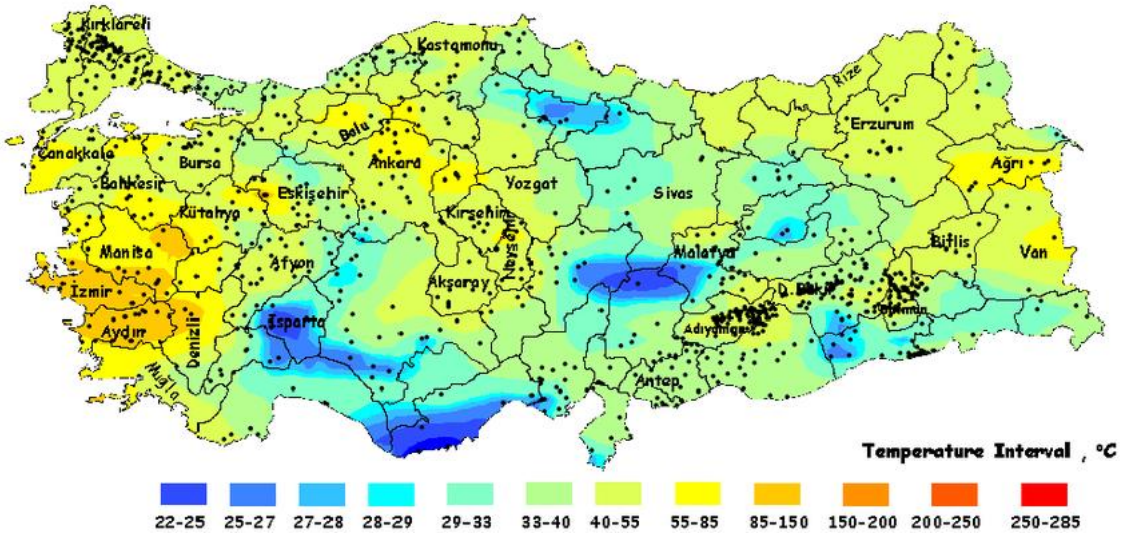


Figure 2.5. 1000 m depth temperature distribution map with kriging interpolation. (Basel et al. 2010).

To add on Turkey's geological and geo-morphological characteristics due to flow, temperature, radioactivity, melted minerals and having different rates from each other in a plurality of thermal source availability (Figure 2.5) With regards to thermal area Turkey ranks first in Europe and is among the top five countries in the world (Özşahin and Kaymaz, 2013).

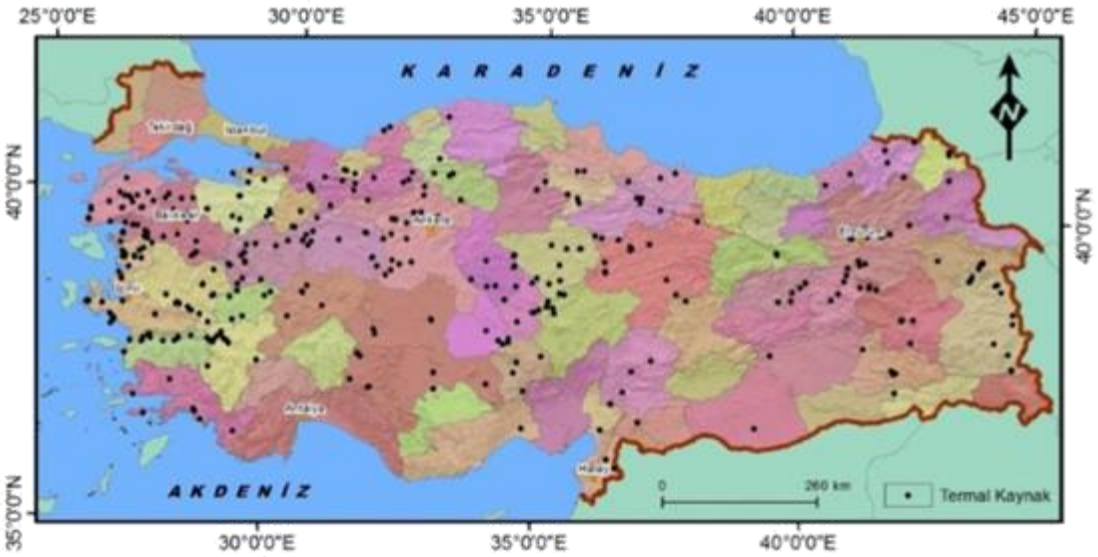


Figure 2.6. The map of hot springs locations in Turkey (Serpen et al. 2008).

The temperatures of the hot springs (45 ^{o}C) in Turkey are generally low when you compare to other places like the Yellow Stone National Park. The number of hot springs with higher temperature has been determined to be 38. The highest temperature of thermal springs in Turkey is about 200 – 242 ^{o} C Kızıldere in Denizli province.

2.6. Thermal Areas in Aydın and Surrounding places.

As mentioned above, Turkey country rich in thermal springs, especially the Aegean region. The most important of these are Denizli thermal areas, which includes the areas of Kızıldere, Tosunlar, Bölmekaya, Yenice, Gölemezli, Karahayıt and Pamukkale. Thermal sources in this region are divided into two basic categories as high temperatures and low temperatures. Low temperatures are used for thermal tourism, house and greenhouse heating whilst high temperatures are powerful enough to run power plants. These areas are also used for the heating of hot springs and spa facilities. Kızıldere and Tekke hamam (Turkish bath) hot springs can also be used for heating Sarayköy (Özşahin and Kaymaz, 2013).

Hot water resources are quite high in Aydın just like in Denizli as stated by geothermal researchers in 1981. Turkey's geothermal potential is also largely located in Aydin. These regions include Kızıldere, Buharkent, Germencik and Kuşadası districts. At

the same time, Buharkent district was declared as "Thermal Tourism Center" in the Official Gazette in 2008.

2.7. The Importance of Thermophilic Cyanobacteria in Biotechnology

Cyanobacteria are considered as one of the most important biomass producers on the Earth and their roles in biogeochemical cycling of elements cannot be ruled out (Häder et al., 2007; Kumar et al. (2019). Cyanobacteria are now widely used as model organisms for studying photosynthetic pathways, the circadian rhythm, nitrogen fixation, biofuel and secondary metabolites production as well as testing responses to various abiotic stresses. They are a vital source of vast array of primary as well as secondary metabolites. The important secondary metabolites which are produced by Cyanobacteria include certain types of toxins, pharmaceutical compounds, bio pesticides, and various growth factors (Abed et al., 2009; Al-Haj et al., 2016; Kumar V., et al., 2018; Kumar, J., et al., 2019).

The metabolites produced by extremophilic Cyanobacteria, give them the ability to cope with both high UV radiation and severe desiccation. They encompass great potential as sources of cosmetics products like sunscreens and moisturizers. Extremophilic species of Cyanobacteria show a number of avoidance, protection and repair mechanisms which makes them better alternatives in production of sunscreens and they also have UV-absorbing/screening compounds such as mycosporine-like amino acids (MAAs) and Scytonemin which serve as natural photo-protectants (Richal et al., 2011, & Derikvand et al, 2017). Cyanobacterial species like *Chroococcidiopsis* and *Nostoc* synthesize both internal and external polysaccharide which enable them to survive within minimal water. *Aphanothece sacrum's* sacran (giant polysaccharide) has 10-fold higher moisture retention capacity than hyaluronic acid which is mostly used in moisturizers (Derikvand, et al., 2017).

Cyanobacteria have been employed in genetic engineering for strain improvement, for example: during the early 2000s, several Cyanobacterial strains have been genetically modified to express mosquitocidal toxins (Bt genes) and they are excellent vector for transferring the mosquitocidal toxins (Carpine et al., 2017). As earlier mentioned, Cyanobacteria are a great source of natural products. Many bioactive compounds with antiviral, antimicrobial, antiprotozoal and anticancer activities have been extracted from Cyanobacterial species (Kumar et al, 2019). When it comes to food supplements,

Cyanobacteria are an excellent option, because they have high content of nutrients such as protein, vitamins, carbohydrates, minerals and are easily digestible (Kumar et al, 2019).

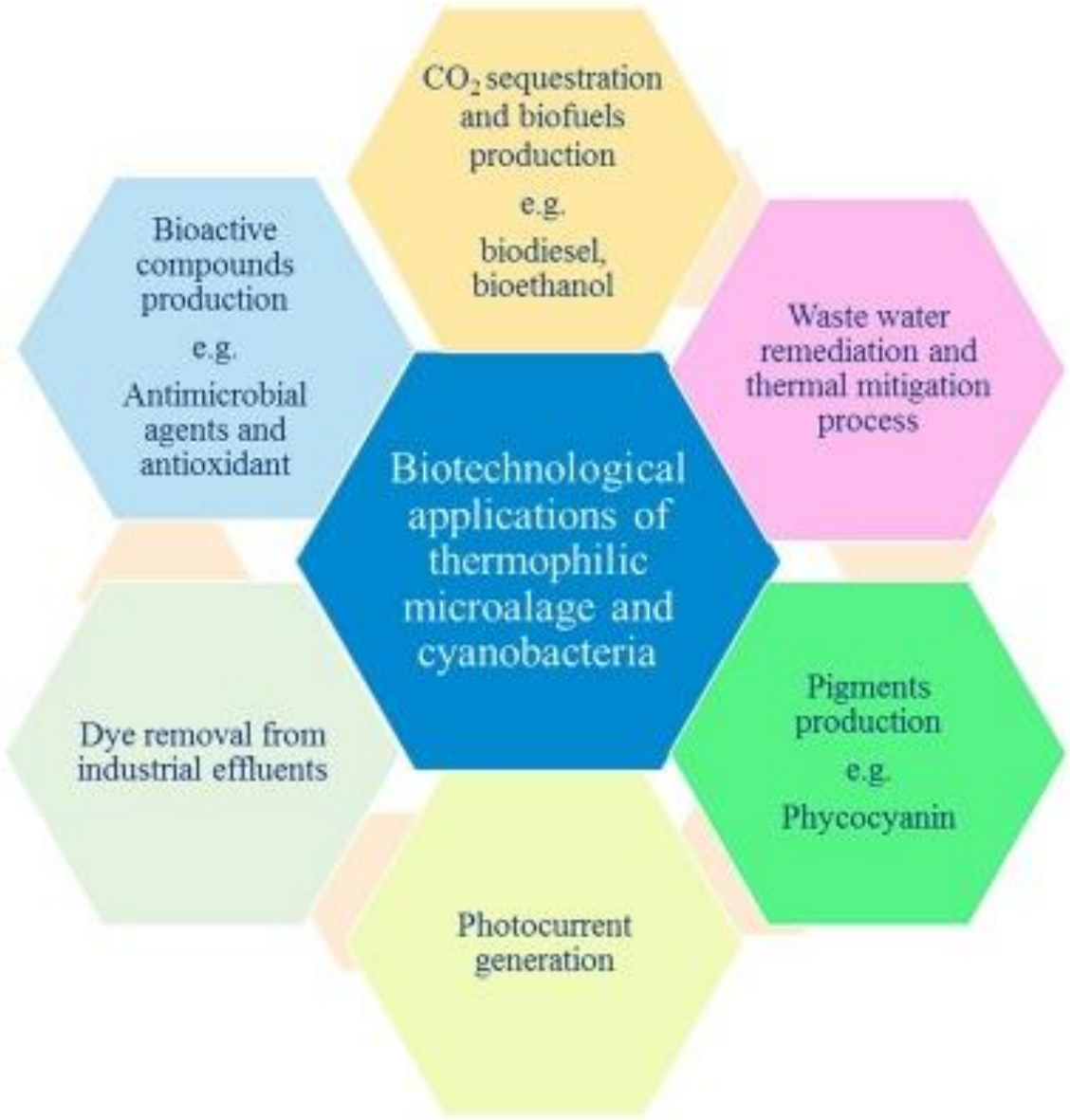


Figure 2.7. Biotechnological applications of thermophilic microalgae and Cyanobacteria (Patel et., al. 2019).

Some Cyanobacteria species such as *Scytonema javanicum*, *Phormidium tenue* and *Microcoleus vaginatus*, have been employed as inoculants for stabilizing and improving sandy soils (Liu et al, 2013, Jose Raul Roman et al, 2018). Cyanobacteria improve soil conditions, soil fertility, and soil stability and enhance water availability in poorly structured and less fertile soils. In addition, they are able to survive prolonged ultraviolet exposure,

long droughts and high salt concentrations (Prasanna et al., Eyal et al., 2015, Roman et al., 2018.) This capacity together with the possibility of being cultured Ex- situ and isolation from soils makes Cyanobacterial inoculation in soil one of the potentially successful bioengineering tool for soil restoration (Roman et al., 2018.)

2.8. 16S Ribosomal RNA and Its Importance in Systematics

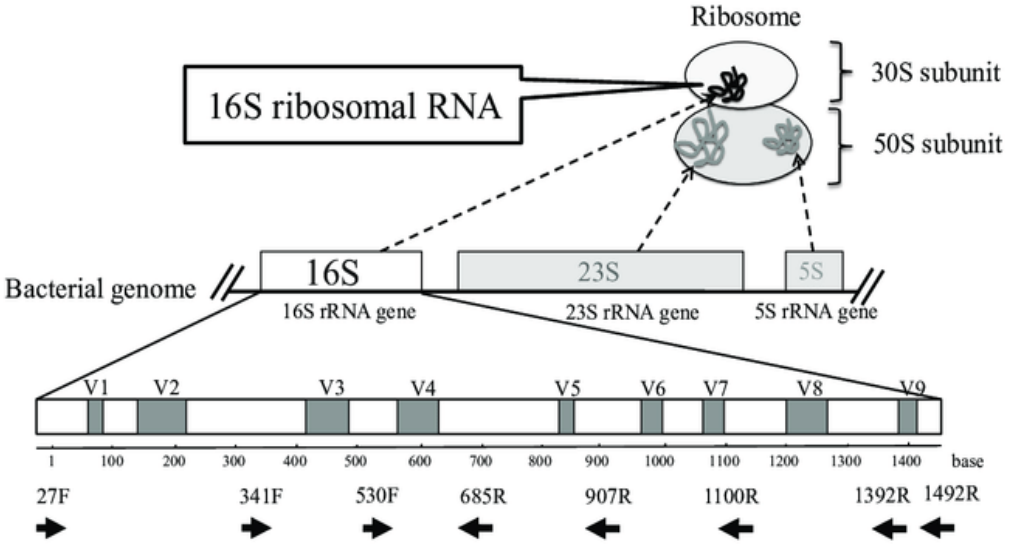


Figure 2.8. Variable regions in the 16S rRNA gene (V1 – V2) (Fukuda et al., 2016)

Ribosomal Ribonucleic Acid (rRNA) is the subunit of the ribosome responsible for protein synthesis in all living things. rRNA contains two subunits: large subunit (LSU) and small subunit (SSU). The large subunit of rRNA functions like a ribosome and catalyzes the formation of peptide bonds. A bacterial ribosome consists of multiple ribosomal proteins and 3 ribosomal RNAs which are 16S rRNA, 23S rRNA, and 5S rRNA. In the rRNA genome, there is an operon called rrn that encodes the related genes arranged in the genome. (Rajendhran and Gunasekaran, 2010).Genome of bacteria can have multiple rrn operons depending on its size and the species. When identifying and classifying bacterial species, the cell size, flagella, capsules, as well as shape, biochemical properties, morphological features are generally used. However, the existence of horizontal gene transfers between bacteria revealed that these features are not very sufficient for phylogenetic classification. Therefore, DNA sequence analysis of evolutionarily stable marker genes is considered as a potential strategy to study bacterial phylogenetics and diversity (Rajendhran and Gunasekaran, 2010).

