POLYPHASIC EVALUATION OF PERIPHYTIC FRESHWATER CYANOBACTERIA IN TUKUN RIVER, PENANG NATIONAL PARK

ASMIMIE ASMAWARNIE BINTI AZIZAN

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by

ASMIMIE ASMAWARNIE BINTI AZIZAN

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LIST OF ABBREVIATIONS

16S	Small Ribosomal Subunit
BLAST	Basic Local Alignment Search Tool
BP	Bootstrap value
bp	Base pair
DNA	Deoxyribonucleic Acid
MEGA	Molecular Evolution and Genetic Analysis
ML	Maximum-Likelihood
MP	Maximum-Parsimony
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
sp.	Species

LIST OF SYMBOLS

°C	Degree Celsius
%	Percent
µl	Microlitre
µm	Micrometre
CCA	Canonical Correspondence Analysis
g	Gram
L	Litre
lx	Lux
mg	Milligram
ml	Millilitre
mm	Millimeter
ppt	Parts per thousand

KAJIAN BERFASA CYANOBAKTERIA PERIFITIK DI SUNGAI TUKUN, TAMAN NEGARA PULAU PINANG

ABSTRAK

Kebanyakan kajian alga di Malaysia telah memberi tumpuan kepada fitoplankton di tasik. Walaupun terdapat banyak sungai dan anak sungai di Malaysia, sebahagian besar komuniti alga dalam ekosistem lotik tidak diketahui. Dalam kajian ini, cyanobakteria makroskopik yang dikutip daripada sembilan tapak di sepanjang Sungai Tukun telah diperiksa untuk mengenal pasti kepelbagaian kumpulan cyanobakteria. Dalam pengetahuan kami, ini adalah kajian perintis cyanobakteria perifitik yang dijalankan di sungai Taman Negara Pulau Pinang. Setakat ini, 24 morfospesies telah dikenal pasti termasuk sembilan famili di mana 10 spesies dijumpai di lapangan dan empat belas morfospesies selebihnya telah dikenal pasti dalam kultur. Kerak adalah bentuk makroskopik paling dominan dengan 7 morfospesies, diikuti dengan lapisan hamparan cyanobakteria (3 morfospesies). *Scytonema* merupakan genus paling dominan dijumpai di 8 daripada 9 kawasan persampelan. Kehadiran cyanobakteria 'heterocytous' (*S. stuposum* atau *S. hofmanni*) dalam 8 daripada 9 kawasan persampelan menunjukkan bahawa sungai itu mempunyai nilai nitrat yang rendah (0.74mg/L) yang dicatatkan sepanjang tempoh kajian. Chroococcales dominan di kedua-dua aliran atasan dan pertengahan sungai. Faktor persekitaran juga di ambil kira dalam mengenal pasti kepelbagaian cyanobakteria di kawasan persampelan. Morfospesies cyanobakteria menunjukkan corak taburan yang berbeza walaupun bacaan parameter ekologi seperti suhu, oksigen terlarut, pH, dan konduktiviti mengalami perubahan yang minima sepanjang persampelan. Analisis 16S rDNA dijalankan ke atas cyanobakteria yang tumbuh di atas kultur untuk proses identifikasi.

Ketiga-tiga strain *Chroococcus* cf. *minor* membentuk sebuah klad dengan genus *Chroococcidiopsis* walaupun data morfologi dan ekologi kedua-dua strain ini tidak menunjukkan persamaan. Strain kajian yang terpisah daripada genus *Chroococcus* mencadangkan bahawa ia adalah mungkin spesies samar. Kajian ini telah menyediakan maklumat asas kepelbagaian cyanobakteria perifitik di Sungai Tukun berhubung dengan faktor persekitaran dan pendekatan molekular untuk pengecaman morfospesies cyanobakteria yang tumbuh di atas kultur.

POLYPHASIC EVALUATION OF PERIPHYTIC FRESHWATER CYANOBACTERIA IN TUKUN RIVER, PENANG NATIONAL PARK

ABSTRACT

Most algae studies in Malaysia have focused on phytoplankton in lakes. Despite the occurrence of numerous streams and rivers in Malaysia, algae community in the lotic ecosystems remained largely unknown. In this study, macroscopic cyanobacteria collected from 9 sites along Tukun River were examined to identify the diversity of the group from December 2014 to November 2015. To the best of our knowledge, this is the pioneer study on periphytic cyanobacteria conducted in the stream of Penang National Park. To date, 24 morphospecies has been identified including 9 family whereby 10 species were encountered from field and the other 14 remaining were identified in cultures. Crust were the most dominant macroscopic forms with 7 morphospecies, followed by mats (3 morphospecies). *Scytonema* was the most dominant genus occurring at 8 out of 9 sampling sites. Presence of heterocytous cyanobacteria (*S. stuposum* or *S. hofmanni*) in 8 out of 9 sampling sites coincide with the low nitrate value (<074mg/L) recorded throughout the study stream. Chroococcales were dominant in both upper and middle stream. Environmental factor were also included in identification of cyanobacterial diversity along the study sites. Morphospecies encountered showed distinct distribution patterns despite minimal fluctuations of ecological parameters such as temperature, dissolved oxygen, pH, and conductivity throughout sampling sites. 16S rDNA analysis were conducted on culturable strains of cyanobacteria for species identification. All three cultured strains of *Chroococcus* cf. *minor* used in the analysis formed a clade with genus *Chroococcidiopsis* in which both morphology and ecology data of both strains did not

conform. The strains were well separated from the genus *Chroococcus* suggesting that it is a possible cryptic species. This study has provided a baseline information of diversity of periphytic cyanobacteria in Tukun River in relation with environmental factors and molecular approach for identification of culturable strains of cyanobacterial morphospecies.

