Assessing Taxonomic Issues with the Genera *Anabaena*, *Aphanizomenon* and *Nostoc* Using Morphology, 16S rRNA and *efp* genes

by

Orietta Beltrami

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2008

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the general public.

ABSTRACT

Cyanobacteria are an ancient lineage of gram-negative photosynthetic prokaryotes that play an important role in the nitrogen cycle in terrestrial and aquatic systems. Widespread cyanobacterial blooms have prompted numerous studies on the classification of this group, however defining species is problematic due to lack of clarity as to which characters best define the various taxonomic levels. The genera *Anabaena*, *Aphanizomenon* and *Nostoc* form one of the most controversial groups and are typically paraphyletic within phylogenetic trees and share similar morphological characters. This study's purpose was to determine the taxonomic and phylogenetic relationships among isolates from these three genera using 16S rRNA and bacterial elongation factor P (*efp*) gene sequences as well as morphological analyses. These data confirmed the non-monophyly of *Anabaena* and *Aphanizomenon* and demonstrated that many of the isolates were intermixed among various clades in both gene phylogenies. In addition, the genus *Nostoc* was clearly not monophyletic and this finding differed from previous studies. The genetic divergence of the genus *Nostoc* was confirmed based on 16S rRNA gene sequence similarities (≥85.1%), and the isolates of *Anabaena* were genetically differentiated, contrary to previous studies (16S rRNA gene sequence similarities ≥89.4%). The morphological diversity was larger than the molecular diversity, since the statistical analysis ANOSIM showed that the isolates were morphologically well differentiated; however, the 16S rRNA gene sequence similarities showed some isolates as being related at the species level. Planktonic and benthic strains were not distinguished phylogenetically, although some well-supported clusters were noted. Cellular

measurements (length and width of vegetative cells, end cells, heterocysts and akinetes) were noted to be the morphological characters that best supported the differentiation among isolates, more than qualitative characterization. Among the metric parameters, the length of akinetes resulted in better differentiation among isolates. The *efp* gene sequence analyses did not appear to be useful for the taxonomic differentiation at lower taxonomic levels, but gave well-supported clusters for *Aphanizomenon* that was supported by the morphological analyses. Both gene regions gave similar trees with the exception of the *Aphanizomenon* isolates which clustered together in phylogenetic trees based on the *efp* gene. This differed from the 16S rRNA gene in which this genus was paraphyletic with *Anabaena* species that were similar in morphology to *Aphanizomenon*. Hence, the application of multiple taxonomic criteria is required for the successful delineation of cyanobacterial species.

ACKNOWLEDGEMENTS

My first acknowledgement is for my supervisor Dr. Kirsten Müller, who gave me the opportunity to work in her lab, provided me with help, advice and constant understanding.

I also want to thank Dr. Stephanie Guildford, Dr. Jonathan Witt and Dr. John Semple, for their support. Particularly, thanks to Hedy Kling for her help, support and advice.

Special thanks to Amanda Poste and Peter Njuru, who collected the samples for this work and to Dr. Susan Watson, Dr. Friedrich Jütter, Mr. George Izaguirre and Dr. Sarah A. Spaulding who provided me with excellent cyanobacterial cultures and samples.

I would also like to thank my labmates: Andrea Gill, Sara Ross, Justin Lorentz, Lynette Lau, Robert Young, Michael Kani and Adam Woodworth for their assistant and their friendly welcoming. Special thanks to Michael Lynch, who provided me with help and advice every time I needed it.

Muchísimas gracias para Pablo Conejeros quien me guió y me acompaño durante todo este difícil periodo, y quien en parte me abrió las puertas de Canadá. También gracias por su apoyo y amor incondicionales.

Gracias a Iván Salinas, quien me dedico muchísimo tiempo y amistad.

A mi papá, mamá y hermanas por su amor y preocupación.

Fue una alegría haber conocido a Rita, Leandro, Maricris, Miguel, Andrea, Andres, Alondra, Alejandro, Macarena, Pancho y María José. Muchísimas gracias por su amistad, la que ojala se siga manteniendo en el tiempo.

A Laura, Pamela, Chica, Guadalupe, Pilar, Eulogio, Gloria, Paola y todos aquellos que me apoyaron a la distancia.

Y especial agradecimiento a mi Flor, que me ha acompañado durante 12 años.

TABLE OF CONTENTS

List of Tables

List of Illustrations

CHAPTER 1: GENERAL INTRODUCTION

1.1 CYANOBACTERIA

Cyanobacteria are a morphologically diverse group of photosynthetic gram-negative prokaryotes that were the dominant form of life on Earth for more than 1.5 billion years (Neilan *et al*., 1995). In addition, cyanobacterial fossils have been dated to be over 2.7 billion years old (Brocks *et al*., 1999). Blue-green algae were the first living organisms to undergo photosynthesis using chlorophylls a and b as well as producing a variety of accessory photosynthetic pigments (xanthophylls, β-carotene; and phycobilins) (Graham and Wilcox, 2000). Moreover, there is strong molecular evidence that cyanobacterial plastids are the ancestors to all other chlorophyll-producing photosynthetic life (Chu *et al*., 2004; Martin *et al.,* 1998; Palmer, 2003). Indeed, the theory of endosymbiosis has been well supported by the comparison of the phylogenetic analysis of nucleic and chloroplast genes, as well as genes related to plastid function such as plastid protein import and plastid targeted solute transport (Reyes-Prieto *et al*., 2007). An example of such an analysis was done by Chu *et al*. (2004) based on the complete chloroplast genome sequences from representative specimens of Archaea, Eubacteria, Chlorophyta, Rhodophyta, Glaucophyta, Embryophyta, and Eukaryota groups. This study demonstrated that all chloroplasts are closely related to the cyanobacteria (Chu *et al*., 2004).

The modern cyanobacteria are recognized for their ability to fix atmospheric nitrogen (Graham and Wilcox, 2000) that enables them to occupy a wide range of environments providing them with a competitive advantage over eukaryotic algae and

plants (Komárek *et al*., 2003). Moreover, the development of heterocysts from vegetative cells, and therefore the differentiation of the heterocystous cyanobacteria, may represent a key event in the evolutionary history of life leading to the presence of an oxygen atmosphere on the planet (Giovanonni *et al*., 1988; Tomitani *et al*., 2006). According to Tomitani *et al*. (2006), between 2.4 and 2.3 billion years ago the partial pressure of oxygen was sufficient to allow for heterocyst differentiation. Heterocysts are specialized cells capable of fixing nitrogen in oxic conditions due to impermeable membranes which prevent the influx of molecular oxygen that inhibits the action of nitrogenase; an enzyme which catalyzes the biological reduction of atmospheric nitrogen. Hence, the heterocystous cyanobacteria do not need anaerobic conditions to fix nitrogen (Tomitani *et al*., 2006). Additionally, cyanobacterial species are essential in soils and fix atmospheric nitrogen for use by plants (Mishra and Pabbi, 2004; Singh and Datta, 2005). In addition, cyanobacteria produce medically useful compounds, like c-phycocyanins, pigments with antioxidant properties (Zhou *et al*., 2005). Moreover, considerable advancement in algal genetic engineering has been achieved using the relatively simple genome structure of cyanobacteria (Elhai, 1994; Graham and Wilcox, 2000).

1.2 TOXICOLOGY OF CYANOBACTERIA

Some cyanobacterial planktonic species are capable of forming massive blooms in mesotrophic and eutrophic water bodies throughout the world (Komárek *et al*., 2003). Such blooms can create anoxic conditions in the water body as well as release cyanobacterial toxins which can cause fish kills, human illness, affect aquaculture, and contribute to the loss of natural biodiversity in aquatic systems. The environmental

factors that control toxic cyanobacterial blooms are not well understood. However, current research suggests that high light intensity, decreased water transparency, high water column stability, relatively high water temperature and pH, low nitrogen to phosphorous (N:P) supply ratio, and higher phosphorous concentrations, can contribute to such blooms (Ferber *et al*., 2004; Jacoby *et al*., 2000; Rapala and Sivonen, 1998). In addition, some species can produce taste and odours that affect drinking water (Steffensen *et al*., 1999; Tang *et al*., 1997; Watson and Ridal, 2004). Moreover, *Nostoc* in symbioses can produce a neurotoxin (ß-methylamino-L-alanine), which is biomagnified by the terrestrial host (e.g. Cycads roots). If these roots are consumed by humans they can cause an illness called amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS/PDC) (Cox *et al.,* 2003; Murch *et al.*, 2003).

Cyanobacteria toxins can be separated in four groups according to their toxicological effects: hepatotoxins, neurotoxins, cytotoxins and dermatotoxins (Codd, 1999). There has been considerably more focus on hepatotoxins since they have been shown to be potent inhibitors of protein phosphatases leading to tumor promotion in the liver (Pereira *et al*., 2004). Among neurotoxins, the saxitoxin group (saxitoxin, neosaxitoxin, and gonyautoxin) is the most studied because it represents the most potent toxins (Pereira *et al*., 2004), which mode of action is blocking the sodium channels in nerve cells (Sivonen and Jones, 1999). Anatoxin-a (s) is another potent neurotoxin in freshwater, which is related to the hipersalivation (Codd, 1999), poisoning and death of animals (Yavasoglu *et al*., 2008). Table 1.1 summarizes the toxin types produced by members of genera *Anabaena*, *Aphanizomenon* and *Nostoc*.

Table 1.1 **Toxins produced by *Anabaena*, *Aphanizomenon* and *Nostoc* species** (Banker *et al*., 1997; Cox *et al*., 2003; Ferreira *et al.,* 2001; Gugger *et al*., 2002; Lyra *et al.,* 1997, 2001; Mahmood and Carmichael, 1986; Murch *et al*., 2003; Negri and Jones, 1995; Pereira *et al*., 2000, 2004; Rolland *et al*., 2005 ; Rouhiainen *et al*., 1995; Shaw *et al.,* 1999; Sivonen *et al.,* 1990; Steffensen *et al*., 1999).

Toxins	***Anabaena***	***Aphanizomenon***	***Nostoc***
Anatoxin A (neurotoxin)	**x**		
Cylindrospermopsin (neurotoxin)		**x**	
BMAA: ß-methylamino-L-alanine (neurotoxin)			**x**
Saxitoxin group (neurotoxic)	**x**	**x**	
Microcystin (hepatotoxic)	**x**		**x**

1.3 ECOGEOGRAPHICAL DISTRIBUTION OF CYANOBACTERIA

Cyanobacteria are a cosmopolitan group and can be observed in almost every habitat on the planet and are the dominant freshwater alga in the Arctic and Antarctic (Sheath *et al*., 1996; Tang *et al*., 1997). In addition, these organisms have the ability to live in extreme conditions such as habitats with high temperatures and salinities (Bell, 1993; Dodds *et al*., 2005; Spaulding *et al*., 1994). But the taxonomic problems at lower levels and the scarcity of species lists related to this group have prompted difficulties in establishing the biogeographic distribution of determined taxa within this group (Hoffmann, 1996; Mollenhauer *et al*., 1999). A low number of cyanobacterial species hold a good description of their distribution, due to the fact that they are the only ones that have been collected and analyzed in several regions, this is the case of *Nostoc commune*, which is a sub-cosmopolitan taxa (Hoffmann, 1996). The next sections will focus on the distribution of the genera *Anabaena*, *Aphanizomenon* and *Nostoc,* all of which are heterocystous cyanobacteria belonging to Nostocales group (Section IV). Members of these genera are the primary focus of this thesis

1.3.1 ECOGEOGRAPHICAL DISTRIBUTION OF *ANABAENA*

Planktonic and benthic *Anabaena* species are observed in brackish, freshwater and marine habitats, in extreme habitats, such as frozen lakes in Antarctic and Arctic (Sheath, 1996, 1997; Spaulding *et al*., 1994), and hot deserts (Bell, 1993), and also include those that are in symbiotic association with terrestrial organisms (Moreno *et al*., 2003; Rajaniemi *et al*., 2005; Rippka *et al*., 2001; Sivonen *et al*., 2007). Although this genus has been considered cosmopolitan, their species within this genus occur only in limited areas or have an endemic distribution, like *A. bituri*, *A. compacta*, *A. fuellebornii*,

A. iyengarii, *A. leonardii, A. nygaardii, A. pseudocompacta* and *A. subtropica* (Hoffman, 1996; Komárek and Zapomělová, 2007). Although there are some species considered truly cosmopolitan, such as *A. flos-aquae* and *A. circinalis*, most species are observed globally except in sub-polar regions (Komárek and Zapomělová, 2007). *Anabaena* species have been reported from most of Europe including Finland (Lyra *et al*., 1997), Czech Republic (Rajaniemi *et al*., 2005), Denmark, France, Norway (Gugger *et al*., 2002), Netherlands, England, Spain (Lyra *et al.,* 2001), Portugal (Pereira *et al*., 2004), German (Stüken *et al.,* 2006), Belgium and Luxembourg (Willame *et al*., 2006) and the Baltic Sea (Sivonen *et al.*, 2007). In addition, there are reports of this genus as well as others in Australia (Fergusson and Saint, 2000), India (Stulp and Stam, 1984); Cuba (Komárek, 2005), Brazil (Gaylarde *et al*., 2005), Argentina (Izaguirre and Vinocur, 1994), Chile (Pereira *et al*., 2000); Canada (Kling, 1997; Rolland *et al*., 2005), U.S.A (Gugger *et al*., 2002) and Africa (Evans, 1997; Krienitz *et al*., 2002; Lung'ayia *et al*., 2000; Sekadende *et al*., 2005).

1.3.2 ECOGEOGRAPHICAL DISTRIBUTION OF *APHANIZOMENON*

The distribution for planktonic and benthic *Aphanizomenon* species is similar to that for *Anabaena* and this genus is also observed in brackish and freshwaters (Gugger *et al*., 2002; Rajaniemi *et al.*, 2005). Species within this genus have been reported from Cuba, Brazil, India, Africa (Hoffmann, 1996), Baltic Sea (Sivonen *et al*., 2007), Finland, Japan, France, The Netherlands, Denmark (Gugger *et al*., 2002), China (Liu *et al*., 2006), German (Stüken *et al.,* 2006); Portugal (Pereira *et al*. 2004), U.S.A. (Mahmood and Carmichael, 1986), Canada (Kling, 1997), Israel (Banker *et al.,* 1997), and Africa (Cronberg and Komárek, 2004).

1.3.3 ECOGEOGRAPHICAL DISTRIBUTION OF *NOSTOC*

The genus *Nostoc* is one of the phototrophic groups that have the most widespread distribution (Dodds *et al*., 1995). Some *Nostoc* species are benthic or planktonic free-living organisms (Hoffmann, 1996; Lyra *et al*., 2001; Mollenhauer *et al*., 1999), but most of them are capable of fixing nitrogen in symbioses with higher plants and fungi (Guevara *et al*., 2002). These symbiotic associations have been observed with different hosts including bryophytes, gymnosperms (cycads), pteridophytes (*Azolla* fern), an angiosperm (genus *Gunnera*), and fungi (lichens) (Baker *et al*., 2003; Bergman *et al*., 1992; Meeks and Elhai, 2002; Rasmussen and Svenning, 1998; Rikkinen *et al*., 2002; West and Adams, 1997).

Nostoc species have a broad distribution and occur in numerous different habitats, which can also depend on their hosts (Table 1.2). For example, *Gunnera* species are usually observed in areas with heavy rainfall and in temperate regions (Guevara *et al.,* 2002). *Nostoc* symbioses with lichens can be observed with bryophytes in moist coniferous forests (Paulsrud *et al.,* 1998). In addition, *Nostoc* has also been observed to form symbioses with liverworts in mineral soils (Costa *et al*., 2001).

With respect to free living *Nostoc*, there are fewer geographical reports with most reports from brackish waters (e.g. Baltic Sea), freshwater (e.g. Chile, Arctic and Antarctic) and marine waters (e.g. Hawaii) (Banack *et al*., 2007; Dodds *et al*., 1995; Hoffmann, 1996; Pereira *et al*., 2000; Sheath *et al*., 1996; Spaulding *et al*., 1994; Vargas *et al*., 1998). For example, the studies of Sheath *et al*. (1996; 1997) demonstrated that *Nostoc commune* was the most widespread species in arctic stream habitats from North

Table 1.2 Geographical distributions of the different *Nostoc* symbiotic relationships.

Symbiotic relationship	Location (Original source)	Reference
Nostoc-*Azolla*	Australia, China, U.S.A., German, Galapagos Islands	Baker *et al*. (2003); Plazinski *et al*. (1990)
Nostoc-Bryophyte	Central Finland	Costa *et al*. (2001)
Nostoc-*Cycads*	Australia, China, Southwest of Asia, America, Japan, South Africa, Cuba, Guatemala, West India	Costa *et al*. (2004); Zheng *et al.* (2002)
Nostoc-Fungi	Northern Europe, western North America and Central China, central Finland	Paulsrud *et al*. (1998, 1999); Rikkinen *et al.* (2002)
Nostoc-*Gunnera*	Central and southern Africa, Madagascar, New Zealand, Tasmania, Indonesia, The Philippines, Hawaii, Mexico, central and south America	Bergman *et al*. (1992); Guevara *et al*. (2002)

America, which was in concordance with the findings in Antarctic streams. Also they observed *N. pruniforme* and *N. verrucosum* species, but in a more limited area.

1.4 PROBLEMS IN TAXONOMY AND PHYLOGENY OF CYANOBACTERIA

Currently, the taxonomy for the cyanobacteria is based either on the International Code of Botanical Nomenclature (ICBN) or International Code for Nomenclature of Bacteria (ICNB); this causes considerable confusion due to a lack of consensus between these two codes. The "Stanier/Rippka" system (Rippka *et al*., 1979) is the main scheme given by ICBN. Although this system is a shift to a system of classification based on a greater number of phenotypic, physiologic and genotypic characters of cultured strains, it is not a phylogenetic classification based on genetic information. Conversely, Bergey's Manual of Systematic Bacteriology 2nd Edition (2001), the ICNB's most used taxonomic scheme, mainly represents a molecular phylogenetic analysis based on sequence analyses of the 16S rRNA gene. But these are not the only problems in the taxonomy and phylogeny of cyanobacteria. As of May 2003, only 13 species names have been proposed in original articles published in International Journal of Systematic and Evolutionary Microbiology (IJSEM)/International Journal of Systematic Bacteriology (IJSB) or validated in the Validation Lists in the journal as of November 2003 (Oren, 2004). Additionally, it is estimated that as many as 50% of cyanobacterial strains existing in culture collections have been identified incorrectly or have been assigned to the wrong taxonomic group (Komárek and Anagnostidis, 1989 *fide in* Willame *et al*., 2006), and relatively few species are maintained in axenic culture conditions that permit the identification of specific characters upon which to base the taxonomy (Casamatta *et al*.,

2005). Therefore, an ever changing classification system and a lack of a consensus phylogeny are considerable issues when attempting to resolve evolutionary relationships and species issues within the cyanobacteria (Litvaitis, 2002), which moreover may affect estimates of cyanobacteria diversity in future studies (Komárek *et al*., 2003). In order to address these issues it is essential to conduct additional studies on the classification of filamentous cyanobacterial at the lower taxonomic levels (genus andspecies), where there appear to be the most significant problems in cyanobacterial classification (Lehtimäki *et al*., 2000; Rajaniemi *et al*., 2005; Zehr *et al*., 1997).

The present thesis is an analysis of the taxonomic and phylogenetic problems of the genera *Anabaena*, *Aphanizomenon* and *Nostoc*. However, it is first necessary to understand that there are taxonomic issues within the entire order of Nostocales. For example, according to Lehtimäki *et al*. (2000), the ability to form gas vesicle in *Nodularia* may not be a useful character to identify cultured strains, since isolates may lose them under growth conditions. *Nodularia* formed a unique cluster by 16S rRNA gene sequence and 16S rRNA RFLP analysis according to Iteman *et al*. (2002). In contrast, Lehtimäki *et al*. (2000) and Moffit *et al*. (2001) said that *Nodularia* genus clustered into two main groups on the basis of 16S rRNA sequences, and they concluded that it is necessary further studies into its phylogeny and evolution. Moreover, according to Iteman *et al*. (2002) the RFLP analysis demonstrated that *Nodularia* strains clustered more closely with members of the genera *Anabaena* and *Aphanizomenon*, which is concordant with the studies of Giovanonni *et al*. (1998), Lehtimäki *et al*. (2000), Lyra *et al*. (2001), and Wilmotte and Herdman (2001). In contrast, Lyra *et al*. (1997) in their 16S rRNA gene and RFLP analysis found that *Nodularia* always clustered with *Nostoc*

strains. Indeed, only two species of *Nodularia* described in the botanical literature should be maintained, *N. spumigera* and *N. harveyana*, and these species remain highly controversial (Rippka *et al*., 2001). Another example is given by Iteman *et al*. (2002), who studied the phylogenetic position of *Cyanospira* and *Anabaenopsis*, and concluded that they belong to a single genus. Additionally, the relationship between *Cyanospira* and *Anabaena* remains unknown since no 16S rRNA sequence data are available. Hence, there are considerable confusing taxonomic relationships among Nostocales members, the Table 1.3 shows the taxonomic and phylogenetic relationships which have not been well established in this order. Basically this is because of the scarcity of distinct and consistent morphologic, biochemistry and molecular characters that support a taxonomic scheme (Baker *et al*., 2003; Boyer *et al*., 2001; Costa *et al*., 2001; Damerval *et al*., 1989; Fergusson and Saint, 2000; Giovanonni *et al*., 1988; Gugger *et al*., 2002; Henson *et al*., 2002; Iteman *et al*., 2002; Lachance, 1981; Lehtimäki *et al*., 2000; Litvaitis, 2002; Lu *et al*., 1997; Lyra *et al*., 2001; Mazel *et al*., 1990; Moffit *et al*., 2001; Nilsson *et al*., 2000; Plazinski *et al*., 1990; Rajaniemi *et al*., 2005; Rasmussen and Svenning, 2001; Rikkinen *et al*., 2002; Rippka *et al*., 2001; Rudi *et al*., 1997, 2000; Seo and Yokota, 2003; Smith *et al*., 1998; Svenning *et al*., 2005; Tamas *et al*., 2000; West and Adams, 1997; Willame *et al*., 2006; Wilmotte and Herdman, 2001; Wilson *et al*., 2000; Wright *et al*., 2001; Zehr *et al*., 1997).

1.4.1 TAXONOMIC AND PHYLOGENETIC PROBLEMS AMONG GENERA *ANABAENA, APHANIZOMENON* AND *NOSTOC*

Genera *Anabaena, Aphanizomenon* and *Nostoc* represent one of the most recurrent problems in taxonomy and phylogeny of the cyanobacteria. To assign independent clades to these genera is very difficult, since they appear to be non-monophyletic in the phylogenetic trees using different molecular markers, like 16S rRNA, *rbcLX* and *rpoB* (Rajaniemi *et al*., 2005; Svenning *et al*., 2005). In addition, they share morphological characters, such as heterocysts and akinete size and as well as the location of these specialized cells within the filaments (Rajaniemi *et al*., 2005). Complicating the matter is that these characteristics can change when these genera are grown in culture (Gugger *et al*., 2002). Herein will be given the most important gaps among these genera within current complexes.

1.4.1.1 The *Anabaena-Aphanizomenon* Complex

The monophyly of genera *Anabaena* and *Aphanizomenon* has been strongly discussed by several authors, and it has been suggested that these two taxa should belong to the same genus (Gugger *et al*., 2002; Iteman *et al*., 2002; Lachance, 1981; Lyra *et al*., 1997, 2001; Rajaniemi *et al*., 2005). The primary phylogenetic problem of this complex is the incongruence between the morphological characterization and the phylogenetic analysis based on molecular markers. Indeed, in this complex it is necessary to re-evaluate the taxonomic criteria with an emphasis on a complete morphological characterization. The real importance of the morphological characterization will be evaluated within this thesis since it is difficult to determine which morphological characters are truly stable to support an adequate taxonomic differentiation (primarily in

Table 1.3 **Nostocales genera that have taxonomic problems.** The X indicates a taxonomic conflict in cases where species in the rows have been ambiguously assigned to different species in the columns.

Nostocales Genera	***Anabaena***	***Anabaenopsis***	***Aphanizomenon***	***Calothrix***	***Cyanospira***	***Cylindrospermopsis***	***Cylindrospermum***	***Nodularia***	***Nostoc***	***Rivularia***	***Scytonema***	***Trichormus***	**References**
Anabaena	-	X	X	X	X		X	X	X		X	X	Baker *et al*. (2003); Boyer *et al*. (2001); Costa *et al*. (2001); Damerval *et al*. (1989); Fergusson and Saint (2000); Giovanonni *et al*. (1988); Gugger *et al*. (2002); Henson *et al*. (2002); Iteman *et al*. (2002); Lachance (1981); Lehtimäki *et al*. (2000); Litvaitis (2002); Lu *et al*. (1997); Lyra *et al*. (2001); Mazel *et al*. (1990); Moffit *et al*. (2001); Nilsson *et al*., (2000); Plazinski *et al*. (1990); Rajaniemi *et al*. (2005); Rasmussen and Svenning (2001); Rikkinen *et al*. (2002); Rippka *et al*. (2001); Rudi *et al*. (1997, 2000); Seo and Yokota (2003); Smith *et al*. (1998); Svenning *et al*. (2005); Tamas *et al*. (2000); West and Adams (1997); Willame *et al*. (2006); Wilmotte and Herdman (2001); Wilson *et al*. (2000); Wright *et al*. (2001); Zehr *et al*. (1997).
Anabaenopsis		-		X	X			X			X		
Aphanizomenon			-	X				X	X		X		
Calothrix				-	X	X	X	X	X	X	X	X	
Cyanospira					-						X		
Cylindrospermo						-	X		X		X		
Cylindrospermu							-		X		X		
Nodularia								-	X		X		
Nostoc									-		X	X	
Rivularia										-	X		
Scytonema											-	X	
Trichormus												-	

culture conditions). To avoid the confusion of changing morphology under culture conditions it is recommended that one only work with species that have been recently isolated. However, that being said, more studies on either are necessary for standardized the morphological taxonomy (Gugger and Hoffmann, 2004; Rajaniemi *et al*., 2005).

Rajaniemi *et al*. (2005) concluded that these genera are not monophyletic on the basis of 16S rRNA gene, *rpoB* and *rbcLX* sequences analysis, and morphological characterization. They obtained in their phylogenetic analyses, nine well supported subclusters within an *Anabaena-Aphanizomenon* cluster, concluding that each subcluster may represent different species, yet the *Anabaena* and *Aphanizomenon* strains were always paraphyletic. Additionally, this phylogenetic distribution was only supported by the akinetes' characterization among seven morphological parameters. Willame *et al*. (2006), based on 16S rRNA gene sequence analysis and morphological characterization could not distinguish among *Anabaena* and *Aphanizomenon* strains. In fact, the strains of both genera formed one well supported cluster, divided in seven subclusters which could be distinguished by the presence of gas vesicles. In addition, they observed that some clusters were highly supported by the morphological characterization, but only by some diacritical characters. These characters were specific for each cluster and different among clusters, however, what is difficult to determine is which character is more stable for an appropriate taxonomic determination. Moreover, some species that presented morphological differences were intermixed, sharing at least 99.6% of internal similarity. This is the case of *Aphanizomenon gracile* and *Anabaena sigmoidea*, which have straight and coiled forms respectively, although they share similarities as well, like the width and length of heterocysts and akinetes. Another case in which the morphological

characterization did not support the molecular phylogenetic analysis is given by Fergusson and Saint (2000), who examined the taxonomy of *Anabaena bergii* and *Aphanizomenon ovalisporum* strains on the base of *rpo*C1 gene sequence analysis. Their sequence analysis demonstrated that these strains are morphological variants of the same cyanobacterium, since they shared 100% similarity. Gugger *et al*. (2002) on the basis of 16S rRNA gene, the spacer region of the ribosomal operon (ITS1) and the *rbcLX* (RubisCO) also support the polyphyly of these genera. In spite of the fact that they concluded that the morphological characterization based on shapes and sizes of vegetative cells, akinetes and heterocysts achieved to separate both genera, their results are similar to the other researchers, this means that only some characters were diacritical for separating both genera.

Strain toxicity has been used as another character for distinguishing between genera *Anabaena* and *Aphanizomenon* (Fergusson and Saint, 2000; Gugger *et al*., 2002; Iteman *et al*., 2002; Lyra *et al*., 2001). Several studies on *Anabaena* species noted that they were separated by their specific toxin in that the hepatotoxic (microcystins) strains were separated from the neurotoxic (anatoxin-a, saxitoxin) ones (Fergusson and Saint, 2000; Gugger *et al*., 2002; Iteman *et al*., 2002; Lyra *et al*., 2001). However, the presence of toxicity was not monophyletic in phylogenetic analyses with the non-toxic *Anabaena* and non-toxic *Aphanizomenon* strains being intermixed in several clades with toxic *Anabaena* strains. Although in these studies were considered toxin-producer strains, it is important to consider that morphologically identical strains can be toxic and non-toxic, since just toxic strains have the gene related with its production (Rantala *et al*., 2004).

1.4.1.2 The *Nostoc-Anabaena* Complex

The main phylogenetic problem in this complex is similar to that noted in the *Anabaena-Aphanizomenon* complex, in that the morphological characterization does not always support the phylogenetic analyses. Indeed, this complex presents more taxonomic problems at strain level than the *Anabaena-Aphanizomenon* one. This could be due to the fact that the two genera, *Nostoc* and *Anabaena,* have been historically differentiated on the basis of morphological and life cycle characteristics (Tamas *et al*., 2000). These characteristics have been demonstrated to be unreliable and can vary according to different growth conditions (Wright *et al*., 2001).

The phylogenetic separation of genera *Anabaena* and *Nostoc* has been questioned using several different molecular markers, in which the data is consistently incongruent with the morphological analyses. For example, Tamas *et al*. (2000) could not discriminate between *Anabaena* and *Nostoc* strains on the base of morphological characterization and *nifH* sequence analysis. This gene did not differentiate the strains which have hormogonia (short filaments formed by fragmentation of the trichome, type of vegetative reproduction (Damerval *et al*., 1991)) and aserial developed stage (cells in packages), typical of the genus *Nostoc*. Svenning *et al*. (2005), in which the 16S rRNA gene sequence analysis depicts a well separated *Nostoc* clade, but other clades were not well supported and show paraphyletic *Anabaena* and *Nostoc* strains. One specific case is given by *Nostoc azollae*, which has been under nomenclatural changes, since it was named *Anabaena azollae* mainly on the base of morphological characterization (Svenning *et al*., 2005). Then it was changed to genus *Nostoc* on the base of RFLP analysis of 16S rRNA gene, and now this is questioned by Svenning *et al*. (2005) on the

base of the entire 16S rRNA gene sequence. But one of the most controversial cases in this complex is *Nostoc* strain PCC7120. First it was considered a species of *Nostoc*, [*N. muscorum* (Adolph and Haselkorn, 1971)], and then it was classified as an *Anabaena* species on the basis of the morphological characterization (Rippka *et al*., 1979). However, it was again assigned to the genus *Nostoc* based on DNA-DNA hybridization (Lachance, 1981), and hybridization pattern with repetitive (STRR) DNA sequences (Mazel *et al*., 1990). Recently, this position has been questioned by Tamas *et al*. (2000) based on a short fragment of *nifH* gene sequence analysis and 16S rRNA gene sequence analysis (Svenning *et al*., 2005). Moreover, this strain has taxonomic issues with the strain *Nostoc* PCC6719, both of which probably belong to the same species on the base of DNA-DNA reassociation (Lachance, 1981) and RFLP analysis of 16S rRNA gene (Lyra *et al*., 1997).

1.4.2 TAXONOMIC AND PHYLOGENETIC PROBLEMS WITHIN GENERA *ANABAENA, APHANIZOMENON* AND *NOSTOC*

The genera *Anabaena*, *Aphanizomenon* and *Nostoc* not only have taxonomic incongruencies among them, they also show identification and phylogenetic problems within each genus. Since the taxonomic position of several strains within the genera, even some species, has been discussed previously, these problems will be separated in gaps.

1.4.2.1 *Anabaena*-strain Gaps

In the genus *Anabaena* there are considerable difficulties separating species and strains. Numerous studies have shown that strains of the same species are dispersed within and among clusters formed by different species, and even different genera, with

high similarity, and then they could be members of the same species, or each cluster could be considered different genera (Fergusson and Saint, 2000; Gill, 2006; Gugger *et al*., 2002; Lyra *et al*., 2001; Rajaniemi *et al*., 2005; Willame *et al*., 2006). For example, Rajaniemi *et al*. (2005), in a phylogenetic analysis based on 16S rRNA gene sequence analysis, observed that different strains of *Anabaena planktonica* and *Anabaena spiroides* among other *Anabaena* species were dispersed and intermixed within a well-supported cluster. Actually, this cluster was unified and differentiated by using some morphological characteristics, like size of heterocysts and akinetes. Moreover, they observed that different *Anabaena flos-aquae* and *Anabaena lemmermannii* strains were dispersed among two high-similarity clusters; however these clusters were not supported by the morphological characterization. The same problem was observed by Willame *et al*. (2006), in which *A. planktonica* and *A. spiroides* strains were not monophyletic but were grouped together in a well-supported cluster. Moreover, two strains of *A. cylindrica* resulted in completely different clusters; these clusters are differentiated by the presence or absence of gas vesicles. Indeed, Willame *et al*. (2006) proposed that the studied *Anabaena* strains without gas vesicles should be a different genus. Other cases are given by Gill (2006), who could not discriminate between one strain of *A. spiroides* and one strain of *A. compacta*, since they have 100% of similarity in the 16S rRNA gene. Moreover, some strains of *Anabaena oscillarioides*, *A. spiroides* and *Anabaena viguieri* resulted dispersed in an *Aphanizomenon-Anabaena* cluster, intermixed with strains of other species, so they did not form a well supported cluster.

1.4.2.2 *Aphanizomenon*-strain Gaps

The genus *Aphanizomenon* is primarily considered a polyphyletic group, since its representatives appear to be intermixed within the 16S rRNA gene phylogenetic analyses. For example, Rajaniemi *et al*. (2005), based on 16S rRNA gene sequence analysis, observed that *Aphanizomenon flos-aquae* clustered separately from the proposed type strain (*A. flos-aquae* PCC 7905). The same non-monophyly in the phylogenetic tree was observed in Gugger *et al*. (2002) for this species and *Aphanizomenon gracile* on the base of ITS sequence analysis. Moreover, on the base of 16S rRNA and phenotypic characterization they concluded that *A. gracile* and *A. flos-aquae* may form only one species (Gugger *et al*., 2002).

1.4.2.3 *Nostoc*-strain Gaps

The Genus *Nostoc* appears to have less taxonomic and phylogenetic problems than the genera *Anabaena* and *Aphanizomenon*. Different molecular markers depict *Nostoc* to form a monophyletic group with high genetic diversity, in which each studied strain may represent individual species (Lachance, 1988; Rajaniemi *et al*., 2005; Rasmussen and Svenning, 2001; Wilmotte and Herdman, 2001). However, Rajaniemi *et al*. (2005) noted an exception to this in which the high similarities of the 16S rRNA sequence and morphological analyses suggested that *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 could be assigned to a single species. Moreover, *N. muscorum* and *N. ellipsosporum* were morphologically and genetically more closely related to each other that to the other *Nostoc* studied strains. Another example is given by Wilmotte and Herdman (2001), in which the *Nostoc* strains GSV 224, ATCC 53789, TDI#AR94,

PCC9709 and *N. punctiforme* PCC73102 can be considered to be members of a single species, whereas *Nostoc* PCC7120 is only distantly related to this group.

1.5 MORPHOLOGICAL IDENTIFICATION FOR TAXONOMIC AND PHYLOGENETIC ANALYSIS ON *ANABAENA*, *APHANIZOMENON* AND *NOSTOC* SPECIES

According to Rajaniemi *et al*. (2005), new phylogenetic studies in cyanobacteria should be carried out by combining morphological and genetic approaches. Although it is widely known that some morphological characters may change or may not be expressed in culture conditions (Lu *et al*., 1997; Svenning *et al*., 2005; Wright *et al*., 2001). For example, gas vesicles presence, colony form, akinetes and heterocysts differentiation, the hormogonia formation, and vegetative cell sizes (Gugger *et al*., 2002; Lehtimäki, 2000; Lu *et al*., 1997; Rippka *et al*., 2001; Svenning *et al*., 2005; Wright *et al*., 2001). For this reason, Rajaniemi *et al*. (2005) proposed the use of recent isolates from samples, and not the use of strains from culture collections. Moreover, there are few molecular studies combined with morphological characterization, so it is essential to conduct more research that relate these two approaches in order to improve the knowledge about which morphological character represents the molecular phylogeny, and in which conditions they should be studied, for example only mature akinetes should be measured and only mature filaments etc. (Willame *et al*., 2006).

The main features used in cyanobacterial morphological classification are: variation in cyanobacterial thallus structure, which include occurrence as unicells, colonies, unbranched filaments, or branched filaments (false and true branches); presence

or absence, form and position of specialized cells such as exospores, baeocytes (endospores), akinetes or heterocysts; presence or absence of mucilaginous sheath, sheath, hormogonium, separation disks or gas vesicles (necridia) (Graham and Wilcox, 2000), as well as life cycle (Rajaniemi *et al*., 2005). In fact, Rajaniemi *et al*. (2005) postulated that the most important characters for the morphological identification of heterocystous cyanobacteria are: the form of colony, shape of terminal cells, presence of sheath and gas vesicle, and life cycle. Hence, it is important to consider that heterocystous cyanobacteria have three possible ways of asexual reproduction: random trichome breaking, formation of hormogonia, and germination of akinetes.

Heterocysts and akinetes appear to have the most useful characteristics for identification of heterocystous cyanobacteria. Heterocysts are specialized cells related with the fixation of nitrogen under oxic conditions, formed by differentiation of a vegetative cell (0-5-10% of the vegetative cell in the filaments are differentiated) (Rippka *et al*., 2001). Their appearance is more granular than vegetative cells and their walls present additional layers (from the inside of the cell: a laminated, a homogeneous, and a fibrous layer) (Castenholz, 2001). The location of the heterocysts in the filaments can be regulated by the necessary interchange of nutrients between heterocystous and vegetative cells, ensuring the efficiency in the distribution of fixed nitrogen along the filaments (Adams and Duggan, 1999), and then they can be at the ends of the filaments or intercalary (Rippka *et al*., 2001). Akinetes, on the other hand, are resting cells in cyanobacteria, and are larger than vegetative cells, have a thicker cell wall surrounding the old wall, and are generally yellowish to brownish (Castenholz, 2001; Meeks *et al*.,

2002). Their location in the filaments is frequently related to the position of heterocysts, either being adjacent to or distant from them (Castenholz, 2001).

1.5.1 *ANABAENA* AND *APHANIZOMENON* MORPHOLOGICAL DIFFERENTIATION

Problems using morphology to differentiate among species of *Anabaena* and *Aphanizomenon* are recurrent. Komárek and Kováčik (1989) postulated that the possible characters for distinguishing species of these two genera are: colonies in fascicle, structure of the trichome, terminal cells, and heterocysts development. But the formation of bundles in some way is an unreliable character as it is only present in some *Aphanizomenon* species (Gugger *et al*., 2002; Hindák, 2000), and it is lost under inappropiate culture conditions (Li *et al*., 2000). Moreover, the structure of the trichome, subsymmetric (lighly attenuated towards ends) in the genus *Aphanizomenon* and metameric (homologous cells lying in a longitudinal series) in the genus *Anabaena*, only can be seen in long filaments (Gugger *et al*., 2002; Hindák, 2000). Sometimes the number and location of heterocysts and akinetes is useful (Gugger *et al*., 2002), although this character can also change according to the environmental conditions (Hindák, 2000). The morphology of the terminal cells may be the most important difference between these two genera in which *Anabaena* has rounded to oval cells, and in *Aphanizomenon* they are elongated-hyaline (translucent or transparent). However, due to breakage and fragmentation these are not always clear, and also this depends on the age and nutrient status of the population (Kling – personal communication).

1.5.2 *ANABAENA* AND *NOSTOC* MORPHOLOGICAL DIFFERENTIATION

One of the primary issues in the morphological identification of *Nostoc* species is differentiating them from *Anabaena* species (Zapomělová, 2006). The primary feature

used to differentiate these two genera is the gelatinous colony formation in *Nostoc*; however this character is usually lost under culture conditions (Rippka *et al.,* 1979). Moreover, some *Anabaena* species present trichomes surrounded with a diffuse mucilaginous sheath (Rajaniemi *et al*., 2005). Rippka *et al*. (1979) suggested that the developmental cycle in these species may be a criterion to differentiate between these two genera. However, this is problematic as many *Nostoc* species in culture do not exhibit natural developmental cycles or even proceed through a developmental cycle (Caudales and Wells, 1992). In addition, according to De Philippis *et al*. (2000) hormogonia are rarely present in species maintained on culture condition. Another characteristic is the presence of motile trichomes in *Anabaena* which differs from *Nostoc*, where the motility is restricted to hormogonia (Rippka *et al.,* 1979), but again strains from culture collections do not always exhibit this characteristic gliding movement (De Philippis *et al*., 2000).

According to Dodds *et al*. (1995), *Nostoc* can be distinguished from *Anabaena* by the development of akinetes and heterocysts in which the akinete formation begins with the differentiation of one vegetative cell midway between two heterocysts, followed by centrifugal formation of the akinete. However, in several species (*N. caeruleum*, *N. commune*, *N. pruniforme* and *N. zetterstedtii)* akinete formation appears not to occur (Mollenhauer *et al*., 1999). With respect to heterocystous formation, the terminal heterocyst is formed by the terminal hormogonia cell after settling and cessation of movement (Dodds *et al*., 1995).

1.5.3 *ANABAENA* SPECIES MORPHOLOGICAL IDENTIFICATION

The usefulness of some characters over others for the species identification within genus *Anabaena* may change according to the authors. For example according to Hindák (2000), the primary characters used in the identification of *Anabaena* species are the position of akinetes, shape of terminal cells, and vegetative cell width. For Rajaniemi *et al*. (2005), the most important features are size and position of akinetes. Stulp and Stam (1984) added to this by noting the characters of heterocyst morphology and the position of akinetes with regard to the heterocysts.

Hiroki *et al*. (1998) developed a database system for the identification of *Anabaena* species based on 26 features. They concluded that trichome form (bundle or solitary) and shape (straight form, regularly coiled, circinate, or irregularly coiled), and akinete morphology (quantity, location, shape, sheath, color, diameter, and length) were the most useful characters. But some of these specific characteristics can be absent in culture. That is the case for example of the coiled trichome in *Anabaena spiroides*, and the akinete aggregation in the center of the colonies in *Anabaena lemmermannii* (Gugger *et al*., 2002). Moreover, some morphological characters may not be monophyletic when compared to molecular phylogenies. For example *Anabaena* isolates with straight, curved and coiled trichomes resulted intermixed in a well supported cluster (Willame *et al*., 2006).

1.5.4 *APHANIZOMENON* SPECIES MORPHOLOGICAL IDENTIFICATION

The morphology of the trichome is the first step for the identification of *Aphanizomenon* species. Komárek and Kováčik (1989) classified *Aphanizomenon* species in four groups:

1) Trichomes arranged in macroscopic bundles.
2) Trichomes curved or flexuous. The terminal cells are narrowed, elongated and hyaline, bluntly or sharply pointed. Akinetes are distant or close to heterocysts.
3) Subsymmetric trichomes with 1–3 heterocysts. The end cells are narrowed but not distinctly elongated or hyaline.
4) Solitary metameric trichomes clearly narrowing towards the ends.

Komárek and Kováčik (1989) concluded that for the differentiation at species level within genus *Aphanizomenon*, vegetative cells, end cells, akinetes and heterocysts sizes and their variation range are the most relevant characters. Additionally, Komárek and Komárková (2006) separated the *Aphanizomenon* species primary based on the tendency to form fascicles or bundles, and secondary according to the appereance of terminal cells and trichome symmetry.

1.5.5 *NOSTOC* SPECIES MORPHOLOGICAL IDENTIFICATION

McGuire (1984) in a study on the morphological classification of *Nostoc* species, using numerical taxonomy, concluded that several species of *Nostoc* could be distinguished on the basis of 30 morphological characteristics. He proposed that the most useful were the size and shape of akinetes, vegetative cells, and heterocysts, color and luster of plant mass, veined plant mass surface, margin fimbriate, and shape of plant mass in nature. Caudales and Wells (1992) talk about the "ambiguous concept of sheath surrounding the trichome", and concluded that this character is unreliable. According to De Philippis *et al*. (2000) this feature need more investigation. They studied 40 *Nostoc* strains from Pasteur Culture Collection, of which only 25 strains showed a significant

sheath or slime, and two more strains released polysaccharides to the media (these sheaths are formed by polysaccharides). Moreover, all hormogonia observed lacked these capsules but can sometimes be surrounded by a viscous slime (De Philippis *et al*., 2000; Mollenhauer *et al*., 1999). In addition, only in some species are hormogonia released from the vegetative trichome (Mollenhauer *et al*., 1999).