Woese and Fox (1977) first pioneered the use of 16S rRNA in species identification and phylogenetics. There are many reasons why 16S rRNA is widely used as a genetic marker in bacterial taxonomy studies: it is found in all bacteria and archaea, and the function of the 16S rRNA gene has not changed over time. However, the 16S rRNA contains some variable regions. The existence of variable regions provides a tool for classification. In addition, the presence of conserved regions used in microbial diversity studies allows effective design of suitable PCR primers and hybridization probes (Figure 2.8) (Woese and Fox 1977; Woese 1987). Less than 97% similarity in the sequence of 16S rRNA along with < 70% DNA-DNA hybridization has been defined as the new species (Stackebrandt and Goebel, 1994; Janda and Abbott, 2007).

The size of 16S rRNA gene (1500 bp) is large enough to give knowledge on informatics. However, there are some limitations in 16S rRNA studies. The number of copies per genome is from 1 to 15 or more. Although copy numbers seem to some extent to be taxon specific and variations have also been noted among strains of the same species. Extremely different 16S rRNA sequences have been observed in some bacterial taxa. Thermophilic bacteria in which 16S rRNA sequences show greater variability have also been identified. In this case, it has been determined that horizontal gene transfer has a high potential (Větrovský and Baldrian, 2013).

2.9. Analysis of 16S rRNA

Culture-independent methods used to determine microbial diversity are as summarized in Figure 2.9. For this reason, different molecules (DNA, RNA etc.) and / or approaches can be selected. Prokaryotic diversities of different habitats can be determined by using either microbiological culture techniques or molecular approaches. A vast fraction of the total bacterial diversity that exists in nature cannot be grown on standard media in the laboratory (Stewart, 2012). For instance, Culture-independent methods can be used to investigate thermophilic species adapted to survive under extreme conditions, regardless of viability.

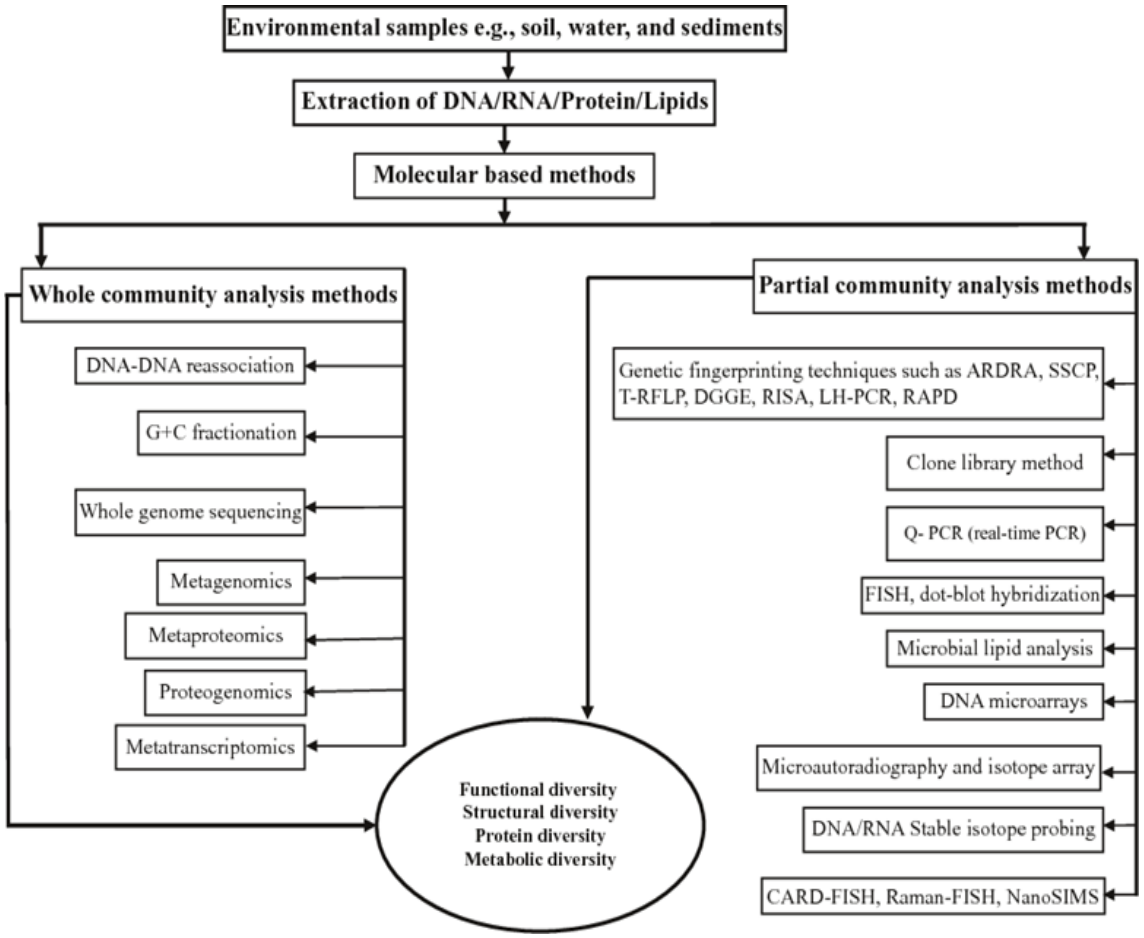


Figure 2.9. Culture-independent molecular toolbox for characterizing the structural as well as functional diversity of microorganisms in Environments. (Gurdeep Rostogi et al., 2011)

Pace et al. (1986) first started environmental research in 1986 by PCR method using primers designed for SSU rRNAs. It is possible to detect bacteria and archaea in various habitats (if previously discovered and entered into the database) on the basis of genus / species. This is made possible by PCR-based molecular techniques that have been used since the nineties and direct 16S rDNAs from the samples are amplified. With Culture-independent methods, unculturable prokaryotic organisms that exist in an environment can be determined by total DNA isolation from samples and sequencing of the final results obtained from PCR. Briefly, DNA is extracted from the samples and all 16S rDNAs in the sample are amplified by PCR. Amplicons are cloned into plasmids and transformed into *E. coli* cells with recombinant plasmids. Subsequently, colonies containing environmental clones are developed in Petri dishes and 16S rDNAs contained in each clone is sequenced and compared with the sequences in the database.

There has been many studies done using molecular identification methods and great results have been recorded. For example, researchers such as Mutlu and Güven (2014), Yılmaz and Arık (2016), Subudhi et al. (2018), Miller et al. (2000), Ozturk et al. (2018), Fewer et al. (2009) and many others determined bacteria diversity using molecular methods.

Miller et al. (2000), using phylogenetic comparative methods such as 16S rRNA gene sequence, were able to analyze the evolutıon of thermotolerance in hot spring Cyanobacteria of the genus *Synechococcus* and among the 20 laboratory clone of *Synechococcus* four different 16S rRNA gene sequences were identified.

Başbülbül and colleagues (2017) used Culture-dependent and Culture-independent methods to assess the prokaryotic diversity of Lake Acıgöl found in Aegean region of Turkey. They used primers specific to the archaeal domain to analyze DNA extracted from these water samples. After 16S rDNA analysis they found fourty-nine different strains belonging to *Aerococcus, Acinetobacter, Bacillus, Enterococcus, Exiquobacterium, Haloalkalibacillus,, Halomonas Piscibacillus, Lysinibacillus, Oceanobacillus, Planococcus, Micrococcus* genera.

Karan et al. (2017) reported *Chroococcus, Geitlerinema, Anabaena, Oscillatoria, Phormidium, Cylındrospermum* and *Nostoc* genera from Tokat province, Turkey.

Fewer et al. (2008), indicated that microcystin producing blooms are persistent phenomenon in the Gulf of Finland through culture independent procedures and the sequence analyses demonstrated that uncultured microcystin-producing *Anabaena*, strains are genetically more diverse than previously demonstrated by cultured strains. Subudhi et al. (2018) collected and analyzed samples from the sulphur rich and alkaline hot springs in India. Based on 16S rRNA analysis they found that Arthronema was more abundant in mesophilic Taptapani whereas *Leptolyngbya* was predominant in thermophilic environment of Atri.

Ozturk et al. (2018) compared Cyanobacteria and Chlorophyta species under culture conditions using classical methods and molecular identification. Phylogenetic relationships of the strains based on 16S rRNA and 18S rRNA gene sequences were determined. According to DNA sequence analysis performed, 3 isolates were found to be similar *to Chodatodesmus mucronulatus* (Chodat) Bock & Krienitz (97%), *Desmodesmus sp*. (Chodat) T.Friedl & Hegewald (98%) and *Pediastrum duplex* Meyen (97%). The other 4 isolates were found to be similar to *Fischerella ambigua* (Kützing ex Bornet & Flahault) Gomont

(99%), *Leptolyngbya* sp. Anagnostidis & Komárek (97%), *Phormidium autumnale* Gomont (99%) and *Rivularia sp*. C. Agardh ex Bornet & Flahault (99%).

Our research aimed at discovering the diversity of Cyanobacteria in selected hot spring areas of Aydin and its surroundings using culture independent analysis.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Water, Mat, and Debris Samples Were Used in This Study.

The samples were collected in Aydın province and its surrounding areas in the years 2016, 2019 and 2020. The samples were then stored at -80 ºC till use.

Table 3.1. Study sites, temperatures of sampling areas and coordinates.

Statıons	Sample Name	Temperatures ºC	Coordinates
Aydın	Alangullu	**55**	37º56’13’’N27º37’39’’E
	Güney Termal hotel	**60**	37º56’46.63’’K28º49’45.69’’D
	Kayta Hotel	**47**	37º56’51.01’’K28º49’42.39’’D
	Karahayıt	**44**	37º57’49.84’’K29º06’13.03’’D
	Ortakçı	**32**	37o56’19’’N27o37’35’’E
Denizli	Umut termal 1	**62**	37º55’18’’N28º49’45’’E
	Umut termal 2	**42**	37o55’18’’N28o49’45’’E
Sarayköy-Denizli	Kabaağaç 1	**57**	37º56’06’’N28º45’39’’E
	Kabaağaç 2	**57**	37º56’06’’N28º45’39’’E
	Kabaağaç 3	**52**	37º56’06’’N28º45’39’’E

*The following are the pictures of the sites where the samples were collected.

Picture 3.1. Umut Termal (Lower pond) 62 º C.

Picture 3.2. Kabaağaç (Kuyu Suyu) 57 º C

Picture 3.3. Kabaağaç (Red sediments) 57º C

Picture 3.4. Alangullu 55 º C.

Picture 3.5. Kayta Thermal Hotel 47 º C

Picture 3.6. Umut Termal (Çamur Havuz) 42 º C

Picture 3.7. Ortakçı (Haman dağ çamur) 32 º C.

3.1.2. Types of Culture Media Used During the Study

Tryptic Soy Agar (TSA)

18.5g of TSA was dissolved in 500 ml distilled water by heating and later autoclaved at 121^0C for 15 min in order to sterilize the medium. After sterilization, the medium was poured into sterile plates.

Tryptic Soy Broth (TSB)

12g of TSB was dissolved in 500 ml distilled water by heating and autoclaved at 121ºC for 15 min to enhance sterilization.

Brain Heart Infusion (BHI) Agar

24.5g of BHI was dissolved in 500 ml distilled water by hearting and autoclaved at 121ºC for 15 min so that it can be sterilized. After sterilization the medium was poured on sterile plates.

Brain Heart Infusion (BHI) Broth

18.5 g of BHI was dissolved in 500 ml distilled water by heating and autoclaved at 121ºC for 15 min to enhance sterilization.

AXI Medium

In 300 ml of TSA, 300 µL Ampicillin, 750 µL X-gal and 1500 µL IPTG were added.

3.1.3. Solvents Used in the Study

50X Tris Acetate EDTA (TAE).

Trizma Base: 242g, Glacial Acetic Acid: 57.1 ml, EDTA (0.5M) pH: 8: 100 ml. All these substances were dissolved and mixed in distilled water using a magnetic mixer. The pH of the prepared solution set was 8 sterilized at 121 °C 15 min by autoclaving.

10X Tris – Borat EDTA (TBE)

Trizma Base: 108g, Boric Acid: 55g: EDTA: 8.3g. All these substances were dissolved and mixed in distilled water using a magnetic mixer. The pH of the prepared solution set was 8 sterilized at 121^{o}C 15 min by autoclaving.

3.1.4. Primers

Two sets of primers were used in this study: the first one was obtained from a study by Saker, (2005) which were used for cyanobacterial diversity and the second we redesigned the first primers by adding restriction enzyme sites.

Table 3.2. Sequences for primers used.

Primer Code	Sequence 5' - 3'	Primers With The Restricted Site	TM	Refference
16S 27F	**5**'AGAGTTTGATCCTGGCTCAG **3'**		60	M.L Saker (2005)
16S 27FBam	**5**'AGAGTTTGATCCTGGCTCAG **3'**	(Bamhi) **atcGGATCC**AGAGTTTGATCCTGGCTCAG	60	This study
16S 809R	**5'**GCTTCGGCACGGCTCGGGTCGATA **3'**		60	M.L Saker (2005)
16S 809RHind	**5'**GCTTCGGCACGGCTCGGGTCGATA **3'**	(HindIII) **atcAAGCTT**GCTTCGGCACGGCTCGGGTCGATA	60	This study

3.2. Method

3.2.1. Total Genomic DNA Isolation From Environmental Samples

The collected samples stored at -20 ° C in sterile bottles and jars were first melted and then centrifuged at -4 ° C, 5000 rpm for 30min. The obtained pellet was the one used for DNA isolation.

Here, two methods for total DNA extraction were used. The first method was the PrestoTm Soil DNA extraction kit, Cat NO SLD100 as stated by the manufacturer. The other method used was the Phenol Chloroform method which involves adding a number of chemicals that are; Phenol; Chloroform; Isomyl alcohol, Proteinase K, Sodium Dodecyl Sulfate (SDS), EDTA, Lysozyme, Sodium acetate, 70% ethanol and TE solution.