CHAPTER 1

INTRODUCTION

Diversity and distribution of periphytic cyanobacteria in running waters have not been intensively studied in Malaysia. One of the earliest studies on diversity of periphytic algae in Malaysian freshwater ecosystem focused specifically on the red algae (Kumano, 1978). Other subsequent studies only reported cyanobacteria as the least common component that co-occured with diatoms and green algae (Anton, 1990; Maznah *et al.,* 2000). Many of these studies only identified prominent cyanobacteria taxa to the genus level with very limited taxonomic information provided.

In comparison, numerous studies have been conducted on planktonic cyanobacteria community in Malaysian lentic environment (Harith & Hasan, 2007; 2009; 2011; Majit *et al.*, 2010; Florence, 2011; Sinang *et al.*, 2015). Both periphytic and planktonic algae community plays the same important role as primary producers in the aquatic ecosystem. They provide valuable assessment of the overall health of these systems and are particularly useful as assessment tools, due to their rapid response to environmental stress.

The presence of cyanobacteria within lotic habitats are useful as biomonitoring unit (Loza *et al.,* 2013). They have vital role in the stream nitrogen, carbon, and oxygen dynamics (Vincent, 2000). They are the main primary producers in the aquatic food webs (Scott & Marcarelli, 2012) and assist in substrate stabilization (Casamatta & Hašler, 2016). Despite their important role in lotic ecosystem, under sampling and

taxonomic uncertainty resulted in the lack of knowledge of the group in the Malaysian stream ecosystem.

Environmental factors governing the distribution of cyanobacteria in Malaysian streams are unknown. Cyanobacteria are able to detect and respond to a various environment. According to Dolman *et al.* (2012) in low nitrate waters, the capacity of some cyanobacterial species to fix nitrogen allows them to outcompete others that are not able to do so. Their success in colonizing such a wide habitat range is due to their resistance to extremes of temperature and dessication and also to their modest nutrient requirements. Most of nitrogen-fixing cyanobacteria thrive photoautotropically in the absence of combined nitrogen (Whitton & Carr, 1982). *Anabaena* and *Nostoc* are the best examples for heterocytous cyanobacteria. Vegetative cells often differentiate into heterocytes only when grown in absence of combined nitrogen (Whitton & Carr, 1982). This observation led Fogg (1949) to suggest that heterocytes are the site of nitragenase, the enzyme complex catalyzing the conversion of dinitrogen to ammonia.

Cyanobacteria are able to grow under low-light conditions due to their accessory pigments; phycobilins, in particular phycocyanin and phycoerythrin (Loeb & Reuter 1981; Reuter & Axler, 1992) allowing them to colonize shaded stream reaches. According to Loza *et al.*, (2013), their vast predominance, diversity and ecosystem importance enables the members of the group to be an excellent bioindicators for monitoring the health of aquatic ecosystems. Increased knowledge of their distribution in response to environmental parameters would enhance the use of cyanobacterial assemblages for biomonitoring of Malaysian stream health.

The introduction of important and more complicated methods (electron-microscopic and, especially, molecular analyses) were necessary to the taxonomic classification of cyanobacteria. This method is called as polyphasic approach, where the genetic evaluation would form the basis, which was combined with other criteria for example, morphological, ecophysiological and ecological analysis. These methods need to be chosen according to the nature of the samples and knowledge of which criteria would provide the most reliable diagnostic characteristics to depict taxonomic groups. It is the most modern method for obtaining a correct review of the diversity of cyanobacteria (Komarek, 2016).

Therefore, the objectives of the present study were:

- To provide thorough descriptions of morphospecies based on descriptions of visible growths in the field and on light microscopy of field-collected specimens.
- To combine the morphological description of cultured isolates and field specimens whenever possible to aid identification.
- To study spatial and temporal patterns in their taxonomic composition along the whole study site in relation to environmental variables.
- To utilize molecular genetics approaches in identification of culturable strains of cyanobacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 The Diversity of morphospecies of cyanobacteria

2.1.1 Introduction to Cyanobacteria

Cyanobacteria are morphologically diverse group of prokaryotic organisms (Komarek, 2003; Sharma *et al.*, 2014). Their morphology ranges from simple unicellular forms to complex filamentous organisms which form true branching and producing differentiated cells types (Waterbury, 2006). They possess prokaryotic cell structure and capable to undergo photosynthesis process. Some of them have specialized cell called heterocyte which are able to fix atmospheric nitrogen (Carr & Whitton, 1982).

