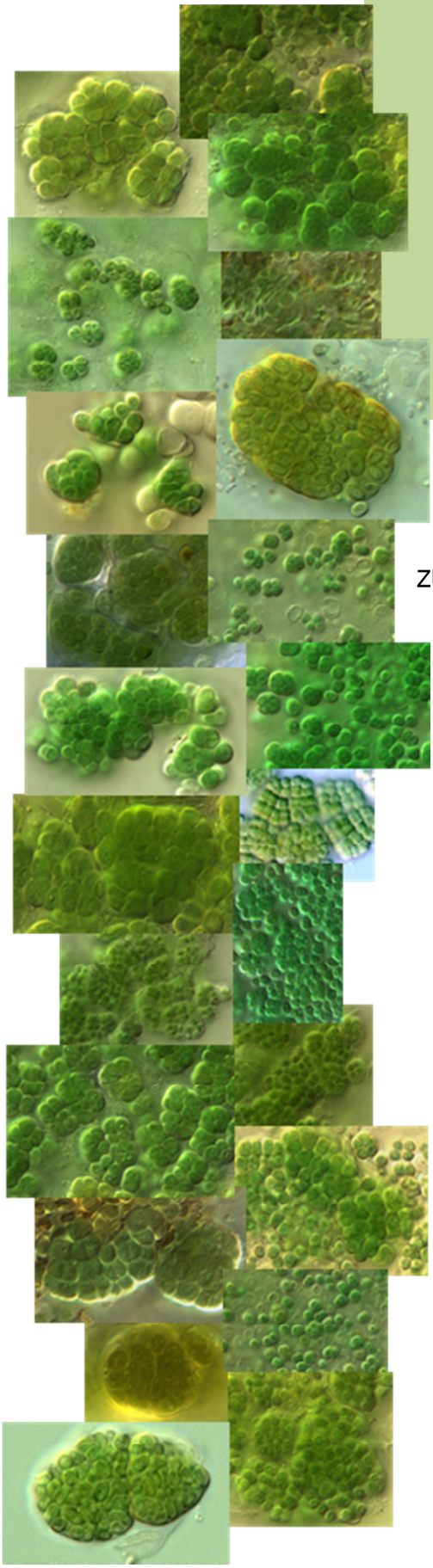

The case of *Chroococcidiopsis*: New phylogenetic and morphological insights into ecologically important Cyanobacteria

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D 386

Für meine Eltern,
meine Geschwister,
und meine Großmütter.

Das Zeichnen von Stammbäumen halte ich,
besonders bei den Blaualgen,
für wenig förderlich.

[I do not consider the construction of evolutionary trees
to be particularly practical,
especially where blue-green algae are concerned.]

Lothar Geitler 1932

For the newcomer,
the taxonomy of cyanobacteria can easily become a nightmare;
for the initiated,
it is a persistent headache.

Ferran Garcia-Pichel 2009

Content

1 Introduction

2. Materials and Methods

3. Results

4. Discussion

Table of figures

Table of Tables

1 Introduction

1.1 A short history and overview on the classification of Cyanobacteria

Cyanobacteria are the only prokaryotes with ability to conduct oxygenic photosynthesis. With this ability they served as a progenitor for the primary symbiosis event, thereby setting the stage for appearance of the green and red algae and to the glaucophytes (Palmer et al. 2003). Moreover, they played a major role in the evolution of the earth, because they were the first organisms performing oxygenic photosynthesis, which caused a sharp rise of the oxygen in the atmosphere about 2.45–2.32 billion years ago (Rasmussen et al. 2008; Schopf 2012). The exact age of cyanobacteria, however, is under current debate. The fossil record indicates an age of between 3.5–2.5 billion years (Hoffmann 1976; Schopf 2012), while the evidence from the amino acid clock trace them back to only 1.5 billion years (Doolittle et al. 1996) and radiocarbon dating to 2.5 billion years (Rasmussen et al. 2008).

Due to their algal way of life (Wilmotte 1994), cyanobacteria had been classified as plants and consequently classified as such under the botanical code of nomenclature. Although Cohn (1853) suggested a close relationship between the cyanobacteria ("blue-green algae") and bacteria, it took almost 130 years to draw conclusions as to their bacterial nature. After the prokaryotic nature of the cyanobacteria was recognized, it was proposed to treat them as bacteria under the rules of nomenclature of bacteria (Stanier et al. 1978). Later Woese (1987) confirmed them as prokaryotes based on rRNA genes and placed them within the bacteria. However, two classification systems were still applied to Cyanobacteria: first, the botanical approach under the botanical code of nomenclature (formerly ICBN, since 2011 ICN–International Code of Nomenclature for algae, fungi, and plants; e.g. Anagnostidis & Komárek 1985) and second, the bacteriological approach under the bacteriological code of nomenclature of the International Code of Nomenclature for Prokaryotes (ICNP; e.g. Rippka et al. 1979). Morphological and ecological features were the base for both approaches and five largely corresponding groups were established: Chroococcales and Pleurocapsales (sections I & II) contained unicellular coccoid single cells or forming colonies, which do not form true filaments. The Chroococcales have reproduction by binary fission. The Pleurocapsales reproduce by multiple fissions or by multiple plus binary fissions. Additionally, they form special cells for the propagation called baeocytes. The remaining groups comprised filamentous, more complex forms with and without true branching.

Oscillatoriales (section III) contained filamentous taxa with vegetative cells only, while Nostocales (section IV) and Stigonematales (section V) included all taxa with heterocytes. No branching or so called “false” branching occurred in the Nostocales while the Stigonematales comprises taxa with false or true branching (Komárek & Anagnostidis 1989; Anagnostidis & Komárek 1990).

Further investigations based on molecular methods, especially on multigene analyses, lead to a more complex picture of cyanobacterial phylogeny. The research revealed that the old concept of treating unicellular and filamentous groups in different taxonomical ranks and categories did not reflect true relationships. Neither the botanical nor the bacteriological systematic approach truly represented the present knowledge of the cyanobacterial phylogeny. In addition, many taxonomic ranks above genera are not yet validated described (Büdel & Kauff 2012). Taking all morphological and molecular information (especially 16S small subunit ribosomal RNA gene, rRNA) together, the most recent phylogeny of the cyanobacteria is presented in Büdel & Kauff (2012). Using that approach the Cyanobacteria can be classified in four subclasses: Gloeobacterophycidae, Synechococcophycidae, Oscillaoriophycidae, and Nostocophycidae, and one group of uncertain order and subclass: the family Chroococcidiopsidaceae.

The subclass of the Gloeobacterophycidae has been suggest by Hoffmann et al. (2005), but so far not validly described. It is composed of one order and the monogeneric family of Gloeobacteraceae. This subclass is characterized by coccoid cell morphology and has no thylakoids. The subclass of coccoid to filamentous, sometimes heteropolar Synechococcophycidae composed of two orders, the Synechococcales and Pseudanabaenales. The subclass and the orders have been proposed, but not validated described (Hoffmann et al. 2005). Both orders contain several families which are mostly occurring in marine and freshwater habitats. The subclass Oscillaoriophycidae contains coccoid, filamentous and sometimes heteropolar species. Again, this subclass is a proposed but not validly described (Hoffmann et al. 2005). It is proposed that it is composed of the three orders Chroococcales, Oscillatoriales and Pleurocapsales, each containing several families and genera. Members of the subclass of the Nostocophycidae are characterized by the appearance of so called akinetes (resting cells) and heterocytes, which are capable of atmospheric nitrogen fixation. This suggested, unvalidated (Hoffmann et al. 2005) monophyletic subclass has only one order: the Nostocales containing several families and genera.

The largest uncertainty is still found in the monogeneric family of the Chroococcidiopsidaceae (Büdel & Kauff 2012), which was formerly described as the genus *Chroococcidiopsis* of the Pleurocapsales due to their baeocyte formation. However, phylogenetic studies on 16S rRNA gene sequences indicate a different picture, *Chroococcidiopsis* is more related to the heterocyte forming cyanobacteria (Fewer et al. 2002; Seo & Yokota 2003). This isolated position leads to the establishment of the familia nova Chroococcidiopsidaceae Geitler ex Büdel, Donner & Kauff (Büdel & Kauff 2012). However, the position of the family Chroococcidiopsidaceae within the Cyanobacteria is still open.

1.2 Characteristics of the family Chroococcidiopsidaceae

The genus *Chroococcidiopsis* was established by Geitler in 1933 (Geitler 1933) and placed in the family Cyanidiaceae (Geitler 1933) together with the genus *Cyanidium*. Later on, Komárek & Anagnostidis (1986) transferred the genus into the family Xenococcaceae Ergec. By the isolated position of the genus in phylogenetic analyses, the familia nova Chroococcidiopsidaceae Geitler ex Büdel, Donner & Kauff (Büdel & Kauff 2012) was established. The family Chroococcidiopsidaceae (monogeneric; *Chroococcidiopsis*) was defined by cell morphology with solitary spherical or irregular cells, which later cluster into non-polarized agglomerations (Fig. 1.1a). Members of this family also have the ability to reproduce by baeocytes, which, when released grow and enlarge to the original cell size (“nanocytes”; Waterbury & Stanier 1978; Fig. 1.1b). Baeocyte production in the family Chroococcidiopsidaceae can be performed by two different modes. First and more common, after one or two binary divisions with their planes rectangular to each other, daughter cells undergo further divisions in different planes without intermediate growth (Büdel & Kauff 2012; Fig. 1.1e). Second and apparently more rare, simultaneous multiple irregular divisions result in numerous cells (Büdel & Kauff 2012; Fig. 1.1f). In both modes small cells called baeocytes are the result. Apparently after the first division there is no mother cell at all, because the cell wall has already disappeared. The sheath envelope of the parental cell ruptures and non-motile baeocytes are released (Fig.1.1c). The only way to distinguish the genus *Chroococcidiopsis* from the morphologically highly similar genus *Myxosarcina* (member of the Pleurocapsales) is the non-motility of baeocytes in the former.

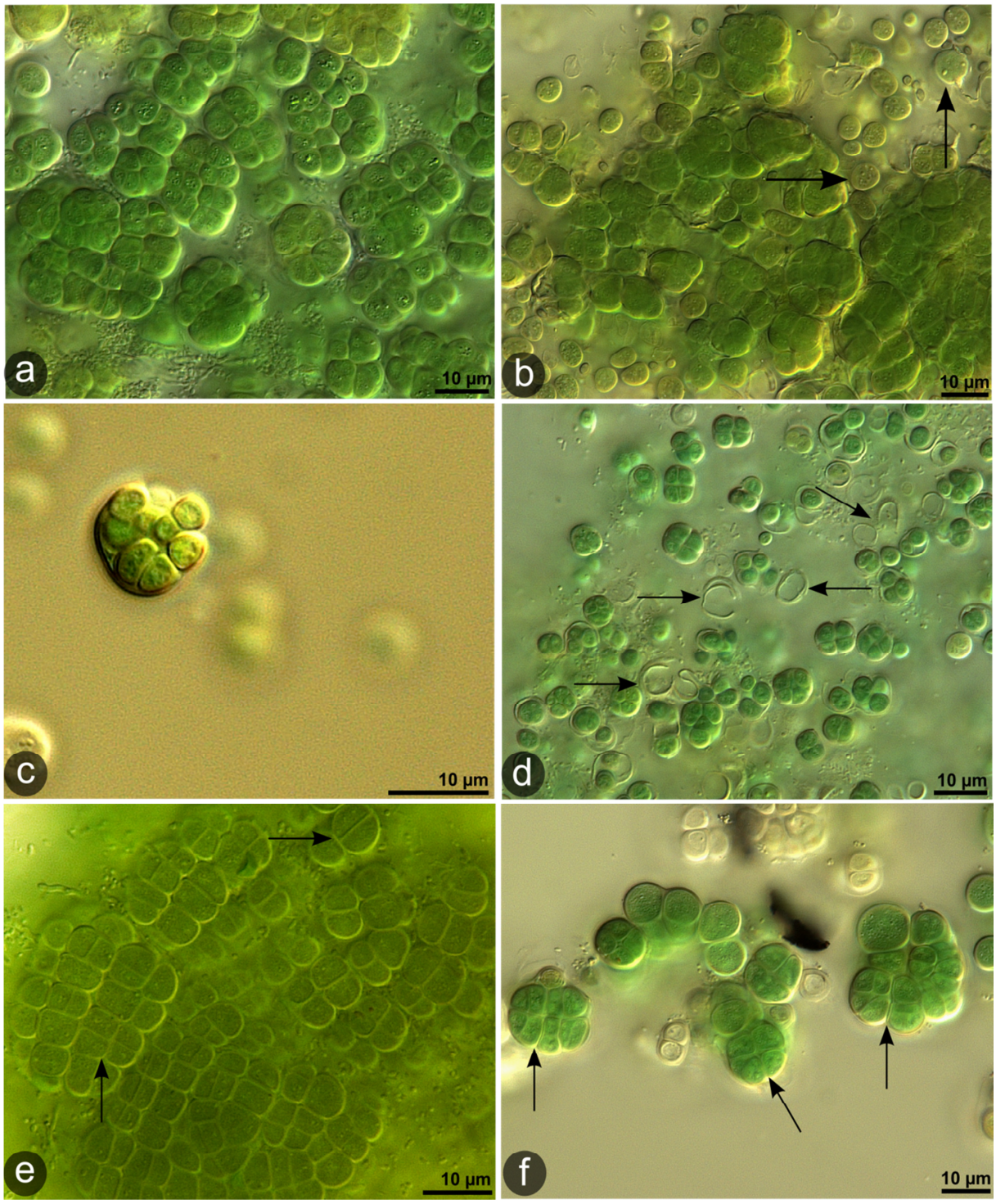


Fig. 1.1: Light micrographs of *Chroococcidiopsis* grown on BG 11 or BG 11 liquid medium. **(a)** - Cells and aggregates at different stages of development (*C.* sp. BB 81.1). **(b)** - Vegetative undivided cells (arrows, *C.* sp. 96.1). **(c)** - Rupture of the sheath envelope and releasing of baeocytes (*C.* thermalis BB 82.2). **(d)** - Emptied sheaths after the release of baeocytes (arrows, *C.* CCMEE 140). **(e)** - Cubic-rounded baeocytes in mother cells after binary fissions at right angles (*C.* sp. BB 79.2). **(f)** - Unregularly shaped baeocytes of different sizes in mother cell after simultaneous divisions (*C.* sp. 84.1).

The non-motile vegetative cells of *Chroococcidiopsis thermalis* Geitler have a diameter of 2–6 µm and occur as single cells with a spherical shape and often in groups of cells forming slightly polygonal, irregular shape (Komárek & Anagnostidis 1999; Büdel & Kauff 2012; Fig. 1.1a–b). Baeocytes are smaller having a diameter of 2-3.5 (4) µm (Komárek & Anagnostidis 1999). Both cell types are enveloped by a thin, firm, colourless and sometimes layered sheath (Komárek & Anagnostidis 1999; Büdel & Kauff 2012; Fig. 1.1 d).

For some cyanobacteria, the existence of a surface-layer (S-layer) is reported, a common feature in bacteria as well as archaea. The S-layer is composed of proteins or glycoproteins, forming a two-dimensional crystalline array of identical proteinaceous subunits (Smarda et al. 2002). It is located at the outer membrane and shows different lattice patterns, such as oblique, square or hexagonal. The occurrence in different taxonomic groups of cyanobacteria is patchy. S-layers are known from the Chroococcales in numerous families: the Oscillatoriales, where they are reported from three families, and from three strains of the genus *Chroococcidiopsis* (Smarda et al. 2002). For *Chroococcidiopsis* strains it is known that the S-layer lattice pattern shows an oblique symmetry of the monomers (Büdel & Rhiel 1985; Smarda et al. 2002). However the scattered distribution within cyanobacteria is presumably due to an incomplete investigation.

The inner structure of the cells of *Chroococcidiopsis* does not differ from other cyanobacteria. Within the cell, the cytoplasma contains storage structures such as cyanophycin granules, carboxysomes, ribosomes, the DNA, and the most important feature for photosynthesis, the thylakoids. These membrane structures carry the light-harvesting complex and photosystems I and II. Only for three *Chroococcidiopsis* strains can one find information about the arrangement of the thylakoid membranes can be found in the literature. For *Chroococcidiopsis* PCC 7203, thylakoids are reported to form fascicles (short parts of the membranes) usually with a radial position within the cell (Komárek & Kastovsky 2003). *Chroococcidiopsis* PCC 7432 and *C.* PCC 7436 show a parietal (orientated along the cell wall) to stacked (small fragments packed together) arrangement (Waterbury & Stanier 1978) in transmission electron microscopy pictures. The thylakoid arrangement is thought to be the most important feature of the inner cell structures which is usable for the taxonomic classification (Komárek & Anagnostidis 1999). While almost nothing is known about the thylakoid

arrangement in *Chroococcidiopsis*, it is difficult to verify the usefulness of this structure for a taxonomic classification of this group.

The earliest morphologically based reports of *Chroococcidiopsis* are derived from 400 million years old lichenized fossil from the Early Devonian Rhynie Chert (Taylor et al. 1995; Taylor et al. 1997). A relaxed-clock phylogenetic analysis however indicates an age between 3.1–1.9 billion years for free-living variants of *Chroococcidiopsis* (Bahl et al. 2011).

1.3 Distribution and Ecology of the family Chroococcidiopsidaceae

The family Chroococcidiopsidaceae is a cosmopolitan cyanobacterium with a wide range of habitats; many of the strains thrive in extreme environments (Fig.1.2, Table A1 appendix). They occur in various habitats, especially where eukaryotic organisms such as vascular plants are inhibited by the environmental factors. *Chroococcidiopsis* species growth as litho-, endo-, chasmoendo-, crypto-, and hypolithis in and on rocks, in fresh, brackish or salt waters, also serve as photobionts of rock and soil inhabiting lichens of the order Lichinales (Henssen & Büdel 1986; class Lichinomycetes Reeb et al. 2004), and rarely free-living in soil. Only *Chroococcidiopsis codiicola* has been described living epiphytic on the marine *Codium fragile* (Chlorophyta) (Beljakova 1987).

Chroococcidiopsis species avoid high light intensities and UV light either by living preferably within soil, rocks, caves, underneath translucent rocks, or as photobionts in lichen symbioses. Whereas the light intensity underneath quartz pebbles can be between 0.005 to 30% of the incoming radiation (Vogel 1955; Berner & Evenari 1978), it is generally less with 0.1 to 2.5% in the endolithic habitat of sandstone (Vestal 1985; Büdel 1987) and 0.005% in quartz (Nienow et al. 1988) or inside the lichen symbioses (Friedmann & Ocampo-Friedmann 1984). In addition, the endo- and hypolithic habitat provides further microclimatic advantages. Rock surfaces provide condensation points for air humidity, thereby improving water availability especially in arid and semi-arid regions (Friedmann et al. 1967). Furthermore they provide more stable temperatures in cold and hot deserts (Broady 1981b; Warren-Rhodes et al. 2006). The substrate mineralogy itself seems to be of minor importance for colonization by *Chroococcidiopsis* species, as they are found across a wide range of geological rock types such as quartz (e.g. Friedmann et al. 1967; Schlesinger et al. 2003; Warren-Rhodes et al. 2006),

Fig. 1.2: Map of the worldwide distribution of proved *Chroococcidiopsis*-species retrieved from literature. Labels of the points see Table A1 (appendix). Overlay of the dark grey area shows the known distribution area (retrieved from the Global Biodiversity Information Facility via: data.gbif.org in October 2012) of lichens of the class Lichinomycetes of which *Chroococcidiopsis* is the frequent photobiont.

sandstone (e.g. Bell et al. 1988; Büdel et al. 2004), dolomite (Cockell & Stokes 2002, 2004), gypsum (Friedmann et al. 1967; Boison et al. 2004), and rarely gneiss (Broady 1981a, b; Cockell et al. 2002). Therefore the physical properties such as porosity, colour and structure seem to be more important microhabitat characteristics.

Hypolithic *Chroococcidiopsis*-species are reported from arid, semiarid to hyperarid regions of all continents except Europe (Table A1 appendix). The temperature apparently is not the crucial factor for the distribution, as they occur not only in warm/hot deserts like the Sonoran Desert (USA) and the Negev Desert (Israel), but also in cold deserts of the Antarctic continent. While hypolithic communities in general are dominated by cyanobacteria (e.g. Schlesinger et al. 2003; Warren-Rhodes et al. 2006; Caruso et al. 2011), the relative abundance of cyanobacterial systematic groups shifts between polar and nonpolar hypolithic desert communities. Within the more diverse non-polar desert communities, *Chroococcidiopsis* strains are the dominating cyanobacteria and seem to be the keystone taxon (Chan et al. 2012). Endolithic (including chasmo- and cryptoendolithic; for detailed definition see Golubic et al. 1981) occurrence in rocks with a porous structure is reported for arid, semiarid, and hyperarid zones as well as hot and cold deserts in America, Australia, Africa, Europe, Asia and Antarctica (Table A1 appendix). The cryptoendolithic living cyanobacteria, such as *Chroococcidiopsis*, form a distinct blue-green layer 0.5-5 mm below the rock surface (Friedmann et al. 1967; Friedmann 1980; Friedmann & Friedmann-Ocampo 1985; Wessels & Büdel 1995; Weber et al. 1996; Büdel 1999; Büdel et al. 2000; Büdel et al. 2004). Similar to the hypolithic communities, *Chroococcidiopsis* is the most abundant organism in these habitats (Friedmann 1980; Bell 1993; Wynn-Williams 2000). Warren-Rhodes et al. (2006) concluded that the decreasing occurrence of the cyanobacterial community along a transect with increasing dryness in the Atacama Desert can be explained by the availability of liquid water such as rain, snow, fog and dew. They concluded further, that other factors like pH and temperature are of minor importance because they remained constant along the transect (Navarro-Gonzales et al. 2003). Hence in environments where liquid water is very rare like in the Atacama Desert, the abundance of overall hypolithic cyanobacterial communities in an arid site (27.6%) drops dramatically compared to an hyperarid site (0.008%; Warren-Rhodes et al. 2006). It is likely that for such a dominant species like *Chroococcidiopsis* the picture may be similar. As the main organism in the arid and semi-arid areas, *Chroococcidiopsis* is the primary producer and hence the sole of the trophic hierarchy in these harsh environments.

Cyanobacteria can have an important influence on the ecosystem processes. For example, endolithic cyanobacteria can cause the weathering of rocks in the form of exfoliated flakes (Friedmann & Weed 1987), which is described for e.g. Antarctica (Friedmann & Weed 1987), South Africa, Australia, USA, and Venezuela (Büdel et al. 2004). The exfoliation can either be caused by biogeophysical effects originated by the growth of organisms (Friedmann & Weed 1987) and the expansion of the extracellular polysaccharides (EPS) during rehydration (Potts 1994), by alkalization of the substrate during the photosynthesis as seen for *Chroococcidiopsis* dominated endolithic communities in sandstone (Büdel et al. 2004). A recent study of Olsson-Francis et al. (2012) found such a pH increase during the growth of cells of an endolithic *Chroococcidiopsis* species in both basalt and rhyolite. Thus, the bio-active weathering process by endolithic living *Chroococcidiopsis* has not only an effect on the immediate vicinity, but also make rock minerals available to other organisms, including cyanobacteria (Olsson-Francis et al. 2012). This process of weathering by *Chroococcidiopsis* affects the landscape geomorphology (Büdel et al. 2004) and is an important first step in the formation of soil.

Chroococcidiopsis can be frequently found not only in rocks, but also in soils where they are a common member of biological soil crusts (BSCs), a soil-surface community consisting of soil particles, cyanobacteria, algae, microfungi, lichens, and bryophytes, living on the top and the upper parts of soil (Belnap et al. 2001a). These species represent only a small part of cyanobacteria occurring in BSCs, and with their small size, between 2–10 (18) µm in diameter (Komárek & Anagnostidis 1999), and immobility, they are often found at the soil surface of the BSCs (Belnap 2001b). While other filamentous cyanobacteria (e.g. *Microcoleus vaginatus*) are stabilizing the soil, the ecological role of *Chroococcidiopsis* within BSCs remains unknown (Belnap 2001b). The large amount of EPS itself can stabilize the particles of the soil and increasing the carbon content of the soil (compare to Mager & Thomas 2011). Within BSCs, *Chroococcidiopsis* strains are reported from Africa, Asia and the Middle East (Dor & Danin 1996; Büdel 2001; Ullmann & Büdel 2001). The following strains of *Chroococcidiopsis* synthesize nitrogenase the main enzyme for N_2 fixation, under anoxic conditions: PCC 6712, PCC 7203, PCC 7431–7434, PCC 7436 and PCC 7439 (Rippka & Waterbury 1977; Rippka et al. 1979) and some strains (from endolithic origin) are capable of acetylene reduction at least in laboratory conditions (Boison et al. 2004). Up to 70% of nitrogen fixed by cyanobacteria is released immediately into the surrounding soil environment and

is available to the associated organisms (Stewart 1970; Millbank 1982). Such a leaching effect might be not only important in BSCs, but also to hypo- and endolithic *Chroococcidiopsis* dominated communities.

Living under and within stones and soil can be seen as a strategy of avoiding, among others reasons, high irradiance and desiccation. An avoidance of high temperatures alone cannot be the reason for these strategies, because in the aquatic environments *Chroococcidiopsis* species do not seem to avoid them. There, they can occur in thermal springs, like for example the typus generis *C. thermalis* Geitler, described from a thermal spring at Sumatra (Indonesia; Geitler 1933). Hayashi et al. (1994) documented *Chroococcidiopsis* species in spring water temperatures of 40 to 80°C with a pH of 7.5-9.0 in Thailand. In contrast *Chroococcidiopsis thermalis* can also be found in thermal springs in the temperate zone as seen for example in Greece and Slovakia (Komárek & Anagnostidis 1999). Besides thermal springs, species of the genus *Chroococcidiopsis* are reported from freshwater habitats (Dor et al. 1991), and as halotolerant organisms occurring in brackish (Compère 1998), marine waters (Komárek & Anagnostidis 1999) or even hypersaline ponds with a salt concentration up to 142 $g*l^{-1}$ NaCl (Dor et al. 1991). Other aquatic habitats reported are wetlands and littoral of standing waters in Florida, Cuba and Mexico (Komárek & Hindák 1975; Komárek & Anagnostidis 1999).

Chroococcidiopsis is a frequent photobiont in lichens of the class Lichinomycetes in arid and semi-arid regions such as savannahs and semi-deserts (Büdel & Henssen 1983; Büdel & Wessels 1991; Büdel et al. 2000), regions where free-living *Chroococcidiopsis* species occur, too. Living in the symbiosis provides advantages such as protection from high irradiance by the pigmentation of the cortex. By this pigmentation and the reflection of the upper parts of lichen, only 7% of the ambient light reaches the zone where the cyanobacteria occur (data for lichen *Peltula euploca*; Büdel 1987). This is a slightly higher to the light intensity which hypo- and endolithic living species of cyanobacteria receive (compare Vestal 1985; Büdel 1987). Due to the specific growth of lichens, water retention for the photobiont may be increased (Büdel et al. 2000). This is especially important because cyanobacteria require liquid water, which is rare in arid and semi-arid regions, for positive photosynthesis.

1.4. Phylogeny of the genus *Chroococcidiopsis*

The current state of the phylogeny of cyanobacteria was summarized recently (Kauff & Büdel 2011; Büdel & Kauff 2012). Special emphasis in this present overview is placed on the relationship of the genus *Chroococcidiopsis* with the order Pleurocapsales and remaining cyanobacteria.

Until now a high number of single gene analyses of different genes have been used to investigate the phylogeny of cyanobacteria. A number of studies used protein coding sequences (e.g. *nifD/H/E/K/N*: Henson et al. 2004a & b, Henson et al. 2008; *gyrB*, *rpoC1*, *rpoD1*: Seo & Yokota 2003). Some of these genes are restricted to certain groups, e.g. the genes related to the fixation of nitrogen (e.g. *nifD/H/E/K/N)* a feature of which not all strains are capable. Consequently, these genes will produce an incomplete picture if used for phylogenetic analysis among all cyanobacteria. The most common gene used for phylogenetically analyses is the 16S rRNA which has been used for larger taxonomic approaches within the cyanobacteria (e.g. Giovannoni et al. 1988; Wilmotte 1994; Honda et al. 1999; Turner et al. 1999; Wilmotte & Herdman 2001) as well as within the phylogeny of the genus *Chroococcidiopsis* (Ishida et al. 2001; Fewer et al. 2002). However it is known that the phylogenetic resolution of 16S rRNA is limited in resolving closely or distantly related organisms (Fox et al. 1992; Stackebrandt & Goebel 1994). The number of studies approaching a multigene analysis to improve resolution within the prokaryotes are rare, both in cyanobacteria in general (e.g. Tanabe et al. 2007) and especially in the genus *Chroococcidiopsis* (e.g. Seo & Yokota 2003).