1.6 INDUCTION OF HETEROCYSTS AND AKINETES DIFFERENTIATION

As noted previously, heterocyst and akinete morphology are important characters in the identification of heterocystous cyanobacteria. Nevertheless, their differentiation depends on the culture conditions (Hindák, 2000), since in natural conditions these celltypes result from stressful environmental changes (Meeks *et al.,* 2002). Therefore, most of strains should be stressed for the induction of the cell differentiation, by changing some environmental factors. Nitrogen depletion (mainly ammonia) (Castenholz, 2001; Meeks *et al*., 2002 ; Rao *et al*., 1987) for heterocyst induction and phosphorous depletion (Dodds *et al*., 1995; Meeks *et al*., 2002; van Dok and Hart, 1996), iron depletion (Hori *et al*., 2002; 2003), lower temperature (10-15°C) (Li *et al*., 1997), and desiccation (Hori *et al*., 2003) for akinetes' induction are among the most successful used factors.

It is important to emphasize that the differentiation of akinetes and heterocysts can be lost by genetic mutation in strains maintained for a long time under laboratory conditions. Moreover, in *Nostoc* the heterocyst formation may depend on the host (Meeks *et al*., 2002).

1.7 MOLECULAR IDENTIFICATION FOR TAXONOMIC AND PHYLOGENETIC ANALYSIS ON *ANABAENA*, *APHANIZOMENON* AND *NOSTOC* SPECIES

1.7.1 SEQUENCE ANALYSIS OF 16S rRNA GENE

The 16S rRNA gene is one of the three genes that form the ribosomal RNA (rRNA) operons in bacteria (Iteman *et al*., 2000) and is approximately 1400 bp long (Casamatta *et al*., 2005). The analysis of this gene has been successfully used in the taxonomic and phylogenetic analysis of cyanobacteria by Giovanonni *et al*. (1988); Lyra *et al*. (1997); Nelissen *et al*. (1996); Nübel *et al*. (1997); Lehtimäki *et al*. (2000); Lyra *et al*. (2001); Litvaitis (2002); Casamatta *et al*. (2005); Rajaniemi *et al*. (2005), and Willame *et al*. (2006). In addition, it is the basis for defining taxonomy groups in the second edition of Bergey's Manual of Systematic Bacteriology (Wilmotte and Herdman, 2001) and appears to be the most promising approach to the phylogenetic classification of cyanobacteria (Nübel *et al*., 1997), due to its highly conservative nature and its universal distribution (Iteman *et al*., 2002). On the other hand, Janson *et al*. (1999) and Iteman *et al*. (2002) have questioned whether sufficient variability exists in 16S rRNA to allow discrimination among species of a genus or strains of a species. For example, as we can see above sometimes it does not clearly differentiate *Nostoc* and *Anabaena* strains (Giovannoni *et al*., 1988; Lyra *et al*., 2001). Even Nübel *et al*. (1997) concluded that it would be necessary to use complete 16S rRNA gene sequences for reliable phylogeny reconstruction.

1.7.2 SEQUENCE ANALYSIS OF *EFP* GENE OF PROTEIN ELONGATION FACTOR P (EF-P)

The three most important systems of cellular information processing (replication, transcription, and translation) are characterized by their universally conserved machinery. The translation process is universal in distribution and has the most conserved components such as RNAs, tRNAs, protein elongation factors and some ribosomal proteins (Kyrpides and Woese, 1998). This process of the protein biosynthesis is initiated by the specific alignment between peptdyl-tRNA and animoacyl t-RNA; following by the peptide bond formation, and translocation of mRNA (Glick and Ganoza, 1975). Each process is mediated by one elongation factor: EF-T (Tu,Ts), EF-P and EF-G respectively (Aoki *et al*., 1997; Glick and Ganoza, 1975; Joe and Park, 1994). Ganoza *et al*. (2002) suggested that EF-P might act particularly like a regulatory molecule for peptide bond formation during protein synthesis, since it promotes the interaction between peptidyl transferase and its aminoacyl-tRNA substrates in conjunction with the 70S ribosome's peptidyl transferase. Moreover, EF-P enhances the synthesis of certain dipeptides initiated by *N*-formylmethionine (Aoki *et al*., 1997).

The gene encoding the EF-P protein, *efp* gene, has been observed to be present in all but two bacterial genomes currently available on GenBank (Lau *et al*., 2008). The two not containing this gene were bacterial parasites and have generally reduced genomes. In addition, EF-P appears to be homologous to archeal initiation factor (aIF-5A) and the eukaryotic initiation factor (eIF-5A) (Aoki *et al*., 1997; Joe and Park, 1994) despite low sequence similarity (Kyrpides and Woese, 1998). EF-P lacks of the most conserved region of the eIF-5A sequence, hypusine, and presents just a residue of lysine, but EF-P has C-terminal section that is highly conserved.

There are no phylogenetic studies based on either *efp* gene or EF-P protein. However, genes for the elongation factors Tu (EF-Tu), and G (EF-G) have been used in studies of universal phylogeny (Baldauf *et al*., 1996). Baldauf *et al*. (1996) concluded that these genes highly support the root of the universal tree between Eubacteria and Archaea/Eucarya. The utility of this gene is confirmed by Lau (2006, M.Sc. Thesis Proposal), who compared phylogenetic trees obtained from *efp* and 16S rRNA sequences of 228 bacteria genomes from NCBI's GenBank. In addition, it appears that there is enough variability in the nucleotide sequences to address taxonomic issues within cyanobacterial species and in turn the protein sequences can be used to address more distant relationships.

1.8 THESIS OBJECTIVES

1. Determine taxonomic and phylogenetic relationships between selected and isolated filamentous cyanobacterial strains from genera *Anabaena*, *Aphanizomenon* and *Nostoc*, by 16S rRNA and bacterial elongation factor P (*efp*) gene sequences. Compare them with available phylogenies in order to solve the gaps among these three genera.

Difficulties in the cyanobacterial classification have been revealed by the phylogenetic studies based on 16S rRNA gene. 16S rRNA gene analysis is the most utilized technique in this group. Therefore, the use of this marker is proposed in this study of the cyanobacterial taxonomy and systematic. However, it is sometimes still not possible to differentiate clearly between closely related strains based on the 16S rRNA

sequences. In this case the use of bacterial elongation factor P (*efp*) gene will be assessed to determine the feasibility for a cyanobacterial phylogenetic marker.

2. Evaluate the use of *efp* gene sequence analysis for distinguishing between closely related strains

There is no data related to this molecular marker on filamentous cyanobacteria in GenBank. We can infer the *efp* sequences only from the species which have had the complete genome sequenced. So, it is necessary to improve this information for a complete phylogenetic analysis; since the application of multiple taxonomic criteria is required for the successful delineation of cyanobacterial species.

3. Detail morphological features of the studied strains in order to compare and compliment the obtained taxonomic and phylogenetic relationships on the base of molecular analysis.

Among the few studies on filamentous cyanobacteria that relate the morphological and molecular analysis it has been recurrently observed that those are not congruent. This has prompted this study to relate both parameters until it is possible to determine which morphological characters are the most important for a clear identification, which molecular marker get a better phylogenetic relationship, and in that way to discover which cyanobacterial group really forms a monophyletic group.

4. Apply phylogenetic and taxonomic analyses from objectives 1, 2 and 3 to obtaining the most accurate identification of new species isolated from Canadian, Chilean and African Lakes.

New *Anabaena*, *Aphanizomenon* and *Nostoc* isolates will be obtained from samples of lakes Ontario (Canada), Malawi (Malawi), and Rupanco (Chile), with the purpose of adding new specimens to the taxonomic and phylogenetic study on cyanobacteria. In this way, the molecular and morphological data of these three genera will be increased, and their taxonomic issues will have more support for being solved.

CHAPTER 2: METHODS

2.1 CYANOBACTERIAL ISOLATES

Fifteen *Anabaena* isolates, three *Aphanizomenon* isolates, and thirteen *Nostoc* isolates (Table 2.1) from Dr. Susan Watson (Environment Canada, University of Calgary), Dr. Friedrich Jüttner (University of Zurich), Mr. George Izaguirre (Metropolitan District of Southern California), Dr. Sarah A. Spaulding (Institute of Arctic and Alpine Research, University of Colorado) and two culture collections: University of Toronto Culture Collection (UTCC) and the University of Texas Culture Collection (UTEX) were amongst the studied cyanobacteria.

2.2 ISOLATION AND CULTURE CONDITIONS

Two isolates from genus *Anabaena* and one from genus *Nostoc* were obtained from water samples of Lake Ontario (Bay of Quinte), Canada; Lake Rupanco, Chile, and Lake Malawi, Malawi (Table 2.1). These strains were isolated by enrichment cultures, mainly as a preliminary step, and single-cell isolation by micropipette (Andersen and Kawashi, 2005). The culture medium used in the isolation process was 50% Cyano Medium (Jüttner *et al*., 1983) and 50% sterilized and filtered water from the corresponding lake. When the culture showed growth the media was changed to 100% Cyano Medium.

All the isolates were maintained in sterile Erlenmeyer flasks (150 ml) containing approximately 50 ml of culture. Chu-10 (Stein, 1973) and Cyano media were used. The

Table 2.1 **Cyanobacterial isolates of genera *Anabaena*, *Aphanizomenon* and *Nostoc* used in the morphometric and 16S rRNA and *efp* gene sequence analyses.**

Taxon	Strain	Origin	Source Collection
Anabaena reniformis	MALW1	Lake Malawi, Africa	Dr. Kirsten Müller (Biology Department, University of Waterloo)
Anabaena compacta		Unknown	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena cf. *cylindrica*		Liverpool, England	Prof. N.G.Carr c/o Dr Friedrich Jüttner (University of Zurich)
Anabaena cf. *flos-aquae*	UTCC64	Western Lake Ontario, Canada	University of Toronto Culture Collection c/o Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena cf. *flos-aquae*	UTEX2383	Burton Lake, Ontario, Canada	University of Texas Culture Collection c/o Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena lemmermannii	GIOL8	California, USA	Mr. George Izaguirre (Metropolitain District of Southern California)
Anabaena lemmermannii	LONT2	Western Lake Ontario, Canada	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena sp.	LONT5	Western Lake Ontario, Canada	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena lemmermannii	ONT1	Bay of Quinte, Lake Ontario, Canada	Dr. Kirsten Müller (Biology Department, University of Waterloo)
Anabaena cf. *oscillariodes*		Unknown	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena cf. *planktonica*		Unknown	Dr. Friedrich Jüttner (University of Zurich)
Anabaena cf. *lemmermannii*		Unknown	Dr. Friedrich Jüttner (University of Zurich)
Anabaena sp.	A2879	Unknown	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena sp.	LOW1	Lake of the Woods, Ontario	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena sp.	7812	Unknown	Dr. Susan Watson (Environment Canada, University of Calgary)
Anabaena variabilis		Unknown	Dr. Susan Watson (Environment Canada, University of Calgary)

Taxon	Strain	Origin	Source Collection
Anabaena cf. *viguieri*		Unknown	Dr. Friedrich Jüttner (University of Zurich)
Aphanizomenon klebahnii	HHAFA	Hamilton, Harbour, Lake Ontario, Canada	Dr. Susan Watson (Environment Canada, University of Calgary)
Aphanizomenon flos-aquae	UTEXLB2384	Warburg, Alberta, Canada	University of Texas Culture Collection
Aphanizomenon cf. *gracile*		Unknown	Dr. Friedrich Jüttner (University of Zurich)
Nostoc calcicola	UTEXB382	Utrecht, Netherlands	University of Texas Culture Collection
Nostoc commune	UTCC74	Scotland	University of Toronto Culture Collection
Nostoc ellipsosporum	UTEX383	Utrecht, Netherlands	University of Texas Culture Collection
Nostoc punctiforme	UTCC41	Utrecht, Netherlands	University of Toronto Culture Collection
Nostoc sp.	RUP1	Lake Rupanco, Chile	Dr. Kirsten Müller (Biology Department, University of Waterloo)
Nostoc sp.	UTCC106	Moderate hot spring, Amparai District, Maha Oya, Sri Lanka	University of Toronto Culture Collection
Nostoc sp.	UTCC314	Sand dune, Presqu'ile, Ontario, Canada	University of Toronto Culture Collection
Nostoc sp.	UTCC355	Unknown	University of Toronto Culture Collection
Nostoc sp.	UTCC387	Unknown	University of Toronto Culture Collection
Nostoc sp.	D1	Taylor Valley, Southern Victoria Land, Lake Frytell, Antarctica	Dr. Sarah A. Spaulding (Institute of Arctic and Alpine Research, University of Colorado)
Nostoc sp.	D2	Taylor Valley, Southern Victoria Land, Lake Frytell, Antarctica	Dr. Sarah A. Spaulding (Institute of Arctic and Alpine Research, University of Colorado)
Nostoc sp.	NWT 150.1	Frozen axel heiberg	Unknown
Nostoc commune	NWT 208.5	Unknown	Unknown
Nostoc verrucosum	CR25	Unknown	Unknown

culture conditions were given by an incubator (23°C, 16:8 h light:dark cycle, irradiance 25 μmol · m^{-2} · s^{-1}).

All the steps of the isolation process and cultures' maintenance were performed in a laminar flow hood using aseptic technique and sterile equipment.

2.3 MORPHOLOGICAL ANALYSIS

Cyanobacterial isolates were identified and photographically documented using an Olympus BX51 light microscope (Olympus Optical Co., LTD). Cell measurements were calculated using the Spot Advanced© version 3.4.5 imaging program for Windows (Diagnostic Instruments, 1997-2002).

For the morphological identification 30 filaments per isolate were described and measured. Per filament at most 3 vegetative cells, 2 end cells, 3 akinetes (when observed) and 3 heterocysts (when observed) were measured and described, then the measurements were averaged. The Table 2.2 summarizes the chosen morphological attributes and characters used in the identification and phylogenetic analysis of the studied isolates. The presence of sheath and mucilaginous sheath were determined by the use of India ink.

2.4 INDUCTION OF AKINETES AND HETEROCYSTS DIFFERENTIATION

Heterocysts and akinetes morphometry are important characters in the identification of heterocystous cyanobacteria. Nevertheless, their differentiation depends on the culture conditions (Hindák, 2000); since in natural conditions these cell types result from stressing environmental changes (Meeks *et al*., 2002). Therefore, most of the

Table 2.2 Morphological attributes and characters used in the identification and phylogenetic analysis of studied isolates (modified from Hiroki *et al*., 1998).

Attributes	**Atribute's characters**
Trichome form	Bundle, solitary
Trichome form	Straight form, slightly curve, regularly coiled, circinate, irregularly coiled
Mucilaginous sheath	Presence, absence, not clear
Sheath	Presence, absence, in vegetative, akinetes and/or heterocysts
Gas vacuole	Presence, absence
Coil diameter (μm)	
Coil distance (μm)	
Cell shape	Spherical, barrel-shaped, short barrel-shaped, cylindrical, ellipsoidal, short ellipsoidal, quadrate, squared-off corners, bent-shaped
Cell width (μm)	
Cell length (μm)	
Apical cell shape	Rounded, conical, obtuse conical, tapered
Apical cell width (μm)	
Apical cell length (μm)	
Heterocyst shape	Spherical, subspherical, cylindrical, barrel-shaped, ellipsoidal, oval, oblong,
Heterocyst width (μm)	
Heterocyst length (μm)	
Akinete rows	Presence or absence
Akinete location	Adjacent to one side of heterocyst, adjacent to both sides of heterocyst, far from heterocyst, rarely far from heterocyst, rarely adjacent to heterocyst, irregularly located
Akinete shape	Lemon-shaped, barrel-shaped, ellipsoidal, subspherical, oval, spherical, oblong, bent-shaped, slightly constrict-shaped at the middle
Akinete width (μm)	
Akinete length (μm)	

Table 2.3 Environmental factors successfully used for the induction of the heterocysts and akinete differentiation.

Cellular type	Environmental factor	Reference
Heterocysts	Nitrogen depletion (mainly NO_3^-)	Rao *et al*. (1987); Castenholz (2001); Meeks *et al*. (2002)
Akinetes	Phosphorous depletion	Dodds *et al*. (1995); van Dok and Hart (1996); Meeks *et al*. (2002)
	Iron depletion	Hori *et al*. (2002; 2003)
	Lower temperature (10-15°C)	Li *et al*. (1997)
	Desiccation	Hori *et al*. (2003)

time the strains were stressed for the induction of the cell differentiation, by change some environmental factors. Table 2.3 summarizes the successful factors for each cellular type. In general, BG-11 Media (Rippka *et al*., 1979) was modified with this purpose. Four modifications were tested: BG-11 medium with no source of nitrogen, BG-11 without any source of phosphorous, BG-11 with no source of nitrogen and phosphorous; and BG-11 without any source of iron.

2.5 DNA EXTRACTION

DNA from cell cultures of cyanobacterial isolates were extracted by transferring 250-1000 µl of each culture into a sterile 1.5 ml microfuge tube and centrifuged at 8000 rpm for 2 min to pellet the cells. The cultures used should be healthy (assessed by buoyancy, colour, and abundance) (Gill, 2006). For cell lysis, the supernatant was removed and the pellet cells were freeze-thawed three times by immersing each tube in liquid nitrogen for 20 s immediately following by immersion in a 78°C water bath for 20s. The protocol given by Saunders (1993), with an RNAse step included, and Wizard Genomic DNA Purification kit (Promega, U.S.A.) were used for the DNA isolation, and DNA was eluted in DNA/RNA free water.

2.6 PCR AMPLIFICATION OF THE 16S RRNA GENE

The 16S rRNA gene (approximately 1300 bp) was PCR amplified. A volume of 2 µl of DNA, 2.5 µl 10x taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 (at 25°C), 1.0% Triton® X-100, and 15 mM $MgCl_2$) (Promega, Canada), 0.2 µl of

homemade Taq DNA polymerase, 200 µM each dNTP (Promega, Canada), 0.5 mM of each primer in a total reaction volume of 25 µl. The used primers were CYA108F (5'-ACGGGTGAGTAACRCGTRA-3') and 16SCYR (5'-CTTCAYGYAGGCGAGTTG CAGC-3') (Hotto *et al*., 2005). A 40 cycle touchdown procedure was followed using the Eppendorf Mastercycler® Gradient 5331 (Eppendorf, USA). The general conditions in the PCR amplification consisted in a denaturation step at 95°C for 1 min 30 s, followed by 40 cycle performed at 95°C for 30 s, 56°C for 1 min, and 65°C for 30 s; and a final extension step at 72°C for 10 min. PCR products were electrophoresed through a 1% agarose gel in 1X TBE buffer at 125 V for 30 min. Products were visualized using the Syngene Bioimaging System (Synoptics Ltd., United Kingdom). PCR product size and concentrations were estimated using a DNA marker (ΦX174 DNA digested with *Hae* III restriction enzyme) that was electrophoresed along side the PCR products.

2.7 SEQUENCING OF THE 16S RRNA GENE

The PCR products were purified using Ultra Clean™ Kit (Mo Bio Laboratories, Ltd.; C. A., U.S.A.), and eluted in 50 µl of biotech grade water (Fisher, Canada). The concentration was estimated used a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., U.S.A.), and when it was necessary DNA was concentrated in a DNA Speed Vacuum Concentrator (Savant Instruments, U.S.A.). DNA concentrations of approximately 50 ng/µL were used for the cloning procedure, which was carry out in order to obtain the nearly completed 16S rRNA gene sequence. The fragments were inserted in pGEM®-T Easy Vector System I (Promega, U.S.A.). 10µL of

ligation reaction consisted in 3 µL of purified DNA, 1 µL pGEM®-T Easy Vector, 5 µL 2X ligation buffer, and 1 µL T4 DNA ligase. This reaction was incubated overnight at 4°C on ice. Then, the transformation of bacteria (*Escherichia coli*) was carried out. 3-10 µL of ligate mix were carefully mixed with 50 µL of competent cells (always the component cell were maintained on ice) and left on ice for 20 min. Then the cells were heat shocked, 45 sec at 42°C and 2 min on ice, and cultured on 950 µL of SOC medium for 90 min, at 37°C and 200 rpm. After that the cells were centrifuge for 10 min at 3300 rpm and plated onto LB medium with ampicillin, X-Gal (50 mg/mL) and IPTG (0.1M). That was maintained at 37°C overnight and store in fridge. Just the white colonies were picked up and grew in 5 ml of LB medium with ampicillin at 37°C. The plasmids DNA were isolated from recombinant *E. coli* with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich Co., U.S.A.). Approximately 100 ng/µL of products were sequenced at the University of Waterloo molecular core facility using the Applied Biosystems 3130XL Genetic Analyzer; the primers used were T7 and Sp6. Sequence reaction products were visualized using Bioedit 7.0.4 sequence alignment editor program (Hall, 1999) (www.mbio.ncsu.edu), aligned using MUSCLE program (Edgar, 2004), similarity to sequences deposited in the GenBank databases were verified by using the program BLAST (Altschul *et al*., 1990) (www.ncbi.nlm.nih.gov/BLAST/).

2.8 PCR AMPLIFICATION OF THE *EFP* GENE

The *efp* gene (approximately 400 bp) was PCR amplified. A volume of 2 µl of DNA, 2.5 µl 10x taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 (at 25°C), 1.0% Triton® X-100, and 15 mM $MgCl_2$) (Promega, Canada), 0.2 µl of homemade

Taq DNA polymerase, 200 µM each dNTP (Promega, Canada), 0.5 mM of each primer in a total reaction volume of 25 µl. The primers were designed by comparative sequence alignment with available GenBank (www.ncbi.nih.gov) sequences, using BioEdit 7.0.4 (Hall, 1999) (www.mbio.ncsu.edu) and MUSCLE 3.6 (Edgar, 2004) programs. The primers were: EFP-F1 (5'-CGACCTGGTGTATCCATTGTC-3'), EFP-R1 (5'-GGAACCATCACAGTTGCACCAG-3'), EFP-F2 (5'-ATGATYTCHAGTAACGAYTTYCG -3') and EFP-R2 (5'-GTRTCDCCYTTRACDCCWGGATC-3'). And three sets were used EFP-F1 and EFP-R1 for *Anabaena* and *Nostoc* species; EFP-F2 and EFP-R2, and EFP-F2 and EFP-R1 for *Aphanizomenon* species. A 30 cycle touchdown procedure was followed using the Eppendorf Mastercycler® Gradient 5331 (Eppendorf, USA). The program (modified from Casamatta *et al.* (2003)) used in the amplification consisted of an initial denaturation step at 95°C for 1 min 30 s, followed by 30 cycles performed at 93°C for 1 min, 55°C for 1min, and 72°C for 1 min 10 s. A final extension step at 72°C for 10 min completed the amplification program. The PCR products were run on a 1% agarose gel in 1X TBE buffer at 125 V for 30 min. Products were visualized using the Syngene Bioimaging System (Synoptics Ltd., United Kingdom). PCR product size and concentrations were estimated using a DNA marker (ΦX174 DNA digested with *Hae* III restriction enzyme) that was electrophoresed along side the PCR products.

2.9 SEQUENCING OF THE *EFP* GENE

The PCR products were purified using Ultra Clean™ Kit (Mo Bio Laboratories, Ltd.; C. A., U. S. A.), and eluted in 50 µl of biotech grade water (Fisher, Canada). Approximately 10-30 ng/µL of products were sequenced at the University of Waterloo molecular core facility using the Applied Biosystems 3130XL Genetic Analyzer. Sequence reaction products were visualized using Bioedit 7.0.4 sequence alignment editor program (Hall, 1999) (www.mbio.ncsu.edu), aligned using MUSCLE program (Edgar, 2004), similarity to sequences deposited in the GenBank databases were verified by using the program BLAST (Altschul *et al*., 1990) (http://www.ncbi.nlm.nih.gov/BLAST/).

2.10 PHYLOGENETIC ANALYSES

For each of the 16S rRNA and *efp* genes a sequence alignment was generated along with sequences of *Anabaena*, *Aphanizomenon* and *Nostoc* obtained from GenBank (www.ncbi.nih.gov). Sequences from genera *Anabaena*, *Aphanizomenon* and *Nostoc* were chosen for comparison (*Anabaena variabilis* ATCC29413, *Aphanizomenon* sp. PCC7905 and *Nostoc* sp. PCC7120), and one sequence from Subsection I (Chroococcales) was used as an outgroup in analyses (*Synechosystis* sp. PCC6803). All the sequences were aligned using the program Muscle v.3.6 (Edgar, 2004) and subsequently manually edited using BioEdit v.7.0.4 (www.mbio.ncsu.edu).

The nucleotide model of evolution used in the analyses was determined by the AIC criterion as implemented in Modeltest v.3.7 (Posada and Crandall, 1998).

The neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses were carried out using the program PAUP* v.4.0b.10 (Swofford, 2003). Maximum likelihood (ML) analysis was carried out with 10 replicates of a heuristic random addition of sequences and the TBR branch swapping algorithm. To assess confidence in tree topologies a bootstrap resampling (1000 replicates) was performed for both NJ and MP.

Bayesian analysis was also performed on each of the 16S rRNA and *efp* gene sequence datasets using MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian posterior probability support for clades was calculated using the GTR model with a gamma distributed rate variation across sites and initiated with default prior parameters. Two parallel analyses of six simultaneous chains of which five were heated (Metropolis-coupled Markov chain Monte Carlo) were run for 1,000,000 generations after convergence below a standard deviation of 0.05 between the two runs. Trees were sampled every 100 generations and trees before convergence of the two independent runs were discarded.

2.11 STATISTICAL ANALYSES OF MORPHOLOGICAL DATA

The statistical analysis of morphometric data was based only on the twenty isolates that developed heterocysts and akinetes as these have been previously considered to be diacritical characters for identification to the species and strain level (Komárek and Zapomělová, 2007; Rajaniemi *et al*., 2005; Willame *et al*., 2006). Thirty filaments of each of the twenty isolates were measured for width and length of vegetative cells, end cells, heterocysts and akinetes. These measurements were then used to further evaluate

the usefulness of these characters for taxonomic delineation through non-metric multidimensional scaling (NMDS) (Kruskal and Wish, 1978 *fide in* Ludwig and Reynolds, 1988). The NMSD is an ordination procedure, which gives a graphic representation of similarities or distance among items, using the least amount of dimensions in the space as is possible. This ordination looks for minimizing the stress, which is a measure of the mismatch between the two kinds of distance (Ludwig and Reynolds, 1988). Hence, the distance between two items in NMDS represents the metric relationship between them. NMDS analysis was assessed by using the analysis of similarities (ANOSIM; Clarke, 1993) function within the Primer 5 software package. ANOSIM contrasts the differences among replicates within each isolate with the differences among replicates of other isolates. So, ANOSIM generates a statistic *R*, which is based on the difference of mean ranks between groups (r_B) and within groups (r_W): $R = (r_B - r_W)/(N(N-1)/4)$. *R* is scaled to lie between -1 and +l, a value of zero representing the null hypothesis. Usually values between 0 and 1 are obtained. $R<0$ values are considered unlikely, indicating a greater dissimilarity among replicates within an isolate than occurs between isolates. $R>0$ values indicate that the isolates showed significant differences (the greater similarity among replicate within isolates than occurs between replicates of other isolates). So, values closer to 0 represent that two isolates are more related on the base of the metric analysis than two isolates which present values closer to 1, although both relationship are significantly differentiated.

A NMDS analysis was also performed on the qualitative data. A presence/absence (1/0) matrix was generated based of the shape of vegetative cells, end cells, heterocysts and akinetes, position of akinetes in relation to the heterocyst and if the akinetes were

observed in rows (placed next to each other in a straight line). The NMDS distance represent the parameter's variability between two isolates, so the major distance represents that any particular morphological parameter is not shared by two replicates.

Additionally, a NMDS analysis was carried out to evaluate the significance of each morphological features and distinguishing which was the most variable character and useful for species and strain delineation. This NMDS was performed in the same way that in the metric analysis. In this case the major distance between two parameters represents that the measurements between them which were more differenced that the measurement between two closer related parameters. Then, the NMDS plot obtained was supported by computation of 95% confidence intervals based on the mean of the measurements for each character. The 95% confidence interval indicates that the mean of the samples will estimate the mean of the population. These confidence intervals were calculated for each sample based on the metric values obtained for each cellular type, and this interval should therefore contain the population mean 95% of the time. The program STATISTICA 6.0 (StatSoft, Inc., 2001) was used to calculate the 95% confidence intervals. In addition, the Kruskal-Wallis test and Dunn's procedure was used as the data set did not exhibit a normal distribution and these non-parametric analyses do not make assumptions about normality (Maxwell and Delaney, 2004). The Kruskal-Wallis test was used to determine the significant difference among the isolates in relation to the different measurement of each cellular type; this indicates that the mean ranks of samples from the populations should be different (alternative hypothesis). This alternative hypothesis is represented by a p value less than 0.05, and a high value of the test statistic H. A Multiple Comparison (Dunn's procedure) was then applied to determine significant difference in

the mean ranks among isolates. For computing Kruskal-Wallis test and Dunn's procedure the program Microsoft® Office Excel 2003 (Stat Macros) for Windows was used.

CHAPTER 3: RESULTS

3.1 ISOLATES DESCRIPTIONS AND IDENTIFICATION

For identification purposes, measurements and morphologies of vegetative cells, end cells, akinetes and heterocysts were compared to the most recent descriptions given by Komárek (2005); Komárek and Komárková (2006), Komárek and Kováćik (1989), Komárek and Zapomělová (2007), Li *et al*. (2002), Pereira *et al*. (2005), Sant'Anna *et al*. (2007), Rajaniemi *et al*. (2005), Stulp and Stam (1984), Hindák (2000) and Willame *et al*. (2006). The characteristics of the studied attributes are shown in Table 3.1.

3.1.1 DESCRIPTIONS OF ISOLATES FROM CULTURE COLLECTIONS

***Anabaena compacta* (Nygaard) Hickel** (Figure 3.1.a)

Solitary trichomes, curved (not coiled) and sometimes straight, clearly constricted at cross walls and not attenuated towards ends. Vegetative cells are spherical, sometimes sub-spherical, 5.03 (4.16-6.02) µm wide and 4.66 (3.37-5.87) µm in length, brownish-blue-green, usually with solitary dark brown granules, a few visible spherical gas vesicles near the cell border. End cells are very similar to vegetative cells, 4.85 (3.65-5.88) µm wide and 4.6 (3.31-5.74) µm in length, rounded at the ends. Heterocysts are spherical, sometimes sub-spherical, 5.33 (4.29-6.54) µm in diameter and 5.03 (3.69-6.23) µm in length, in intercalary and terminal positions, with evident colorless pores and homogeneous yellowish-brown content. Akinetes are spherical, 9.59 (6.73-11.38) µm wide and 9.61 (6.99-12.19) µm in length, solitary, distant from heterocysts, in intercalary and terminal positions within the filament, more orange than vegetative cells.

Table 3.1 Morphological attributes and characters of studied isolates.

Ak=akinete, het=heterocysts, NO=not observed.

Attribute / Isolate	Trichome form	Trichome shape	Mucilaginous sheath	Gas vesicle	Cell shape	Apical cell shape	Heterocyst shape	Akinete rows	Akinete location	Akinete shape
An. compacta	solitary	Straight to slightly curve	absence	Presence	spherical	rounded	spherical	absence	Far from hets	spherical
An. cf. *cylindrica*	solitary	straight	absence	Absence	Barrel-shaped	Rounded (also obtuse conical to conical)	Barrel-shaped to cylindrical	absence	Adjacent to both sides of hets	Cylindrical to barrel-shaped
An. cf. *flos-aquae* UTCC64	solitary and in bundles	Straight to circinate	absence	Presence (in few cells)	Ellipsoidal to cylindrical	rounded	spherical to ellipsoidal	NO	NO	NO
An. cf. *flos-aquae* UTEX 2383	solitary and in bundles	Straight to circinate	absence	Presence (in few cells)	Ellipsoidal to cylindrical	rounded	spherical	NO	NO	NO
An. lemmermannii GI0L8	solitary	straight to slightly curve	absence	Presence	Cylindrical to barrel-shaped	rounded	Ellipsoidal to spherical	absence	Adjacent to both sides of hets	Ellipsoidal to long ellipsoidal
An. lemmermannii LONT2	solitary	straight	absence	presence (in some cells)	Barrel to cylindrical	rounded	Spherical (few ellipsoidal)	absence	Adjacent to both sides of hets	long ellipsoidal
An. cf. *oscillariodes*	Solitary (old cultures in mats)	Straight to circinate	absence	Absence	cylindrical	rounded	NO	NO	NO	NO
An. cf. *planktonica*	solitary	straight	absence	Presence	Cylindrical to barrel-shaped	rounded	ellipsoidal	absence	Far from hets	ellipsoidal
An. cf. *lemmermannii*	solitary	straight to slightly curve	absence	Presence	Barrel-shaped to ellipsoidal	rounded	Ellipsoidal to spherical	absence	adjacent to one side of hets	ellipsoidal
Anabaena sp A2879	Solitary and bundles	Straight to curve	absence	Absence	Barrel-shaped	Conical or rounded	Barrel-shaped	NO	NO	NO
Anabaena sp. LONT5	solitary	straight	absence	Absence	Barrel-shaped	conical	Spherical to subspherical	NO	Ellipsoidal	NO
Anabaena sp. LOW1	bundles, few solitariy	Irregularly coiled to slightly curve	absence	Presence	cylindrical	Rounded to tapered	Ellipsoidal	absence	Adjacent to both sides of hets	Cylindrical to kidney-shaped
Anabaena sp. 7812	Solitary	straight to circinate	absence	Presence (in few cells)	Spherical to sub-spherical	rounded	NO	NO	NO	NO
An. variabilis	bundle	straight	absence	Absence	Cylindrical to barrel-shaped	Rounded, few conical	Ellipsoidal to barrel-shaped	NO	Far from hets	Ellisoidal to long ellipsoidal
An. cf. *vigueri*	solitary (few in bundles)	straight	absence	Absence	Barrel-shaped to sub-spherical	Rounded, few conical	spherical	NO	NO	NO
***Aph. flos-aquae* UTEXLB2384**	bundle	straight	absence	Presence	cylindrical, squared-off corner	tapered	NO	NO	NO	NO

Attribute / Isolate	Trichome form	Trichome shape	Mucilaginous sheath	Gas vesicle	Cell shape	Apical cell shape	Heterocyst shape	Akinete rows	Akinete location	Akinete shape
Aph.* cf. *gracile	bundle	straight	absence	Presence	cylindrical	Rounded to tapered	Cylindrical to ellipsoidal	NO	Far from hets	Cylindrical to elipsoidal
***Aph. klebahnii* HHAFA**	bundle	straight	absence	Presence	cylindrical, squared-off corned	tapered	Ellipsoidal to barrel shaped	absence	adjacent to one side of hets	cylindrical
***N. calcicola* UTEXB382**	bundle and solitary	straight to amorphous	presence	Absence	barrel-shaped to ellipsoidal and cylindrical	rounded	spherical (few elliposoidal)	presence	adjacent to one side of hets	ellipsoidal
***N. commune* UTCC74**	bundle and solitary	straight to amorphous	presence	Absence	Barrel-shaped	rounded	ellipsoidal	NO	NO	NO
***N. ellipsosporum* UTEX383**	bundles	straight to amorphous	presence	Presence	barrel-shaped and cylindrical	Rounded (few conical)	Ellipsoidal (few spherical)	presence	Far from hets	ellipsoidal
***N. punctiforme* UTCC41**	bundles	straight to amorphous	presence	Absence	Spherical to sub-spherical	rounded	Spherical to sub-spherical	NO	NO	NO
***Nostoc* sp. UTCC106**	solitary (few in bundles)	straight to slightly curve	absence	Presence (in few cells)	Ellipsoidal to spherical	rounded	NO	NO	NO	NO
***Nostoc* sp. UTCC314**	bundles	straight to slightly curve	absence	Absence	barrel-shaped	rounded	Barrel-shaped to ellipsoidal	presence	Far from hets	ellipsoidal
***Nostoc* sp. UTCC355**	bundle and solitary	straight to slightly curve	Absence	presence (few per cell)	Barrel-shaped (few ellipsoidal)	rounded	Barrel-shaped to ellipsoidal	presence	adjacent to one side of hets	ellipsoidal
***Nostoc* sp. UTCC387**	solitary (few in bundles)	curve	absence	Absence	Barrel-shaped	rounded	Spherical to ellipsoidal	NO	NO	NO
***An. lemmermannii* ONT1**	solitary (few in bundles)	slightly curve	absence	Presence	Ellipsoidal or kidney-shaped	rounded	ellipsoidal	absence	adjacent to both sides of hets	kidney-shaped to ellipsoidal
***Nostoc* sp. RUP1**	Bundles	curve	presence	Absence	Barrel-shaped to cylindrical	rounded	Spherical to subespherical	presence	Far from hets	ellipsoidal
***An. reniformis* MALW1**	solitary	irregularly coiled to slightly curve	absence	Presence	Kidney-shaped and cylindrical	rounded	spherical	presence	adjacent to both sides of hets	Spherical

***Anabaena* cf. *cylindrica* Lemmermann** (Figures 3.1.b, 3.1.c)

Solitary filaments that sometimes form mats. Straight trichomes that are clearly constricted at cross walls and sometimes lightly attenuated towards ends. Vegetative cells are barrel or long-barrel shaped, 3.85 (2.56-5.28) µm wide and 7.36 (4.59-10.42) µm in length, blue-green, no gas vesicles, more or less homogeneous. End cells are rounded to conical (conical end cells are typical in short filaments), 3.46 (2.37-4.63) µm wide and 7.36 (4.36-10.78) µm in length. Heterocysts are typically barrel shaped, although are sometimes ellipsoidal, 4.74 (3.85-6.12) µm wide and 8.12 (5.68-11.21) µm in length, in intercalary and terminal positions within the filament, yellowish-brown, with colourless pores that are sometime visible and two evident membranes (Figures 3.1.b, 3.1.c). Figure 3.1.c shows akinetes almost cylindrical to ellipsoidal, 5.75 (4.06-8.27) µm wide and 18.55 (12.13-28.28) µm in length, on both sides of heterocysts, in intercalary and terminal positions within the filaments, with granular green content.

***Anabaena* cf. *flos-aquae* (Lyngbye) Brébisson ex Bornet & Flauhault UTCC 64**

(Figure 3.1.d)

Solitary filaments that tend to form bundles. Straight to circinate trichomes clearly constricted at cross walls and not attenuated towards ends. Vegetative cells are ellipsoidal or cylindrical with rounded ends, 4.28 (3.34-6.56) µm wide and 6.15 (4.13-8.81) µm in length, green to blue-green; very few filaments have gas vesicles, homogeneous contents. End cells are similar in appearance to vegetative cells, rounded, 4.19 (3.27-5.00) µm wide and 5.60 (4.16-7.13) µm in length. Heterocysts are spherical to shortly ellipsoidal, 5.09 (3.82-6.51) µm wide and 5.58 µm (4.36-7.14) µm in length, in intercalary and

terminal positions within the filament, yellowish-brown, with small brown pores. Akinetes were not observed.

***Anabaena* cf. *flos-aquae* (Lyngbye) Brébisson ex Bornet & Flauhault UTEX 2383** (Figure 3.1.e)

Solitary filaments that tend to form bundles generally when they are under extreme condition, like under iron and phosphorous depletion. Straight to circinate trichomes clearly constricted at cross walls and not attenuated towards ends. Vegetative cells are ellipsoidal or cylindrical with rounded ends, 4.31 (3.42-5.77) µm wide and 6.26 (3.41-8.78) µm in length, green to blue-green, some gas vesicles present and homogeneous contents. End cells are similar in appearance to vegetative cells, rounded, 4.13 (3.46-5.26) µm wide and 5.24 (3.28-6.89) µm in length. Heterocysts are spherical, 5.25 (4.04-6.82) µm wide and µm 5.47 (3.65-7.37) µm in length, in intercalary and terminal positions within the filament, yellowish-brown, with small colorless pores. Akinetes were not observed.

***Anabaena lemmermannii* Richter GIOL8** (Figure 3.1.f)

Solitary, curved trichomes clearly constricted at cross walls and not attenuated towards ends. Vegetative cells are cylindrical with rounded ends or long-ellipsoidal in shape, 4.68 (3.63-6.42) µm wide and 7.12 (5.04-9.03) µm in length, green, usually with solitary dark brown granules, evident gas vesicles dispersed within the cell. End cells are very similar to vegetative cells, rounded, 4.51 (3.66-5.92) µm wide and 6.72 (4.55-9.43) µm in length. Heterocysts are shortly ellipsoidal almost spherical, 5.01 (3.99-6.07) µm

wide and 5.95 (3.68-7.69) µm in length, in intercalary and terminal positions within the filament, greener and more homogeneous than vegetative cells, two colourless pores. Akinetes are cylindrical to ellipsoidal 7.93 (6.22-9.40) µm wide and 12.68 (9.84-17.52) µm in length, adjacent to heterocysts, the content is very similar to vegetative cells but more brownish in colour.

***Anabaena lemmermannii* Richter LONT2** (Figure 3.1.g)

Solitary, straight trichomes clearly constricted at cross walls and not attenuated towards ends. Vegetative cells are primarily barrel shaped and cylindrical with rounded ends, sometimes spherical, 5.73 (4.40-7.13) µm in diameter and 6.54 (4.30-8.56) µm in length, blue-green, usually with solitary dark brown granules, old cultures have brownish-green cells and homogenous contents, gas vesicles are exhibited by some filaments but never in filaments of old cultures. End cells are rounded, have the same inner structures as vegetative cells, 5.33 (4.58-6.56) µm wide and 6.42 (3.90-9.3) µm in length. Heterocysts are spherical, slightly smaller than vegetative cells, 6.18 (4.27-8.6) µm wide and 6.24 (5.06-8.27) µm in length, in intercalary and terminal positions within the filament and similar in appearance to vegetative cells, slightly browner with no visible pores. Akinetes are almost cylindrical to ellipsoidal 8.41 (6.47-11.39) µm wide and 17.98 (10.54-23.5) µm in length, on one side of a heterocyst or on both sides, terminal or intermediated; contents are very similar to the contents of vegetative cells.

***Anabaena* cf. *oscillariodes* Bory de Saint-Vincent, nom. illeg.** (Figure 3.2.a)

Typically solitary filaments but may form mats in old cultures similar to some species of *Nostoc*. Straight and slightly circinate trichomes lightly constricted at cross walls and not attenuated towards ends. Cylindrical vegetative cells, sometime a little curved resembling a bean, 3.12 (2.55-3.59) µm wide and 4.92 (3.84-6.09) µm in length, blue-green, browner in old cultures, no gas vesicles, and homogenous contents. End cells have the same appearance as vegetative cells, rounded at the ends, 3.08 (2.34-3.76) µm wide and 4.93 (3.46-6.33) µm in length. Heterocysts and akinetes were not observed.

***Anabaena* cf. *planktonica* Brunnthaler** (Figures 3.2.b, 3.2.c, 3.2.d)

Solitary, straight trichomes slightly constricted at cross walls and narrowed towards ends. Cylindrical vegetative cells, barrel shaped when they are in division, 5.52 (2.79-7.73) µm wide and 13.21 (7.08-18.65) µm in length, brownish-green, gas vesicles and dark brown granules scattered. Figure 3.2.c shows the end cells, they are rounded and occasionally longer than vegetative cells, 5.23 (2.69-7.53) µm in diameter and 12.87 (6.18-22.79) µm in length, sometimes they show a hyaline tapered extreme similar to *Aphanizomenon* species. Heterocysts are spherical to ellipsoidal, 7.15 (6.00-8.93) µm wide and 8.52 (7.04-10.15) µm in length, in intercalary and terminal positions within the filament, greener than cells, homogenous, no visible pores and only one membrane (Figure 3.2.b). Akinetes are ellipsoidal to shortly ellipsoidal, 14.33 (6.59-19.56) µm wide and 21.79 (17.07-31.31) µm in length, greener than vegetative cells and with dark brown granules, were never observed near heterocysts, and always observed in the middle of the filaments (Figure 3.2.d).

***Anabaena* cf. *lemmermannii* Richter** (Figures 3.2.e, 3.2.f)

Solitary, straight to slightly curved trichomes, clearly constricted at the cross walls and slightly thinner toward the ends. Vegetative cells are ellipsoidal to barrel-shaped, 5.38 (4.25-6.98) µm wide and 6.74 (4.14-10.61) µm length, brownish-blue-green, usually with solitary dark brown granules, with gas vesicles. End cells are very similar in appearance to vegetative cells, sometime with hyaline extreme, rounded ends, 5.19 (3.37-6.85) µm wide and 6.34 (4.04-10.74) µm in length. Heterocysts are spherical to ellipsoidal 6.27 (4.75-9.69) µm wide and 6.75 (5.06-8.87) µm in length, smooth yellowish-brown contents, with two visible colourless pores, and observed in intercalary and terminal positions within the filaments (Figure 3.2.e). Akinetes are ellipsoidal, 8.27 (5.80-9.50) µm in diameter and 12.35 (10.00-17.97) µm in length, brownish-green, fine granular contents, on one side of heterocysts (Figure 3.2.f).