These microorganisms can occur in a wide variety of habitats. According to Cohen and Gurevitz (2006), cyanobacteria are being dominant in a broad spectrum of aquatic environments and terrestrial habitats, including deserts and are even found in extreme environments such as hot springs and polar lakes. Whitton (1992) stated that, cyanobacteria that are morphologically complex are more adapted to environments that are particularly heterogenous in space or time.

There are many excellent detailed studies conducted on periphytic cyanobacteria around the world, (Sheath & Cole, 1992; Sheath and Cole, 1996; Branco & Necchi Jr, 1996; Perona *et al.*, 1998; Lindstrøm *et al.*, 2004; Sherwood, 2006; Yang *et al.*, 2009; Krupek & Branco, 2012; Loza *et al.*, 2013). However, the diversity and distribution of benthic cyanobacteria from lotic ecosystems in Malaysia are still

poorly known. The first study on algae in Malaysia was recorded in 1978. Kumano (1978) studied freshwater red algae from streams and lakes in West Malaysia. In 1990, Anton conducted a water quality study in Langat River, Selangor by using two *Chlorella* species. Later in year 2000, Wan Omar et al., (2000) investigated the relationship between periphyton biomass and water pollution in the Pinang River basin, Malaysia. Other studies on algae in the Malaysian freshwater body have been focused towards the diversity and ecology of phytoplankton community in lakes (Wan Omar *et al*., 2002; Sidik *et al*., 2008). The limited publications on algae in stream and the extensive literature available for phytoplankton studies in lakes indicated a gap of knowledge on the diversity and ecology of the stream algae community. This is particularly true for cyanobacteria whereby, to the best of my knowledge, remained as the most understudied group lacking literature and records in Malaysian stream ecosystem.

2.1.2 Taxonomic Classification of Cyanobacteria

Cyanobacteria in botanical tradition have been characterized by phenotypic properties (Palinska & Surosz, 2014). Taxonomic reference was started by Thuret (1875), Bornet and Flahaut (1888; 1889) and Gomont (1892). They wrote the first comprehensive taxonomic monograph for blue green algae which was recognised by phycologist as the starting point in taxonomic referencing. An updated taxonomic review and determination manual that recognised 1 300 species, classified into 145 genera, 20 families and 3 orders were made by Geitler (1932).

Based on morphology of field collected specimens, Geitler's classification system marked the beginning of the modern era of cyanobacterial systematics which

recognized by both phycologists and microbiologists. Since then, numerous revised systems were proposed including Elenkin (1938, 1949), Desikachary (1959), Starmarch (1966), Kondrateva (1968), Bourelly (1970) and Golubic (1999).

The taxonomic system of cyanobacteria was changed vigorously with the introduction of electron microscopy and of molecular and genetic methods for classification of cyanobacterial taxa (Komarek *et al.*, 2014). Earlier, some researchers also claimed that the systematic classification of cyanobacteria should be based on bacteriological criteria since they are unquestionably prokaryotes (Waterbury & Stanier, 1977; Krumbein, 1979; Rippka *et al.*, 1979). According to Castenholz and Waterbury (1989), Stanier relied largely on morphological properties and used Geitlerian designations in revising the cyanobacteria genera by altering many generic definitions in line with properties expressed in culture

Updated and revised system which included ultracultural properties has been proposed (Anagnostidis & Komarek, 1985; Komarek & Anagnostidis, 1999, 2002; Komarek, 2013). Incorporation of Bacteriological and Botanical approaches have been developed by Komarek and Anagnostidis (1995, 2002) to solve the confusion in cyanobacteria taxonomy.

The method of modern classification of cyanobacterial diversity respecting all of phenotypic, molecular and ecological approaches is commonly called as the "polyphasic approach" (Komarek, 2011). This approach has been used worldwide in recent cyanobacterial studies (Boutte *et al.*, 2005; Ballot *et al.*, 2008; Berrendero *et al.*, 2008; Zapomělová *et al.*, 2009; 2010; Heath *et al.*, 2010; Dadheech *et al.*, 2012;

Sciuto *et al.*, 2012; Loza *et al.*, 2013; Martineau *et al.*, 2013; Liu et al., 2013; Mühlsteinová *et al.*, 2014a, 2014b; Gaget *et al.*, 2015; Bravakos *et al.*, 2016) to confidently identify and characterized the group.

2.2 Spatial and Temporal Patterns in the Distribution of Cyanobacteria

2.2.1 Periphytic cyanobacteria in stream

Algae gain prominence among periphyton as they play basic role as primary producer in the food chain of continental aquatic systems (Goldsborough & Robinson, 1996; Lam & Lei, 1999; Cattaneo & Kalf, 1978). Studies on periphytic algae community have increased due to their variation of tolerances habitat in environments (Rodrigues *et al.*, 2003). Periphytic algae refer to algae that grow on various substrata. According to Round (1981) epilithon are groups of algae occuring on the surface of exposed rock. Meanwhile, epipsammon refer to the growth of algae communities attached to sand grains. Epipelon is the growth of algae on accumulation of sediments and epiphyton refer to the algal flora grow on other plant parts (Round, 1981).