Chroococcidiopsis was traditionally classified within the Pleurocapsales (see part 1.2). Phylogenetic studies indicated another picture of the evolutionary relationship and based on the isolated position (e.g. Fewer et al. 2001; Seo & Yokota 2003), the genus *Chroococcidiopsis* was just recently transferred into a separate familia nova Chroococcidiopsidaceae Geitler ex. Büdel, Donner & Kauff (Büdel & Kauff 2012). It is apparent that taxon sampling plays an important role in resolving the position of *Chroococcidiopsis* and the Pleurocapsales. However, there is no study which included all genera in their analysis. Giovannoni et al. (1988) studied three genera of the Pleurocapsales, *Dermocarpa* PCC 7437, *Pleurocapsa* PCC 7321, and *Myxosarcina* PCC 7312 with an additional 26 other cyanobacteria strains. From the position of the three genera in the resulting tree and their common mode of reproduction (baeocyte formation), they concluded that the Pleurocapsales are monophyletic. Wilmotte

(1994) confirmed this classification by examining the same Pleurocapsales genera and other cyanobacteria strains (in total 54 taxa). However no statistical support was given. The analysis presented by Turner et al. (1999) used 53 taxa, three of which belonged to the Pleurocapsales (*Pleurocapsa* PCC 7516, *Stanieria* PCC 7437, and *Xenococcus* PCC 7305). The analysis placed these three genera together within the order Chroococcales and hence the Pleurocapsales seemed to be monophyletic. Of these studies, only Wilmotte (1994) emphasized that other genera of the Pleurocapsales should additionally be investigated. In fact there are later studies which included members of the genus *Chroococcidiopsis* (e.g. Rudi et al. 1997; Turner 1997; Garcia-Pichel at al. 1998; Ishida et al. 2001; Wilmotte & Herdman 2001; Fewer et al. 2002; Seo & Yokota 2003). Although some of these studies had not focused on the relation of the genus *Chroococcidiopsis* and the Pleurocapsales, they demonstrated a statistical support for the separation of the genus *Chroococcidiopsis* from the other genera, and hence the polyphyly of the Pleurocapsales. Only Rudi et al. (1997) and Wilmotte & Herdman (2001) found no significant support for a polyphyly; this was probably related to the taxa chosen within these studies. The separation of *Chroococcidiopsis* was apparent even if only one taxon of the genera *Chroococcidiopsis* (e.g. *C.* PCC 7431 in Seo & Yokota 2003) or more taxa (e.g. *C.* SAG 2023, *C.* SAG 2024, *C.* SAG 2025, *C.* SAG 2026, and *C.* PCC 7203 in Fewer et al. 2002) were chosen. Despite the fact that all those studies point to the same direction, Fewer et al. (2002) with their on 16S rRNA based study were the only one to highlight the relationship of the genus *Chroococcidiopsis* with the heterocyte forming cyanobacteria (Nostocales and Stigonematales) as each other´s closest living relatives. Furthermore, they concluded that the reproduction mode with the formation of baeocytes has been developed multiple times during the evolution of cyanobacteria (Fewer et al. 2002). Consequently, the high morphological similarity between the genera *Myxosarcina* (as a true member of the Pleurocapsales) and *Chroococcidiopsis* must be very likely a result of convergent evolution (Fewer et al. 2002). Both genera expose an extremely high degree of morphological similarity. The only characteristic which distinguishes them is the motility of the baeocytes which are motile in *Myxosarcina* and immotile in *Chroococcidiopsis* (Waterbury & Stanier 1978). The findings of Fewer et al. (2002), based on 16S rRNA gene were strongly supported by Seo & Yokota (2003), who also used the 16S rRNA gene in addition to the protein coding genes *gyrB*, *rpoC1* and *rpoD1*, and partial combinations of these sequences. A recently published analysis of 126 cyanobacterial genomes seems to support the studies of

Fewer et al. (2002) and Seo & Yokota (2003) with a separation of the strain *Chroococcidiopsis* PCC 7203 of remaining Pleurocapsales (Shih et al. 2013).

When studying the phylogenetic relationships of *Chroococcidiopsis,* taxon sampling is critical, especially if only one species of the genus is used. For example, *Chroococcidiopsis* PCC 6712, the reference strain of the Cluster 2 of the form-genus *Chroococcidiopsis* in the bacteriological system (Rippka et al. 2001b), always clusters within the Pleurocapsales (Ishida et al. 2001; Fewer et al. 2002; Shih et al. 2013). Moreover, this strain differs morphologically from other *Chroococcidiopsis* taxa. With a size of 6.3–5 µm for vegetative cells and 4–3 µm for baeocytes, this strain has larger cells than *Chroococcidiopsis* strains (Waterbury & Stanier 1978). Also, PCC 6712 presents a lower GC content (40 mol%) and a smaller genome size (3.31 Gdal) than other *Chroococcidiopsis* taxa (Herdman et al. 1979a, b). The misidentification of *Chroococcidiopsis* PCC 6712 is supported by the percentage of its polyunsaturated acids. In contrast to "true" *Chroococcidiopsis* strains, PCC 6712 has a lower level of polyunsaturated acids with the same basic acid profile like the other Pleurocapsales (Caudales et al. 2000). This fits to the suggestion that PCC 6712 might be a different species, if not a separate genus (Rippka et al. 2001b) and justifies the suggested placement of strain PCC 6712 into the *Pleurocapsales* (Fewer et al. 2002).

The question of monophyly of the remaining Pleurocapsales is still open. While there are numerous studies indicating monophyly (e.g. Giovannoni et al. 1988; Wilmotte 1994; Turner et al. 1999; Fewer et al. 2002) a few suggest heterogeneity (e.g. Rudi et al. 1997; Ishida et al. 2001). For example, the strain *Pleurocapsa* PCC 7327 clusters apart from the Pleurocapsales in the Chroococcales (next to *Microcystis*; Rudi et al. 1997; Ishida et al. 2001; Rippka et al. 2001a; Seo & Yokota 2003; Shih et al. 2013).

Because *Chroococcidiopsis* occurs in many biomes all around the world and, moreover, it is a frequent photobiont in lichens of the Lichinomycetes, the question arises if it is possible to distinguish taxa with different life-strategies such as free-living and symbiotic. A small dataset of 16S rRNA genes of *Chroococcidiopsis* could not differentiate between those two strategies and the results suggested that lichenization with *Chroococcidiopsis* happened more than one time (Fewer et al. 2002) with more than one strain or species. Whereas DNA–DNA hybridization (Friedmann et al. 1987; Nienow & Friedmann 1993) and the exclusive use of 16S rRNA gene analysis (Fewer et al. 2002) failed to differentiate between strains of different geographical origin, an analysis of 16S rRNA

gene sequences together with the 5.8S internal transcribed spacer (ITS) and 23S rRNA regions clearly distinguished between hot and cold desert hypolithic *Chroococcidiopsis* variants (Bahl et al. 2011). All samples from cold deserts were monophyletic and samples from hot deserts clustered into two regionally separated distinct groups. The last common ancestor of all existing hot and cold variants lived 2.5 billion years (range 3.1-1.9 billion years) and the separation of the both hot desert clusters took place ~2.4–~2.5 billion years ago (Bahl et al. 2011). This is on the eve of the great oxygenic event 2.45–2.32 billion years ago (Bekker et al. 2004), which was caused by the photosynthetic activity of cyanobacteria (Rasmussen et al. 2008). The phylogenetic relationship between geographical very distinct regions is owed to a founder population and not by a recent inter-regional gene flow (Bahl et al. 2011).

1.5 Aims of this study

Morphological data were traditionally used for the classification of cyanobacteria (e.g. Geitler 1932; Rippka et al. 1979; Komárek & Anagnostidis 1986), which often did not reflect their evolutionary history. The unicellular genus *Chroococcidiopsis* is known as a globally distributed component of desert photoautotrophic communities (see part 1.3), and thus the evolution of this genus is of major interest. The thylakoid arrangement is thought to be most important feature of the inner cell structures which is suitable for the taxonomic classification (Komárek & Anagnostidis 1999). However, only little is known about the arrangements of thylakoids in *Chroococcidiopsis* strains (see part 1.2). It is also unclear if there are differences between the genus *Chroococcidiopsis* and the Pleurocapsales. Therefore, there is a great deal of uncertainty whether there is a clear correlation between the phylogenetic data and the thylakoid arrangement. To answer this question, this study examined the thylakoid arrangement of 42 selected taxa using Low Temperature Scanning Electron Microscopy (LT-SEM). Together with data from literature 66 strains were used for the analysis of the thylakoid structure. The combination of morphological data and phylogenetic methods will estimate the position of *Chroococcidiopsis* within the cyanobacteria.

Presently, available genomic data for the *Chroococcidiopsis* in public databases such as National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/; accessed January 2013) are restricted to *C.* PCC 6712 and *C.* PCC 7203; while the former is not a "true" member of the genus *Chroococcidiopsis* (see part 1.4). The same restricted genome availability applies for the Pleurocapsales, where only *Pleurocapsa* PCC 7319,

P. PCC 7327, *Stanieria* PCC 7437 and *Xenococcus* PCC 7305 are being sequenced. Hence, genome availability does not sufficiently represent neither the genus *Chroococcidiopsis* nor the order Pleurocapsales. Therefore, the phylogenetic analysis in this study falls back to gene sequences. The most used gene in the phylogeny for cyanobacteria is the 16S rRNA (e.g. Giovannoni et al. 1988; Wilmotte; Honda et al. 1999; Turner et al. 1999), which is the "gold-standard" for all prokaryotes. For this reason, this study try to establish a phylogeny for *Chroococcidiopsis* based on 16S rRNA gene sequences and two additional genes. While the 16S rRNA gene analysis might not resolve the relationship of closely related organisms (Fox et al 1992; Stackebrandt & Goebel 1994), other genes appear to be suitable to clarify the phylogeny of this group. Within the genomes of cyanobacteria there is a stable core and a variable shell of genes according to evolutionary events like horizontal gene transfer (HGT; Shi & Falkowski 2008). While the latter is more is affected by HGT, the former involved genes are highly conserved, related to important metabolic pathways of the photosynthesis and ribosomal apparatus, and hence they are less affected by HGT. Such core related single copy gene is at least the subunit B protein of the DNA gyrase (*gyrB*). The beta subunit of the RNA polymerase (*rpoC2*) is such core related gene, too (Shi & Falkowski 2008). So it might be that the gamma subunit of the RNA polymerase (*rpoC1*) is part of the core genome.

Single gene analysis in cyanobacteria often leads to unresolved phylogenies, especially in basal branches of the tree. The resolution can be improved by the use of multigene analysis (Brown et al, 2005; Blank 2004). Additionally, such a multigene analysis can reveal a high diversity of cyanobacteria (Tanabe et al. 2007). Furthermore, single gene analysis might be affected by a false signal caused by horizontal gene transfer. This effect can be reduced by the use of multiple genes (Suchard 2005). By this, a multigene analysis using the three genes 16S rRNA, *gyrB* and *rpoC1* should give new insights into the evolution of *Chroococcidiopsis* and the Pleurocapsales. In contrast to former studies a higher number of *Chroococcidiopsis* and Pleurocapsales strains will be investigated. All together approximately 100 strains of the previously named, and heterocyte-forming and other cyanobacteria were identified for analysis. Available data from GenBank and completed genomes were combined with new sequences and analysed using Maximum Likelihood and Bayesian inference methods.

Large organisms (>2mm) have distinct spatial pattern of distribution over space, which are called biogeographies. Such patterns by smaller organisms like prokaryotes are subsumed by the "everything is everywhere" hypothesis (EiE; Baas Becking 1934) and

are so far unresolved (Fenchel et al. 1997). While a study based on multi-locus sequencing of the worldwide distribution of hypolithic living *Chroococcidiopsis* variants was able to discriminate between hot and cold deserts origins (Bahl et al. 2011), others failed. The well-established method of DNA-DNA hybridization failed to discriminate between *Chroococcidiopsis* strains from different geographic regions (Friedmann et al. 1987; Nienow & Friedmann 1993). The same was revealed by a 16S rRNA gene phylogeny of *Chroococcidiopsis*, where no specific patterns between geographic origins were observable (Fewer et al. 2002). Even the life-strategy of lichenization within the genus *Chroococcidiopsis* arose more than one times during the evolution (Fewer et al. 2002). The question as to how far a differentiation within not only one life strategy (hypolithic living), but between more strategies (e.g. endolithic vs. free-living vs. lichenized) using phylogenetic analyses is possible is still open. Thus biogeographical distribution patterns will be compared to the phylogenetic relationships, in order to understand the distribution patterns of *Chroococcidiopsis*.

To summarize, this work presents an approach to understanding the evolutionary relationships within the genus *Chroococcidiopsis* and the family Chroococcidiopsidaceae using three lines of evidence. Firstly, thylakoid structures, as a morphological characteristic, of 42 selected taxa are analysed using Low Temperature Scanning Electron Microscopy (LT-SEM). These data will be combined with data from literature, all together data from 66 strains will be analysed. Second, this study reconstructs phylogenetic trees based on a multi-locus sequence analysis using three genes (16S rRNA, *rpoC1* and *gyrB*) for a wide range of *Chroococcidiopsis*, Pleurocapsales and remaining cyanobacteria. Thirdly, biogeographical distribution patterns are compared to the phylogenetic relationships, in order to understand distribution patterns of *Chroococcidiopsis*. The consequences of the conceptual view of the evolutionary relationships of *Chroococcidiopsis* are highlighted and future research perspectives are discussed.

2 Materials and Methods

2.1 Cyanobacterial cultures and their origin

The herein newly investigated strains were obtained from public cultures collections, CCALA (Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic, Centre of Phycology, Trebon, Czech Republic), SAG (Sammlung von Algenkulturen at the University of Göttingen, Germany), PCC (Pasteur Culture Collection of Cyanobacteria, Institut Pasteur, Paris, France) and CCMEE (Culture Collection of Microorganisms from Extreme Environments, Center for Ecology and Evolutionary Biology, University of Oregon, Eugene, USA) as listed in Table 2.1. Strains from these culture collections are named with their corresponding abbreviations. Additionally, strains named "BB", from the personal culture collection of Prof B. Büdel, at Plant Ecology and Systematics Department, University of Kaiserslautern, Germany, were used.

Strains were maintained on BG11 and $BG11_0$ growth medium (Stanier et al. 1971), Basal medium, Z, Z2, Z4, and Z454 liquid medium (all Schlösser 1994). The required medium for each strain can be found in Table 1. Strains were cultured at 16–18°C under a lights/dark regime of 12:12 h at a light intensity of about 20-50 µmol photons * m^{-2} * s^{-1}.

For the analysis of biogeographic pattern, additional sequences of *Chroococcidiopsis* strains from Bahl et al. (2011) were used (GenBank accession numbers FJ805842–FJ805957).

Table 2.1 (pages 21-25): Overview of all processed strains with theirs required growth medium and the source of the sequences for the phylogenetic analyses. The origin of the strains is named in the abbreviations CCALA = Culture Collection of Autotrophic Organisms (Institute of Botany, Academy of Sciences of the Czech Republic, Centre of Phycology, Trebon, Czech Republic), SAG = Sammlung von Algenkulturen at the University of Göttingen (Germany), PCC = The Pasteur Culture Collection of Cyanobacteria (Institut Pasteur, Paris, France), CCMEE = Culture Collection of Microorganisms from Extreme Environments (Center for Ecology and Evolutionary Biology, University of Oregon, Eugene, USA), IAM = Microbial Culture Collection, National Institute for Environmental Studies (Tsukuba, Japan) and BB = personal culture collection of Prof B. Büdel University of Kaiserslautern (Germany). Sequences which were not obtained by this study are named by their GenBank accession number. Type strains are marked by a T. ►

Strain	Medium	16S rRNA	*rpoC1*	*gyrB*
Anabaena cylindrica IAM M-253	not in culture*	AF247592	AB074793	AB074770
Anabaena flos-aquae (Lyngb.) Breb BB 97.35	Z liquid	this study	—	—
Anabaena solitaria f. planctonica (Brunnth.) Komárek BB 97.102	Z liquid	this study	this study	—
Anabaena variabilis IAM M-204	not in culture*	AB074502	AB074789	AB074766
Anabaena variabilis IAM M-3	not in culture*	AB016520	AB074795	AB074772
Anabaenopsis circularis IAM M-4	not in culture*	AB074502	AB074789	AB074766
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.111	Z liquid	this study	—	—
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.25	Z liquid	this study	this study	—
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.85	Z liquid	this study	this study	—
Calothrix brevissima IAM M-249	not in culture*	AB074504	AB074768	AB074791
Chamaesiphon PCC 7430	not in culture*	AY170472	—	—
Chlorogloeopsis fritschii CCALA 039	not in culture*	AB093489	AB096735	AB096725
Chlorogloeopsis PCC 6718	not in culture*	AF132777	AB074801	AB074778
Chroococcidiopsis BB 79.1	BG11	this study	—	—
Chroococcidiopsis BB 82.1b	BG11	this study	this study	this study
Chroococcidiopsis CCMEE 10	BG11 liquid	this study	this study	—
Chroococcidiopsis CCMEE 140	BG11 liquid	this study	this study	—
Chroococcidiopsis PCC 7203	not in culture*	AB039005	—	—
Chroococcidiopsis cubana PCC 7431	not in culture*	AB074506	AB074809	AB074786
Chroococcidiopsis cubana CCALA 045	not in culture*	AJ344558	—	—
Chroococcidiopsis sp. BB 79.2	BG11	AJ344552	this study	—
Chroococcidiopsis sp. BB 80.1	BG11	this study	this study	this study
Chroococcidiopsis sp. BB 81.1	BG11	this study	—	this study
Chroococcidiopsis sp. BB 82.3	BG11	AJ344553	—	—
Chroococcidiopsis sp. BB 84.1	BG11	AJ344554	—	—
Chroococcidiopsis sp. BB 90.1 ◊	BG11	—	—	—

Strain	Medium	16S rRNA	*rpoC1*	*gyrB*
Chroococcidiopsis sp. BB 90.5	BG11	this study	—	—
Chroococcidiopsis sp. BB 95.6 ◊	BG11	—	—	—
Chroococcidiopsis sp. BB 96.1	BG11	AJ344555	this study	—
Chroococcidiopsis thermalis BB 82.2	BG11	this study	this study	—
Chroococcidiopsis sp. BB 96.19	BG11	this study	this study	—
Chroococcidiopsis sp. BB 97.116	Z liquid	this study	this study	this study
Chroococcidiopsis cf. CCMEE 167	BG11 liquid	this study	this study	—
Chroococcidiopsis (Lichinella iodopulchra)	not in culture	this study■	—	—
Chroococcidiopsis (Lichinella cribellifera)	not in culture	this study■	—	—
Chroococcidiopsis (Anema decipiens)	not in culture	this study■	—	—
Chroococcidiopsis (Lichinella nigritella)	not in culture	this study■	—	—
Chroococcus sp. CCALA 057	BG11	GQ375045	—	—
Cyanothece PCC 7424	not in culture*	AF132932	CP001291	CP001291
Cylindrospermum BB 97.12	Z liquid	this study	—	—
Cylindrospermum PCC 7417	not in culture*	AF132789	AF159371	—
Dermocarpella sp. PCC 7326^T	$BG11_0$	AJ344559	this study	—
Dichotrix spec. SAG 32.92	Z liquid	this study	—	—
Fischeralla ambigua (Näg.) Gom. BB 97.28	Z4 liquid	this study	this study	this study
Fischerella muscicola BB 98.1	Basal liquid	AJ344560	—	—
Fischerella PCC 73103	not in culture*	AB074505	AB074804	AB074781
Geitlerinema PCC 7105	not in culture*	AB039010	FJ042944	FJ042943
Geitlerinema sp. CCALA 138	Z liquid	EU196626	this study	—
Gloeobacter PCC 7421^T	not in culture*	AF132790	BA000045	BA000045
Gloeotrichia longicauda SAG 32.84	Z liquid	this study	this study	—
Hassalia byssoidea CCALA 823	not in culture*	AM905327	—	—
Lyngbya sp. BB 97.64	Z liquid	this study	—	—

Strain	Medium	16S rRNA	*rpoC1*	*gyrB*
Lyngbya sp. BB 97.65	Z liquid	this study	—	—
Mastigocladus laminosus SAG 4.84	not in culture*	EU116035	—	—
Microcoleus chtonoplastes BB 92.3	BG11	this study	—	—
Microcoleus sp. BB 97.74	Z liquid	this study	—	—
Microcystis aeruginosa NIES 104	not in culture*	AJ133174	AB074794	AB074771
Mojavia pulchra CCALA 691	Z liquid	AY577534	—	—
Myxosarcina CCMP 1489 ‡	not in culture	this study■	—	—
Myxosarcina PCC 7312	not in culture*	AJ344561	—	—
Myxosarcina sp. BB 86.6	not in culture*	AJ344562	—	—
Nostoc sp. BB 89.12	BG11 liquid	this study	this study	this study
Nostoc BB 94.2	BG11/ BG11-N 1:1	AJ344563	—	—
Nostoc sp. BB 98.3	BG11 liquid	this study	—	—
Nostoc linckia IAM M-251	not in culture*	AB074503	AB074792	AB074769
Nostoc muscorum BB 90.3	BG11 liquid	AB075992	—	—
Nostochopsis lobatus BB 92.1	BG11 liquid	this study	this study	—
Oscillatoria agardhii IAM M-244	not in culture*	AB074507	AB074790	AB074767
Oscillatoria sp. BB 96.17	BG11	this study	—	this study
Oscillatoria PCC 7112	not in culture*	AB074509	AB074802	AB074779
Petalonema alatum SAG 44.87 ◊	Z liquid	—	—	—
Pleurocapsa PCC 7314	not in culture*	AB074511	AB074806	AB074783
Pleurocapsa PCC 7319	not in culture*	AB039006	—	—
Pleurocapsa PCC 7327	not in culture*	AB039007	AB074807	AB074784
Pleurocapsa PCC 7516	not in culture*	X78681	—	—
Pleurocapsa sp. BB 01.1	BG11 liquid	this study	this study	—
Pleurocapsa sp. BB 97.117	Z liquid	this study	this study	this study
Pleurocapsa sp. SAG 31.84	not in culture*	X78681	—	—

Strain	Medium	16S rRNA	*rpoC1*	*gyrB*
Pseudanabaena catenata Lauterborn BB 97.2	Z liquid	this study	this study	—
Pseudanabaena PCC 7367	not in culture*	AB039018	AB074799	AB074776
Pseudanabaena PCC 7403	not in culture*	AB039019	AB074810	AB074787
Rivularia PCC 7116	Z liquid	AM230677	this study	—
Scytonema BB 97.127	Z2 liquid/ Z4 liquid	this study	this study	—
Scytonema ocellatum BB 02.1	BG11 liquid	this study	—	—
Scytonema PCC 7110	not in culture*	AB075996	—	—
Spirulina platensis IAM M-135	not in culture*	AB074508	AB074788	AB074765
Stanieria PCC 7301	not in culture*	AB039009	—	—
Stanieria PCC 7437ᵀ	not in culture*	AF132931	AB074777	AB074800
Starria zimbabweensis SAG 74.90	not in culture*	AB115962	—	—
Stigonema mamillosum (Lyngb.) Ag. BB 97.104	Z454 liquid	this study	—	—
Stigonema ocellatum Thuret BB 97.103	Z454 liquid	this study	—	—
Symploca PCC 8002	not in culture*	AB075997	—	—
Synechocystis PCC 6803	not in culture*	NC_000911	BA000022	BA000022
Tolypothrix BB 97.100	Z2 liquid	this study	—	—
Tolypothrix sp. BB 97.26	Z liquid	this study	—	—
Trichormus variabilis CCALA 205	BG11	this study	this study	—
Westelliopsis prolifica SAG 23.96	not in culture*	AJ544087	—	—
Xenococcus sp. BB 97.118	Z liquid	this study	this study	—
Xenococcus PCC 7305	not in culture*	AF132783	—	—
Xenococcus PCC 7307	not in culture*	AB074510	AB074803	AB074780

* "Not in culture" meaning that for these taxa genomes or single gene sequences where available, therefore no cultivation and extracts out of these taxa where necessary.
■ Sequences provided by J. Jaeger and M. Schultz.
T: type strain.
◊ Strain was only used for the survey of the thylakoid structure.
ǂ Originally identified as *Chroococcidiopsis polansiana*, Fewer et al. 2002 confirmed identity as "*Myxosarcina*"; hence this study labeled the strain "*Myxosarcina* CCMP 1489".

2.2 Investigation of thylakoid arrangements with Low Temperature Scanning Electron Microscopy

To investigate the thylakoid arrangements in the cyanobacterial cells a Low Temperature Scanning Electron Microscope (LT–SEM; Zeiss, Oberkochen, Germany) was used. Additionally, literature was searched for thylakoid arrangement, especially for those strains where genetic information was taken from genomes (Table 2.2–2.3). The investigated strains cover a wide systematic range of cyanobacteria (Table 2.2). Cyanobacteria were grown on their required media (solid 1% agar media; see Table 2.1) The strains were cultured at ca. 24°C under a lights/dark regime of 12:12 h at a light intensity of about 20–50 µmol photons * m^{-2} * s^{-1} until their biomass was considered to be enough for microscopy (3–12 weeks). The samples, small blocks of agar with cells on top, were embedded into a mixture of Vaseline and graphite, which were used as a glue to fix the samples at the sample holder. The mixture was proofed in previous investigations as best glue and to have the best main. Samples were cryofixed freeze fractured to detect the details of the thylakoids within the cells. The samples were coated with palladium in a sputter coater (Gala Instruments, Bad Schwalbach, Germany), which had a power of 20–35 mA for 1:30–5:00 min. Prepared samples were analysed using the Supra 55VP Scanning Electron Microscope (Zeiss, Oberkochen, Germany) at 3–7 kV. The structures of the thylakoids were categorized in different groups. Single cells with special orientations of the thylakoids were not included into the analyses.

Table 2.2 (pages 27-28): Names of genera and strain numbers of which the thylakoid arrangements were studied with the LT-SEM, and where information was obtained from literature.

	Names of genera	Number of strains	
		This study	Literature
Gloeobacterophycidae			
Gloeobacterales			
Gloeobacteraceae	*Gloeobacter*	–	1
Synechoccophycidae			
Synechoccales			
Chamaesiphonaceae	*Chamaesiphon*	–	1
Merismopediaceae	*Aphanothece*	1	–
	Synechocystis	–	1
Synechoccaceae	*Cyanobium*	–	1
Pseudanabaenales			
Pseudanabaenaceae	*Geitlerinema*	–	1
	Pseudanabaena	–	3
	Wilmottia♠	–	1
Oscillatoriophycidae			
Chroococcales			
Chroococcaceae	*Chroococcus*	1	–
Cyanobacteriaceae	*Cyanothece*	–	2
Oscillatoriales			
Gomotiellaceae	*Starria*	–	1
Oscillatoriaceae	*Lyngbya*	2	–
Phormidiaceae	*Microcoleus*	2	–
	Tychonema	–	1
Pleurocapsales			
Dermocarpellaceae	*Dermocarpella*	–	1
	Stanieria	–	2
Hydrococcaceae	*Pleurocapsa*	1	1
Xenococcaceae	*Xenococcus*	1	3
Nostocophycidae			
Nostocales			
Scytonemataceae	*Brasilonema*	–	1
	Scytonema	2	–
Microchaetaceae	*Petalonema*	1	–
	Tolypothrix	3	–
Nostocaceae	*Anabaena*	1	–
	Aphanizomenon	3	–
	Cylindrospermum	1	–
	Mojavia	1	–
	Nostoc	3	–
	Trichormus	1	–
Fischerellaceae	*Fischerella*	2	–

	Names of genera	Number of strains	
		This study	Literature
Nostochopsidaceae	*Nostochopsis*	1	–
Stigonemataceae	*Stigonema*	2	–
Chroococcidiopsidaceae	*Chroococcidiopsis*	13	3

♠ The genus Wilmottia have been described by Strunecky et al. 2011 and was not included in the systematic of Büdel & Kauff 2012. According to the results of Strunecky et al. 2011 this genus is assigned to the Pseudanabaenales.

Table 2.3 (pages 28-31): Investigated strains for the arrangement of their thylakoids using a LT-SEM. Besides these ones the information of some other strains were obtained via the given literature.

Strain	Origin
Anabaena flos-aquae (Lyngb.) Breb BB 97.35	This study
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.111	This study
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.25	This study
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.85	This study
Aphanothece cf. krumbeinii	Smarda & Roussomoustakaki 2000
Brasilonema bromeliae	Fiore et al. 2007
Chamaesiphon PCC 7430	Waterbury 1976
Chroococcidiopsis BB 79.1	This study
Chroococcidiopsis BB 82.1b	This study
Chroococcidiopsis sp. BB 79.2	This study
Chroococcidiopsis sp. BB 80.1	This study
Chroococcidiopsis sp. BB 81.1	This study
Chroococcidiopsis sp. BB 82.3	This study
Chroococcidiopsis sp. BB 84.1	This study
Chroococcidiopsis sp. BB 90.5	This study
Chroococcidiopsis sp. BB 96.1a	This study

Strain	Origin
Chroococcidiopsis sp. BB 96.1b	This study
Chroococcidiopsis thermalis BB 82.2	This study
Chroococcidiopsis cf. BB 96.19	This study
Chroococcidiopsis sp. BB 97.116	This study
Chroococcidiopsis PCC 7203	Komárek & Kastovsky 2003
Chroococcidiopsis PCC 7432	Waterbury & Stanier 1978
Chroococcidiopsis PCC 7436	Waterbury & Stanier 1978
Chroococcus sp. CCALA 057	This study
Cyanobium PCC 7001	Rippka et al. 1974
Cyanothece PCC 7424	Komárek & Anagnostidis 1999
Cyanothece halobia	Roussomoustakaki & Anagnostidis 1991
Cylindrospermum BB 97.12	This study
Dermocarpella PCC 7326	Waterbury & Stanier 1978
Fischeralla ambigua (Näg.) Gom. BB 97.28	This study
Fischerella muscicola BB 98.1	This study
Geitlerinema splendidum	Anagnostidis 1989
Gloeobacter PCC7421	Rippka et al. 1974
Lyngbya sp. BB 97.64	This study
Lyngbya sp. BB 97.65	This study
Microcoleus chtonoplastes BB 92.3	This study
Microcoleus sp. BB 97.74	This study
Mojavia pulchra CCALA 691	This study
Nostoc BB 94.2	This study
Nostoc muscorum BB 90.3	This study

Strain	Origin
Nostoc sp. BB 89.12	This study
Nostochopsis lobatus BB 92.1	This study
Petalonema alatum SAG 44.87	This study
Pleurocapsa PCC 7314	Waterbury & Stanier 1978
Pleurocapsa sp. BB 97.117	This study
Pseudanabaena PCC 7367	Guglielmi & Cohen-Bazire 1982
Pseudanabaena PCC 7403	Guglielmi & Cohen-Bazire 1982
Pseudanabaena PCC 7408	Cohen-Bazire & Bryant 1982
Scytonema BB 97.127	This study
Scytonema ocellatum BB 02.1	This study
Stanieria PCC 7304	Waterbury 1976
Stanieria PCC 7437	Waterbury 1976
Starria zimbabweensis SAG 74.90	Lang 1977
Stigonema mamillosum (Lyngb.) Ag. BB 97.104	This study
Stigonema ocellatum Thuret BB 97.103	This study
Synechocystis PCC 6803	van de Meene et al. 2006
Tolypothrix BB 97.100	This study
Tolypothrix sp. BB 97.26	This study
Tolypotrix distorta var. penicillata (Ag.) Lemm. BB 97.17	This study
Trichormus variabilis CCALA 205	This study
Tychonema bourellyi CCAP 1967/1459-10	Komárek & Albertano 1994
Wilmottia murrayi (W. et. G.S. West)	Strunecky et al. 2011
Xenococcus PCC 7305	Waterbury 1976
Xenococcus PCC 7306	Waterbury 1976

Strain	Origin
Xenococcus PCC 7307	Waterbury & Stanier 1978
Xenococcus sp. BB 97.118	This study

2.2.1 Statistical analysis of thylakoid arrangements

To test the relationship between the type of thylakoidal arrangement and the group assignment, a Chi² test was done with Statistica v7 (Stat Soft Inc., Tulsa, USA). Thylakoid arrangements were attributed to seven different groups (coiled, parietal, stacked, coiled to radial, parietal to coiled, stacked to parietal, stacked to radial), while taxa (order and family, respectively) were used for group assignments (Table 3.5). Order and family assignment based on Büdel & Kauff (2012). Additionally, corrected contingency coefficient (C_{corr}) was calculated after Köhler et al. (2007) as an estimate for the strength of the relationship:

$$C\ corr = \sqrt{\frac{\chi^2 \times m}{(\chi^2 + N)(m-1)}}$$

χ^2 = estimate the strength of the relationship
m = lower number of the groups of the two factors were chosen
N = sample size

This coefficient ranges from 0.0 to 1.0 (i.e. no to relationship between the two factors).