***Anabaena* sp. A2879** (Figure 3.2.k)

Solitary filaments that tend to produce a ball like colonies, straight or curved trichomes clearly constricted and only in some filaments gradually narrowed towards the ends (just in short filaments). Vegetative cells are barrel shaped, 6.14 (4.15-7.80) µm wide and 5.80 (3.73-7.58) µm in length, green with dark brown granules. End cells may be rounded or conical, 5.14 (3.76-7.17) µm wide and 6.17 (4.34-8.54) µm in length, present similar texture than vegetative cells. Barrel-shaped heterocysts in intercalary positions within the filaments, but when in the terminal position they may be conical 7.68 (6.18-8.97) µm wide and 8.05 (5.55-9.72) µm in length, browner than vegetative cells,

homogeneous in appearance. The heterocysts are connected with the vegetative cells by mucilaginous connections. Akinetes were not observed.

***Anabaena* sp. LONT5** (Figure 3.2.g, 3.2.h)

Solitary, straight trichomes, sometimes curved or arcuate, clearly constricted at cross walls and not attenuated towards ends. Vegetative cells are barrel to short barrel shaped, 5.99 (4.25-7.56) µm wide and 5.24 (4.00-7.06) µm in length, brownish-green, without gas vesicles and with granules on the cell walls between cells. The contents of end cells are very similar to vegetative cells, but they are a slightly narrowed cone shape, 5.56 (5.01-6.59) µm wide and 5.30 (3.87-7.29) µm in length. Heterocysts are spherical and sub-spherical sometimes with one flat side, 6.82 (5.03-8.49) µm wide and 6.60 (5.06-8.72) µm in length, sometimes in rows of two or more, in intercalary and terminal positions within the filament, browner than vegetative cells, homogeneous, without visible pores (Figure 3.2.g). The akinetes are shown in Figure 3.2.h, they are ellipsoidal, 9.57 (6.21-12.49) µm wide and 14.90 (11.36-18.03) µm in length. The position of the akinetes in relation to the heterocysts was not noted since just free akinetes were observed.

***Anabaena* sp. LOW1** (Figures 3.2.i, 3.2.j)

Trichomes gathered in small clusters, sometimes solitaries, lightly constricted at cross wall and same diameter along the filament. Trichomes are irregularly coiled, but they may be straight and shorted under cultures condition. Cylindrical vegetative cells, 2.97 (2.15-4.55) µm wide and 9.68 (3.73-14.57) µm in length, blue-green, dispersed gas

vesicles and dark brown granules. End cells are similar to vegetative cells, rounded to tapered, 2.77 (2.06-3.88) µm wide and 9.47 (6.47-13.26) µm in length. These features are shown in Figure 3.2.i. Ellipsoidal heterocysts are browner and more homogeneous, evident pores and one membrane, 4.11 (2.53-6.06) µm wide and 7.87 (5.70-10.77) µm in length (Figures 3.2.i, 3.2.j). Akinetes are cylindrical to bean shaped, 5.96 (4.27-7.85) µm wide and 25.25 (16.67-30.95) µm in length, arising of both sides of heterocysts (Figure 3.2.j).

***Anabaena* sp. 7812** (Figures 3.3.a, 3.3.b, 3.3.c)

Trichomes are straight, but sometimes circinate since the cells are not organized on the base of an axis, clearly constricted at a cross wall and the same diameter extends the length of the filaments. Vegetative cells are spherical to sub-spherical, 5.8 (4.4-6.7) µm wide and 5.0 (4.0-8.8) µm in length, dark green, gas vesicles only in some filaments, and a few dark brown granules. End cells are shown in Figure 3.3.a, they are similar to vegetative cells, rounded, 5.5 (4.0-6.5) µm wide and 4.9 (3.4-5.9) µm in length. In old cultures the filaments release many free brown vegetative cells. Heterocysts and akinetes were not observed. Possible heterocysts and akinetes can be observed in Figures 3.3.b and 3.3.c respectively.

***Anabaena variabilis* (Kutzing) Bornet et Flahault** (Figures 3.3.d, 3.3.e, 3.3.f)

Mats of straight trichomes attenuated gradually towards the ends and clearly constricted across wall. Vegetative cells are cylindrical to barrel shaped, 3.55 (2.29-5.23) µm wide and 5.35 (2.61-8.48) µm in length, dark greyish-green, homogeneous, without

gas vesicles. End cells are similar to vegetative cells, rounded sometimes lightly conical (mainly in young filaments), 3.39 (2.38-4.63) µm wide and 5.54 (3.43-8.24) µm in length (Figure 3.3.d). Heterocysts are ellipsoidal to barrel shaped, browner and bigger than vegetative cells, 4.37 (2.94-6.65) µm wide and 5.85 (3.56-9.51) µm in length, intercalary or terminal within the filaments (Figure 3.3.f). Akinetes are ellipsoidal, 6.18 (4.63-7.77) µm wide and 10.94 (8.98-14.50) µm in length, distant from heterocysts (Figures 3.3.e, 3.3.f).

***Anabaena* cf. *viguieri* Denis et Frémy** (Figure 3.3.g)

Solitary straight to curved trichomes that under culture conditions tend to form bundles, visible constricted at cross wall, slightly attenuated at the ends. Vegetative cells are barrel-shaped to sub-spherical, 5.25 (3.19-7.32) µm wide and 5.14 (3.50-6.74) µm in length, brownish-green, and without gas vesicles. End cells are rounded, but sometimes conical, 3.80 (2.52-5.48) µm wide and 4.85 (2.56-6.87) µm in length, of the same inner structure than vegetative cells. Heterocysts are almost spherical, 6.57 (3.65-9.93) µm wide and 7.02 (4.80-9.90) µm in length, smooth browner contents, in intercalary and terminal position within the filament. Akinetes were not observed.

***Aphanizomenon flos-aquae* (Linneaus) Ralfs ex Bornet et Flahault UTEX LB2384** (Figure 3.3.h)

Similar description to *Aphanizomenon* sp. HHAFA, although heterocysts and akinetes were never observed, and the vegetative cells in division are square-shaped.

Vegetative cells have 4.98 (3.14-8.31) µm wide and 11.46 (4.56-22.58) µm in length, and end cells have 4.75 (2.82-6.12) µm wide and 12.92 (3.51-25.79) µm in length.

***Aphanizomenon* cf. *gracile* Lemmermann** (Figures 3.3.i, 3.3.j, 3.3.k)

Straight trichomes in fascicles (macroscopic bundles), but solitary filaments were also observed. Trichome slightly narrowed to the ends and more constricted at cross wall than *Aphanizomenon flos-aquae and Aphanizomenon* sp. HHAFA (Figure 3.3.i). Vegetative cells are cylindrical, 3.59 (2.34-5.45) µm wide and 11.51 (7.60-19.66) µm in length, blue-green, with dispersed gas vesicles and brown granules. End cells are rounded or slightly tapering, 3.46 (2.22-5.05) µm wide and 10.58 (6.88-16.64) µm in length, hyaline appearance was observed in few filaments, in general very similar to vegetative cells. Heterocysts are cylindrical to ellipsoidal, 4.64 (3.17-7.79) µm wide and 10.22 (6.64-13.56) µm in length, intercalary, browner than vegetative cells (Figure 3.3.k). Figure 3.3.j shows the cylindrical akinetes, 6.72 (5.54-8.68) µm wide and 30.15 (24.70-43.94) µm in length, distant from heterocysts.

***Aphanizomenon* klebahnii HHAFA** (Figures 3.3.l, 3.3.m)

Straight trichomes in fascicles slightly or not constricted at cross wall and attenuated towards the ends. Vegetative cells are cylindrical, squared-off corned, barrel-shaped when the cell is in division, 4.03 (2.92-6.21) µm wide and 9.76 (6.02-17.45) µm in length, blue-green, with scattered gas vesicles and brown granules. Very slightly tapering end cells, 3.77 (2.45-5.59) µm wide and 13.23 (6.33-26.87) µm in length, hyaline, with plasma (Figure 3.3.l). Heterocysts are barrel-shaped or cylindrical, 4.71

(3.39-6.40) µm wide and 9.53 (7.99-13.03) µm in length, always intermediate, with visible pores and smoother in appearance than vegetative cells. Long-cylindrical akinetes, 7.05 (5.43-8.59) µm wide and 41.04 (27.21-66.34) µm in length, similar in appearance than cells, close to heterocysts and intermediate within the filament, the akinetes were observed positioned only at one side of the heterocyst. Heterocysts and akinetes are shown in Figure 3.3.m.

***Nostoc calcicola* Brébisson UTEX B382** (Figures 3.4.a, 3.4.b)

Curved trichomes, sometimes in mucilaginous amorphous mats, visible constricted at cross wall and no narrowed at the ends. Vegetative cells are usually barrel-shaped, cylindrical in young filaments, 4.71 (3.37-6.43) µm wide and 6.77 (4.82-8.47) µm in length, brownish black to brown, smooth surface, without gas vesicles. End cells similar to vegetative cells, rounded at the end, 4.54 (3.21-6.42) µm wide and 6.33 (4.31-8.88) µm in length. In Figure 3.4.a the heterocysts are shown, they are spherical to ellipsoidal, barrel-shaped in young filaments, 4.54 (3.86-5.89) µm wide and 5.10 (3.62-6.39) µm in length, yellowish green, no visible pores, intercalary and terminal within the filament, sometimes rows of 5 heterocysts were observed. Akinetes are ellipsoidal, 6.83 (5.31-9.36) µm wide and 10.20 (9.13-13.19) µm in length, in chains or groups of four or more, never in young filaments, brown with granular content, dark grey epispore, they were observed only on one side of heterocysts (Figure 3.4.b). The mucilaginous sheath only is observed in mature filaments.

***Nostoc commune* Vaucher UTCC 74** (Figures 3.4.c, 3.4.d)

Mucilaginous colonies are amorphous and dark blue-green (Figure 3.4.c). Trichomes constricted, younger trichomes are smaller and narrowed at the ends than mature filaments. Vegetative cells are barrel shaped, 3.30 (2.56-4.16) μm wide and 5.01 (3.20-6.87) μm in length, greyish-brown, no gas vesicles, homogeneous contents. End cells are smaller than vegetative cells, but they have the same appearance, 3.20 (2.39-3.95) wide and 4.89 (2.79-7.40) in length, rounded at the end. Heterocysts are ellipsoidal, terminal or intermediate, greener than vegetative cells, 4.12 (4.08-4.16) μm wide and 5.23 (5.02-5.43) μm in length (Figure 3.4.d). Akinetes were not observed.

***Nostoc ellipsosporum* Rabenhorst ex Bornet & Flahault UTEX 383** (Figures 3.4.e, 3.4.f)

Amorphous mucilaginous colonies, dark blue-green. Trichomes are straight or curved depending on the culture age, constricted, with the same diameter along the filament. Vegetative cells are barrel-shaped in mature cultures and cylindrical in young cultures, 3.84 (2.65-5.19) μm wide and 5.55 (3.48-8.18) μm in length, yellowish-green, with dark brown granules and gas vesicles. End cells are similar to vegetative cells, 3.82 (2.98-4.68) μm wide and 5.63 (3.78-7.23) μm in length, rounded and sometimes conical in young filaments. Heterocysts are ellipsoidal to spherical, shown only for young filaments, 4.35 (3.37-5.60) μm wide and 5.72 (4.31-7.06) μm in length, terminal or intercalary within the filament, more yellow than vegetative cells, with finely granular content, evident little pores (Figure 3.4.e). Akinetes are shown in Figure 3.4.f, they are ellipsoidal, 6.45 (5.60-8.11) μm wide and 8.88 (7.32-10.88) μm in length, browner than

vegetative cells, with roughly granular content, in rows, remote from heterocysts, intercalary or terminal position within the filament.

***Nostoc punctiforme* (Kützing) Hariot UTCC 41** (Figures 3.4.g, 3.4.h)

Amorphous mucilaginous colonies, dark blue-green. Trichomes are constricted and no narrowed at the ends. They are straight in young filaments, however, this organization is lost in mature cultures. Vegetative cells are spherical to sub-spherical, 4.54 (2.62-5.98) µm broad and 4.26 (2.82-5.83) µm long, blue-green, smooth in texture and without gas vesicle. End cells are rounded, 4.18 (2.94-5.21) µm wide and 4.05 (2.80-5.66) µm in length, similar appearance than vegetative cells. Heterocysts are spherical to sub-spherical, conical when they are in the filament end, smaller than vegetative cells, 4.29 (2.90-5.62) µm broad and 3.88 (2.15-5.32) µm long, intercalary and terminal within the filament, yellowish-green, homogeneous contents, and two small evident pores. Akinetes were not observed.

***Nostoc* sp. UTCC 314** (Figures 3.4.i, 3.4.j, 3.4.k)

Trichomes are straight to slightly curved, since they are too long (>200 cells) and in mats, evidently constricted at cross wall and with the same diameter along the filament. Vegetative cells are barrel to short barrel-shaped, 5.48 (3.73-6.59) µm wide and 7.52 (4.34-11.38) µm in length, green or greyish-blue, without gas vesicles and more or less homogeneous. End cells are rounded; of the same inner structure as in the middle cells, but a little smaller, 4.96 (3.45-6.33) µm wide and 7.39 (4.08-10.54) µm long (Figure 3.4.k). Heterocysts are barrel-shaped to ellipsoidal, 7.02 (5.59-8.65) µm wide and

8.08 (6.12-9.67) µm in length, sometimes in chains of two (Figure 3.4.k), intercalary within the filament (Figure 3.4.i), yellowish-green, smooth in surface and one pore more evident that the other one. Akinetes are ellipsoidal, 6.71 (5.57-8.70) µm wide and 12.01 (7.93-31.13) µm in length, with distinct, widened radially striated epispore, granular contents, sometimes in rows (of 2-7), remote from heterocysts, intercalary and terminal positions within the filament (Figures 3.4.i, 3.4.j).

***Nostoc* sp. UTCC 106** (Figure 3.5.a)

Straight to slightly curved trichomes, sometimes in mats, clearly constricted at cross walls and with the same diameter along the filament. Vegetative cells are ellipsoidal to spherical, but whole the filament shows the same shape, 3.51 (2.64-5.00) µm broad and 3.58 (2.66-4.84) µm long, blue-green, homogeneous contents, gas vesicles present in some filaments. End cell are very similar to vegetative cells, rounded ends, 3.44 (2.63-4.50) µm broad and 3.49 (2.50-4.72) µm long. Heterocysts and akinetes were not observed.

***Nostoc* sp. UTCC 387** (Figures 3.5.b, 3.5.c)

Trichomes are curved, sometimes in mats, clearly constricted at cross wall, and not attenuated towards ends. Vegetative cells are barrel-shaped, 3.35 (2.67-4.21) µm wide and 3.68 (2.44-4.97) µm in length, blue-green, smooth contents, without gas vesicles. End cells are similar in appearance than vegetative cells, 3.16 (2.57-4.03) µm wide and 3.23 (2.08-4.42) µm in length, with rounded ends (Figure 3.5.b). Figure 3.5.c shows the heterocysts, they are spherical, sometimes ellipsoidal, 3.09 (2.18-4.14) µm

wide and 3.21 (2.46-4.35) µm in length, almost always in terminal position, browner than vegetative cells, smooth contents, and one pore more visible than the other one. Akinetes were not observed.

***Nostoc* sp. UTCC 355** (Figures 3.5.d, 3.5.e)

Curved trichomes that tend to form mats, visibly constricted at cross walls, and not attenuated towards ends. Vegetative cells are barrel-shaped, 5.14 (3.00-6.91) µm wide and 6.18 (3.63-9.64) µm in length, blue-green, granular contents, few gas vesicle in some cells within the filaments. Ends cells have similar sizes, shape and inner structure than vegetative cells, 4.61 (2.46-6.12) µm broad and 6.64 (3.59-9.71) µm long, rounded, sometimes conical. Heterocysts are barrel-shaped to ellipsoidal, 5.95 (4.34-7.83) µm broad and 7.19 (5.15-9.99) µm long, yellowish-brown, with granular appearance, intercalary and terminal within the filaments, pores slightly visible (Figures 3.5.d, 3.5.e). Akinetes are ellipsoidal, 7.02 (5.68-10.05) µm wide and 9.61 (6.85-15.76) µm in length, sometimes in row of until ten akinetes, intercalary or terminal, sometimes on one side of heterocysts, yellowish-brown, with slightly granular content, and dark epispore (Figure 3.5.e).

3.1.2 Descriptions of Isolates Obtained from Lake Samples

***Anabaena lemmermannii* Richter ONT1** (Figures 3.5.f, 3.5.g)

Solitary trichomes that tend to form clumps, curved clearly constricted at cross walls, not attenuated towards ends, slightly coiled, with a coiled diameter of 33.86 (17.63-54.33) µm and a distance diameter of 21.33 (6.68-36.19) µm. Vegetative cells are

cylindrical with rounded ends, kidney-shaped 5.72 (4.08-8.50) µm wide and 9.64 (6.30-16.91) µm in length, blue-green to greyish-blue, usually with solitary dark brown granules, evident gas vesicles dispersed within the cell (Figure 3,5,f). End cells are very similar to vegetative cells, rounded, 5.42 (3.65-9.22) µm wide and 8.74 (5.74-11.19) µm in length. Heterocysts are ellipsoidal, sometimes spherical, 6.32 (4.85-7.92) µm wide and 8.11 (6.48-10.60) µm in length, in intercalary and terminal positions within the filament, greener than vegetative cells, homogeneous contents, two colourless pores. Akinetes are kidney-shaped, but sometimes they are ellipsoidal 8.70 (6.81-11.73) µm wide and 18.91 (13.24-31.03) µm in length, adjacent to heterocyst and usually in both side, sometimes in clusters, the content is very similar to vegetative cells but browner. Akinetes and heterocysts are shown in Figures 3.5.f and 3.5.g.

***Nostoc* sp. RUP1** (Figures 3.5.h, 3.5.i, 3.5.k)

Curved trichomes in mats, not densely entangled in younger cultures, surrounded by a mucilaginous sheath, visibly constricted at cross walls and no narrowing at the ends. Vegetative cells are usually barrel shaped, cylindrical in young filaments, 5.7 (4.3-6.9) µm wide and 5.4 (3.9-7.1) µm in length, brownish black to brown, smooth surface, gas vesicles were observed in some filaments. End cells similar to vegetative cells, rounded at the end, 5.2 (3.3-6.0) µm wide and 5.4 (3.7-7.4) µm in length (Figure 3.5.i). Heterocysts are spherical to subspherical, ellipsoidal in young filaments, 6.5 (4.3-8.3) µm wide and 6.3 (4.5-8.1) µm in length, yellowish green, smooth to finely granular contents, no visible pores, intercalary and terminal positions within the filament, sometimes in chains of two (Figure 3.5.h). Akinetes are ellipsoidal, 7.5 (6.4-9.1) µm wide and 10.7

(8.6-12.9) µm in length, in chains or groups of four or more, never in young filaments, with the same colour as vegetative cells but more granular contents, not observed next to heterocysts (Figure 3.5.k). The mucilaginous sheath present only in mature filaments.

Anabaena reniformis Lemmermann **MALW1** (Figure 3.5.j)

Solitary trichomes, coiled and sometimes straight, slightly constricted at cross walls and not attenuated towards ends. The diameter of the coil is 14.69 (9.86-19.82) µm and the distance is 10.11 (1.69-16.20) µm. Vegetative cells are cylindrical and kidney-shaped, 3.41 (2.28-4.65) µm wide and 7.21 (4.64-18.01) µm in length, blue-green to brownish-green, finely granular contents, visible gas vesicles only in some filaments. End cells are very similar to vegetative cells, 3.48 (2.56-5.37) µm wide and 7.32 (4.92-11.46) µm in length, rounded at the ends. Heterocysts are spherical, sometimes ellipsoidal, 5.36 (4.42-6.25) µm in diameter and 6.12 (4.55-7.55) µm in length, in intercalary and terminal positions, browner than vegetative cells, with evident browner pores. Akinetes are spherical, 7.82 (6.62-12.11) µm wide and 8.19 (6.64-11.14) µm in length, solitary, at both side of heterocysts, in intercalary and terminal positions within the filament, yellowish-brown content with scattered green granules.

Figure 3.1 **Morphology of cyanobacterial isolates used in this study.** *Anabaena compacta* (a); *Anabaena* cf. *cylindrica* (b, c)*; Anabaena* cf. *flos-aquae* UTCC 64 (d); *Anabaena* cf. *flos-aquae* UTEX 2383 (e); *Anabaena lemmermannii* GIOL8 (f); *Anabaena lemmermannii* LONT2 (g). Heterocysts are labeled as "H" and akinetes as "A". Bars, 10 μm.

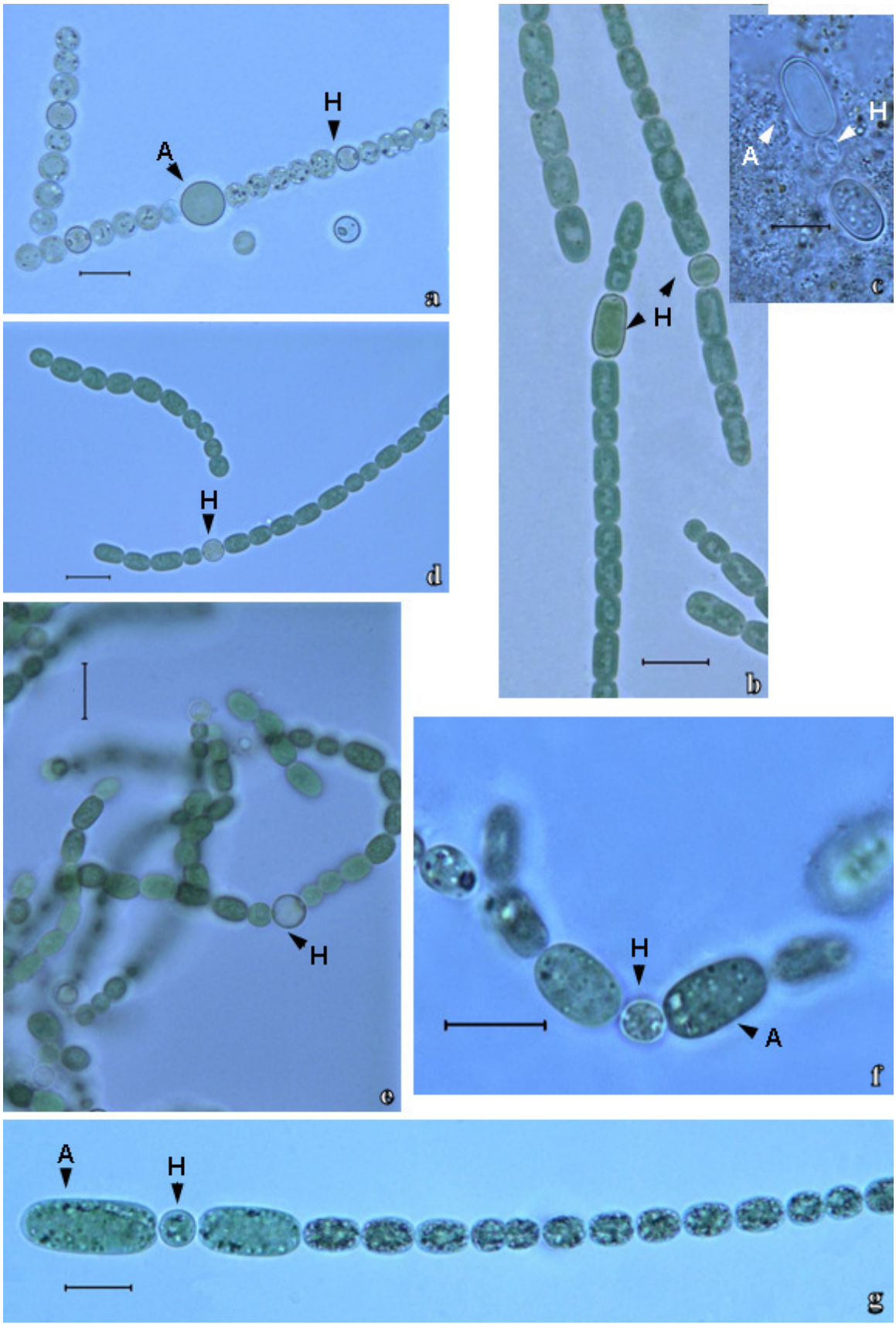

A
H
a
H
d
H
e
H
b
A
H
c
H
A
f
A
H
g

Figure 3.2 Morphology of cyanobacterial isolates used in this study. *Anabaena* cf. *oscillariodes* (a); *Anabaena* cf. *planktonica* (b, c, d); *Anabaena* cf. *lemmermannii* (e, f); *Anabaena* sp. LONT5 (g, h); *Anabaena* sp. LOW1 (i, j); *Anabaena* sp. A2879 (k). Heterocysts are labeled as "H" and akinetes as "A". Bars, 10 µm.

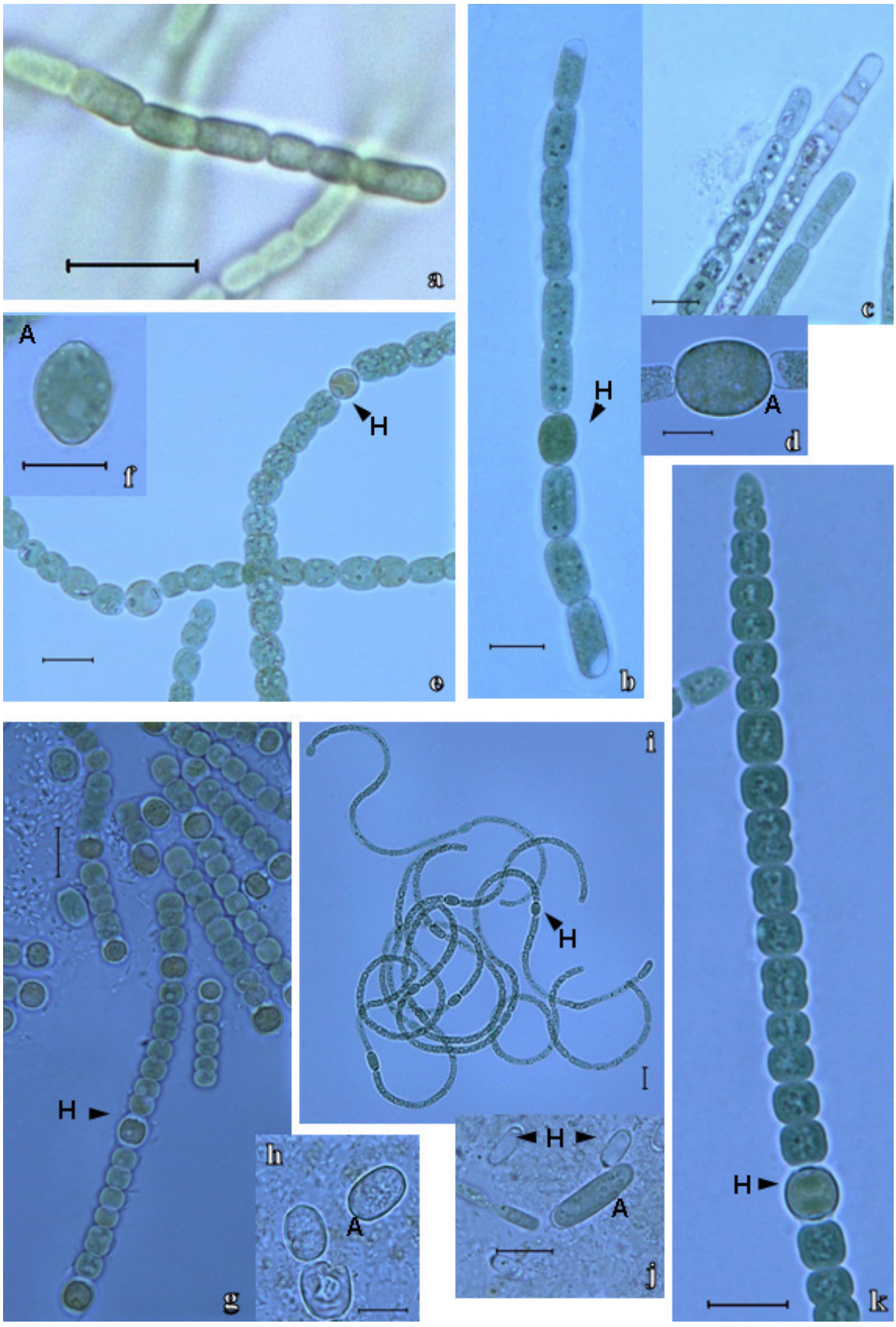

a
b
H
c
d
A
e
H
f
A
g
H
h
A
i
H
j
H
A
k
H

Figure 3.3 Morphology of cyanobacterial isolates used in this study. *Anabaena* sp. 7812 (a, b, c); *Anabaena variabilis* (d, e, f); *Anabaena* cf. *viguieri* (g); *Aphanizomenon flos-aquae* UTEX LB2384 (h); *Aphanizomenon* cf. *gracile* (i, j, k); *Aphanizomenon klebahnii* HHAFA (l, m). Heterocysts are labeled as "H" and akinetes as "A". Bars, 10 µm.

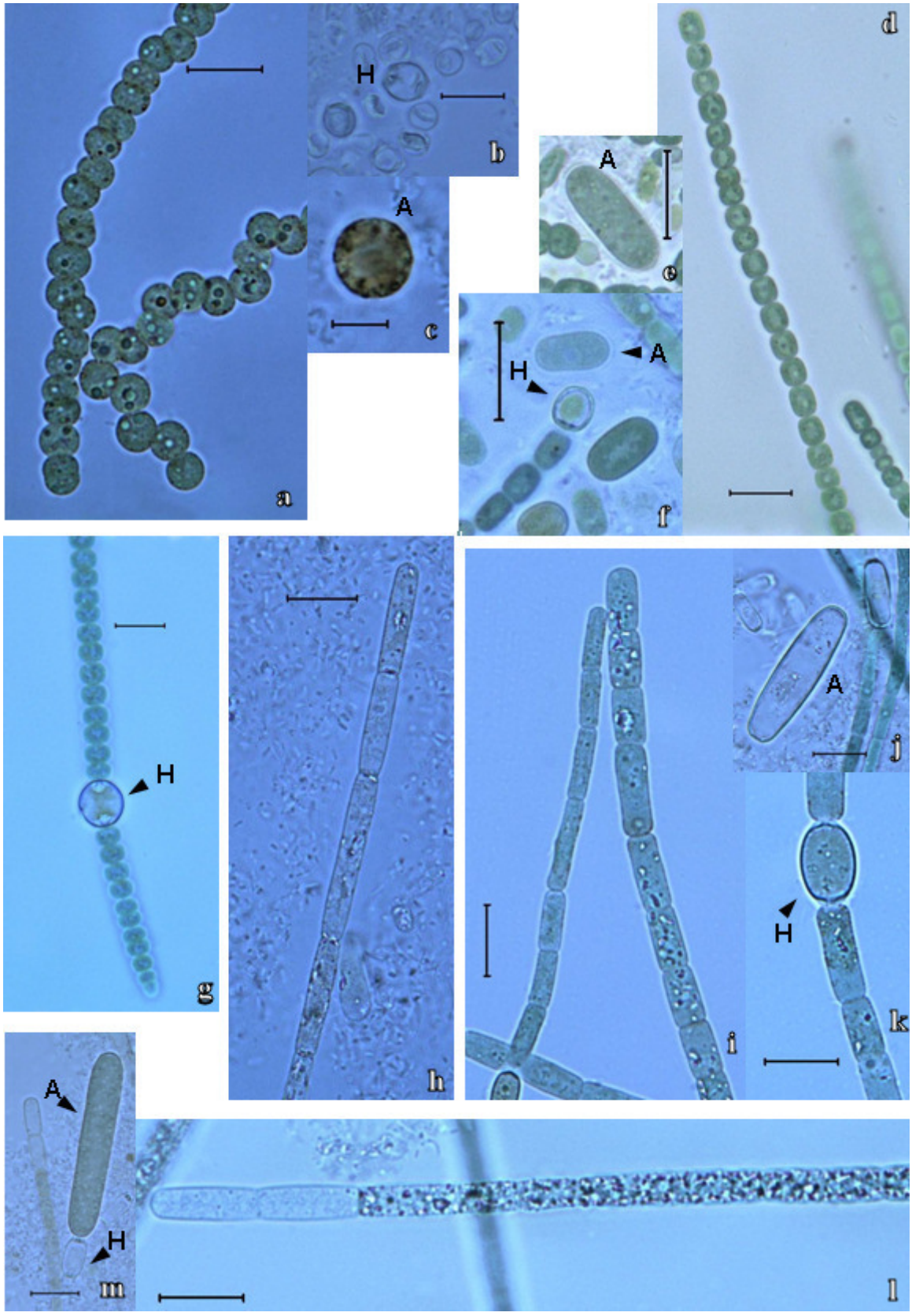

a
H
b
A
c
d
A
e
H
A
f
H
g
h
A
j
i
H
k
A
H
m
l

Figure 3.4 Morphology of cyanobacterial isolates used in this study. *Nostoc calcicola* UTEX B382 (a, b); *Nostoc commune* UTCC 74 (c, d); *Nostoc ellipsosporum* UTEX 383 (e, f); *Nostoc punctiforme* UTCC 41 (g, h); *Nostoc* sp. UTCC 314 (i, j, k). Heterocysts are labeled as "H" and akinetes as "A". Bars, 10 µm.

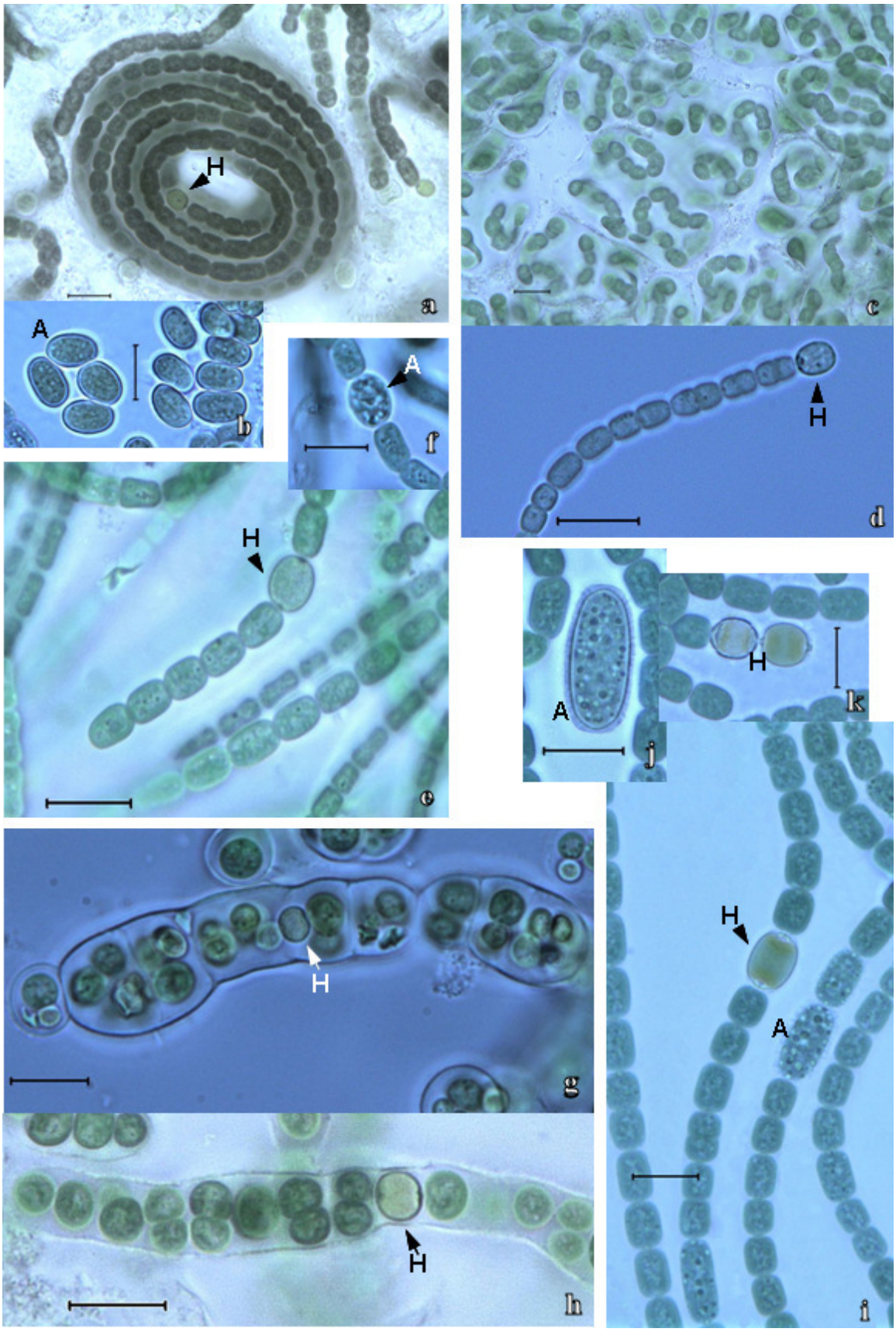
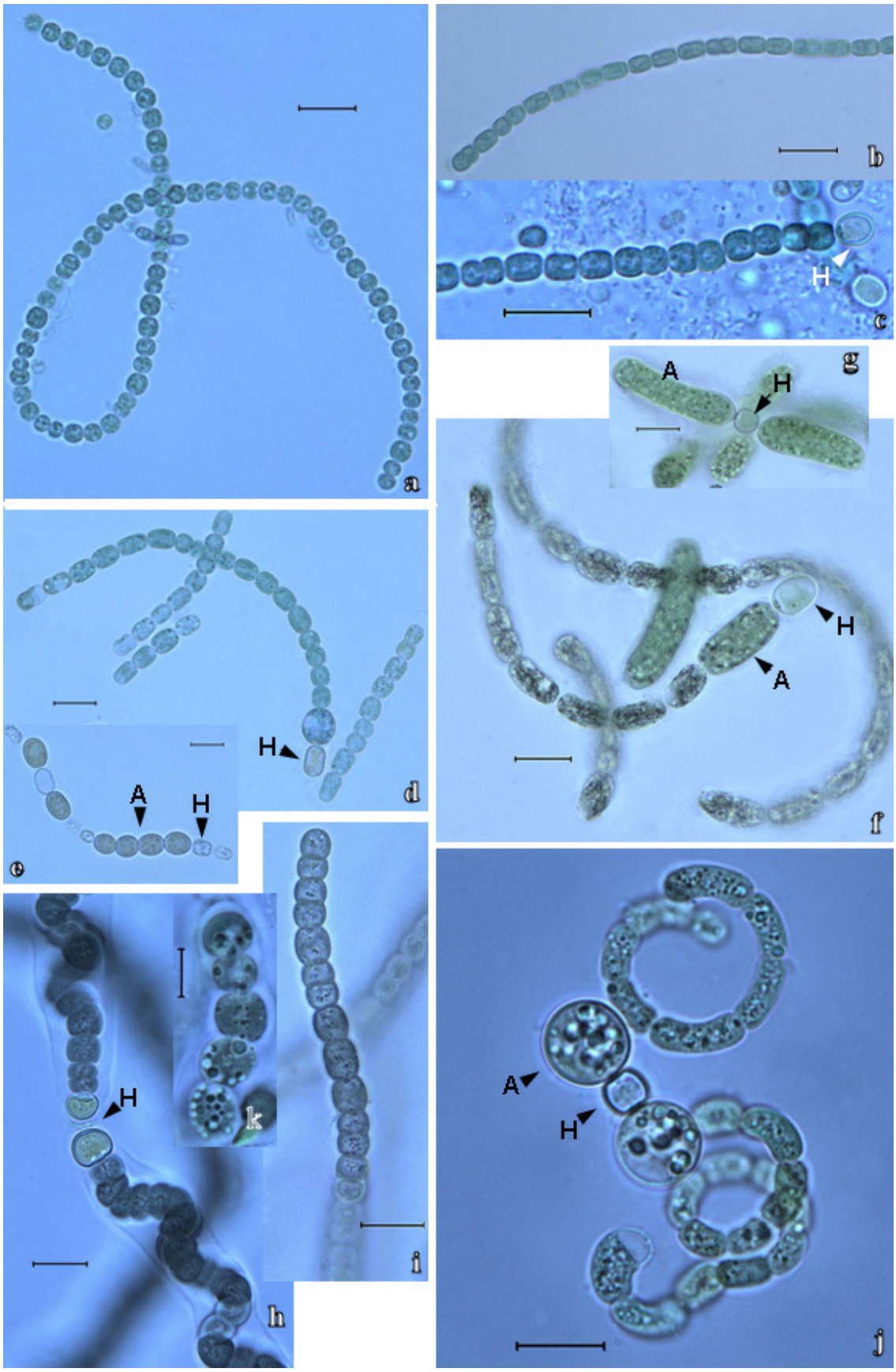

H
a
c
A
b
A
f
H
d
H
e
A
j
H
k
H
A
i
H
g
H
h

Figure 3.5 Morphology of cyanobacterial isolates used in this study. *Nostoc* sp. UTCC 106 (a); *Nostoc* sp. UTCC 387 (b, c); *Nostoc* sp. UTCC 355 (d, e); *Anabaena lemmermannii* ONT1 (f, g); *Nostoc* sp. RUP1 (h, i, k); *Anabaena reniformis* MALW1 (j). Heterocysts are labeled as "H" and akinetes as "A". Bars, 10 µm.


a
b
H
c
A
H
g
H
A
f
H
d
A
H
e
H
k
i
h
A
H
j

3.2 STATISTICAL ANALYSES OF MORPHOLOGICAL DATA

ANOSIM test (analysis of similarities) was used for validating the significance of the groups generated by NMDS on the base of the metric features and the separation of these isolates is depicted in Figure 3.6.a. Table 3.2 summarized the ANOSIM test results, which had a global *R* of 0.799 and was validated through 5000 permutations. The test showed that the studied isolates had significant differences (p=0.0) for an *R* different to 0, indicating that the isolates separation on the base of the metric analysis was well statistically supported. Three primary groups can be distinguished in Figure 3.6.a. Group 1 was formed by *Aphanizomenon* isolates and *Anabaena* sp. LOW1 and was a well-supported group with *R*=0.982, where the lowest *R*s are given ($R \geq 0.721$) by the relationship with *Anabaena* cf. *cylindrica*, which overlapped with this group. The difference between the two *Aphanizomenon* isolate was *R*=0.384, value which reflects a major similarity between them. *Anabaena* sp. LOW1 appeared to be more related to *Aphanizomenon* cf. *gracile* than *Aphanizomenon klebahnii* HHAFA (*R*= 0.37 and 0.7 respectively), indicating strong similarity between *Aphanizomenon* cf. *gracile* and *Anabaena* sp. LOW1. The second group was formed by *Anabaena* cf. *planktonica,* which was close to *Anabaena lemmermannii* ONT1 (*R*=0.761). In addition, *Anabaena* cf. *planktonica* appeared as a separate entity, supported by its *R* value (≥0.939). This isolate showed the major similarity with *Aphanizomenon* cf. *gracile*, therefore according to the ANOSIM it was closer related to group 1 than group 3. The third group was formed by the remaining isolates in which *Anabaena compacta*, *Anabaena* cf. *cylindrica, Anabaena* sp. LONT5, *Anabaena reniformis* MALW1 and *Anabaena variabilis* were in the periphery of the group and their individuality was confirmed by *R* values. These values

were 0.788 between *Anabaena compacta* and *Nostoc* sp. RUP1; 0.794 between *Anabaena* cf. *cylindrica* and *Anabaena lemmermannii* ONT1; 0.68 between *Anabaena* sp. LONT5 and *Anabaena lemmermannii* LONT2; 0.642 between *Anabaena reniformis* MALW1 and *Nostoc* sp. UTCC355, and 0.423 between *Anabaena variabilis* and *Nostoc ellipsosporum* UTEX2383. The remaining isolates appeared to overlap in the center of this group (*Anabaena* cf. *flos-aquae* UTCC64, *Anabaena flos-aquae* UTEX2383, *Anabaena* cf. *lemmermannii, Anabaena lemmermannii* GIOL8, *Anabaena lemmermannii* LONT2, *Anabaena variabilis*, *Nostoc calcicola* UTEXB382, *Nostoc ellipsosporum* UTEX383, *Nostoc* sp. UTCC314, *Nostoc* sp. UTCC355 and *Nostoc* sp. RUP1) presented *R*s less than 0.5, reflecting their considerable similarity with each other than with the other isolates.