Cyanobacteria are one of the most important photosynthetic microorganisms which can be found in both periphytic and planktonic algae community (Felisberto *et al.*, 2014). They may thrive in extreme environments such as, hot springs, rocky shores, drought, desiccation, osmotic, various salinity, UV stresses and others (Sinha & Hader, 1996; Zehr *et al.*, 2000; Kalib, 2002; Saha *et al.*, 2003). According to Saha et al., (2007) cyanobacteria can grow on surfaces of exposed rock or the rock that submerged in the water. The presence of cyanobacteria on bare rock can be identified by the slipperiness due to exopolysaccharides after exposed to water splashed (Saha

et al., 2007). Sinha et al., (1995; 1997) stated that cyanobacteria are vital in global nutrient cycling especially due to their inherent capacity to fix atmospheric carbon dioxide. Moreover, some of them which possess specialized cell called heterocytes enables them to fix atmospheric nitrogen (Sinha *et al.*, 1995; 1997).

2.2.2 Cyanobacteria in Tropical Stream

In tropical stream, benthic algae including cyanobacteria are controlled by similar factors such as water flow, light, nutrient supply and grazing as of temperate streams. But in some cases, tropical stream may differ in water flow or alternation between dry and wet states associated with seasonal rainfall (Flecker, 1992: 1996). Scott et al., (2012) stated that, the phenomenon of high water flows during the wet season disturbed stream bed frequently and increased turbidity. Meanwhile, during the dry period, streams benthic community experienced low water flow, warmer temperature and higher water clarity as the effect of decreased sedimentation. These factors usually resulted in the dominance of periphyton, organic detritus and associated epipelon (Pringles & Hamazaki, 1998; Flecker & Taylor, 2004).

According to Goulden (2011), cyanobacteria are commonly used in biomonitoring activities of lakes and streams in Asia. The organisms are very important in studies of lake's eutrophication. Hence, reliable identification is important because some forms of cyanobacteria can synthesize and accumulate toxic compounds. Their presence can cause serious problems especially when abundant in lakes and reservoirs used as drinking water sources (Goulden, 2011).

2.2.3 Cyanobacteria as Biological Indicator

In the year 1908, Kolkwitz and Marsson made the first serious attempt to use cyanobacteria as bioindicators of water quality through saprobic system. This system showed that water conditions determined the composition of the algal flora. Later on, a range of methods were developed even though the principal tools for monitoring rivers, which are vastly employed, are indices based on diatom communities (Wyatt & Stevenson, 2010). Nevertheless, a number of researchers have suggested that besides diatoms, the floristic composition of other groups in the benthos could be helpful for monitoring rivers (Kelly & Whitton 1998). Moreover, Kelly and Whitton (1998) stated that many upland rivers have slow-growing cyanobacterial species which are colonial that may eventually prove to be good indicators of certain combinations of nutrient conditions.

The obvious difference in morphology of hairs produced by a particular strain of Rivulariaceae under different deficiencies was the greater length of the hairs under phosphate deficiency (Sinclair & Whitton, 1977). The hairs not only enhance the surface area for phosphatase activities, but also aid in P absorption from the environment. A large number of hairs has been observed due to phosphorus limitation in streams (Berrendero *et al.*, 2008; Mateo *et al.*, 2010; Mun˜oz-Martı´n *et al.*, 2014a; Stancheva *et al.*, 2013). Increased values of phosphatase activity have also been recorded as good indicator of P limitation (Mateo *et al.*, 2010; Mun˜oz-Martı´n *et al.*, 2014; Sabater *et al.*, 2000; Whitton *et al.*, 1998).

Heterocyte formation happened if there are low concentrations of nitrate and/or ammonium in the growth medium. The presence of many heterocytes in a filament is an indication that the organism is growing in an environment relatively deficient in combined nitrogen compared to other nutrients (Whitton & Mateo 2012). Analyses of nitrogenase gene expression to rate N2 fixation along a NO3-N gradient showed that nitrogenase activities specific to *Nostoc* and *Calothrix* were only detected in streams with low values of nitrates (Stancheva *et al.*, 2013). Those species which have both heterocysts and well developed hairs when present in stream environment can act as reliable bioindicators reflecting the deficient in combined nitrogen during part of the time, and of phosphorus for the rest of time (Whitton, 2002; Whitton & Potts, 2000 Mateo *et al.*, 2015).

2.3 Molecular analysis of cyanobacteria

2.3.1 Introduction of molecular analysis of cyanobacteria

Diversity of cyanobacteria was firstly discovered during the 19th century when cyanobacteria were eventually recognized as a different group of organisms. The classification was based solely on morphology of isolated strains and specimens from the field. The most important diacritical characters were cell dimension, cell/filament morphology, type of cell division and presence of sheath or envelope (Geitler, 1932).

The trend continued till nearly 20th century. Since then, molecular markers especially 16S rRNA have transformed cyanobacterial systematics. However, it was a problem to standardize cyanobacteria species identified using botanical nomenclature to those described based on bacteriological nomenclature (Komarek 2010, Komarek &

Mareš, 2012). To date, there is still no consensus of nomenclature that has been universally accepted (Komarek & Mareš, 2012).