2.3 Cell size measuring

For morphological characterization, 15 *Chroococcidiopsis* strains were investigated (Table 2.4). Strains were identified morphologically using Komárek & Anagnostidis (1999). Each culture was min. two weeks old and maintained on required media, nutrients were not depleted (Table 2.1). Of each strain, 50 randomly chosen cells were measured with a microscope (AxioCam MRc, Carl Zeiss, Germany) and imaging software AxioVision v4.7.2.0 (Carl Zeiss Imaging Solutions GmbH, Germany).

For all *Chroococcidiopsis* strains, mean cell size and their standard deviations were calculated (Table 2.4). In order to detect differences in the cell sizes of differences a one-way analysis of variance (ANOVA) was performed. Further, Tukey´s *post hoc* was computed test to determine which strains are significantly different. Statistical analyses were done with Statistica v7 (Stat Soft Inc., Tulsa, USA).

Table 2.4: Strains investigated for cell size using light microscopy.

Strain
Chroococcidiopsis BB 79.1
Chroococcidiopsis BB 82.1
Chroococcidiopsis CCMEE 10
Chroococcidiopsis CCMEE 140
Chroococcidiopsis cf. CCMEE 167
Chroococcidiopsis cf. BB 96.19
Chroococcidiopsis sp. BB 79.2
Chroococcidiopsis sp. BB 80.1
Chroococcidiopsis sp. BB 81.1
Chroococcidiopsis sp. BB 82.3
Chroococcidiopsis sp. BB 84.1
Chroococcidiopsis sp. BB 90.5
Chroococcidiopsis sp. BB 96.1
Chroococcidiopsis sp. BB 97.116
Chroococcidiopsis thermalis BB 82.2

2.4 Molecular work

2.4.1 DNA Extraction

The cultures were dried in a sterile Eppendorf tube on silica gel for 2–3 days and manually crushed using a micropestle on liquid nitrogen. DNA was extracted using the "Invisorb Nucleo Spin Plant Extract II" (Invitrogen, Life Technologies, Carlsbad, USA) as recommended by the manufacturer. The DNA concentrations from the extractions were controlled using a NanoDrop (Thermo-Scientific, Waltham, USA). DNA extracts and their aliquots were stored at -20°C.

2.4.2 PCR amplification

A typical 50 µl PCR for the DNA amplification was done using "Taq DNA Polymerase", "HotStar Taq DNA Polymerase" or "HotStar Taq Plus DNA Polymerase" (all Qiagen, Hilden, Germany; see Table 2.5). For each PCR reaction a positive control (containing a template that is certain to amplify under these conditions) and negative control (containing H_2O dd instead of the template DNA) was used. If the signal of a PCR product gave weak fluorescence signal in gel electrophoresis, higher $MgCl_2$ volumes were used (up to 3.5 mM). If DNA template had a low concentration, then the template volume was increased and, therefore, the volume of the H_2O dd was decreased in order to keep always a final volume of 50 µl.

The 16S rRNA was amplified by PCR using the primer pair 1 and 18 (Table 2.6; Wilmotte et al. 1993). The *rpoC1* gene was amplified by PCR using the primer rpoC1-1 and rpoC1-T (Table 2.6; Palenik 1994). And the *gyrB* gene was amplified by PCR using the primer GB/3MF and GB/CR-2 (Table 2.6; Seo & Yokota 2003). All primers were synthesized by Microsynth (Blagach, Switzerland) or Eurofins MWG GmbH (Ebersberg, Germany). Every primer was diluted from 100 pmol/µl to 1:10 concentration with H_2O dd. The PCR cycling parameters with specific temperatures for the primers can be found in Table 2.5–2.9.

Table 2.5: Ingredients of a 50 µl PCR for the DNA amplification. Primer pairs are for the 16S rRNA: 1 and 18 (Wilmotte et al. 1993); for *rpoC1*: rpoC1-1 and rpoC1-T (Palenik 1994), and for *gyrB*: GB/3MF and GB/CR-2 (Seo & Yokota 2003).

Ingredients	16S rRNA Volume [µl]	*gyrB* or *rpoC1* Volume [µl]
10x PCR Buffer (Qiagen)	5	5
dNTP Mix (10 mM each, Qiagen)	1	1
$MgCl_2$ (2,5 mM, Qiagen)	2	—
Primer 1	1.25	2.5
Primer 2	1.25	2.5
Taq/HotStar Taq/HotStar Taq Plus (Qiagen)	0.25	0.5
H_2O dd	38.25	37.5
DNA template	1	1

Table 2.6: Primer sequences for the PCR and sequencing for the genes 16S rRNA, *rpoC1*, *gyrB* and M13.

Gene	Primer	Sequence 5´→3´	bp	Reference
16S rRNA	Wil1	AGAGTTTGATCCTGGCTCAG	20	Wilmotte et al. 1993
	Wil4*	AGGCAGCAGTGGGGAAT	18	Wilmotte et al. 1993
	Wil5*	CTGCTGCCTYCCGTA	15	Wilmotte et al. 1993
	Wil10*	GAATTGACGGGGRCCC	16	Wilmotte et al. 1993
	Wil11*	CCGTCAATTYYTTTRAGTTT	20	Wilmotte et al. 1993
	Wil16*	AAGGAGGTGATCCAGCCGCA	20	Wilmotte et al. 1993
	Wil18	TTTGCGGCCGCTCTGTGTGCCTAGGTATCC	30	Wilmotte et al. 1993
rpoC1	rpoC1-1	GAGCTCYAWNACCATCCAYTCNGG	24	Palenik et al. 1994
	rpoC1-T	GGTACCNAAYGGNSARRTNGTXGG	24	Palenik et al. 1994
gyrB	GB/3MF	AAGCGHCCNGSNATGTAYATHGG	23	Seo & Yokota 2003
	GB/CR-2	CCNGCNGARTCNCCYTYNAC	20	Seo & Yokota 2003
M13	M13 rev (-29)*	CAGGAAACAGCTATGACC	18	—
	M13 uni (-21)*	TGTAAAACGACGGCCAGT	18	—

* Primers were used for the sequencing step.

Table 2.7: PCR program for the amplification of the 16S rRNA.

Step	Temperature [°C]	Time [min]	Cycles [No.]
Initial Denaturation	94 / 95*	3 / 15 / 5*	
Denaturation	94	1	35
Annealing	50	1	
Extension	72	1	
Final Extension	72	10	
Pause	4	∞	

*Time for the initial denaturation: 3 min at 94 °C using Taq DNA Polymerase, 15 min at 95 °C using HotStar Taq DNA Polymerase and 5min at 95 °C with HotStar Taq Plus DNA Polymerase (all Qiagen, Hilden, Germany).

Table 2.8: PCR program for the amplification of *rpoC1* gene.

Step	Temperature [°C]	Time [min]	Cycles [No.]
Initial Denaturation	95	5	
Denaturation	94	1	30
Annealing	47	1	
Extension	72	2	
Final Extension	72	10	
Pause	4	∞	

Table 2.9: PCR program for the amplification of *gyrB* gene.

Step	Temperature [°C]	Time [min]	Cycles [No.]
Initial Denaturation	95	5	
Denaturation	94	1	35
Annealing	52	1	
Extension	72	1	
Final Extension	72	10	
Pause	4	∞	

Amplified PCR products were verified by a gel electrophoresis on a 1% agarose gel labelled by GelStar® (Lonza, Basel, Switzerland). PCR products with the appropriate size, in terms of gene length , were purified using the "Nucleo Spin Extract II" kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. The DNA concentrations were controlled using a NanoDrop (Thermo-Scientific, Waltham, USA). PCR products were stored at -20°C.

2.4.3 Cloning of PCR products

For the sequencing of the *rpoC1* and *gyrB* genes an additional cloning step was necessary. The fresh purified PCR products of *rpoC1* and *gyrB* genes were cloned in competent *Escherichia coli* cells using the TOP10 ONE SHOT Kit (Invitrogen, Life Technologies, Carlsbad, USA) and pGEM®-T Vector System I (Promega Corporation, Madison, USA) as recommended by the manufacturers. The 10 µl mixture for the ligation step contained 5 µl 2x Rapid Ligation Buffer, 1 µl pGEM®-T Vector (50 ng), 1 µl T4 DNA Ligase (3 Weiss units/µl) and 3 µl of the PCR product or for the positive control 2 µl Control Insert DNA (all products Promega Corporation, Madison, USA). Transformed cells were spread on LB agar plates which were prepared with 50 µg/ml ampicillin (Sigma-Aldrich, St. Louis, USA) and 40 mg/ml X-Gal (Peqlab, Erlangen, Germany) and incubated over night at 37°C. While the TOP10 ONE SHOT Kit (Invitrogen, Life Technologies, Carlsbad, USA) have a white/blue screening for efficient cloning reaction, at minimum 5 white coloured cloning products were picked and incubated in liquid LB medium containing 50 µg/ml ampicillin (Sigma-Aldrich, St. Louis, USA) overnight at 37°C. These liquid cultures were taken for checking the length of the insert by a PCR with an end volume of 25 µl and a Taq polymerase (Axon, Kaiserslautern, Germany). PCR compounds can be seen in Table 2.10 and PCR programs for the cloning steps are given in Table 2.11.

Table 2.10: Ingredients of a 25 µl PCR for checking the length of the cloning inserts. Primer 1 and 2 were M13 rev (-29) and M13 uni (-21).

Ingredients	Volume [µl]
10x PCR Buffer (Qiagen)	2.5
dNTP Mix Qiagen (10 mM each)	0.5
Primer 1	0.5
Primer 2	0.5
Taq (Axon)	0.25
H_2O dd	19.5
Liquid culture	1

Table 2.11: PCR program in the cloning steps of the *rpoC1* and *gyrB* gene PCR products for the check of the length of the clones.

Step	Temperature [°C]	Time [min]	Cycles [No.]
Initial Denaturation	95	2	
Denaturation	94	0:45	
Annealing	52	0:45	35
Extension	72	1:30	
Final Extension	72	5	
Pause	4	∞	

To verify the presence of the cloned insert on the amplified PCR, cloning products were detected for the right size by a gel electrophoresis on a 1% agarose gel labelled by GelStar® (Lonza, Basel, Switzerland). From the samples with the right size, 1ml each of the liquid culture were purified using the "Invisorb© Spin Plasmid Mini Two" kit (Stratec Biomedical, Birkenfeld, Germany) as recommended by the manufacturer. After the cleaning, the DNA concentrations of purified recombinant clones were measured with a Nano-Drop (Thermo-Scientific, Waltham, USA). Left over cloning products were stored at -20°C.

2.4.4 Sequencing

The purified PCR products of 16S rRNA as well as the purified cloning products were sequenced by a commercial supplier (SeqIT GmbH, Kaiserslautern, Germany). For the 16S rRNA the set of primers 1, 4, 5, 10, 11, and 16 were used (Wilmotte et al. 1993), and the cloning products of *rpoC1* and *gyrB* genes were sequenced (both strands) using vector-specific M13 rev (-29) and M13 uni (-21) primers (sequences see Table 2.6).

2.5 Phylogenetic analyses

2.5.1 Alignment preparation

Sequences were assembled with the Sequencer Software v4.5 (GeneCodes, Ann Harbor, USA) and corrected manually if necessary. To corroborate the cyanobacterial origin of sequenced samples, a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul 1997)) was performed using the program "blastn" against the "Nucleotide collection (nr/nt)" database. All non-cyanobacterial sequences were excluded from further analyses. In order to increase the database size, GenBank (Benson et al. 2011) was used to retrieve cyanobacterial genomes and sequences. The source of all used sequences can be found in Table 1. For testing biogeographic patterns of *Chroococcidiopsis* strains sequences from Bahl et al. (2011) were obtained from GenBank and add to alignments (NCBI GenBank accession numbers FJ805842–FJ805957).

2.5.2 Likelihood-mapping of the alignments

To test the phylogenetic signal that is present in the genes and alignments a likelihood-mapping was performed with TreePuzzle v5.2 (Schmidt et al. 2002). This *a priori* test visualizes the phylogenetic signal of a phylogenetic tree in form of a triangle with the likelihood-mapping method (Strimmer & von Haeseler 1997). Values indicate percentage in the centre and on the lateral of the triangle represents unresolved phylogenies, whereas values located in the corners indicate well-resolved phylogenies of all possible quartets (Fig. 2.1).

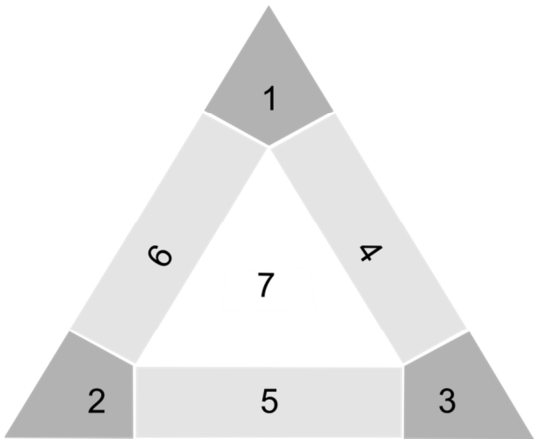


Fig. 2.1: The seven main areas of the likelihood-mapping in the triangle supporting different evolutionary information. Area 1 + 2 + 3 represents resolved quartets, 4 + 5 + 6 partly resolved quartets and 7 unresolved quartets. Adopted from Schmidt & von Haeseler 2009.

In brief, as more values are located in the corners of the triangle (area 1–3), the more are the data phylogenetically informative. While the program supports only a limited number of evolutionary models, GTR (Lanave et al. 1984) was the nearest method supporting the data from the alignments. Not only a likelihood mapping for each single gene, additionally a combination of three genes and a combination of 16S rRNA and *rpoC1* were performed. The number of puzzling steps was set to 0, meaning all possible quartets were estimated.

2.5.3 Phylogenetic analyses

For each gene, a multiple sequence alignment was obtained using the approximation described in Huerta-Cepas et al. (2011). Briefly, three different programs, MUSCLE v3.7, MAFFT v6.712b and DIALIGN-TX, were used to align sequences in forward and reverse directions. Resulting six alignments were combined into a meta-alignment using M-Coffee v9.01 (Wallace et al. 2006). Final alignment was generated after removing unreliable columns, in terms of poor consistency (<0.1667) across generated alignments and/or high percentage of gaps (>90%), using trimAl v1.3 (Capella-Gutierrez et al. 2009). So that at the end of this process all regions that are unalignable were excluded for the analyses.

As the outgroup taxon *Gloeobacter violaceus* PCC 7421 was chosen. This cyanobacterium lacks the thylakoid membranes and shows differences in the metabolism which distinguish them from all other cyanobacterium (Rippka et al. 1974; Mangels et al. 2002; Nakamura et al. 2003). This species was described the most basal cyanobacteria (e.g. Nelissen et al. 1995; Ishida et al. 1997; Honda et al. 1999; Turner et al. 1999; Seo & Yokota 2003; Schirrmeister et al. 2011).

The phylogenetic reconstruction was performed using the Maximum Likelihood (ML) and Bayesian inference (BI) methods. The ML method evaluates the probability of obtaining a phylogenetic tree given a hypothesis (meaning a multiple sequence alignment and a set of parameters, including an evolutionary model). The evolutionary model GTR+I+G and parameters were given as input to RAxML v7.3.0 (Stamatakis 2006; Stamatakis et al. 2008) and run at the cluster "Elwetritsche" of the University of Kaiserslautern. The statistical support for the ML trees was computed by bootstrap with replacement analyses of 1000 replicas.

The Bayesian analysis is as well a statistical-based method, but it works different. The method samples a set of trees given using a Metropolis-coupled Markov Chain Monte

Carlo algorithm (MCMCMC or MC^3) then the posterior probability of getting such distribution is computed. The Bayesian analyses were performed using MrBayes v3.2.1 (Huelsenbeck & Ronquist 2001; Ronquist et al. 2012). The runs were performed for 2-5 million generations, sampling every 500th generation, a print frequency of 1000 and with 4 parallel chains in order to evaluate convergence criteria. To be sure that the analysis has reached the plateau phase, all analyses were checked with a graph showing number of generations versus posterior probabilities. For getting this graph, the script "plot.py" provided by Frank Kauff (University of Kaiserslautern) was used.

2.5.4 Data sets

The phylogenetic analyses pursued two objectives. First, the evolutionary relationships of the genus *Chroococcidiopsis,* the order Pleurocapsales and the order Nostocales should be reconstructed. Second, biogeographical and life-strategy pattern of *Chroococcidiopsis* should be examined. For the reconstruction of the evolutionary relationships single gene analyses of the three genes 16S rRNA, *rpoC1* and *gyrB* have been performed. Afterwards a combination of the gene sequences was done to increase the phylogenetic signal of the single genes. To test the influence of missing data in the concatenated alignment, an additional data set with 16S rRNA and *rpoC1* gene sequences was done with taxa for which sequences of both genes were available. Biogeographical and life-strategy pattern of the genus *Chroococcidiopsis* were analysed in a combined data set with 16S rRNA data from hot and cold desert originated *Chroococcidiopsis* strains from Bahl et al. (2011). Finally the 16S rRNA, *rpoC1* gene and *gyrB* gene were combined and compared with information's about the geographical origin and life-strategy of the strains.

2.5.5 Determination of Operational taxonomic units

The traditional classification of Cyanobacteria is based greatly on morphological characters (e.g. Anagnostidis & Komárek 1985; Rippka et al. 1979). This morphology classification might be not sufficient enough to discriminate between phylogenetic distinguishable taxa (e.g. Rajaniemi et al. 2005; Taton et al. 2006). One approach to estimate the bacterial phylogenetic diversity is the defining of phylotypes or Operational Taxonomic Units (OTUs). The 16S rRNA sequences were grouped into OTUs on the basis

of their similarity, which was defined on this study as sharing a level of 97% of similarity, meaning a cut-off of 0.03 were chosen. With a level of similarity greater than 97% the sequence is assigned to the same species (Stackebrandt & Goebel 1994; Schloss & Handelsman 2005). A distance matrix was calculated based on the 16S rRNA of 37 *Chroococcidiopsis* and Pleurocapsales strains, with the DNADIST program in the PHYLIP package v3.69 (Felsenstein 1989) using default parameters and Jukes-Cantor as substitution model. This matrix served as input for Mothur v1.27.0 (Schloss et al. 2009) to assign sequences to OTUs.

3. Results

3.1 Results Morphology

3.1 Cell size as a potential trait for morphological discrimination of *Chroococcidiopsis* strains

In general, mean cell sizes varied between 2.75-5.43 µm in diameter (Table A2 appendix). Some strains had very regular cell sizes, indicated by small standard deviations (e.g. *Chroococcidiopsis* BB 97.116 and *C.* sp. BB 90.5; Fig. 3.1). In contrast, some strains had very variable cell sizes, indicated by large standard deviations (> 1.25 µm, e.g. *Chroococcidiopsis* sp. BB 96.1 and *C.* sp. BB 84.1). Cell sizes differed significantly between strains (Fig. 3.1) and can be explained at 47% by strain type (Table 3.1). While, there were strains with significantly different cell sizes, e.g. *Chroococcidiopsis* sp. BB 80.1 and *C.* sp. BB 90.5 (Fig. 3.1), there were others, which did not differ in cell size, e.g. *Chroococcidiopsis* sp. BB 84.1 and *C.* sp. BB 82.3.

Table 3.1: ANOVA Results of for differences in cell size between 15 different *Chroococcidiopsis* strains (each n = 50).

df	n	F	p	Adjusted R^2
14	750	48.23	**0.000**	0.468853

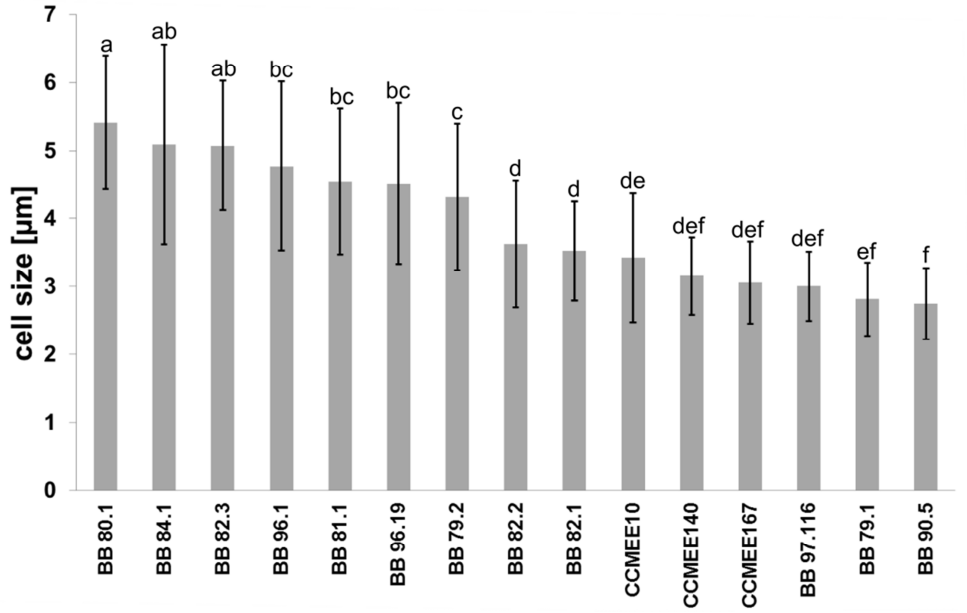


Fig. 3.1: Mean values and standard deviations of the cell sizes of 15 *Chroococcidiopsis* strains (each n = 50). Different letters indicate differences between strains revealed by an ANOVA (Table 3.3) with Tukey's *post hoc* test at p ≤0.05.

3.2 Thylakoids

3.2.1 Basic pattern of thylakoid arrangement

The thylakoids were clearly recognizable as a double layer membrane with small glycogen granules located between the membranes. In the cells of the strains *Mojavia pulchra* CCALA 691, *Tolypothrix* sp. BB 97.26 and *Tolypotrix distorta var. penicillata* (Ag.) Lemm. BB 97.17, holes were regularly observed (Fig. 3.2a). Some of these holes contained small particles of unknown nature and variable sizes. The thylakoids were not only arranged in one plane, but in a complex three-dimensional structure. In some cells of *Chroococcidiopsis* strains fingerlike, tubular thylakoids appeared (Fig. 3.2b). The opening of the double layer and forming of intra-thylakoid spaces in forms of loops (Fig. 3.2c) were noticed in cells of the order Oscillatoriales. In *Trichormus variabilis* CCALA 205, *Chroococcus* sp. CCALA 057, *Chroococcidiopsis* BB 82.1b and *Nostoc sp.* BB 89.12 some cells with net-like thylakoids were observed (Fig. 3.2d).

Three basic patterns of thylakoid arrangements within the cell were found and termed hereafter as: (1) parietal, (2) stacked and (3) coiled (Fig. 3.3a-c). In the parietal arrangement the thylakoids were arranged parallel to the cytoplasmic membrane in varying numbers. Stacked thylakoids formed fascicles of short sections in different numbers (not to be confused with connected and stacked thylakoids of higher plants). Coiled thylakoids were irregularly distributed in a wavy and dense structure, and in most cases appeared very long.

It was difficult to count the number of thylakoid membranes, especially in parietal and coiled arrangements. This was due to difficulties in determining the beginning and end of a membrane. Additional to the three basic patterns, two main orientations of thylakoids were observed. One group showed a more wall bound orientation towards the periphery of the cell (Fig. 3.4a-c), and in the second group the thylakoids were distributed throughout the whole cell (Fig. 3.4d-f). The dominating orientation in all systematic groups was throughout the cell. For 45 strains it was possible to investigate the orientation, where the distribution peripheral was counted 10 times and throughout the cell 35 times (Table 3.2). Within the Oscillatoriales, Pleurocapsales, Nostocales, and in the genus *Chroococcidiopsis* both orientations were apparent. In contrast, only one type of orientation was observed in the Synechococcales, Pseudanabaenales and Chroococcales, the former having a throughout the cell and the latter two a peripheral orientation.

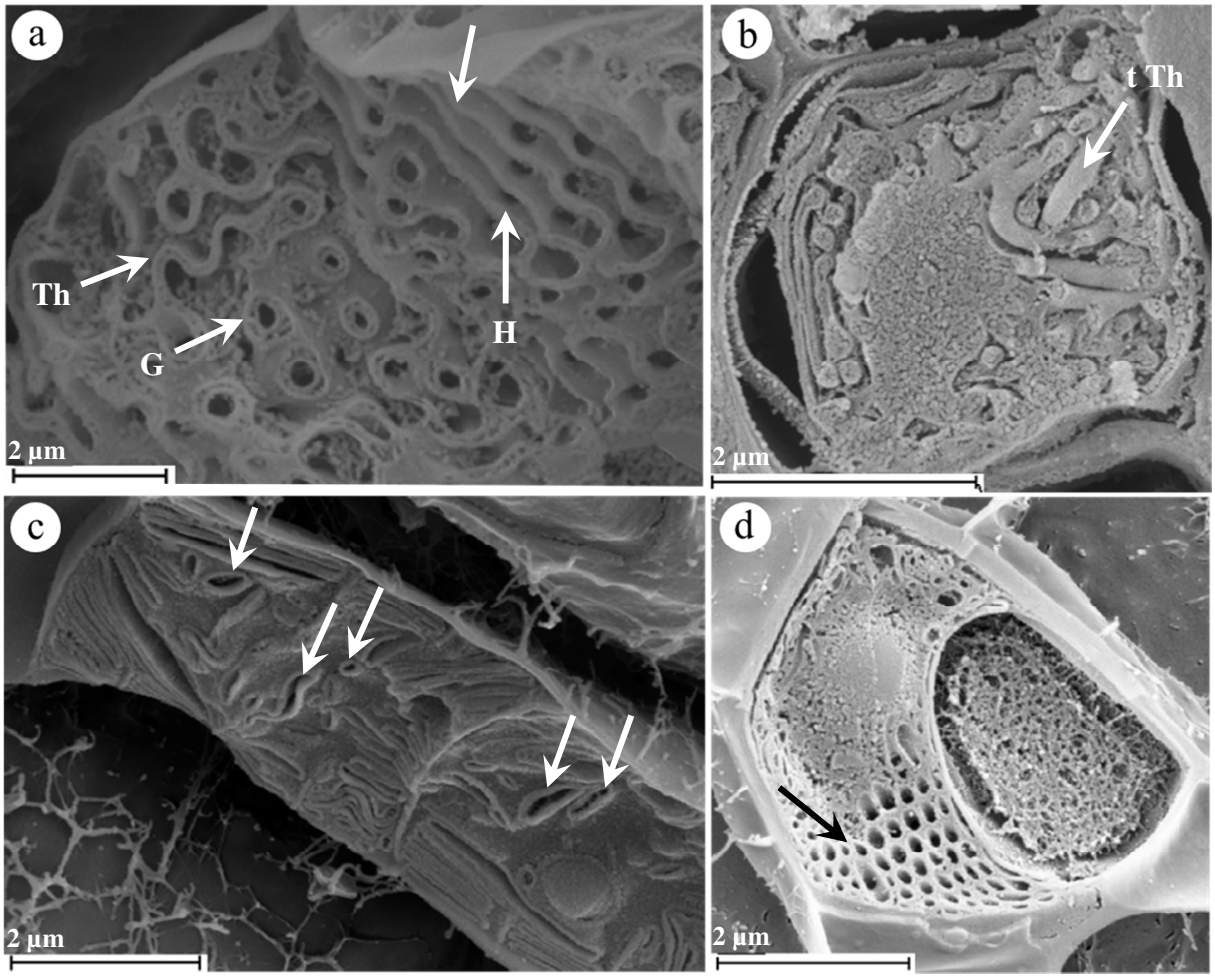


Fig. 3.2: Thylakoid arrangements in cyanobacterial cells. **(a) -** Thylakoids (**Th**) arranged as double layer membranes with glycogen granules (**G**) located between the thylakoids. The 3-D structure (arrow) and membranes are sometimes penetrated regularly by holes (**H**) (in *Tolypotrix distorta var. penicillata* (Ag.) Lemm. BB 97.17). **(b) -** Tubular thylakoids (**t Th**) observed in some cells (here *Chroococcidiopsis* sp. BB 96.1b). **(c) -** Also winded thylakoids forming loops appeared (arrows; in *Lyngbya* sp. BB 97.64). **(d) -** And sometimes thylakoids were arranged very regular and net-like in the cells (arrow; in *Nostoc* sp. BB 89.12).

Fig. 3.3: Schemes of three observed thylakoid arrangements in cyanobacteria. **(a) -** Parietal: parallel orientation to the cytoplasmic membrane. **(b) -** Stacked: short sections forming fascicles, and **(c) -** Coiled: thylakoids wavy and dense structure.