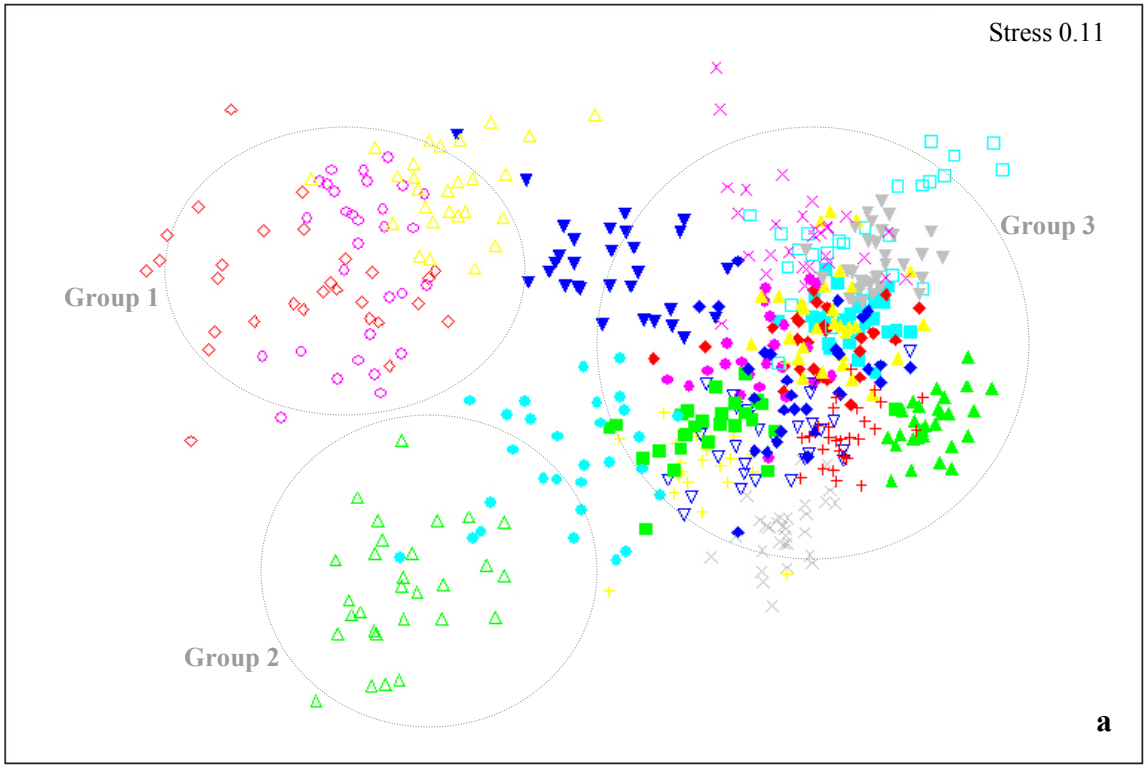
Additionally, an NMDS analysis was performed on the qualitative parameters (Figure 3.6.b). The results showed similarities with the NMDS for metric features. For example, *Aphanizomenon* isolates and *Anabaena* sp. LOW 1 form one independent group and *Anabaena compacta, Anabaena* cf. *cylindrica*, *Anabaena reniformis* MALW1, and *Anabaena variabilis* were separated from the main group (Figure 3.6.a, 3.6.b). One noted difference between the two analyses is that *Anabaena compacta* and *Anabaena reniformis* MALW1 form an independent group whereas in the metric analysis (Figure 3.6.a) they were in the periphery of the second group. Similarly, *Anabaena* cf. *lemmermannii* and *Nostoc* UTCC314 were completely integrated in the group 2 of metric analysis (Figure 3.6.a) and in the qualitative analysis formed independent groups (Figure 3.6.b). Another notable difference was the position of *Anabaena* cf. *planktonica*, which did not form an independent group (Figure 3.6.b). In fact, the qualitative analysis gave

Figure 3.6.a NDMS plot of isolates differentiation on the base of metric features for all the isolates which presented heterocysts and akinetes.

Figure 3.6.b NDMS plot of isolates differentiation on the base of qualitative features for all the isolates which presented heterocysts and akinetes.

.

- *Anabaena compacta*
- *Anabaena* cf. *cylindrica*
- *Anabaena* cf. *flos-aquae* UTCC64
- *Anabaena* cf. *flos-aquae* UTEX2383
- *Anabaena* cf. *lemmermannii*
- *Anabaena lemmermannii* GIOL8
- *Anabaena lemmermannii* LONT2
- *Anabaena* cf. *planktonica*
- *Anabaena* sp. LONT5
- *Anabaena variabilis*
- *Aphanizomenon klebahnii* HHAFA
- *Aphanizomenon* cf. *gracile*
- *Anabaena sp.* LOW1
- *Nostoc calcicola* UTEXB382
- *Nostoc ellipsosporum* UTEX383
- *Nostoc* sp. UTCC314
- *Nostoc* sp. UTCC355
- *Anabaena lemmermannii* ONT1
- *Nostoc* sp. RUP1
- *Anabaena reniformis* MALW1


Stress 0.11
Group 1
Group 2
Group 3
a

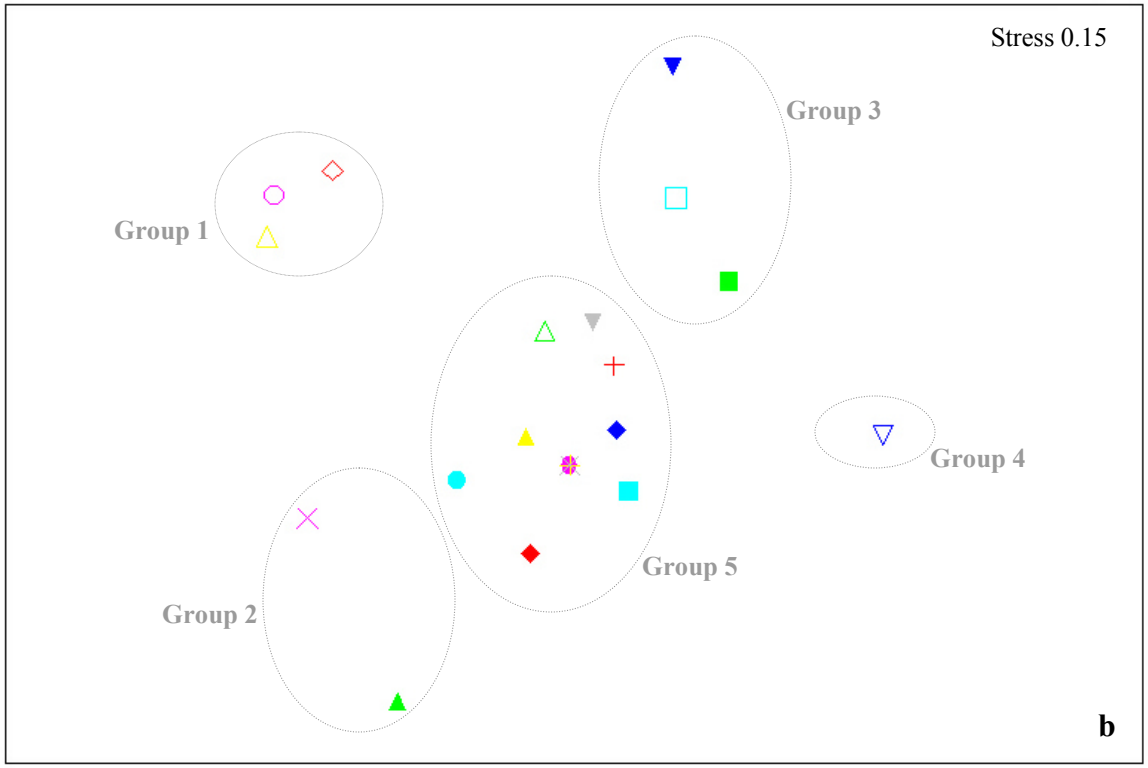

Stress 0.15
Group 1
Group 2
Group 3
Group 4
Group 5
b

Table 3.2 **One-way analysis of similarities (ANOSIM), based on NMDS, and measured from values of Bray-Curtis for the differentiation of the isolates.**

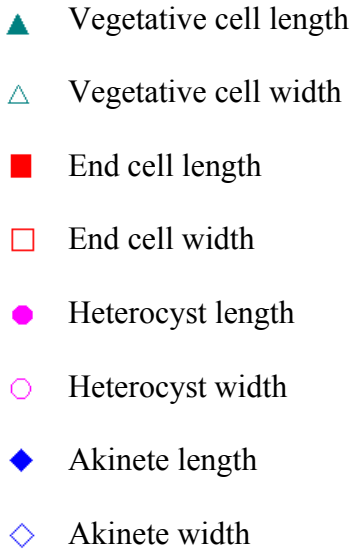
Global Test	
Sample statistic (Global *R*)	0.799
Statistics' significance level (*p*%) for rejecting H_0	>5
Significance level (*p*%) of sample statistic	0.0
Number of permutations	5000
Number of permuted statistics greater than or equal to Global *R*	0

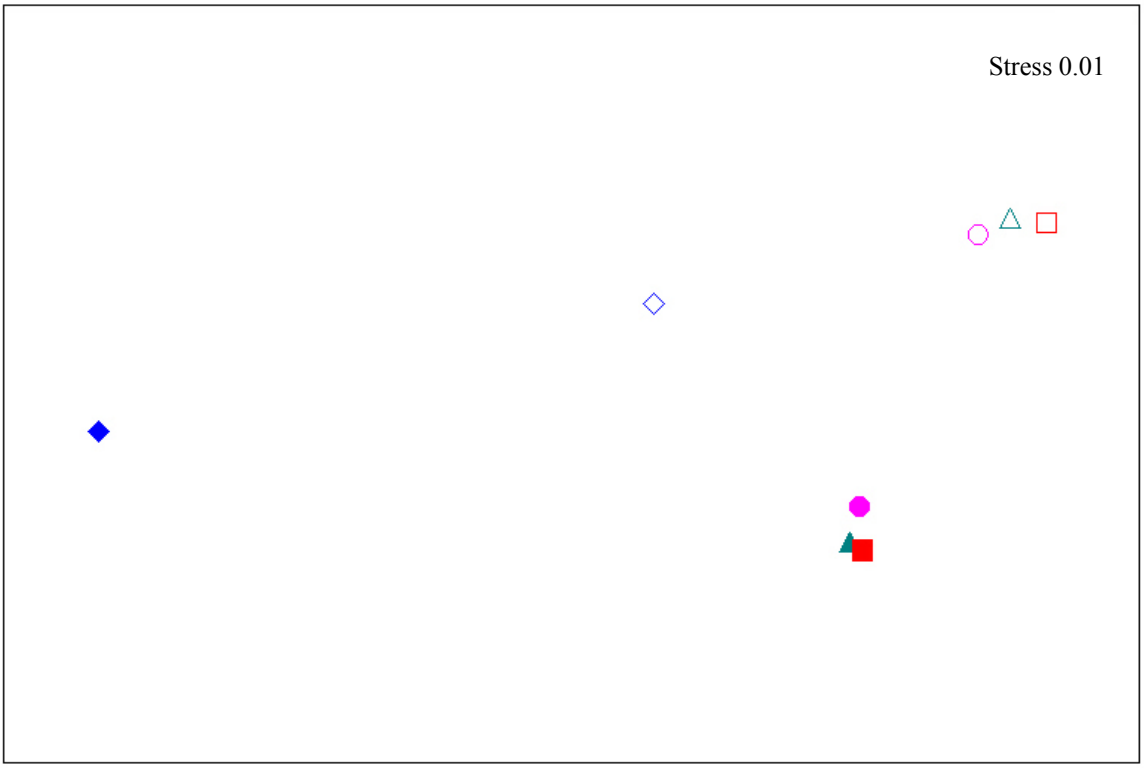
five possible groups (Figure 3.6.b) in which group 1 is formed by *Aphanizomenon* isolates and *Anabaena* sp. LOW 1; group 2 by *Anabaena reniformis* MALW1 and *Anabaena compacta*; group 3 by *Anabaena* cf. *cylindrica, Anabaena variabilis* and *Nostoc* sp. UTCC314; group 4 by *Anabaena* cf. *lemmermannii*, and group 5 for the remaining isolates.

The NMDS analysis (Figure 3.7) determined that akinete length was the most variable character and the most useful for species and strain delineation, since the difference among the measurement of this parameter was greater than the difference among the measurement of the other parameters. This parameter was followed by the akinete width and the rest did not show significant differences for species and strain delineation.

The Kruskal-Wallis test and Dunn's procedure were used to validate the significance of the groups generated by NMDS on the basis of metric parameters (Figure 3.7) and the 95% confidence intervals plotting on the base of the mean results (Figures 3.8-3.11). The Dunn's procedure results were not shown since they were completely coincident with the results of the 95% confidence interval plotting on the base of the mean. Table 3.3 summarized the Kruskal-Wallis test results for each metric parameter. This test had in general, high H values, which were validated through 1000 randomizations and showed that the metric parameters were significantly different ($p<0.05$). Hence, each metric parameter itself was significantly different for each isolate and hence useful for differentiation. Akinete length had the highest value of H (504.76), which confirms that this character is the most useful for the isolate differentiation. This

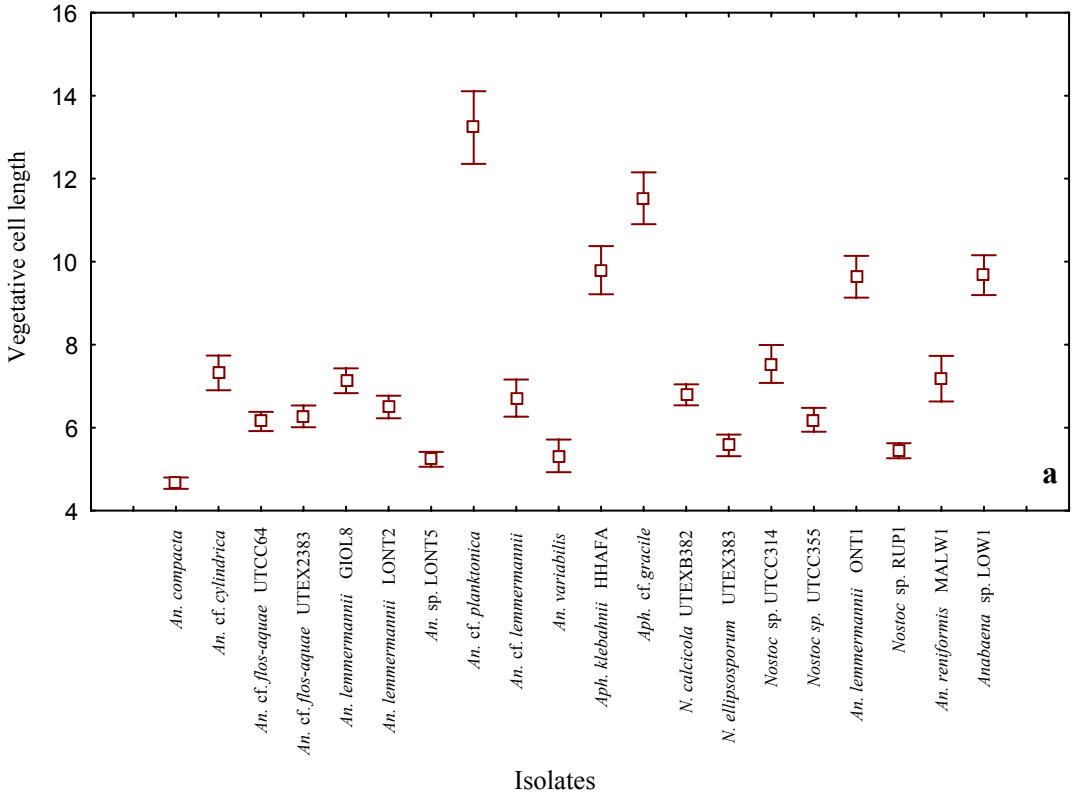
Figure 3.7 **NDMS plot of metric parameters differentiation on the base of the isolates measurements for all the isolates for heterocysts and akinetes.**




Stress 0.01

higher value was due to the *Aphanizomenon* isolates, which had akinetes longer than the remaining isolates (Figure 3.11.a). In general, Figures 3.8-3.11 show the distribution (mean and 95% confidence interval) of a specific parameter measurement among the isolates. For example, the vegetative cell length (Figure 3.10.a) showed the *Anabaena* cf. *planktonica* isolate grouping with the *Aphanizomenon* isolates; a relationship not observed in vegetative cells' width plotting. In fact this *Anabaena* isolate was completely separated from the genus *Aphanizomenon* based on this metric parameter. Indeed, this was observed in each metric parameter, where *Anabaena* cf. *planktonica* was closer related to *Aphanizomenon* isolated in lengths measurements and completely separated to them in width measurements. Another example was *Anabaena* cf. *cylindrica*, which grouped closer to *Aphanizomenon* isolates in almost all metric parameters with the exception of vegetative cells, end cells and akinetes lengths. And *Nostoc calcicola* UTEXB382 was always related to *Nostoc* sp. RUP1, less in vegetative cells and heterocysts widths.

Figure 3.8 **Mean plot (Confidence Interval: ±0.95) of vegetative cell length (a) and width (b).**


Vegetative cell length
16
14
12
10
8
6
4
a
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

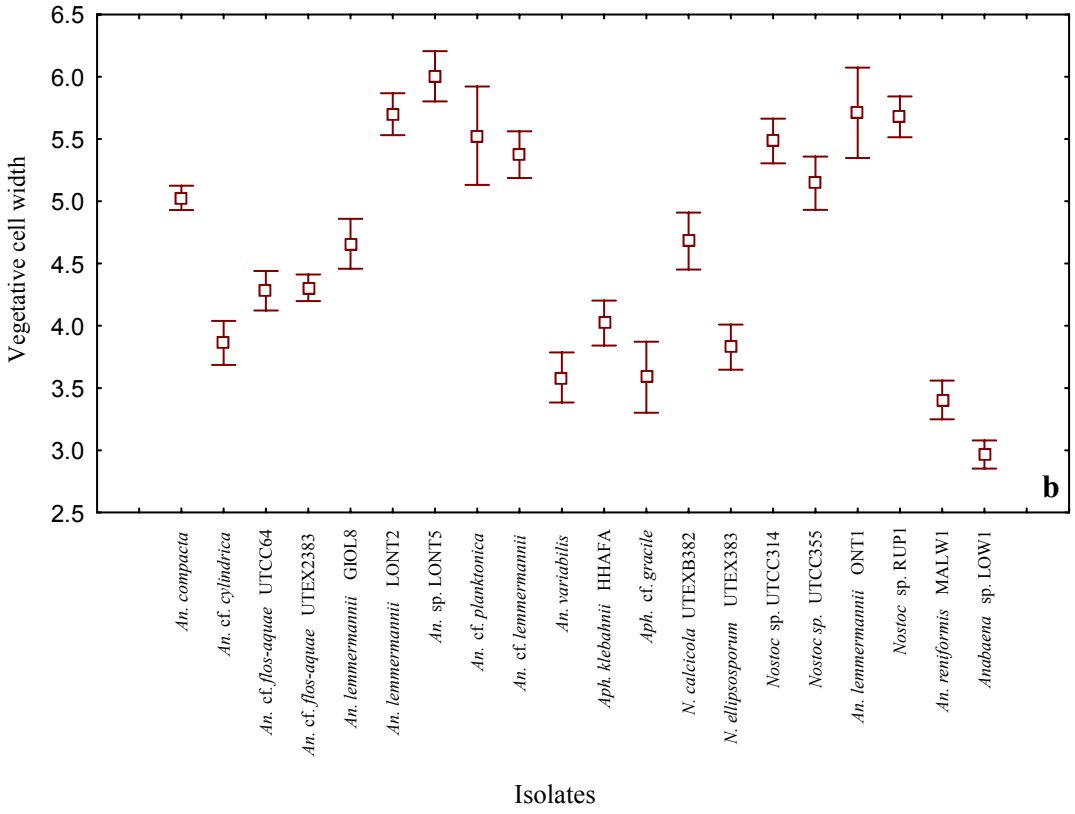
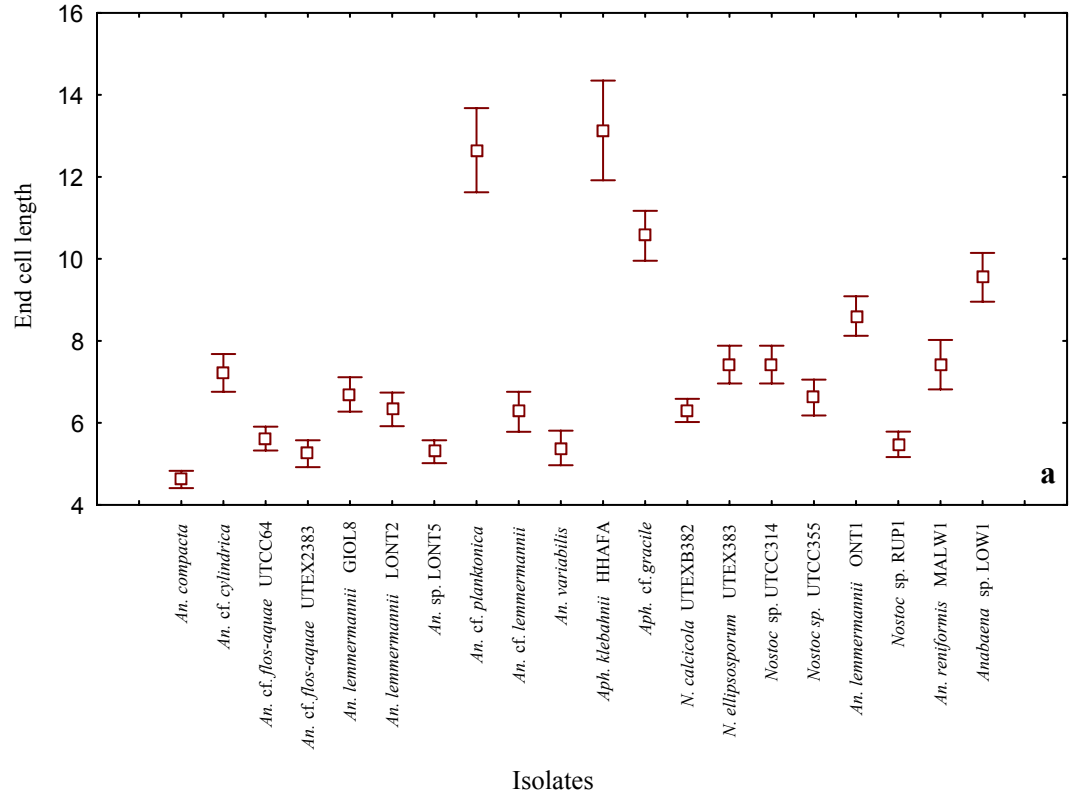

Vegetative cell width
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
b
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

Figure 3.9 **Mean plot (Confidence Interval: ±0.95) of end cell length (a) and width (b).**


End cell length
16
14
12
10
8
6
4
a
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

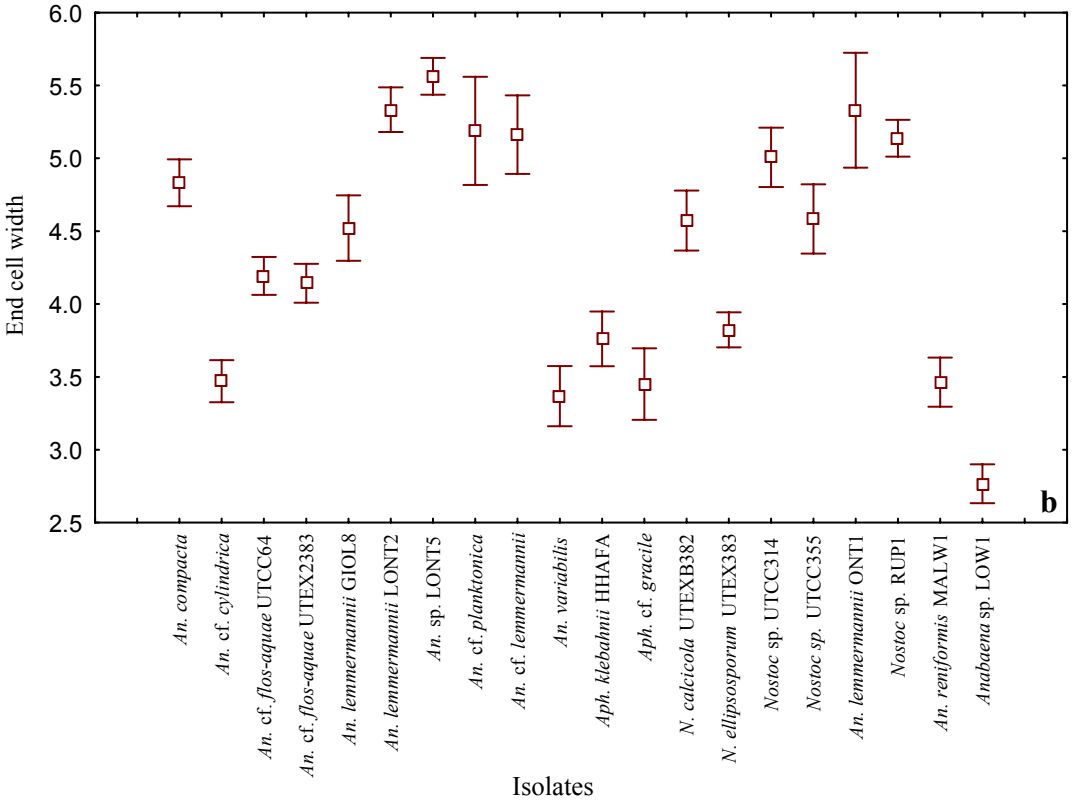
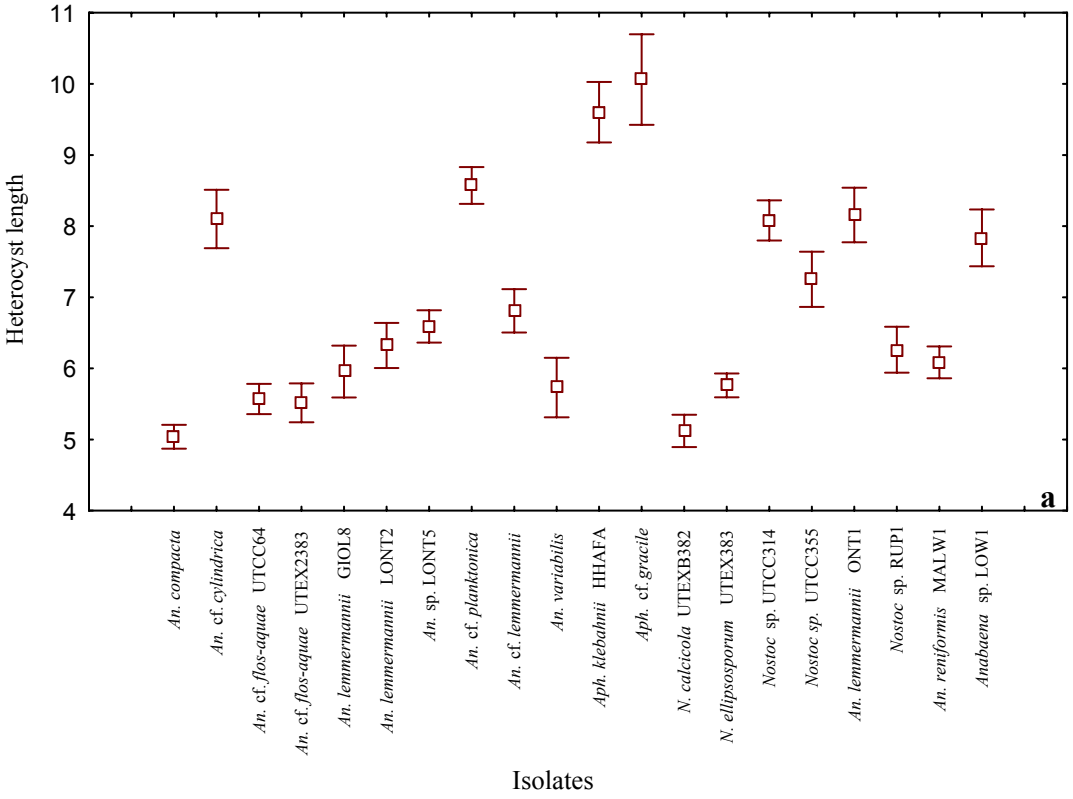

End cell width
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
b
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

Figure 3.10 **Mean plot (Confidence Interval: ±0.95) of heterocyst length (a) and width (b).**


Heterocyst length
11
10
9
8
7
6
5
4
a
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

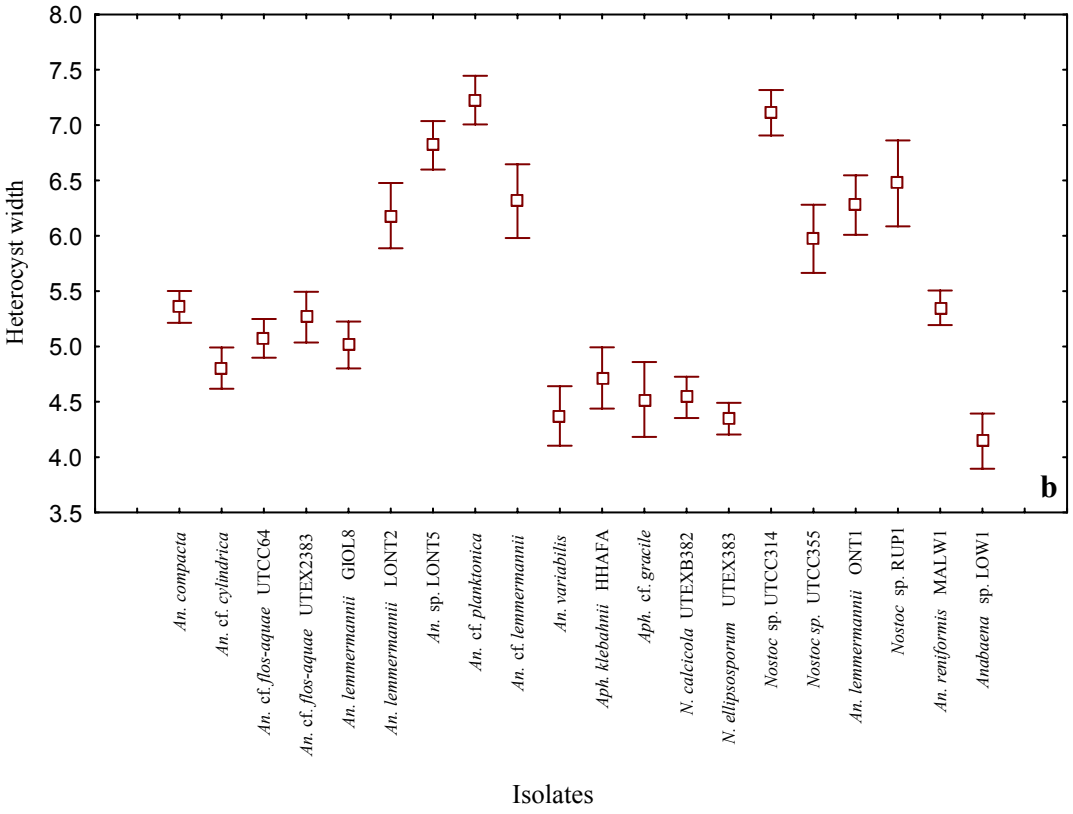
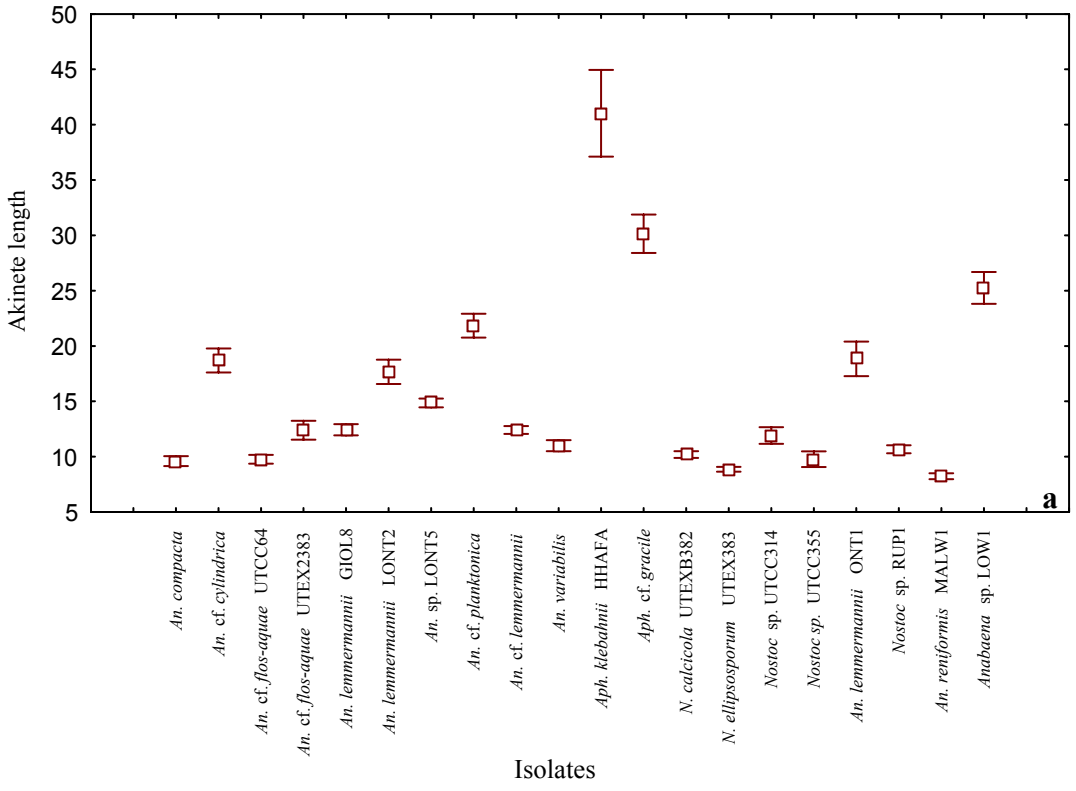

Heterocyst width
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
b
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

Figure 3.11 **Mean plot (Confidence Interval: ±0.95) of akinete length (a) and width (b).**


Akinete length
50
45
40
35
30
25
20
15
10
5
a
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

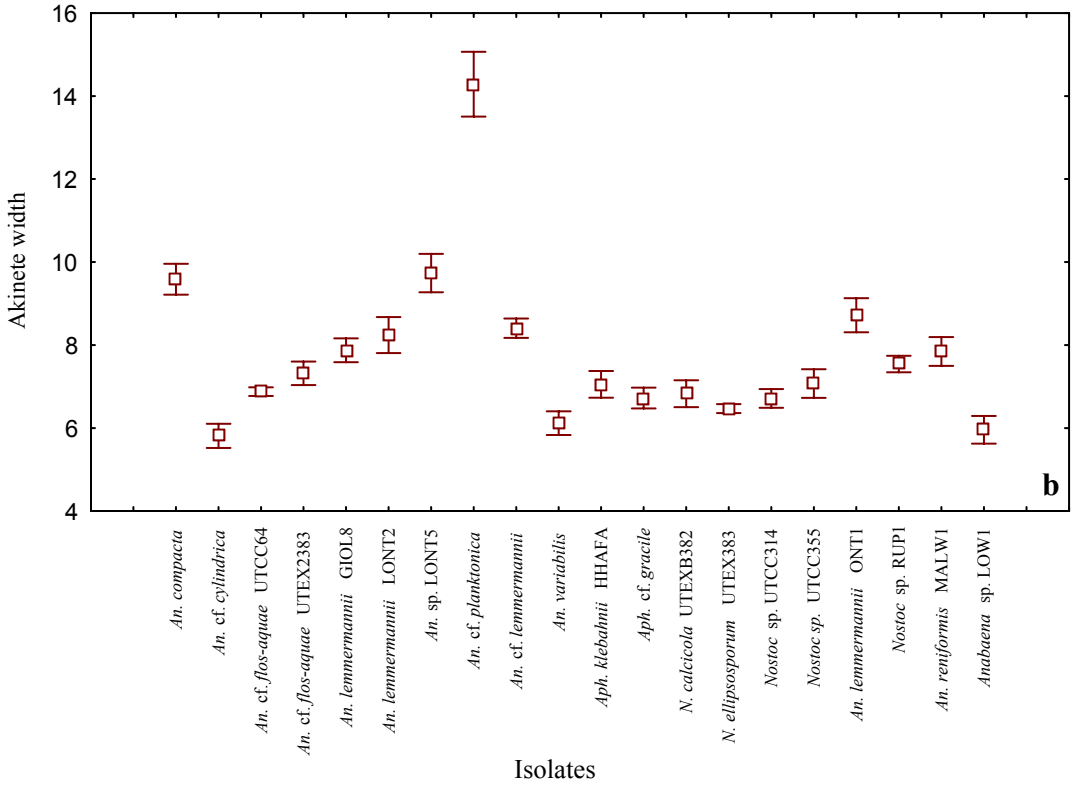

Akinete width
16
14
12
10
8
6
4
b
An. compacta
An. cf. cylindrica
An. cf. flos-aquae UTCC64
An. cf. flos-aquae UTEX2383
An. lemmermannii GIOL8
An. lemmermannii LONT2
An. sp. LONT5
An. cf. planktonica
An. cf. lemmermannii
An. variabilis
Aph. klebahnii HHAFA
Aph. cf. gracile
N. calcicola UTEXB382
N. ellipsosporum UTEX383
Nostoc sp. UTCC314
Nostoc sp. UTCC355
An. lemmermannii ONT1
Nostoc sp. RUP1
An. reniformis MALW1
Anabaena sp. LOW1
Isolates

Table 3.3 Kruskal-Wallis Test results for each metric parameter.

Cellular parameter	N	H	*p*
Vegetative cell length	567	438.86	<0.05
Vegetative cell width	566	432.37	<0.05
End cell length	562	402.45	<0.05
End cell width	567	409.03	<0.05
Heterocyst length	567	426.19	<0.05
Heterocyst width	567	404.44	<0.05
Akinete length	566	504.76	<0.05
Akinete width	567	403.61	<0.05

3.3 PHYLOGENETIC RELATIONSHIPS OF THE STUDIED ISOLATES

The 16S rRNA gene was amplified and sequenced for 29 isolates of the genera *Anabaena*, *Aphanizomenon* and *Nostoc* and are noted in table 2.1. Additional sequences obtained from GenBank including the one outgroup sequence. The *efp* gene was sequenced for 34 isolates of the three studied genera (Table 2.1), and 2 sequences and one outgroup were obtained from GenBank. The obtained sequences were approximately 1300 bp and 415 bp for 16S rRNA and *efp* genes respectively.

3.3.1 16S rRNA GENE SEQUENCE ANALYSES

In the Neighbour-joining (NJ) analysis five clusters were formed (Figure 3.12). Cluster 1 contained only benthic *Nostoc* species with mucilaginous sheaths. In this cluster *Nostoc* sp. RUP1 and *Nostoc punctiforme* UTCC41 clustered together with a high bootstrap value (98%), and these two species formed a cluster with *Nostoc calcicola* UTEXB382 with a lesser support (65%). Cluster 2 contained isolates from the three studied genera with evident gas vesicles and hence formed a well-supported (100% bootstrap) planktonic cluster. Cluster 3 was a highly supported cluster as well (100% bootstrap) that contained *Anabaena* and *Nostoc* isolates without gas vesicles, with the exception of *Anabaena lemmermanni* LONT2, which showed evident gas vesicles within the filaments (Figure 3.1.g). In spite of this, the sequence divergence among *Anabaena lemmermanni* LONT2 and the remaining isolates of this cluster was very low (between 0.007 and 0.036), and the bootstrap with the closer isolate (*Nostoc* sp. UTCC314) was 85%. Cluster 4 contained the isolates that had few and small gas vesicles within the cell and just in some cells within the filament and in some filaments of the culture but not all filaments. They were considered like “no-evident gas vesicles”, since the isolates with

"evident gas vesicles" (Cluster 2) showed the cells completely covered with large gas vesicles, which occupy at least the 50% of the cell surface. There were two exceptions to this, for example *Anabaena reniformis* MALW1, which clearly had gas vesicles but was not supported by bootstrap (<50%). Moreover, this was the isolate with the high divergence with the rest of the cluster (between 0.074 and 0.1). And the other one was *Nostoc* sp. UTCC387 in which gas vesicles were never observed despite moderate bootstrap support (80%) and low sequence divergences (between 0.008 and 0.078). Finally, cluster 5 contained only one isolate, *Anabaena* cf. *cylindrica*, which did not appear to have gas vesicles and it was separated from the remainder of the cluster and only weakly associated with a low bootstrap value (60%) and high sequence divergence (between 0.033 and 0.082).

The Maximum-parsimony (MP) showed some differences in comparison to the NJ analysis; these differences were among clusters and among isolates within a cluster (Figure 3.13). First, *Nostoc calcicola* UTEXB382 was not included in cluster 1 of NJ analysis, this isolate was closer related to cluster 3 and 5, although this questionable position was reflected in the low bootstrap support (<50%). Cluster 2 was maintained in both topologies. But, some relationships were different, like the position of *Anabaena lemmermannii* GIOL8 and *Anabaena lemmermannii* ONT1. In the case of the MP analysis, the clade formed by these isolates clustered with the rest of the cluster with 71% bootstrap value (77 Bayesian posterior probability). Instead, in the NJ they clustered with *Anabaena* sp. LOW1, *Aphanizomenon* cf. *gracile* and *Aphanizomenon* sp. PCC7905, the remainder of the isolates was outside of this cluster. This ambiguous position was supported by a bootstrap lesser than <50%. In the MP analysis, the isolates of cluster 3,

Nostoc calcicola UTEXB382 and *Anabaena* cf. *cylindrica* were not contained in a consistent clade, and it appears that *Anabaena* cf. *cylindrica* and *Nostoc calcicola* UTEXB382 were more closely related to cluster 3 than was seen in the NJ analysis. This would reflect the absence of gas vesicles in these isolates and the low divergences among them (0.032753 and 0.040769). Finally, *Anabaena reniformis* MALW1 clustered completely out of the tree, although this relationship was not well supported (<50%). The *Anabaena reniformis* MALW1 position was different in the three topologies, since in the NJ analysis was related to cluster 4, and in ML with *Anabaena* cf. *cylindrica* (Cluster 5), which clade was related with cluster 3. Clusters 3, 4, and 5 never presented evident gas vesicles, and *Anabaena reniformis* MALW1 showed them in culture, so MP analysis could reflect the separation of this isolate better in relation to the presence of gas vesicle.

The Maximum-likelihood (ML) and Bayesian analysis were very similar, with the only exception that in the ML analysis cluster 1 resulted more closely related to the remaining clusters than cluster 4, instead in the Bayesian analysis cluster 1 and 4 were related in the same way with the rest of the isolates, although this relationship was low supported (<50 Bayesian posterior probability) (Figures 3.14, 3.15). Another difference was that *Anabaena* sp. LONT5 and *Anabaena* sp. A2879 were more closely related in the Bayesian analysis with 61 Bayesian posterior probabilities. In general both topologies were similar to NJ analysis in terms of the isolates that formed each cluster, but the relationship among cluster was more similar to MP analysis. One case was *Nostoc calcicola* UTEXB382, which was not included in cluster 1; what was in concordance with MP analysis, in fact in the Bayesian analysis this relationship was supported by a Bayesian posterior probability of 61 and in the MP with a bootstrap value of 51%. Cluster

2 was maintained in the three topologies, but the relationship with the other clusters was different. In ML and Bayesian analysis cluster 2 was related to clusters 3, 5 and *Anabaena reniformis* MALW1, instead in the NJ analysis it was related to clusters 1 and 3. In fact the relationships shown in ML and Bayesian analysis were better supported than the results obtained in the NJ analysis (62 Bayesian posterior probability and >50% respectively). On the other hand, in MP analysis Cluster 2 was related with the rest of the isolate with a 57% bootstrap value.

Cluster 3 contained the same isolates that NJ analysis, but here it presented a closer relationship to *Anabaena reniformis* MALW1 and *Anabaena* cf. *cylindrica*, like MP analysis, where cluster 3 did not form a consistent cluster. And cluster 4 was the same as well, but it was not related with *Anabaena reniformis* MALW1. The relationship of *Anabaena* cf. *cylindrica*, cluster 5 of NJ, was completely different, since in this case it clustered within cluster 3, and specifically with *Anabaena reniformis* MALW1.