Johansen & Casamatta (2005) suggested few practical criteria for identification of cyanobacterial species. According to the authors, finding evidence of separation from present species may be accomplished by (1) evaluation of morphological differences, (2) genetic distance in 16S rRNA sequence, (3) differences in 16S- 23S ITS secondary structures, (4) composition of secondary metabolites, and (5) physiological ecology (biotope of studied strain). The real biodiversity obtained from morphological evaluation alone is expected to be underestimated.

Based on the phylogenetic analysis of the 16S rRNA in a study, the researchers proposed the unification of five species of the genus *Microcystis* into a single species of *M. aeruginosa* under the Rules of the Bacteriological Code. The five species were *M. aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. viridis* and *M. wesenbergii* whereby DNA–DNA hybridization and phylogenetic data both showed no clear distinction among strains with the first method having more than 70% homology. The data from the study proved that all five species belong to the same species of the cyanobacterium *M. aeruginosa* (Otsuka *et al*., 2001)

Borges and others (2015) in their finding stated that isolates of *Geitlerinema amphibium*, *Geitlerinema splendidum* and *Geitlerinema lemmermannii*, whose identifications were supported by morphological characters, showed cell dimensions, apical cell and motility conform with Komarek and Anagnostidis (2002). However, phylogenetic analyses based on 16S rDNA sequences revealed that these species

actually form distinct lineages, suggesting that the genus behave as paraphyletic and polyphyletic, as identified in previous studies (Willame et al., 2006; Bittencourt-Oliveira et al., 2009; Perkerson et al., 2010; Hasler et al., 2012). Hasler and others (2012) in a phylogeny study with species of Geitlerinema, Phormidium and Microcoleus, identified that the genera behave polyphyletic and also suggest the taxonomic revision of these groups.

Gugger *et al*. (2004) conducted a study using the 16S rRNA applied to 16 strains of order Stigonematales to evaluate whether true branching is a monophyletic characteristic in the cyanobacterial order. The order revealed a polyphyletic nature of the branching type since the three types of true branching were intermixed in the phylogenetic tree. In another study, evaluation of four genera of cyanobacteria belonging to the Order Nostocales (*Nostoc*, *Anabaena*, *Aphanizomenon* and *Trichormus*) has been carried out based on their genetic relationships along with morphological criteria (Rajaniemi *et al*., 2005). Their study reported that the taxonomic classification should be revised since the planktonic *Anabaena*/*Aphanizomenon* and benthic *Anabaena* were not monophyletic strains in all three genetic markers studied. In view of the retrieved data, morphological criteria obtained by both botanical and bacteriological codes did not conform to the obtained genetic relationships for these three genera (Rajaniemi *et al*., 2005).

There are various examples of cyanobacteria which are morphologically indistinguishable but do not share a common evolutionary history to which their molecular phylogeny is more diverse than morphology. This incompatibility resulted

in the concept of “cryptic species” complexes which often occur in cyanobacteria (Flechtner *et al.* 2002; Casamatta *et al.,* 2003; Siegesmund *et al.,* 2008; Hasler *et al.,* 2014).

2.3.2 Molecular marker used in taxonomy of cyanobacteria

The best method for understanding the evolution of organisms is through analysis of DNA (protein) sequences and other molecular markers. Similar trend occurred in molecular systematics and population genetics of cyanobacteria (Giovanonni *et al.,* 1988; Boyer *et al.*, 2001, Castenholz 2001, Komárek 2010). 16S rRNA (SSU) which codes small ribosomal subunits is the most widely used gene. The 16S rRNA gene is reliable, generally accepted, and a highly conserved region (Chakravorty *et al.,* 2007). Hugenholtz *et al.* (1998) stated that rRNA sequences, especially 16S rRNA is the current most important targets of study in bacterial evolution and ecology. It includes the establishment of phylogenetic relationships among taxa, the discovery of bacterial diversity in the environment and the quantification of the relative abundance of taxa of various ranks.

There are few reasons 16S rRNA is suitable for this purpose. One of them is the gene is universally distributed, which allowed the analysis of phylogenetic relationships among distant taxa. 16S rRNA gene is expected to be only weakly affected by horizontal gene transfer as the gene is a crucial part of the core gene set (Daubin *et al.,* 2003). This feature of the gene is very useful for phylogenetic studies. Sequence analysis of the 16S ribosomal RNA (rRNA) gene has been widely used to identify bacterial species and perform taxonomic studies (Clarridge, 2004; Munson *et al.,* 2004; Petti *et al.*, 2007).

Although 16S rRNA is a conserved gene region, slight are evident in the gene region. Presence of variable regions allows adequate diversification required as a tool for classification. Meanwhile, presence of conserved regions enabled the design of suitable PCR primers or hybridization probes for various taxa at different taxonomic levels ranging from species to whole phyla (Head *et al.*, 1998).

16S rRNA genes generally contain nine "hypervariable regions" that display considerable sequence diversity among different species that can be utilized for species identification (Van de Peer *et al.*, 1996). Hypervariable regions are boarded by conserved stretches in most bacteria which enables PCR amplification of target sequences using universal primers (Baker *et al.*, 2003; Munson *et al.*, 2004).

