OPTIMIZATION OF MICROBIAL DNA EXTRACTION FROM *Dendrocalamus asper* (BULUH BETUNG) FOR METAGENOMIC SEQUENCING

by

YAMUNAA D/O SANGAYA

A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Natural Resources Science) with Honours

FACULTY OF EARTH SCIENCE
UNIVERSITI MALAYSIA KELANTAN

2017

DECLARATION

I declare that this thesis entitled "title of the thesis"is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : _____

Name : _____

Date : _____

ACKNOWLEDGEMENTS

First and foremost, a deep sincere thanks to the God for keeping me strong during this period of time and for allowing me to complete this project with ease. Secondly, I would to like convey my heart felt gratitude to my lovely final year project supervisor, Dr. Suganthi Appalasamy for all her kind advices and guidance from the very beginning till the end of this study. She is also one of the women that I look up upon as she had taught me the true definition of resilience and hard work which is needed in order to accomplish success in life. Thirdly, I also owe a piece of appreciation to Dr. Darika Kongrit from Prince of Songkla University, Thailand as she had never failed to guide me through the initial part of this project besides the time and energy that she had spend to teach me the basic techniques of molecular science which was very crucial for me to conduct my project.

Next, I am greatly in debt with my family members for being my pillar of strength all this while. They never failed to be by my side when I needed moral support to lift up my spirit. Apart from that, a big thank you also goes to my dearest friends and seniors who have shared their knowledge, experience and ideas that have kept me motivated and inspired while conducting my project. I would also like to thank the Lab assistants who were kind enough to provide all the needed chemicals and equipments when I was in need.

Without the help that I received from all those I have mentioned above, I could not have come this far. Thanks to all.

TABLE OF CONTENT

	PAGE
DECLARATION	ii
ACKNOWLEDGEMENT	Iii
TABLE OF CONTENT	Iv
LIST OF FIGURES	Vii
LIST OF ABBREVIATION	Viii
LIST OF SYMBOLS	X
LIST OF TABLE	Xi
ABSTRAK	Xii
ABSTRACT	Xiii
CHAPTER 1: INTRODUCTION	
1.1 Background of the Study	1
1.2 Problem Statement	4
1.3 Objective	4
CHAPTER 2: LITERATURE REVIEW	
2.1 Metagenomics	5
2.2 *Ailuropoda melanoleuca* (Giant Panda)	6
2.3 The feeding habit of giant panda	10
2.4 *Dendrocalamus asper* (Buluh Betung)	12
2.5 Gut Microbiota	14

2.6 Illumina-Next Generation Sequencing 16

2.7 MG-RAST Server 17

2.8 DNA Extraction using CTAB 18

CHAPTER 3: MATERIALS AND METHOD

3.1 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* for metagenomic using different method 20

3.1.1 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* using SDS-based buffer method 20

3.1.2 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* using Wizard Genomic DNA Purification Kit 24

3.1.3 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* using liquid nitrogen and Wizard Genomic DNA Purification Kit 26

3.1.4 Extraction of surface microbiota genomic DNA *Dendrocalamus asper* using CTAB-based extraction method 29

3.2 DNA quality and quantity checking using Agarose Gel Electrophoresis and Nanodrop Spectrophotometer 32

3.2.1 Agarose Gel Electrophoresis 32

CHAPTER 4: RESULT AND DISCUSSION

4.1 Importance of DNA extraction protocol in identifying genomic DNA from *Dendrocalamus asper* for metagenomic using different method 34

4.1.1 Effect of surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using SDS-based extraction Method 38

4.1.2 Differences in the preparation for surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using Wizard Genomic DNA Purification Kit and its importance 41

4.1.3 Effect of surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using liquid nitrogen and Wizard Genomic DNA Purification Kit 42

4.1.4 Effect of surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using CTAB-based extraction buffer 45

4.2 Comparisons of the gel electrophoresis and spectrophotometer Nanodrop reading for different DNA extraction method for *Dendrocalamus asper* (Buluh Betung) 47