The Protocol for Phenol Chloroform DNA extraction was performed as follows:

1. The obtained pellet after centrifugation was resuspended in 100 µl TES (50 µl 1M Tris (pH: 8), 25 µl 0.25M EDTA (pH: 8), 700 µl %50 sucrose, final volume had to be 5 mL). The solution was mixed till homogenization.

2. Later we added lysozyme enzyme 5 µl (10mg/mL), 1 µl RNAse (10mg/mL) and the Eppendorf was placed on 37 ºC incubation while shaking for 30 min.

3. After incubation we added 2 µl (5mg/ml) proteinase K and 15 µl of %20 SDS then placed it on 56 ºC incubation for 30 mins.

4. We added volume/ volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the sample, and then we vortex and inverted the tubes upside down about 8 times till a white cloud was formed.

5. We centrifuged the tubes for 5 minutes at 4ºC 12000 rpm, and carefully removed and transferred the upper aqueous phase approx. 100 µl, to a fresh tube.

6. We added 1/10 µl 3M pH5 Sodium Acetate and also 2-3 volume of 220 µl Isopropanol, approx. 220 µl.

7. The tubes were placed overnight at –20°C to precipitate the DNA from the mixture.

8. The next day we centrifuged the samples at 4°C for 30 minutes at 15,000 rmp.

9. We carefully removed the supernatant without disturbing the DNA pellet and added volume / volume of 70% ethanol. The sample mixture was then centrifuged at 4°C for 5 minutes at 12,000 rmp.

10. We then discarded the supernatant and dried the DNA pellet in a Thermo Scientific™ SpeedVac ™ concentrator for 2 minutes and another option was to leave the tubes at room temperature for 10 - 20 minutes.

11. Finally, we resuspended the DNA pellet in 50 – 100 µl of TE buffer or dH_2O by pipetting up and down 30–40 times and placed the tubes in store at -20 ^{o} C degrees.

3.2.2. Amplification of Cyanobacterial 16S rRNA Genes Using PCR (Polymerase Chain Reaction).

Table 3.3. Conditions used for PCR amplification.

Temperature ºC		Time	Cycles
Denaturation	95	4 min	x 35
Denaturation	95	55 sec	
Annealing	60	45 sec	
Extension	72	1 min	
Last extension	72	30 min	

The PCR machines used was from Long gene®, A 300 Fast Thermal Cycler, BIO-RAD T100™ Thermal Cycler and Applied Biosystems by life technologies Simpli Amp Thermal Cycler.

3.2.3. Purification of PCR Amplicons.

1. After the amplification of DNA by PCR method, the obtained amplicons were then purified using the Gene Mark Plus PCR Clean-Up Kit. Following PCR amplification or other enzymatic manipulations, we transferred the reaction mixture (containing the DNA to be purified) into a clean Eppendorf tube.

2. 3 volume of Binding Solution was added to the reaction mixture (e.g. 50 µl reaction mixture add 150 µl Binding Solution) and was vortexed briefly in order to mix.

3. We inserted the Spin Column into a Collection Tube, transferred the solution into the Spin Column and centrifuged at top speed (14~16000xg) for 1 min, and discarded the filtrate in the collection tube.

4. 700 µl of Wash Solution was added to the Spin Column, and waited for 1 min for equilibration with the membrane. Then we centrifuged at 14~16000xg for 1 min and discarded the filtrate. This step was repeated as commanded.

5. The filtrate was discarded and the tubes were centrifuged at top speed for additional 5 min in order to remove residual traces of ethanol. (If centrifugation speed is lower than 14 000xg or residual ethanol must be removed completely, incubate the spin column in a heat oven (60~65^{o}C) for 5 min to evaporate any residual ethanol).

6. The Spin Column was transferred into a new microcentrifuge tube and 30~ 100 µl of Elution Solution or H_2O (pH 7.0~ 8.5) was added to the column and waited for 1~2 min.

7. Finally, we centrifuged at top speed for 2 min to elute the DNA and stored the eluted DNA at -20^{o}C.

3.3. Cloning of the 16S rRNA gene

Before performing the cloning process, the obtained amplicons as well as the plasmid to be used were first restricted (cut) using restriction enzymes. For the plasmid, *Escherichia coli* strain containing pUC19 plasmid was selected for use.

3.3.1. pUC19 Plasmid Restriction

10 µl pUC19 plasmid.

6 µl distil water (dH_2O)

2 µl FastDigest Enzyme Buffer

1 µl FastDigest Bamhi restriction enzyme

1 µl FastDigest HindIII restriction enzyme

3.3.2. Amplicon Restriction

10 µl Total DNA amplicon.

6 µl distil water (dH_2O)

2 µl FastDigest Enzyme Buffer

1 µl FastDigest Bamhi restriction enzyme

1 µl FastDigest HindIII restriction enzyme

After mixing the mixtures, the tubes were incubated at 37 ºC for 30 min in incubator (Thermo Shaker) and later precipitation process was performed in which the amplicon together with the plasmid were then combined into one tube and by using Phenol Chloroform, Sodium Acetate, Isopropanol and 70% ethanol, were precipitated.

3.3.3. Forming of the TA Cloning Vector and TA Cloning

Plasmid DNA Restriction

10 µl pUC19 Plasmid DNA.

7µl distil water (dH_2O).

2 µl 10X Enzyme Buffer.

1 µl FastDigest SmaI restriction enzyme.

The sample is then incubated for 1 hour at 37 º C and later after restriction is completed, 5 µl is electrophoresed in Electrophoretic gel system. The remaining sample is then precipitated.

3.3.4 Making of TA Vector (Plasmid) By Adding of dTTP (deoxyThymidine Triphosphate) to Plasmid with Blunt Ends.

34 µl Plasmid DNA.

10 µl 10mM dTTP (Final Concentration 2mM).

5 µl 10X PCR Buffer.

1 µl Taq polymerase enzyme (1u/ µl).

The prepared reaction was then left for 2-hour incubation at 72^{o}C. After this stage the process of precipitation was repeated. The DNA was then dissolved in 10 µl of distil water.

In this method, the condition for PCR slightly differ, the last extension time is 30 min and the amplicons to be used in ligation process have to be newly obtained in the sense that the PCR for total DNA has to be done on the same day as the 72^{o}C incubation stage.

Ligation:

17 µl vector – total DNA mixture.

1 µl T4 ligase buffer (10X).

1 µl T4 ligase enzyme (5U/ µl)

After adding the buffer and enzyme, the tubes were incubated at 16 ^{o}C 1 hour and then left for whole night at 22 ^{o} C. The next day, preparation of competent cells was performed as follows:

3.3.5. Competent Cell Preparation and Chemical Transformation of Recombinant DNA

Competent Cell Preparation:

E. coli is the common bacterial species used in the transformation step of a cloning workflow.

The preparation of competent cell was performed using the chemical principle.

1. A single fresh colony of DH10 from *E. coli* was taken from an agar plate and inoculated into a liquid medium (5ml) at 37 ^{o}C for 24hrs.

2. The next day 1ml from the 5ml inoculated into a 50ml liquid medium. It was left in the incubator for 3hrs.

It is important to keep the cells at 4 ^{0}C and so after removing from the incubator, the cells were placed in the ice for 30 mins.

3. The cells were harvested by centrifugation at 5000rpm for 10mins using the cold centrifuge at 4 ^{0}C. The supernatant was decanted, resuspended and then centrifuged at 5000rpm at 4 ^{0}C for 10mins. We repeated this stage twice.

4. 10 mL cold (%10) glycerol was then added to the pellet and centrifuged at 5000 rpm for 10 mins at 4 ^{0}C.

5. The supernatant was decanted, resuspended with 30 mL of cold solution mixture of (80mL $MgCl_2$ and 20 mL $CaCl_2$) and centrifuged at 5000 rpm for 10 mins.

6. The supernatant was decanted. 1mL of 0.1M $CaCl_2$ was added to the pellet and with the aid of pipete it was gently mixed in order to obtain a homogenised solution. Chang et al., (2017).

3.3.6. Recombinant DNA Chemical Transformation

1. 10 – 5 µl recombinant plasmid was added to 100 µl of chemical competent cells and then mixed by pipetting. The tube was incubated on ice for about 20 – 30 mins.

2. We applied heat shock by placing the tube on the incubator that was already set at 42^{o}C for 2 min.

3. When the time elapsed, we placed the tubes on ice for 2 min.

4. 900 µl of TSB was added into the tube and incubated for 1 hour at 37 ^{o}C and later inoculated 50 µl as well as 200 µl on AXI Selective growth media.

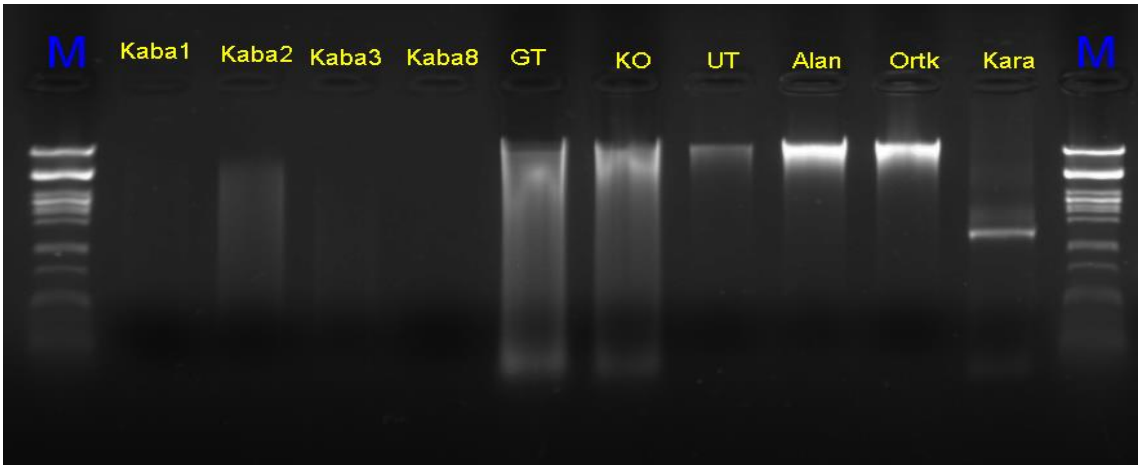
*The plate was incubated for overnight at 37^{o}C and the following day it was observed for any formation of white/ blue colonies.

At least 30-50 colonies were selected from white clones (transformants) formed on AXI medium containing Ampicillin, IPTG and X-Gal as a result of cloning. Colony PCR was performed using primers M13F and M13R to reproduce 16S fragments. If the DNA fragment sizes obtained as a result of PCR are suitable (> ~ 500 bp), sequence analysis was done by service purchase. M13F and M13R primers were used for sequence analysis. The obtained sequences were BLAST analysed using the BLAST program through the NCBI (http://www.ncbi.nlm.nih.gov) site and the similarity ratios / the groups belonging to the clones were determined according to the sequences obtained from the clones.

4. RESULTS

4.1 Total DNA isolation from Water samples

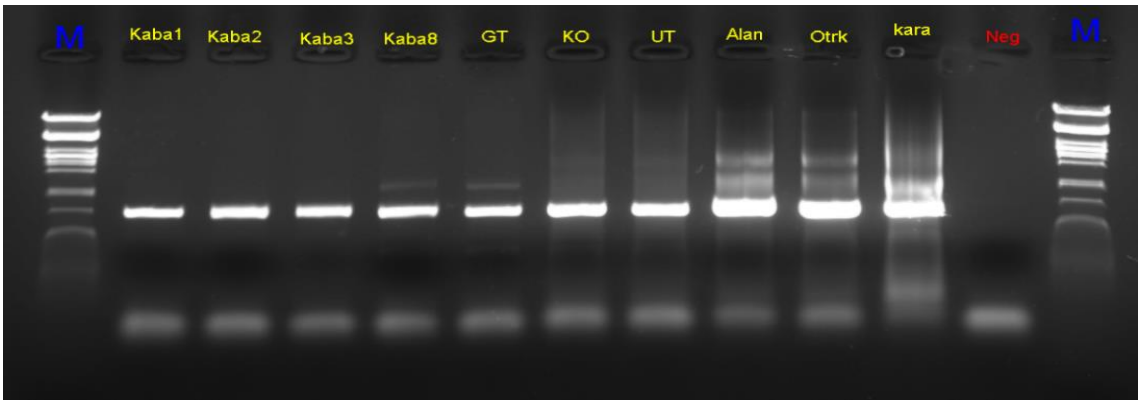
DNA was isolated from the collected samples of Kabaağaç 1, Kabaağaç 2, Kabaağaç 3, Kabaağaç 8, Güney Termal, Kayta Otel, Umut Termal, Alangullu, Ortakçı and Karahayıt stations. Five µl sample of each DNA was run on agarose gel. As shown below in the image, some stations like Kabaağaç 1 and Kabaağaç 8 did not show any band though after 16S rRNA PCR they were all positive.



Picture 4.1. Electrophoresis image for Total DNA of all selected samples

4.2. Amplification of 16S rDNA with PCR

The isolated DNA was amplified using the selected primers 16S 27F, 16S 809R, 16S 27FBamhI and 16S 809RHindIII. The results are shown in picture 4.2 below.



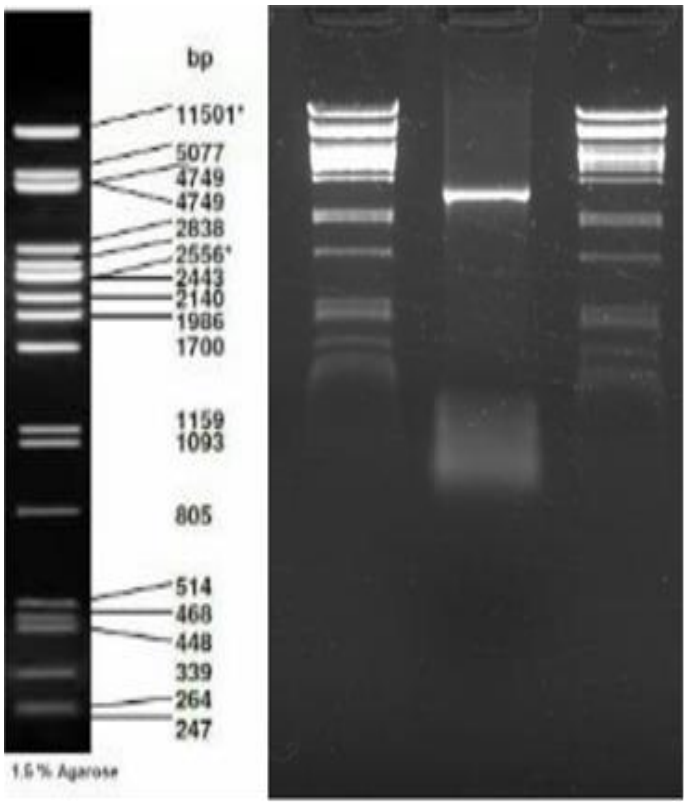
Picture 4.2. Showing the image of electrophoresis image for all selected samples' PCR.