Identification process through the sequences of 16S-rRNA is conducted by extraction of genomic DNA, amplification and sequencing. Sequence analysis software are able to compare the analysed genes in 16S-rRNA sequence library allowing a more confident identification to be carried out (Tang *et al.*, 1998).

2.3.3 Phylogenetic sequence analysis

Phylogenetic analyses are used to estimate the evolutionary relationships of cyanobacteria. The analyses comprising of alignment of sequences, construction of a phylogenetic tree, and reliability test of the constructed tree through bootstrapping (Ludwig and Klenk, 2001). Sequence alignment is a crucial step in phylogenetic analysis. This is because, only the sequences with a common ancestor (homologous positions) can be used in phylogenetic analysis (Swofford *et al.*, 1996). In alignment, other than deletion, the sequences from different strains are organised by inserting

gaps so that homologous positions of the sequences are placed in the same columns of the data matrix. Several computer programs for instance ClustalW, MUSCLE, RDP, T-Coffee have been created for aligning the sequences purposes (Tamura *et al.*, 2013).

The relationships of the aligned sequences are usually shown as a tree, in which the branching pattern of the tree (topology) displays the evolutionary relationships of the strains (Nei & Kumar 2000). The most commonly applied tree construction methods are distance, maximum parsimony (MP), and maximum likelihood (ML) (Nei & Kumar 2000; Ludwig and Klenk 2001). Distance methods such as neighbour joining (NJ) (Saitou & Nei 1987) use pair-wise distances (i.e. the number of base differences between two sequences), calculated from aligned sequences and usually corrected to evolutionary distances within a substitution model (Nei & Kumar 2000). The sequences with the shortest distances are clustered together in a tree, where the tree length is optimised to correspond to the distance matrix (Nei & Kumar 2000).

The MP method uses the actual sequence data instead of distances and searches for the tree(s) with minimum length, i.e., topology of the tree can be explained with a minimum number of transformations from one character state to another (Swofford *et al.*, 1996; Nei & Kumar, 2000). ML method estimates the likelihood for tree topology that could have resulted in the sequence alignment under the given model of evolution and searches for the tree with maximum likelihood (Swofford *et al.*, 1996; Nei & Kumar, 2000). Mathematical background and more detailed discussion of tree construction methods are presented in Swofford *et al.* (1996) and Nei and Kumar (2000)

CHAPTER 3

MATERIALS AND METHODS

3.1 Description of the Study Site

3.1.1 Features of Penang National Park

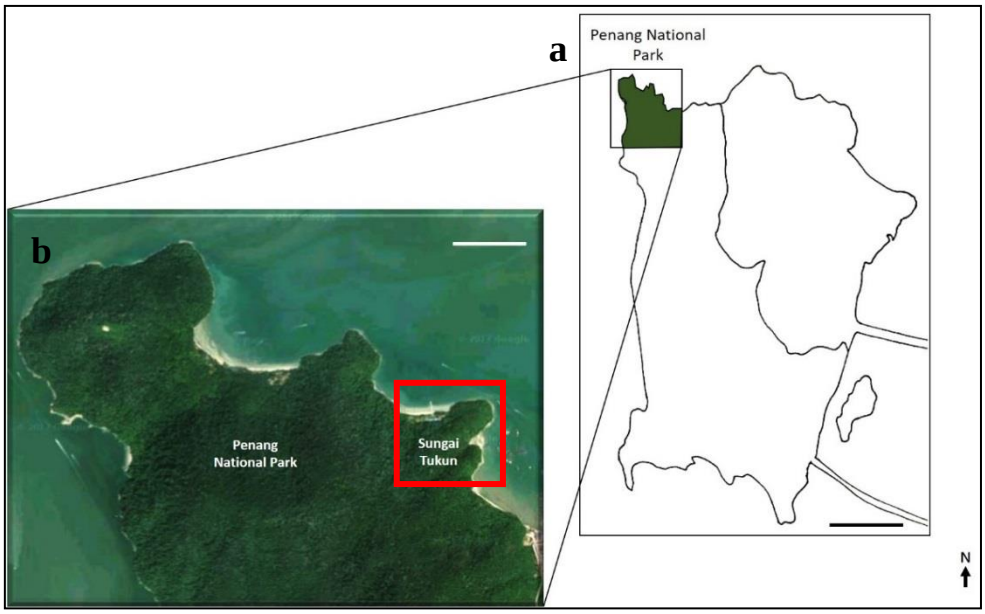

Figure 3.1: Locality map of study site. **a,** location of Penang National Park in Penang Island, **b,** location of Tukun River, Penang National Park (red box) Scale bar: **a,** 4 km, **b,** 500 m. (Modified from GoogleMaps)

Figure 3.2: Locality map of Tukun River of Penang National Park from the human settlement. Scale bar: 200 m. (Modified from GoogleMaps).