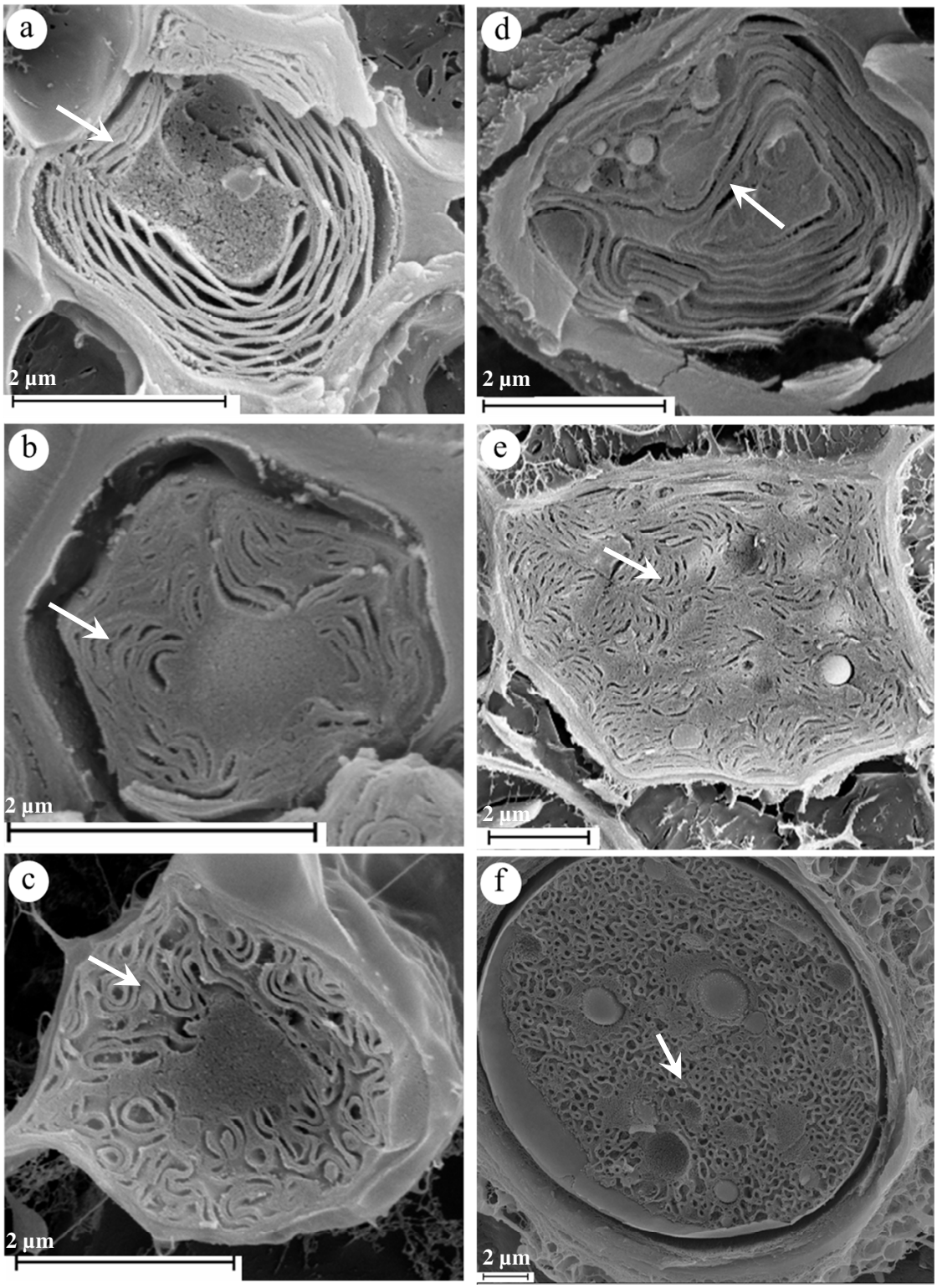


Fig. 3.4: Examples of observed thylakoid arrangements and orientations in cyanobacteria. Peripheral distribution of thylakoids (arrows) (**a-c**) and throughout the whole cell (**d-f**). **(a) -** Parietal, peripheral in *Nostoc* sp. BB 89.12, **(b) -** Stacked, peripheral in *Chroococcidiopsis* sp. BB 90.1, **(c) -** Coiled, peripheral in *Nostoc muscorum* BB 90.3, **(d) -** Parietal, throughout the whole cell in *Chroococcidiopsis* BB 79.1, **(e) -** Stacked, throughout the whole cell in *Pleurocapsa* sp. BB 97.117, and **(f) -** Coiled, throughout the whole cell in *Tolypotrix distorta var. penicillata* (Ag.) Lemm. BB 97.17.

3.2.2 Distribution of thylakoid arrangement in systematic groups

The strains of the family Chroococcidiopsidaceae showed all three basic and two intermediate forms of thylakoid arrangements (Table 3.2 & Fig. 3.5). The majority showed a coiled and stacked structure. Strain *Chroococcidiopsis* BB 79.1 had a parietal to coiled thylakoid arrangement. Three strains showed intermediate forms of stacked to parietal arrangement. In pictures of *C.* PCC 7436 from literature parietal in bigger cells and stacked arrangement in smaller cells can be seen (Waterbury & Stanier 1978).

The only cyanobacterium investigated that lacks thylakoids was *Gloeobacter violaceus* PCC 7421 (Table 3.2). Components for photosynthesis are located at the cytoplasmic membrane (Rippka et al. 1974).

The four strains of the order Pseudanabaenales had a parietal arrangement. Thylakoids in cells of *Geitlerinema splendidum* had a peripheral orientation (Table 3.2 & Fig. 3.5).

Within the four strains of Synechococcales all three main arrangements of the thylakoids have been observed (Table 3.2 & Fig. 3.5). In one strain (*Aphanothece cf. krumbeinii*) the orientation of the thylakoids was spread through the whole cell, with a slight tendency to peripheral parts.

The three strains of the order Chroococcales had a coiled or intermediate form between coiled and radial (Table 3.2 & Fig. 3.5). The thylakoid distribution could only be determined for *Chroococcus* sp. CCALA 057 and *Cyanothece halobia*: distributed throughout the whole cell.

In the order of the Oscillatoriales the arrangements of the thylakoids were equally distributed, stacked and parietal, and transitions between stacked to parietal, and stacked to radial were possible (Table 3.2 & Fig. 3.5). Only for some strains that showed a distribution throughout the whole cell could the thylakoid distribution be determined. The only exception was *Microcoleus chonoplastes* BB 92.3, where the thylakoids had a peripheral to longitudal orientation.

The LT-SEM observations revealed parietal and stacked arrangements within the strains of the four genera of the order Pleurocapsales (Table 3.2 & Fig. 3.5). All strains had a stacked arrangement, except *Xenococcus* sp. 97.118, which had a parietal arrangement. In general, the strains of the subclass Oscillatoriophycidae showed all three thylakoidal arrangements as well with some intermediate forms (Table 3.2 & Fig. 3.5).

Almost all strains from the subclass Nostocophycidae had a coiled arrangement (Table 3.2 & Fig. 3.5). The exception was strain *Anabaena flos-aquae* (Lyngb.) Breb BB 97.35, which had a stacked to parietal arrangement and *Nostoc* sp. BB 89.12, which had a parietal arrangement of the thylakoids.

Table 3.2 (sites 50-53): Observed thylakoid arrangement and orientations within the cells using LT-SEM. "▲" Additional information of strains obtained from literature. "–" Unable to determine the orientation.

Strain	Arrangement	Orientation
Anabaena flos-aquae (Lyngb.)Breb BB 97.35	stacked to parietal	peripheral
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.111	coiled?	throughout the cell
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.25	coiled?	throughout the cell?
Aphanizomenon flos-aquae (L.) Ralfs. BB 97.85	coiled	throughout the cell
Aphanothece cf. krumbeinii	coiled [1]	throughout the cell, tendency to peripheral parts
Brasilonema bromeliae ▲	coiled	–
Chamaesiphon PCC 7430 ▲	stacked	–
Chroococcidiopsis sp. BB 97.116	parietal?	–
Chroococcidiopsis BB 79.1	parietal to coiled	throughout the cell
Chroococcidiopsis BB 82.1b	stacked	peripheral
Chroococcidiopsis sp. BB 79.2	stacked to parietal	peripheral
Chroococcidiopsis sp. BB 80.1	coiled	throughout the cell
Chroococcidiopsis sp. BB 81.1	coiled	throughout the cell?
Chroococcidiopsis sp. BB 82.3	coiled	peripheral
Chroococcidiopsis sp. BB 84.1	coiled	throughout the cell
Chroococcidiopsis sp. BB 90.5	stacked	throughout the cell
Chroococcidiopsis sp. BB 96.1a	stacked?	throughout the cell

Strain	Arrangement	Orientation
Chroococcidiopsis sp. BB 96.1b	stacked	throughout the cell
Chroococcidiopsis thermalis BB 82.2	coiled	throughout the cell
Chroococcidiopsis cf. BB 96.19	coiled	throughout the cell
Chroococcidiopsis PCC 7203 ▲	stacked [2]	–
Chroococcidiopsis PCC 7432 ▲	stacked to parietal	–
Chroococcidiopsis PCC 7436 ▲	parietal in bigger cells, stacked in smaller cells	–
Chroococcus sp. CCALA 057	coiled	throughout the cell
Cyanobium PCC 7001 ▲	parietal	–
Cyanothece PCC 7424 ▲	coiled to radial	–
Cyanothece halobia ▲	coiled [3]	throughout the cell, tendency to peripheral parts
Cylindrospermum BB 97.12	coiled	throughout the cell
Dermocarpella sp. PCC 7326 ▲	stacked, small	–
Fischeralla ambigua (Näg.) Gom. BB 97.28	coiled?	–
Fischerella muscicola BB 98.1	coiled	throughout the cell
Geitlerinema splendidum ▲	parietal	peripheral
Gloeobacter PCC 7421 ▲	no thylakoids	–
Lyngbya sp. BB 97.64	stacked	throughout the cell
Lyngbya sp. BB 97.65	stacked	throughout the cell
Microcoleus sp. BB 97.74	stacked to parietal	throughout the cell
Microcoleus chtonoplastes BB 92.3	parietal	peripheral, longitudal
Mojavia pulchra CCALA 691	coiled	throughout the cell
Nostoc sp. BB 89.12	parietal	throughout the cell
Nostoc BB 94.2	coiled	throughout the cell - peripheral

Strain	Arrangement	Orientation
Nostoc muscorum BB 90.3	coiled	peripheral
Nostochopsis lobatus BB 92.1	coiled	throughout the cell
Petalonema alatum SAG 44.87	coiled?	–
Pleurocapsa PCC 7314 ▲	stacked	throughout the cell
Pleurocapsa sp. BB 97.117	stacked	peripheral
Pseudanabaena PCC 7367 ▲	parietal	–
Pseudanabaena PCC 7403 ▲	parietal	–
Pseudanabaena PCC 7408 ▲	parietal	–
Scytonema BB 97.127	coiled	throughout the cell
Scytonema ocellatum BB 02.1	coiled	throughout the cell
Stanieria PCC 7304 ▲	stacked	–
Stanieria PCC 7437 ▲	stacked, small	–
Starria zimbabweensis SAG 74.90 ▲	parietal	–
Stigonema mamillosum (Lyngb.) Ag. BB 97.104	coiled	throughout the cell
Stigonema ocellatum Thuret BB 97.103	coiled	throughout the cell
Synechocystis PCC 6803 ▲	parietal	–
Tolypothrix BB 97.100	coiled	throughout the cell
Tolypothrix sp. BB 97.26	coiled	throughout the cell
Tolypotrix distorta var. penicillata (Ag.) Lemm. BB 97.17	coiled	throughout the cell
Trichormus variabilis CCALA 205	coiled	throughout the cell
Tychonema bourellyi CCAP1967/1459-10▲	stacked to radial	throughout the cell
Wilmottia murrayi (W. et G.S.Wes) ▲	parietal	–
Xenococcus PCC 7305 ▲	stacked	peripheral

Strain	Arrangement	Orientation
Xenococcus PCC 7306 ▲	stacked, small	–
Xenococcus PCC 7307 ▲	stacked	throughout the cell
Xenococcus sp. BB 97.118	parietal	peripheral

[1] Described by Smarda & Roussomoustakaki 2000 as “irregular”
[2] Described by Komárek & Kastovsky 2003 as “Fascicles and usually in radial position”
[3] Described by Roussomoustakaki & Anagnostidis 1991 as “irregular”

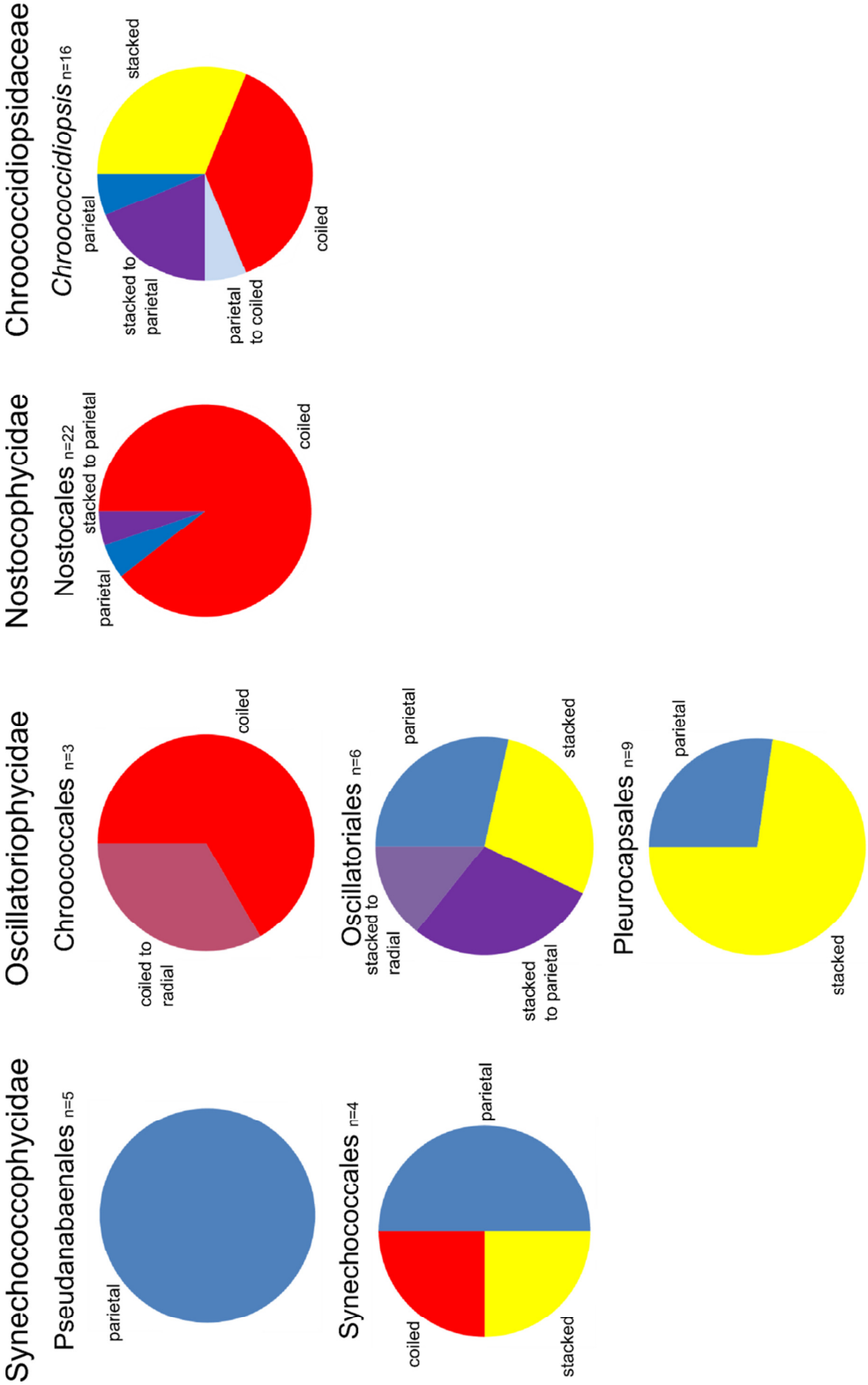


Fig. 3.5: Distribution of thylakoidal arrangements within the different subclasses Synechococcophycidae, Oscillatoriophycidae, Nostocophycidae and their orders, and the family *Chroococcidiopsidaceae*.

3.2.3 Thylakoid arrangements as a potential feature for morphological identification of cyanobacteria

The overall comparison of the thylakoid arrangements of all investigated orders (Pseudanabaenales, Synechococcales, Chroococcales, Oscillatoriales and Pleurocapsales) and the family Chroococcidiopsidaceae had the highest C_{corr} at 0.852 (Table 3.3). Hence, there is a strong relationship between the thylakoidal arrangement and the group assignment of an organism. The pairwise comparison between the family Chroococcidiopsidaceae and the order Nostocales had a much lower C_{corr} at 0.733 (Table 3.2). This means that the thylakoidal arrangement of the Chroococcidiopsidaceae and Nostocales is more similar than that of other groups.

Table 3.3: Results of the Chi^2 test for the relationship between taxa assignment (order and family, respectively) and thylakoid arrangement. Additionally, the corrected contingency coefficient is given, which ranges from 0 to 1 (1 representing maximum relationship between the factor "thylakoidal arrangement" and the factor "taxon").

	n	Chi^2	df	p	C_{corr}
All taxa	65	107.24	36	**0.000**	0.852
Chroococcidiopsidaceae, Nostocales and Pleurocapsales	47	34.81	8	**0.000**	0.799
Chroococcidiopsidaceae and Pleurocapsales	25	9.48	4	**0.050**	0.741
Chroococcidiopsidaceae and Nostocales	38	13.94	4	**0.008**	0.733

3.2.4 Special thylakoid arrangements

The nostocalean strain *Stigonema ocellatum* Thuret BB 97.103 exhibited a special arrangement. One intercalary cell showed a different thylakoid arrangement in the form of two whorls at one pole of the cell (Fig. 3.6a). In contrast, all other cells had a coiled thylakoid arrangement (Fig. 3.6b), In comparison to other cells, this special cell had a thickened cell wall (Fig. 3.6a-b).

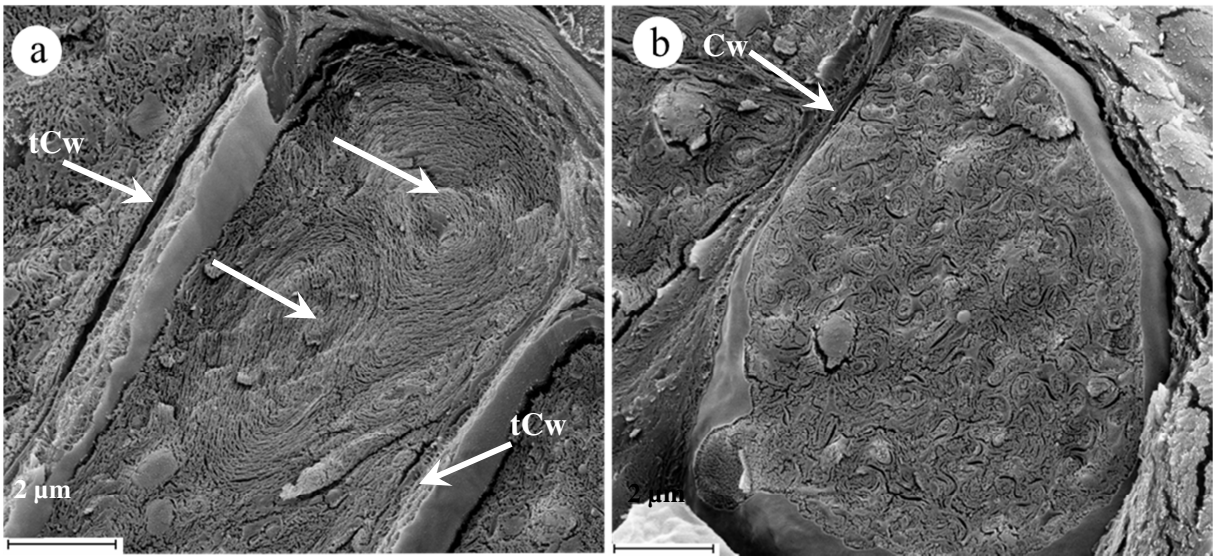


Fig. 3.6: Two types of thylakoid aggregations in *Stigonema ocellatum* Thuret BB 97.103. **(a) -** The thylakoids formed two centric whorls (arrows) at one pole of the cell. Notice the thickened cell wall (**tCw**). **(b) -** In comparison a cell with a coiled arrangement throughout the whole cell and a thinner cell wall (**Cw**).

3.2.5 General structure of cells

The cell itself was surrounded by a multi-layered cell wall and in the cell a couple of components such as cyanophycin granules and polyhedral carboxysomes were frequently apparent (Fig. 3.7a). The cyanophycin granules were small and sometimes numerous, but in a few cells they reached very large sizes occupying almost one third of the cell volume (Fig. 3.7c). Such big cyanophycin granules had of course effects on the distribution of thylakoids. In all cells the DNA was more or less densely concentrated and distinct in the nucleoplasm (or “nucleoid region”) (Fig. 3.7b). In an aquatic living strain cylindrical, aggregated clusters of gas-vesicles (aerotopes) were observed (Fig. 3.7c).

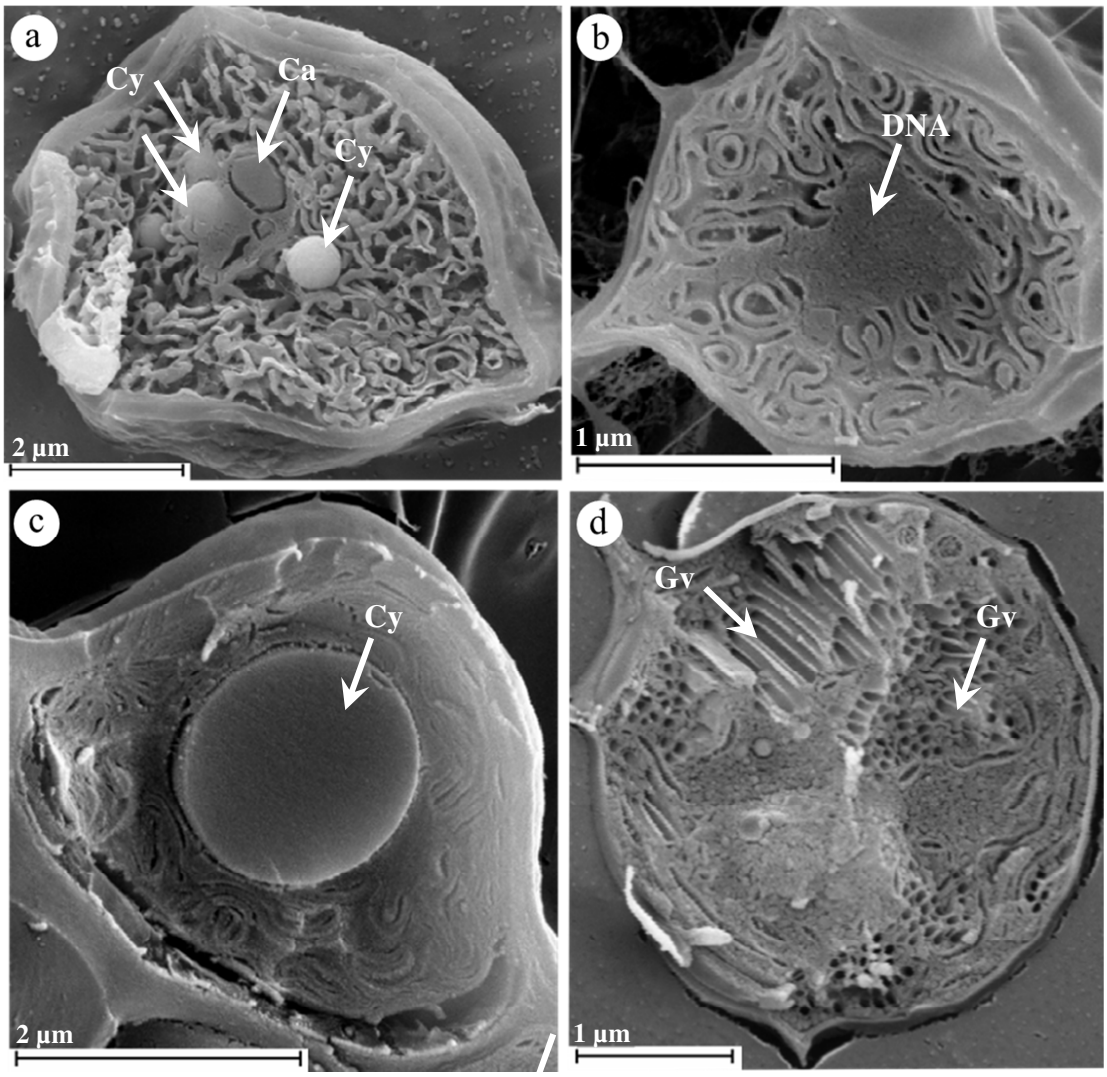


Fig. 3.7: Components in the cytoplasma of cyanobacterial cells. **(a) -** Round cyanophycin granules (**Cy**) consisting of polymers of arginine and aspartic acid. And the polyhedral carboxysomes (**Ca**), containing the ribulose-1,5-bisphophate-carboxylase (RUBISCO) as the main enzyme for the carboxylation (in *Chroococcidiopsis* sp. BB 84.1). (**b) -** A region with complex folded DNA (in *Nostoc muscorum* BB 90.3). **(c) -** A very large cyanophycin granule (**Cy**) (in *Chroococcidiopsis* sp. BB 90.1). **(d) -** Aggregated parallel clusters (aerotopes) of cylinder-shaped gas vesicles (**Gv**) in the planktonic living *Anabaena flos-aquae* (Lyngb.) Breb BB 97.35.

3.3 Results Phylogeny

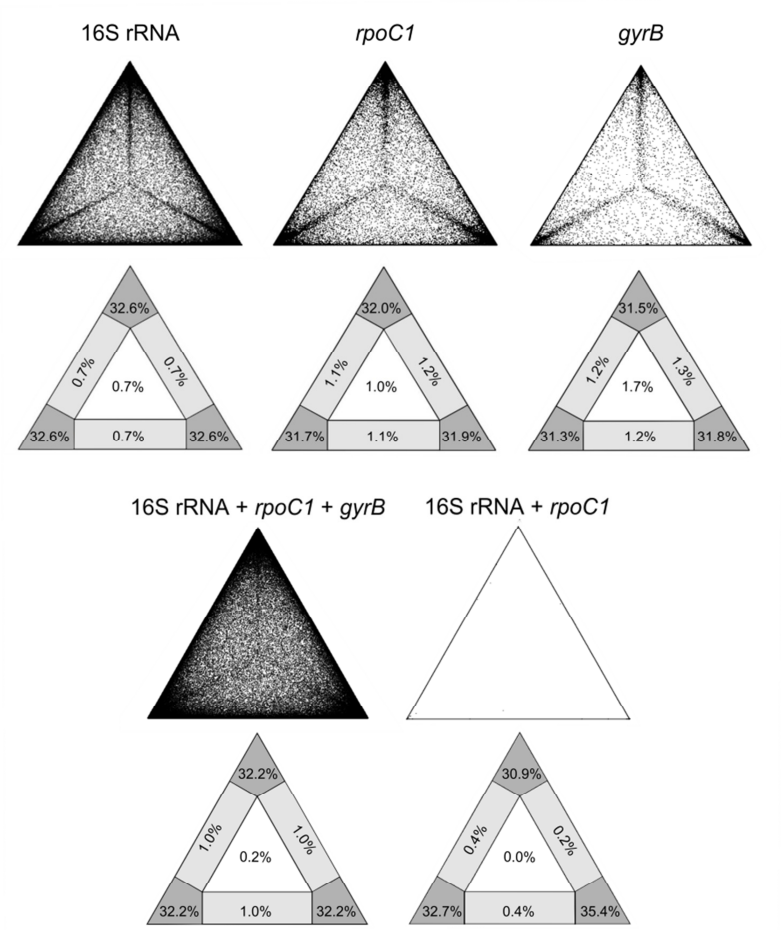
The phylogenetic analysis of this study generated 77 new sequences. Specifically, 45 sequences for the 16S rRNA, 24 for the *rpoC1* gene and 8 for the *gyrB* gene. Additional sequences from GenBank were used to cover a broader taxon sampling. Taking both sets together, a total of 97 sequences for 16S rRNA, 49 for *rpoC1* gene and 32 for *gyrB* gene were used to perform different phylogenetic analyses. This study doubled the number of *rpoC1* cyanobacterial gene sequences available in GenBank, contributing 24 to the existing 23, in the context of this thesis.

All analyses were performed using the Maximum Likelihood (ML) method with RAxML software and Bayesian inference (BI) method using the MrBayes software package, to reconstruct the evolutionary relationships between different strains of cyanobacteria. The previously described alignment optimization excluded all unalignable regions. Bootstrap values (BV) below 75% and Posterior probabilities (PP) below 0.95 are not displayed. Those branches which are statistically significant and supported by both methods (BV ≥75% and BI ≥0.95) are indicated by with thick lines in the following graphs.

3.3.1 Tree Puzzle

To visualize the phylogenetic content in an alignment a maximum-likelihood mapping analysis was performed (Strimmer & von Haeseler 1997). For this, the alignment is stripped into groups of four sequences (quartets), representing the smallest set of taxa for which more than one unrooted tree topology exists (Schmidt & von Haeseler 2010). The likelihood-mapping of the sequences gave the following numbers of all possible quartets: 3,464,840 for 16S rRNA, 211,876 for *rpoC1*, 52,360 for *gyrB*, 3,321,960 for the combined data set of the three genes, and 495 for the combination of 16S rRNA and *rpoC1*.

The resulting likelihood values of the quartets were visualized in an equilateral triangle. This triangle sectored into seven areas, assembled into three groups, which are supporting different evolutionary information (Fig. 3.8). The combination of the 16S rRNA and *rpoC1* gene sequences showed the highest phylogenetic signal, with 99% fully resolved quartets and without fully unresolved quartets. A slightly lower phylogenetic signal showed the pure 16S rRNA, with 97.8% fully resolved quartets and 0.1% fully unresolved quartets. Followed by the combination of all three genes (96.6% of quartets were fully and 0.2% were fully unresolved). The lowest phylogenetic signal showed the *gyrB* gene, with 94.6% of fully resolved, 3.7% of partly resolved and 1.7% of fully unresolved quartets. In general, the phylogenetic signal of all genes and combinations did not differ greatly from each other.