4.3. Restriction of Amplicons and the Plasmid.

For the primers 16S 27FBam and 16S 809RHindIII, the plasmid as well as the amplicons were restricted using the enzymes BamhI and HindIII.

4.4. TA cloning Vector

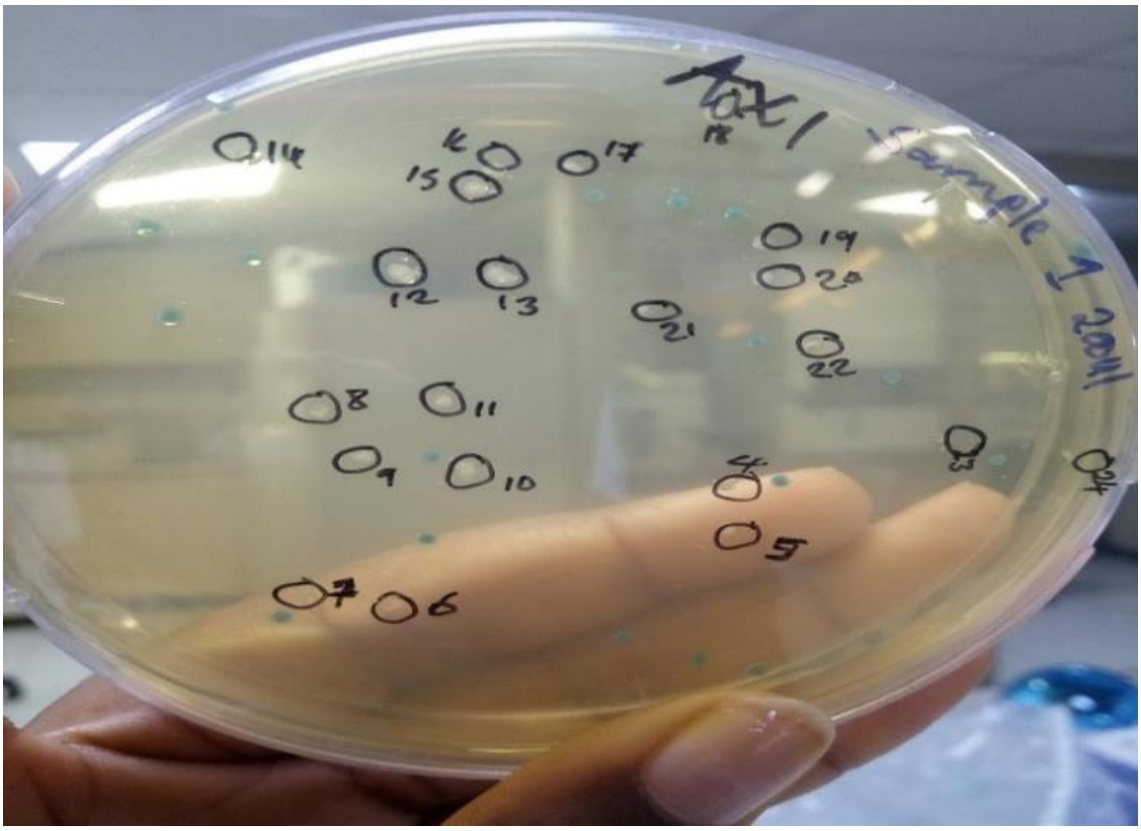
TA cloning kit was used for the cloning of amplicons obtained with primers 16S 27F and 16S809R. We also prepared the TA vector, using our own protocol found in laboratory. The electrophoresis picture of the Puc19 plasmid that we had cut with Sma1 enzyme is given in Fig 4.4. Subsequently, the plasmid was incubated overnight at 22 ° C for ligation.



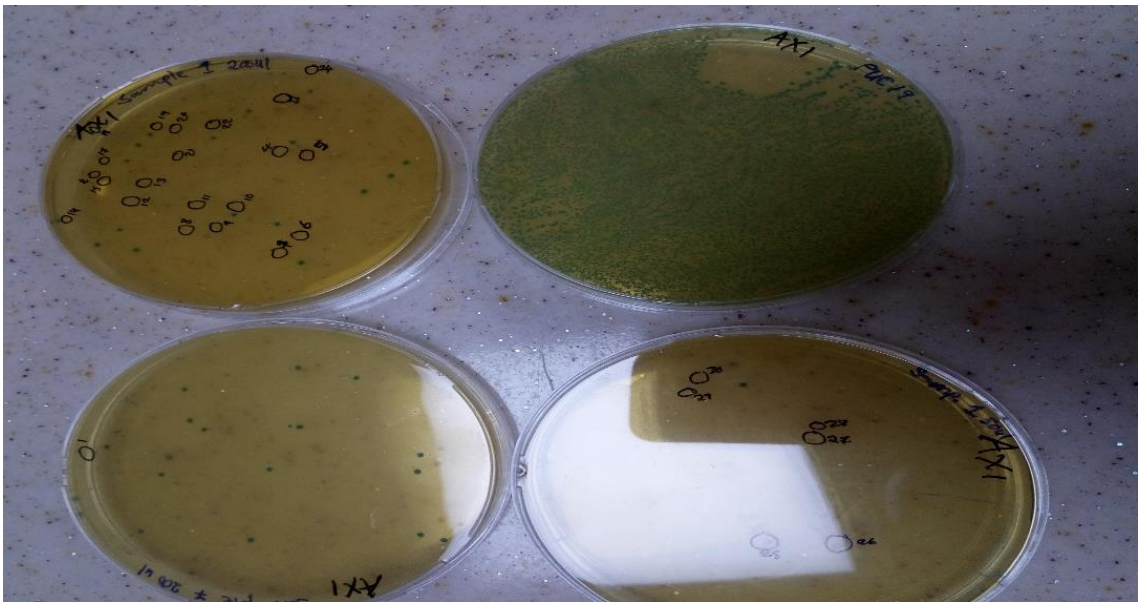
Picture 4.3. TA Vector (Restriction of pUC19 plasmid with Sma1 enzyme).

4.5. Selection of Colonies and M13 PCR.

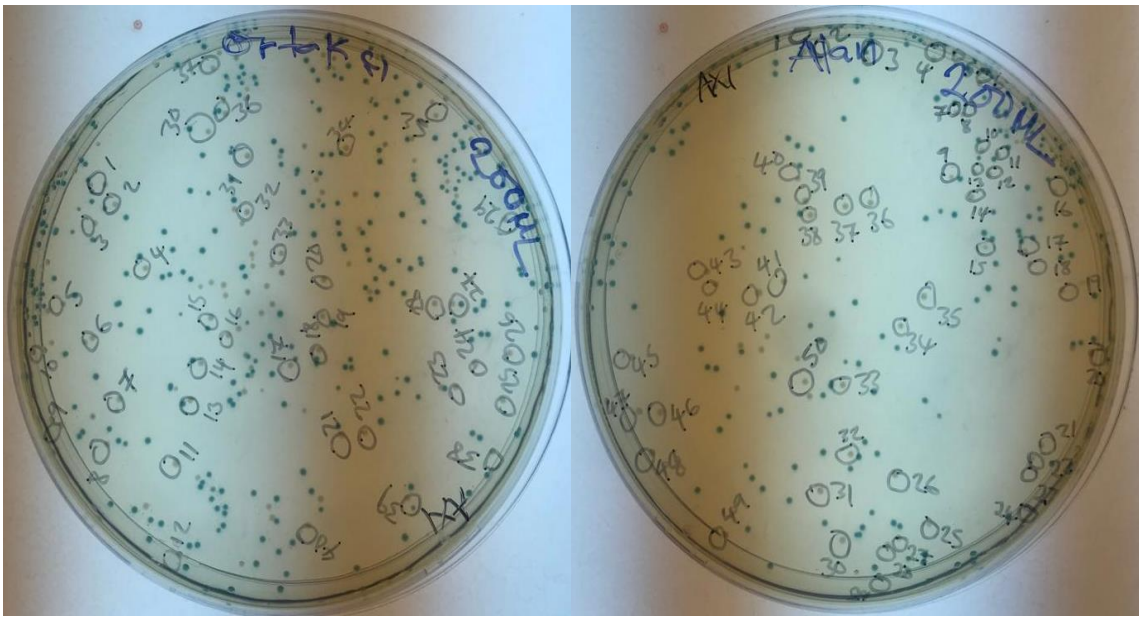
White colonies growing on AXI medium as a result of transformation were selected. (Pictures 4.4 - 4.7) As a result of PCR with M13 primers, amplicons of around 800 bp were sent for sequence analysis. M13 PCR results of clones belonging to different samples are given in picture 16 - 22. Amplicons sent for the sequence analysis are shown with an asterisk in electrophoresis images. Clones have been given different codes.



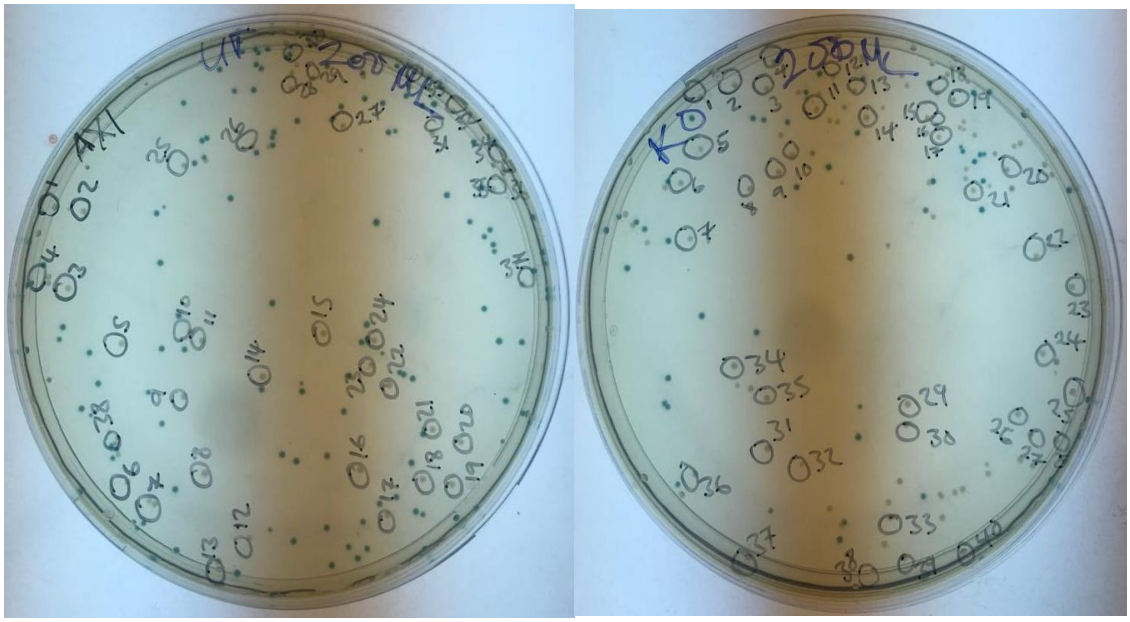
Picture 4.4. White colonies obtained from Kabaağaç sample



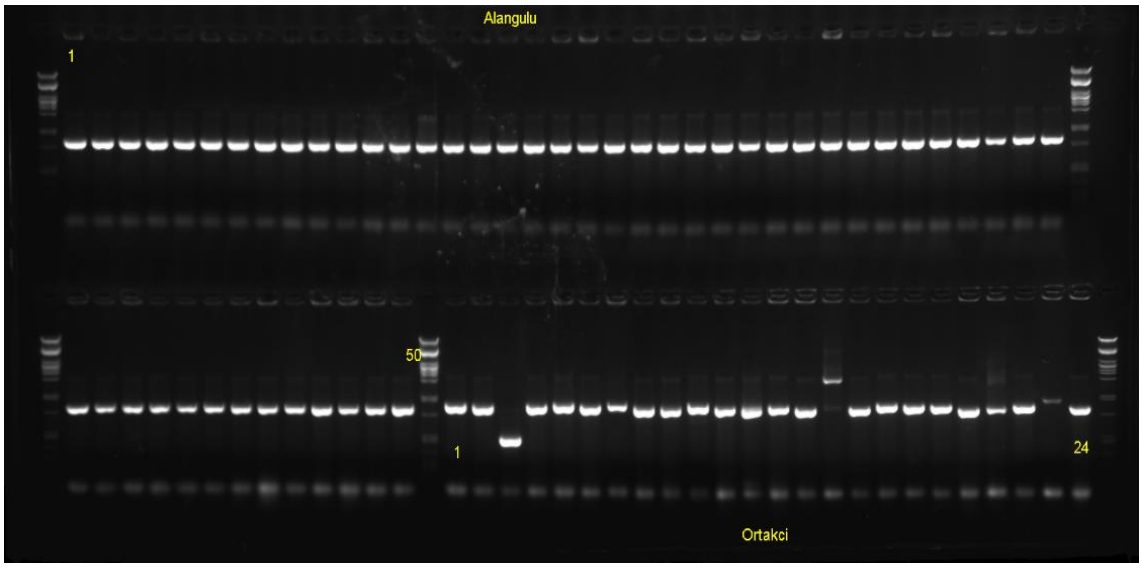
Picture 4.5. Showing some plates of control and formed white colonies of selected samples.



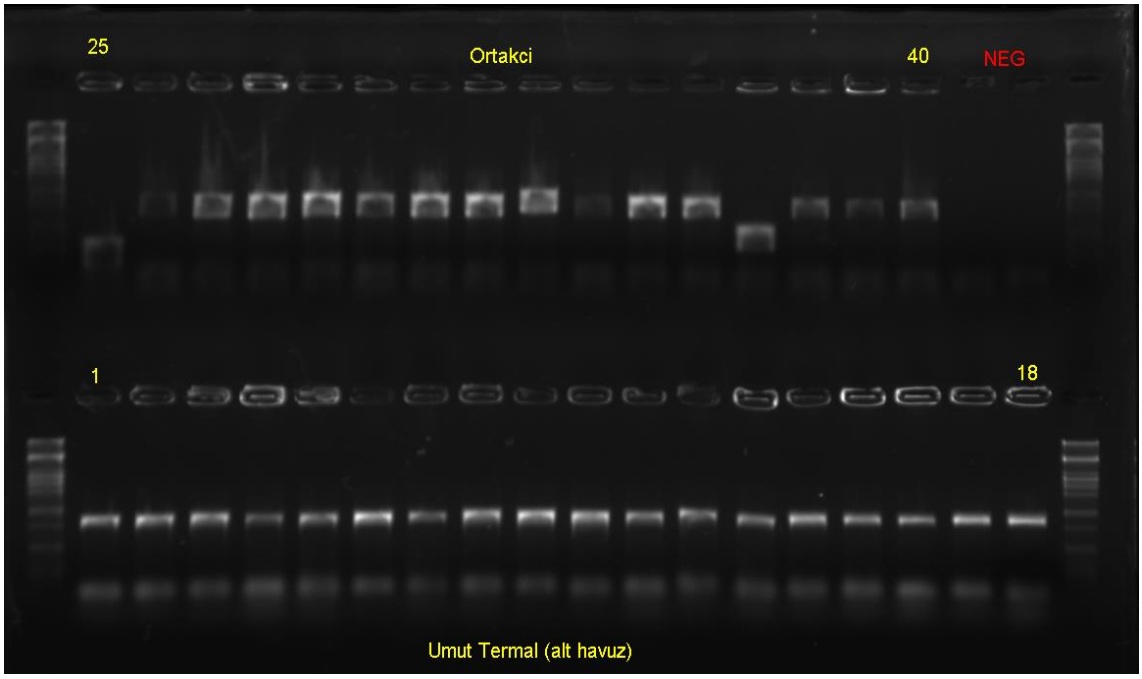
Picture 4.6. Selected white colonies from cloned (Ortakçı and Alangullu) samples.