Penang National Park is located in the northwest of Penang Island in latitude of 5° 28’ N and longitude of 100° 12’ E. The area was formerly known as Pantai Acheh Forest Reserve. It is a tropical rain forest intact and undisturbed nature. Penang National Park was gazetted under the National Parks Act 1980, No. 226 and has a total area of 2, 562 963 hectares. It covers a land area of 1, 182.6 hectares of land and marine ecosystems of 1, 379.3 hectares. Penang National Park is the smallest national park in Malaysia and one of the smallest national park in the world (Ahmad, 2007).

Geology

Penang National Park consists of two types of granites; Feringgi Granite and Muka Head Granite. Both phases of these rocks belong to North Pluton Penang. The highest peak of the region is Bukit Batu Hitam, which is 464 meters. Land of this area is in series of Bukit Temiang. Half of Penang National Park’s area are cover by the sea. The park is surrounded by shallow waters. The depth of the sea at a distance of one nautical mile from the coast are mostly 5 meters at most (Ahmad, 2007).

Climate

Average annual rainfall in the park is 2 600 mm. Rainfall is influenced by the Northeast Monsoon. Dry season is usually occurred from December to March every year. Normally, range of temperature is between 23°C - 30° C. Ambient temperature will not exceed 28° C in the area under the forest canopy (Ahmad, 2007).

Vegetation

Penang National Park consists of Lowland dipterocarp forest ecosystem, Mangrove Forest and Forest Hill Dipterocarp Coast. The land consists of sloping hills overgrown by Seraya (*Shorea curtisii*), Resak (*Vatica* sp.), palms, rattan, climbers, orchids, fungi and more than 50 species of ferns. A total of more than 1000 species of plants have been recorded in Penang National Park. Of the total, 428 species from 69 families are dicotyledonous plants. While 49 species from 10 families are monocotyledonous plants. Along the forest trails, there are two types of pitcher. The park also has more than 90 herbs (Ahmad, 2007).

Land use

In spite of to maintain and to protect wildlife, vegetation and others, Penang National Park also is opened for recreational activities. Construction of facilities available for visitors were focused on two areas which are lower part of Tukun River and Pantai Kerachut before it was gazetted as a national park in 2003 (Ahmad, 2007).

3.1.2 The study site

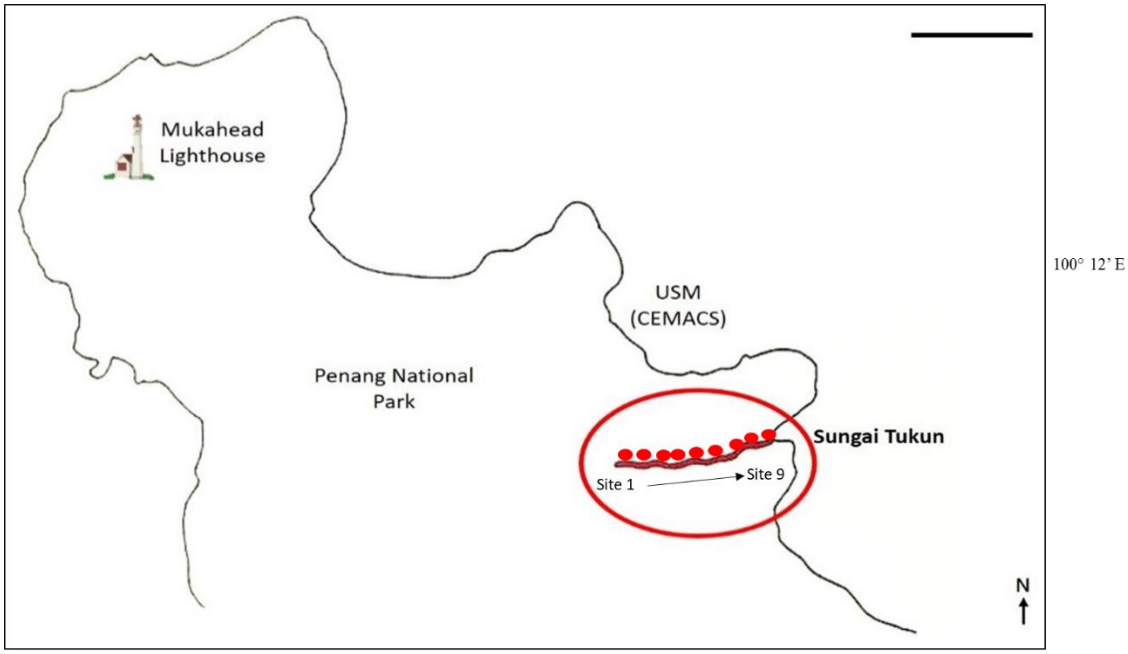

Figure 3.3: Map of Penang National Park. Tukun River is located in red circle area. Scale bar: 500 m.

Tukun River is a small stream, approximately 700 m long. Sampling locations consist of three sites in each upstream, middle stream and lower stream respectively. Sampling locations were numbered from 1- 9 with 1 being at the uppermost of the site in Tukun River and 9 at the lowest part of stream. Within each sampling sites, runs, riffles and pools were identified based on water flow (Harding *et al.*, 2009). Substrata comprised of bedrock, boulders, cobbles and silt were noted in each sampling locations. The upstream and middle stream are free from anthropogenic activities.