Data set	Number of possible quartets	Fully resolved quartets [%]	Partly unresolved quartets [%]	Fully unresolved quartets [%]
16S rRNA	3,464,840	97.8	2.1	0.1
rpoC1	211,876	95.6	3.4	1.0
gyrB	52,360	94.6	3.7	1.7
16S rRNA + *rpoC1* + *gyrB*	3,321,960	96.6	3.0	0.2
16S rRNA + *rpoC1*	495	99.0	1.0	0.0

Fig. 3.8: Likelihood mapping results for the different alignments. For each alignment all possible quartets were calculated (with the function n = 0). Fully resolved quartets are shaded dark grey, partly unresolved quartets are shaded light grey and fully unresolved quartets are shaded white.

Fig. 3.9: Phylogenetic tree based on 16S rRNA gene sequences from 97 cyanobacteria strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the evolutionary model GTR+I+G. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. Sequences obtained by this study marked with a star. A1-A3, B, C1-C2 and D denote clades described in the text

3.3.2 Evolutionary relationships of the genus *Chroococcidiopsis* and the order Pleurocapsales

3.3.2.1 Single gene analysis of the 16S rRNA

From 97 strains a phylogenetic analysis of the 16S rRNA was performed (fig. 3.9, sequences obtained by this study indicated by a star). The 16S rRNA data set consisted of 1989 characters including gaps. In general more nodes were supported by the BI method than by the ML method. Internal nodes were often only supported by BI, with the ML method receiving no significant support. Support by both methods could often be only observed at the tips of the nodes.

The phylogenetic tree of the 16S rRNA showed several distinguishable clades. The genus *Chroococcidiopsis* and the order Nostocales were separated from all the other cyanobacteria, whereas *Chroococcidiopsis* was a sister clade to the Nostocales (clade C1-C2, and D, respectively; PP = 0.98, BI = -). The genus *Chroococcidiopsis* (clade D1 & D2) and the order Pleurocapsales (clade B) were clearly separated from each other. Four *Chroococcidiopsis* strains showed almost no evolutionary distances and were located at the top of the clade D2 (*C. cubana* PCC 7431, *C.* cf. *cubana* CCALA 045, *C. thermalis* BB 82.2 and *C.* PCC 7303).

The order Pleurocapsales (clade B) is a sister group of the orders Oscillatoriales, Chroococcales and Synechococcales (clade A1 & A2). A separation of the Pleurocapsales from the remaining strains was only given by BI method (PP = 0.97). Surprisingly the pleurocapsalean strain *Pleurocapsa* PCC 7327 was not part of this group, instead it was placed on one node together with *Cyanothece* PCC 7424, a member of the Oscillatoriales (BV = 89%, PP = 0.98). These evolutionary separations would suggest the Pleurocapsales are polyphyletic. Additionally, the remaining genera of the Pleurocapsales did not formed a monophyletic clade. Strains from the order Pleurocapsales, Oscillatoriales, Chroococcales and Synechococcales were basal to *Chroococcidiopsis* and the Nostocales.

The Nostocales formed one clade (D; BV = -, PP = 0.99), which implies monophyly of this order. The strain *Scytonema ocellatum* BB 02.1 showed a very long branch.

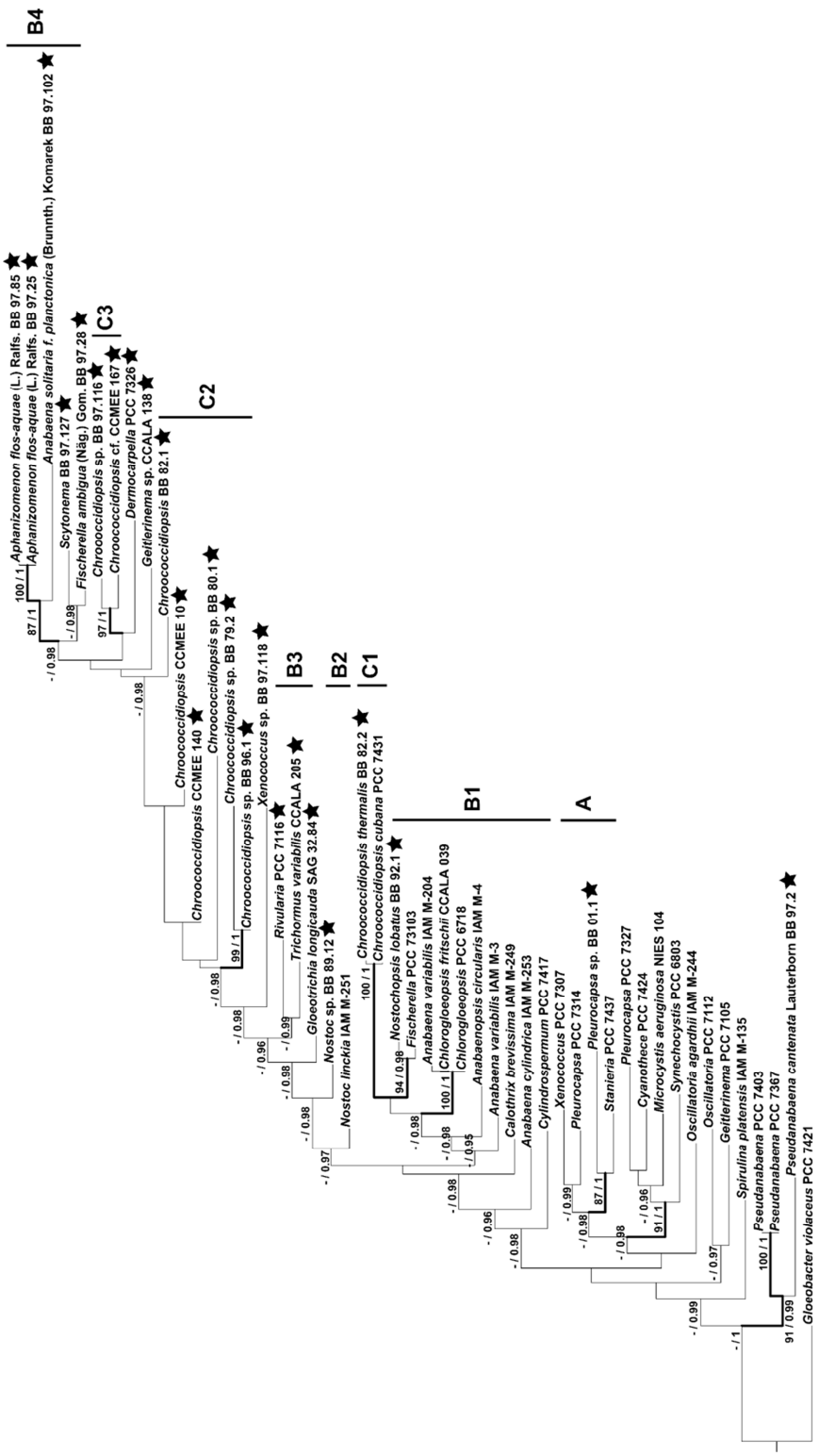


Fig. 3.10: Phylogenetic tree based on *rpoC1* gene sequences from 49 cyanobacteria strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the GTR+I+G evolutionary model. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. A, B1-B4 and C1-C3 denote clades described in the text. Sequences obtained by this study marked with a star.

3.3.2.2 Single gene analysis of the *rpoC1* gene

Phylogenetic analysis of the *rpoC1* gene was performed with 49 strains (fig. 3.10, sequences obtained by this study marked by a star). The *rpoC1* gene data set consisted of 1061 characters, including gaps. In general, the nodes showed a higher support by the BI method, which was the only analytical method to give a statistical significant signal. No differences in the statistical support between the deep and the tip nodes were observed.

No differentiated clade structure was observed for *Chroococcidiopsis* species, neither for the orders Pleurocapsales, nor Nostocales. The *Chroococcidiopsis* strains did not group together (clade C1-C3). Instead they were spread over the tree and mixed within the Nostocales, Pleurocapsales and several other cyanobacteria orders. The pleurocapsalean strains *Pleurocapsa* sp. BB 01.1, *P.* PCC 7314, *Stanieria* PCC 7437, and *Xenococcus* PCC 7307 were clustered together with members of the orders Oscillatoriales, Chroococcales and Synechococcales (clade A; PP = 0.98). Pleurocapsa PCC 7327 was situated apart from these pleurocapsalean. In fact, a complete separation of the pleurocapsalean strains was not obtained, with two strains nested within the Nostocales, *Chroococcidiopsis* and other cyanobacteria. Therefore, a clear separation of the pleurocapsalean and *Chroococcidiopsis* strains, as seen for the 16S rRNA analysis, could be not observed. In addition, the members of the Nostocales grouped not in a single clade, but were dispersed throughout the tree (B1–B4).

Actually, the *rpoC1* tree had a different position from those of the 16S rRNA tree for most of the strains. The disparity in the placement of the strains might represent an insufficient phylogenetic signal carried by this gene to clearly establish evolutionary relationships among different strains, or a methodological artifact. Nevertheless, there is a trend in general topology of the tree. *Pseudanabaena*, coccalean cyanobacteria and Pleurocapsales are at the base, while *Chroococcidiopsis* species group together within the Nostocales.

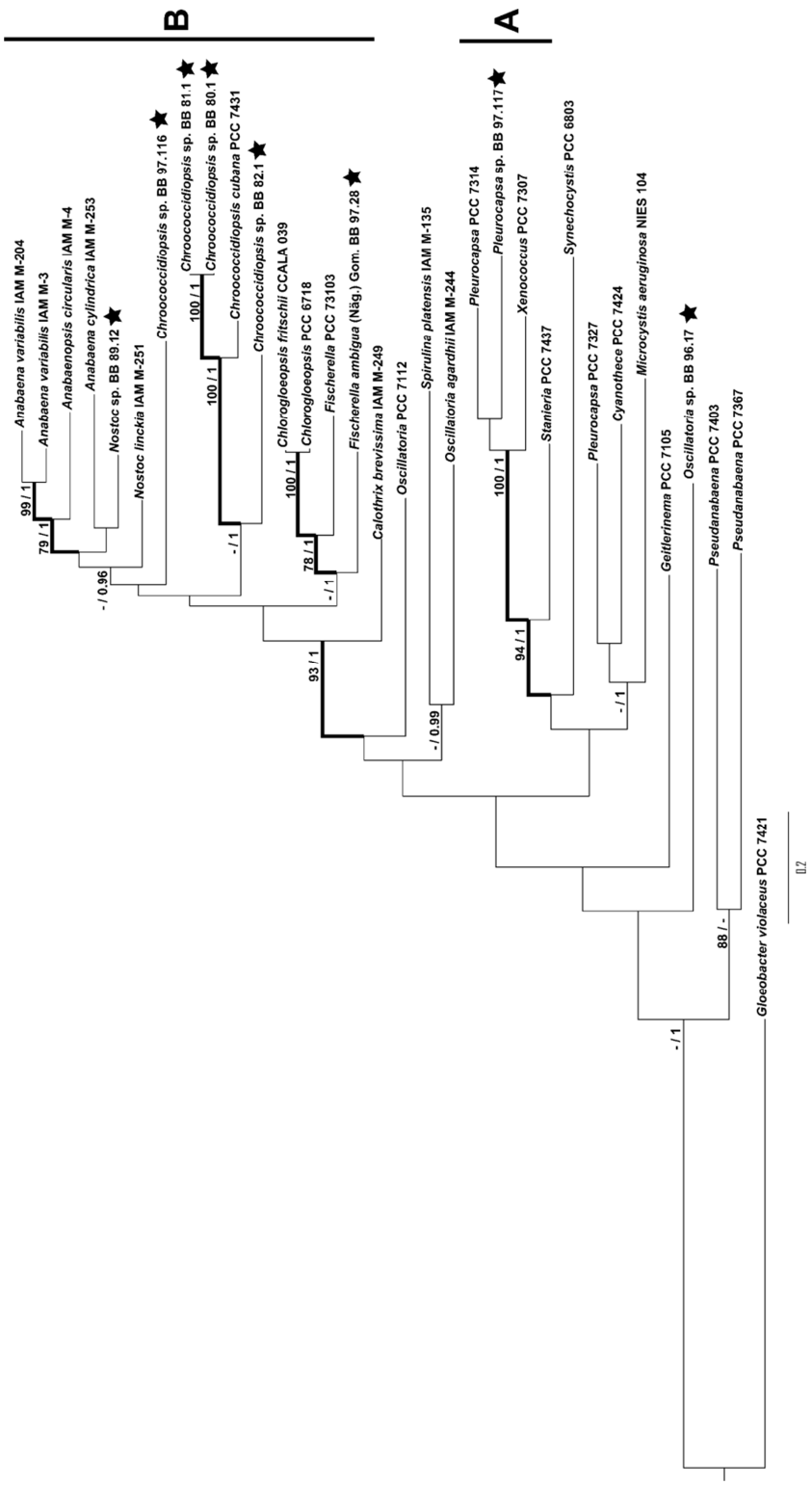


Fig. 3.11: Phylogenetic tree based on *gyrB* gene sequences from 32 cyanobacteria strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the GTR+I+G evolutionary model. Bootstrap values (≥ 75%) and Bayesian posterior probabilities (≥ 0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. A and B denote clades described in the text. Sequences obtained by this study marked by a star.

3.3.2.3 Single gene analysis of the *gyrB* gene

The phylogenetic analysis with the *gyrB* gene was performed with sequences of 32 strains (fig. 3.11, sequences obtained by this study indicated by a star). The *gyrB* gene data set consisted of 1232 characters, including gaps. Similarly as seen in the analysis of the 16S rRNA, more nodes were supported by the BI method. The support was almost only found at the tip of the tree.

The analysis of the *gyrB* gene confirmed the previous results of the other single gene analysis. All Nostocales and *Chroococcidiopsis* strains formed a distinct clade (B; BV = 93%, PP = 1). Within this clade more nodes were supported than in the rest of the tree. A separation of the *Chroococcidiopsis* strains and the Nostocales was not observed, as deep nodes of this clade did not receive statistically significant support.

Pleurocapsales, Oscillatoriales, Chroococcales and Synechococcales were placed basal to *Chroococcidiopsis* strains and the Nostocales. Within this mixed group *Stanieria* PCC 7437, *Xenococcus* PCC 7307, *Pleurocapsa* sp. BB 97.117 and *P.* PCC 7314 were found within one clade (clade A; BV = 94%, PP = 1). *Pleurocapsa* PCC 7327 was separated from other the Pleurocapsales, and clustered instead with two Chroococcales strains (BV = -, PP = 1).

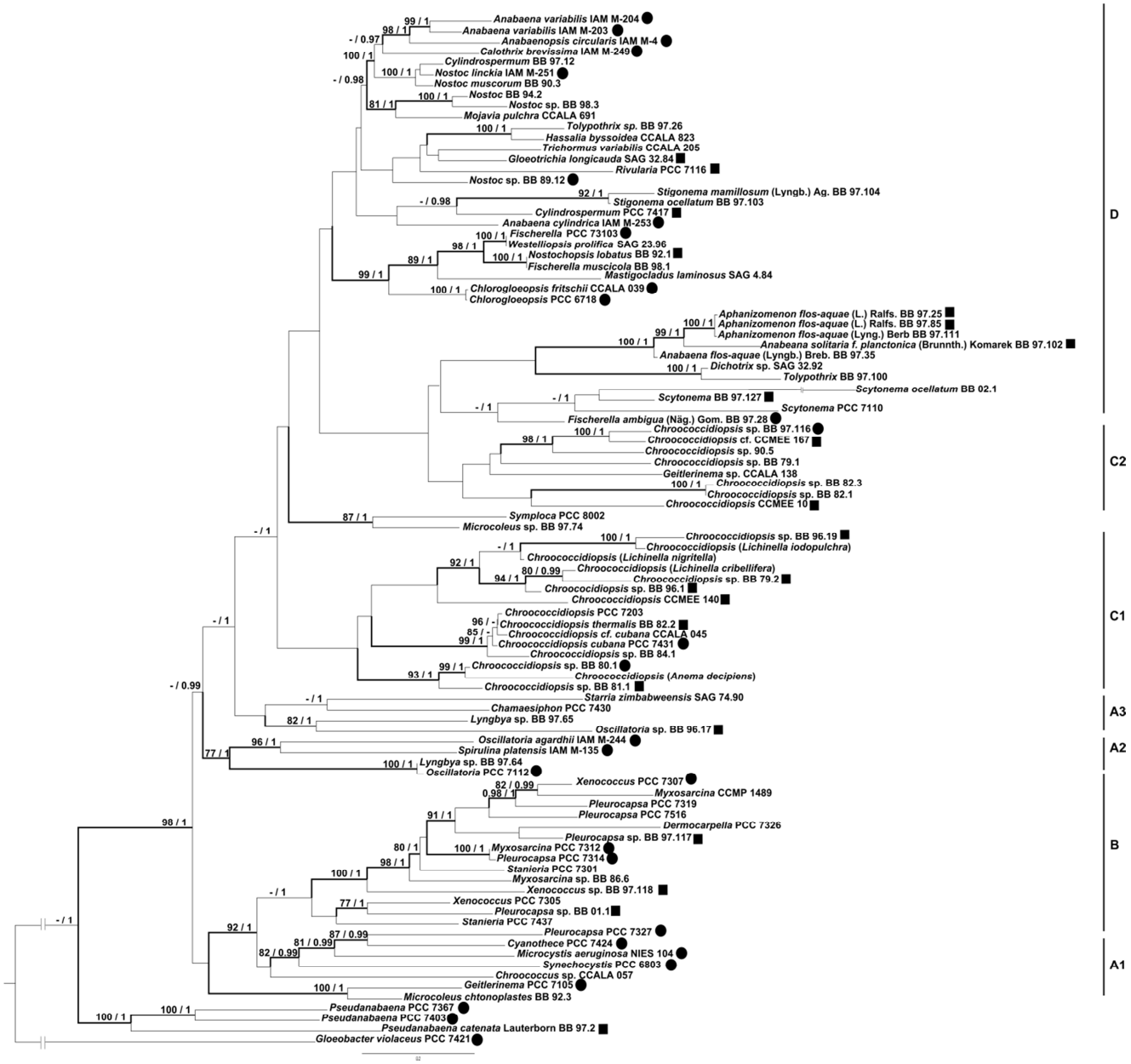


Figure 3.12: Phylogenetic tree based on the concatenated data set of the 16S rRNA, *rpoC1* and *gyrB* gene sequences from 97 cyanobacteria strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the GTR+I+G evolutionary model. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. Strains with sequences for three genes marked by a dot, strains with sequences for two genes marked by a square. A1-A3, B, C1-C2 and D denote clades described in the text.

3.3.2.4 Multigene analysis of 16S rRNA, *rpoC1* gene and *gyrB* gene sequences of all cyanobacteria

The phylogenetic analysis of single genes failed to resolve the phylogenetic relationships of the deep nodes. Analysis of a concatenated data set was considered as an option to overcome the low phylogenetic signals observed for the single analyses. The concatenated data set was compromised of the three genes of 97 strains, without accounting for partially missing data (fig. 3.12). The data set of 16S rRNA, *rpoC1* gene and *gyrB* gene consisted of 1985, 1058 and 1230 characters, respectively, including gaps. Strains with sequences for all three genes were marked with a dot and strains with sequences for two genes were marked by a square. The majority of all nodes were statistically significant supported by BI. However, fewer nodes were statistically significant supported by ML. As well as a high number of the deep nodes, as the tip nodes were statistical supported by both phylogenetic methods.

The result from this combined analysis is congruent with the result from the 16S rRNA analysis (fig. 3.9). In comparison, the number and distribution of statistical significant supported nodes of the single gene tree based on 16S rRNA differed only slightly. The genus *Chroococcidiopsis* and the order Nostocales were separated from the remaining cyanobacteria (BV = -, PP = 1), although both formed no separated cluster. Basal to these strains was a mixed group of Oscillatoriales, Chroococcales and Synechococcales (A2 & A3). *Chroococcidiopsis* clustered into two groups (C1 & C2), but in contrast to the 16S rRNA tree (fig. 3.9) with no statistical significant support.

The Pleurocapsales (clade B) confirmed clade B in the 16S rRNA tree (fig. 3.9), basal to *Chroococcidiopsis* and Nostocales, with slightly different positioning of single strains. Almost all nodes had a high statistical support. Like in the 16S rRNA tree *Pleurocapsa* PCC 7327 was not part of the Pleurocapsa clade (B), instead it was on one node together with *Cyanothece* PCC 7424 of the Oscillatoriales (BV = 87%, PP = 0.99). The remaining genera of the order Pleurocapsales are not each monophyletic.

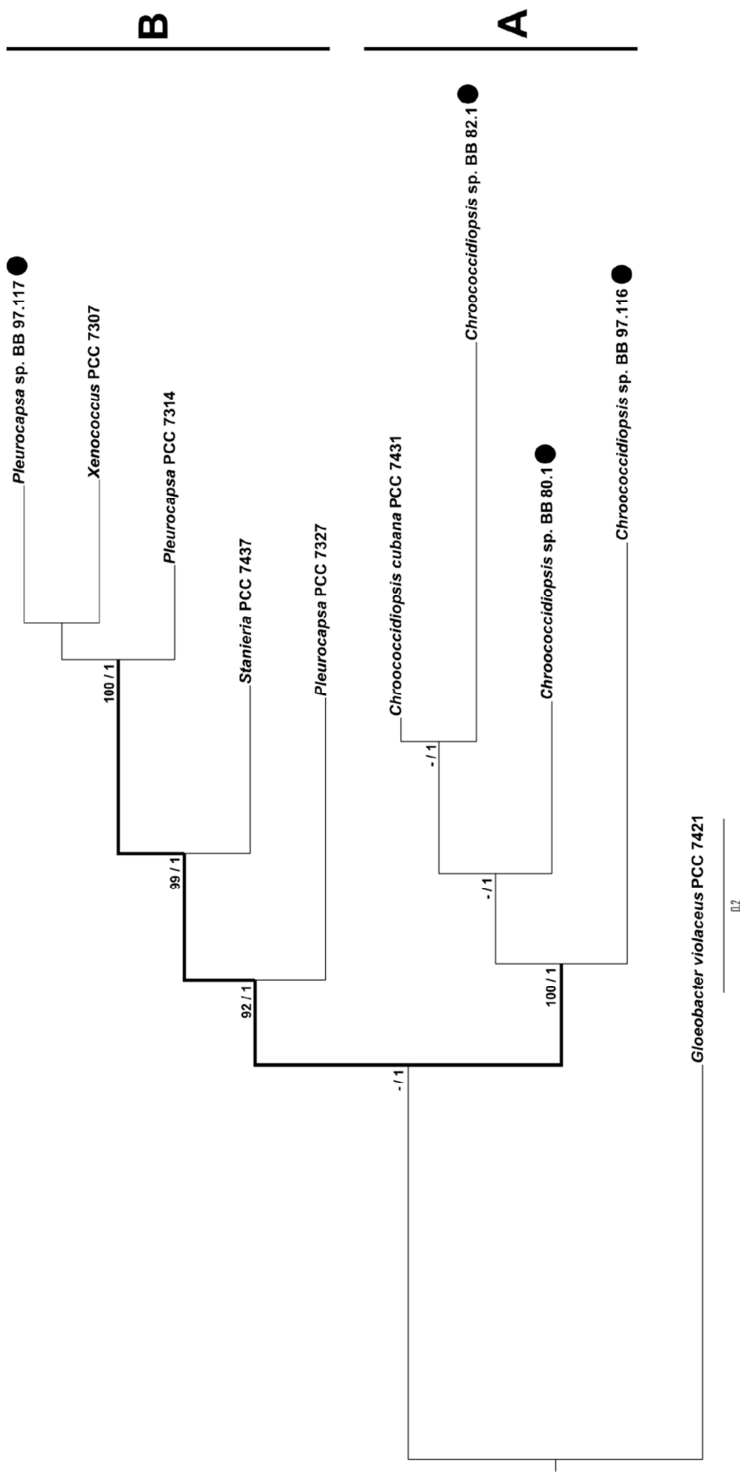


Fig. 3.13: Phylogenetic tree based on the concatenated data set of the 16S rRNA, *rpoC1* and *gyrB* gene sequences from 10 *Chroococcidiopsis* and Pleurocapsales strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the GTR+I+G evolutionary model. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. Strains with sequences obtained by this study marked by a dot. A and B denote clades described in the text.

3.3.2.5 Multigene analysis of the 16S rRNA, *rpoC1* gene and *gyrB* gene sequences of the genus *Chroococcidiopsis* and the order Pleurocapsales

The single gene analyses of the 16S rRNA, *rpoC1* and *gyrB* gene revealed a separation of the genus *Chroococcidiopsis* and the order Pleurocapsales (fig. 3.9–3.12). For verification of this result and to see whether partially missing data did not influenced the phylogeny, a multigene analysis of the 16S rRNA, *rpoC1* gene and *gyrB* gene was performed (fig. 3.13). The data set encompassed all strains of both *Chroococcidiopsis* and Pleurocapsales which had sequences for all three genes. The analysis was done with 10 strains and 16S rRNA (1981 characters), *rpoC1* gene (1052 characters) and *gyrB* gene (1211 characters; including gaps). Strains with sequences obtained by this study marked by a dot. A larger number of nodes had statistical support by the BI method. Three nodes had no support by ML, but were highly supported by BI.

The multigene analysis confirmed the general trend of the single gene analyses (fig. 3.9-3.11). The tree showed a clear separation of *Chroococcidiopsis* (clade A; BV = 100%, PP = 1) and Pleurocapsales (clade B; BV = 92%, PP = 1). The three *Pleurocapsa* strains were separated at different branches; *Pleurocapsa* PCC 7327 was basal to all Pleurocapsales and separated from the other two *Pleurocapsa* strains.

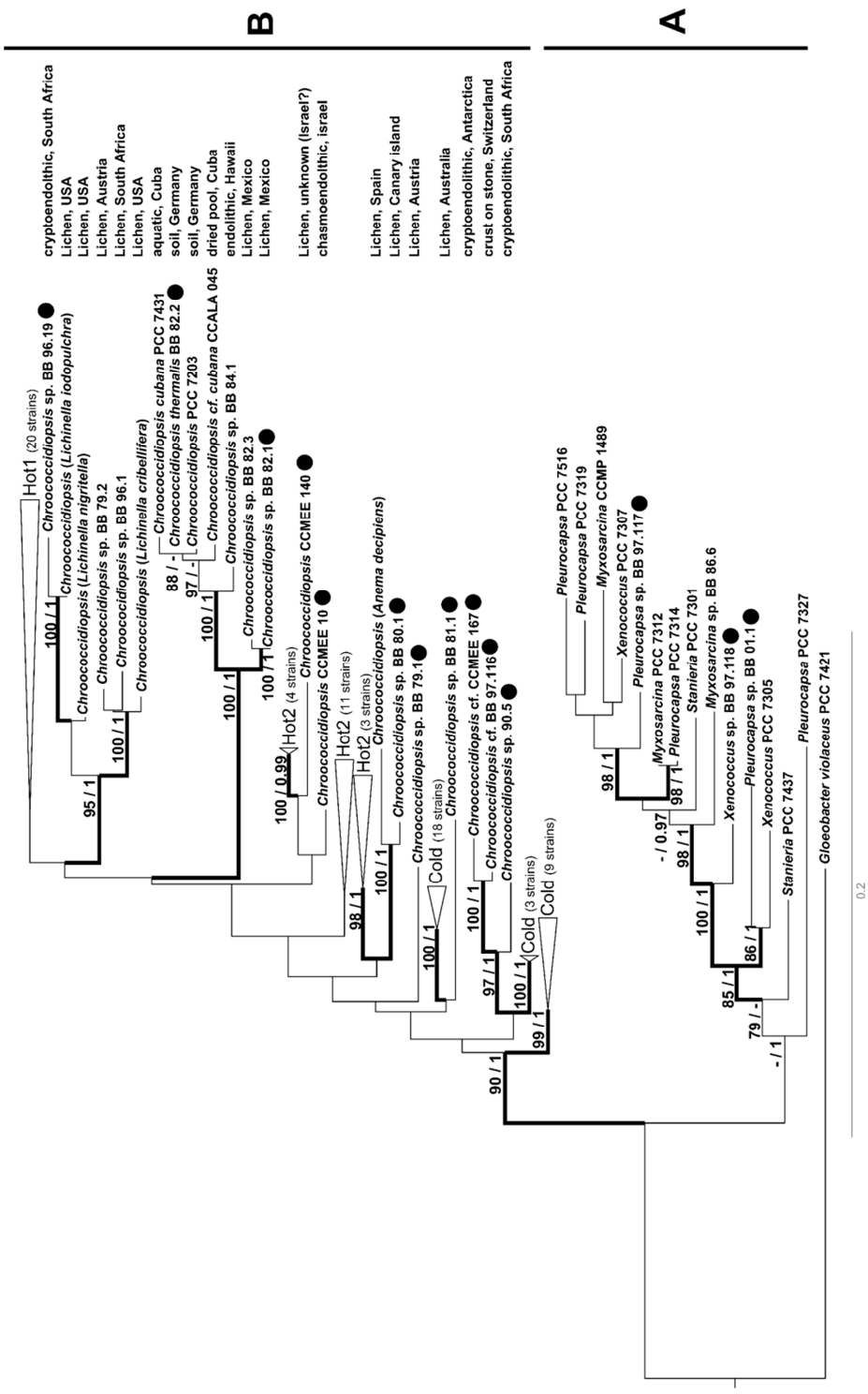


Fig. 3.14: Phylogenetic tree based on 16S rRNA gene sequences from 105 *Chroococcidiopsis* strains resulting from this study, from GenBank and Bahl et al. (2011). Tree was reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the GTR+I+G evolutionary model. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. Sequences obtained by this study marked by a dot. A and B denote clades described in the text. Cold, Hot1 and Hot2 reference to sections described by Bahl et al. (2011).