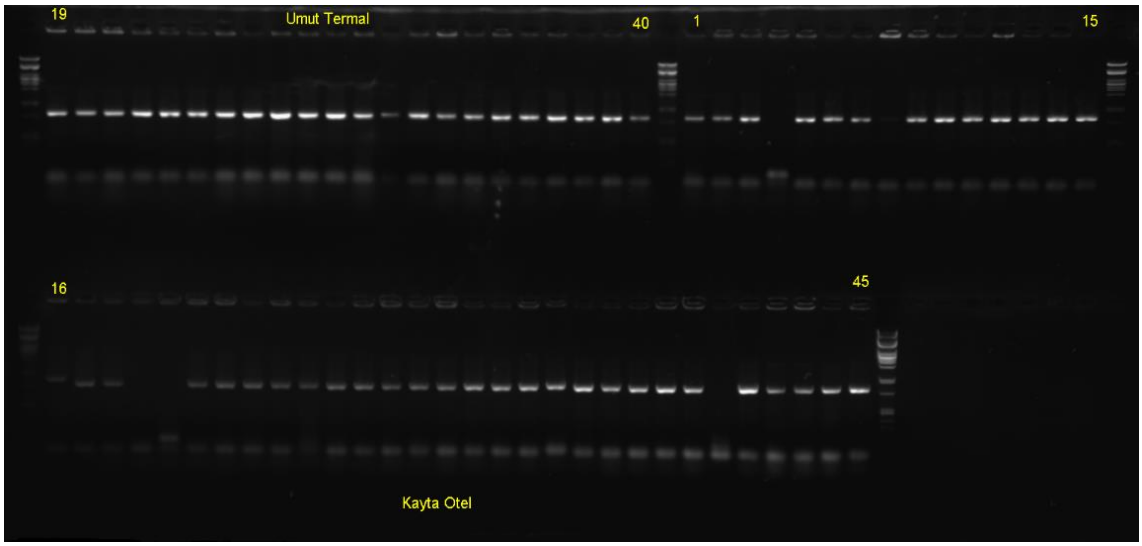
Picture 4.7. Selected white colonies cloned from (Umut Termal and Kayta) Otel.



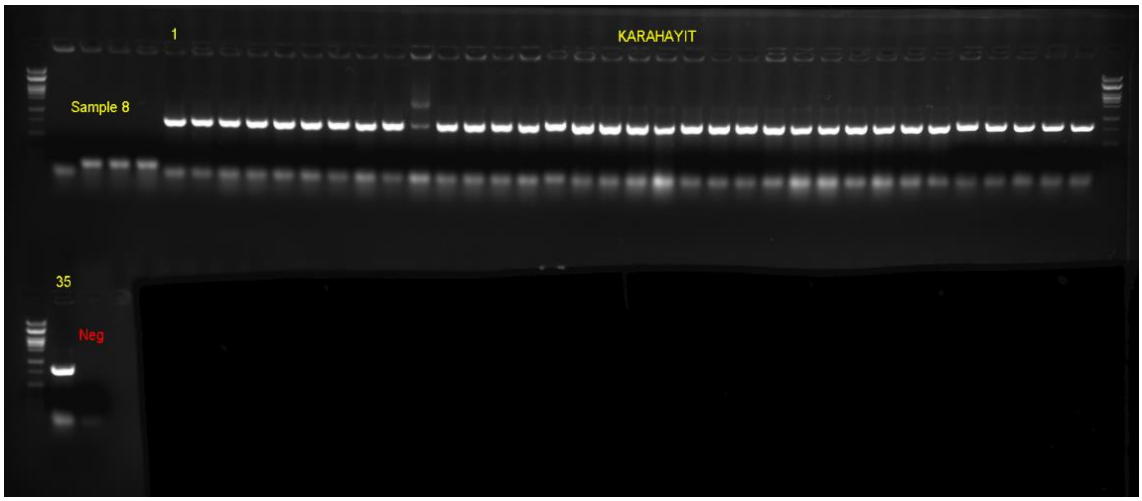
Picture 4.8. M13 PCR results for Alangullu samples.



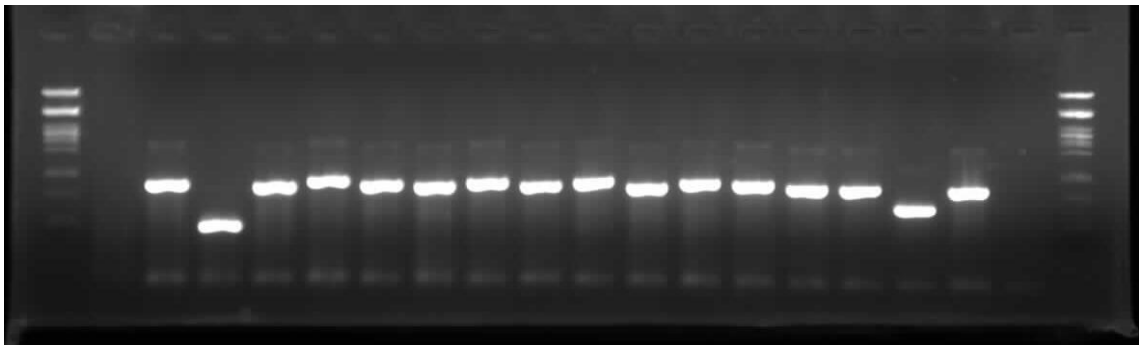
Picture 4.9. M13 PCR results of Ortakçı samples (clone 25 -40) and Umut Termal samples.



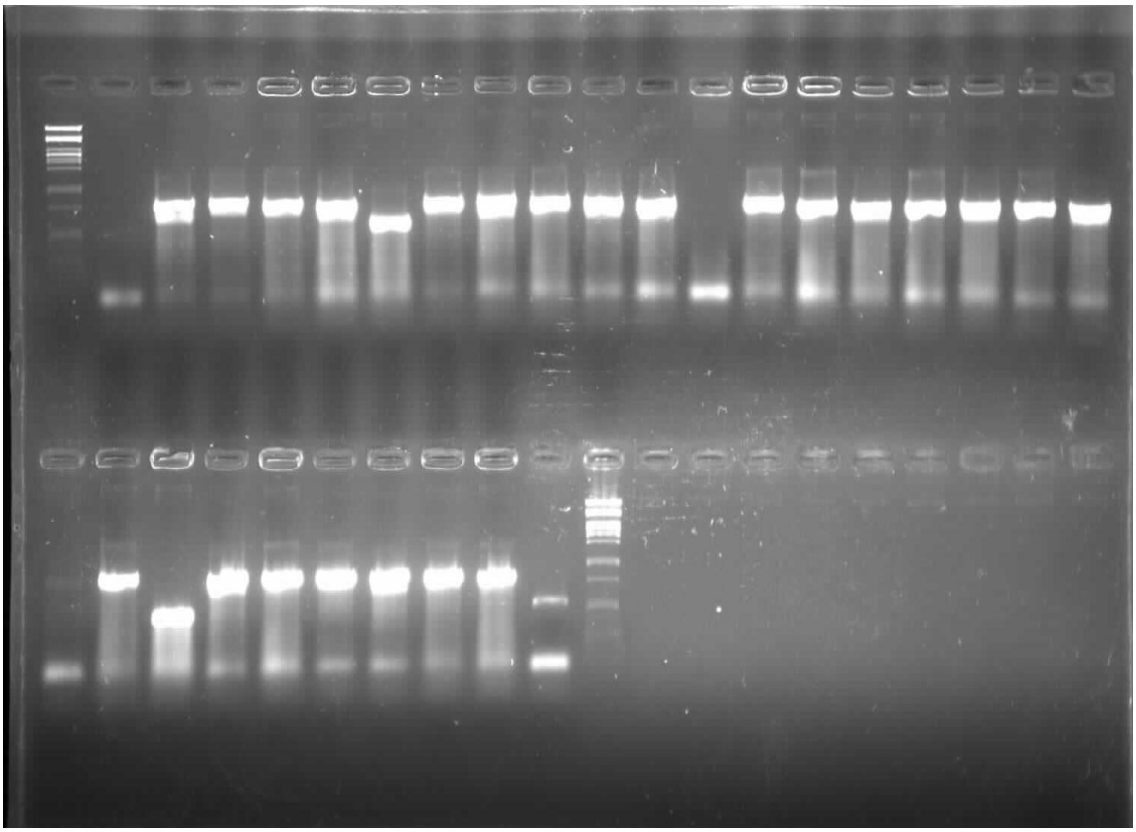
Picture 4.10. M13 PCR results of Umut Termal and Kayta Otel samples.



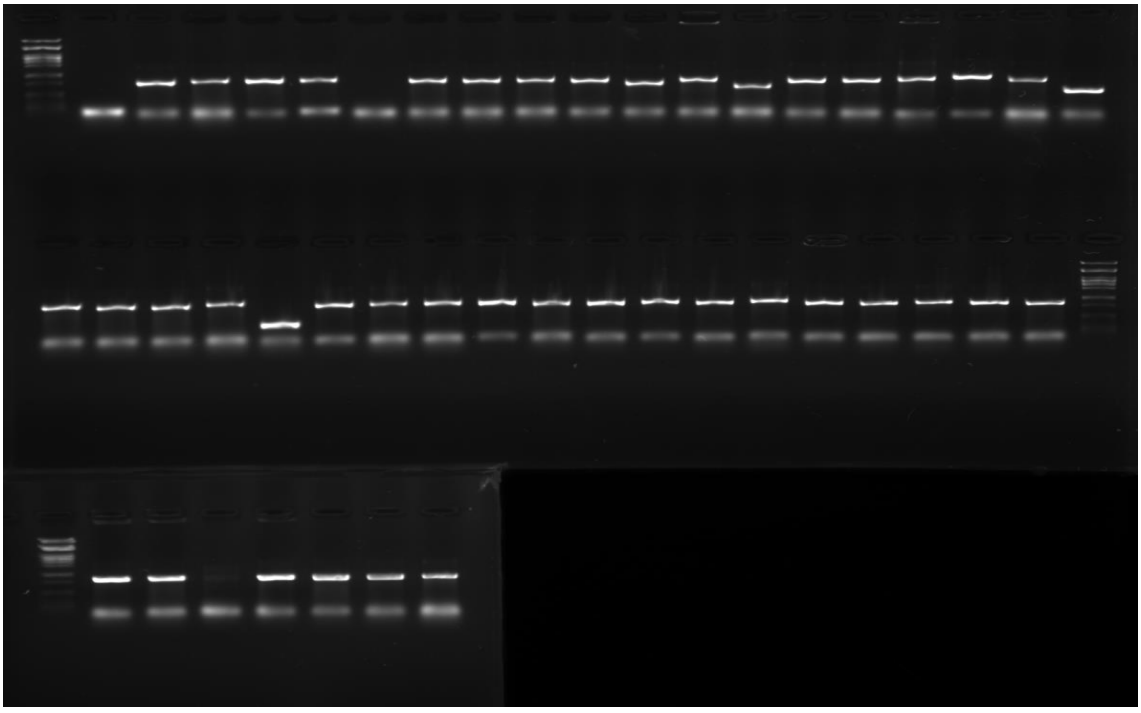
Picture 4.11. M13 PCR results of Karahayıt sample.



Picture 4.12. M13 PCR results for Kabaağaç 2.



Picture 4.13. M13 PCR results of Kabaağaç 1 samples.



Picture 4.14. M13 PCR results of Güney Termal, Kabaağaç 2 and 3.

4.6. Sequence Analysis and Determination of Homologies.

Sequences of 16S rRNA genes amplified by M13 PCR were compared with other sequences in the database using the BLAST program in GenBank. The highest similarity rates, bases compared and microbial classes are given in the tables below.

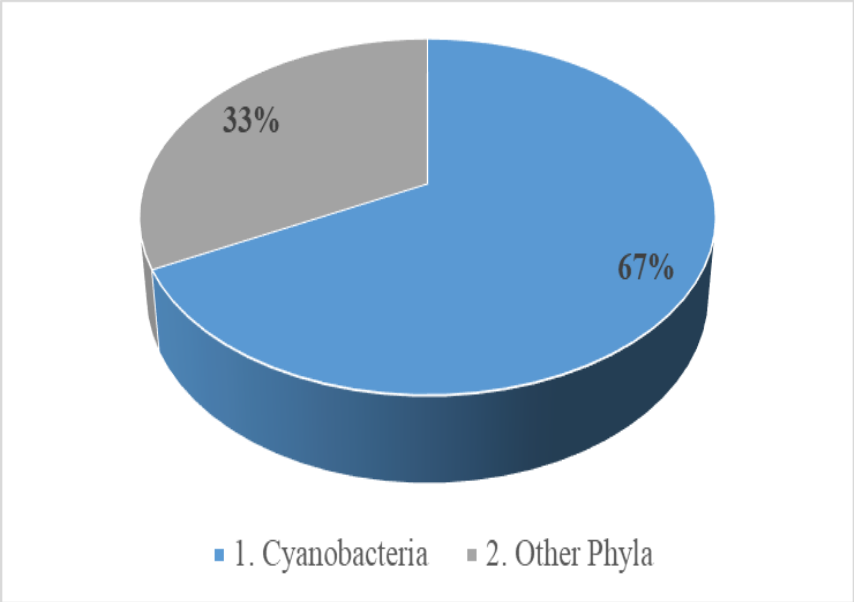


Figure 4.1. Total Percentage of Cyanobacteria and other Phyla for all samples

Table 4.1. Sequence Analysis Results for Alangullu sample

Station	Tem °C	No: of Colonies	n	Species	Per. % Identity	Class
Alangullu	55	50	16	*Trichocoleus desertorum*	719/785 (92%)	Trichocoleusaceae
			32	*Planktothricoides raciborskii*	98% 756/771	Microcoleaceae
			1	*Sodaleptolyngbya stromatolitii*	98% 746/758	Sodaleptolyngbya
			1	*Amazoninema brasiliense*	84% 583/691	Pseudanabaenaceae