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion 53

5.2 Recommendations 54

REFERENCES 55

APPENDIX 60

LIST OF FIGURE

Figure	Caption	Page
Figure 1.1	*Ailurus fulgens* (Red Panda)	3
Figure 2.1	*Ailuropoda melanoleuca* (Giant Panda)	9
Figure 2.2	Microbial flora of giant panda	9
Figure 2.3	Bamboo flowering	11
Figure 2.4	*Dendrocalamus asper* (Buluh Betung)	13
Figure 2.5	The culm of *Dendrocalamus asper* (Buluh Betung)	13
Figure 2.6	Distribution of *Dendrocalamus asper* (Buluh Betung) in South East Asia	14
Figure 4.1	Fragments of extracted DNA from *Dendrocalamus asper* (Buluh Betung) using CTAB –based extraction buffer	40
Figure 4.2	Fragments of extracted DNA from *Dendrocalamus asper* (Buluh Betung) using SDS-based extraction buffer	44
Figure 4.3	Fragments of extracted DNA from *Dendrocalamus asper* (Buluh Betung) using Wizard Genomic DNA purification Kit	46

LIST OF ABBREVIATIONS

µL	nanolitre
µg	microgram
mg	milligram
TAE Buffer	Tris-Acetate-EDTA Buffer
COMT	Catechol-o-methyltransferase
CTAB Buffer	Cetylmethlyammonium Bromide Buffer
DNA	Deoxyribonucleic Acid
EBA	Extraction Buffer A
EBB	Extraction Buffer B
EDTA Buffer	Ethyltrimethylammonium bromide Buffer
gDNA	Genomic Deoxyribonucleic Acid
IUCN	INTERNATIONAL UNION FOR CONSERVATION OF NATURE
MGI	Malaysian Genomic Institute
MG-RAST	MetagenomicRapidAnnotations using Subsystems Technology
mL	millilitre
NGS	Next Generation Sequencing

no.	number
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrollidone
RNA	Ribonucleic Acid
rpm	rotation per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium Dodecyl Sulfate
sp	species
TE buffer	Tris-EDTA buffer
UV	Ultraviolet

LIST OF SYMBOL

%	percentage
° C	Degree Celcius

LIST OF TABLE

Table No.	Title	Page
Table 4.0	Nanodrop Spectrophotometer Reading for Extracted DNA from *Dendrocalamus asper* (Buluh Betung) using three different extraction method	43

PENGOPTIMUMAN PENGASINGAN DNA MIKROB DARIPADA *Dendrocalamus asper* (BULUH BETUNG) UNTUK PENJUJUKAN METAGENOMIK

ABSTRAK

Pengasingan DNA mikrob daripada pokok berkayu telah memberikan cabaran khas untuk kerja molekul kerana beberapa metabolit tumbuhan seperti sebatian fenolik mempunyai sifat kimia yang serupa dengan asid nukleik yang menyebabkan sebatian ini untuk bersama-mendakan dengan DNA dan RNA sehingga mengakibatkan kuantiti dan kualiti asid nukleik untuk terjejas. Memandangkan hakikat bahawa yang tidak ada satu pun protokol yang telah diterbitkan sehingga kini boleh digunakan sebagai protokol yang universal untuk mengekstrakkkan DNA mikrob daripada pelbagai jenis tumbuhan, teknik-teknik yang biasa digunakan memerlukan penyesuaian sebelum ia boleh digunakan dengan sampel tumbuhan. Dalam laporan ini, beberapa pendekatan telah diaplikasikan untuk mendapatkan DNA yang berkualiti baik sampel berkayu yang dikenali sebagai *Dendrocalamus asper* (Buluh Betung) termasuklah penggunaan kit konvensional seperti Wizard Genomic DNA Extraction Kit disamping penggunaaan pendekatan tradisional seperti Cetyl trimethyl ammonium bromida (CTAB) dan sodium dodecyl sulfat penampan (SDS). Selain itu, penekanan terhadap kesan penggunaan pelbagai jenis bahan kimia di samping faktor-faktor luaran yang boleh mengubah keadaan DNA yang telah diekstrakkan juga telah dilakukan dalam kajian ini. DNA genomik yang diekstrakkan akhirnya tertakluk kepada agarose gel elektroforesis dan spektrofotometer untuk menentukan tahap hasil dan kualiti DNA. Apabila membandingkan semua protokol yang berbeza , didapati bahawa kaedah konvensional CTAB yang diubah suai dengan teliti telah membuahkan hasil yang konsisten dan lebih tinggi dengan kepekatan DNA sebanyak 19.66 ng/μL manakala hanya sebanyak 8.90 ng/μL telah diekstrakkkan melalui kaedah SDS dan kit konvensional. Kaedah pengekstrakan menggunakakan CTAB terbukti boleh dipercayai dalam menjana kualiti DNA yang baik untuk genera tertentu.