3.3.3 Biogeographical and life-strategy pattern of the genus *Chroococcidiopsis*

3.3.3.1 Biogeographic and life-strategy pattern of the genus *Chroococcidiopsis* by a single gene analysis of the 16S rRNA

Macroscopic organisms show distinctive biogeographic patterns. However, for microorganisms it is a controversial discussion, if they have restricted distributions or not. To test the spatial pattern of the worldwide distributed genus *Chroococcidiopsis* newly generated sequences, as well existing sequences retrieved from GenBank and Bahl et al. (2011), respectively, were analysed. Sequences of the latter indicated with “Cold”, “Hot1” and “Hot2”. “Hot1” are from hot arid deserts in Asia, Africa, Australia and America, and “Hot2” are from hot arid deserts in Africa, Asia and Australia. “Cold” are from cold arid deserts around the world. The comprehensive analysis included 105 taxa and 1510 characters, including gaps (fig. 3.14, sequences obtained by this study marked with a dot, Table A3 appendix). The majority of statistically significant supported nodes were confirmed by both methods. Evolutionary distances of all strains were small given the average substitutions per site in the alignment (indicated by the scale: 0.2 substitutions per site).

Chroococcidiopsis strains revealed no biogeographical pattern, because of the lack of support for most of the deep nodes. The same held true for the life-strategy. Strains with different habitats and strategy were mixed together. Strains from Bahl et al. (2011) were split into more subgroups in comparison to the original study. Furthermore, the newly generated sequences formed distinct clades, separated from the strains of Bahl et al. (2011). Besides the aspect of the biogeography of *Chroococcidiopsis*, the dramatic extension of sequences supported the phylogenetic trees of this study with less strains from this genus (e.g. fig. 3.9), the Pleurocapsales (Clade A; BV = -, PP = 1) being separate from the *Chroococcidiopsis* strains (Clade B; BV = 90%, PP = 1).

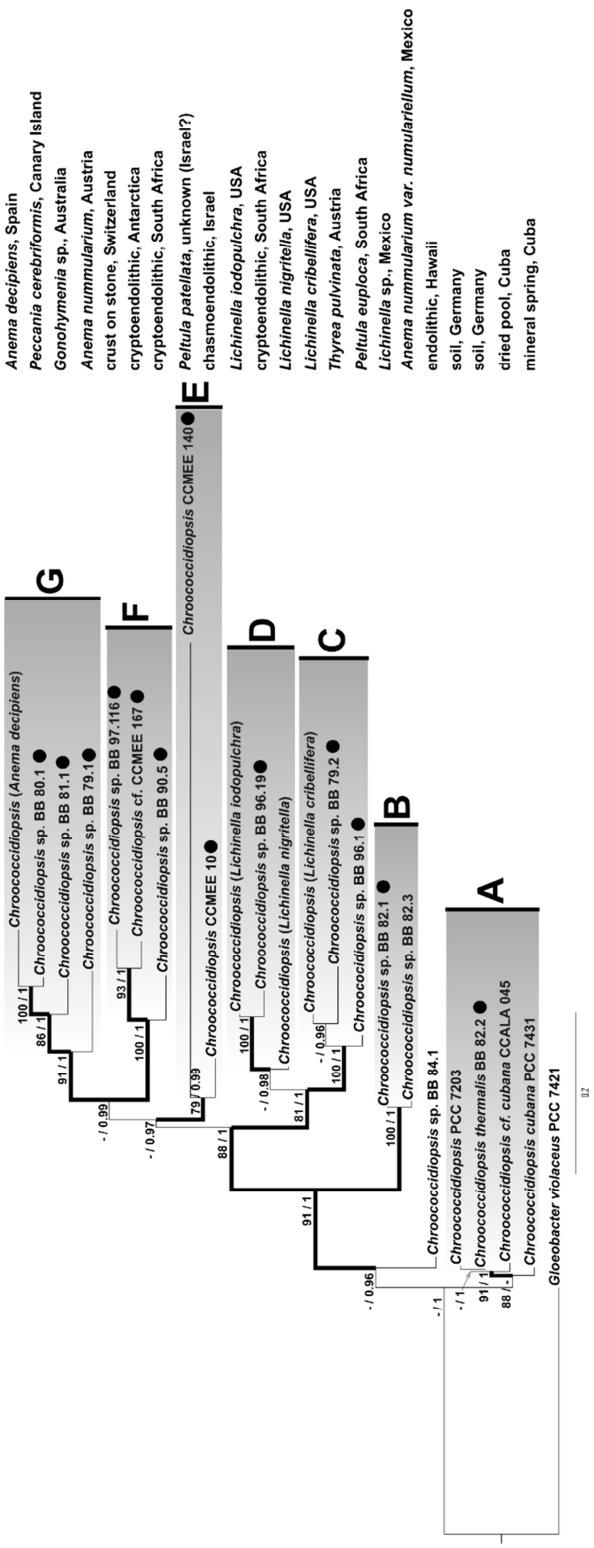


Fig. 3.15: Phylogenetic tree based 16S rRNA, *rpoC1* and *gyrB* gene sequences from 23 *Chroococcidiopsis* strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method by the GTR+I+G evolutionary model. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. Strains with sequences obtained by this study marked by a dot. Grey boxes and A-G denote clades described in the text. Additionally life-strategy and geographical origin are given.

3.3.3.2 Biogeographic and life-strategy pattern of the genus *Chroococcidiopsis* by a multigene analysis of 16S rRNA, *rpoC1* and *gyrB* gene sequences

The single gene analysis showed no patterns of strains from different geographical origin or life-strategies (fig. 3.14). In contrast, a previous multigene study of Bahl et al. (2011) showed spatial patterns of *Chroococcidiopsis* strains originated from hot and cold deserts. This raised the question as to whether the 16S rRNA sequences alone provide enough information to answer the question on the biogeography of *Chroococcidiopsis*. To answer this question, a concatenated date set of 16S rRNA (1495 characters), *rpoC1* gene (797 characters) and *gyrB* gene (1231 characters) from 23 *Chroococcidiopsis* strains with no concern about missing data for single taxa was done (in total 3525 characters, including gaps, fig. 3.15, newly obtained sequences by this study marked by a dot, Table A3 appendix). The majority of all nodes were supported by both methods.

As seen for the single gene analysis, the multigene analysis could not reveal a general pattern in either geographic origin or life-strategies. Several nodes with the highest statistical significant support suggested that strains from very distant geographical regions were closely related. Seven separated clades were clearly distinguishable, which had support at least by one method (clade A-G). *Chroococcidiopsis* sp. BB 84.1 did not cluster together with any other strain. An example of extreme geographical distances were *Chroococcidiopsis* sp. BB 90.5 (South Africa), *C.* cf. CCMEE 167 (Antarctica) and *C.* cf. BB 97.116 (Switzerland) joined at one node with the highest statistical significant support (clade F). A second example with distances of thousands of kilometres and high statistical support were clade C with *Chroococcidiopsis* sp. BB 96.1 (South Africa), *C* (*Lichinella cribellifera)* (USA) and *C.* sp. BB 79.2 (Austria). In contrast to those clades, the only clade with strains of very close geographical origin (Mexico) was clade B (BV = 100%, PP = 1) consist of *Chroococcidiopsis* sp. 82.3 and *C*. sp. 82.1.

For the life-strategy two types of clades were distinguishable. There were clades, which included strains with exclusively one life-strategy, e.g. clade C with lichenized and clade F (BV = 100%; PP = 1) with free-living strains. In contrast, there were two clades with strains performing different life-strategies. For example clade D, containing two lichenized and one cryptoendolithic strain.

Most basal were species in clade A containing the strains *Chroococcidiopsis* PCC 7431 (mineral spring, Cuba), *C*. cf. *cubana* CCALA 045 (dried pool, Cuba), *C*. *thermalis* BB 82.2 (soil, Germany) and *C*. PCC 7203 (soil, Germany). These four strains had almost identical

sequences of the three genes, which resulted in nearly no resolution. Interestingly, this group was already observed in other phylogenetic trees of this study, sometimes at a different position within the trees (fig. 3.9, 3.12 & 3.14). Overall the evolutionary distance of all *Chroococcidiopsis* strains was very small (indicated by the scale; 0.2 substitutions per site). *Chroococcidiopsis* CCMEE 140 had a longer branch length.

3.3.3.3 Biogeographic and life-strategy pattern of the genus *Chroococcidiopsis* by a gene analysis of 16S rRNA and *rpoC1* gene sequences

The previous concatenated data set included all *Chroococcidiopsis* strains without accounting for missing data for single genes in each taxon, which lead to partially incomplete data sets. In order to avoid the impact of missing data a combined data set of the 16S rRNA and *rpoC1* gene sequences was constructed from taxa for which sequences of both genes were available. The data set included 12 strains, 1924 characters for 16S rRNA and 1055 characters for the *rpoC1* gene, including gaps (fig. 3.16, Table A3 appendix). Strains with sequences obtained by this study were marked by a dot.

Similar to the previous analyses, in this data set no pattern in geographic origin and life-strategy could be observed. Strains from geographically distant regions clustered together and suggested a very close relationship, the same was revealed for the different life-strategies. For example, the strains *Chroococcidiopsis* sp. 97.116 (Switzerland) and *C.* cf. CCMEE 167 (Antarctica) clustered together with the highest support. The basal strains *Chroococcidiopsis* PCC 7431 (Cuba) and *C. thermalis* BB 82.2 (Germany) had almost identical sequences, resulting in no resolution. All other strains separated from these two strains (clade A, BV = 100%, PP = 1).

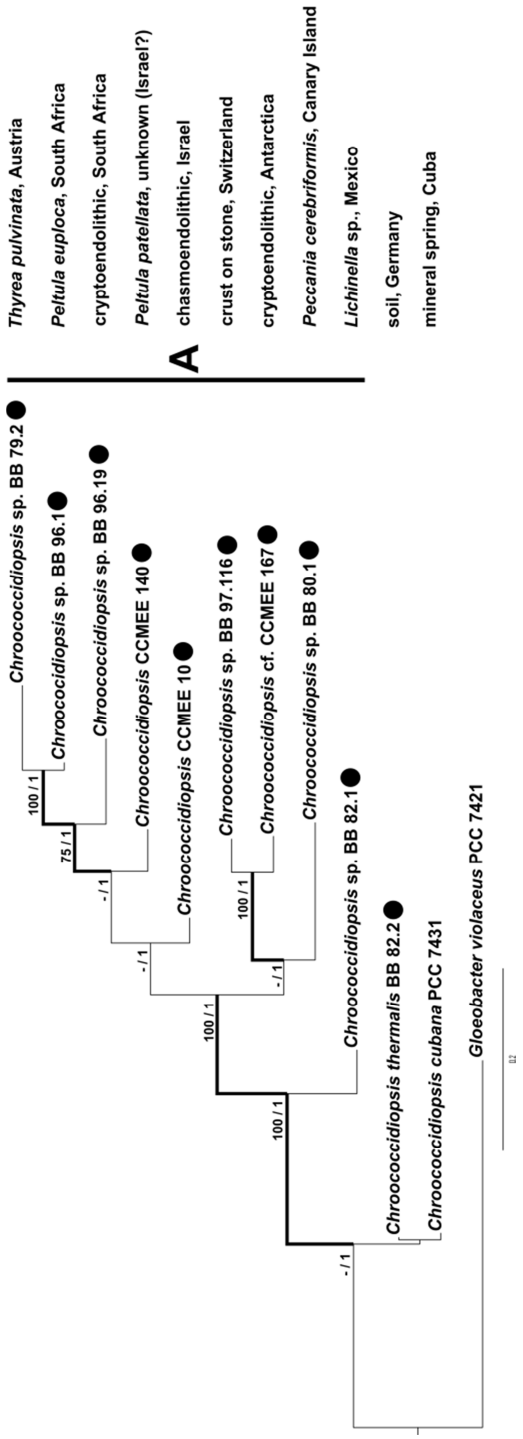


Fig. 3.16: Phylogenetic tree based on the concatenated data set of 16S rRNA and *rpoC1* gene sequences from 12 *Chroococcidiopsis* strains reconstructed using Maximum Likelihood (ML) and Bayesian inference (BI) method based on the GTR+I+G evolutionary model. Bootstrap values (≥75%) and Bayesian posterior probabilities (≥0.95) are given (ML first, BI second number). Thick lines marks internal nodes that were statistical significant supported by both methods. Bar represents 0.2 nucleotide substitutions per site. Strains with sequences obtained by this study marked by a dot. A denote to a clade described in the text. Additionally life-strategy and geographical origin are given.

3.3.4 Similarity analysis of the 16S rRNA within the genus *Chroococcidiopsis* and the order Pleurocapsales

Based on sequence similarity of a 16S rRNA data set of 37 *Chroococcidiopsis* and Pleurocapsales strains a search for operational taxonomic units (OTU) was performed. One OTU was defined as a group of sequences at the threshold of 97% similarity to each other and to allow discrimination between different bacterial species (Stackebrandt & Goebel 1994; Schloss & Handelsman 2005).

Overall the analysis of 37 *Chroococcidiopsis* and Pleurocapsales strains revealed 27 OTUs (Table 3.5, Table A4 appendix). 13 OTUs belonged to the genus *Chroococcidiopsis*, 13 OTUs belonged to the order Pleurocapsales and one OTU was the outgroup taxon *Gloeobacter violaceus* PCC 7421.

At a threshold of 97% of similarity, six OTUs were observed containing more than one strain (fig. 3.17). The remaining *Chroococcidiopsis* and Pleurocapsales strains each formed a single OTU. The strains *Chroococcidiopsis* sp. BB 84.1, *C*. PCC 7431, *C*. cf. c*ubana* CCALA 045, *C*. PCC 7203 and *C*. *thermalis* BB 82.2 grouped into OTU Chr13, which was stable up to a threshold of 98% sequence similarity and could be observed in several other phylogenetic trees of this study (compare Table 3.5). The only stable OTU up to a threshold of 99% of similarity in the genus *Chroococcidiopsis* was OTU Chr12 (Table A4 appendix). Within the Pleurocapsales, *Pleurocapsa* PCC 7314 and *Myxosarcina* PCC 7312 formed OTU Pleu07, which was stable up to a threshold 99% sequence similarity (Table A4 appendix).

Comparing the results of the phylogenetic analysis of the multigene data set (fig. 3.15) and the OTU (fig. 3.17) search resulted in two arrangements in groups: one which is in accordance with both methods and one which is in conflict with the methods (Table 3.5). The first group consisted of strains united in one OTU, which were already together in a distinctive and well supported clade (Table 3.5, fig. 3.15 & 3.17). For example, *Chroococcidiopsis* BB 82.1 and *C.* sp. BB 82.3 clustered in the multigene analysis in clade B (fig. 3.15) and in the similarity analysis into the OTU Chr12 (Table 3.5). To the second group contained strains, which fell together in one supported clade during phylogenetic analyses and in the similarity analysis were resolved into single OTUs, e.g. clade E (fig. 3.15) with *Chroococcidiopsis* CCMEE 10 and *C*. CCMEE 140, were split into Chr10 and Chr11 (Table 3.5).

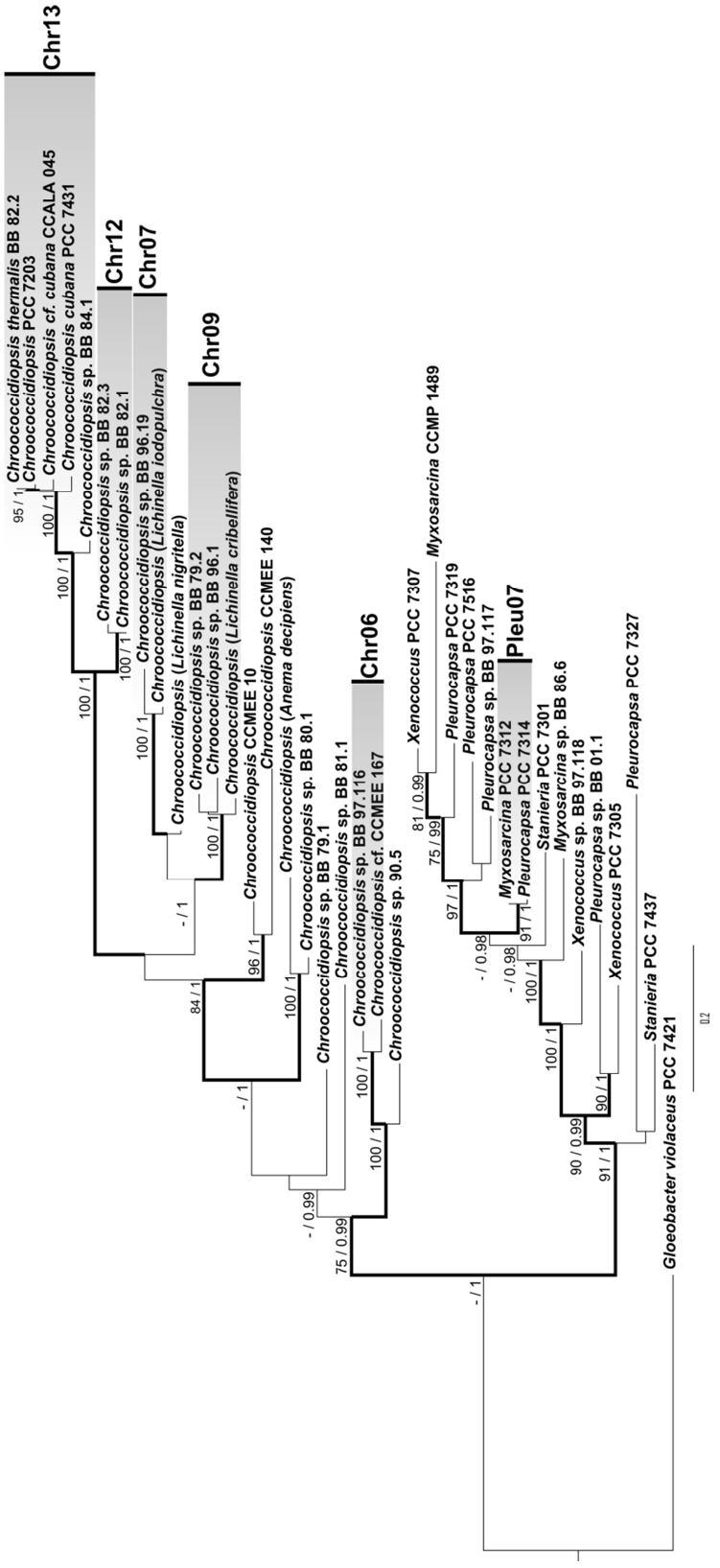


Fig. 3.17: OTUs which included more than one strain (grey boxes) based on 16S rRNA sequences of *Chroococcidiopsis* and Pleurocapsales. One OTU was defined on 97% similarity. Bootstrap values are indicated for ≥75% and posterior probability ≥0.95. Values below are indicated by "-". Branches which were supported by both methods are indicated in thick lines.

Table 3.5: Summary of the labels from the phylogenetic analysis of the concatenated data set (16S rRNA, *rpoC1* and *gyrB* genes; fig. 3.15) and the OTU analysis (fig. 3.17) for *Chroococcidiopsis* strains.

Strain name	Label in phylogenetic analysis	Label in OTU analysis
Chroococcidiopsis cubana PCC 7431	A	Chr13
Chroococcidiopsis cubana CCALA 045		
Chroococcidiopsis thermalis BB 82.2		
Chroococcidiopsis PCC 7203		
Chroococcidiopsis BB 82.1	B	Chr12
Chroococcidiopsis sp. BB 82.3		
Chroococcidiopsis (Lichinella cribellifera)	C	Chr09
Chroococcidiopsis sp. BB 96.1		
Chroococcidiopsis sp. BB 79.2		
Chroococcidiopsis (Lichinella nigritella)	D	Chr08
Chroococcidiopsis cf. BB 96.19		Chr07
Chroococcidiopsis (Lichinella iodopulchra)		
Chroococcidiopsis CCMEE 10	E	Chr10
Chroococcidiopsis CCMEE 140		Chr11
Chroococcidiopsis sp. BB 90.5	F	Chr05
Chroococcidiopsis cf. CCMEE 167		Chr06
Chroococcidiopsis sp. BB 97.116		
Chroococcidiopsis BB 79.1	G	Chr04
Chroococcidiopsis sp. BB 81.1		Chr03
Chroococcidiopsis sp. BB 80.1		Chr02
Chroococcidiopsis (Anema decipiens)		Chr01

4. Discussion

4.1 Discussion Morphology

The thylakoid arrangement is considered to be most important feature of the inner cell structures usable for the taxonomic classification (Komárek & Anagnostidis 1999). In fact, literature about the thylakoid arrangement in the cyanobacteria is most frequently restricted to orders (or subsection in the bacteriological systematic), and in decreasing frequency to genera and single taxa (e.g. Komárek & Caslavska 1991; Komárek & Anagnostidis 1999; Komárek & Anagnostidis 2005). Furthermore, information for a certain order does not necessarily imply information about all the families, genera and taxa it encompasses. Additionally, the competition and ongoing revision of the botanical and bacteriological classification systems make it more challenging.

This present study provided new insights into the thylakoid arrangement by giving information for 66 cyanobacteria strains, with emphasis on the genus *Chroococcidiopsis*, the orders Pleurocapsales and Nostocales. Three basic pattern (parietal, radial and coiled) and two orientations (throughout the cell, cell wall bound) were found. Additionally, this study undertook a statistical test of the thylakoid arrangement of several taxa.

4.1.1 Basic pattern of thylakoids

Thylakoids are not randomly distributed in the cyanobacterial cell, but show various distribution patterns. Three basic patterns in different systematic groups are described and consist with the following terms: parietal, radial and coiled (Lang & Whitton 1973; Komárek & Anagnostidis 1986; Komárek & Anagnostidis 1989; Anagnostidis & Komárek 1999). In the parietal arrangement the thylakoids are arranged along the cytoplasmatic membrane, in the radial orientation they are arranged towards the cell centre and in the coiled distribution they are irregular distributed. The herein observed parietal and coiled patterns are consistent with the descriptions reported in the literature. However, a radial orientation was not found in the LT-SEM investigated strains; instead, fascicles were found. Fascicles consist of short and more or less stacked thylakoids for which no special orientation in the cells could be observed. The formation of fascicles in a radial or peripheral arrangement in the cell has been described by Komárek & Anagnostidis (1999).

Besides the three basic patterns described in the literature, two major orientations of the thylakoids have been observed in this study. The thylakoids were either bound to the cytoplasmic membrane or filled the complete cell; both orientations were observed for

each arrangement. Such distributions have been described before (e.g. Lang & Whitton 1973; Komárek & Anagnostidis 1986; Komárek and Anagnostidis 1999). The reasons for different orientations are unknown. At least in one case an external influence might be the cause. A peripheral distribution of stacked thylakoids was observed in one of cell of *Xenococcus* PCC 7305 (Waterbury 1976). This cell was infected by cyanophages, located in the centre of the cell and caused the peripheral orientation (Waterbury 1976). Even the dominating orientation in all systematic groups was throughout the cell, the low number of strains from single orders or/and genera makes it difficult to make general statements regarding to the usefulness of this feature for taxonomical classification purposes.

4.1.2 Thylakoids in the genus *Chroococcidiopsis*

The genus *Chroococcidiopsis* sensu Geitler (1933) was described as having irregular (=coiled) thylakoids which were distributed throughout the whole cell (Komárek & Anagnostidis 1999). The present extended investigation revealed two completely new and unknown arrangements for this genus. The parietal and stacked arrangements have been found several times. This underlines the importance of a broad survey of strains from one genus to be sure which arrangements are present or not. Actually only a very limited number of pictures of the inner structure of *Chroococcidiopsis* cells have been published (Waterbury & Stanier 1978; Komárek & Anagnostidis 1999; Büdel & Kauff 2012). Therefore, the results of this study expand the knowledge about the thylakoid arrangement for this genus by more than 400%.

My analysis not only revealed clearly separated arrangements, but also intermediate forms that were observed in three cases. Such an unclear picture between parietal and coiled arrangement can be seen in a freeze etched TEM picture of *Chroococcidiopsis* sp. strain BB 80.2 (B. Büdel, pers. comm.) in Büdel & Kauff (2012). Another intermediate form was found in two strains (*C.* sp. BB 79.2 and *C.* PCC 7432), where the arrangement was varying between parietal and stacked. Interestingly, bigger cells of strain *Chroococcidiopsis* PCC 7436 seems to have a parietal arrangement, whereas in smaller cells the thylakoids were stacked (Waterbury & Stanier 1978). Such differences between young and old grown cells can be observed in other orders, too, e.g. *Pleurocapsa* sp. CCALA 1126 (Pinevich et al. 2008) and *Gloeotrichia* sp. (Miller & Lang 1971). Furthermore, in strains from Büdel & Kauff (2012) and Waterbury & Stanier (1978) the intermediate form appeared not only in one single separated cell, but also in cells which

were originated by the same mother cell sharing one sheath. Thus, the number and arrangement of the thylakoids seem to vary according to the stage of development of the cell. Younger cells of *Chroococcidiopsis* have parietal arranged and older cells have coiled or stacked thylakoids. This explains the existence of intermediate forms. One could interpret the parietal arrangement as a longer version of stacked thylakoids, because in both cases the membrane layers are arranged in parallel form, in contrast to the coiled arrangement. However, as long as no time series of dividing and developing cells are investigated, the relations between different arrangements are theoretical.

4.1.3 Thylakoids in other systematic groups of cyanobacteria

Waterbury & Stanier (1978) described for the pleurocapsalean cyanobacteria the thylakoids as dispersed throughout the cytoplasm in irregular parallel groups. The results of this actual study showed such a stacked arrangement and confirm the literature. Only one strain differs from that pattern, with a parietal arrangement. It is unclear, if the different arrangement was caused by environmental influence or the result of developmental stage or if the genus *Xenococcus* has more than one arrangement. This result clearly supports the need for additional studies in more strains to more fully resolve the structure.

According to the literature, the thylakoid arrangement in the subclass Nostocophycidae (Hoffmann et al. 2005) seems to be uniform having a coiled arrangement (Komárek & Anagnostidis 1989; Anagnostidis & Komárek 1990). The results of this study confirmed the coiled arrangement for the investigated genera. For several genera the thylakoid arrangement could be described for the first time, such as in *Aphanizomenon*, *Cylindrospermum*, *Mojavia*, *Petalonema*, and *Scytonema,* all had coiled arrangements, thereby supporting the trend in the literature about the complex filamentous cyanobacteria.

The order Chroococcales sensu Komárek & Anagnostidis (1999) contains all baeocyte producing cyanobacteria and five different thylakoid arrangements have been described for this order. This general statement about this order is redundant if it is compared with the systematic of Büdel & Kauff (2012), because several genera changed the orders or even the subfamily. If the left two examined chroococalean sensu Komárek & Anagnostidis (1999) genera *Chroococcus* and *Cyanothece* are considered, only *Cyanothece* with an irregular to radial arrangement is consistent with the literature (Komárek & Anagnostidis 1999). The arrangement in *Chroococcus* was described by

Potts and colleagues (1983) as tightly appressed and cell filling in a more or less random arrangement. This is consistent with the results for *Chroococcus* sp. CCALA 057 herein (coiled and throughout the cell spread thylakoids). A coiled arrangement was not described by Komárek & Anagnostidis (1999).

The thylakoids of the genera *Geitlerinema* and *Pseudanabaena*, former members of the Oscillatoriales sensu Anagnostidis & Komárek (1988), and now part of the Pseudanabaenales sensu Büdel & Kauff (2012) agree with the literature. Together with the genus *Wilmottia* (Strunecky et al. 2011), the Pseudanabaenales sensu Büdel & Kauff (2012) show a consistent picture with parietal thylakoids.

4.1.4 Thylakoid arrangements as a potential feature for morphological identification of cyanobacteria

There is a strong relationship between the group assignment of cyanobacteria and their thylakoid arrangements (all taxa C_{corr} = 0.852). Hence, it is in general possible to conclude from this certain phenotypic character the affiliation to a certain family, order or genus.

The coiled arrangement seems to be a shared feature of the family Chroococcidiopsidaceae and the order Nostocales. Thus, the phylogenetic close relationship of these two groups is to some extent reflected in the thylakoid arrangements. However, the genus *Chroococcidiopsis* also shares thylakoid arrangements with other orders that are not closely related as the former two, e.g. parietal arrangement in *Chroococcidiopsis* and Pleurocapsales. Consequently, thylakoid arrangements do not completely reflect evolutionary relationships. In this study, Chroococcidiopsidaceae and Nostocales on the one hand and Chroococcidiopsidaceae and Pleurocapsales on the other hand could be told apart by thylakoid arrangement to the same extent (C_{corr} = 0.733 versus C_{corr} = 0.741). This suggests that phylogenetic relationships cannot be derived from thylakoid arrangements only. However, especially in the latter case, sampling numbers were very low (n = 25) considering the group variability. Therefore, it is likely that differences between Chroococcidiopsidaceae and Pleurocapsales will increase at greater sampling rates and might better reflect the phylogenetic distance.

With a known thylakoid arrangement it is possible to rule out some orders or families, e.g. the coiled arrangement is a shared feature of the genus *Chroococcidiopsis* and the order

Nostocales, but not of *Chroococcidiopsis* and the order Pleurocapsales. Or at lower taxonomic ranks such as genera, e.g. a parietal arrangement can be only found in the genus *Xenococcus*, but not in other genera of the order Pleurocapsales.

Making conclusions to the evolutionary relations between single thylakoid arrangements are premature and daring (compare Komárek & Kastovsky 2003; Hoffmann et al. 2005), unless all taxonomic ranks have been examined.

4.1.5 Special observations in the arrangement of thylakoids

Thylakoids can show variations in their arrangement and structure due to environmental factors, cell type, life cycle and the species (e.g. Fogg et al. 1973; Lang & Whitton 1973; Kunkel 1984; Cmiech et al. 1986; Komárek & Anagnostidis 1999). One deviation is, for example, widening and fingerlike structures, which appear in non-healthy cells (Castenholz 2001). Such fingerlike structures have been observed in single cells of *Chroococcidiopsis* BB 79.1 and *C.* sp. BB 96.1b. The net-like structures in single cells in *Trichormus variabilis* CCALA 205, *Chroococcus* sp. CCALA 057, *Chroococcidiopsis* BB 82.1b and *Nostoc* sp. BB 89.12 could be an extreme form of the tubular thylakoids. These structures showed a clear double layer membrane and had the lattice structure of stacked tubes. Lattice structures have been observed in aged *Anabaena* sp. cells (Lang & Rae 1967) and heterocytes of *Trichormus variabilis* (Smarda & Hindák 2005). In contrast to those patterns, which occupy only small areas of the cells, the net-like structures in previously mentioned strains always fill larger parts of the cell. The question is whether such a lattice structure is caused by age, or is an artifact, remains open.