3.3.2 *EFP* GENE SEQUENCE ANALYSES

Similar to the 16S rRNA gene sequence analyses, the isolates from genera *Anabaena*, *Aphanizomenon* and *Nostoc* were intermixed in the phylogenetic analysis of *efp* gene sequences. Hence, both genes cannot discriminate among strains of these genera. However, with the *efp* gene there appears to be a better resolution of *Aphanizomenon* isolates, since they formed a well supported cluster (100%) in the four topologies, considering that this cluster was within the complete tree in the NJ and ML analysis. (Figures 3.16, 3.18), although in the MP and Bayesian analyses it was related in the same way with clusters 2, 3 and 4, although these relationships always resulted with low bootstrap values and Bayesian posterior probability (>50%) (Figures 3.17, 3.19).

Moreover, this gene could not discriminate among benthic and planktonic species, although some clusters were only formed by planktonic strains (cluster 1) and benthic strains (cluster 4).

In the NJ analysis four clusters were formed (Figure 3.16). Cluster 1 contained all the studied *Aphanizomenon* isolates and was a well supported cluster with bootstrap support of 100%. Cluster 2 was formed by *Anabaena* and *Nostoc* isolates, where almost all the isolates formed a clade with a bootstrap support value of 100%, with the exception of *Anabaena reniformis* MALW1 and *Nostoc* sp. UTCC355, which clustered out of this well-supported clade. Additionally, in this cluster almost all the isolates maintained in culture conditions were characterized by the presence of gas vesicles, with the exception of *Anabaena* cf. *oscillariodes* and *Nostoc* sp. UTCC387. The bootstrap value support for these isolates was 71% for the clade that contained *Anabaena* cf. *oscillariores* and <50% for the clade that contained *Nostoc* sp. UTCC387. Cluster 3 contained the *Anabaena* isolates without gas vesicles and was a well-supported cluster (bootstrap value of 100%), with the exception of *Anabaena lemmermannii* LONT2, which showed evident gas vesicle under culture conditions. Finally, cluster 4 contained *Nostoc* isolates with mucilaginous sheath and without gas vesicles, and was well-supported by bootstrap (100%), although it did not include *Nostoc ellipsosporum* UTEX383 and *Nostoc calcicola* UTEXB382, which were characterized for the presence of both morphological characters. These two isolates had divergences in relation to the other isolates that form mucilaginous sheath within 0.292 and 0.416, which was high in relation to the whole divergence matrix (0-1.006).

The MP analysis gave similar results to NJ analysis, since the clusters were maintained, although there were some differences in the relationships within the clades (Figure 3.17). The main difference was that *Anabaena reniformis* MALW1 clustered completely out of Cluster 2, although in NJ analysis this relationship was related but was not well supported (<50%). Cluster 1, in this case had lower bootstrap values, and although *Aphanizomenon* continued being a well-defined clade. Cluster 2 was very similar in both topologies, although some relationships were now better defined. One case was the well supported relationship (bootstrap value of 99%) between *Nostoc ellipsosporum* UTEX383 and *Nostoc* sp. UTCC106, which were not closely related in the NJ, where it was clustered with *Nostoc* sp. UTCC387 with a bootstrap value lower than 50%, although these differences between both topologies were not reflected in the divergences (0.409 and 0.407 for NJ and MP respectively). Another case was *Anabaena lemmermanni* GIOL8 and *Anabaena compacta* (74% bootstrap value); instead in NJ they were no closely related.

The ML analysis was more similar to NJ than MP analysis, only some small intra-cluster differences could be observed (Figures 3.16-3.18). For example, *Nostoc* sp. UTCC387 and *Nostoc ellipsosporum* UTEX383 were related to *Anabaena* cf. *planktonica* and formed one cluster. The close relationship between *Nostoc* sp. UTCC87 and *Anabaena* cf. *planktonica* was supported by their divergence (0.03), contrary the divergence between *Nostoc ellipsosporum* UTEX383 and *Anabaena* cf. *planktonica* was higher, supporting their separation in the NJ analysis.

The Bayesian tree was more similar to MP analysis than NJ and ML analysis, mainly in the relationship among clusters, although these relationships always resulted in

low support (<50) (Figures 3.16-3.19). But the isolates' distribution within the cluster was similar among all topologies, although these showed some differences. For example, *Aphanizomenon klebahnii* HHAFA and *Aphanizomenon flos-aquae* UTEXLB2384 were better supported in NJ and Bayesian analyses (100%/ Bayesian posterior probability) than MP analysis (55%). The same could be observed in cluster 4, which showed supports of 100% in NJ analysis, 99 Bayesian posterior probability and 97% in MP analysis. In relation with cluster 3, Bayesian analysis, like MP analysis, considered *Anabaena variabilis* more closely related to the rest of the isolates than *Anabaena* cf. *cylindrica*, instead the NJ and ML topologies considered *Anabaena* cf. *cylindrica* closer related than *Anabaena variabilis*. In fact these relationships were better supported in Bayesian analyses (100 Bayesian posterior probability and 94% respectively) than NJ analysis (50%). In general cluster 2 was very similar to NJ, MP and ML analysis, although it was closer related to ML analysis. Since in both topologies *Anabaena* cf. *planktonica*, *Nostoc* sp. UTCC387 and *Nostoc ellipsosporum* UTEX383 formed one independent cluster (66 Bayesian posterior probabilities).

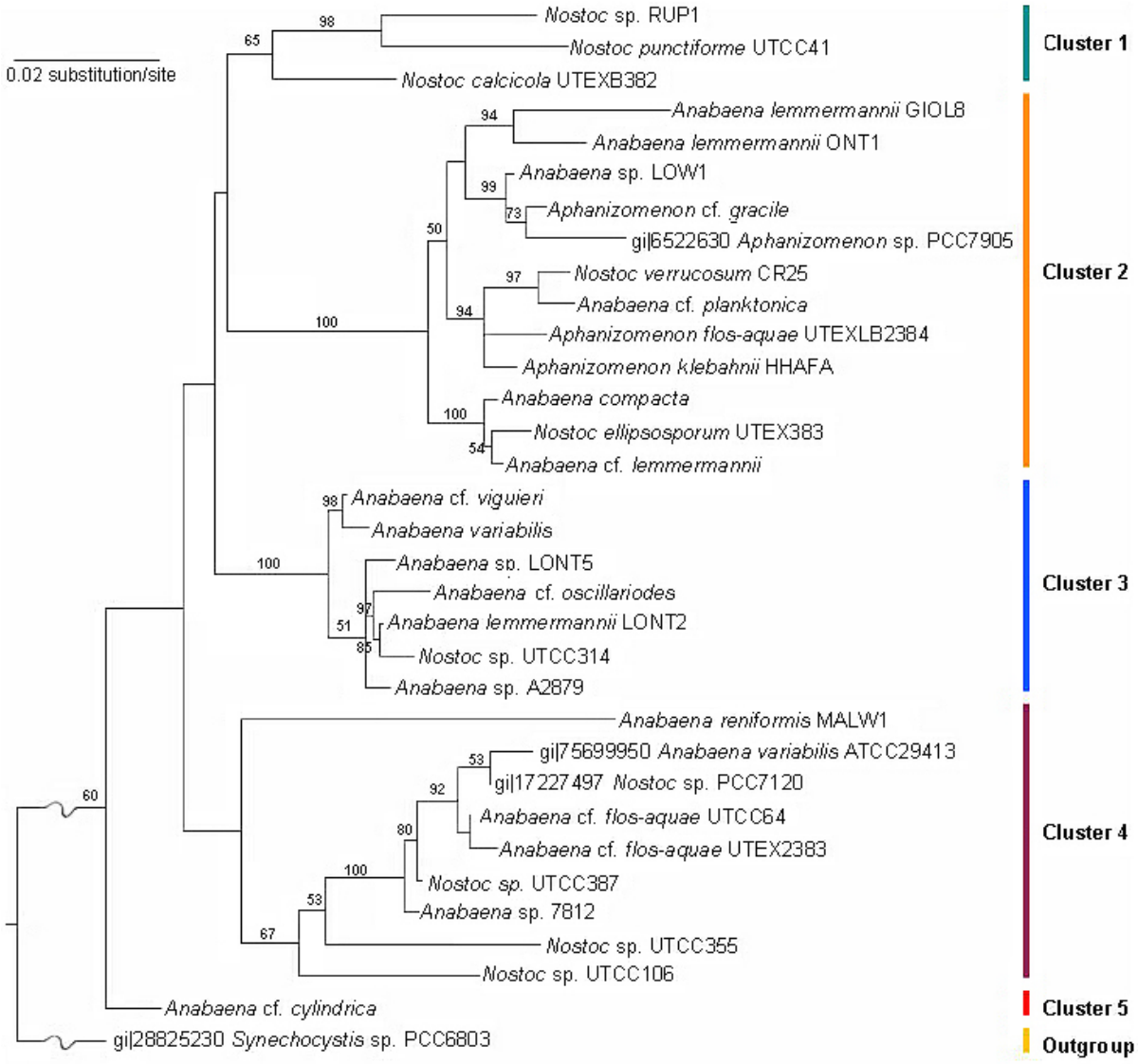


Figure 3.12 Neighbour-joining tree of 32 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using 16S rRNA gene sequences (1308 bp), divided in 5 main clusters. Numbers at nodes represent bootstrap percentages of 1000 replicates; only values ≥ 50% are shown. Tree length=680.

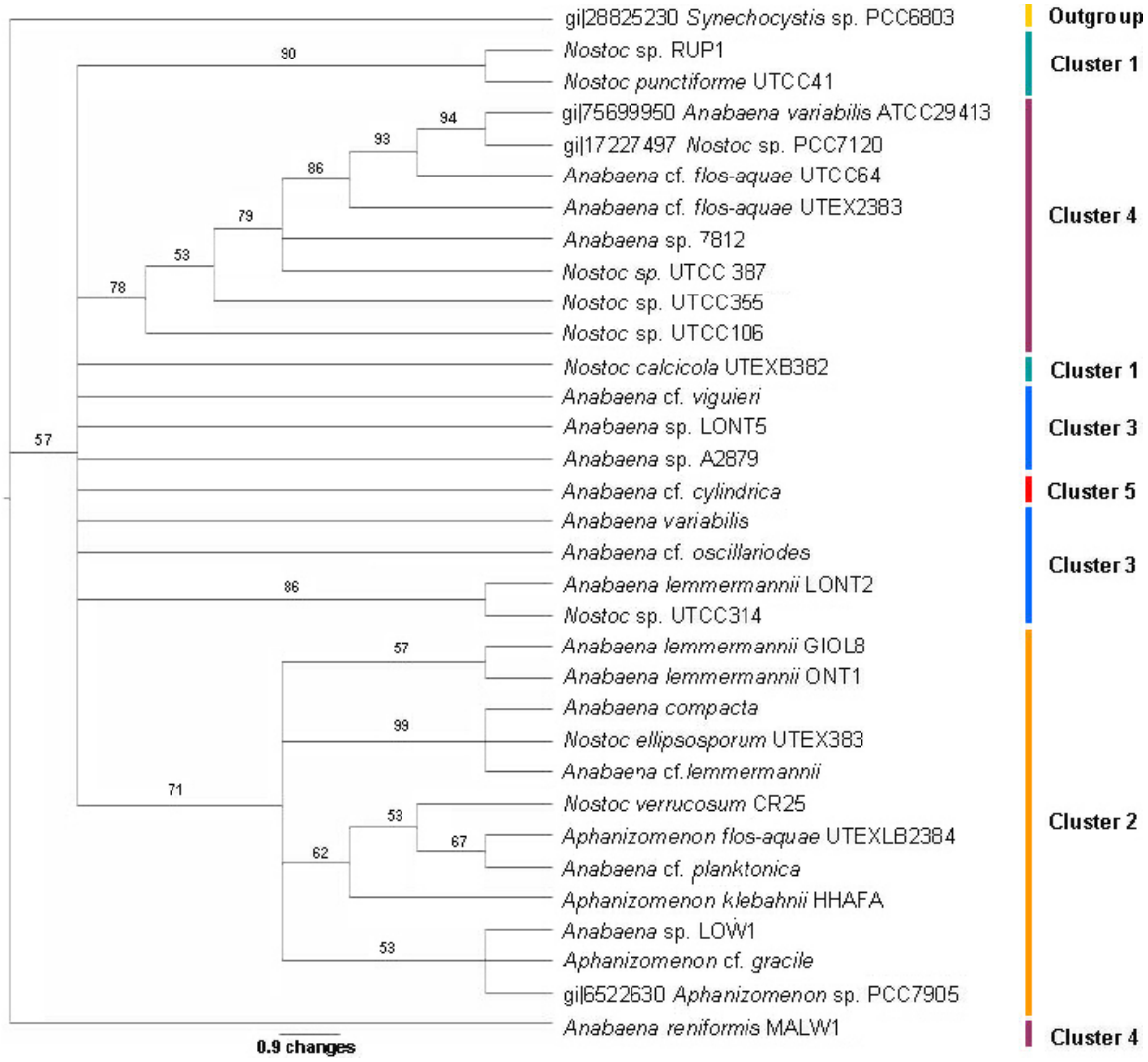


Figure 3.13 **Maximum-parsimony phylogenetic tree of 32 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using 16S rRNA gene sequences (1308 bp), showing the difference of clustering in relation with the NJ analysis.** Numbers at nodes represent percentages of 1000 bootstrap replicates; only values ≥ 50% are shown. Tree length=682. Number of parsimony-informative characters=175. CI value=0.5147.

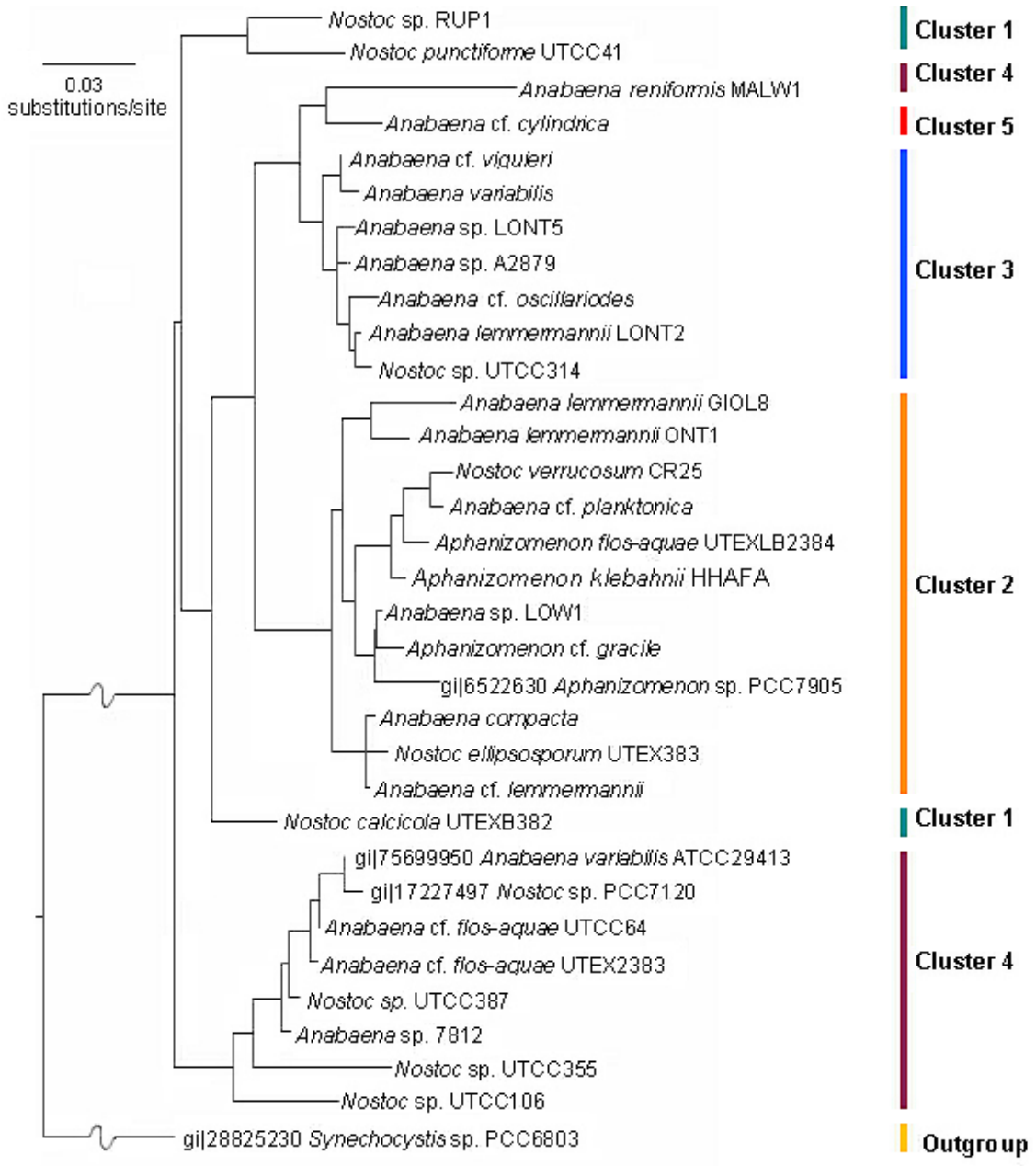


Figure 3.14 **Maximum-likelihood phylogenetic tree of 32 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using 16S rRNA gene sequences (1308 bp), showing the difference of clustering in relation with the NJ analysis**. Tree length=674. Ln=5262.65963.

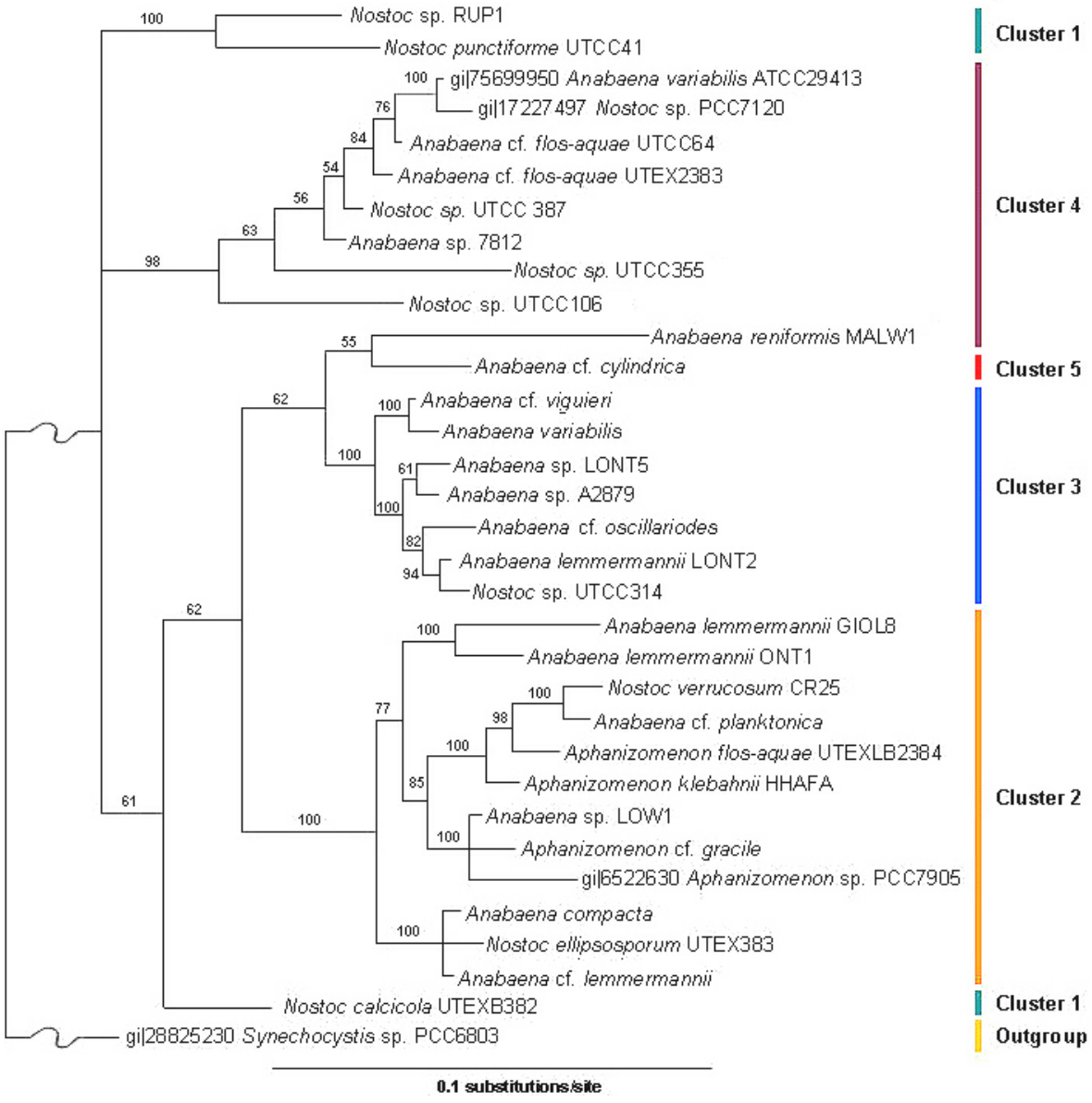


Figure 3.15 Bayesian phylogenetic tree of 32 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using 16S rRNA gene sequences (1308 bp), showing the difference of clustering in relation with the NJ analysis. Numbers at nodes represent bootstrap percentages of 1000 replicates; only values ≥ 50% are shown. Tree length=680.

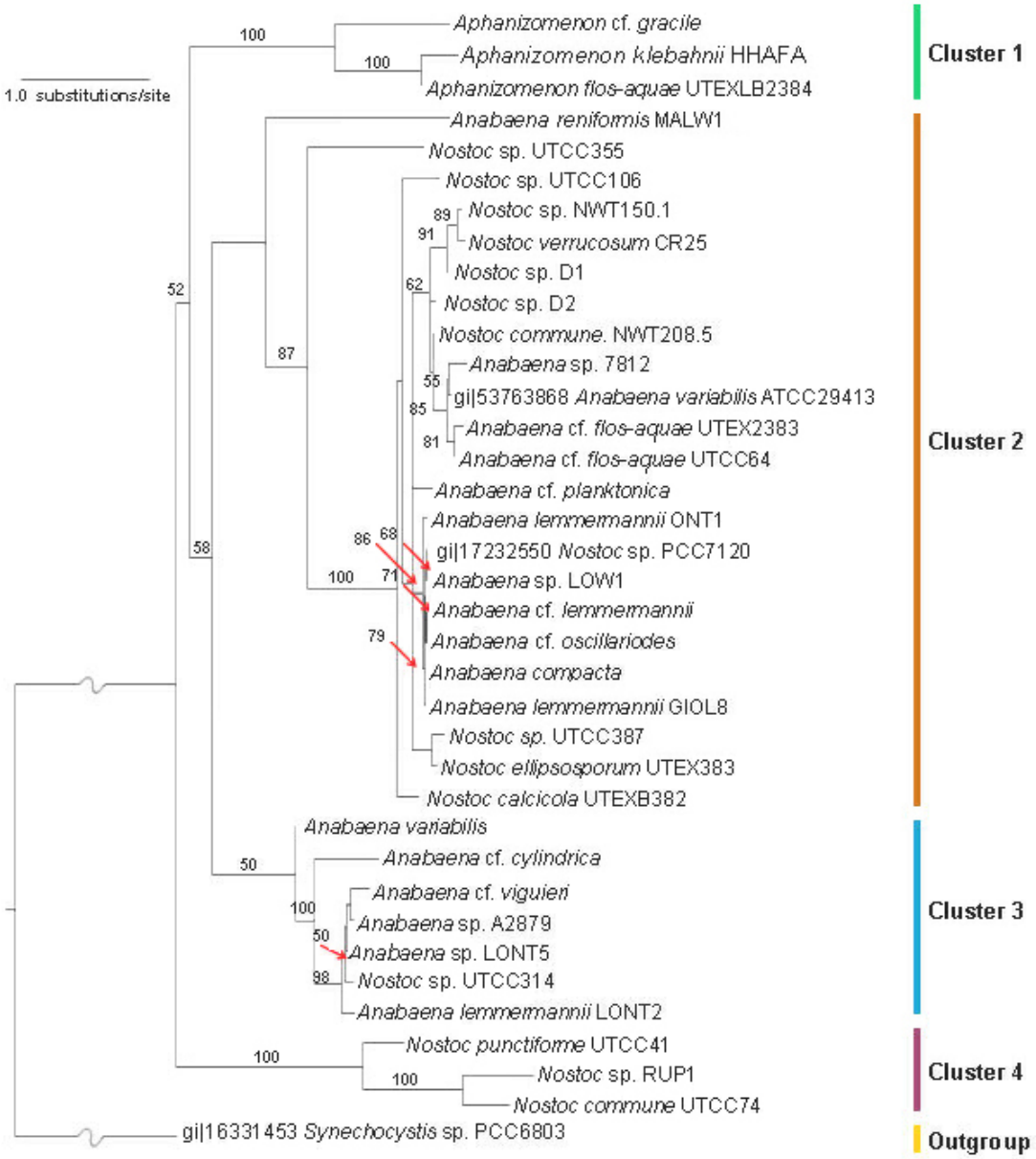


Figure 3.16 **Neighbour-joining tree of 36 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using *efp* gene sequences (419 bp), divided in 4 main clusters.** Numbers at nodes represent bootstrap percentages of 1000 replicates; only values ≥ 50% are shown. Tree lengh=520.

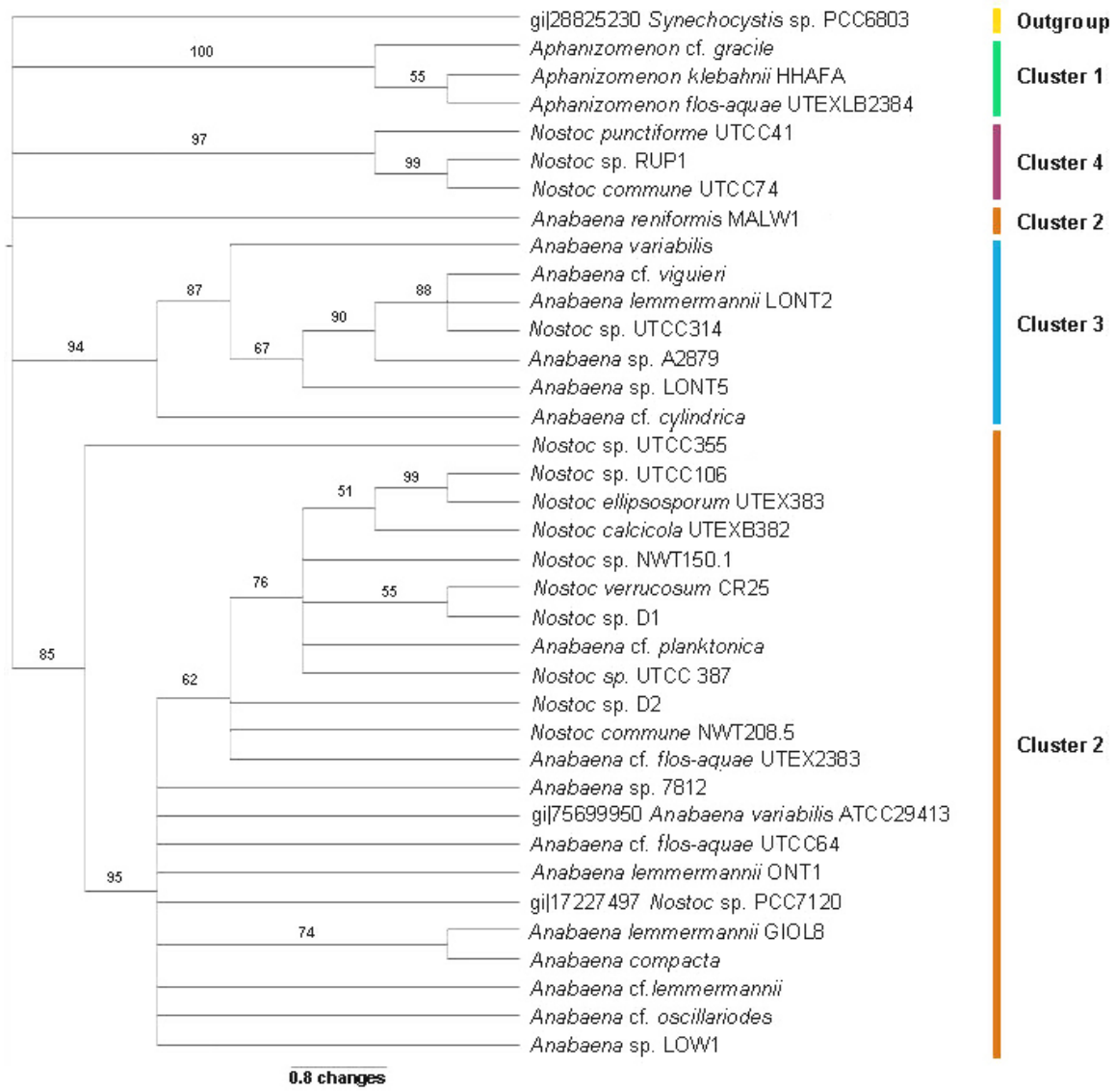


Figure 3.17 Maximum-parsimony phylogenetic tree of 36 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using *efp* gene sequences (419 bp), showing the difference of clustering in relation with the NJ analysis. Numbers at nodes represent percentages of 1000 bootstrap replicates; only values ≥ 50% are shown. Tree length=531. Number of parsimony informative characters=145. CI value=0.6271.

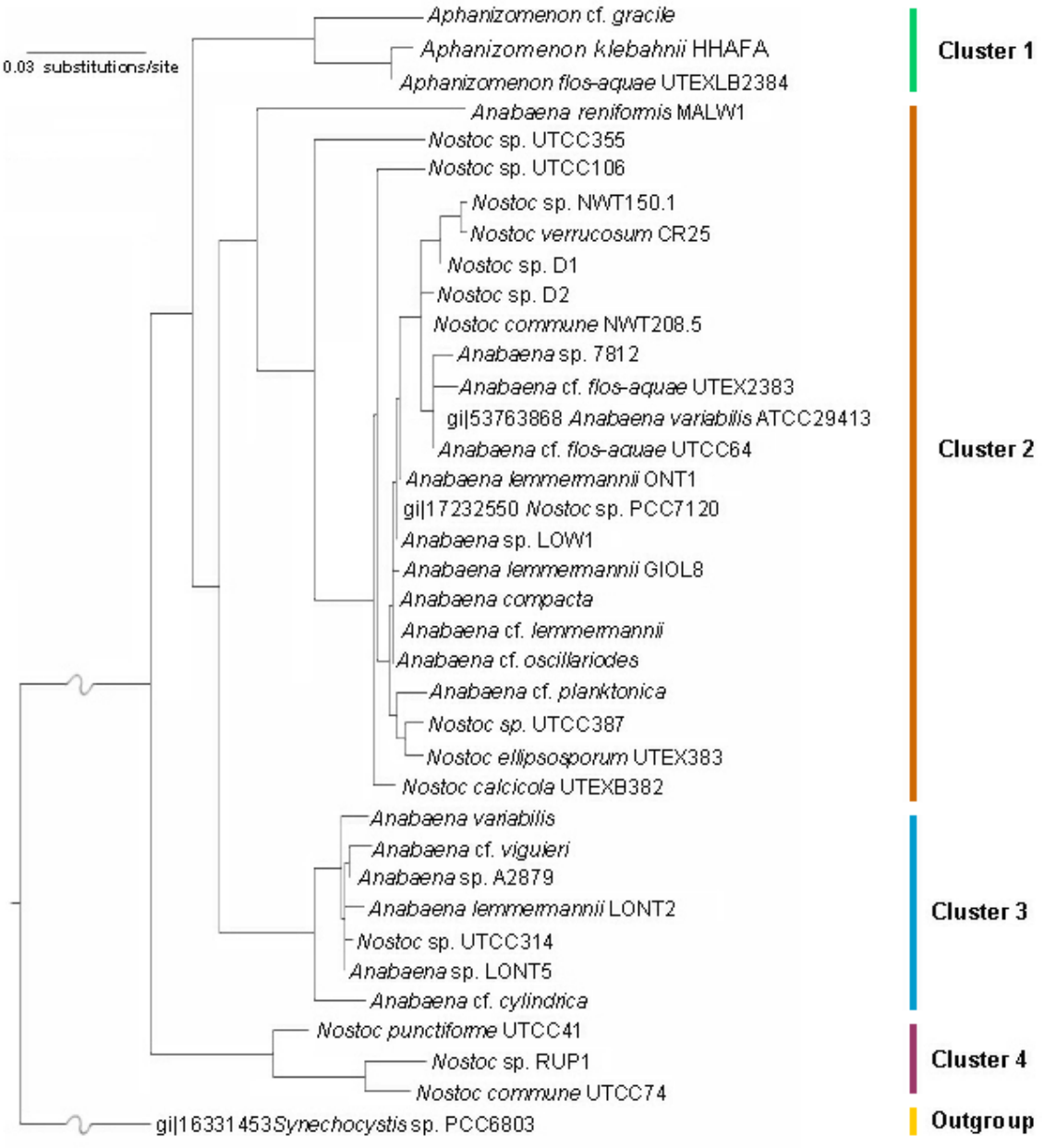


Figure 3.18 Maximum-likelihood phylogenetic tree of 36 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using *efp* gene sequences (419 bp), showing the difference of clustering in relation with the NJ analysis. Tree length=514. Ln=2905.34782.

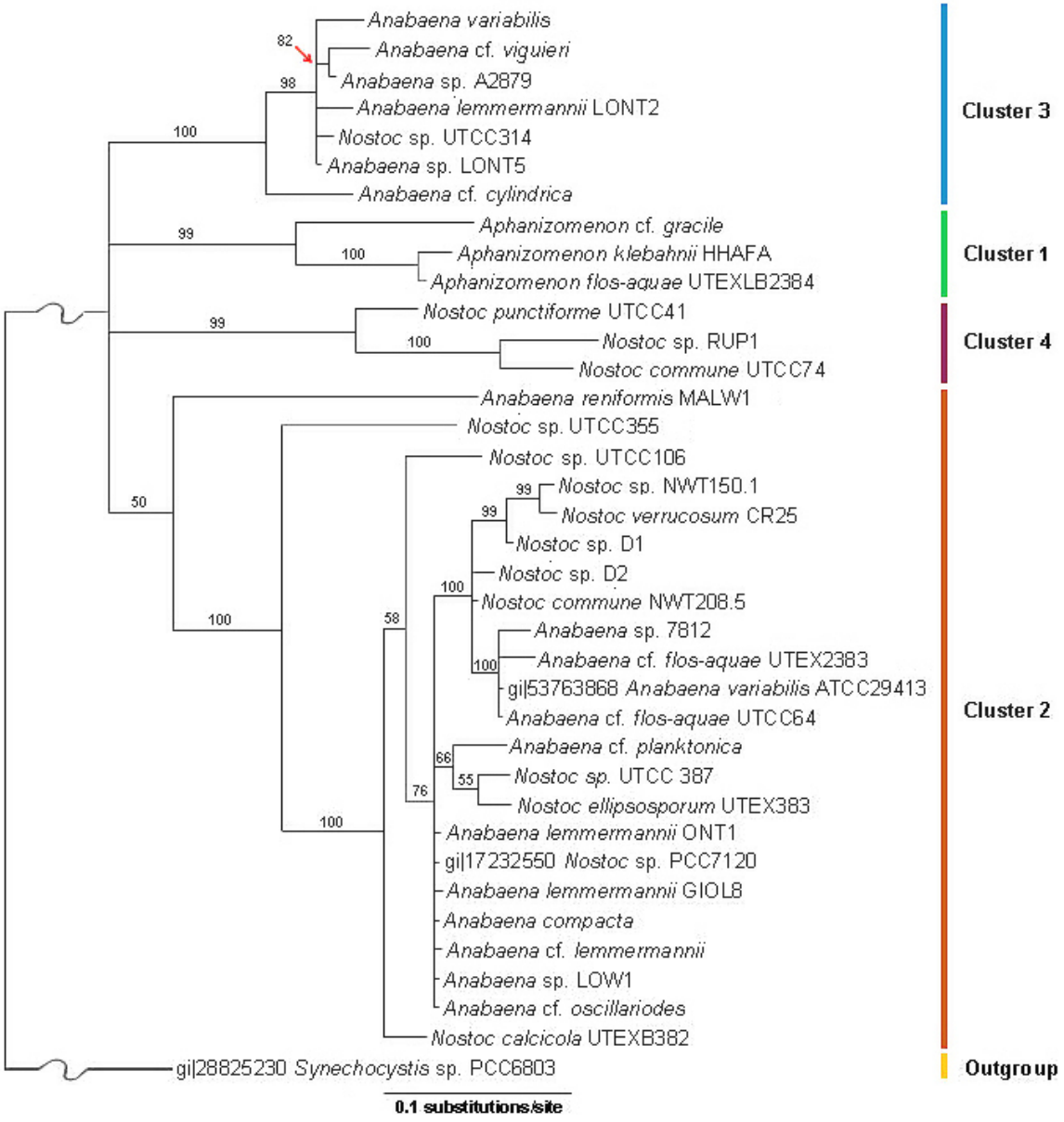


Figure 3.19 **Bayesian phylogenetic tree of 36 *Anabaena, Aphanizomenon* and *Nostoc* species and strains, constructed using *efp* gene sequences (419 bp), showing the difference of clustering in relation with the NJ analysis**. Numbers at nodes represent bootstrap percentages of 1000 replicates; only values ≥ 50% are shown. Tree length=527.

CHAPTER 4: DISCUSSION

4.1 ISOLATES DESCRIPTIONS AND IDENTIFICATION

The identification of some isolates was problematic due to differences among published descriptions and actual features noted in the cyanobacterial cultures, such as the cellular and filament appearances, and width and length of vegetative cells, end cells, heterocysts and akinetes. In addition, some of the attributes varied or changed under culture conditions and akinetes and heterocysts induction was not always successful. This latter point is crucial as these characteristics are essential for the identification of heterocystous cyanobacteria. These difficulties and challenges will be discussed in detail as will the discrepancies with the published literature. Table 4.1 summarizes the isolates which presented difficultes in their identification and their possible specific identification.

***Anabaena compacta* (Nygaard) Hickel**

According to Komárek and Zapomělová (2007) the dense coiling is a diacritical character in the identification of this species. In the present study, the isolates exhibited straight to curved trichomes and never dense coiling (Figure 3.1.a). This is in agreement with Rajaniemi *et al*. (2005) who defined the taxonomic position of *Anabaena compacta* on the basis of 16S rRNA and noted that the trichomes were straight in culture. Other diacritical characters emphasized by Komárek and Zapomělová (2007) are the vegetative cell width and akinete dimensions. In our collections of *A. compacta* these characters corresponded to the dimensions given by Komárek and Zapomělová (2007) for this species. However, the akinetes in our species were smaller than noted by Rajaniemi *et al*.

Table 4.1 Error! Bookmark not defined.**Isolates with identification (ID) problems based on morphological analysis, the main problem for there ID and possible ID.**

Isolate	Main ID problem	Possible ID
Anabaena cf. *cylindrica*	Cell dimensions	*Anabaena augstumalis*
Anabaena cf. *flos-aquae* UTCC64	No akinetes	No ID
Anabaena cf. *flos-aquae* UTEX2383	No akinetes	No ID
Anabaena lemmermannii LONT2	Immature akinetes	*Anabaena subcylindrica*
Anabaena cf. *oscillariodes*	No heterocysts and akinetes	No ID
Anabaena cf. *planktonica*	Cell dimesions and shapes	*Anabaena planktonica* *Aphanizomenon schindlerii*
Anabaena cf. *lemmermannii*	Shape of akinetes	*Anabaena lemmermannii*
Anabaena sp. A2879	No akinetes	*Anabaena bergiii*
Anabaena sp. LONT5	Just free akinetes where observed	*Nostoc* sp.
Anabaena sp. LOW1	Cell dimensions	*Anabaena lemmermannii*
Anabaena sp. 7812	No heterocysts and akinetes	*Anabaena smithii*
Anabaena cf. *viguieri*	No akinetes	*Anabaena viguieri* *Anabaena austro-africana*
Aphanizomenon cf. *gracile*	Cell dimesions Shape of akinetes and end cells	*Aphanizomenon flos-aquae*
Nostoc sp. RUP1	Cell dimensions	*Nostoc ellipsosporum*
Nostoc sp. UTCC106	No heterocysts and akinetes	No ID
Nostoc sp. UTCC314	Shape of akinetes	No ID
Nostoc sp. UTCC355	Hormogonia formation, cell dimension and discolouration	*Nostoc muscorum* *Nostoc viride*
Nostoc sp. UTCC387	No akinetes	No ID

(2005), 9.11 x 9.59 µm versus 10.0 x 11.5 µm respectively. On the other hand, all the cellular dimensions were larger than the observations of Willame *et al*. (2006), with the exception of the terminal cell length, which we observed to be 4.6 µm compared to 9.8 µm by Willame *et al.* (2006). According to Li *et al*. (2000) and Komárek and Zapomělová (2007), the shape of the akinetes is also important and are typically widely-oval to almost spherical. In the present *A. compacta* collections, the akinetes were nearly spherical, longer than wide but never widely-oval (Figure 3.1.a). This is in concordance with Rajaniemi *et al*. (2005) and Willame *et al*. (2006), who described a rounded to oval or slightly oval akinetes. In addition, these researchers concluded that spherical akinetes are a diagnostic character that unified the cluster containing *A. compacta*. Moreover, according to Komárek and Zapomělová (2007) the akinetes can occur in pairs, however this characteristic was not noted in our collections and is not considered a diacritical character. Additionally, this identification was completely supported by a BLAST analysis of 16S rRNA gene sequences (99% identity, E-value=0.0) with *Anabaena compacta* GenBank sequences (strains 1403/24, 189, 118, and ANACOM-KOR, which accession numbers are AY701569, AJ293111, AJ293109, AJ630418 respectively).

***Anabaena* cf. *cylindrica* Lemmermann**

The vegetative cells in our collections of *Anabaena* cf. *cylindrica* were a little larger than expected; being 3-4.5 µm wide and 5-8.5 µm long for the type strain (Gill, 2006). The heterocysts were slightly longer by approximately 3 µm hence they were not spherical as noted for the type species. A nearly invisible sheath has been noted for this species; however, in this case it was not observed but may be a consequence of culture

conditions. Moreover, in the studied isolates the trichomes sometimes were attenuated towards ends which were not noted in the original description of this species (Figure 3.1.b).

The BLASTn query of 16S rRNA gene sequences resulted in sequence matches with *Anabaena cylindrica* strains NIES19 and DC-3 (AF247592, EU780157) and *Anabaena augstumalis* Schmidke Jahnke strain SCHMIDKE JAHNKE/4a (AJ630458) (99% identity, E-value=0.0 in both cases). In fact, the isolate was similar in morphology to descriptions of *A. augstumalis* (Rajaniemi *et al*., 2005), in that conical terminal cells were observed and the dimensions of the vegetative cells (Figure 3.1.b) and akinetes dimensions were similar. For example, the vegetative cell widths of our isolate ranged from 2.56 to 5.28 µm similar to *A. augstumalis* (1.5-6.1 µm), and the akinete dimensions were 4.06-8.27 µm wide and 12.13-28.28 µm in length, and for the described species were 5.3-9.2 and 13-22 µm respectively. Therefore, the primary difference between *A. cylindrica* and *A. augstumalis* is the morphology of the terminal cells; as a result, this isolate can be identified as *A. augstumalis* on the basis of this morphological parameter.

***Anabaena* cf. *flos-aquae* (Lyngbye) Brébisson ex Bornet & Flauhault**

UTCC64 and UTEX2383

Despite that these strains are from culture collections, the name was changed by adding the epithet "cf.", since there are differences relative to the type species description. It is possible that this is the result of being grown under laboratory growth conditions for a prolonged time (Svenning *et al*., 2005). In addition, akinetes were not observed, which is crucial in their identification (Komárek and Zapomělová, 2007).

These strains also did not show the typical spherical vegetative cells, which is a diacritical character in their classification according to Komárek and Zapomělová (2007) (Figures 3.1.d, 3.1.e). But, comparing our isolate with *Anabaena flos-aquae* UTEX LB2558 (www.utex.org), both of them show ellipsoidal to cylindrical vegetative cells. Moreover, Li *et al*. (2000) described barrel-shaped vegetative cells for this species; hence the diacritical position of this character should be questionable. Another difference was the coiling; however it has been documented that this trait can be lost under culture conditions (Komárek and Zapomělová, 2007). Moreover, amorphous mats were observed in some cultures but this is not representative of this species, although under culture condition the coiling can be irregular (Komárek and Zapomělová, 2007). The mucilaginous sheath noted in Li *et al*. (2000) was not observed in our strains, although according to Komárek and Zapomělová (2007) there should be no mucilage in *Anabaena flos-aquae*. Finally, the morphological identification was supported by BLAST analysis of 16S rRNA gene sequence AY218829 of *Anabaena flos-aquae* UTCC 64 (99% identity, E-value=0.0).