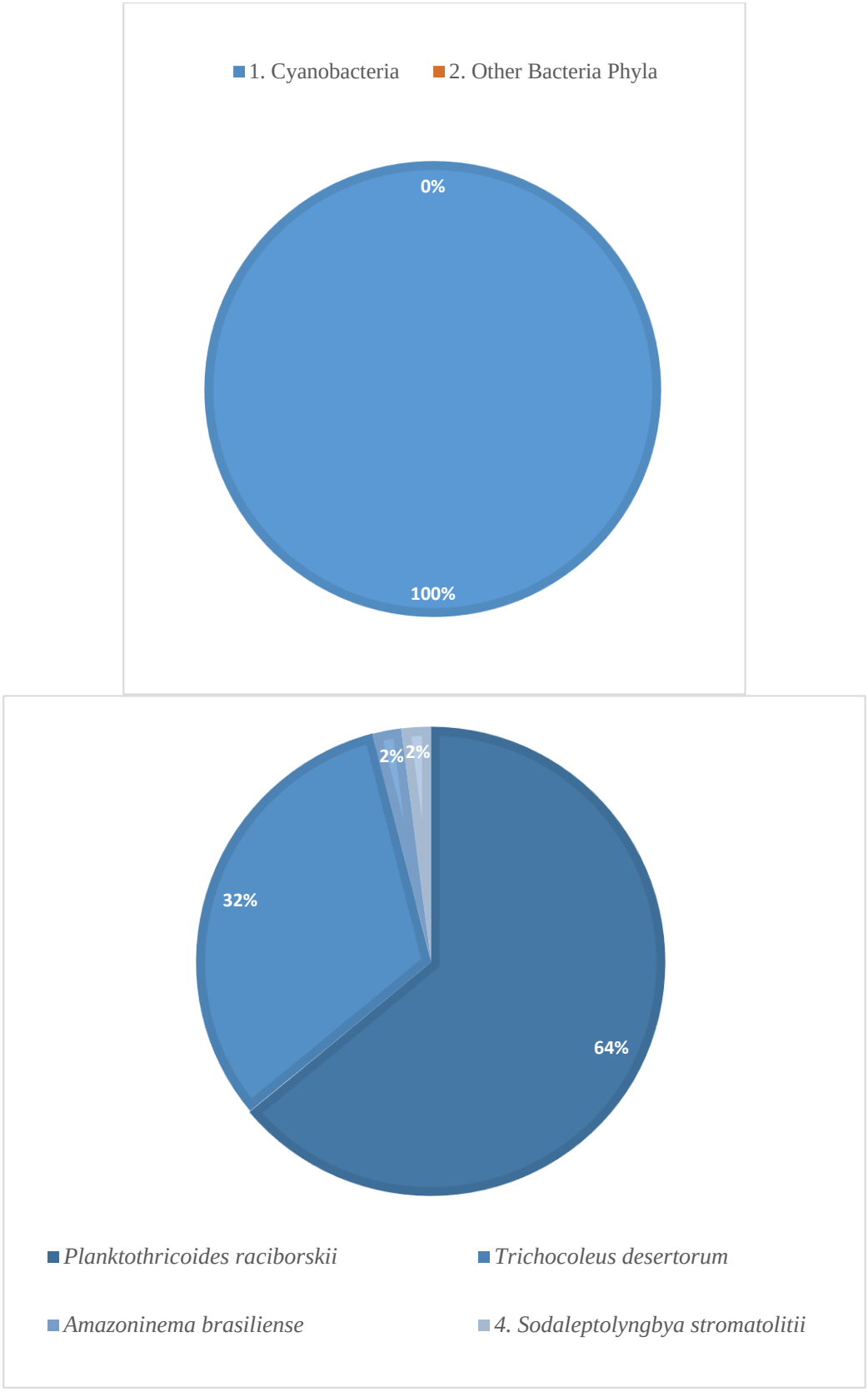


Figure 4.2. a. showing the percentage of Cyanobacteria and other phyla in Alangullu sample. b, percentage of species similarity.

Table 4.2. Sequence Analysis Results for Güney Termal sample

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
Güney Termal	62	2	1	*Oscillatoria sancta* PCC 7515	98% 632/683	Oscillatoriaceae

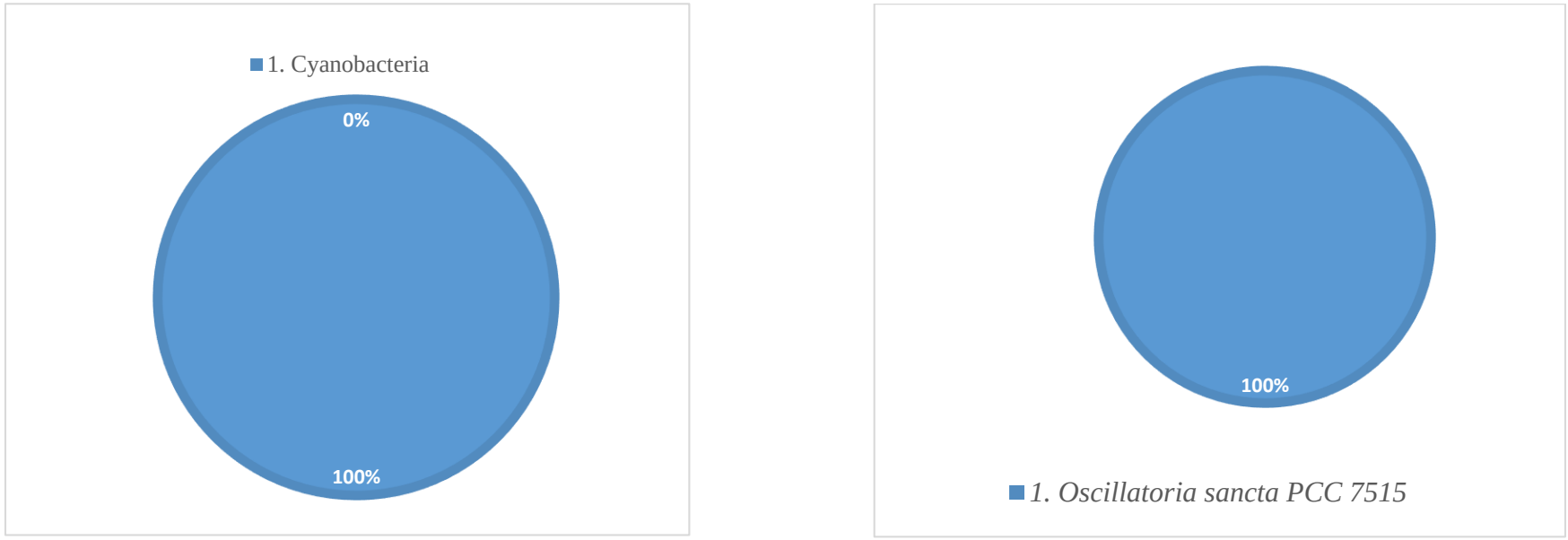


Figure 4.3. Showing the percentage of Cyanobacteria and other phyla in Güney Termal sample. b, percentage of species similarity.

Table 4.3. Sequence Analysis results for Kabaağaç 2

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
			14	*Armatimonadetes bacterium*	86% 661/772	Armatimonadetes
			7	*Candidatus Planktophila sulfonica*	463/561(83%)	Nanopelagicaceae
			2	*Geothermobacter ehrlichii*	88% 733/836	Geobacteraceae
	57	36	**2**	*Candidate division* CPR2	80% 622/779	Candidate division CPR2
			2	*Candidatus Planktophila versatilis*	713/747(95%)	Nanopelagicaceae
Kabaağaç			**3**	*Desulfosoma profundi*	95% 770/811	Syntrophobacteraceae
RedSediments)			**2**	*Thermobispora bispora*	81% 666/822	Actinobacteria incertae sedis
			1	*Dissulfurirhabdus thermomarina*	88% 736/839	Dissulfurirhabdaceae
			1	*Thermoanaerobacter yonseiensis*	85% 476/561	Thermoanaerobacteraceae
			1	*Hydrogenedentes bacterium*	97% 777/800	Candidatus Hydrogenedentes
			1	*Delta proteobacterium*	83% 647/778	Deltaproteobacteria
			1	*Candidatus Endomicrobium pyrsonymphae*	83% 651/789	Endomicrobiaceae

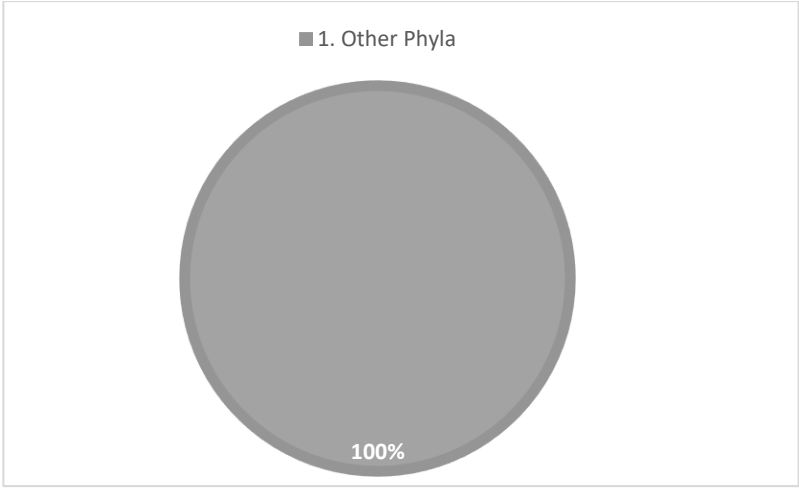


Figure 4.4. Showing the percentage of other phyla in Kabaağaç 2 sample

Table 4.4. Sequence Analysis results for Kabaağaç 1.

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
Kabaağaç (kuyu suyu)	57	25	**13**	*Spirulina subsalsa*	98% 710/766	Spirulinaceae
			5	*Candidatus curcubocaldaceae cyanobacterium*	99% 762/767	Cyanobacteria
			3	*Stanieria cyanosphaera*	92% 708/772	Dermocarpellaceae
			2	*Filamentous thermophilic cyanobacterium*	99% 740/746	Cyanobacteria
			2	*Oscillatoriales cyanobacterium*	92% 702/761	Oscillatoriaceae

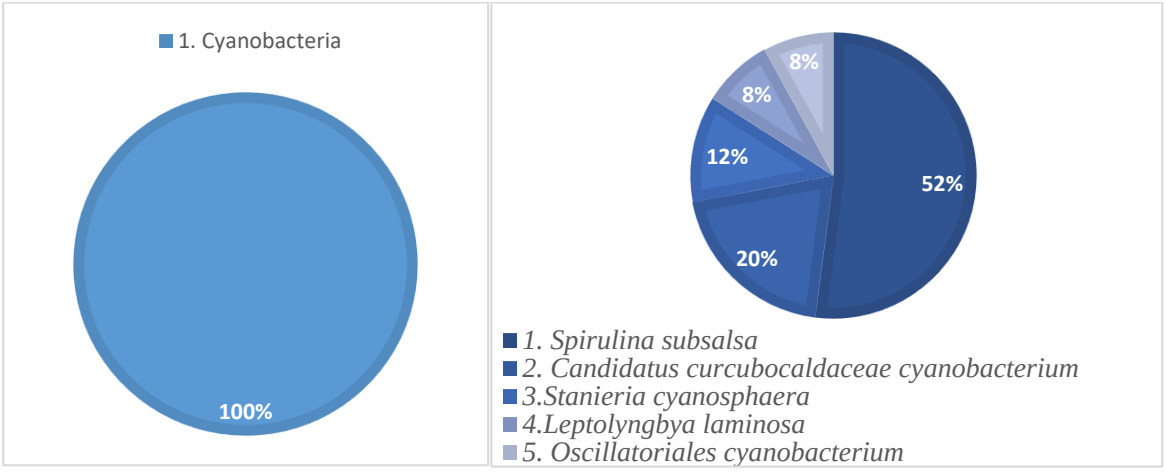


Figure 4.5. Showing the percentage of Cyanobacteria and other phyla in Kabaağaç 1 sample. b, percentage of species similarity.

Table 4.5. Sequence Analysis results for Umut Termal (1)

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
UT. (Altı havuz)	60	44	27	*Gloeobacter violaceus*	88% 692/782	Gloeobacteraceae
			7	*Leptolyngbya laminosa*	96% 728/759	Oculatellaceae
			3	*Filamentous thermophilic cyanobacterium*	99% 744/752	Unclassifield Cyanobacteria
			2	*Candidatus curcubocaldaceae cyanobacterium*	93% 708/761	Unclassifield Cyanobacteria
				Thermophilic cyanobacterium	99% 408/409	Unclassifield Cyanobacteria
			1	*Koinonema pervagatum*	98% 753/766	Oscillatoriales incertae sedis
			1	*Thermoleptolyngbya oregonensis*	99% 586/591	Oculatellaceae
			1	*Chloroflexi bacterium*	98% 764/779	Unclassifield bacteria

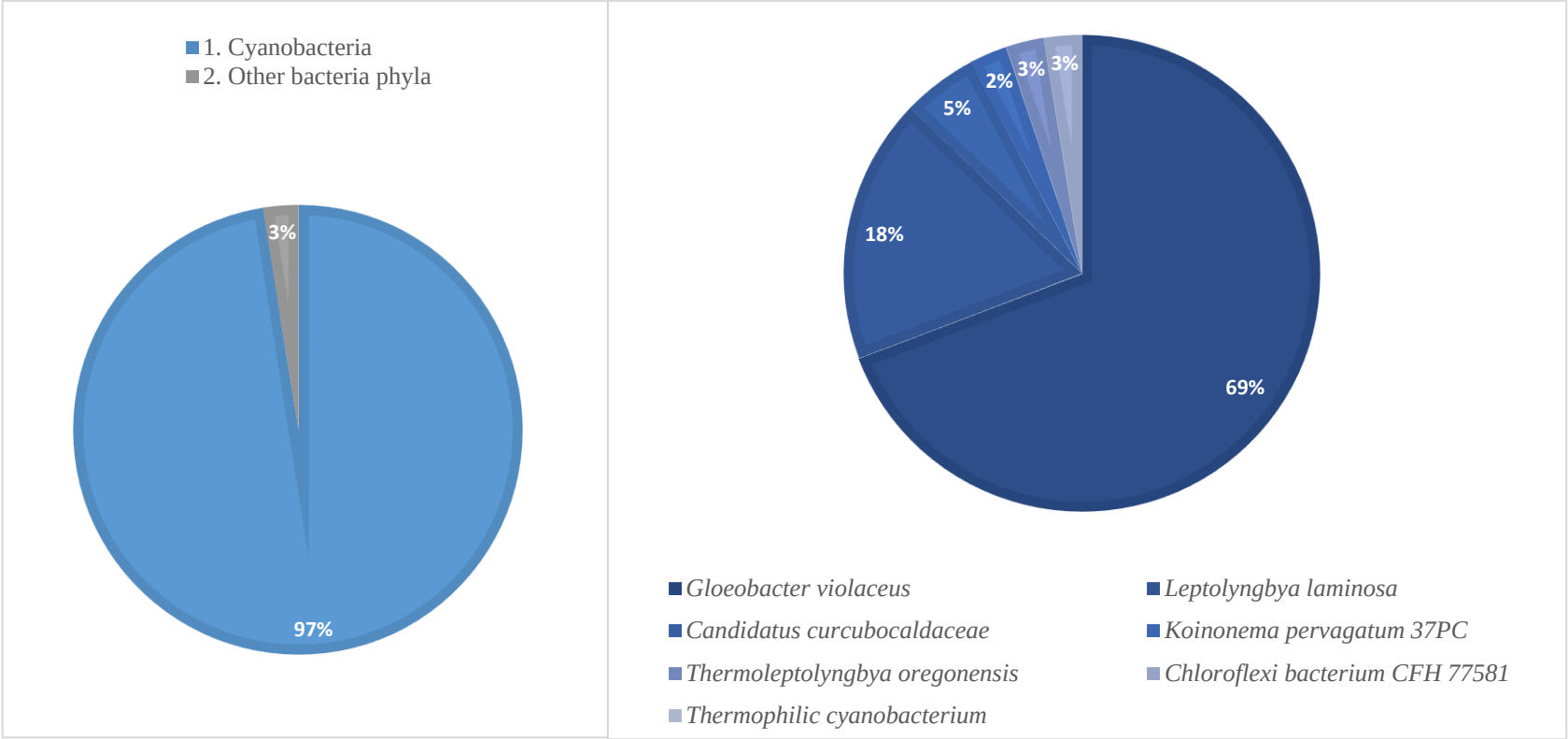


Figure 4.6. Showing the percentage of Cyanobacteria and other phyla in Umut Termal 1 sample. b, percentage of species similarity.