OPTIMIZATION OF MICROBIAL DNA EXTRACTION FROM *Dendrocalamus asper* (BULUH BETUNG) FOR METAGENOMIC SEQUENCING

ABSTRACT

Isolation of genomic DNA of microbial cells in the woody plants presents a special challenge to molecular work especially when several plant metabolites such as phenolic compound that have chemical properties similar to those of nucleic acids which cause these compounds to co-precipitate with DNA and RNA thus resulting in low quantity and quality of the nucleic acid. In view of the fact that none of the published protocol has established itself as universally applicable to extract DNA of plant microbes from all plant varieties, the commonly used techniques require adaptation before it can be used with plant samples. In this work, attempts were made to extract a good quality DNA from woody sample of *Dendrocalamus asper* (Buluh Betung) using different protocols including conventional and extraction kit involving the use of Cetyltrimethylammonium Bromide (CTAB) and Sodium Dodecyl Sulphate (SDS) buffers together with Wizard Genomic DNA purification Kit. Apart from that, the types of chemicals beside the external factors that have significant effects on the condition of the extracted were also emphasized in this review. The extracted genomic DNA was eventually subjected to agarose gel electrophoresis and spectrophotometer to determine its yield and purity levels. In comparison of all the four different protocol, it was found that the carefully modified CTAB conventional method generally produces a consistent and higher yield of DNA with a concentration of 19.66 ng/µL in relative to only 8.90 ng/µL of DNA concentration for DNA extracted using SDS-based DNA extraction buffer as well as the extraction kit. Therefore, the CTAB- based extraction buffer method was proven to be reliable in generating a good quality of DNA from these particular genera.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Ailuropoda melanoleuca or commonly known as giant panda among humankinds is an endangered mammalian species that originally have evolved from omnivorous bear of Ursidae family (Wei *et al*., 2014). Based on the previous studies, giant pandas are carnivore by phylogeny but herbivore by diet as they have retained carnivorous gastrointestinal tract even though their diet is dominated by bamboo (Zhua *et al*., 2012). The presence of short and straight colon with the absence of caecum in the digestive tract of giant pandas are similar to other animals belonging to Order Carnivore and it is ideal and suited for meat processing rather than for bamboo diet (Wei *et al*., 2014). According to Zhao*et al*. (2010), the dietary switch in giant pandas are probably due the malfunction of umami taste receptor leading to Tas 1R1 gene pseudogenization which causes the giant pandas to lose their ability to sense and taste protein compound. Therefore, giant pandas are believed to consume bamboo of low nutrition content as their main food source to promote growth and survivorship.

It is also believed that 99% of their diet is composed of highly fibrous bamboo even though giant pandas are genetically deficient in cellulose digesting enzymes as well as lignin-degrading genes in their genome (Fang *et al*., 2012). The capability of the giant

pandas to partially digest lignin and lignin-related phenolic compound such as cellulose and hemicellulose from bamboo has puzzled scientist as it is impossible to do so based on the giant pandas genetic composition (Zhua *et al*., 2012). Additionally, the ability for giant pandas to sustain living with such a low digestion coefficients for bamboo with only 17% of dry matter consumed through their simple digestive system have led to various speculations because even more than 40% of ruminants faces difficulties to optimize plant fibre digestion even though they have the enzymes and bacteria to degrade cellulose component (Hofmann,1989). This situation leads to speculations that the digestion of bamboo fiber is contributed by symbiosis relationship of the microbial in the gut of giant pandas. This hypothesis was then supported by findings in earlier researches whereby the scientists have detected the presence of 13 operational taxonomic units closely related to *Clostridium* group I and XIVa including putative genes coding for two cellulose-digesting enzymes, one hemicelluloses digesting enzyme, cellulase, β-glucosidase, and xylan 1,4-β-xylosidase which is known for lignin degradation in the guts of giant pandas through metagenomic analysis (Fang *et al*., 2012).