***Anabaena lemmermannii* Richter GIOL8**

The morphology described for this isolate was consistent with the described strains (Komárek and Zapomělová, 2007; Li *et al*., 2000; Rajaniemi *et al*., 2005). Nevertheless, there were some differences. For example, the width of the trichome was 0.57 µm smaller than suggested by Komárek and Zapomělová (2007). The akinetes in our strains were also smaller and ranged between 6.22-9.4 µm in width by 9.84-17.52 µm in length compared to 6.3-11 x 13-25.26 noted by Komárek and Zapomělová (2007). In

addition, the kidney-shaped akinetes described by these authors was not observed (Figure 3.1.f). It is possible that this is another variation of this species, since its morphology can be variable among populations and can also change under laboratory growth conditions (Komárek and Zapomělová, 2007). For example, the observed ellipsoidal to cylindrical akinete shape (Figure 3.1.f) was in concordance with the observed by Rajaniemi *et al*. (2005) in which akinete length and trichome width are smaller than we noted (12-16.3 μm and 3-5 μm respectively). The coiling of the trichomes also appears to be a variable character in this species, although Komárek and Zapomělová (2007) consider it a diacritical character. However, the coiling has also not been observed by Li *et al*. (2000). With respect to the heterocysts, they were smaller (3.99-6.07 x 3.68-7.69 μm) than the described by Li *et al*. (2000) (6-8.7 x 7.5-11 μm). Lastly, the mucilaginous envelope described by Li *et al*. (2000) was not observed and this characteristic was also not noted by Komárek and Zapomělová (2007). In addition, the identification of this isolate as *Anabaena lemmermannii* was supported by the BLAST analysis of its 16S rRNA gene sequence (identity 98%, E-value=0.0 with *A. lemmermannii* Ana Dalai AY701571). Also was closer to *Anabaena flos-aquae* PCC 9302 (AY038032) (identity 98%, E-value=0.0), but the studied isolate did not correspond to this species because always showed akinetes at both sides of heterocysts, which is a diacritical character for *Anabaena lemmermannii,* and in *Anabaena flos-aquae* the akinetes are rarely observed at one side of the heterocysts (Komárek and Zapomělová, 2007).

***Anabaena lemmermannii* Richter LONT2**

This isolate was very similar to *Anabaena lemmermanni* GIOL8, although in general all the cell kinds were larger (Figure 3.1.g). The width of the trichome was 0.23 µm larger than described by Komárek and Zapomělová (2007) and the vegetative cell sizes were more closely related to the description given by Li *et al*. (2000). This was also the same with the heterocysts. Akinetes were comparable to that of *Anabaena lemmermanni* GIOL8, although their sizes were similar to the description given by Komárek and Zapomělová (2007). Finally, *A. lemmermanni* LONT2 showed barrel-shaped vegetative cells as described by both Komárek and Zapomělová (2007) and Li *et al*. (2000). The BLASTn query of the 16S rRNA gene sequence analysis supported this identification with 94% identity and an E-value=0.0 in relation to *Anabaena lemmermannii* BC strain Ana 0005 (DQ023199).

This isolate also had some similarities with *Anabaena subcylindrica* Borge. Although the dimensions were congruent with descrition given by McGuire (1984) it showed some morphological differences. This type species neither have cylindrical and spherical vegetative cells nor spherical heterocysts, instead in the studied isolates these cell shapes were observed. In addition, there are no sequences available of *Anabaena subcylindrica* on GenBank for a Blastn query.

***Anabaena* cf. *oscillariodes* Bory de Saint-Vincent, nom. illeg.**

Anabaena cf. *oscillariodes* was very difficult to identify due to the absence of heterocysts and akinetes (Figure 3.2.a). The morphology was similar to the description in the literature for *Anabaena oscillariodes* Bory (Rajaniemi *et al*., 2005), just the

vegetative cells were longer in 0.64 µm. Rajaniemi *et al*. (2005) suggested that only the width of the trichome is important in the classification of this species, and the measurements for this strain were within the range that they proposed (2.3-5.4 µm). Additionally, this identification was supported by a BLASTn query of 16S rRNA gene sequences in which there was a match to *Anabaena oscillariodes* BO HINDAK (AJ630428) with an identity of 95% (E-value=0.0).

***Anabaena* cf. *planktonica* Brunnthaler**

The morphological description of this isolate was compared with the descriptions given by Çelekli *et al*. (2007), Li *et al*. (2000), Rajaniemi *et al*. (2005), and Wood *et al*. (2005). Some differences were observed, for example the hyaline mucilaginous envelope and shorter-than-wide vegetative cells were not observed. The width of vegetative cells was smaller and ranged between 2.79-7.73 µm compared to 6.7-14.6 µm in Li *et al*. (2000) and 8-14 µm in Wood *et al*. (2005) (Table 4.1). The heterocysts were not only spherical; ellipsoidal heterocysts were also observed and the dimensions were within the range given by these authors (Figure 3.2.b). In addition, the akinete dimensions were smaller (approximately 3 µm in width and 7.5 µm in length) overall than the expected size of 8.8-23.7 x 14.7-39.8 µm noted in Li *et al*. (2000). But, they were closer to the expected of 11-17 x 15-27 µm in Wood *et al*. (2005). Çelekli *et al*. (2007) described similar characteristics to that of Li *et al*. (2000), but the dimensions of this strain were similar to that given by Rajaniemi *et al*. (2005). For example, the vegetative cells width was 4.1-11 µm and the akinetes have 9.1-19.1 µm in width and 12-

Table 4.2 **Dimensions of vegetative cells, heterocysts and akinetes in *Anabaena* cf. *plaktonica* and the related species in literature.**

	Vegetative cells		Heterocysts		Akinetes		References
	Width (µm)	**Length (µm)**	**Width (µm)**	**Length (µm)**	**Width (µm)**	**Length (µm)**	
Anabaena* cf. *planktonica	2.79-7.73	7.08-18.65	6.00-8.93	7.04-10.15	6.59-19.56	17.07-31.31	This study
Anabaena planktonica	6.7-14.6	-	5.5-14.6	-	8.8-23.7	14.7-39.8	Li *et al.* (2000)
Anabaena planktonica	8-14	6-12	9-13	9-13	11-17	15-27	Wood *et al.* (2005)
Aphanizomenon schindlerii	1.6-4.2	4.2-11.7	2.1-6.5	2.8-8.6	7.0-12.6	13-22.4	Kling *et al*. (1994)

17.6 in length. The isolate's identification as *Anabaena planktonica* was supported by a BLASTn query of 16S rRNA DQ264160 and DQ264159 GenBank sequences (98-99% identity, E-value=0.0).

This isolate was also similar to *Aphanizomenon schindlerii* Kling *et al.* (1994), as both have the similar akinetes (Figure 3.2.d) and have the hyaline end in the terminal cells (Figure 3.2.c), however these cells were longer than expected and did not show the typical bulbose tip (Kling *et al*., 1994). Additionally, the dimensions of the cells, in general, were smaller by an average of 3.5 µm than the expected sizes for *Aphanizomenon schindlerii* noted by Kling *et al*. (1994). But these dimensional differences were not useful for identifying this isolate neither as *Anabaena planktonica* Brunnthaler nor as *Aphanizomenon schindlerii*, since the size of the isolate was intermediate between the size of these two type species (Table 4.1).

***Anabaena* cf. *lemmermannii* Richter**

Morphologically, this strain conformed to many of the characteristics of the type strain given by Komárek and Zapomělová (2007). The most important character was the sizes of akinetes, which were approximately 4 µm smaller than the type strain (6.3-11 x 13-25.6 µm) and the shape of akinetes, which were ellipsoidal and not kidney-shaped (Figure 3.2.f). But as seen above, the morphology of this species can change under culture conditions and among populations (Komárek and Zapomělová, 2007). This is confirmed by Rajaniemi *et al*. (2005), who described oval akinetes with dimensions of 7-8.5 µm in width and 12-16.3 µm in length, which fit with ours. The mucilaginous sheath described by Li *et al*. (2000) was not observed, but this character could have lost under

laboratory growth conditions since other isolates loss it as well. The heterocysts were smaller (4.75-9.69 x 5.06-8.87 µm) than described (6-8.7 x 7.5-11 µm) in the literature (Li *et al*., 2000). This isolate had 97% identity (E-value=0.0) with *Anabaena lemmermannii* strain BC Ana 0005 (DQ023199) in a BLAST analysis of 16S rRNA gene sequences. However, this isolate had a closer match (100% identity, E-value=0.0) to *Anabaena compacta* strains 1403/24, 189,118, and ANACOM-KOR (AY701569, AJ293111, AJ293109, and AJ630418 respectively), but was morphologically different from the description given by Komárek and Zapomělová (2007). *Anabaena compacta* (Nygaard) Hickel show spherical vegetative cells, akinetes distant from heterocysts whereas the isolate studied had ellipsoidal and barrel-shaped vegetative cells and akinetes at one side of the heterocyst (Figures 3.2.e, 3.2.f). In addition, the akinetes were almost 5 µm longer than the expected of 8.2-12.5 µm (Komárek and Zapomělová, 2007).

***Anabaena* sp. LONT5**

This isolate was first identified as *Anabaena lemmermanni* Richter according to its morphology however there some morphological differences despite the dimensions still being within the range given for the type species (Komárek and Zapomělová, 2007; Li *et al*., 2000; Rajaniemi *et al*., 2005). For example, the isolate has slightly narrowed-toward-ends filaments, conical end cells, and heterocysts in rows. These characteristics and a BLAST search based on 16S rRNA gene sequence (99% identity, E-value=0.0) suggested this isolate to be *Trichodesmium* (*T. variabilis* strain HINDAK 2001/4, accession number: AJ630456). However, the cells are larger than expected for this genus being 9.57 µm in width by 14.9 µm in length compared to 7.4 x 13.1 µm according to Rajaniemi *et al*. (2005) for *Trichodesmium variabilis* and 5.8 x 8.3 µm as described by

Willame *et al*. (2006). Hence, this isolate remains unidentified though it could be a species of *Nostoc*, which can have heterocysts in rows (Rippka *et al*., 1979) similar to *N. calcicola* UTEXB382 of this study. The identification of this isolate as a *Nostoc* strain is supported by 99% identity (E-value=0.0) in the BLASTn query, where in matched with *Nostoc* sp. 8941 (AY742448.1). And it had 94-95% identity with *Nostoc calcicola* (E-value=0.0) (strains TH2S22 and VI, accession numbers: AM711529 and AJ630448).

***Anabaena* sp. LOW1**

This isolate can be tentatively identified as *Anabaena lemmermannii* Richter however it was difficult to determine this conclusively as the cell dimensions were different than described for *Anabaena lemmermannii* Richter. However, this isolate had akinetes on both sides of heterocysts, which is an important diagnostic character for this species (Li *et al*., 2000). With respect to the cell dimensions, Komárek and Zapomělová (2007) noted that the width of the vegetative cells should range between 2.5-6.9 µm and Li *et al*. (2000) suggested that these cells should range between 5.1-7.3 µm in diameter and 7.0-11.9 µm in length. However, this strain ranged between 1.32-4.55 µm in width and 3.73-14.57 µm in length resulting in cells that are thinner and longer than the described ones. Actually, Komárková-Legnerová (1988 *fide in* Li *et al*., 2000) established two varieties for this species: *Anabaena lemmermanni* var. *lemmermamannii* Richter and *Anabaena lemmermanni* var. *minor* Utermoehl, in which length:width ratio is 1.7-2.5:1 and 1.4-1.7:1 respectively, which differs considerably from our ratio of 3.5:1. It is possible that this strain may be considered *Anabaena lemmermanni* var. *lemmermamannii* due to the longer vegetative cells. With respect to the akinetes, these

were kidney-shaped but were longer than previously described by Rajaniemi *et al*. (2005) (maximum length of 12 µm) and Komárek and Zapomělová (2007) (maximum length of 25.6 µm), and shorter than the described by Li *et al*. (2000) in approximately 15 µm. On the other hand, the heterocysts fitted within the expected sizes for this species. The identification of this isolate as *Anabaena lemmermanni* was supported by the BLAST analysis of 16S rRNA gene sequence (97% identity, E-value=0.0), in relation with the same species (strain BC Ana 0005), which accession number is DQ023199.

It is also possible that this isolate could be *Anabaena mendotae* Trelease, since the dimensions were within the range given by Komárek and Zapomělová (2007). In addition, the morphological appearance was very similar to this species. However, in *A. mendotae* the akinetes are distant from heterocysts, which this is considered a diacritical character (Komárek and Zapomělová, 2007; Li *et al*. 2000). Additionally, this identification was better supported than *Anabaena lemmermanni* one by the BLASTn query of 16S rRNA gene sequences, with a 99% identity (E-value=0.0) in relation to *A. mendotae* strain 57 (AJ293107). Additionally, the 16S rRNA gene sequence of this isolate was related in the BLASTn query with a 99% identity (E-value=0.0) to *Aphanizomenon flos-aquae* PCC7905 (AY038035), *Aphanizomenon* sp. PCC7905 (AJ133154) and *Aphanizomenon gracile* strain 219 (AJ293124). Although some physical measurement were coincident with the description of some *Aphanizomenon* isolates (*Aphanizomenon yezoense*, Komárek and Komárková, 2006), *Anabaena* sp. LOW 1 can not be considered an *Aphanizomenon* species because the akinetes were observed at both sides of the heterocysts. Contrarily, this genus is characterized for having akinetes distant from the heterocysts (Komárek and Komárková, 2006).

***Anabaena* sp. A2879**

Due to the absence of akinetes it was not possible to accurately identify this isolate. However, the morphological characteristics were consistent with the strain of *Anabaena bergii* Ostenfeld described in Hindák (2000). For example, the dimensions of vegetative cells and heterocysts were within the diameter range expected of 5-7 μm and 7-8 μm respectively. The only difference noted was that the gas vesicles described for the type species were not observed in our strains. Again it is a possibility that this could be a morphological change produced from being under culture conditions for a prolonged time. Moreover, the short filaments gradually narrowed towards the filament ends, which was never described for the type species and was only observed in very short filaments (Figure 3.2.k). Hindák (2000) did not describe the formation of mats, which was observed in our strains and confirmed by Çelekli *et al*. (2007), although the latter noted larger cells and heterocysts by approximately 2 μm. The identification of this isolate as *Anabaena bergii* Ostenfeld (AF160256) was supported in a 95% identity in the BLASTn query of its 16S rRNA gene sequence.

***Anabaena* sp. 7812**

This isolate cannot be positively identified as it did not develop heterocysts or akinetes under induction conditions. However, one possible akinete was observed (Figure 3.3.c), which is very similar to the akinetes of *Anabaena smithii* (Komárek) Watanabe as described by Li *et al*. (2000) in being spherical and having a diameter ranging between 15.0-15.5 μm that is within the previously described range of 12.1-24.2 μm. Moreover, spherical heterocysts were observed (Figure 3.3.b) with dimensions of 6.8-8.5 μm in

diameter, which was also within the expected diameter of 7.7-14.9 µm of *Anabaena smithii* and vegetative cell shape was similar to that for the description of this species. The gas vesicles were observed in some filaments; however, a mucilaginous sheath was not observed and could possibly be character lost due to the culture conditions. Moreover, this isolate could belong to other genus as *Komvophoron* Anagnostidis et Komárek. For example, it could be *Komvophoron schmidlei* (Jaag) Anagnostidis et Komarek (Komárek, 1992) if we supposed that this strain never formed specialized cells, although the trichome width given for the type species is larger than ours (up to 10 µm wide). However, Willame *et al*. (2006) obtained a thinner strain for this genus, with 1.8-2.4 µm wide, but this showed no spherical to subspherical vegetative cells like our isolate. Matula *et al*. (2007) studied four species of this genus, where only *Komvophoron minutum* showed spherical vegetative cells, but they are very thin in relation to ours (2.5-2.6 µm), and *Komvophoron constrictum*, which has a closer wide of 3.5-6.4 µm, but the vegetative cells are cylindrical. However, if we consider the observed akinete and heterocysts, the dimensions and morphological analysis fitted better in the description given for *Anabaena smithii* than the one given for genus *Komvophoron*. Moreover, the BLASTn query of the 16S rRNA gene sequences supported better matches with the *Anabaena smithii* strains TAC428, TAC431, TAC432, TAC450, and 1tu39s8 (GenBank: AY701553, AY701554, AY701555, AY701556, and AJ630436 respectively) (93% identity, E-value=0.0) than the *Komvophoron* one (strain ORO36S1, accession number=DQ264227) (85% identity, E-value=0.0).

***Anabaena variabilis* (Kutzing) Bornet et Flahault**

According to Komárek and Anagnostidis (1989 *fide in* Rajaniemi *et al*., 2005) *Anabaena variabilis* (Kutzing) Bornet et Flahaultand and *Trichormus variabilis* (Ralfs ex Bornet *et* Flahault) Komárek *et* Anagnostidis correspond to the same species. Our isolate had similar appearance to the described species by Rajaniemi *et al*. (2005) and Willame *et al*. (2006), but with some differences in the quantitative characters. The position of akinetes within the filament can not be discussed as all akinetes were observed to be free in the culture and not on the filament (Figure 3.3.e). Although the vegetative cells dimensions fall within the expected range, the heterocysts and akinetes were larger. The heterocysts were approximately 1 µm wider and longer than the expected overall of 4.9-5.9 x 5.6-7.2 µm (Willame *et al*., 2006), and the akinetes approximately 2 µm wider and longer than the expected overall of 4.9-5.9 x 5.6-7.2 µm (Willame *et al*., 2006). Conversely, the isolate's akinetes fitted very well in the description given by Rajaniemi *et al*. (2005). Finally, the end cells were longer in approximately 2 µm than expected of 2.6-4.6 µm given by Willame *et al*. (2006), although it was in the range 2.1-9.6 µm given by Rajaniemi *et al*. (2005). The identification of this strain as *Trichormus variabilis* was confirmed by a 99% identity (E-value=0.0) in the BLASTn query with 16S rRNA gene sequences of *T. variabilis* HINDAK 2001/4 (AJ630456).

In addition, this isolate had some similarities with the type strain *Anabaena orientalis* S. C. Dixit (Komárek, 2005). Nevertheless the heterocysts were smaller by approximately 2 µm overall compared to the expected values of 5.0-8.0 x 5.8-12 µm and the akinetes were smaller by approximately 4 µm overall from the expected values of 9.2-

10.8 x 13.0-18.0 µm. Unfortunately, there are no sequences of *Anabaena orientalis* in GenBank that this sequence could match to.

***Anabaena* cf. *viguieri* Denis et Frémy**

Despite that the morphology of this strain appears to be consistent with the type description of *Anabaena viguieri* Denis et Frémy (Li *et al*., 2000) the name of this isolate was changed adding the epithet "cf." as akinetes were never observed and some characters (gas vesicles and mucilaginous sheath) were possibly lost under culture conditions. However, the presence of a mucilaginous sheath as a diagnostic character has been questioned by Komárková-Legnerová and Eloranta (1992 fide in Li *et al*., 2000). The vegetative cells were smaller compared to the expected diameter by 0.6 µm, and the heterocyst's diameter was bigger by 1.1 µm to the expected one (Li *et al*., 2000). This identification was supported in just 94% identity in the BLASTn query of the 16S rRNA available sequence in GenBank (strain TAC433, accession number=AY701559).

This isolate was also similar to the species *Anabaena austro-africana* Cronberg et Komárek described by Komárek (2005), in that the filaments are gradually attenuated towards the ends and the apical cell can be conical or rounded (Figure 3.3.g). Although there were some small differences in the dimensions such as the width being 1 µm smaller than the diameter of our isolate, this isolate was more similar to *Anabaena austro-africana* than *Anabaena viguieri* Denis et Frémy. Though as noted previously, no gas vesicles were observed in our isolate. Unfortunately, there are no 16S rRNA gene sequences of *Anabaena austro-africana* in GenBank for supporting this identification by a BLAST analysis.

Aphanizomenon flos-aquae (Linneaus) Ralfs ex Bornet et Flahault **UTEX LB2384**

This isolate was obtained recently from UTEX culture collection, so the identification was maintained despite that the heterocysts and akinetes were not observed (Figure 3.3.h). The filaments were not observed in fascicles; however this arrangement is usually lost under laboratory growth conditions (Rajaniemi *et al*., 2005). In addition, the average trichome width was just 0.03 µm bigger than the expected of 4.5-8 µm. The taxonomic identity of this isolate was confirmed by the BLASTn query by 99% identity and 0.0 E-value to *Aphanizomenon flos-aquae* strains LMECYA 10, Aph Zayi and Aph Inba (accession numbers EU078537, AY196082 and AY196083 respectively).

***Aphanizomenon* cf. *gracile* Lemmermann**

Two kinds of filaments or morphotypes were observed in this culture, which only difference was the dimensions (Figure 3.3.i). One morphotype was visibly smaller than the other one. The 16S rRNA gene was sequenced on three separate occasions to ensure monoalgal condition and identical sequences were obtained hence this size difference did not correspond to the presence of two different species but a highly variable morphology depending on the culture conditions as the dominance of the morphotypes changed within this culture. Because of this variability the epithet "cf." was added to the name of this isolate. Moreover, the morphological characterization did not fit with the description of *Aphanizomenon gracile* Lemmermann given by Komárek and Kováčik (1989). The most important difference with the *Aphanizomenon gracile* is that this present a cup-shaped sheath on the ends of the akinetes (Komárek and Kováčik, 1989), which was not observed in our isolate (Figure 3.3.j). In addition some hyaline-end cells

were observed (Figure 3.3.i), and this species is characterized by the absence of this characteristic (Komárek and Komárková, 2006). It also had the slightly bulbous feature on the end cells, character observed in *Aphanizomenon schindlerii* (Kling et al. 1994). Moreover, the dimensions of our isolate were larger than *Aphanizomenon gracile*. For example, the vegetative cells were longer in approximately 12.6 µm and the end cells was 9.3 µm than the expected of 2.6-7.1 and 3.6 7.4 µm respectively. In fact, the dimensions were closer to *Aphanizomenon flos-aquae* var. *klebahnii* Elenkin (Komárek and Kováčik, 1989). The vegetative cells length showed the biggest difference, with 8.3 µm overall the expected of 4.3-11.4 µm. The other cell types fall better in the ranges given by Komárek and Kováčik (1989). On the other hand, the BLASTn query of the 16S rRNA gene sequence analysis supported better the relation with *Aphanizomenon gracile* (strains LMECYA 148, LMECYA 64 and LMECYA 33, accession numbers EU078533, EU078532, and EU078531 respectively) (97-99% identity, E-value=0.0) than the relation with *Aphanizomenon flos-aquae* var. *klebahnii* (strains 218 and 83, accession numbers AJ293123 and AJ293122) (97% identity, E-value=0.0).

***Aphanizomenon klebahnii* (Elenkin) Pechar et Kalina HHAFA**

This isolate can be identified as *Aphanizomenon klebahnii* (Elenkin) Pechar et Kalina. The morphological appearance was very consistent in relation with the type species, although this isolate appears to have some size differences. The width of the trichome was in the range of 2.92-6.21 µm, which is wider than the expected of 3.2-5.2 µm. The akinetes had a size of 5.43-8.59 x 27.21-66.34, which actually is closely related to the type species (5.4-9.3 x 20-54(113) µm). Although, there were some similarities

with *Aphanizomenon flos-aquae* Ralfs ex Bornet et Flahault (Komárek and Komárková, 2006), the trichome width of 4.5-6.5(8) µm is closer to this isolate than to the description of *Aphanizomenon klebahnii*. This controversial position of our isolate between *flos-aquae* and *klebahnii* has been analyzed by Komárek and Kováčik (1989), who divided the genus *Aphanizomenon* into three groups. These groups are divided mainly according to the morphology of the trichomes and the fascicle formation. *Aphanizomenon flos-aquae* species represent the group which trichomes are in fascicles and the vegetative cells show similar wide along the whole filaments. Although, the filament aggregation in the studied isolate was not observed (this isolate has been in culture for a prolonged period), the filaments were not narrowed towards ends (Figure 3.3.l). Additionally, the *Aphanizomenon flos-aquae* group presents two verities of *Aphanizomenon flos-aquae*: var. *klebahnii* Elenkin and var. *flos-aquae* Ralfs ex Bornet et Flahault, which main differences are in the quantitative features. Moreover, it is important to note that *Aphanizomenon flos-aquae* var. *klebahnii* is a synonymy of *Aphanizomenon klebahnii* and *Aphanizomenon flos-aquae* var. *flos-aquae* is indeed *Aphanizomenon flos-aquae* (Komárek and Komárková, 2006). As we can see in Table 4.2, our isolate was in between these two varieties when comparing the metric analysis and sometimes larger than the var. *flos-aquae*. For example, the terminal cells were longer by approximately 2.5 µm than var. *flos-aquae*, and much longer than var. *klebahnii*, in more than 9 µm. In addition, the vegetative cells were thinner in approximately 1.4 µm than var. *klebahnii*, and wider in 0.5 µm than var. *flos-aquae*. The BLASTn query of the 16S rRNA gene sequences matches with both varieties with 99% identity and 0.0 E-value (*Aphanizomenon flos-*

Table 4.3 **Dimensions of vegetative cells, end cells, heterocysts and akinetes of *Aphanizomenon klebahnii* HHAFA and the related species in literature.**

	Vegetative cells		End cells		Hetetocysts		Akinetes		Reference
	Width (µm)	**Length (µm)**	**Width (µm)**	**Length (µm)**	**Width (µm)**	**Length (µm)**	**Width (µm)**	**Length (µm)**	
A. klebahnii HHAFA	2.92-6.21	6.02-17.45	2.45-5.59	6.33-26.87	3.39-6.40	7.99-13.03	5.43-8.59	27.21-66.34	This study
A. flos-aquae var. *klebahnii*	4.3-5.7	4.3-11.4	2.8-5	7.1-17.1	3.1-5.7	6.4-11.4	5.4-9.3	30-54.3	Komárek and Komárková (2006)
A. flos-aquae var. *flos-aquae*	4.6-7.8	5-13.6	4-6.4	7.1-24.3	5-6.4	10-17.8	7.1-10	42.8-88.5	Komárek and Komárková (2006)

aquae LMECYA 129 (EU078543), and *Aphanizomenon flos-aquae* var. *klebahnii* strain 218 (AJ293123)).

***Nostoc calcicola* Brébisson UTEXB382**

This isolate was obtained recently from the UTEX culture collection, so the identification was maintained despite some differences with the description of the type species (Hrouzek *et al*., 2003). The vegetative cells were smaller than the expected of 2.4-5.4 x 2.2 -6.9 µm (Hrouzek *et al*., 2003), since they measured 3.37-6.43 x 4.82-8.47 µm. The heterocysts and akinetes dimension falling in the expected of 2.4-5.8 x.2.6-8 µm and 2.7-6.4 x.2.7-7.7 respectively (Hrouzek *et al*., 2003). Although, they were slightly larger than described by McGuire (1984), this author also gave an error range of plus or minus 5 µm or less. Instead, the analyzed strain, the measures in general were between 3.37 and 13.19 µm. The akinetes dimension were the primary difference compared to the study by McGuire (1984), with a range of 5.31-9.36 µm in width and 9.13-13.19 µm in length. This identification was supported by a 96% identity in the BLAST analysis of 16S rRNA sequence analysis with *N. calcicola* strains TH2S22 and VI (AM711529 and AJ630448 respectively).

***Nostoc commune* Vaucher UTCC74**

This isolate was recently obtained from CPCC culture collection; hence the identification was maintained despite some differences with the described type strain (Novis and Smissen, 2006). This species is characterized by exhibiting a high diversity among populations (Novis and Smissen, 2006). For example, the vegetative cells can be

near spherical to ellipsoidal, and measure 2.2-6.4 x 3.6-5.6 (width x length). Our strain was larger than this with a range of 2.56-4.16 x 3.20-6.87 (width x length) and the vegetative cells were barrel-shaped (Figure 3.4.d), although they were be similar to the described ellipsoidal vegetative cells by Novis and Smissen (2006). McGuire (1984) also described spherical vegetative cells, which diameter and length are less than 5 µm. The rest of the features were in concordance with McGuire (1984) and Novis and Smissen (2006).

***Nostoc ellipsosporum*. Rabenhorst ex Bornet & Flahault UTEX383**

This isolate was obtained recently from UTEX culture collection, but this strain showed some differences in the cellular dimensions with the type strain (Hrouzek *et al*., 2003). In general, vegetative cells, heterocysts and akinetes were smaller than the expected for *N. ellipsosporum*. For example, the heterocysts were 2.65-5.19 x 3.48-8.18 µm, which is smaller by approximately 2 -7 µm than the expected of 4.1-11 x 6.1-15.9 µm. However, according to Hrouzek *et al*. (2003), the large heterocysts were rarely observed (frequency of less than 5%). On the other hand, using the *N. ellipsosporum*'s description of McGuire (1984), there were only differences in the length of the vegetative cells. McGuire (1984) noted vegetative cell lengths of 5 µm or less, however our isolate ranged between 3.48 to 8.18 µm. Moreover, this strain exhibited several of the other cellular shapes observed by this author, with the exception of spherical vegetative cells, spherical and cylindrical heterocysts, and spherical akinetes (Figures 3.4.e, 3.4.f). Additionally, this identification was supported just in a 93% identity (E-value=0.0) by a BLAST analysis of the 16S rRNA gene sequences of *Nostoc ellipsosporum* strains V and

LCR14 (AJ630450 and EU446014, respectively). The closer species in the BLAST query was *Anabaena compacta* ANACOM-KOR (AJ630418) (99%), *Anabaena* cf. *circinalis macrospore* strains 0tu25s6, 1tu28s13, 1tu27s5, 1tu26s10, and 1tu23s3 (AJ630412, AJ630445, AJ630410, AJ630409, AJ630408) and *Anabaena sigmoidea* strains 0tu36s7 and 0tu38s4 (AJ630434 and AJ630435) with 99, 97 and 97%.

***Nostoc punctiforme* (Kützing) Hariot UTCC41**

This isolate was obtained recently from CPCC culture collection, so the species epithet was maintained despite differences with previously described strains (McGuire, 1984). Generally, this strain was very similar to the McGuire (1984) description however, the barrel-shaped and cylindrical vegetative cells were not observed (Figures 3.4.g, 3.4.h). On the other hand, it is quite different from the strain presented by Meeks *et al*. (2001), wherein amorphous colonies were not described, the vegetative cells and heterocysts width were close to the range given for these researchers of 5-6 µm and 6-10 µm respectively, although just slightly smaller. The identification of this strain was well supported by the BLAST analysis of 16S rRNA gene sequence (99% identity, E-value=0.0) in relation with *Nostoc punctiforme* SAG 71.79 (DQ185258).

***Nostoc* sp. UTCC 314**

This strain was recently obtained from the CPCC culture collection and has not been identified in the literature. The cellular dimensions do not fit in any of the available descriptions. But the most remarkable character was the radially striated epispore around the whole akinete (Figure 3.4.j). This is similar in appearance to what was described in

Nostoc alatosporum (Sant'Anna *et al*., 2007), but in this case the epispore is not surrounding the complete akinete and the cellular dimensions were different. Sant'Anna *et al*. (2007) first described this kind of mature akinete with striated epispore in the genus *Nostoc* which had previously only been observed in the genera *Cylindrospermum* (Pereira *et al*., 2005) and *Anabaena* (Komárek, 2005). However, this strain cannot be *Cylindropermum* because there are intercalary heterocysts (Figure 3.4.i) and genus *Cylindrospermum* is characterized by the presence of terminal heterocysts. On the other hand, it cannot be identified as *Anabaena* since it presented hormogonia and akinetes in rows (Figure 3.4.i), which are exclusive characteristics of the genus *Nostoc* (Rippka *et al*., 1979). The BLASTn query of the 16S rRNA analysis related this isolate with *Nostoc muscorum* (96% identity, E-value=0.0), but this species does not show the distinctive akinete observed in our isolate. Unfortunately, the 16S rRNA sequence for *Nostoc alatosporum* is not available in GenBank.

Nostoc sp. UTCC 106

Nostoc sp. UTCC 106 is a strain recently obtained from the CPCC culture collection, and it corresponds to *Nostoc* sp. ATCC 240911 and PCC 7524. It was very difficult to identify as it never developed heterocysts and/or akinetes under induction culture conditions (Figure 3.5.a). In fact, Rippka *et al*. talked in 1979 about the lost of its capability to fix nitrogen aerobically after being for a prolonged time in medium BG-11. Moreover, it showed other morphological changes as a result of prolonged time under growth conditions, such as very short filaments, free cells and some discoloration. One difference with the description of this strain to our collection is that gas vesicles were

observed in some but not all cells. In the BLASTn query the 16S rRNA gene sequence of this isolate matched with *Nostoc* sp. PCC 7120 sequence with 96% identity (E-value=0.0).

Nostoc sp. UTCC 387

Nostoc sp. UTCC 387 is a strain recently obtained from the CPCC culture collection. It was impossible to identify and even give a tentative identification as it never developed akinetes (Figures 3.5.b, 3.5.c). Moreover, this strain exhibited other morphological changes that are probably the result for being under prolonged culture conditions, such as hormogonia formation, and cells with different sizes and discolouration. Distinguishable features were not observed and antecedent from the culture collection are not available. According to BLAST analysis, this strain is closely related to *Nostoc* sp. PCC7120 and *Anabaena variabilis*, with 99% of maximum identity and 0.0 E –value. The isolate resulted in smaller than the expected sizes for these species, although hormogonia were not observed. The appearance was very similar to the pictures of *Anabaena* sp. PCC7120 presented by Adolph and Haselkorn (1971) and Rippka *et al*. (1979). Actually, as we can see in the first chapter of this thesis, the generic position of this strain always has been controversial. Initially it was considered a species of *Nostoc*, *N. muscorum* (Adolph and Haselkorn, 1971), and then it was classified as an *Anabaena* species on the basis of morphological characters (Rippka *et al*., 1979) and then was formally considered to be a species of *Anabaena* by Henson *et al*. (2002), although this is rejected by Tamas *et al*. (2000). In comparison to *Anabaena variabilis* (*Trichormus variabilis*), the vegetative cells and heterocysts fall within the ranges given by Willame *et*

al. (2006) and were smaller by an average of 3 µm than the description given by Rajaniemi *et al*. (2005), moreover it did not showed coiling trichomes and conical terminal cells.

***Nostoc* sp. UTCC 355**

This strain was recently obtained from the CPCC culture collection and has not been identified in the literature. In spite of developed heterocysts and akinetes, this strain has morphological discrepancies due to being under culture conditions for a prolonged time (hormogonia formation, and cells with different sizes and discolouration). This strain could be tentatively identified as *Nostoc muscorum* Agardh, since the vegetative cells, heterocysts and akinetes dimension fall within the range given by Hrouzek *et al*. (2003), although the heterocysts and akinetes were approximately 2.5 µm longer than the expected of 5.3-11.1 and 4.5-12.5 respectively. Moreover, they share some characteristics, such as the akinete formation process between two heterocysts, although transverse akinetes to the filament's axis were never noted. Moreover, short filaments with a terminal spherical heterocysts were observed, which correspond to the akinete germination product (Hrouzek *et al*., 2003). This possible identification was supported as well by the BLAST analysis with *N. muscorum* CENA61 (AY218828), were 99% of maximum identity and 0.0 E-value were obtained. On the other hand, it is also similar to *Nostoc viride* Sant'Anna *et al*. (2007), because of the dimensions differences were similar to the differences with *Nostoc muscorum*, presented the akinetes in rows between heterocysts as well and the akinetes are not traversed to the axis filament.

***Anabaena lemmermannii* Richter ONT1**

This strain was recently isolated from Lake Ontario (Canada) and has not been under the influence of the laboratory growth conditions for a long time period. For example, this isolate did not lose the trichome coiling (a character usually lost in these conditions) and its morphology was very consistent with the type description for *Anabaena lemmermannii* Richter (Komárek and Zapomělová, 2007). However, the trichome width was 1.5 µm above the expected of (2.5) 4-6.9 µm given by Komárek and Zapomělová, (2007), conversely it fell into the range of 5.1-9.2 µm given by Rajaniemi *et al*. (2005). In the same way, the heterocysts dimensions fitted very well in the range of 6.0-8.7 x 7.5-11 µm (Rajaniemi *et al*., 2005). And the akinetes presented the diacritical shape and position characteristics of this species (kidney-shaped and adjacent to heterocysts) (Figures 3.5.f, 3.5.g), although they were bigger then the expected ones by Komárek and Zapomělová (2007). These are (6.3)7.9-11(13.3?) x (13)15-25.6 and ours was 6.81-11.73 x 13.24-31.03, although Li *et al*. (2000) described longer akinetes with 13.4-33.9 µm, which are better related to ours. Moreover, the identification of this isolate was confirmed by the BLAST analysis of 16S rRNA gene sequence of *A. lemmermannii* strain 202A2 (AJ293104) (99% identity, E-value=0.0).

***Anabaena reniformis* Lemmermann MALW1**

This isolate is from the east African Lake Malawi, Malawi, and similar to *Anabaena lemmermannii* ONT1 it was simple to identify as it was a new strain and had not been under prolonged culture conditions. Its morphology was consistent with the type description of *Anabaena reniformis* Lemmermann (Komárek and Zapomělová, 2007).

The vegetative cell's shape was the same, and width was in the expected range of 3.2(3.6)-5.5 µm. Other concordances are the shape, the position at both sides of heterocysts, the spherical akinetes and the dimensions of the akinetes falling in the expected one of 8.5-11 µm (Figure 3.5.j). However, it did show some differences compared with the species described by Komárek (2005). For example, the heterocysts were bigger in 2.5-3 µm than the expected of ± 4.5 x 3.4-4 µm. The isolate showed smaller vegetative cells and barrel-shaped or oval heterocysts in relation to the type species proposed by Lemmermann, which present spherical heterocysts and vegetative cells of 4.0 x 7-8 µm. The vegetative cell length of the type description is smaller in relation to the isolate studied, with lengths between 4.64-18.01 µm, but this character is not considered diacritical by Komárek and Zapomělová (2007). Unfortunately, the 16S rRNA gene sequence of *Anabaena reniformis* was not available in GenBank for supporting this identification with a BLAST analysis.

***Nostoc* sp. RUP1**

This isolate from Lake Rupanco (Chile) can be tentatively identified as *Nostoc ellipsosporum* Rabenhorst ex Bornet & Flahault, as it exhibited some characteristic life cycle phases described previously and morphological features noted by Hrouzek *et al*. (2003). For example, the germination of heterocysts by fragmentation of a triad of heterocysts as well as the absence of mucilaginous sheath in some terminal heterocysts were observed (Figure 3.5.h). Despite this strong evidence, the isolate had smaller cells than described for the type species (Hrouzek *et al*., 2003). The most important difference was the length of heterocysts, which was approximately 8 µm smaller than expected

(15.9 μm), although according to the authors, the presence of these long heterocysts were rarely observed. Our dimensions were closer to the description given by Pereira *et al*. (2005), where the heterocysts length was 2 μm longer than ours, and the rest of the dimensions falling within the range described for these researchers. The taxonomic position of this strain in genus *Nostoc* was confirmed by the BLASTn query of the 16S rRNA gene sequences, where all the matches resulted with this genus (96-97%, E-value=0.0) and with *Nostoc ellipsosporum* strains LCR14 and V (EU446014, AJ630450) the identity was 94% and an E-value of 0.0.

4.2 PHYLOGENETIC RELATIONSHIPS OF THE STUDIED ISOLATES

Based on the 16S rRNA and *efp* gene sequence analyses, the genera *Anabaena*, *Aphanizomenon* and *Nostoc* were not monophyletic and were intermixed within the phylogenetic trees. In addition, these genera had divergent sequence similarities in which some isolates had similarities as low as 85.1% (92.2% on average) in the 16S rRNA and 54.4% (82.5% on average) in the *efp* gene sequence analyses. The 16S rRNA divergence values were in agreement with Gugger *et al*. (2002), Henson *et al*. (2003), Iteman *et al*. (2002), Lachance (1981), Lyra *et al*. (2001), and Rajaniemi *et al*. (2005) and among others. However, Rajaniemi *et al*. (2005) and Rikkinen *et al*. (2002) concluded that the genus *Nostoc* was a monophyletic group based on 16S rRNA gene, *rpoB* and *rbcLX* sequences. Conversely, *Anabaena*, *Aphanizomenon* and *Nostoc* strains were paraphyletic in the *nif*H and 16S rRNA gene sequence analyses of Iteman *et al*. (2002) and Lyra *et al*. (2001) respectively.

Additionally, according to Ludwig *et al*. (1998) and Stackebrandt and Goebel (1994), the levels of sequence similarities that define bacterial species and genera utilizing the 16S rRNA gene sequence similarities should be above 97.5 and 95% respectively. Based on this, the studied isolates of the genera *Anabaena* and *Nostoc* were genetically divergent and should be considered separate species as well as genera as the sequence divergence values fell well below these thresholds. Conversely, the isolates of the genus *Aphanizomenon* can be considered one species since the sequence similarity was above 97.5%. According to the DNA-DNA reassociation of Lachance (1981) and the 16S rRNA gene sequence analyses of Lyra *et al*. (2001) and Rajaniemi *et al*. (2005) the genus *Nostoc* was more divergent than *Anabaena* and *Aphanizomenon.* Rajaniemi *et al*. (2005) obtained similarities of ≥93.9% for *Nostoc* strains and ≥95.4% for *Anabaena* and *Aphanizomenon* strains. Analyzing cluster by cluster in the NJ of 16S rRNA gene sequence analysis (Figure 3.12), cluster 1 had a similarity above 94.2% (average of 94.7%), cluster 2 above 94.9% (average of 96.6%), cluster 3 above 98.2% (average of 98.9%) and cluster 4 above 90% (average of 95.9%). Hence, based on these sequence divergences, isolates in cluster 1, 2 and 4 may have issues with generic delineation. On the other hand, isolates of cluster 3 should correspond to the same species. In fact, on the basis of these values 46 specific level relationships were observed (Table 4.3). For example, *Anabaena* sp. LOW1 may be the same species as *Anabaena compacta, Anabaena lemmermannii* ONT1, *Anabaena* cf. *lemmermannii, Anabaena* cf. *planktonica, Nostoc ellipsosporum* UTEX383, *Aphanizomenon* cf. *gracile,* and *Aphanizomenon klebahnii* HHAFA. In addition, 51 generic relationships were obtained (Table 4.3) where *Aphanizomenon klebahnii* HHAFA should belong to the same genus than *Anabaena*

Table 4.4 **Generic (G) and specific (S) relationships of the studied isolates according to the consensus given by Ludwig *et al*. (1998) and Stackebrandt and Goebel (1994) for bacterial species and genus definition based on 16S rRNA gene sequences.** The colours indicate the different clusters obtained in the NJ analysis of 16S rRNA gene sequences (● Cluster 1; ● cluster 2; ● cluster 3; ● cluster 4; and ● cluster 5).

Isolates	*An. reniformis* MALW1	*Nostoc* sp. UTCC106	*Nostoc* sp. UTCC355	*An.* cf. *flos aquae* UTCC64	*An.* cf. *flos aquae* UTEX2383	*Anabaena* sp. 7812	*Nostoc* sp. UTCC387	*Nostoc* sp. RUP1	*N. punctiforme* UTCC41	*N. calcicola* UTEXB382	*An.* cf. *viguieri*	*Anabaena* sp. LONT5	*Anabaena* sp. A2879	*An. variabilis*	*An.* cf. *oscillariodes*	*An. lemmermannii* LONT2	*Nostoc* sp. UTCC314	*An. lemmermannii* GIOL8	*An. compacta*	*An. lemmermannii* ONT1	*N. ellipsosporum* UTEX383	*An.* cf. *lemmermannii*	*Aph. flos aquae* UTEXLB2384	*An.* cf. *planktonica*	*Aph. klebahnii* HHAFA	*Anabaena* sp. LOW1	*An.* cf. *cylindrica*
***An. reniformis* MALW1**																											
***Nostoc* sp. UTCC106**																											
***Nostoc* sp. UTCC355**																											
***An.* cf. *flos- aquae* UTCC64**		G	G																								
***An.* cf. *flos aquae* UTEX2383**			G	S																							
***Anabaena* sp. 7812**		G	G	S	S			G																			
***Nostoc* sp. UTCC387**		G	G	S	S	S																					
***Nostoc* sp. RUP1**																											
***N. punctiforme* UTCC41**								G																			
***N. calcicola* UTEXB382**																											
An.* cf. *viguieri																											
***Anabaena* sp. LONT5**											S																
***Anabaena* sp. A2879**											S	S															
An. variabilis											S	S	S														G
An.* cf. *oscillariodes											S	S	S	S													G
***An.lemmermannii* LONT2**											S	S	S	S	S												G
***Nostoc* sp. UTCC314**											S	S	S	S	S	S											G

Isolates	*An. reniformis* MALW1	*Nostoc* sp. UTCC106	*Nostoc* sp. UTCC355	*An.* cf. *flos aquae* UTCC64	*An.* cf. *flos aquae* UTEX2383	*Anabaena* sp. 7812	*Nostoc* sp. UTCC387	*Nostoc* sp. RUP1	*N. punctiforme* UTCC41	*N. calcicola* UTEXB382	*An.* cf. *viguieri*	*Anabaena* sp. LONT5	*Anabaena* sp. A2879	*An. variabilis*	*An.* cf. *oscillariodes*	*An. lemmermannii* LONT2	*Nostoc* sp. UTCC314	*An. lemmermannii* GIOL8	*An. compacta*	*An. lemmermannii* ONT1	*N. ellipsosporum* UTEX383	*An.* cf. *lemmermannii*	*Aph. flos aquae* UTEXLB2384	*An.* cf. *planktonica*	*Aph. klebahnii* HHAFA	*Anabaena* sp. LOW1	*An.* cf. *cylindrica*
***An.lemmermannii* GIOL8**																											
An. compacta																		G									
***An.lemmermannii* ONT1**																		G	G								
***N. ellipsosporum* UTEX383**																		G	S	G							
An.* cf. *lemmermannii																		G	S	G	S						
***Aph. flos aquae* UTEXLB2384**																		G	G	G	G	G					
An.* cf. *planktonica																		G	G	G	G	G	S				
***Aph. klebahnii* HHAFA**																		G	G	G	G	G	S	S			
***Anabaena* sp. LOW1**																		G	S	S	S	S	G	S	S		
Aph.* cf. *gracile																		G	G	G	G	G	S	G	S	S	
An.* cf. *cylindrica											G	G	G														

compacta, Anabaena lemmermannii ONT1, *Anabaena lemmermannii* GIOL8, *Anabaena* cf. *lemmermannii*, and *Nostoc ellipsosporum* UTEX383.