Another special observation was single membrane connections. These connections between have been described for several cyanobacterial strains (Lang & Whitton 1973; Nevo et a. 2007; Liberton et al. 2011). Such fusions or bridges of thylakoids result in a complex 3-D network similar to higher plants chloroplast structures and thus avoid numerous completely independent lumenal areas, which would be a challenge for translocation of metabolic products.

A third special observation was holes in the membranes. These have also been described in literature (Nevo et al. 2007; Liberton et al. 2011) and are known to an intracellular and intralumenal transport of metabolites and cell compounds. Such perforations within the thylakoid membranes are especially necessary in cells where thylakoids are distributed

throughout the whole cell and fill these almost completely. While Nevo et al. (2007) and Liberton et al. (2011) observed single holes in cyanobacteria which had parallel arranged thylakoids, the holes in the strains *Mojavia pulchra* CCALA 691, *Tolypothrix* sp. BB 97.26 and *Tolypotrix distorta var. penicillata* (Ag.) Lemm. BB 97.17 studied here were much more numerous and regularly distributed. These holes are not just single perforations, which allow a transport from one side of stromata to the other. In this special case the holes form a complex sponge like system, which has never been described before. Simultaneously, densely packed thylakoids and the perforation within them, allow an effective use of the limited space while ensuring the transport within the cell is maintained. The ability for fusions and holes in thylakoid membrane might be a requirement in forming the complex networks as seen in the above mentioned strains. While changes in number and location of glycogen and cyanophycin granules appear during different times in a dark-light-cycle (Liberton et al. 2011) and with varying age (Miller & Lang 1971), the question of how and if holes are responding to environmental changes remains open.

A fourth special observation of thylakoid arrangements are found on heterocytes. Such a heterocyte was observed in *Stigonema ocellatum* Thuret BB 97.103. This single cell had a thickened cytoplasmic membrane, to prevent the diffusion of oxygen from the surrounding environment into the heterocytes, which would inactivate the main enzyme (nitrogenase) of the nitrogen fixation process. It is known that a multi-layered cell envelope can be observed in desiccated (Caiola et al. 1996) or nitrogen starving cells (Billi & Caiola 1996). It is unlikely that this special cell was stressed, because all other cells of this culture showed no stress symptoms. Furthermore, the majority of the cells had a coiled thylakoid structure. In contrast, in this special cell two centric whorls are formed at one pole of the cell. The forming of whorls at one pole of a cell has been observed for *Anabaena cylindrica* and *A. azollae* heterocytes (Lang & Whitton 1973). Further evidence is related to the intercalary position of the heterocyte in the filament of *Stigonema ocellatum* Thuret BB 97.103, which is characteristic for this genus (Hoffmann 1991; Komárek et al. 2003).

4.1.6 Conclusions

This study provided new insights into thylakoid arrangements in cyanobacteria strains of several systematic groups; some of them are previously undescribed and unknown. The examination of a high number of *Chroococcidiopsis* strains revealed two undescribed thylakoid arrangements for this genus. This shows that using a limited number of observations might lead to a false assumption for an entire genus. In any case, it is necessary to investigate a larger number of strains of one genus, as well all representatives of all genera of a family. This is challenging, because not all described taxonomical ranks are available in culture collections. It is crucial to study these morphological characteristics under controlled culture conditions to allow for synchronization between cells of the same growth stage. After the investigation of lower systematically ranks, one might make conclusions about the thylakoid arrangement in higher ranks such as orders.

The here presented results of a statistical analysis of a broad spectra of cyanobacteria suggested a correlation between thylakoid arrangement and affiliation to systematic groups. Nevertheless, it is important to consider the thylakoid arrangement not as an isolated feature for taxonomic classification, but as a guide to be used in conjunction with other key morphological characteristics, as well as molecular derived taxonomic information.

The technique of the low-temperature scanning electron microscopy allows visualization of the interior of the cyanobacterial cells in 3-D, while a transmission electron microscopy provides only a 2-D view, due to the random cutting into ultrathin slices. The spatial view allows new insights on the arrangement and orientation of thylakoid membranes and a better understanding of their complex arrangements. The great advantage of the LT-SEM is that the samples need no chemical preparation, thereby minimizing artifacts. A use of different fixatives can lead to different results in the appearance of some cell inclusions (compare Hoare et al. 1971). Furthermore, the LT-SEM provides the picture immediately and requires neither reconstruction nor image analysis.

4.2 Discussion Phylogeny

4.2.1 Evolutionary relationships of the genus *Chroococcidiopsis*

4.2.1.1 Single gene analysis of the 16S rRNA, *rpoC1* and *gyrB* gene

The 16S rRNA analysis showed the separation of the genus *Chroococcidiopsis* from the order Pleurocapsales sensu Waterbury & Stanier (1978). Furthermore, *Chroococcidiopsis* was found to be the sister group of the order Nostocales. These results confirm previous phylogenetic studies based on this gene (Rudi et al. 1997; Turner et al. 1999; Garcia-Pichel et al. 1998; Ishida et al. 2001; Wilmotte & Herdman 2001; Fewer et al. 2002; Seo & Yokota 2003; Schirrmeister et al. 2011; Schirrmeister et al. 2013). By extending the analysis to a larger number of *Chroococcidiopsis* strains, I identified two new sub-clades (C1 and C2, fig. 3.9) within the genus. These sub-clades may originate from two oscillatorian strains within the *Chroococcidiopsis* clade. The bigger sub-clade (C2) contains *Chroococcidiopsis* PCC 7203, which is the reference strain for the form-genus *Chroococcidiopsis* in the bacteriological system (Rippka et al. 2001b), so that it can be assumed that all other strains which are included in this sub-clade are true *Chroococcidiopsis* strains.

The reason for a clustering of two oscillatorian strains within one *Chroococcidiopsis* could be explained by the reconstruction of the alignment, which is a critical step in phylogenetic analyses, and which might eliminate differences and lead to highly similar sequences between the *Chroococcidiopsis* and the two oscillatorian strains. This makes sense in the context where the identity, as oscillatorian taxa, was confirmed by microscopy and a BLAST search of the sequences against public databases (data not shown), which may lead to misidentification problems. Interestingly, in a phylogenetic tree of 16S rRNA of Bahl et al. (2011) two oscillatorian strains (*Microcoleus* sp. and *Oscillatoria kawamurae*) were found within hot and cold habitat originated *Chroococcidiopsis* strains. However, the polyphyly of the order Oscillatoriales and some of their families is supported by several single and multigene analyses (Wilmotte 1994; Ishida et al. 2001; Marquardt & Palinska 2007; Palinska & Marquardt 2008; Engene et al. 2010; Thomazeau et al. 2010).

The results of the present analysis showed the monophyly of the order Nostocales, which is also supported by 16S rRNA analyses of several others studies (e.g. Giovannoni et al. 1988; Turner 1997; Castenholz 2001; Wilmotte & Herdman 2001; Fewer et al. 2002; Seo & Yokota 2003).

The 16S rRNA belongs to the core genome of cyanobacteria (Shi & Falkowski 2008) and has a highly functional importance, related to the translation process of the cells. This results in the 16S rRNA being less affected by horizontal gene transfer. Being such an essential part of the genome, means that the gene must be highly conserved. The highly conserved nature was confirmed for other bacteria (Fox et al. 1992; Stackebrandt & Goebel 1994), as well as for cyanobacteria (Schirrmeister et al. 2012). This implies that the 16S rRNA may be not sufficient for the analysis of closely related strains. Nevertheless, the comparison between the clustering of the higher systematic ranks and a comprehensive genome analysis of 126 cyanobacteria strains (Shih et al. 2013) and 16S rRNA analyses (e.g. Honda et al. 1999; Turner et al. 1999; this study), showed a high similarity of the main clades. An alternative to the 16S rRNA could be to work with complete genomes, but their numbers have just recently started to grow (Shih et al. 2013). Therefore, they are still limited and far away from covering the entire diversity in cyanobacterial morphology and evolution. In contrast, the 16S rRNA sequences cover a broad range of the Cyanobacteria biodiversity and have been widely used in phylogenetic analyses (e.g. Giovannoni et al. 1988; Wilmotte 1994; Turner 1997; Honda et al. 1999; Taton et al. 2003; Schirrmeister et al. 2011).

The *rpoC1* gene analysis of the present study did not show complete separation of pleurocapsalean strains from the *Chroococcidiopsis*. Surprisingly, two strains of the Pleurocapsales clustered within the *Chroococcidiopsis* and Nostocales group. These results contradict the results of Seo & Yokota (2003), whose phylogenetic reconstruction based on *rpoC1* gene sequences showed a separation of *Chroococcidiopsis* PCC 7431 and pleurocapsalean strains.

Still it is not clear if *rpoC1* is a useful marker in phylogenetic analysis of cyanobacteria. Phylogenetic analyses based on this gene were not able to distinguish *Cylindrospermopsis raciborskii* strains from different localities (e.g. Wilson et al. 2000; Gugger et al. 2005; Haande et al. 2008; Wu et al. 2011), the same applies for different species of the *Nostoc* genus (Han et al. 2009) and the *Synechococcus* genus (Dall´Agnol et al. 2012). In contrast, closely related *Planktothrix* (Lin et al. 2010) and *Lyngbya* species (Engene et al. 2010) could be distinguished with this gene.

The phylogenetic signal of the *rpoC1* gene obtained herein by the likelihood mapping method (Strimmer & von Haeseler 1997) was below those of the 16S rRNA and their possible combinations. However, the proportion of the noise is far below 20-30% and suggests the data are not reliable for phylogenetic inference (Schmidt &

von Haeseler 2009). Nevertheless, the gene showed synonymous substitutions at the third-codon position and signs of homoplasy, suggesting that this gene provides limited cladistic signal and causes incongruence between 16S rRNA and *rpoC1* gene (Han et al. 2009). Contrasting results can be found in Rantala et al. (2004), where the *rpoC1* gene gave the same tree topology as the 16S rRNA. Rantala et al. (2004) suggested that such a congruent topology was caused by limited taxon sampling. The reason for the contradictory topology between Seo & Yokota (2003) and the present study is unclear although the limited taxon sampling in the former may play a role for the incongruence. Furthermore, Seo & Yokota (2003) used the distance based neighbour-joining method for the reconstruction of the phylogenetic tree, whereas this present study used the Maximum Likelihood and Bayesian inference method. It is known that conflicting phylogenies of cyanobacteria can be a result of artifacts of phylogenetic reconstruction (Shi & Falkowski 2008). However, while the causes for the contradictory results remain vague at the intra-generic level, this gene should be used with care in phylogenetic analysis (Han et al. 2009). For this present study this means, that phylogenetic analysis should be combined with other loci. This corroborates with the highest phylogenetic signal, which was given by the likelihood mapping method for the combination of the *rpoC1* gene and 16S rRNA.

The phylogenetic tree based on the *gyrB* gene of the present study confirms the separation of the genus *Chroococcidiopsis* and the order Pleurocapsales. *Chroococcidiopsis* strains clustered together with members of the order Nostocales and confirmed the results of Seo & Yokota (2003). The node, which separates *Chroococcidiopsis* and Nostocales from the rest of the Cyanobacteria, is the only deep node that was supported by the Maximum Likelihood and Bayesian inference methods. The trend of low or no statistical support for most of the nodes was already observed in the study of Seo & Yokota (2003).

The *Chroococcidiopsis* strains were not clearly separated from the nostocalean strains, but found within the middle. This is only supported by the Bayesian inference method and might be owed to the limited number of *gyrB* sequences. Such a clustering was not observed in the *gyrB* gene tree of Seo & Yokota (2003).

The *gyrB* gene has been previously used in a limited number of studies for inferring phylogenies in cyanobacteria (e.g. Seo & Yokota 2003; Tanabe et al. 2007; Sciuto et al. 2011). The gene seems to have a limited phylogenetic signal, reflected by the lack of support in phylogenetic trees (this study; Seo & Yokota 2003). Additionally,

limited phylogenetic signal for cyanobacteria is supported by the lower percentage of fully resolved quartets in the likelihood-mapping in comparison with e.g. 16S rRNA (this study; Sciuto et al. 2011). This may be explained by the high degree of conservation at sequence level of the *rpoC1* gene (Seo & Yokota 2003). Due to this and the lack of a phylogenetic signal, an unlimited recommendation given for the usability in phylogenies of cyanobacteria by Seo & Yokota (2003) must be seen critically. Nevertheless, this gene has proved to be a useful tool in phylogenetic analysis of other bacteria, e.g. Actinobacteria (Yamamoto & Harayama 1996), *Micromonospora* (Actinobacteria; Kasai et al. 2000) and *Flavobacteria* (Bacteroidetes; Peeters & Willems 2011). To resolve the issue of the usability off the phylogeny of cyanobacteria further sequences are needed.

Despite the problems with the phylogenetic analysis of this gene, there was a more methodological problem to get new sequences using the primer pair GB/3MF and GB/CR-2 (Seo & Yokota 2003). Seo & Yokota (2003) designed the forward primer for the *gyrB* gene from gene sequences of *Escherichia coli* K-12, *Bacillus subtilis*, and *Synechocystis* sp. PCC 6803. The reverse primer was designed from *Synechocystis* sp. PCC 6803, *Escherichia coli* K-12, *Bacillus subtilis*, *Micromonospora olivasterospora* IFO14304 and *Micromonospora carbonacea* IFO14107. The Actinobacteria *Bacillus subtilis* and both *Micormonospora* species are ubiquitous in the natural environment, occurring in soil and water. Including such common bacteria in the primer design might have led to unspecific primers, which might be especially problematic in the usage of non-axenic cyanobacteria cultures. Other primers like *gyrF* and *gyrR* (Tanabe et al. 2007) designed by using sequences of *Microcystis aeruginosa* could be limited on this genus and hence not be useful in a broader spectrum of cyanobacteria. The future design of new primers should be based upon an improved coverage of cyanobacteria.

By comparing the single gene trees, the phylogenetic tree based on *rpoC1* gene resulted in a different topology than the 16S rRNA and *gyrB* gene tree, with the latter two confirming each other. Both genes showed the separation of the genus *Chroococcidiopsis* from the order Pleurocapsales sensu Waterbury & Stanier (1978). Additionally, the sister group relationship of the genus *Chroococcidiopsis* and the order Nostocales was confirmed (Turner 1997; Fewer et al. 2002). The exact position of the genus *Chroococcidiopsis* is still unclear. While the analysis of the 16S rRNA gene of the present study placed it basal to the Nostocales, the study of Fewer et al. (2002) suggested a reverse position. These results are in contrast to the *rpoC1* and *gyrB* gene analysis of the

present study, which showed no separation of the genus *Chroococcidiopsis* and the order Nostocales. Furthermore the single markers reflects only small parts of the entire genomes and hence the evolution of the organisms. Thus the combination of the three loci should reveal new insights, by alleviating problems due to the lack of phylogenetic signal and avoiding false signals from possible horizontal gene transfer (Suchard 2005).

4.2.1.2 Multigene analysis of the 16S rRNA, *rpoC1* and *gyrB* gene

This study performed a phylogenetic analysis using combinations of the previous used single genes. The combined data sets showed the separation of the genus *Chroococcidiopsis* and the order Pleurocapsales sensu Waterbury & Stanier (1978). Furthermore, the results showed a sister group relationship of the genus *Chroococcidiopsis* and the order Nostocales, with *Chroococcidiopsis* basal to the Nostocales. Therefore the result of the present study confirmed the single gene analyses of previous studies (Rudi et al. 1997; Turner et al. 1999; Garcia-Pichel et al. 1998; Ishida et al. 2001; Wilmotte & Herdman 2001; Fewer et al. 2002; Seo & Yokota 2003; Schirrmeister et al. 2011; Schirrmeister et al. 2013). The basal position of the genus *Chroococcidiopsis* contrasts with Fewer et al. (2002), where the opposite was the case. Recently, the basal position and sister group relationship was confirmed by an analysis of 126 cyanobacterial genomes, in which *Chroococcidiopsis* PCC 7203, the type strain for *Chroococcidiopsis* in the bacteriological system (Rippka et al. 2001b), clustered basal towards the Nostocales (Shih et al. 2013). All together this justifies the familia nova Chroococcidiopsidaceae Geitler ex Büdel, Donner & Kauff (Büdel & Kauff 2012).

The classification of the bacteriological and botanical systems (e.g. Rippka et al 1979; Komárek & Anagnostidis 1999; Rippka et al. 2001c) based on the assumption that the reproduction mode multiple fission and baeocyte formation evolved only once in the Cyanobacteria, is not supported by this study. The results from this work, multigene trees as well single trees, suggest the opposite. By the clustering of strains with these characteristics into different groups, it is likely that baeocytes have arisen more than once in the evolution of the Cyanobacteria. The high morphological similarity between *Chroococcidiopsis* and *Myxosarcina* seems to be a result of convergent evolution (Fewer et al. 2002). The only way to distinguish both is the motility of baeocytes in *Myxosarcina* and the immobility of those in *Chroococcidiopsis* (Waterbury & Stanier 1978).

The comparison of the statistical support of the phylogenetic tree based on 16S rRNA and the combined data set show a small increase in number and support of internal nodes. In fact, the likelihood-mapping of the two different data sets suggest a slightly lower phylogenetic signal for the combined alignment including the three gene sequences, than for the 16S rRNA only. The opposite was observed in the study of Sciuto et al. (2011), where the 16S rRNA alone gave a lower phylogenetic signal. However, even the combination of gene sequences in the present study didn´t substantially increase the support of internal nodes; the general possibility of horizontal gene transfer and resulting false signal was reduced by the use of more than one gene (Suchard 2005). Furthermore, all three genes have a high functional importance for the cells. Hence they have a high degree of conservation, which makes it quite unlikely of being horizontally rather than vertically transferred.

The results of the single gene analysis of the *rpoC1* gene gave a deviate picture from the other two single gene analyses. However, the result of the multigene analysis shows that the signal of the *rpoC1* gene did not overcome the signal of the other. This shows that a phylogenetic reconstruction in the Cyanobacteria should never rely on a single gene only, but instead multigene approaches should be applied. Until now, only a very limited number of studies used more than one gene (e.g. Robertson et al. 2001; Seo & Yokota 2003). Even if these studies utilized more than one gene a combination of the several genes into one concatenated data set were not (Robertson et al. 2001) or only partly included (Seo & Yokota 2003). Studies which performed real multilocus sequence are restricted on single genera such as *Microcystis* (Tanabe et al. 2007) or *Cylindrospermopsis* (Wu et al. 2011).

Most molecular markers are used to infer phylogenetic relations in bacteria, generally speaking, and cyanobacteria, specifically, because for historic reasons and their simplistic application. However, only very few studies investigated the suitability of these markers on a large taxonomic scale, including evolutionary behaviour (e.g. Han et al. 2009). To overcome the obstacles of unknown reliability of single markers and the still limited coverage of genomes, the utilization of a robust set of genes, detected by a phylogenomic approach as presented by Capella-Gutierrez (2012) may be promising.

4.2.2 Diversity of the genus *Chroococcidiopsis*

4.2.2.1 Genetic entities

There are 14 species described for the genus *Chroococcidiopsis* Geitler (1933), with an additional six species unrevised and their systematic assignment is unresolved (Komárek & Hauer 2013). Because of difficulties in the discrimination of single species, most identifications in literature end at the genus level (compare to fig.1.2; 58 of 90 entries have been identified as "*Chroococcidiopsis* sp."). Additionally, the morphology of single cells and cell aggregates of the genus *Chroococcidiopsis* are highly similar to the genus *Myxosarcina*. However, the separation of the genera *Chroococcidiopsis* and *Myxosarcina* were clearly demonstrated by phylogenetic analyses (e.g. Fewer et al. 2002; this study).

In the present study, 22 *Chroococcidiopsis* strains were used for phylogenetic analysis, of which only three have been identified at species level. In the multigene phylogenetic analyses seven distinctive clades have been observed (clade A–G; fig. 3.15). Partly similar clustering of the strains has been observed in the single gene analysis and the different arrangements of the sequences. Interestingly, the only strains which have been identified to the species level (BB 82.2, CCALA 045 and PCC 7431, as *C. thermalis* and *C. cubana*, respectively), form a constant clade across several trees in this study (e.g. fig. 3.15, clade A). Although these strains have been identified as two different species, the phylogenetic analysis showed almost no evolutionary distances, which has been already observed by Fewer et al. (2002). This suggests that *Chroococcidiopsis thermalis* and *C. cubana* seem to be the same species. The remaining *Chroococcidiopsis* strains were less stable in their relative positions relative to each other. This instability can be explained partially by the reconstruction of the alignments, a critical step in phylogenetic analyses. A multiple sequence alignment aims to identify and put together residues, either amino-acids or nucleotides, with a common evolutionary origin but this not an easy task. Additionally, the different numbers of sequences per strain might have affected the result of the multigene analysis, due to the phylogenetic signal of the different genes.

The approach to estimate the diversity of *Chroococcidiopsis* strains with the similarity of the 16S rRNA sequences revealed 13 different OTUs. By this, the genetic diversity seems to be higher than in the phylogenetic analysis. At a similarity level of 97%, only five OTUs corresponded to more than one strain. Only three OTUs encompassed the same strains, which were already together in distinctive and well supported clades in the phylogenetic analysis (Chr09, Chr12 and Chr13). One of these multi-sequence OTUs was Chr13, which

was congruent with clade A in the phylogenetic analysis (fig. 3.15). This OTU implies that the species *Chroococcidiopsis cubana* and *C. thermalis* are highly similar and not distinguishable on the basis of 16S rRNA. Additionally, even if cut-offs for similarity were increased these strains remained in the same OTU.

The OTU-based method uses arbitrary cut-off levels to define OTUs in bacteria (e.g. 97% by Stackebrandt & Goebel 1994), and cyanobacteria, especially (e.g. 97% by Tracy et al. 2010; 97.5% by Taton et al. 2003; 98% by de la Torre 2003). However, that application of increasing cut-off values in the present study increased the number of OTUs for the *Chroococcidiopsis* strains. This implicates a higher number of different genetic entities at a higher degree of similarity. The number of OTUs depends on the other sequences in the data set, because this method is cluster based. Furthermore, at 98% similarity of the 16S rRNA, two ecological different *Prochlorococcus* ecotypes can be detected (Moore et al. 1998). This means, that even at high similarity the true diversity may be underestimated.
The lack of separation of *Chroococcidiopsis cubana* and *C. thermalis* is in strong contrast with differences in the mode of cell divisions and their ecology. *Chroococcidiopsis thermalis* undergoes simultaneous multiple divisions (Komárek & Anagnostidis 1999) and *C. cubana* follows successive multiple divisions (Komárek & Hindák 1975). The former occurs in mineral and thermal springs (Komárek & Anagnostidis 1999) and latter occurs in mineral springs, pools and puddles (Komárek & Hindák 1975). In addition, the phylogenetic analysis resulting from the generation of OTUs based on 16S rRNA similarity (a conserved locus), underestimates the actual phylogenetic diversity that accompanies both their physiological and ecological diversity. This may not be a surprise, because both methods were based on the analysis of highly conserved genes, which may not have contained enough data to discriminate between closely related strains (Fox et al. 1992; Casamatta et al. 2005). Additionally, the OTUs are based on identity only and do not take the phylogenetic information of the gene itself into account (e.g. Martin 2002).

4.2.2.2 Combination of genetic entities with morphological characters

In the present study not only genetic features were estimated, additionally morphological characters such as thylakoid arrangement and cell sizes were taken into account to compare different strains. The comparison of the phylogenetic results with the thylakoid arrangement showed no special pattern for the clustering. In addition, phylogenetically distinct clades A–G (fig. 3.15) as illustrated by the multi-sequence OTUs, had

heterogeneous thylakoid arrangements. Furthermore, the observed intermediate thylakoid arrangements indicate a partial dependence on the life cycle.

The result of the cell size measurements in the present study suggests a discrimination of single strains by this character (e.g. *Chroococcidiopsis* sp. BB 80.1 and *C.* sp. BB 90.5). Although cell size depends on the age of the culture and sheath structure, it seems to be one reliable trait for the morphological discrimination of *Chroococcidiopsis* strains used in this study. The same can be observed for validly described *Chroococcidiopsis* species. Some of them have partially very different cell sizes (e.g. *Chroococcidiopsis kashaii* with ~4.8 µm (Friedmann 1961) and *Chroococcidiopsis bourrellyana* with 9–16 µm (Compère 1998)). Moreover, several herein examined strains can be assembled into groups, in which the strains do not differ from each other (e.g. BB 96.1, BB 81.1 and BB 96.19). This can be observed for some validly described *Chroococcidiopsis* species, as well. For example, *Chroococcidiopsis cubana* has a cell size of 10-13 µm (Komárek & Hindák 1975) and *Chroococcidiopsis mysorensis* of 8–14 µm (Tiwari 1972). However, a discrimination of species cannot be done solely on the feature cell size.

The combination of the phylogenetic distinctive clades A–G (fig. 3.15) and measured cell sizes resulted in no discernible classification pattern. None of the clades had a fixed cell size. A combination of the OTUs and cell sizes revealed a more complex picture. Most of the multi-sequence OTUs encompassed strains with significantly variable cell sizes, the minority encompassed strains with no significant different cell sizes. This shows once again, that the analysis seems to be inappropriate for registration and clarification of the diversity of the *Chroococcidiopsis* strains.

The presented results suggest that several genetic clusters exist in the genus *Chroococcidiopsis*. However, the characters thylakoid arrangement and cell size does not necessarily correspond with these genetic distinguishable strains. This confirms the assumption that the taxonomy of cyanobacteria should not be based solely on morphological characters (Wilmotte 1994). This study clearly illustrated the shortcomings in using either current genetic markers or morphological makers alone in cyanobacteria classification, particularly with respect to classifying *Chroococcidiopsis cubana* and *Chroococcidiopsis thermalis*. It is evident that further research is essential to identify reliable genetic and morphological markers to aid the systematic analysis of the Cyanobacteria systematics, particularly with respect to the genus *Chroococcidiopsis*.

4.2.3 Biogeography of the genus *Chroococcidiopsis*

The results of the phylogenetic analysis of this study suggested that the genus *Chroococcidiopsis* has no biogeographical patterns; instead these results imply close relationships between strains from very distant geographical origins (fig. 3.15 & 3.16). This is in contrast with a recent study, which indicated that *Chroococcidiopsis* variants from different hot and cold deserts around the world are specific to their habitat, as a result of the ancient legacy due to a very early separation of these lineages (Bahl et al. 2011). This means that the distribution of hypolithic *Chroococcidiopsis* taxa is based upon historical events, which leads to the pattern observed today. In contrast to Bahl et al. (2011) and in accordance with this study, Fewer et al. (2002) confirmed the non-existence of biogeographical patterns suggesting close relationships between strains from very distant geographical origins (e.g. Antarctica and Israel; Fewer et al. 2002). However, these results contradict a recent meta-analysis, which found in 86% of phylogeographic analyses actual biogeographic patterns for microorganisms, hence rejecting the EiE-hypothesis (Jenkins et al. 2011).

However, it appears that the non-detection of biogeographical patterns in the examined *Chroococcidiopsis* strains are comparatively related to the genetic loci used, rather than a non-existence of patterns. Bahl et al. (2011) used 16S-ITS-23S rRNA, in contrast Fewer et al. (2002) used 16S rRNA only. Also the single gene analysis of 16S rRNA, as the inclusion of *rpoC1* and *gyrB* gene sequences in this study did not lead to any detection of biogeographical patterns. This suggests that neither 16S rRNA nor *rpoC1* gene nor *do gyrB* gene contain biogeographical information. In fact, there are studies indicating that the choice of genetic marker influences the results in the detection of biogeographic relationships. Cho & Tiedje (2000) used fluorescent *Pseudomonas* strains from soil samples of four continents for analysis three molecular typing methods: 16S rRNA restriction analysis (ARDRA), 16S-23S rRNA intergenic spacer-restriction fragment length polymorphism (ITS-RLFP), and repetitive extragenic palindromic PCR genomic fingerprinting with a BOX primer set (BOX-PCR). One method showed no differences among sites (ARDRA), another showed weak differences among sites (ITS-RFLP) and the third method revealed a high endemicity of genotypes (BOX-PCR). The same influence of a marker can be seen in a study by Fernandez-Carazo et al. (2011) on endemism of cyanobacteria strains from continental Antarctica. In this study the analysis of ITS sequences revealed a higher diversity and endemism than the results of the 16S rRNA marker suggested.

However, the use of ITS sequences for phylogeny may be problematic. It is known that bacteria can have multiple copies of ITS (Cho & Tiedje 2000; Stewart & Cavanaugh 2007), and have cyanobacteria (Lu et al. 1997; Neilan et al, 1997; Iteman et al. 2000; Boyer et al. 2001; Finsinger et al. 2008). Furthermore, these multiple copies can be non-identical (Boyer et al. 2001; Stewart & Cavanaugh 2007), which could cause errors in the analyses (Boyer et al. 2001; Brown et al. 2005). Nevertheless, there are several studies where ITS was successfully used for the discrimination of cyanobacteria on intra- and interspecific levels (e.g. Rocap et al. 2002; Ernst et al. 2003; Brown et al. 2005; Taton et al. 2006; Cadel-Six et al. 2007) and between different geographical distributions (Finsinger et al. 2008; Bruno et al. 2009; Bahl et al. 2011; Fernandez-Carazo et al. 2011).

Chroococcidiopsis occurs not only throughout a wide geographic range, but also in different habitat types, encompassing different life-strategies such as hypolithic, endolithic, free-living and lichenized. The phylogenetic analysis of the present study showed lichenized strains at different positions within the trees, suggesting that lichenization occured multiple times. These results support Fewer et al. (2002). The same is for the other strategies, which occurred in different clades. However, it is noticeable that strains which came from soils (BB 82.2 and PCC 7203), a dried pool (CCALA 045) and a mineral spring (PCC 7431) were basal to the other *Chroococcidiopsis* strains, which had a lichenized or rock associated life-strategy (fig. 3.15 & 3.16). These results may be explained by the limited information for such pattern in the data set, similar to the biogeographic patterns.