According to Fang *et al*. (2012), seven out of the 13 operational taxonomic units (OTUs) were unique to pandas compared with other mammals and the high proportion of *Firmicutes* OTUs in gut of giant pandas is the same as to other herbivores. However, Xue *et al*. (2015) argued that the contribution of these microbes in the digestion of bamboo is still not clear because proper mechanism of the intestinal microbes on the bamboo digestion is yet to be developed. Interestingly, red panda or also known as *Ailurus fulgens* (Figure 1.1) is also a carnivore that developed several similar morphological features as in

giant pandas such as the false thumb in adaptation to the same dietary switch to bamboo (Li *et al*., 2015). Even though red panda and giant panda have the same diet, but it has been found that the gut microbiota of giant panda diverged rather than converged with that of red panda (Li *et al*., 2015). The differences in gut microbes in both of red panda and giant panda might be due to the differences in habitat and also due to the difference in the species of bamboo plant that they consume. Furthermore, the comparative studies on the microbe community in the guts of red pandas and giant pandas have revealed that the gut microbes vary according to the seasons. Hence, this leads to the possibilities that not the gut microorganisms that are actually involved in the degradation of the bamboo fibers but it is the microbes from the bamboo plant itself that contributes in digestion of cellulose compounds of the bamboo in giant pandas gut.

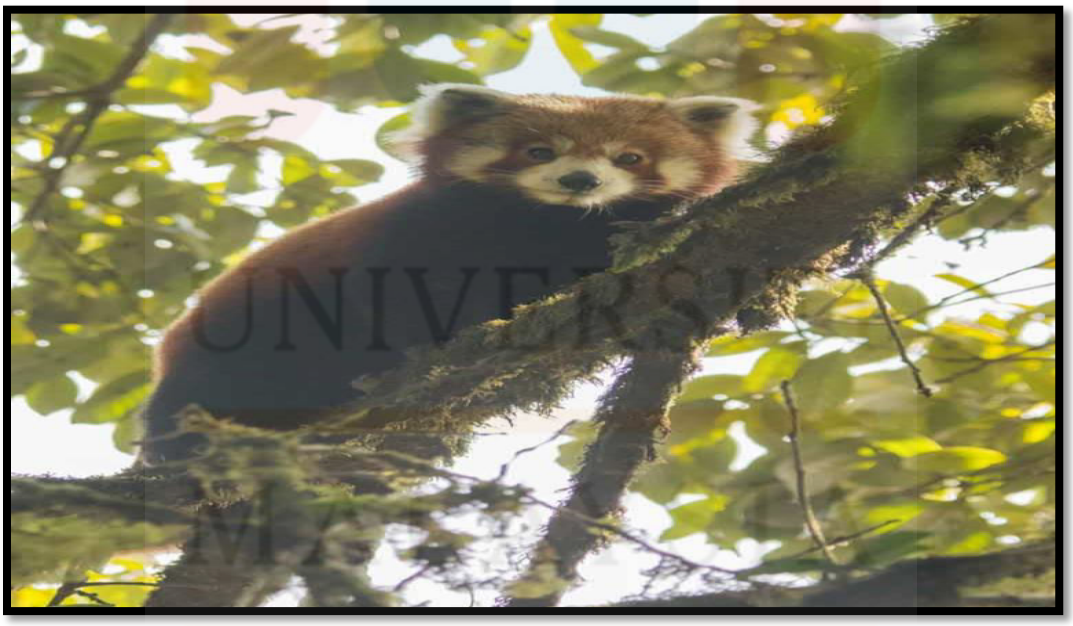


Figure 1.1: *Ailurus fulgens* (Red Panda)

(Source: Paul McDougall, October 2016)

1.2 Problem Statement

This study aimed to identify the type of microorganisms in *Dendrocalamus asper* (Buluh Betung) through metagenomics analysis. This is because a limited number of researches have been done on the potential contribution of the microorganisms found in the bamboo with the guts of giant pandas in digestion of bamboo.