Table 4.6. Sequence Results for Kayta Hotel

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
			30	*Synechococcus sp*.	97% 736/762	Synechococcaceae
			5	*Chloroflexi bacterium*	98% 753/769	Unclassifield Chloroflexi
Kayta Hotel	47	43	**3**	*Geitlerinema sp*. Bal-2	99% 743/745	Coleofasciculaceae
			1	*Thermus scotoductus*	97% 932/956	Thermaceae
			1	*Nodosilinea sp*.	89% 680/767	Prochlorotrichaceae
			1	*Thermus brockianus*	100% 291/291	Thermaceae
			1	*Thermus aquaticus*	100% 291/291	Thermaceae
			1	*Paenibacillus illinoisensis*	100% 31/31	Paenibacillaceae

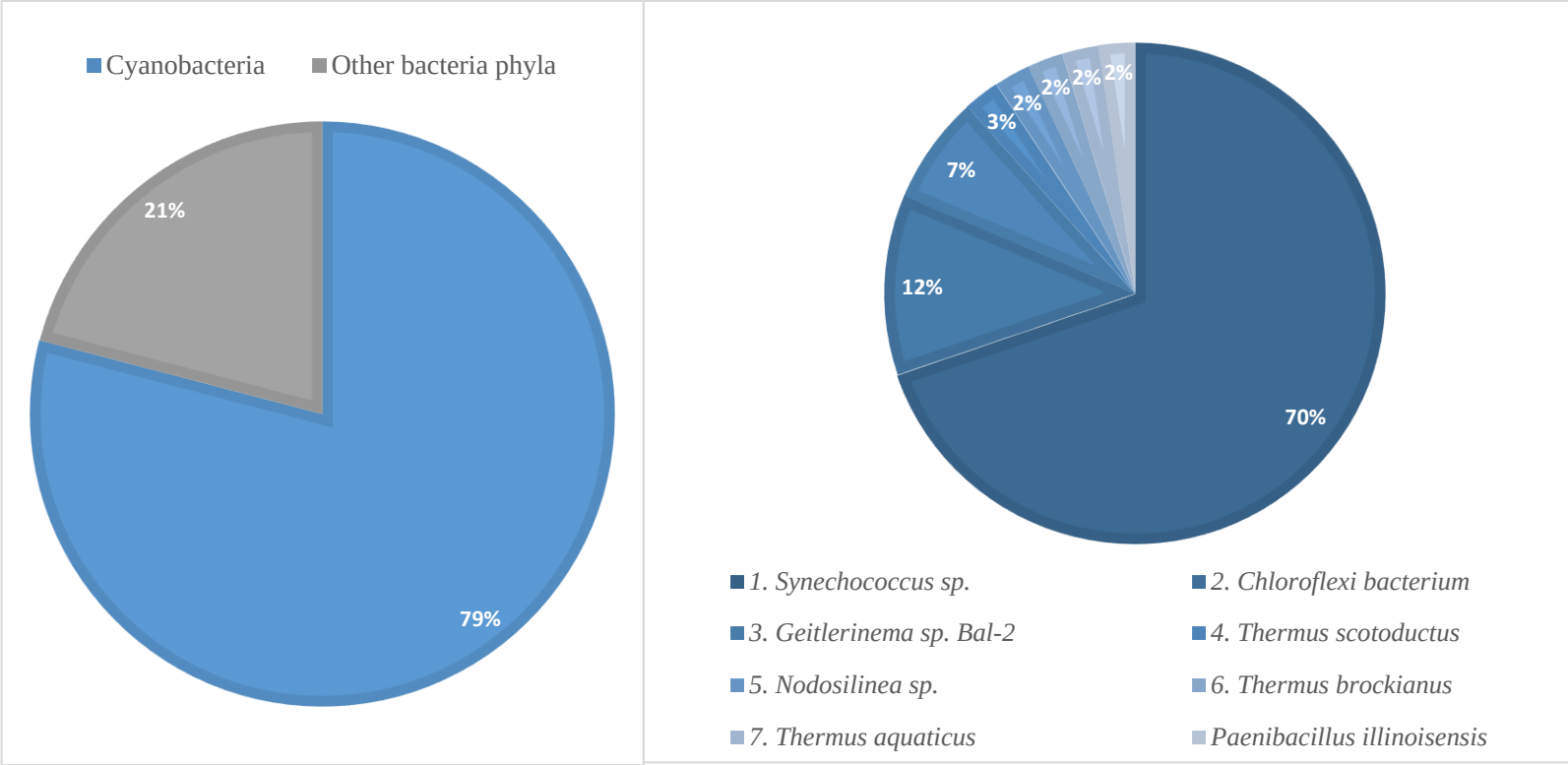


Figure 4.7. Showing the percentage of Cyanobacteria and other phyla in Kayta Otel sample. b, percentage of species similarity.

Table 4.7. Sequence Results of Umut Termal 2

Station	Tem ºC	Number of Colonies	Species	Per. % Identity	Class
Umut Termal (Çamur havuz)	42	4	*Arthrospira platensis*	92% 708/770	Microcoleaceae

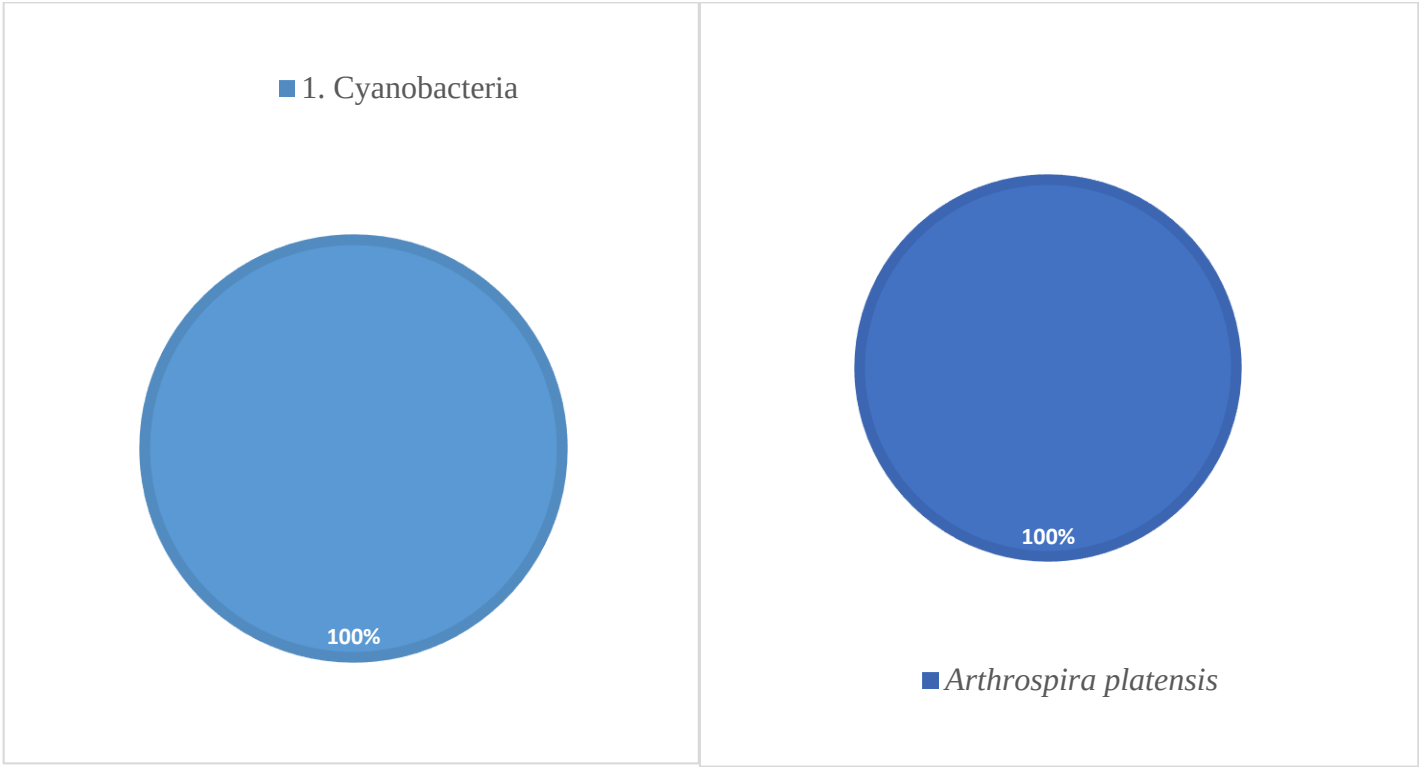


Figure 4.8. Showing the percentage of Cyanobacteria and other phyla in Umut Termal 2 sample. b, percentage of species similarity.

Table 4.8. Sequence Results for Kabaağaç 3

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
			5	*Armatimonadetes bacterium*	86% 634/741	Unclassified Armatimonadetes
			4	*Actinobacterium* YJF1-30	84% 489/581	Unclassified Actinobacteria
			3	*Candidate division* CPR2	82% 569/696	candidate division CPR2
Kabaağaç			**2**	*Berkelbacteria bacterium*	83% 472/570	Candidatus Berkelbacteria
(Dere)	52	24				
			1	Desulfotomaculum sp. MPNeg1	94% 615/651	unclassified Desulfotomaculum
			1	*Chloroflexi* bacterium	97% 753/774	Chloroflexi
			1	*Weissella paramesenteroides*	90% 55/61	Leuconostocaceae
			1	*Caldicoprobacter algeriensis*	82% 476/580	Caldicoprobacteraceae
			1	*Syntrophaceae* bacterium	82% 682/833	Syntrophaceae
			1	*Chloroflexi* bacterium	88% 689/786	Chloroflexi
			1	Thermobispora bispora	85% 695/819	Actinobacteria incertae sedis
			1	*Brevibacterium iodinum*	79% 274/347	Brevibacteriaceae
			2	*Delta proteobacterium*	83% 645/77	Deltaproteobacteria

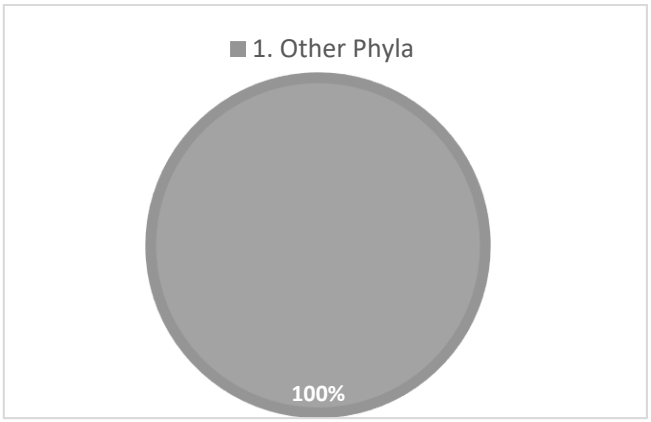


Figure 4.9. Showing the percentage of other phyla in Kabaağaç 3 sample

Table 4.9. Sequence Results for Ortakçı

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
Ortakçı (Hamam dağ çamur)	32	39	10	*Gracilibacter thermotolerans*	673/750(90%)	Gracilibacteraceae
			6	*Acidobacteria bacterium*	*783/817(96%)*	Unclassifield Acidobacteria
			4	*Inanidrilus exumae Delta*	751/812(92%)	Deltaproteobacteria
			3	*Vicinamibacter silvestris*	769/818(94%)	Vicinamibacteraceae
			2	*Desmonostoc sp. SA49*	761/764(99%)	Nostocaceae
			3	*Nostoc carneum NIES-*	761/775(98%)	Nostocaceae
			2	*Desulfatitalea tepidiphila*	694/826(84%)	Desulfobacteraceae
			1	*Syntrophomonas zehnderi*		Syntrophomonadaceae
			1	*Desulfatirhabdium butyrativorans*	681/773(88%)	Desulfobacteraceae
			1	*Streptacidiphilus hamsterleyensis*	691/774(89%)	Streptomycetaceae
			1	*Candidatus Sumerlaeaceae bacterium*	631/779(81%)	Candidatus Sumerlaeaceae
			1	*Hydrogenedentes bacterium*	700/830(84%)	Candidatus Hydrogenedentes
			1	*Desulfococcus sp.*	770/824(93%)	Desulfobacteracea
			1	*Dehalogenimonas alkenigignens*	677/792(85%)	Dehalococcoidia
			1	*Thermodesulfobacteriales bacterium*	660/786(84%)	Thermodesulfobacteriacea
			1	*Streptacidiphilus hamsterleyensis*	700/783(89%)	Streptomycetaceae