This study can neither exclude nor prove the possibility of biogeographic and life-strategy patterns in the genus *Chroococcidiopsis*. Further investigations based on appropriate marker selection (e.g. ITS) may resolve the contrariness of the current results. Future research on biogeographical relationships should continue with the search for mechanisms and testing hypothesis that help to understand the patterns that have already begun to emerge the evolution and ecology of the genus *Chroococcidiopsis*.

4.2.4 The polyphyly of Pleurocapsales

The present study showed a clear separation of *Pleurocapsa* PCC 7327 from the remaining Pleurocapsales in both the single and multigene analysis. While the former use of limited numbers of strains and genera of the order Pleurocapsales sensu Waterbury & Stanier (1978) implied the monophyly of the order (Giovannoni et al. 1988; Wilmotte 1994; Turner 1997; Garcia-Pichel et al. 1998; Bhattacharya et al. 1999; Turner et al. 1999), the present study shows that *Pleurocapsa* PCC 7327 clustered together with strains from the Synechococcophycidae sensu Hoffman et al. (2005) and Oscillatoriophycidae sensu Hoffman et al. (2005). Similar clustering can be seen in the study of Rudi et al. (1997), Ishida et al. (2001) and Seo & Yokota (2003), which proofs the polyphyly of the Pleurocapsales.

My results are supported by a recent comprehensive genome analysis, which showed the same pattern for a set of 126 cyanobacterial genomes (Shih et al. 2013). The *Pleurocapsa* PCC 7327 strain was separated from the other *Pleurocapsa* strains. The used 16S rRNA, *rpoC1* and *gyrB* sequences BLAST (data not shown) to the newly obtained genome of Shih et al. (2013) and by this confirming their identity. Not only the phylogenetic analysis separates *Pleurocapsa* PCC 7327, but also other characteristics such as metabolism, ecology and physiology, differ from remaining Pleurocapsales. For example the analyses of secondary metabolites showed that *Pleurocapsa* PCC 7327 has a pure polyketide synthase (PKS) gene cluster, whereas other pleurocapsalean strains contain mixed gene clusters consisting of two or three PKS gene cluster (Shih e al. 2013). Furthermore, *Pleurocapsa* PCC 7327 came originally from a freshwater hot spring with a temperature of 50°C (Castenholz 1969). In contrast, other members of the genus *Pleurocapsa* were isolated from different marine environments (Waterbury & Stanier 1978; Rippka & Herdman 1992), with moderate water temperatures.

Overall, the results regarding *Pleurocapsa* PCC 7327 can have several reasons. Some authors assumed that up to 50% of strains in culture collections are misidentified (Komárek & Anagnostidis 1989). In this case misidentification seems to be unlikely, because intensive research was done on growth pattern and development of cells by Waterbury & Stanier (1978). These investigations identified this strain unequivocally as a *Pleurocapsa* strain. There is a little chance of confusion with the morphological similar genera *Solentia* and *Hyella* (both Pleurocapsales; Büdel & Kauff 2012). The ecological characters of *Pleurocapsa* PCC 7327 speak against this assumption, because *Pleurocapsa* PCC 7327 growths in mats (Castenholz 1969), whereas the other two

genera, both pseudofilamentous, grow into the substrate (Komárek & Anagnostidis 1999).

The separation of *Pleurocapsa* PCC 7327 from the other *Pleurocapsa* and hence Pleurocapsales is of special interest for this study, because it underlines the hypothesis that multiple fission and baeocyte forming has not only arisen two times, but (at least) three-times during the evolution of cyanobacteria (with the separation of the genus *Chroococcidiopsis* and the Pleurocapsales). This statement is also supported by genome analysis of Shih et al. (2013), where no specific and unique genes underlying morphological complex phenotypes, such as baeocyte formation, were detected.

However, prokaryotes exchanges genetic material by transduction, conjugation and transformation, known as horizontal gene transfer (HGT) (Jain 1999; Koonin et al. 2000; Zhaxybayeva & Gogarten 2002). I suggest that the development of baeocytes happened three times independently and appoint the phylogenetic analysis. Especially the multigene analysis referring to the so-called core genome (Koonin et al. 2000; Shi & Falkowski 2008) together with the analysis at genome-scale of Shih et al. (2013) support this suggestion and show that possibility of HGT in this special case is unlikely. The explanatory power of the manifold confirmed phylogenetic analysis is stronger than the assumption of HGT, although lab experiments showed the possibility of experimental conjugation of plasmids into cyanobacteria (e.g. Sode et al. 1992; Billi et al. 2001) or the transduction of genetic material via viruses is very likely, because cyanophages are very abundant in the natural environment of cyanobacteria (Mann & Clokie 2012).

4.2.5 Conclusions

The phylogenetic trees of the single gene analyses of the 16S rRNA, *rpoC1* and *gyrB* genes and a multigene analysis of the present study clearly revealed the separation of the genus *Chroococcidiopsis* from the order Pleurocapsales. Therefore, the familia nova Chroococcidiopsidaceae Geitler ex Büdel, Donner & Kauff is justified (Büdel & Kauff 2012). The results of the current study indicate that this monogeneric family with the genus *Chroococcidiopsis* is a sister group to the Nostocales (e.g. Fewer et al. 2002; Seo & Yokota 2003).

The comparison of the genetic markers used, suggests various conclusions on their usefulness. Previously the *rpoC1* gene was considered to cause problems on the intra-generic level and incongruences with the 16S rRNA (Han et al. 2009). This was confirmed by a diverging topology from the 16S rRNA tree. Nevertheless, the basic picture, which separates the genus *Chroococcidiopsis* from the order Pleurocapsales, was confirmed. The same was observed from the phylogenetic analysis of the *gyrB* gene. This gene had problems to provide new sequences, which may be related to unspecific primers of Seo & Yokota (2003). In contrast to the single gene analysis of the 16S rRNA, the *rpoC1* and *gyrB* gene analysis did not showed a clear separation of the genus *Chroococcidiopsis* from the order Nostocales (e.g. Fewer et al. 2002; Seo & Yokota 2003).

The combination of the single genes into a concatenated data set confirmed the 16S rRNA analysis. A comparison of the statistical support between the single gene and multigene analysis showed a slightly increase. However, the multigene approach to investigate the phylogenetic relationship of the genus *Chroococcidiopsis* was successfully used in this study. The results underline the hypothesis that the multiple fission and especially the baeocyte formation have arisen several times during the evolution of cyanobacteria.

The diversity of the genus *Chroococcidiopsis* remains unclear. The results of this study suggest the existence of several genetic clusters. A combination of two investigated phenotypic features (thylakoid arrangement and cell size) did not corresponded with these genetic entities. Future research should investigate further features and genetic markers to link between genotypic and phenotypic characters.

The phylogenetic analysis suggested that the genus does not have biogeographical patterns, which is in contrast with a recent study on hypolithic living *Chroococcidiopsis*

strains (Bahl et al. 2011) and the majority of phylogeographic analysis of microorganisms (Jenkins et al. 2011). Only a separation of soil and aquatic–living strains from lichenized and rock associated were observed. This may be related to the genetic markers utilized. Results of previous studies suggest that these markers may not contain biogeographical information, it may be contained in the ITS sequence (e.g. Bahl et al. 2011). Currently this study can neither exclude nor prove the possibility of biogeographic and life-strategy patterns in the genus *Chroococcidiopsis*. To resolve this problem, future research should focus on the investigation of appropriate marker selection.

The investigation on a higher number of pleurocapsalean strains in this study showed in the phylogenetic analyses a clear separation of the strain *Pleurocapsa* PCC 7327 from the remaining Pleurocapsales. Based on these results it can be assumed that the unique and complex formation of baeocytes has evolved at least three times in Cyanobacteria. This clearly shows that a broad range of cyanobacterial strains within various taxonomic ranks should be investigated to reveal the evolution of these organisms.

5. Summary

Cyanobacteria are the only prokaryotes with the ability to conduct oxygenic photosynthesis, therefore having major influence on the evolution of life on earth. Their diverse morphology was traditionally the basis for taxonomy and classification. For example, the genus *Chroococcidiopsis* has been classified within the order Pleurocapsales, based on a unique reproduction modus by baeocytes. Recent phylogenetic results suggested a closer relationship of this genus to the order Nostocales. However, these studies were based mostly on the highly conserved 16S rRNA and a small selection of *Chroococcidiopsis* strains. One aim of this present thesis was to investigate the evolutionary relationships of the genus *Chroococcidiopsis*, the Pleurocapsales and remaining cyanobacteria using 16S rRNA, *rpoC1* and *gyrB* gene. Including the single gene, as the multigene analyses of 97 strains clearly showed a separation of the genus *Chroococcidiopsis* from the Pleurocapsales. Furthermore, a sister relationship between the genus *Chroococcidiopsis* and the order Nostocales was confirmed. Consequently, the monogeneric family Chroococcidiopsidaceae Geitler ex. Büdel, Donner & Kauff familia nova is justified. The phylogenetic analyses also revealed the polyphyly of the remaining Pleurocapsales, due to the fact that the strain *Pleurocapsa* PCC 7327 was always separated from other strains. This is supported by differences in their metabolism, ecology and physiology.

A second aim of this study was to investigate the thylakoid arrangement of *Chroococcidiopsis* and a selection of cyanobacterial strains. The investigation of 13 strains with Low Temperature Scanning Electron Microscopy revealed two unknown thylakoidal arrangements within *Chroococcidiopsis* (parietal and stacked). This result revised the knowledge of the thylakoid arrangement in this genus. Previously, only a coiled arrangement was known for three strains. Based on the data of 66 strains, the feature thylakoid arrangement was tested as a potential feature for morphological identification of cyanobacteria. The results showed a strong relationship between the group assignment of cyanobacteria and their thylakoid arrangements. Hence, it is in general possible to conclude from this certain phenotypic character the affiliation to a particular family, order or genus.

The third aim of this study was to investigate biogeographical patterns of the worldwide distributed genus *Chroococcidiopsis*. The phylogenetic analysis suggested that the genus

do not have biogeographical patterns, which is in contrast with a recent study on hypolithic living *Chroococcidiopsis* strains and the majority of phylogeographic analysis of microorganisms. Further analysis showed no separation of different life-strategies within the genus. These results could be related to the genetic markers utilized, which may not contain biogeographical information. Hence the present study can neither exclude nor prove the possibility of biogeographic and life-strategy patterns in the genus *Chroococcidiopsis*.

Future research should be focused on finding appropriate genetic markers investigate of evolutionary relationships and biogeographical patterns within *Chroococcidiopsis*.

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7. Appendix

Table A1: The worldwide distribution and the habitats of proved *Chroococcidiopsis* species from literature (pages 142-146). Labels are indicating the location in fig.1.2.

Continent	Location	Strategy	Species	Label	Reference
Africa	Kalahari Desert, Botswana	Hypolithic	*C.* sp.	1	Bahl et al. 2011
	Lake Chad, Chad	Aquatic (hypersaline)	*C. cf. thermalis*	2	Iltis 1969
	Djibouti, Djibouti	Aquatic	*C. fissurarum*	3	Silva et al. 1996
	Libyan Desert, Egypt	Hypolithic	*C.* sp.	4	Bahl et al. 2011
	Libyan Desert, Egypt	Hypolithic	*C.* sp.	5	Bahl et al. 2011
	Namib Desert, Namibia	Biological Soil Crust	*C.* sp.	6	Büdel et al. 2009
	Namib Desert, Namibia	Hypolithic	*C.* sp.	7	Büdel & Wessels 1991
	Golden Gate National Park, South Africa	Cryptoendolithic	*C.* sp.	8	Büdel 1999
	Kalahari Highveld, South Africa	Biological Soil Crust	*C.* sp.	9	Büdel et al. 2009
	Karoo Namib, South Africa	Biological Soil Crust	*C.* sp.	10	Büdel et al. 2009
	Langjan Nature Reserve, Limpopo, South Africa	Cryptoendolithic	*C.* sp.	11	Büdel 1999
	North-Transvaal, South Africa	Lichenized	*C.* sp.	12	Fewer et al. 2002
	Transvaal, South Africa	Cryptoendolithic	*C.* sp.	13	Büdel & Wessels 1991; Weber et al. 1996
	Sine & Saloum River, Senegal	Aquatic (brackish)	*C. bourrellyana*	14	Compère 1998
	Zarzis, Tunesia	Biological Soil Crust	*C.* sp.	15	Ullmann & Büdel 2001
America	Bolivian Desert, Bolivia	Hypolithic	*C.* sp.	16	Bahl et al. 2011
	South East Brazil, Mata Atlantica, Brazil	unknown	*C. cubana, C. fissurarum*	17	Werner 2010
	Devon Island, Canada	Hypolithic	*C.* sp.	18	Cockell et al. 2002; Cockell & Stokes 2004; Bahl et al. 2011

Continent	Location	Strategy	Species	Label	Reference
	Cornwallis Island, Canada	Hypolithic	*C.* sp.	19	Cockell & Stokes 2004
	Atacama Desert, Chile	Chasmoendolithic	*C.* sp.	20	Billi et al. 2001
	Atacama Desert, Chile	Endolithic	*C.* sp.	21	De los Rios et al. 2010
	Atacama Desert, Chile	Endolithic	*C.* sp.	22	De los Rios et al. 2010
	Atacama Desert, Chile	Hypolithic	*C.* sp.	23	Lacap et al. 2011
	Atacama Desert, Chile	Hypolithic	*C.* sp.	24	Warren-Rhodes et al. 2006
	Atacama Desert, Chile	Hypolithic	*C.* sp.	25	Bahl et al. 2011
	Havana, Cuba	Aquatic (fresh)	*C. cubana*	26	Komarek & Hindák 1975
	San Diego, Cuba	Soil	*C. cubana*	27	Fewer et al. 2002
	San Diego, Cuba	Aquatic (fresh)	*C. thermalis*	28	Komarek & Hindák 1975
	Santa Fee, Cuba	Aquatic (fresh)	*C. thermalis*	29	Komarek & Hindák 1975
	Near Galapagos Islands, Ecuador	Aquatic (marine)	*C. polansiana*	30	Komarek & Anagnostidis 1999
	Campeche, Gulf of Mexico, Mexico	Rocky shore, intertidal zone	*C.* sp.	31	Narváez-Zapata et al. 2005
	Vizcaino Desert, Baja California, Mexico	Cryptoendolithic, Chasmolithic	*C.* sp.	32	Büdel & Wessels 1991
	Vizcaino Desert, Baja California, Mexico	Epilithic	*C.* sp.	33	Garcia-Pichel et al. 1991
	Baboquivari Mountains, USA	Cryptoendolithic	*C.* sp.	34	Büdel & Wessels 1991
	Colorado Plateau, USA	Cryptoendolithic	*C.* sp.	35	Bell et al. 1986
	Death Valley, USA	Hypolithic	*C.* sp.	36	Bahl et al. 2011
	Mojave Desert, USA	Hypolithic	*C.* sp.	37	Schlesinger et al. 2003
	Oahu, Hawaii, USA	Endolithic	*C.* sp.	38	Büdel & Rhiel 1985
	San Nicolas Island, USA	Soil (?)	*C. edaphica*	39	Flechtner et al. 2008

Continent	Location	Strategy	Species	Label	Reference
	Utah Desert, USA	Hypolithic	*C.* sp.	40	Bahl et al. 2011
	Yellowstone National Park, USA	Hypolithic	*C.* sp.	41	Bahl et al. 2011
	Parque Nacional Canaima, Venezuela	Epilithic, Cryptoendolithic	*C.* sp.	42	Büdel 1999
Antarctica	Davis Station, Vestfold Hills, Antarctica	Chasmolithic, Hypolithic	*C.* sp.	43	Broady 1981a, b; Smith et al. 2000
	Edward VII. Peninsula, Antarctica	Chasmolithic, Hypolithic	*C.* sp.	44	Broady 1989
	Mawson Station, Antarctica	Chasmolithic	*C.* sp.	45	Broady 1981a
	Ross Desert, Antarctica	Cryptoendolithic, Chasmoendolithic	*C.* sp.	46	Friedmann et al. 1988; Billi et al. 2000; de la Torre et al. 2003; Büdel & Veste 2008; Bahl et al. 2011
Asia	Qaidam Basin, China	Hypolithic	*C.* sp.	47	Pointing et al. 2007
	Taklimakan Desert, China	Hypolithic	*C.* sp.	48	Pointing et al. 2007; Bahl et al. 2011
	Tibet, China	Endolithic	*C.* sp.	49	Wong et al. 2010
	Tibet, China	Hypolithic	*C.* sp.	50	Wong et al. 2010; Bahl et al 2011
	Turpan Depression, China	Hypolithic	*C.* sp.	51	Pointing et al. 2007; Bahl et al 2011
	Sinai Desert, Egypt	Chasmoendolithic	*C.* sp.	52	Billi et al. 2000
	Sumatra, Indonesia	Aquatic (thermal hot spring)	*C. thermalis*	53	Geitler 1933
	Dehradun, India	Epilthic	*C. doonensis*	54	Singh 1968
	Karnatak, India	Epilithic	*C. indica*	55	Tripathy et al. 1999
	Madras, India	Aquatic	*C. indica*	56	Desikachary 1959
	Naganahalli, Mysore, India	Aquatic	*C. mysorensis*	57	Tiwari 1972
	Orissa, India	Epilithic	*C. indica*	58	Tripathy et al. 1999
	Tamil Nadu, India	Epilithic	*C. indica*	59	Tripathy et al. 1999
	Dead Sea, Israel	Aquatic (hypersaline)	*C. versatilis*	60	Dor et al. 1991, Billi et al. 2000

Continent	Location	Strategy	Species	Label	Reference
	Dead Sea Valley, Israel	Soil, Biological Soil Crust	*C.* sp.	61	Dor & Danin 1996
	En Kerem (Jerusalem), Israel	Epilithic	*C. umbratilis*	62	Dor et al. 1991
	Mount Carmel & Judaean Mountains, Israel	Epilithic (Cave)	*C. kashaii*	63	Friedmann 1961, 1962
	Negev Desert, Israel	Hypolithic, Chasmoendolithic, Biological Soil Crust	*C.* sp.	64	Friedmann 1967; Potts & Friedmann 1981; Caiola et al. 1993; Billi et al. 2000; Kidron & Büdel 2012
	Sea of Galilee, Israel	Aquatic (hypersaline)	*C. supralittoralis*	65	Dor et al. 1991
	Gimhae, South Korea	Epilithic	*C.* sp.	66	Tripathi et al. 2007
	Gobi Desert, Mongolia	Hypolithic	*C.* sp.	67	Billi et al. 1998; Billi et al. 2000
	Popov Island, Sea of Japan, Russia	Epiphytic on aquatic chlorophyta	*C. codiicola*	68	Beljakova 1989
	Chiang Mai, Thailand	Aquatic (thermal hot spring)	*C.* sp.	69	Hayashi et al. 1994; Sompong et al. 2005
	Dubai Desert, United Arab Emirates	Hypolithic	*C.* sp.	70	Bahl et al. 2011
Europe	Austria	Lichenized	*C.* sp.	71	Fewer et al. 2002
	Dalmatia, Croatia	Chasmoendolithic	*C. fissurarum*	72	Komarek & Anagnostidis 1995
	Greifswald, Germany	Soil	*C. thermalis*	73	Komarek & Hindák 1975
	Bad Sachsa, Germany	Chasmoendolithic	*C.* sp.	74	Boison et al. 2004
	Euboea, Greece	Aquatic	*C. thermalis*	75	Komarek & Anagnostidis 1999
	Hymettos, Greece	Epilithic (Cave)	*C. doonensis*	76	Lamprinou et al. 2009
	Jenne, Italia	Epilithic	*C. kashaii*	77	Abdelahad 1989
	Padron Region, Spain	Endolthic	*C. doonensis*	78	Noguerol-Seoane & Rifon-Lastra 1999

Continent	Location	Strategy	Species	Label	Reference
	Murcia Region, Spain	Epilithic (Caves and surfaces of buildings)	*C. kashaii, C. doonensis*	79	Asencio & Aboal 1996; Asencio & Aboal 2000; Uher et al. 2005
	Mamaia, Romania	Soil	*C.* sp.	80	Komarek & Hindák 1975
	Bratislava, Slovakia	Epilithic	*C. umbratilis*	81	Uher et al. 2005
	Piestany & Sklene Teplice, Slovakia	Aquatic (thermal spring)	*C. thermalis*	82	Hindák 1978
	Lanzarote Island, Spain	Lichenized	*C.* sp.	83	Büdel et al. 1983
Oceania	Atherton Tablelands, Queensland, Australia	Chasmolithic	*C.* sp.	84	Büdel 1999
	Broken Hill, New South Wales, Australia	Hypolithic	*C.* sp.	85	Billi et al. 2000
	Heron Island, Queensland, Australia	Marine epilithic	*C.* sp.	86	Diez et al. 2007
	Ayers Rock, Northern Territory, Australia	Cryptoendolithic, Chasmolithic	*C.* sp.	87	Büdel & Wessels 1991
	Kimberleys, Northern Territory, Australia	Cryptoendolithic	*C.* sp.	88	Büdel et al. 2004
	Simpson Desert, Northern Territory, Australia	Hypolithic	*C.* sp.	89	Dor et al. 1991; Bahl et al 2011
	South Island, New Zealand	Epilithic	*C. cf. kashaii*	90	Broady & Marican 2012 (pers. comm. P. Broady)

Table A2: Measured cell sizes of *Chroococcidiopsis* strains (n=50).

Strain	Average cell size [µm]
Chroococcidiopsis BB 79.1	2.81
Chroococcidiopsis BB 82.1	3.53
Chroococcidiopsis CCMEE 10	3.43
Chroococcidiopsis CCMEE 140	3.16
Chroococcidiopsis cf. CCMEE 167	3.06
Chroococcidiopsis cf. BB 96.19	4.52
Chroococcidiopsis sp. BB 79.2	4.33
Chroococcidiopsis sp. BB 80.1	5.42
Chroococcidiopsis sp. BB 81.1	4.55
Chroococcidiopsis sp. BB 82.3	5.07
Chroococcidiopsis sp. BB 84.1	5.09
Chroococcidiopsis sp. BB 90.5	2.75
Chroococcidiopsis sp. BB 96.1	4.78
Chroococcidiopsis sp. BB 97.116	3.00
Chroococcidiopsis thermalis BB 82.2	3.63

Table A3: Geographical origin and habitat of investigated *Chroococcidiopsis* strains.

Strain	Geographical Origin	Habitat
Chroococcidiopsis BB 79.1	Austria	Lichen
Chroococcidiopsis BB 82.1	Mexico	Lichen
Chroococcidiopsis CCMEE 10	Israel	Chasmoendolithic
Chroococcidiopsis CCMEE 140	Unknown, Israel?	Lichen
Chroococcidiopsis cf. CCMEE 167	Antarctica	Cryptoendolithic
Chroococcidiopsis cf. BB 96.19	South Africa	Lichen
Chroococcidiopsis sp. BB 79.2	Austria	Lichen
Chroococcidiopsis sp. BB 80.1	Canary Island	Lichen
Chroococcidiopsis sp. BB 81.1	Australia	Lichen
Chroococcidiopsis thermalis BB 82.2	Germany	Soil
Chroococcidiopsis sp. BB 82.3	Mexico	Lichen
Chroococcidiopsis sp. BB 84.1	Hawaii	Endolithic
Chroococcidiopsis sp. BB 90.5	South Africa	Cryptoendolithic
Chroococcidiopsis sp. BB 96.1	South Africa	Lichen
Chroococcidiopsis sp. BB 97.116	Switzerland	Crust on stone
Chroococcidiopsis thermalis BB 82.2	Cuba	Soil
Chroococcidiopsis cubana PCC 7431	Cuba	Mineral spring
Chroococcidiopsis cf. *cubana* CCALA 045	Cuba	Dried pool
Chroococcidiopsis PCC 7203	Germany	Soil
Chroococcidiopsis (Lichinella cribellifera)	USA	Lichen
Chroococcidiopsis (Lichinella nigritella)	USA	Lichen
Chroococcidiopsis (Lichinella iodopulchra)	South Africa	Lichen
Chroococcidiopsis (Anema decipiens)	Spain	Lichen

Table A4: Summary of the labels from the OTU analysis for *Chroococcidiopsis* and Pleurocapsales strains at the threshold values of 95%, 97%, 98% and 99% similarity based on the 16S rRNA.

Strain	Similarity level of 95%	97%	98%	99%
Chroococcidiopsis cubana PCC 7431	Chr10	Chr13	Chr15	Chr18
Chroococcidiopsis cubana CCALA 045	Idem	Idem	Idem	Idem
Chroococcidiopsis thermalis BB 82.2	Idem	Idem	Idem	Idem
Chroococcidiopsis PCC 7203	Idem	Idem	Idem	Idem
Chroococcidiopsis sp. BB 84.1	Idem	Idem	Idem	Chr17
Chroococcidiopsis BB 82.1	Idem	Chr12	Chr14	Chr16
Chroococcidiopsis sp. BB 82.3	Idem	Idem	Idem	Idem
Chroococcidiopsis (Lichinella cribellifera)	Chr07	Chr09	Chr11	Chr12
Chroococcidiopsis sp. BB 96.1	Idem	Idem	Idem	Chr11
Chroococcidiopsis sp. BB 79.2	Idem	Idem	Idem	Chr13
Chroococcidiopsis (Lichinella nigritella)	Idem	Chr08	Chr10	Chr10
Chroococcidiopsis cf. BB 96.19	Chr06	Chr07	Chr09	Chr08
Chroococcidiopsis (Lichinella iodopulchra)	Idem	Idem	Chr08	Chr09
Chroococcidiopsis CCMEE 10	Chr08	Chr10	Chr12	Chr14
Chroococcidiopsis CCMEE 140	Chr09	Chr11	Chr13	Chr15
Chroococcidiopsis sp. BB 90.5	Chr04	Chr05	Chr05	Chr05
Chroococcidiopsis cf. CCMEE 167	Chr05	Chr06	Chr06	Chr06
Chroococcidiopsis sp. BB 97.116	Idem	Idem	Chr07	Chr07
Chroococcidiopsis BB 79.1	Chr03	Chr04	Chr04	Chr04
Chroococcidiopsis sp. BB 81.1	Chr02	Chr03	Chr03	Chr03
Chroococcidiopsis sp. BB 80.1	Chr01	Chr02	Chr02	Chr02
Chroococcidiopsis (Anema decipiens)	Idem	Chr01	Chr01	Chr01
Myxosarcina CCMP 1489	Pleu01	Pleu04	Pleu04	Pleu04
Myxosarcina PCC 7312	Pleu02	Pleu07	Pleu07	Pleu07
Pleurocapsa PCC 7314	Idem	Pleu07	Pleu07	Pleu07
Myxosarcina sp. BB 86.6	Idem	Pleu08	Pleu08	Pleu08
Pleurocapsa PCC 7319	Idem	Pleu05	Pleu05	Pleu05
Pleurocapsa PCC 7516	Idem	Pleu01	Pleu01	Pleu01
Pleurocapsa sp. BB 97.117	Idem	Pleu03	Pleu03	Pleu03
Stanieria PCC 7301	Idem	Pleu06	Pleu06	Pleu06
Xenococcus PCC 7307	Idem	Pleu08	Pleu02	Pleu02
Xenococcus sp. BB 97.118	Pleu03	Pleu09	Pleu09	Pleu09
Xenococcus PCC 7305	Pleu04	Pleu10	Pleu10	Pleu10
Pleurocapsa sp. BB 01.1	Pleu05	Pleu11	Pleu11	Pleu11
Stanieria PCC 7437	Pleu06	Pleu12	Pleu12	Pleu12
Pleurocapsa PCC 7327	Pleu07	Pleu13	Pleu13	Pleu13

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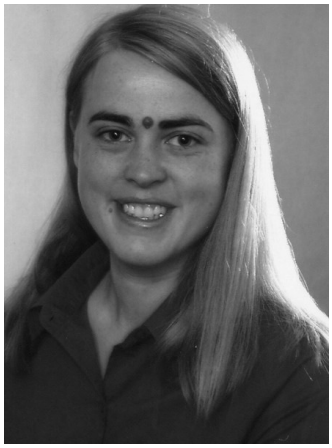
CV



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CONFERENCES & CONTRIBUTIONS

02/2013	Oral presentation at the „BioSyst.EU 2013 Global Systematics!" University Vienna, Austria
02/2012	Poster at the "13th Jahrestagung der Gesellschaft für Biologische Systematik (GfBS)" Zoologisches Forschungsmuseum Alexander König Bonn, Germany
12/2011	Oral presentation at the "11th Young Systematists´ Forum" Natural History Museum London, UK
02/2011	Poster at the "BioSystematics Berlin 2011", Free University of Berlin, Germany
12/2010	Poster at the "10th Young Systematists´ Forum" Natural History Museum London, UK

GRANTS & AWARDS

02/2013	Travel Grant Award from the Gesellschaft für Biologische Systematik (GfBS) to participate at the Conference: "BioSyst.EU 2013 Global Systematics!" University Vienna, Austria
02/2012	Travel Grant from the Gesellschaft für Biologische Systematik (GfBS) to participate at the Conference: "13th Jahrestagung der Gesellschaft für Biologische Systematik (GfBS)" Bonn, Germany
02/2011	Travel Grant from the Gesellschaft für Biologische Systematik (GfBS) to participate at the Conference: "BioSystematics Berlin 2011" Free University of Berlin, Germany
11/2002	2. Place "Dahlberg-Medal", Students work at the University of Rostock, Project title: "Quantification of algae on thermally insulated fronts by using of chlorophyll *a* in plaster samples"

Publications

2012	Fam. Chroococcidiopsidaceae Geitler ex Büdel, Donner & Kauff familia nova" in Syllabus of Plant Families Part 1/1, Frey, W. (Hrsg.), Gebr. Bornträger Verlagsbuchhandlung

Declaration

Hiermit versichere ich, dass die vorliegende Dissertation von mir in allen Teilen selbstständig angefertigt wurde und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden.

Darüber hinaus erkläre ich, dass die Dissertationsschrift weder vollständig, noch teilweise einer anderen Fakultät mit dem Ziel vorgelegt worden ist, einen akademischen Grad zu erlangen.

Kaiserslautern, den