4.2.1 16S rRNA Gene Sequence Analyses

The genera *Anabaena*, *Aphanizomenon* and *Nostoc* were paraphyletic in the three obtained topologies for the 16S rRNA gene sequence analyses. This confirmed the problematic taxonomic assignment based on this molecular marker noted by numerous authors (Costa *et al*., 2001; Damerval *et al*., 1989; Fergusson and Saint, 2000; Gugger *et al*., 2002; Iteman *et al*., 2002; Lachance, 1981; Litvaitis, 2002; Lu *et al*., 1997; Lyra *et al*., 1997, 2001; Mazel *et al*., 1990; Nilsson *et al*., 2000; Rajaniemi *et al*., 2005; Rasmussen and Svenning, 2001; Rikkinen *et al*., 2002; Rudi *et al*., 1997, 2000; Svenning *et al*., 2005; Tamas *et al*., 2000; West and Adams, 1997; Willame *et al.* 2006; Wilmotte and Herdman, 2001; Wright *et al*. 2001). The neighbor-joining analysis differed from the topologies in the other analyses, as it included *Nostoc calcicola* UTEXB382 in cluster 1 (bootstrap support - 65%) (Figure 3.12). Whereas in the ML and Bayesian topologies this isolate was associated with clusters 2, 3 and 5 (bootstrap >50%) (Figures 3.14 and 3.15) and similarly in the MP analysis (bootstrap 57%) (Figure 3.13). Moreover, the taxonomic position of *Nostoc calcicola* UTEXB382 is questioned when examining the sequence similarity of this taxon compared to those in cluster 1 (94.2-95.3%) and in clusters 2, 3 and 5 (85.1-98.4%). In all topologies, *Nostoc punctiforme* UTCC41 maintained the same position within cluster 1, which was in agreement with the results of Lyra *et al*. (2001) and Rajaniemi *et al*. (2005) in which the strain *N. punctiforme* PCC73102 was included in a well-supported *Nostoc* clade and was closely related to *N. calcicola* strains III and VI (Rajaniemi *et al*., 2005). However, in their REP and ERIC fingerprinting analyses, Lyra

et al. (2001) obtained opposite results in which *N. punctiforme* PCC73102 clustered with *Anabaena* strains.

Cluster 2 was the same in the three 16S rRNA topologies and had high sequence similarities (94.9-99.7%) and was moderately (MP-71%) to strongly supported (NJ-100%) by bootstrap resampling. Furthermore, based on the sequence divergence values and the criteria noted by Ludwig *et al*. (1998) and Stackebrandt and Goebel (1994); members of this cluster could be considered to be part of one genus and some isolates probably represent the same species (Table 4.3). Cluster 2 was a mixing of *Anabaena*, *Aphanizomenon* and *Nostoc* isolates, although the morphological analysis of *Nostoc verrucosum* CR25 was not available, so it was impossible to determine if this was correctly identified; and *Nostoc ellipsosporum* UTEX383 was the only *Nostoc* species with evident gas vesicles. In fact, the identification of this latter strain is questioned by a BLASTn query, which resulted in a match with *Anabaena compacta* strains 1403/24, 189 and 118 (E value=0.0, identity=99%), moreover the morphometric analysis of the different cell types indicated that the classification of this strain is likely belonging to the species *Anabaena compacta* and *Nostoc* isolates in similar way (Figures 3.8-3.11). Conversely, the NMDS of the qualitative data separated *Nostoc ellipsosporum* UTEX383 from *Anabaena compacta*, groups 5 and 2 respectively (Figure 3.6.b).

Without considering the *Nostoc* species in cluster 2, *Aphanizomenon* isolates clustered with *Anabaena* cf. *planktonica*, *Anabaena* sp. LOW 1, *Anabaena lemmermannii* GIOL8 and *Anabaena lemmermannii* ONT1. However, this subcluster was not well supported in the NJ analysis (<50%), which is in concordance with ANOSIM based in the cellular metric characterization that treated each isolate as an independent species. In

the phylogenetic analyses, *Aphanizomenon* isolates were intermixed with *Anabaena* strains (Figures 3.12-3.15) and contradicted the ANOSIM in which they formed a completely different group (Figure 3.6.a). *Anabaena* cf. *planktonica* was one of the isolates mixed with the genus *Aphanizomenon* in the phylogenetic analyses and this genetic position was not supported by the ANOSIM, since the *R* values between them was 0.949, which indicates that they form well-differentiated morphological entities, in fact *Anabaena* cf. *planktonica*, formed a completely separate group in the NMDS metric-analysis (Figure 3.6.a). Indeed *Anabaena* cf. *planktonica* was closer to *Anabaena lemmermannii* ONT1 (R=0.761) than to *Aphanizomenon klebahnii* HHAFA and *Aphanizomenon* cf. *gracile* (*R*=0.958 and 0.939). Therefore, there were more metric similarities between *A*. cf. *planktonica* and *A. lemmermannii* ONT1 than with the *Aphanizomenon* isolates, although *Anabaena* cf. *planktonica* had characteristics unique to the genus *Aphanizomenon*, like hyaline-end cells and heterocysts distant to akinetes (Komárek and Komárková, 2006) and this isolate is similar to *Aphanizomenon schindlerii* (Kling *et al.,* 1994). According to the mean plot (confidence interval: ±0.95) of the isolates measurements, the relationship of *Anabaena* cf. *planktonica* and *Aphanizomenon* isolates was confirmed by the width of vegetative cells, end cells and heterocysts, and rejected by the length of the each cell kind and akinetes width (Figures 3.8-3.11). Hence, the controversial position of *Anabaena* cf. *planktonica* between genera *Anabaena* and *Aphanizomenon* was supported by the phylogenetic and morphological analyses.

On the other hand, the questionable identification of *Aphanizomenon* cf. *gracile* was confirmed by the fact that this isolate clustered with *Aphanizomenon flos-aquae* PCC7905 in the four obtained topologies for 16S rRNA gene. Rajaniemi *et al*. (2005)

noted that *Aphanizomenon gracile* did not cluster with *Aphanizomenon flos-aquae* strains, which is in agreement with the morphological character hyaline end cells. Typically, *A. flos-aquae* has hyaline end cells and *A. gracile* does not. However, *Aphanizomenon* cf. *gracile* in this study had hyaline end cells. On the other hand, the relationship noted among *Aphanizomenon* cf. *gracile*, *Anabaena compacta* and *Anabaena* cf. *cylindrica* isolates (cluster 2) was also observed by Willame *et al*. (2006) in their 16S rRNA gene sequence analyses. *Anabaena* sp. LOW1 was the other isolate closely related to *Aphanizomenon* isolates and in the quantitative and qualitative NMDS analyses (Figures 3.6.a, 3.6.b) this isolate grouped with *Aphanizomenon* cf. *gracile* and *Aphanizomenon klebahnii* HHAFA (R=0.37 and 0.7 respectively) and the BLASTn query matched *Aphanizomenon* strains. However, based on the morphological analysis, *Anabaena* sp. LOW1 should not be considered *Aphanizomenon* as it showed heterocysts at both sides of akinetes and this is not characteristic of *Aphanizomenon* (Komárek and Komárková, 2006). In general, these results were in concordance with Rajaniemi *et al*. (2005), who rejected the monophyly of the genera *Anabaena* and *Aphanizomenon* on the basis of 16S rRNA gene sequence analyses as these two genera were intermixed in the phylogenetic tree and the sequence similarities were above 94.8%. This is contrary to the present study in which the sequence divergence values in the *Anabaena-Aphanizomenon* cluster (cluster 2) was smaller (95.1-99.7%), with some isolates belonging to the same genus and other belonging to the same species. Gugger *et al*. (2002) obtained similar results and noted similarities above 97%, concluding that these two genera belong to the same species.

Nostoc ellipsosporum UTEX383 formed a subclade with *Anabaena compacta* and *Anabaena* cf. *lemmermannii* (100% and 99% supports in NJ and MP respectively) within cluster 2 (Figures 3.12-3.15). However, *Anabaena compacta* in ANOSIM of metric data was closer to *Nostoc* sp. RUP1 than to *Nostoc ellipsosporum* UTEX383 (R=0.778 and 0.993 respectively) (Figure 3.6.a). In fact *Nostoc ellipsosporum* UTEX383 was neither related to *Anabaena compacta* in the 16S rRNA analyses (Figures 3.12-3.15) nor NMDS of qualitative data (Figure 3.6.b). This was confirmed by the difference between *Anabaena compacta* with *Nostoc ellipsosporum* UTEX383 in vegetative cell, end cell, heterocyst and akinete widths (Figures 3.8.b, 3.9.b, 3.10.b, 3.11.b). In addition, *Anabaena compacta* and *Nostoc* sp. RUP1 were closely related in the mean plot (Confidence Interval: ±0.95) of the isolates measurements, showing some differences in heterocyst and akinete widths (Figures 3.10.b and 3.11.b).

Rajaniemi *et al*. (2005) noted a well-supported cluster formed by *Anabaena flos-aquae* and *Anabaena lemmermannii* strains with high sequence similarities (≥99.2%), although they concluded that this relationship was not supported by the morphological analysis. In the present study, these strains clustered separately with *Anabaena lemmermannii* isolates in clusters 2 and 3, and *Anabaena* cf. *flos-aquae* isolates in cluster 4 (Figures 3.12-3.15). In addition, these morphospecies have low 16S rRNA gene sequence similarities (89.1-93.1%). These clusters, however, were not supported by the quantitative and qualitative NMDS and the mean plot (Confidence Interval: ±0.95) analyses (Figures 3.6-3.11). In addition, two important exceptions can be observed: first, according to these analyses *Anabaena lemmermannii* LONT2 should belong to cluster 2, as they grouped together and had low R values in relation to the rest of *Anabaena flos-*

aquae and *Anabaena lemmermannii* isolates and second, *Anabaena lemmermannii* ONT1 should be separate from the remaining isolates since it was between group 2 and 3 (Figure 3.6.a) and showed major differences according to morphological parameter (Figures 3.8-3.11).

Cluster 3 within the neighbor-joining analysis was associated with a clade formed by cluster 1 and 2. However, in the ML and Bayesian analyses it was also associated with a clade formed by *Anabaena* cf. *cylindrica* (NJ-cluster 5) and *Anabaena reniformis* MALW1 (NJ-cluster 4). *Anabaena* cf. *cylindrica* could also be identified as *Anabaena augstumalis* based on morphological analyses (Rajaniemi *et al*., 2005), and formed a cluster in the periphery of the NJ tree in the 16S rRNA gene sequence analysis related with a huge cluster formed for the remaining isolates studied with a bootstrap value of 60% (in the rest of the trees it was associated to cluster 3). A similar result was obtained by Gugger *et al*. (2002) and Rajaniemi *et al*. (2005), where this species (strains XP6B and PH133 respectively) formed a cluster completely separate from the primary *Anabaena-Aphanizomenon* cluster in the analyses of the 16S rRNA, *rbcLX* , and *rpo* genes as well as the ITS regions. These results were also observed by Lyra *et al*. (2001) using the same molecular marker. However, in their RFLP analysis of the 16S-23S rDNA spacer region they obtained opposite results in which *Anabaena cylindrica* PCC7122 was within a clade formed by *Anabaena* and *Aphanizomenon* strains. Hence, they concluded based on low similarities and low bootstrap values that this position was ambiguous. The peripheral position of *Anabaena cylindrica* was also supported based on the presence of conical end cells and terminal heterocysts (Rajaniemi *et al*., 2005); results not observed in this study. According to Gugger *et al*. (2002), *Anabaena* cf. *cylindrica* without gas

vesicles should be distinguished from other benthic isolates, which agrees with the results observed in the NJ analysis. However, in the remaining analyses, this isolate grouped with the benthic strains. Additionally, compared with the results given by Henson *et al*. (2002) based on the nitrogen fixation gene *nif*D, *Anabaena cylindrica* (PCC7122) clustered separated from *Nostoc* sp. PCC7120, similar to this study (clusters 4 and 5 respectively). Similar results were showed by Lyra *et al*. (2001) based on 16S rRNA, although in their RFLP study of the 16S-23S rDNA spacer region and REP and ERIC analyses, *Anabaena cylindrica* PCC7122 grouped with other *Anabaena* strains and not with *Nostoc* sp. PCC7120. According to the cellular-metric analysis ANOSIM, the controversial position of *Anabaena* cf. *cylindrica* was supported as this isolate had high *R* values with some members of cluster 3 (*Anabaena* sp. LONT5, *Nostoc* sp. UTCC314 and *Anabaena reniformis* MALW; $R = 0.913$ to 0.986). This suggests that these isolates are completely differentiated at morphological level and this is reflected in the separation of *Anabaena* cf. *cylindrica* from the rest of the isolates in the NJ analysis. Conversely, the *R* values were lower in relation to *Anabaena variabilis* (R=0.846) and *Anabaena lemmermannii* LONT2 (R=0.856) and implies that they form an independent species but with some metric relationship, which was noted in the clustering of the ML analysis. This was opposite to the NMDS analysis of qualitative parameters, which grouped *Anabaena* cf. *cylindrica* with *Nostoc* sp. UTCC314 and *Anabaena variabilis*.

Anabaena reniformis MALW1 was another species with controversial phylogenetic results as it was the only isolate in cluster 4 (NJ analysis) that had evident gas vesicles (Figure 3.12, Table 3.1). On the other hand, in the ML and Bayesian analyses it clustered with *Anabaena* cf. *cylindrica* and was associated with cluster 3 and in the MP

analysis it did not group with any clade. This unresolved position of this isolate was also supported by the cellular-metric analysis (ANOSIM), where it was more closely related to *Anabaena* cf. *cylindrica* than with the remaining isolates. On the other hand, cluster 3 had the highest 16S rRNA sequence similarity (98.9%) and its members can be considered to be part of the same species despite some morphological differences. However, in the case of ML and Bayesian analyses of the 16S rRNA, were cluster 3 included *Anabaena reniformis* MALW1 and *Anabaena* cf. *cylindrica*, the sequence similarity was as low as 97% and hence they may not belong to the same species. In fact *Anabaena reniformis* MALW1 had a sequence similarity between 91.7 and 93.6% with the remaining members of cluster 3.

Cluster 4 was very similar in the all 16S rRNA topologies, although MP, ML and Bayesian analyses did not include *Anabaena reniformis* MALW1 (Figures 3.12-3.15). The 16S rRNA gene sequence similarity (average=95.9%) could support the definition of some isolates in this clade as unique genera (Table 4.3). If we exclude *Anabaena reniformis* MALW1 (like the ML and MP analysis), the isolates still can not be considered the same species as a group (average=97.1%). Conversely this cluster had the lowest *R* values in the cellular-metric analysis ANOSIM (*R*=0.345-0.789), indicating a closer morphological relationship than the genetic one. For example, *Anabaena* cf. *flos-aquae* UTCC64 and UTEX2383 isolates had an *R* value of 0.345, and *Anabaena* cf. *flos-aquae* UTCC64 with *Nostoc* sp. UTCC355 had an *R*=0.447. The morphological parameters that best represent this cluster was the akinete length and width (Figures 3.11.a, 3.11.b), and vegetative cell length (Figure 3.8.a). In fact *Anabaena reniformis* MALW1 had the largest difference from *Anabaena* cf. *flos-aquae* UTCC64 and

UTEX2383 in vegetative cell and end cell widths (Figures 3.8.b, 3.9.b), this was also represented in the qualitative NMDS analysis (Figure 3.6.b). *Nostoc* sp. PCC7120 has been a controversial strain with the phylogenetic assignment intermediate between genera *Anabaena* and *Nostoc* (Henson *et al*. 2002). In this study, this strain clustered with isolates of both genera, in fact it formed a clade with *Anabaena variabilis* ATCC29413 with 100% support. This was opposite to the results obtained by Tamas *et al*. (2002), where *Nostoc* PCC 7120 was in a different clade than *A. variabilis* ATT29413, although *A. variabilis* isolate analyzed in this thesis was in cluster 3, which would be in concordance with Tamas *et al*. (2002) results. On the other hand, based on their RFLP study Lyra *et al*. (2001), *Nostoc* sp. PCC7120 clustered with *Nostoc* strains completely separate from *Anabaena* and *Aphanizomenon* isolates. Conversely, these authors observed that the same *Nostoc* subclade that contains *Nostoc* sp. PCC7120 formed an independent subclade but within a major clade formed by *Anabaena* and *Aphanizomenon* ones in their 16S rRNA gene sequence analyses.

4.2.2 *EFP* GENE SEQUENCE ANALYSES

The genera *Anabaena*, *Aphanizomenon* and *Nostoc* were paraphyletic in the three topologies obtained for *efp* gene, which agrees with the 16S rRNA gene sequence analyses and was also supported by the ANOSIM cellular-metric analysis. Nevertheless, some well defined clusters were obtained. For example, cluster 1 was a well supported entity of *Aphanizomenon* isolates (not observed in the 16S rRNA gene sequence analyses) and was similar to the findings of Li *et al*. (2003), who proposed the monophyly of *Aphanizomenon* strains based on 16S rRNA gene sequence analyses. However, they did not include planktonic *Anabaena* sequences, which should be included

in future analyses considering that *Aphanizomenon* is also a planktonic genus and usually clusters with these types of *Anabaena* strains (Gugger *et al*., 2002; Lyra *et al*., 2001; Rajaniemi *et al*., 2005; Willame *et al*., 2006). On the other hand, this *Aphanizomenon* cluster was supported by the ANOSIM statistical analysis in which *Aphanizomenon klebahnii* HHAFA and *Aphanizomenon* cf. *gracile* formed an independent group with a high *R* value in relation with the rest of the analyzed isolates (between 0.905 and 1), and a low *R* value between them (0.384). This *Aphanizomenon* cluster (cluster 1) was noted in the four topologies with high bootstrap support (99-100%), though there were some differences in the relationships between this clade and others in the MP and Bayesian analyses. For example, in the NJ and ML analyses cluster 1 was in between a *Nostoc* cluster (cluster 4) and the remaining isolates, whereas in the MP and Bayesian analyses it was associated with *Anabaena reniformis* MALW1 and clusters 2, 3 and 4 in a similar manner (Figures 3.16-3.19).

Cluster 4 in the *efp* gene sequence analyses was also well-supported and only included *Nostoc* isolates (100%-NJ, 97%-MP and 99%-Bayesian) and observed in all obtained topologies. This is in agreement with Svenning *et al*. (2005), but this was contrary to the phylogenetic position of *Nostoc commune* NWT208.5 in this study, which was included in cluster 2 and not in cluster 4 like *Nostoc punctiforme* UTCC41. On the other hand, this cluster is comparable to cluster 1 of 16S rRNA gene sequence analysis; with the exception that it did not contain *Nostoc calcicola* UTEXB382. In fact, *N. calcicola* UTEXB382 was one of the isolates in cluster 2 with lower *R* values in the ANOSIM cellular-metric analysis (*R*=0.751) in relation with *Nostoc* sp. RUP 1 (*efp*-cluster 4 and 16S rRNA-cluster 1), and with members of *efp*-cluster 2 showed a wide

range between 0.295 and 0.999 (with *Anabaena* cf. *flos-aquae* UTEX2383 and *Anabaena planktonica* respectively). So, *N. calcicola* UTEXB382 was one of the strains that shared more morphometry characters with the rest of the isolates, in fact in NMDS analysis of quantitative and qualitative data this isolate belonged to the main group (Figures 3.6.a, 3.6.b).

The phylogenetic position of *Anabaena reniformis* MALW1 was more consistent in these analyses compared to the 16S rRNA gene sequence analyses and was weakly supported as grouping with cluster 2 in the all the phylogenetic analyses. In the 16S rRNA analyses, this taxon was closely related to *Anabaena* cf. *cylindrica* whereas in the *efp* analyses these two isolates were in different clusters. In fact, the *efp* results were supported by the quantitative analysis ANOSIM, since *Anabaena* cf. *cylindrica* and *Anabaena reniformis* MALW1 share an *R* value of 0.966, indicating that they formed a well differentiated isolates on the base of their cellular dimensions. Instead, according to this parameter, *Anabaena reniformis* MALW1 should be related with *Nostoc* sp. UTCC355 (*R*=0.642), which correspond to the relationship showed in NJ, ML and Bayesian analyses of *efp* gene sequences. On the other hand, similar results were observed in the NMDS analysis of the quantitative and qualitative data (Figures 3.6.a, 3.6.b), where the relationship of *Anabaena reniformis* MALW1 with *Anabaena* cf. *cylindrica* was not supported, indeed these two isolates were in different clusters, instead *Anabaena reniformis* MALW1 was more closely related to *Nostoc* sp. UTCC355.

Cluster 3 was well-supported with high bootstrap values in both the *efp* (50%-NJ, 94%-MP and 100%-Bayesian) and 16S rRNA (100%-NJ and 62%-Bayesian) gene sequence analyses, but did not appear to be related to *Anabaena* cf. *cylindrica* in the NJ

and MP analyses as discussed previously. In general, *Anabaena* cf. *cylindrica* corresponds to a well-supported species based on the ANOSIM cellular-metric analysis, since the *R* values were between 0.846 (with *Anabaena variabilis*-cluster 3) and 0.998 (with *Anabaena compacta*-cluster 2). The lowest *R* value was with *Anabaena lemmermannii* ONT1 (0.794) and indicated that they share more morphological similarities than with the rest of the isolates. The relationship of *Anabaena* cf. *cylindrica* and *Anabaena variabilis* (*R*=0.846) was also observed in the NMDS analysis of qualitative parameter, where these isolates formed the group 3 (Figure 3.6.b).

The large Cluster 2 in the *efp* gene sequence analyses included a mixing of *Anabaena* and *Nostoc* isolates (Figures 3.16-3.19). However, two main clades are present within this clade in both the NJ and ML analyses. The first one was formed by *Nostoc* sp. NWT150.1, *Nostoc verrucosum* CR25, *Nostoc* sp. D1, *Nostoc* sp. D2, *Nostoc commune* NWT208.5, *Anabaena* sp. 7812, *Anabaena variabilis* ATCC29413, *Anabaena* cf. *flos-aquae* UTEX2383, and *Anabaena* cf. *flos-aquae* UTCC64. The second one was formed by *Anabaena lemmermannii* ONT1, *Nostoc* sp. 7120, *Anabaena* sp. LOW1, *Anabaena* cf. *lemmermannii*, *Anabaena* cf. *oscillariodes*, *Anabaena compacta* and *Anabaena lemmermanii* GIOL8. The first clade was also observed in the ML and Bayesian analyses (Figures 3.17, 3.18). In this clade some *Nostoc* strains formed a well supported subclade, which was different from the well supported clade in the MP analysis, which included more *Nostoc* isolates (*Nostoc* sp. UTCC106, *N. ellipsosporum* UTEX383, *N. calcicola* UTEXB382, *Nostoc* sp. UTCC387), and even one specie of genus *Anabaena* (*A.* cf. *planktonica*). However, according to the cellular-metric analysis (ANOSIM) this relationship was not supported, since *A.* cf. *planktonica* showed *R* values of 0.999 and 1.0

with *N. calcicola* UTEXB382 and *N. ellipsosporum* UTEX383 respectively. Conversely in the 16S rRNA gene sequence analysis *Nostoc* sp. UTCC387 was related to *A. variabilis* ATCC29413, *A.* cf. *flos-aquae* UTCC64, *A.* cf. *flos-aquae* UTEX2383 and *Anabaena* sp. 7812. In fact, *Anabaena* sp. 7812, *A. variabilis* ATCC29413, *A.* cf. *flos-aquae* UTEX2383, and *A.* cf. *flos-aquae* UTCC64 were well related by both gene sequence analyses, what was confirmed by the cellular-metric analysis ANOSIM (R=0.345-0.525). Although the *efp*-MP analysis did not related them closely, since *A.* cf. *flos-aque* UTCC64 was out of a clade with a bootstrap value of 62%.

In the *efp*-gene sequence analysis *Nostoc* sp. UTCC387 formed a clade with *Nostoc ellipsosporum* UTEX383, although this was not well-supported (<50%-NJ, 55%-Bayesian) (Figures 3.16, 3.19). This relationship of *N. ellipsosporum* UTEX383 was not observed in the 16S rRNA gene sequence analysis (Figures 3.12-3.15) and in the *efp*-MP analysis (Figure 3.17). In fact, in the 16S rRNA gene sequence analysis *N. ellipsosporum* UTEX383 was always associated with *Anabaena compacta* and *Anabaena* cf. *lemmermannii*, although according to the ANOSIM cellular-metric analysis each of these isolates represent independent species (R=0.938-0.983).

The phylogenetic relationship among the isolates of the second subcluster in the NJ-cluster 2 (Anabaena *lemmermannii* ONT1, *Nostoc* sp. 7120, *Anabaena* sp. LOW1, *Anabaena* cf. *lemmermannii*, *Anabaena* cf. *oscillariodes*, *Anabaena compacta* and *Anabaena lemmermanii* GIOL8) was not maintained in the MP and Bayesian analyses. In the NJ analysis each isolate was subdivided in clades of two, instead in the ML and Bayesian analyses each isolate formed individual clades, which have the same phylogenetic position than the first subclade and a clade formed by *Anabaena* cf.

planktonica, *Nostoc* sp. UTCC387 and *Nostoc ellipsosporum* UTEX383 (Figures 3.17, 3.19). In the 16S rRNA gene sequence analyses, *Anabaena lemmermannii* ONT1 was always associated with *A. lemmermannii* GIOL8 (bootstraps: NJ= 94%, MP= 57%, Bayesian=100%) (Figures 3.12-3.15), but in the *efp* gene sequence analysis was also associated with *Anabaena* cf. *lemmermannii*, *A.* cf. *oscillariodes*, *A. compacta*, and *Anabaena* sp. LOW1 (Figures 3.16-3.19). According to the ANOSIM cellular-metric analysis, *A. lemmermannii* ONT1 had some similarities with *A. lemmermannii* GIOL8 (R=0.756) but was similar to *A*. cf. *lemmermannii* (R=0.684) as well. This relationship was also reflected in the 16S rRNA analysis as all were in cluster 2.

4.3 MORPHOLOGICAL DIFFERENTIATION OF THE STUDIED ISOLATES

The molecular phylogenetic analyses did not support the distinction among isolates with and without gas vesicles (planktonic and benthic isolates respectively). First, the clusters with (clusters 2 of 16S rRNA, cluster 1 of *efp*) (Figures 3.12-3.19) and without gas vesicles (clusters 1 of 16S rRNA, cluster 4 of *efp*) (Figures 3.12-3.19) were paraphyletic within the phylogenetic trees. In addition, some clusters showed exceptions, such as cluster 3 of 16S rRNA and *efp* (Figures 3.12-3.19) which will be analyzed below, and some clusters had a mixing of isolates with gas vesicles in the whole filament, with gas vesicles in some cells within the filament and without gas vesicles (cluster 2 of 16S rRNA and *efp*) (Figures 3.12-3.19). Similar results were obtained by Rajaniemi *et al*. (2005) and Willmotte and Herdman (2001) on the basis of 16S rRNA gene sequence analysis. Therefore the presence of gas vesicles does not appear to be useful for the differentiation of *Anabaena*, *Aphanizomenon* and *Nostoc* isolates. In addition, it is

important to note that this is a morphological character that is easily lost under culture conditions (Lehtimäki *et al*., 2000; Rajaniemi *et al*., 2005; Willame *et al*., 2006), hence it is unreliable as a diacritical character unless the isolates are characterized immediately after isolation (Rajaniemi *et al*., 2005). In fact, most of the studied isolates have been in culture for a prolonged time and hence the presence or absence of gas vesicles was not considered in the statistical analysis (Figures 3.6.b). Examining the phylogenetic trees in more detail (Figures 3.12-3.19), cluster 3 in both the 16S rRNA and *efp* gene sequence analyses was clearly evident (NJ-bootstrap=50 and 100 respectively, Bayesian=100) and this was a cluster characterized by the absence of gas vesicles, with the exception of *Anabaena lemmermannii* LONT2. However, this was not confirmed by the 16S rRNA gene sequence similarities above 98.2% (98.9% on average), which related the members of this cluster at species level. Comparable results were observed by Willame *et al*. (2006), who noted a cluster containing only benthic strains, although they concluded that the *Anabaena* strains which formed this cluster probably were related to each other based on generic level relationships rather than species. Cluster 2 in the 16S rRNA gene sequence analysis was formed by planktonic *Anabaena*, *Aphanizomenon* and *Nostoc* isolates that showed sequence similarities that may support a generic relationship (>94.2, 96.6% on average). This is in concordance with Gugger *et al*. (2002), who on the basis of 16S rRNA, ITS and *rbc*L*X* regions showed that planktonic *Anabaena* and/or *Aphanizomenon* isolates should belong to the same genus. In the *efp* gene sequence analysis the only cluster containing gas vesicles was cluster 1, which was formed only by *Aphanizomenon* isolates (Figures 3.16-3.19). The 16S rRNA gene analysis exhibited the benthic isolates in cluster 1 whereas they were observed in cluster 4 of the *efp* gene

analysis. On the other hand, identification problems could support the difficulties in separating the planktonic and benthic isolates. For example *Nostoc ellipsosporum* UTEX383 should not have gas vesicles (Hrouzek *et al*., 2003), but our isolate showed evident gas vesicles and clustered with the other strains that formed gas vesicle (cluster 2) (Figures 3.12, 3.16).

Other morphological characters that were not included in the statistical analysis included the following: presence of mucilaginous sheath, coiling of the trichome and the fascicle-like colonies. All of these characters can be lost under culture conditions or develop depending on growth conditions (Gugger *et al*., 2002; Komárek and Zapomělová, 2007; Li *et al*., 2000; Rajaniemi *et al*., 2005; Willame *et al*., 2006). Moreover, other researchers have demonstrated that molecular analyses do not support their usefulness for classification. For example, Rajaniemi *et al*. (2005), Willame *et al*. (2006) and Zapomělová *et al*. (2008a) observed that strains with straight and coiling trichomes formed one large cluster in their 16S rRNA gene and *rpoB* and *rbcLX* sequences.

The quantitative and qualitative NMDS analyses showed a close relationship among *Aphanizomenon* isolates and *Anabaena* sp. LOW1 as well as *Anabaena* cf. *cylindrica* (Figures 3.6.a, 3.6.b). This latter relationship is controversial position but also observed in the 16S rRNA gene sequence analyses (Figures3.12-3.15). The relationship among the *Aphanizomenon* isolates was supported by each morphological character (length and width of vegetative cells, end cells, heterocysts, and akinetes) (Figures 3.8-3.11). However, the characters that best supported their differentiation from the remaining isolates were the lengths of end cells, heterocysts and akinetes (Figures 3.9.a,

3.10.a, 3.11.a). Willame *et al*. (2006) observed that the characters that best represent the *Aphanizomenon* strains was the narrowest vegetative cells and heterocysts, results not observed in this study (Figures 3.8.b, 3.10.b). For example, *Anabaena* sp. LOW1 was characterized by the narrowest vegetative cells in the same way of *Anabaena reniformis* MALW1 (Figure 3.8.b), and *Anabaena* sp. LOW1 and *Anabaena variabilis* had the narrowest heterocysts (Figure 3.10.b). Hindák (2000) demonstrated high morphological heterogeneity in the genus *Aphanizomenon* and again, this result was not observed in this study. However, two different morphotypes described by Komárek and Komárková (2006) (*Aphanizomenon flos-aquae* and *Aphanizomenon gracile*) were observed. The *efp* gene sequence analyses separated *Aphanizomenon* from *Anabaena* and *Nostoc* isolates (Figures 3.16-3.19). Conversely, in the 16S rRNA *Aphanizomenon* isolates clustered with other isolates characterized by narrow cylindrical cells (*Anabaena* sp. LOW1 and *Anabaena* cf. *planktonica*) and with two *Anabaena lemmermannii* isolates (strains GIOL8 and ONT1), which present ellipsoidal vegetative cells, completely different to the cylindrical ones (Figure 3.12). Hence, the width of vegetative cells and heterocysts, characters proposed by Willame *et al*. (2006) were supported by neither the 16S rRNA nor the *efp* gene phylogenetic analyses. Moreover, the qualitative distribution of *Aphanizomenon* isolates was supported by all the analyzed parameters (Table 3.1), where the only difference between them was the akinete location in relation to the heterocysts, since *A.* cf. *gracile* had akinetes located far from the heterocyst and *A. klebahnii* HHAFA had akinetes at one side of the heterocyst. On the other hand, the morphology of end cells has been considered an important character in the *Aphanizomenon* species identification (Willame *et al*. 2006), which was in concordance with our results. For example, end cells

of *A. klebahnii* HHAFA were much longer that the *A.* cf. *gracile* ones (Figure 3.9.a), and exhibited the hyaline appearance in every filament. On the other hand, this was only observed in some end cells in a few filaments in *A.* cf. *gracile*.

Anabaena sp. LOW1 was related to *Aphanizomenon* isolates in the 16S rRNA gene sequence and in the morphological analyses. These isolates had similar end cell lengths but were separated on the basis of the shape of the akinetes; where *Anabaena* sp. LOW1 had cylindrical and kidney-shaped akinetes and were observed on both sides of the heterocysts, instead *A.* cf. *gracile* had cylindrical to ellipsoidal akinetes distant to heterocysts and *A. klebahnii* HHAFA had cylindrical akinetes at one side of heterocysts. On the other hand, the morphological analysis (Figures 3.6.a, 3.6.b) showed a relationship between the *Aphanizomenon* isolates and *Anabaena* cf. *cylindrica*, which was not observed in the phylogenetic analyses (Figures 3.12-3.19). The parameters that best grouped *Anabaena* cf. *cylindrica* with *Aphanizomenon* isolates was heterocyst length and cylindrical akinetes (Figure 3.10.a; Table 3.1). Another species closely related to *Aphanizomenon* isolates was *Anabaena* cf. *planktonica*, this relationship was supported by the 16S rRNA gene sequence analyses (Figures 3.12-3.15). In terms of the morphological analyses, this relationship was evident in the mean plot of the morphological parameters, where they were related and differentiated from the rest of the isolates by the length of vegetative cell, end cells and heterocyst (Figures 3.8.a, 3.9.a, 3.10.a). *Anabaena* cf. *planktonica* also formed a completely separate group in the quantitative NMDS analysis (Figure 3.6.a), which may be attributed to some larger differences with *Aphanizomenon* isolates in terms of the width of each cell kind and in akinete length (Figures 3.8.b, 3.9.b, 3.10.b, 3.11.a, 3.11.b). In fact, with respect to akinete

width, this isolate was completely separated from the rest. Moreover, these isolates were differentiated on the base of the presence of barrel-shaped vegetative cells and ellipsoidal akinetes in *Anabaena* cf. *cylindrica*, and cylindrical vegetative cells and akinetes in *Aphanizomenon* isolates (Table 3.1).

According to Zapomělová *et al*. (2007), *Anabaena compacta* populations were the only morphospecies clearly defined on the basis of width of vegetative cells, shape of akinetes and regularity of coiling. Similar results were obtained in the qualitative NMDS analysis as these isolates formed an independent group with *Anabaena reniformis* MALW1 (Figure 3.6.b). In addition, they were the only isolates with spherical akinetes (Table 3.1). However, Zapomělová *et al*. (2007) did not include *Anabaena reniformis* in the 2007 analyses but it was considered later (Zapomělová *et al*., 2008c), where they noted these two species appeared to be closely related at morphological level and the differences in their coiling was the only distinguishing character. In this thesis *A. reniformis* MALW1 was differentiated from *A. compacta* in the shape of vegetative cells and the position of the akinetes in relation to the heterocysts (Table 3.1). However, it is possible that these differences could be due to culture conditions, since a high variability in the vegetative cell morphology has been demonstrated in *A. reniformis* (Zapomělová *et al*., 2008c). In addition, the position of the akinetes distant from heterocysts in *A. compacta* and at both sides of the heterocysts of *A. reniformis* MALW1 was in agreement with the description of the type species (Komárek and Zapomělová, 2007). On the other hand, this grouping was not supported by the phylogenetic analyses, since in the 16S rRNA gene sequence analysis *A. compacta* was in cluster 2 and *A. reniformis* MALW1 was in cluster 4 (Figures 3.12-3.15). However, in the *efp* gene sequence analysis these

two isolates were in the same cluster but had only 78.2% sequence similarity (Figures 3.16-3.19). But in the quantitative NMDS analysis *A. compacta* was in the periphery of group 2 and overlapped with *Nostoc* sp. RUP1 (Figure 3.6.a). This position may be more defined by the length of vegetative and end cells, and the width of heterocysts than by the width of vegetative cells (Figures 3.8.a, 3.9.a, 3.10.b, 3.8.b respectively) and it was not supported by the phylogenetic analysis since they clustered in completely separate clades (Figures 3.12-3.19). The regular coiling on *A. compacta* observed by Zapomělová *et al*. (2007) is a character that was lost under culturing and only straight to slightly curve filaments were observed (Table 3.1). In addition, in the 16S rRNA and *efp* gene sequence analyses *A. compacta* was completely different from other isolates in its cluster as it was the only isolate with spherical vegetative cells, heterocysts and akinetes (Table 3.1), which was supported by the quantitative and qualitative NMDS analysis (Figures 3.6.a, 3.6.b).

Anabaena lemmermannii ONT1 was intermediate between group 1 and 3 in the metric NMDS analysis (Figure 3.6.a), and *Anabaena* cf. *lemmermannii* formed an independent group (group 4, Figure 3.6.b) in the qualitative NMDS analysis. This morphological heterogeneity among populations of *Anabaena lemmermannii* morphospecies has been shown by Zapomělová *et al*. (2007), however all the population of these morphospecies showed the same position of the akinetes (at both sides of heterocysts), and were differentiated primarily by the vegetative cell width and length:width ratio. In this study, three of the four studied *A. lemmermannii* had two akinetes on both sides of heterocysts; one on each side of the heterocyst. However, *Anabaena* cf. *lemmermannii* had only one next to the heterocyst, which contributed to an

ambiguous identification and position in a different group in the NMDS analysis (group 4, Figure 3.6.b). Moreover, *Anabaena* cf. *cylindrica* and *Anabaena* sp. LOW1 also had akinetes on both sides of the heterocyst, but this character was not crucial in their distribution in the NMDS analysis (Figure 3.6.b) since *Anabaena* cf. *cylindrica* and *Anabaena* sp. LOW1 were in completely different groups than the *A. lemmermannii* isolates. The importance of this character for the identification of this species has been questioned by Gugger *et al*. (2002), which was confirmed by the 16S rRNA and *efp* phylogenetic analyses (Figures 3.12-3.19) in which *A. lemmermannii* LONT2 clustered in a different clade than the remaining isolates that had akinetes on both sides of heterocysts. With respect to the metric analyses, Figures 3.8-3.11 demonstrated that *A. lemmermannii* isolates were morphologically heterogeneous and the parameter that showed more differences among isolates was the vegetative cells width similar to the findings by Zapomělová *et al*. (2007).

Anabaena cf. *flos-aquae* isolates (UTCC64 and UTEX2383) were difficult to identify due to the absence of akinetes and some differences from the type species such as the shape of the vegetative cells. In the metric NMDS analysis these two isolates overlapped (Figure 3.6.a) whereas in the qualitative analysis they were slightly separated but in the same group (Figure 3.6.b). According to Zapomělová *et al*. (2007), the *A. flos-aquae* populations are unified by the width of vegetative cells, shape of vegetative cells, and length:width ratio of akinetes. The two *A.* cf. *flos-aquae* in this study were virtually identical and the only difference was that *A.* cf. *flos-aquae* UTCC64 sometimes had ellipsoidal heterocysts whereas *A.* cf. *flos-aquae* UTEX2383 always had spherical heterocysts (Table 3.1). In the metric analysis the major differences between this two

isolates was the width of heterocysts (Figure 3.10.b) but the widths of the vegetative cells were very similar (Figure 3.8.b). The two *A*. cf. *flos-aquae* clustered together in both gene sequence analyses, therefore the morphological characters (width and shape of vegetative cells) that define them had phylogenetic support, which was previously demonstrated by Zapomělová *et al*. (2007).

The *Nostoc* isolates were spread out in the qualitative NMDS analyses (Figures 3.6.a, 3.6.b) but were unified by the shape of vegetative cells (Table 3.1). In the phylogenetic analyses they were also intermixed among various clusters with some species differentiated in well-supported clusters (e.g. cluster 1 of 16S rRNA and cluster 4 of *efp* had bootstrap values of 65 and 100% respectively) (Figures 3.12, 3.16). In the metric NMDS analysis the *Nostoc* isolates overlapped in group 3 and were intermixed with *Anabaena* isolates (Figure 3.6.a). However, in the qualitative NMDS analysis just *Nostoc* UTCC314 formed a different group (Figure 3.6.b). This relationship was supported by the 16S rRNA and *efp* gene sequence analyses, where *Nostoc* UTCC314 was the only *Nostoc* strain in cluster 3 and was clearly separate from the remaining *Nostoc* strains (Figures 3.12, 3.16). *Nostoc calcicola* UTEXB382 clustered with *Nostoc* RUP1 in the 16S rRNA gene sequence analyses but this relationship was not observed in the *efp* gene sequence analyses. In fact, *Nostoc calcicola* UTEXB382 clustered with *N. ellipsosporum* UTEX383 and *Nostoc* sp. UTCC355 despite divergent sequences (similarities ≥ 86.38%). This was not supported by the metric NMDS analysis in which the five *Nostoc* isolates overlapped, or by the qualitative NMDS analysis, where *Nostoc* RUP1, *N. ellipsosporum* UTEX383 and *Nostoc* sp. UTCC355 were closer together than with *Nostoc calcicola* UTEXB382. There are few studies that combine morphological

data with the phylogenetic analyses on the genus *Nostoc* or other cyanobacterial genera (e.g. Rajaniemi *et al*. 2005) and very few in which a complete morphological analysis had been carried out (e.g. *Anabaena* by Zapomělová *et al*., 2007). In general, the morphological differentiation of *Nostoc* species and strains has been primarily based on hormogonia development and shape of mucilaginous sheath, as the works of Li *et al*. (2005) and Wright *et al*. (2001), but these characters are considered unreliable as they vary according culture conditions (Wright *et al*., 2001).

In summary, the NMDS analysis of metric parameters demonstrated that akinete length is an important taxonomic character, following by the width of akinetes (Figure 3.7) and is in agreement with the results of Rajaniemi *et al*. (2006). This was also noted for *Aphanizomenon* isolates, which had the largest akinetes (Figure 3.11.a). This was similar to the findings by Zapomělová *et al*. (2008b), although they considered all the dimensions of akinetes and vegetative cells the most variable characters. In fact, the H value of Kruskal-Wallis test noted that all differences of the morphological parameters were significant; hence each parameter is useful for isolate differentiation. On the other hand, some researchers suggest that the studied parameter (width and length of each cell kind) depend on growth conditions, in the same way that the differentiation of heterocysts and akinetes, presence and absence of mucilaginous sheath and gas vesicles, fascicle-like colonies, etc. (Hrouzek *et al*., 2003). Although, Zapomělová *et al*. (2008a) concluded that the experimental conditions have little effect on the dimensions of vegetative cells and heterocysts, but do affect in the coiling of the trichomes, and heterocysts developing.