The upper area of the stream comprised of slow running water at the first sampling sites, riffle and rapid at the second and third sampling locations. The water current rapidly flows down towards the middle part. The middle part area are partly shaded with boulders, cobble and pebble as the main substrata for algae attachment (Figure

3.3a). The middle parts of the stream are comprised of run, riffle and pools. Apart from boulder, cobble and pebble, (Figure 3.3b) middle stream are covered with few bedrocks and also sand which served as substrate for periphyton attachment (Figure 3.3c). Same with upper stream area, middle stream also is partly shaded.

The lower parts of the stream are comprised of run and riffle. This area is different from the others as the last two sampling sites are exposed to anthropogenic activities (Figure 3.3d). Therefore, apart from biological substrata, there are also artificial substrata for periphytic community. In term of shading, the area consisted of partly shaded part at site 7 and site 8. The last sampling locality, site 9 has area that completely exposed to sunlight and subjected to intrusion of seawater during high tide.

Figure 3.4: Stream order. **a,** upper stream area with boulder and cobble **b,** middle stream area with very few boulder and cobble **c,** middle stream area with dominant sand and silt **d,** lower stream with artificial substrata under unshaded condition

3.2 Sampling Procedure

Macroscopic algae collection

Sampling was conducted in Tukun River, Penang National Park from December 2014 to November 2015. Prior floristic survey was carried out at the beginning of the study to identify all macroscopic growth forms along the stream. All accessible locations along the stream were included in the study. A total of nine sampling sites were identified for subsequent monthly survey. Samples of visible growths of cyanobacteria were collected from different substrata. Substrata sampled were bedrocks, boulders, cobbles, pebbles, gravels, sand and silt. Crusts were scraped from rock surfaces using autoclave-sterilized spatulas. Mats, gelatinous colonies, other macroalgae and other vegetation with possible epiphytic cyanobacteria were collected by hand. Each sample was stored in separate sterile polycarbonate screw-top containers (60 mL) with water from the collecting site. The containers were kept in ice chest and transported back to the laboratory for further analysis.

At each site, stream bed substrata (Table 3.1), degree of shading (Table 3.2) and variation of water flow (Table 3.3). Type of subrata were classified based on modified Wentworth scale (Harding *et al*.2009).

Table 3.1: Type of substrata.

Type of substrata	Scale
Bedrock	> 4000 mm
Boulders	> 256-4000 mm
Cobbles	> 64-256 mm
Pebbles	> 16-64 mm
Gravel	> 2-16 mm
Sand	> 0.063-2 mm
Silt	< 0.063 mm

The shade is assessed at the water surface. Shading is considered at all points across the water surface throughout the reach and through the full 180°, so that the influence of stream banks, stream bank vegetation, and hill slopes are included (Harding *et al*.2009).

Table 3.2: Type of shading

Type of shading	Percentage (%)
Unshaded	0-30% shade
Partly shaded	30-60 % shade
Shaded	> 80% shade

Surfaces flow patterns are often associated with different substrate types. The frequency and length of these habitats are usually predictable and correlated with channel width (Gordon *et al.* 2004). They are determined by the local channel slope, shape, structure, flow depth and mean water velocity (Harding *et al*. 2009).

Table 3.3: Type of water flow.

Type of water flow	Explanation
Rapid	Shallow to moderate depth, swift flow and strong currents, surface broken with white water
Riffle	Shallow depth, moderate to fast water velocity, with mixed currents, surface rippled but unbroken
Run	Area in between riffle/rapid and pool, slow-moderate depth and water velocity, uniform-slightly variable current, surface unbroken, smooth-rippled
Pool	Deep, slow flowing with smooth water surface, usually where the stream widens and/ or deepens.

Water Collection

Temperature, dissolved oxygen, total dissolve solid, salinity, nitrate and ammonium concentration were measured using YSI professional multi probes parameter. pH was measured using Hach 5465010 Sension 156 pH meter and light intensity was measured using Center 337 lightmeter. All these ecological parameters were measured *in situ* at every sampling site on every sampling occasion. For Ortho phosphate analysis, three replicates of 120 ml water from each site were collected into separate 120 ml acid cleaned polyethylene bottles on every sampling occasion. These were then transported back to the laboratory in an ice chest.

Ortho phosphate analysis was carried out by using ascorbid acid or Murphy-Riley method based on APHA standard (Rice *et al.*, 2012). Special reagents such as Armstrong reagent, ascorbid acid, hydrochloric acid (HCL) and stock potassium ($PO4^{-3}$-P) were prepared as instructed by the manual. For standardization, 1.0 mL of the 1mg/mL stock $PO4^{-3}$-P solution was diluted up too 100 mL therefore the solution would be 10 mg/L. The standard that is closest to the sample concentration was prepared (2.0 mL of 10 mg/L was diluted up to 200 mL). Then, 50 mL duplicates of the standard and two 50 mL deionized distilled water as blank were poured into the containers. The standards and blanks were treated exactly as the following procedure. With a 1 cm cell at 880 nm, the absorbance of the 100 µg/L standard minus the blank should be in the range of 0.298 - 0.320. If the standard's absorbance does not fall within this range, then there must be a problem with the analysis.