a

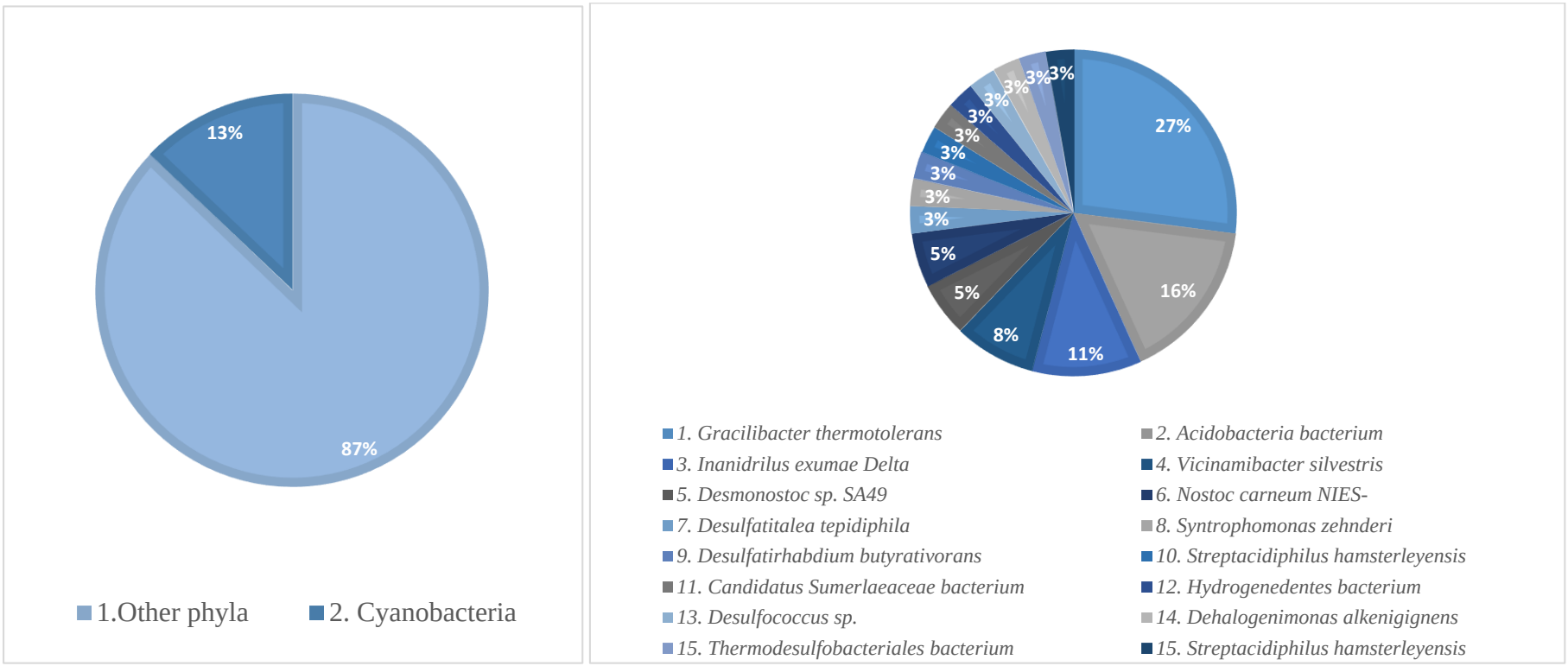


Figure 4.10. Showing the percentage of Cyanobacteria and other phyla in Ortakçı sample. b, percentage of species similarity

Table 4.10. Sequence Results of Karahayıt.

Station	Tem ºC	No: of Colonies	n	Species	Per. % Identity	Class
			13	*Alkalinema pantanalense*	91% 709/778	Leptolyngbyaceae
Karahayıt	44	34	9	*Leptolyngbya laminosa*	91% 742/758	Leptolyngbyaceae
			6	*Spirulina subsalsa*	99% 745/750	Spirulinaceae
			5	*Oculatella ucrainica*	90% 625/695	Oculatellaceae
			1	*Stanieria cyanosphaera*	91% 713/782	Dermocarpellaceae

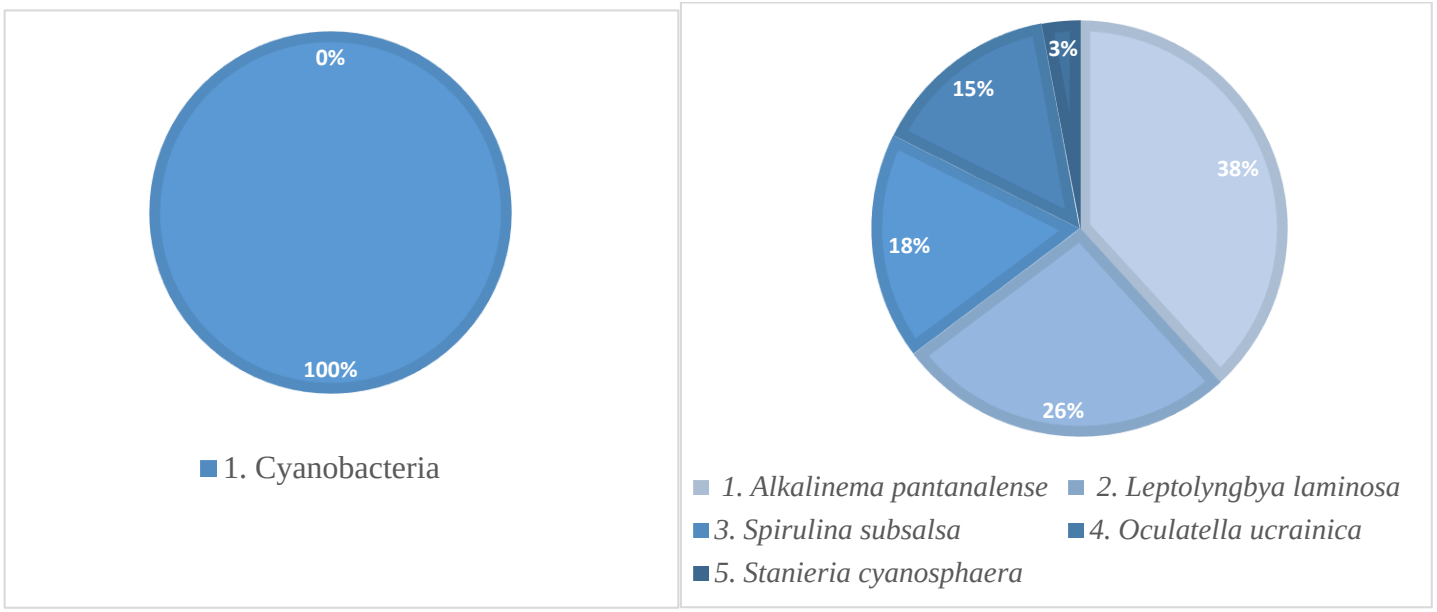


Figure 4.11. Showing the percentage of Cyanobacteria and other phyla in Karahayıt sample. b, percentage of species similarity.

5. DISCUSSION

This thesis investigated the cyanobacterial diversity from hot spring samples collected from Aydin province and its surroundings. As the first study, we used culture independent methods with two sets of primers which have the same sequences and one pair of primers with restriction sites for two restriction enzymes BamhI and HindIII to analyze the Cyanobacterial diversity from extreme habitats. Generally, culture dependent method and analysis of the 16S rRNA gene which contains functionally conserved regions ubiquitous to bacteria and archaea are used to isolate and screen different samples for microbial life. Because traditional methods are tedious recently culture independent methods are being preferred (Větrovský & Baldrian, 2013). This technique allows the direct exploration of the DNA or RNA in samples without culturing the microorganisms, making it possible to study the total diversity in a single step. Moreover, classical physiological and biochemical tests are substandard in rapidly analyzing and identifying the bacteria such as Cyanobacteria from microbial communities, because such bacterial populations involved are fastidious and usually similar nutritional requirements and environmental conditions cannot be replicated in the laboratory (Su et al., 2012; Tayaban et al., 2018; Damatac and Cao 2021).

We recorded positive PCR results for all samples after amplification of DNA. The total number of cloned colonies were 298, and among these, 201 (67.4%) belonged to Cyanobacteria phyla and 97 (33%) to other phyla. Among our clones, some showed lower similarities in the range (80 – 97 %) while others were over (99 %). In Alangullu sample 50 ^{o} C, all 50 formed colonies showered a 100% sequence similarity and the most dominant species we detected was *Planktothricoides raciborskii*, which is a member of Cyanobacteria Class Microcoleaceae; this followed by *Trichocoleus desertorum* from the Class Trichocoleusaceae . As for samples from Karahayıt (44^{o}C) and Umut termal (42 ^{o}C) 100% of the sequence results were Cyanobacterial in origin and the most abundant species found were *Alkalinema pantanalense and Arthrosipra plantensis These seem to be recently discovered species.*

Several research has been conducted assessing diversity of extremophilic Cyanobacteria and other extremophilic organisms around the world using culture independent methods. For example, Amarouche et al., (2014) examined water, concretions

and mat samples with the temperatures ranging from 39 °C to 93 °C from Algerian hot springs using 16S rRNA molecular analysis, and they found Cyanobacteria mostly belonging to *Synechococcus*, *Candidatus Leptolyngbya* and *Synechococcus* – like Cyanobacteria and Gleo-margarita. Similarly, McGregor and Rasmussen (2007) studied the microbial mats from Australian alkaline thermal springs to evaluate the Cyanobacteria composition. Temperature range for this site ranged 43 ºC – 71ºC. They used polyphasic approach like denaturing gradient gel electrophoresis and 16S rRNA specific Cyanobacteria PCR primers. According to their results, eight genera and 10 species belonging to three Cyanobacteria orders were identified with species such as *Oscillatoria amphigranulata*, *Leptolyngbya* and *Geilterinema* being the most detected.

Compared to the rest of the world few studies have been conducted in Turkey using culture independent methods. Yılmaz and Arık (2018) analyzed and compared the samples using culture independent and dependent methods like DGGE (denaturing gradient gel electrophoresis) and Cloning of PCR amplified fragments of 16S rRNA genes of environmental samples collected in Afyonkarahisar, Turkey. According to the culture independent method results, they recorded a total of 97 clones from 16S rRNA gene library analyzed by Amplified Ribosomal DNA Restriction Analysis (ARDRA). 16S rRNA sequence analysis of these clones demonstrated that the Cyanobacterial clones retrieved from environmental samples were similar to 16S rRNA gene sequences of *Geilterinema* spp. and *Leptolyngbya laminosa.* These results are similar to our results of sample Kayta Otel with Temperature of 44 º C. Yılmaz (2016), determined the Cyanobacterial composition in Kütahya Turkey from Eynal hot spring. According to his findings, molecular analysis results detected *Synechococcus*, *Geilterinema*, *Phormidium* and filamentous Cyanobacterium. He further stated that the Cyanobacteria diversity in this hot spring was low.

In a study conducted by Roeselers et al. (2007), heterocystous and non- heterocystous filamentous cyanobacteria which dominated the microbial mat communities from Arctic hot springs (Greenland) were detected using specific oligonucleotide primers for Cyanobacterial 16S rRNA gene fragments. Denaturing gradient gel electrophoresis (DGGE) was used to separate the amplified products. The sequences related to filamentous Cyanobacteria were of the genera *Chlorogloeopsis, Fischerella* and *Leptolyngbya.* Olsson- Francis et al. (2010) used culture independent (16S rRNA) and culture dependent methods and by using Exposure to Low earth orbit showed that a rock dwelling microbial community composed

Cyanobacteria community of Pleurocapsales, Oscillatoriales and Chroococcales. These communities survived exposure to extreme radiation and desiccating conditions which are associated with space. This ability of the organism to survive such conditions may be linked to the formation of dense colonies. Another study by Burns et al. (2004) using molecular approaches like polyphasic approach of culture – dependent and culture independent methods examined the microbial diversity of extant stromatolites in the hypersaline marine environment of shark Bay in Australia. The results showed the community being characterized by organisms of the Cyanobacterial genera *Synechococcus, Xenococcus, Microcoleus, Leptolyngbya plectonema, Symploca, Cyanothece, Pleurocapsa and Nostoc.* Damatac II and Cao, (2021) showed Cyanobacterial diversity from the order Psuedoanabaenales, *Arthronema africanum* (60.1 %) and *Leptolyngbya* ssp. 97.0 % using 16S r RNA gene sequencing and isolation independent method with samples collected from some mine tailing sites in Benguet province of the Philippines. Other recovered taxa were *Calothrix, Halomicronema, Nostoc, Phormidium, Planktothrix, Psuedoanabaena, Acaryochloris* and *Thermosynechococcus.*

Though, according to sequence results, some locations such as Kabaağaç 2 and 3 had no Cyanobacteria sequences. We reasoned that no Cyanobacteria species occur at these sites or the conditions in the area are not favorable for Cyanobacterial species. The bacterial species sequence results we got belonged to the Classes Armatimonadetes, Geobacteraceae, Syntrophobacteraceae, Dissulfurirhabdaceae, Thermoanaerobacteraceae and Nanopelagicaceae for Kabaağaç 2 while Armatimonadetes, Actinobacteria, Chloroflexi, Leuconostocaceae, Caldicoprobacteraceae, Syntrophaceae, Brevibacteriaceae, Deltaproteobacteria for Kabaağaç 3. A recent work (Samarasinghe et al., 2021), assessing the bacterial diversity of Sri Lankan geothermal hot spring samples, found eight genera belonging to Proteobacteria (57.39%), Firmicutes (23.7%) and Chloroflexi (4.14%). They also detected other Phyla; Actinobacteria, Planctomycetes and Bacteroidetes with a percentage less than 3%. The average temperature of the water samples was 44.5^{o}C.

Among our selected study sites, the location Ortakçı had the lowest temperature of 32 ^{o}C and we recorded species belongıng to many different Phyla such as; Acidobacteria (31%), other combined phyla (26%), Proteobacteria (22%), Cyanobacteria (13%), Firmicutes (5%), Thermodesulfobacteria (3%) and Candidatus sumerlaeota (3%). The sequence similarity for Cyanobacteria species were *Nostoc carneum*, and *Desmonostoc* sp.

SA49. At the highest temperature 62 ºC for our samples, we detected *Oscillatoria santa* PCC 7515 belonging to Cyanobacteria class Oscillatoriaceae.

6. CONCLUSION

As earlier mentioned that in Turkey, there are rare thermophilic Cyanobacteria studies using culture independent methods thus, this study will contribute to the literature deficiency in Turkey as many locations have not been adequately studied and to add on, it is important for being first culture- independent approach to determine cyanobacteria from thermal environments in Aydın province and surrounding regions. Our project may provide new insights into the Cyanobacteria researches from extreme habitats in our country and the other parts of the world. Another important output of the study is clones have relatively low similarities with those ones in databases, meaning they may represent new Cyanobacterial species and may contribute to the biodiversity and new metabolite studies.

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SCIENTIFIC ETHICAL STATEMENT

I hereby declare that I composed all the information in my master's thesis entitled Determination of Cyanobacteria Diversity From Hot Springs Around Aydin Province By Culture Independent Methods within the framework of ethical behavior and academic rules, and that due references were provided and for all kinds of statements and information that do not belong to me in this study in accordance with the guide for writing the thesis. I declare that I accept all kinds of legal consequences when the opposite of what I have stated is revealed.

Ruth Maseko Phiri

20 /08 /2021