CHAPTER 5: CONCLUSIONS

The taxonomic differentiation of the genera *Anabaena*, *Aphanizomenon* and *Nostoc* was not supported by the morphological and genetic data, and the phylogenetic analysis was not congruent with the morphological analyses, although some important conclusions should be noted.

1. **The genera *Anabaena*, *Aphanizomenon* and *Nostoc* are not monophyletic** - Isolates in these genera are paraphyletic in phylogenetic trees. Nevertheless, the *efp* gene sequence analyses resulted in a well-supported clade of *Aphanizomenon* isolates (cluster 1, bootstrap=99-100%) which was also supported by the morphological analysis.
2. **The morphological diversity is higher than genetic diversity** - The ANOSIM metric-analysis showed that each isolate was well differentiated however; the 16S rRNA and *efp* gene sequence analysis depicted some of these as being closely related and could be considered one species.
3. **Sequence divergence is higher than expected in the genera *Anabaena* and *Nostoc*** -The sequence similarity was above 85.1% (92.5% in average) and 89.4% (average of 91.5%) for *Anabaena* and *Nostoc*, respectively and indicates separate species and genera in some cases. Conversely, the isolates of genus *Aphanizomenon* can be considered one species as sequence similarity was above 97.5%.
4. **Only members of cluster 3 can be considered the same species** - Based on the 16S rRNA gene sequences analysis only members of cluster 3 should be

considered the same species. This conclusion is on the basis of the parameter given by Ludwig *et al*. (1998) and Stackebrandt and Goebel (1994). In which for bacterial species and genus definition the 16S rRNA gene sequence similarities should be above 97.5 and 95% respectively. Therefore *Anabaena* cf. *viguieri*, *Anabaena variabilis*, *Anabaena* sp. LONT5, *Anabaena* cf. *oscillariodes*, *Anabaena lemmermannii* LONT2, *Nostoc* sp. UTCC314 and *Anabaena* sp. A2879 should be considered one species since had a 16S rRNA gene sequence similarities above 98.2%. Within the remaining clusters the generic level of taxonomy can be questioned (>94.2% in cluster 1, >94.9% in cluster 2 and >90% in cluster 4).

5. **The presence or absence of gas vesicles is not a diacritical character** - In the 16S rRNA and *efp* gene sequence analyses the isolates were not separated into separate groups based of presence and absence of gas vesicles. Since clusters with (16S rRNA-clusters 1 and 2, and *efp*-cluster 1), without (16S rRNA-cluster 5, and *efp*-clusters 4) and cluster with a mixing of planktonic and benthic isolates (16S rRNA-cluster 3 and 4, and *efp*-cluster 2 and 3) were paraphyletic in the phylogenetic trees.
6. **Akinete length is the morphological character that best differentiates isolates** - although every metric character was useful for their differentiation (width and length of vegetative cells, end cells and heterocysts, and width of akinetes). The length of akinetes as a diacritical character for species delineation was mainly supported by the presence of *Aphanizomenon* isolates, which presented the longest akinetes.

7. **Long-term maintenance of cyanobacteria in culture can result in mis-identification** - This thesis confirmed that the time of the cultures under controlled condition is crucial for genus and species identification and the characterization of the isolates should be done immediately after isolation since some morphological characters may change or be lost under culture conditions. For example, the mucilaginous sheath, the coiling of the trichome and the fascicle-like colonies are characteristics typically lost in culture. This also raises further support for the cryopreservation of key isolates.
8. **Numerous strains and isolates need to be revised in terms of their taxonomic identification** - *Anabaena* cf. *cylindrica, Anabaena* cf. *flos-aquae* UTCC64 and UTEX2383, *Anabaena* cf. *oscillariodes, Anabaena* cf. *planktonica, Anabaena* cf. *lemmermannii, Anabaena* sp. A2879, *Anabaena* sp. LONT5, *Anabaena* sp. LOW1, *Anabaena* sp. 7812, *Anabaena* cf. *viguieri, Aphanizomenon* cf. *gracile, Nostoc* sp. UTCC 106, *Nostoc* sp. UTCC 314, *Nostoc ellipsosporum* UTEX383, *Nostoc* sp. UTCC 355, *Nostoc* sp. UTCC 387, and *Nostoc* sp. RUP1 require revision in identification as there appear to be clear differences from the type species, or heterocysts and/or akinetes were never observed, or the BLASTn query did not presented high similarities with their type species. In fact the generic position of some species was questioned on the base of the morphological data (*Anabaena* cf. *planktonica* was similar to *Aphanizomenon schindlerii*) or by the BLASTn query (*Anabaena* sp. LONT5, *Anabaena* sp. LOW1 and *Nostoc* sp. UTCC 387 showed the highest similarities with members of genus *Trichodesmium, Aphanizomenon* and *Anabaena* respectively).

9. ***Anabaena* sp. LOW1 appears to be a member of the genus *Aphanizomenon*** This position supported by the BLASTn query, qualitative and quantitative analyses, and 16S rRNA gene sequence analyses. However, the one difference is that this isolate had heterocysts at both sides of the akinetes, whereas the genus *Aphanizomenon* is characterized by akinetes distant from heterocysts.
10. **Confirmation of close relationship between *Anabaena compacta* and *Anabaena reniformis*** – This was initially proposed by Zapomělová *et al*. (2008) and was confirmed by the morphological analyses but not by the phylogenetic analyses.
11. **First report of *Anabaena reniformis* Lemmermann in African waters** – This thesis contains the first report of this taxon from Africa (Lake Malawi) which was previously only reported from the Canada, Ukraine, Germany, Cuba and Japan (Komárek 2005; Komárek and Zapomělová, 2007).
12. ***Anabaena lemmermannii* is morphologically heterogeneous** - This is in agreement with Zapomělová *et al*. (2007). Additionally, *Anabaena lemmermannii* isolates clustered separately from *Anabaena flos-aquae* isolates, which differs from the findings by Rajaniemi *et al*. (2005) and was not supported by the morphological analyses.
13. **The position of *Anabaena* cf. *cylindrical* continues to be controversial** - Both the 16S rRNA gene sequence analysis and NMDS analysis confirmed the controversial position of *Anabaena cylindrica* demonstrated by Gugger *et al*. (2002) and Rajaniemi *et al*. (2005).

14. ***Anabaena* cf. *planktonica* was the isolate best differentiated from the rest in the metric NMDS analysis –** Since it did not group with other isolates. This result was mainly based on the length of vegetative cells, since this isolates had the longest ones. This result was not supported by the qualitative analysis, whereas the phylogenetic analysis showed different relationships but not a completely separation, since it clustered with *Aphanizomenon* isolates in the 16S rRNA and with *Anabaena* and *Nostoc* isolates in the *efp*.
15. **The genus *Nostoc* requires a major revision** – Genus *Nostoc* is the studied group which displays the greatest deficiency of studies that combine morphological and molecular approaches, and it has been shown that a polyphasic studies are required for an accurate identification on cyanobacteria (Rajaniemi *et al*. 2005, Zapomělová *et al*., 2007).
16. **First study using *efp* gene sequences for phylogenetic analyses** - The results of this thesis confirmed the importance of improving the taxonomy and phylogeny of genera *Anabaena*, *Aphanizomenon* and *Nostoc* on the basis of a combination of morphological and genetic data, especially considering the lack of studies s that combine these two approaches (e.g. Rajaniemi *et al*., 2005; Willame *et al*., 2006). Additionally, this thesis confirms the necessary re-evaluation of the separation of these three genera.

FUTURE RESEARCH

Future works on the taxonomy and phylogeny of genera *Anabaena*, *Aphanizomenon* and *Nostoc* are required since the strain, specific and even generic delineations are weak. These studies must combine genetic and morphological data. In addition it is mandatory to find one molecular markers or a combination of different molecular markers which give a better differentiation at lowest taxonomic level (species) and strain level. These could include the PCR fingerprinting with STRR and LTRR (short and long tandemly repeated repetitive) sequences, since Rasmussen and Svenning (1998) concluded that these techniques are useful for clustering of even closely related strains, and are a valuable and rapid alternative to other methods used for classification and diversity studies. In addition, genes related with the heterocysts and akinetes differentiation may be useful in the taxonomy and phylogeny of these three genera. *HetA*, *hetK*, *hetN*, *hetR*, *hetS*, *ntcA*, *patA* and *hanA* are regulatory genes for heterorocyst maturation (Janson *et al*., (1999); Lechno-Yossef *et al*., 2006; Meeks *et al*., 2002). NpF0062, NpR4070 and NpF6000 are regulatory genes for akinetes differentiation (Argueta *et al*., 2006) and *hetA*, *devR* and *hetR* are heterocyst genes associated with akinete differentiation (Meeks *et al*., 2002).

In relation with the morphological analysis, it is necessary to do a more accurate study of the life cycle and development and germination of akinetes, since genus *Nostoc* is defined by obligatory presence of hormogonia, while *Anabaena* strains are defined by obligatory absence of hormogonia, and the development of akinetes is a stable character and it could have important taxonomic value for *Anabaena* classification (Stulp and Spam 1982, 1985). In addition, it is important to determine which isolates never produce

or are potential producers of gas vesicles or a sheath. Unfortunately, since these characters can change when the strains have been cultured in the laboratory and thus the lack of observation of these morphological characters can result in mistakes when determining the phylogenetic relationships (Tamas *et al*. 2000). Therefore it is necessary to standardize the culture condition under which the species are evaluated.for morphology and phylogenetics. So, it is first necessary to understand which environmental conditions are the most influential in the sheath, gas vesicles, akinetes and heterocysts differentiation and any other character which usually change under culture conditions. Preliminary studies on this topic are given by Zapomělová *et al*. (2008a). On the other hand, the morphological analysis should be done before isolation, although there will always be some source of error presents, since the isolation procedure may not ensure the isolation of the described specimen, particularly when the specimen is in a minority in the sample. So a description of the isolate should be also done shortly after isolation, although it already could show some changes due to culturing conditions. Cryopreservation may be another solution to this problem. Although when a culturing is required for obtaining the required amount of material for molecular analysis, it is difficult to ensure that the studied specimen corresponds to the species cryopreserved in the initial sample, but in this way the sample is always available for comparison.

Finally, it is important to understand the evolutionary relationships among the isolates of genera *Anabaena*, *Aphanizomenon* and *Nostoc* based on the *efp* gene, and in this way recognize possible horizontal gene transfer (HGT) and gene duplication that could explain their polyphyly, considering HGT and gene duplication have been demonstrated in the bacterial *efp* gene (Lau, 2008). In addition, it is essential to analyze a

large number of isolates that represent each genus. In that sense, it is necessary to increase the number of the analyzed *Aphanizomenon* isolates to more accurately assess phylogentic relationships within this genus and confirm or refute the well-supported cluster obtained in the *efp* gene sequence analysis will be confirmed.

REFERENCES

Adams D. G. and P. S. Duggan (1999). Heterocyst and akinete differentiation in cyanobacteria. New Phytologist 144(1): 3-33.

Adolph, K. W. and R. Haselkorn (1971). Isolation and characterization of a virus infecting the blue-green algae *Nostoc muscorum*. Virology 46: 200.208.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Andersen, R. A. and M. Kawashi (2005). Traditional microalgae isolation techniques. In: Algal culturing techniques. Elsevier Academic Press.

Argueta, C., K. Yuksek, R. Patel and M. L. Summers (2006). Identification of *Nostoc punctiforme* akinete-expressed genes using differential display Molecular Microbiology 61(3): 748-757.

Aoki, H., K. Dekany, S-L. Adams and M. C. Ganoza (1997). The Gene Encoding the Elongation Factor P Protein Is Essential for Viability and Is Required for Protein Synthesis. The Journal of Biological Chemistry 272(5): 32254-32259.

Baker, J. A., B. Entsch and D. B. McKay (2003). The cyanobiont in an *Azolla* fern is neither *Anabaena* nor *Nostoc*. FEMS Microbiology Letters 229, 43-47.

Baldauf, S. L., J. D. Palmer and W. F. Doolittle (1996). The root of the universal tree and the origin of eukaryotes based on elongation factor phylogeny. Proceedings of the National Academy of Science 93: 7749-7754.

Banack, S. A., H. E. Johnson, R. Cheng and P. A. Cox (2007). Production of the Neurotoxin BMAA by a Marine Cyanobacterium. Marine Drugs 5: 180-196.

Banker R., S. Carmeli, O. Hadas, B. Teltsch, R. Porat and A. Sukenik (1997). Identification of cylindrospermopsin in *Aphanizomenon ovalisporum* (Cyanophyceae) isolated from Lake Kinneret, Israel. Jorunal of Phycology 33: 613-616.

Bell R. A. (1993). Cryptoendolithic algae of hot semiarid lands and deserts. Journal of Phycology 29: 133-139.

Bergman B., C. Johansson and E. Söderbäck (1992). The *Nostoc*-*Gunnera* symbiosis. Tansley Review no 42. New Phytologist 122:379-400.

Boyer, S. L., V. R. Flechtner and J. R. Johansen (2001). Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in Cyanobacteria. Molecular Biology and Evolution 18(6): 1057-1069.

Brocks J. J., G. A. Logan, R. Buick and R. E. Summons (1999). Archean Molecular Fossils and the Early Rise of Eukaryotes. Science, New Series 285 (5430): 1033-1036.

Casamatta D. A., M. L. Vis and R. G. Sheath (2003). Cryptic species in cyanobacterial systematics: a case study *Phormidium retzii* (Oscillatoriales) using RAPD molecular marker and 16S rDNA sequence data. Aquatic Botany 77(4): 95-309.

Casamatta, D. A., J. R. Johansen, M. L. Vis and S. T. Broadwater (2005). Molecular and morphological characterization of ten polar and near-polar strains within the Oscillatoriales (Cyanobacteria). Journal of Phycology 41: 421-438.

Castenholz, R. W. (2001). General Characteristics of the Cyanobacteria. In: Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 1, pp. 474-487. Edited by D. R. Boone and R. W. Castenholz. Springer, New York.

Caudales, R. and J. M. Wells (1992). Differentiation of free-living *Anabaena* and *Nostoc* cyanobacteria on the basis of fatty acid composition. International Journal of Systematic Bacteriology 42(2): 246-251.

Çelekly, A., O. Obali and O. Külköylüoğlu (2007). The phytoplankton community (except Bacillariophyceae) of Lake Abant (Bolu, Turkey). Turkish Journal of Botany 31: 109-124.

Chu K. H., J. Qi, Z. G. Yu and V. Anh (2004). Origin and phylogeny of chloroplasts revealed by a simple correlation analysis of complete genomes. Molecular Biology and Evolution 21:200-206.

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18:117-143.

Codd G. A. (1999). Cyanobacterial toxins: their occurrence in aquatic environments and significance to health. Bulletin de l'Institut océanographique, Monaco, n° special 19: 483-500.

Costa, J. L., P. Paulsrud, J. Rikkinen and P. Lindblad (2001). Genetic diversity of *Nostoc* symbionts endophytically associated with two bryophytes species. Applied and Environmental Microbiology 67(9): 4393-4396.

Costa J. L., E. Martínez Romero and P. Lindblad (2004). Sequence based data supports a single *Nostoc* strain in individual coralloid roots of cycads. FEMS Microbiology Ecology 49: 481–487.

Cox P. A., S. A. Banack and S. J. Murch (2003). Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. PNAS 100(23): 13380–13383.

Cronberg G. and J. Komárek (2004). Some nostocalean cyanoprokaryotes from lentic habitats of Eastern and Southern Africa Nova Hedwigia 78(1-2): 71-106 Abstract.

Damerval, T., A-M. Castets, G. Guglielmi, J. Houmard and N. Tandeu de Marsac (1989). Occurrence and distribution of gas vesicle genes among cyanobacteria. Journal of Bacteriology 171(3): 1445-1452.

Damerval, T., G. Guglielmi, J. Houmard and N. Tandeau de Marsac (1991). Hormogonium differentiation in the cyanobacterium *Calothrix:* A photoregulated developmental process. The Plant Cell 3: 191-201.

De Philippis R., C. Faraloni, M. C. Margheri, C. Sili, M. Herdman and M. Vincenzini (2000). Morphological and biochemical characterization of the exocellular investments of polysaccharide-producing *Nostoc* strains from the Pasteur Culture Collection. World Journal of Microbiology & Biotechnology 16: 655-661.

Dodds W. K., D. A. Gudder and D. Mollenhauer (1995). The ecology of *Nostoc*. Journal of Phycology 31: 2-18.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792-1797.

Elhai, J. (1994). Genetic techniques appropriate for the biotechnological exploitation of cyanobacteria. Journal of Applied Phycology 6: 177-186.

Evans J. H. (1997). Spatial and seasonal distribution of phytoplankton in an African Rift Valley Lake (L. Albert, Uganda, Zaire). Hydrobiologia 354: 1–16.

Ferber, L. R., S. N. Levine, A. Lini and G. P. Livingstone (2004). Do cyanobacteria dominate in eutrophic lakes because they fix atmospheric nitrogen? Freshwater Biology 49: 690–708

Fergusson, K. M. and C. P. Saint (2000). Molecular phylogeny of *Anabaena circinalis* and its identification in environmental samples by PCR. Applied and Environmental Microbiology 66(9): 4145-4148.

Ferreira F. M. B., J. M. F. Soler, M. L. Fidalgo and P. Fernández-Vila (2001). PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro River, northern Portugal). Toxicon 39: 757-761.

Ganoza, M. C., M. C. Kiel and H. Aoki (2002). Evoltuionary Conservation of Reactions in Translation. Microbiology and Molecular Biology Reviews 66(3): 460-485.

Gaylarde P. M., C. A. Crispim, B. A. Neilan and C. C. Gaylarde (2005). Cyanobacteria from Brazilian building walls are distant relatives of aquatic genera. Source: OMICS-A Journal of Integrative Biology 9(1): 30-42 Abstract..

Gill, A. (2006). Molecular Characterization of potential geosmin-producing cyanobacteria from Lake Ontario. Thesis presented to the University of Waterloo in fulfillment of the thesis requirement for the degree of Master of Science in Biology.

Giovannoni S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane and N. R. Pace (1988). Evolutionary relationships among cyanobacteria and green chloroplasts. Journal of Bacteriology 170(8): 3584-3592.

Glick B. R. and C. Ganoza (1975). Identification of a soluble protein that stimulates peptide bond synthesis. Proceedings of the National Academy of Science 72(11): 4257-4260.

Graham L. E. and L.W. Wilcox (2000). Algae. Upper Saddle River, Prentice Hall.

Guevara, R., J. J. Armesto and M. Caru (2002). Genetic diversity of *Nostoc* microsymbionts from *Gunnera tinctoria* revealed by PCR-STRR fingerprinting. Microbial Ecology 44, 127-136.

Gugger, M., C. Lyra, P. Henriksen, A. Coute, J-F. Humbert and K. Sivonen (2002). Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. International Journal of Systematic and Evolutionary Microbiology 52: 1867-1880.

Gugger, M. F. and L. Hoffmann (2004). Polyphyly of true branching cyanobacteria (Stigonematales). International Journal of Systematic and Evolutionary Microbiology 54: 349–357

Guindon, S. and O. Gascuel (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52(5): 696-704.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Henson, B. J., L. E. Watson and S. R. Barnum (2002). Molecular differentiation of the heterocystous cyanobacteria, *Nostoc* and *Anabaena*, based on complete *NifD* sequences. Current Microbiology 45: 161-164.

Hindák, F. (2000). Morphological variation of four planktic nostocalean cyanophytes –members of the genus *Aphanizomenon* or *Anabaena*? Hydrobiologia 438: 107–116, 2000.

Hiroki, M., A. Shimizu, R. Li, M. Watanabe and M. M. Watanabe (1998). Development of a database system useful for identification of *Anabaena* spp. (Cyanobacteria). Phycological Research 46: 85–93.

Hoffmann, L. (1996). Geographic distribution of freshwater blue-green algae. Hydrobiologia 336: 33-40.

Hori, K., S. Ishii, G. Ikeda, J. Okamoto, Y. Tanji, C. Weeraphasphong and H. Unno (2002). Behavior of filamentous cyanobacterium *Anabaena* spp. In water column and its cellular characteristics. Biochemical Engineering Journal 10: 217–225.

Hori, K., J. Okamoto, Y. Tanji and H. Unno (2003). Formation, sedimentation and germination properties of *Anabaena* akinetes. Biochemical Engineering Journal 14: 67–73.

Hotto, A., M. Satchwell and G. Boyer (2005). Seasonal Production and Molecular Characterization of Microcystins in Oneida Lake, New York, USA. Environmental Toxicology 20(3): 243-248.

Hrouzek, P., M, Šimek and J. Komarek (2003). Nitrogenase activity (acetylene reduction activity) and diversity of six soil *Nostoc* strains. Algological Studies 108: 87-101.

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

Iteman, I., R. Rippka, N. Tandeau de Marsac and M. Herdman (2000). Comparison of conserved structural and regulatory domains within divergent 16S rRNA–23S rRNA spacer sequences of cyanobacteria. Microbiology 146: 1275–1286.

Iteman, I., R. Rippka, N. Tandeau de Marsac and M. Herdman (2002). rDNA analyses of plaktonic heterocystous cyanobacteria, including members of the genera *Anabaenopsis* and *Cyanospira*. Microbiology 148: 481-495.

Izaguirre, I. and A. Vinocur (1994) Algal assemblages from shallow lakes of the Salado River Basin (Argentina). Hydrobiologia 289(1-3): 57-64 Abstract.

Jacoby, J. M., D. C. Collier, E. B. Welch, J. Hardy and M. Crayton (2000). Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. Canadian Journal of Fisheries and Aquatic Sciences 57:231-240.

Janson, S., B. Bergman, E. J. Carpenter, S. J. Giovanonni and K.Vergin (1999). Genetic analysis of natural populations of the marine diazotrophic cyanobacterium *Trichodesmium*. FEMS Microbiology Ecology 30: 57-65.

Joe, Y. A. and M. H. Park (1994). Structural Features of the eIF-5A Precursor Required for Posttranslational Synthesis of Deoxyhypusine. The Journal of Biological Chemistry 269(41):25916-25921.

Jüttner, F., J. Leonhardt and S.Moehren (1983). Environmental factors affecting the formation of mesityl oxide di methyl allylic alcohol and other volatile compounds excreted by *Anabaena cylindrica*. Journal of General Microbiology 129: 407-412.

Kling, H. J. (1997). A summary of past and recent plankton of LakeWinnipeg, Canada using algal fossil remains. Journal of Paleolimnology 19: 297–307.

Kling, H. J., D. L. Findlay and J. Komárek (1994). *Aphanizomenon schindleri* sp.nov. : a new nostocacean cyanoprokaryote from the Experimental Lakes Area, northwestern Ontario. Canadian Journal of Fisheries and Aquatic Sciences 51: 2267- 2273.

Komárek, J. (1992). Komvophoron. http://www.cyanodb.cz/Komvophoron

Komárek, J. (2005). Studies on the cyanophytes (Cyanobacteria, Cyanoprokaryota) of Cuba 11. Freshwater *Anabaena* species. Preslia, Praha, 77: 211–234.

Komárek, J. and L. Kováčik (1989). Trichome structure of four *Aphanizomenon* taxa (Cyanophyceae) from Czechoslovakia, with notes on the taxonomy and delimitation of the genus. Plant Systematics and Evolution 164: 47-64.

Komárek J., H. Kling and J. Komárková (2003). Filamentous Cyanobacteria. In: Freshwater Algae of North America. Pp. 117-174. Edited by Wehr, J. D. and Sheath R. G. Elsevier Science. Academic Press.

Komárek J. and J. Komárková (2006). Diversity of *Aphanizomenon*-like cyanobacteria. Czech Phycology, Olomouc 6:1-32.

Komárek, J. and E. Zapomělová (2007). Planktic morphospecies of the cyanobacterial genus *Anabaena*= subg. *Dolichospermum* – 1. part: coiled types. Fottea, Olomouc 7(1): 1–31.

Krienitz, L., A. Ballot, C. Wiegand, K. Kotut, G. A. Codd and S. Pflugmacher (2002). Cyanotoxin-producing bloom of *Anabaena flos-aquae*, *Anabaena discoidea* and *Microcysits aeruginosa* (Cyanobacteria) in Nyanza Gulf of Lake Victoria, Kenya. Journal of Applied Botany - Angewandte Botanik 76: 179-183.

Kyrpides, N. C. and C. Woese (1998). Universally conserved translation initiation factors. Proceedings of the National Academy of Sciences 95: 224-228.

Lachance, M-A. (1981). Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation. International Journal of Systematic Bacteriology 31(2): 139-147.

Lau, L. (2006) A Bioinformatics Analysis on the Evolutionary Relationships of the Bacterial Protein elongation Factor P (EF-P) and its Homologs. Thesis Proposal presented to the University of Waterloo in fulfillment of the thesis requirement for the degree of Master of Science in Biology.

Lau, L. (2008). Sequence analysis of the bacterial protein elongation factor P. Thesis presented to the University of Waterloo in fulfillment of the thesis requirement for the degree of Master of Science in Biology.

Lechno-Yossef, S., Q. Fan, S. Ehira, N. Sato and C. P. Wolk (2006). Mutations in four regulatory genes have interrelated effects on heterocyst maturation in *Anabaena* sp. Strain PCC 7120. Journal of Bacteriology 188(21): 7387-7395.

Lehtimäki, J., C. Lyra, S. Suomalainen, P, Sundman, L. Rouhiainen, L. Paulin, M. Salkinoja-Salonen and K. Sivonen (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. International Journal of Systematic and Evolutionary Microbiology 50: 1043-1053.

Li, R., M. Watanabe and M. M.Watanabe (1997). Akinete formation in planktonic *Anabaena* spp. (Cyanobacteria) by treatment with low temperature. Journal of Phycology 33: 576-584.

Li, R, W. W. Carmichael, Y. Liu and M. M. Watanabe (2000). Taxonomic re-evaluation of *Aphanizomenon flos-aquae* NH-5 based on morphology and 16S rRNA gene sequences. Hydrobiologia 438: 99–105.

Li, D., L. Chen, G. Li, G. Wang, L. Song and Y. Liu (2005). Photoregulated or Energy dependent Process of Hormogonia Differentiation in *Nostoc sphaeroides* Kützing (Cyanobacterium). Journal of Integrative Plant Biology Formerly Acta Botanica Sinica 47(6): 709-716.

Litvaitis, M. K. (2002). A molecular test of cyanobacterial phylogeny: inferences from constraint analyses. Hydrobiologia 468, 135-145.

Liu, Y., W. Chen, D. Li, Y. Shen, Y. Liu and L. Song (2006). Analysis of paralytic shellfish toxins in *Aphanizomenon* DC-1 from Lake Dianchi, China. Environmental Toxicology 21(3): 289-295.

Lu, W., E. H. Evans, S. M. McColl and V. A. Saunders (1997). Identification of cyanobacteria by polymorphisms of PCR-amplified ribosomal DNA spacer region. FEMS Microbiology Letters 153: 141-149.

Ludwig, J. A. and J. F. Reynolds (1998). Statistical ecology: a primer on methods and computing, 337 pp. John and Sons, USA.

Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner and K. H. Schleifer (1998). Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19: 554-568.

Lung'ayia, H. B. O., A. M'Harzy, M. Tackx, J. Gichuki and J. J. Symoens (2000). Phytoplankton community structure and environment in the Kenyan waters of Lake Victoria. Freshwater Biology 43:529-543.

Lyra, C., J. Hantula, E. Vainio, J. Rapala, L. Rouhiainen and K. Sivonen (1997). Characterization of cyanobacteria by SDS-PAGE of whole-cell proteins and PCR/RFLP of the 16S rRNA gene. Archives of Microbiology 168, 176-184.

Lyra, C., S. Suomalainen, M. Gugger, C. Vezie, P. Sundman, L. Paulin and K. Sivonen (2001). Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. International Journal of Systematic and Evolutionary Microbiology 51: 513-526.

Mahmood N. A. and W. W. Carmichael (1986). Paralytic shellfish poisons produced by the freshwater cyanobacterium *Aphanizomenon flosaquae* NH-5. Toxicon 24(2): 175-186.

Martin, W., B. Stoebe, V. Goremykin, S. Hansmann S, M. Hasegawa and K. V. Kowallik (1998). Gene transfer to the nucleus and the evolution of chloroplasts. Nature 393:162-165.

Matula, J., M. Pietryka, D. Richter and B, Wojtuń (2007). Cyanoprokaryota and algae of Arctic terrestrial ecosystems in the Hornsund area, Spitsbergen. Polish Polar Research 28(4): 283-315.

Maxwell, S. E. and H. D. Delaney (2004). Designing experiments and analyzing data. Lawrence Erlbaum Associates, Publishers. London.

Mazel, D., J. Houmanrd, A. M. Castets and N. Tandeau de Marsac (1990). Highly repetitive DNA sequences in cyanobacterial genomes. Journal of Bacteriology 172(5): 2755-2761.

McGuire, R. F. (1984). A numerical taxonomic study of *Nostoc* and *Anabaena*. Journal of Phycology 20: 454-460.

Meeks, J. C., J. Elhai, T. Thiel, M. Potts, F. Larimer, J. Lamerdin, P. Predki and R. Atlas (2001). An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynthesis Research 70: 85–106.

Meeks, J. C. and J. Elhai (2002). Regulation of cellular differentiation in filamentous cyanobacterial in free-living and plant-associated symbiotic growth states. Microbiology and Molecular Biology Reviews 66: 94-121.

Meeks, J. C., E. L. Campbell, M. L. Summers and F. C. Wong (2002). Cellular differentiation in the cyanobacterium *Nostoc punctiforme*. Archives of Microbiology 178: 395-403.

Mishra, U. and S. Pabbi (2004). Cyanobacteria: A potential biofertilizer for rice. Resonance June 2004: 6-10.

Moffitt, M. C., S. I. Blackburn and B. A. Neilan (2001). rRNA sequences reflect the ecophysiology and define the toxic cyanobacteria of the genus *Nodularia*. International Journal of Systematic and Evolutionary Microbiology 51: 505-512.

Mollenhauer, D., R. Bengtsson and E. Lindstrém (1999). Macroscopic cyanobacteria of the genus *Nostoc*: a neglected and endangered constituent of European inland aquatic biodiversity. European Journal of Phycology 34:349-360.

Murch, S. J., P. A. Cox and S. A. Banack (2003). A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. PNAS 101(33): 12228–12231.

Negri, A. P. and G. J. Jones (1995). Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium *Anabena circinalis* by the freshwater mussel *Alathyria condola*. Toxicon 33(5): 667-678.

Neilan, B. A., D. Jacobs and A. E. Goodman (1995). Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus. Applied and Environmental Microbiology 61(11): 3875-3883.

Nilsson, M., B. Bergman and U. Rasmussen (2000). Cyanobacterial diversity in geographically related and distant host plants genus *Gunnera*. Archives of Microbiology 173: 97-102.

Novis P. M. and R. D. Smissen (2006). Two genetic and ecological groups of *Nostoc commune* in Victoria Land, Antarctica, revealed by AFLP analysis. Antarctic Science 18 (4), 573–581.

Nübel, U., F. Garcia-Pichel and G. Muyzer (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. Applied and Environmental Microbiology 63(8): 3327-3332.

Oren, A. (2004). A proposal for further integration of the cyanobacteria under bacterial code. International Journal of Systematic and Evolutionary Microbiology 54: 1895-1902.

Palmer, J. D. (2003). The symbiotic birth and spread of plastid: how many times and whodunit? Journal of Phycology 39: 4-11.

Paulsrud, P., J. Rikkinen and P. Lindblad (1998). Cyanobiont specificity in some *Nostoc* containing Lichens and in a *Peltigera aphthosa* photosymbiodeme. New Phytologist 139: 517-524.

Paulsrud, P., J. Rikkinen and P. Lindblad (1999). Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens. New Phytologist 146: 291-299.

Pereira P., H. Onodera, D. Andrinolo, S. Franca, F. Araújo, N. Lagos and Y. Oshima (2000). Paralytic shellfish toxins in the freshwater cyanobacterium *Aphanizomenon flos-aquae*, isolated from Montargil reservoir, Portugal. Toxicon 38(12): 1689-1702.

Pereira, P., R. Li, W. W. Carmichael, E. Dias and S. Franca (2004). Taxonomy and production of paralytic shellfish toxins by the freshwater cyanobacterium *Aphanizomenon gracile* LMECYA40. European Journal of Phycology (2004), 39: 361-368.

Pereira, I., M. Moya, G. Reyes and V. Kramm (2005). A survey of heterocystous nitrogen-fixing cyanoacteria in Chilean rice fields. Gayana Botánica 62(1): 26-32.

Plazinski, J., Q. Zheng, R. Taylor, L. Croft, B. G. Rolfe and B. E. S. Gunning (1990). DNA probes show genetic variation in cyanobacterial symbionts of the Azolla fern and a closer relationship to free-living *Nostoc* strains than to free-living *Anabaena* strains. Applied and Environmental Microbiology 56(5): 1263-1270.

Posada, D. and K. A. Crandall (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics. 14:817-818.

Rajaniemi, P., P. Hrouzek, K. Kastovska, R. Willame, A. Rantala, L. Hoffmann, J. Komárek and K. Sivonen (2005). Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). International Journal of Systematic and Evolutionary Microbiology 55: 11-26.

Rantala, A., D. P. Fewer, M. Hisbergues, L. Rouhiainen, J. Vaitomaa, T. Borner and K. Sivonen (2004). Phylogenetic evidence for the early evolution of microcystin synthesis. Procceding of the National Academic of Science of the U. S. A. 101: 568-73.

Rao, V. V., R. Ghosh and H. N. Singh (1987). Diazotrophic regulation of akinete development in the cyanobacterium *Anabanea doliolum*. New Phytologist 106: 161-168.

Rapala, J. and K, Sivonen (1998). Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. Microbial Ecology 36: 181-192.

Rasmussen, U. and M. M. Svenning (1998). Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. Applied and Environmental Microbiology, 64(1): 265-272.

Rasmussen, U. and M. M. Svenning (2001). Characterization by genotypic methods of symbiotic *Nostoc* strains isolated from five species of *Gunnera*. Archives of Microbiology 176, 204-210.

Reyes-Prieto, A., A. P. M. Weber and D. Bhattacharya (2007). The origin and establishment of the plastid in algae and plants. Annual Review of Genetics 41:147–68.

Rikkinen, J., I. Oksanen and K. Lohtander (2002). Lichen guilds share related cyanobacterial symbionts. Science 297: 357.

Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman and R. Y. Stanier (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111: 1-61.

Rippka, R., R. W. Castenholz and M. Herdman (2001). Subsection IV. (Formerly Nostocales Castenholz 1989 sensu Rippka, Deruelles, Waterbury, Herdman and Stanier 1979). In: Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 1, pp. 562-589. Edited by D. R. Boone and R. W. Castenholz. Springer, New York.

Rolland, A., D. F. Bird and A. Giani (2005). Seasonal changes in composition of the cyanobacterial community and the occurrence of hepatotoxic blooms in the eastern townships, Québec, Canada. Journal of Plankton Research 27(7): 683-694.

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

Rouhiainen, L., K. Sivonen, W. J. Buikema and R. Haselkorn (1995). Characterization of toxin-producing cyanobacteria by using an oligonucleotide probe containing a tandemly repeated heptamer. Journal of Bacteriology 177(20): 6021-6026.

Rudi, K, O. M. Skulberg, F. Larsen and K. S. Jacobsen (1997). Strain characterization of oxyphotobacteria in clone culture on the basis of 16S rRNA sequences from the variable regions V6, V7, and V8. Applied and Environmental Microbiology 63(7): 2593-2599.

Rudi, K, O. M. Skulberg, R. Skulberg and K. S. Jacobsen (2000). Application of sequence-specific labeled 16S rRNA gene oligonucleotide probes for genetic profiling of cyanobacterial abundance and diversity by array hybridization. Applied and Environmental Microbiology 66(9): 4004-4011.

Saunders, G. W. (1993). Gel purification of red algal genomic DNA: An inexpensive and rapid method for the isolation of polymerase chain reaction-friendly DNA. Journal of Phycology 29: 251-254.

Sekadende, B. C., T. J. Lyimo and R. Kurmayer (2005). Microcystin production by cyanobacteria in the Mwanza Gulf (Lake Victoria, Tanzania). Hidrobiologia 543: 299-304.

Seo, P.-S. and A. Yokota (2003). The phylogenetic relationships of cyanobacteria inferred from 16S rRNA, *gyrB*, *rpoC1*, and *rpoD1*. Journal of General Applied Microbiology 49: 191-203.

Shaw, G. R., A. Sukenik, A. Livne, R. K. Chiswell, M. J. Smith, A. A. Seawright, R. L. Norris, G. K. Eaglesham and M. R. Moore (1999). Blooms of the Cylindrospermopsin Containing Cyanobacterium, *Aphanizomenon ovalisporum* (Forti), in Newly Constructed Lakes, Queensland, Australia. Environmental Toxicology 14: 167-177.

Sheath, R. G., L. V. Morgan, J. A. Hambrook and K. M. Cole (1996). 7. Tundra stream, macroalgae of North America: composition, distribution and physiological adaptations. Hydrobiologia 336: 67-82.

Sheath, R. G. and K. M. Muller. (1997). Distribution of stream macroalgae in four high Arctic drainage basins. Arctic 50(4): 355-364.

Singh, S. and P. Datta (2005). Growth and survival potentials of immobilized diazotrophic cyanobacterial isolatesexposed to common ricefield herbicides. World Journal of Microbiology & Biotechnology 21:441-446.

Sivonen, K., W. W. Carmichael, M. Namikoshi, K. L. Rinehart, A. M. Dahlem and S. I. Niemela (1990). Isolation and Characterization of Hepatotoxic Microcystin Homologs from the Filamentous Freshwater Cyanobacterium *Nostoc* sp. Strain 152. Applied and Environmental Microbiology 56(9): 2650-2657.

Sivonen, K. and G. Jones (1999). Cyanobacterial toxins. In: Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring, and Management. Chorus, I. and Bartram, J. [Eds.], pp. 41-111. E. & F. N. Spon., London & New York.

Sivonen, K., K. Halinen, L. M. Sihvonen, K. Koskenniemi, H. Sinkko, K. Rantasärkkä, P. H. Moisander and C. Lyra (2007). Bacterial Diversity and Function in the Baltic Sea with an Emphasis on Cyanobacteria. Ambio 36: 2–3.

Smith, J. K., J. D. Parry and R. J. Smith (1998). A PCR technique based on the *Hip1* interspersed repetitive sequence distinguishes cyanobacterial species and strains. Microbiology 144: 2791-2801.

Spaulding, S. A., D. M. McKnight, R. L. Smith and R. Dufford (1994). Phytoplankton population dynamics in perennially ice-covered Lake Fryxell, Antactica. Journal of Plankton Research 16: 527-541.

Stackebrandt, E. and B. M. Goebel (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in Bacteriology. International Journal of Systematic Bacteriology 44: 846–849.

Steffensen, D., M. Burch, B. Nicholson, M. Drikas and P. Baker (1999). Management of toxic blue-green algae (Cyanobacteria) in Australia. Environmental Toxicology 14(1): 183-195.

Stein, J. E. (1973). Handbook of Phycological Methods: Culture Methods and Growth Measurements, Cambridge University Press.

Stulp, B. K. and W. T. Stam (1984). Growth and morphology of *Anabaena* strains (Cyanophyceae, Cyanobacteria) in cultures under different salinities. British Phycological Journal 19: 281-286.

Stüken, A., J. Ruecker, T. Endrulat, K. Preussel, M. Hemm, B. Nixdorf, U. Karsten and C. Wiedner (2006). Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. Phycologia 45(6): 696-703.

Svenning, M. M., T. Eriksson and U. Rasmussen (2005). Phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16sDNA sequence analyses. Arch Microbiol 183: 19-26.

Swofford, D. L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4.

Tamas, I., Z. Svircev and G. E. Andersson (2000). Determinative value of a portion of the *nifH* sequence for the genera *Nostoc* and *Anabaena* (Cyanobacteria). Current Microbiology 41: 197-200.

Tang, E. P. Y., W. F. Vincent, D. Proul, P. Lessard and J. de la Noüe (1997). Polar cyanobacteria versus green algae for tertiary waste-water treatment in cool climates. Journal of Applied Phycology 9: 371–381.

Tomitani, A., A. H. Knoll, C. M. Cavanaugh and T. Ohno (2006). The evolutionary diversification of cyanobacteria: Molecular–phylogenetic and paleontological perspectives. Proceedings of the National Academy of Sciences of the United States of America 103(14): 5442-5447.

van Dok, W. and B. T. Hart (1996). Akinete differentiation in *Anabaena circinalis* (Cyanophyta). Journal of Phycology 32: 557-565.

Vargas, M. A., H. Rodríguez, J. Moreno, H. Olivares, J. A. Del Campo, J. Rivas and M. G. Guerrero (1998). Biochemical composition and fatty acid content of filamentous nitrogen fixing cyanobacteria. Journal of Phycology. 34, 812–817.

Watson, S. B. and J. Ridal (2004). Periphyton: a primary source of widespread and severe taste and odour. Water Science and Technology 49(9): 33-39.

West, N. J. and D. G. Adams (1997). Phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from a single field site. Applied and Environmental Microbiology 63(11), 4479-4484.

Willame, R., C. Boutte, S. Grubisic, A. Wilmotte, J. Komárek and L. Hoffmann (2006). Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. Journal of Phycology 42: 1312-1332.

Wilmotte, A. and M. Herdman (2001). Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. In: Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 1, pp. 487-589. Edited by D. R. Boone and R. W. Castenholz. Springer, New York.

Wilson, K.M, M.A. Schembri, P.D. Baker and. C.P. Saint (2000). Molecular Characterization of the Toxic Cyanobacterium *Cylindrospermopsis raciborskii* and Design of a Species-Specific PCR. Applied and Environmental Microiology 66(1): 332-338.

Wood, S. A., Crowe A. L. M., Ruck J. G. and Wear R. G. (2005). New records of planktonic cyanobacteria in New Zealand freshwaters. New Zealand Journal of Botany 43: 479-492.

Wright, D., T. Prickett, R. F. Helm and M. Potts (2001). Form species *Nostoc commune* (Cyanobacteria). International Journal of Systematic and Evolutionary Microbiology 51: 1839-1852.

Yavasoglu, A., M. A. Karaaslan, Y. Uyanikgil, F. Sayim and U. Ates (2008). Toxic effects of anatoxin-a on testes and sperm counts of male mice. Experimental and Toxicologic Pathology 60(4-5): 391-396.

Zapomělová, E. K. (2006) Current taxonomic issues with planktonic representatives of the genus *Anabaena* (Cyanobacteria) with special reference to their morphological features; literary review. Czech Phycology 6: 33-47.

Zapomělová, E., K. Řeháková, P. Znachor and J. Komárková (2007). Morphological diversity of coiled planktonic types of the genus *Anabaena* (cyanobacteria) in natural populations– taxonomic consequences. Cryptogamie, Algologie 28 (4): 353-371.

Zapomělová, E., P. Hrouzek, K. Řeháková, M. Šabacká, M. Stibal, L. Caisová, J. Komárková and A. Lukešová (2008a) Morphological variability in selected heterocystous cyanobacterial strains as a response to varied temperature, light intensity and medium composition. Folia Microbiologica 53 (4): 333-341.

Zapomělová, E., J. Jezberová, K. Řeháková, P. Hrouzek, , E. Soldati, D. Hisem, P. Znachor, J. Komárková and S. Ventura (2008b). Phenotypic and genotypic diversity of planktonic *Anabaena*-like cyanobacteria in fishponds and reservoirs of the Czech Republic. Manuscript In: Zapomělová, E. (2008). *Anabaena* phenotypic and genotypic diversity of planktonic strains in fishponds and reservoirs of the Czech Republic. Thesis presented to the University of South Bohemia fulfillment of the thesis requirement for the degree of PhD in Biology.

Zapomělová, E., J. Jezberová, P. Hrouzek, D. Hisem, K. Řeháková and J. Komárková (2008c). Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (cyanobacteria) and their re-classification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana).* Journal of Phycology (submitted).

Zehr, J. P., M. T. Mellon and W. D. Hiorns (1997). Phylogeny of cyanobacterial *nifH* genes: evolutionary implications and potential applications to natural assemblages. Microbiology 143: 1443-1450.

Zheng, W., T. Song, X. Bao, B. Bergman and U. Rasmussen (2002). High cyanobacterial diversity in coralloid roots of cycads evealed by PCR fingerprinting. FEMS Microbiology Ecology 40: 215-222.

Zhou, Z., L. Liu, X. Chen, J. Wang, M. Chen, Y. Zhang and U. B. Zhou (2005). Factors that effect antioxidant activity of c-phycocyanins from *Spirulina platensis*. Journal of Food Biochemistry 29: 313–322.