1.3 Objectives

To identify the diversity of microorganisms that can be found in *Dendrocalamus asper* (Buluh Betung) through metagenomic analysis

CHAPTER 2

LITERATURE REVIEW

2.1 Metagenomics

Metagenomics is the study of genetic material obtained directly from environmental samples including the gut, soil, and water (Rahul *et al*., 2015). According to Leveau *et al*. (2007), metagenomics use DNA as a genetic information carrier in research field especially to facilitate the development biocatalyst as well as novel genes for being used in various sectors such as pharmaceutical, biotechnology and also industry. Metagenomic analysis can be performed based on few procedures in an arranged manner starting with isolation of metagenomic DNA, cloning of metagenomic DNA, screening of metagenomic library, DNA sequencing and sequence analysis.

According to Rahul *et al*. (2015), a few studies have been done on the mammals using metagenomic analysis. This includes research on gut microbial community structure and its functions in different host species such as human, mouse, cow, giant panda, cow, feline, yak and canine. Through this research, the diversity of the microbial community in the guts of different organism is identified through metagenomic analysis. Besides that, the healthy microbiota and the deviations related to etiopathogenesis of disease were also identified through metagenomic analysis. This discovery is important to predict the development of disease as well to generate microbiota-targeted therapies

mainly to digestive system related diseases such as inflammatory bowel disease apart from neurological disorders like Alzheimer's disease, obesity related metabolic disease like atherosclerosis as well as cancer. In the case of giant panda, the evidence for lignin oxidation by giant panda fecal microbiome was from 16S rRNA pyrosequencing method (Rahul *et al*., 2015).

Not only that, the evidence for cellulose metabolism by giant pandas gut microbiota besides the evolution of bacteria in giant pandas and red pandas were also studied through metagenomic analysis. As for research on bamboo, early research on the presence of endosymbiotic microbiota of the bamboo pseudococcid known as *Antonina crawii* has been done (Takema *et al*., 2000). Not only that, the bacterial communities for the bamboo retting process was also analyzed by culture-independent tools based on the PCR amplification of the 16S rRNA fragments (Fu *et al*., 2011).

2.2 *Ailuropoda melanoleuca* (Giant Panda)

The giant panda with scientific name *Ailuropoda melanoleuca* (Figure 2.1) refers to black and white cat-footed bear that has been listed as an endangered mammalian species in the World Conservation Union's (IUCN's) Red List of Threatened Species. Based on studies by Xue *et al*. (2015), it is proven that giant pandas are omnivorous bear with a carnivorous gastrointestinal tract but they have undergone adaptive evolution as herbivore over time when their diet is mainly dominated by bamboo. This statement is

supported by the evidence that gut microbiotas of the giant pandas are clustered closer to those of the carnivorous Asian black bears which are phylogenetically closer to the giant panda (Li *et al*., 2015).

Besides that, the DNA samples of omnivorous bear and carnivorous tiger showed gut microbiota structure similar to that of these giant pandas, but samples from other omnivores and herbivores diverged remarkably. The switch in diet has occurred because of diminished attraction of returning to meat consumption in the absence of Tas 1R1 gene in giant pandas. Not only that, but the deletion in the catechol-o-methyltransferase (COMT) gene in the appetite reward system, which is probably result in loss of function in giant pandas catechol- amine metabolic pathways may also govern the species' food choice (Wei *et al*., 2014). Furthermore, the unique diet of giant pandas have also resulted in morphological adaptation such as the development of pseudothumb that is crucial in grasping bamboo besides the large and flat teeth with an elaborated crown pattern that provides efficient crushing surface to enable effective digestion of coarse bamboo (Zhua *et al*., 2012).

Apart from that, the ability for giant pandas to survive by only obtaining a low amount of nutrient from the digestion of highly fibrous bamboo plant has amazed the researches. This is because the recent genome analysis of giant panda has revealed that its gene encodes all the enzymes needed for carnivorous digestive system but lacking of those which are responsible for the digestion of cellulose and hemicelluloses in the gut of

giant panda (Zhua *et al*., 2012). The scientists have detected the presence of 13 operational taxonomic units closely related to *Clostridium* group I and XIVa including putative genes coding for two cellulose-digesting enzymes, one hemicelluloses digesting enzyme, cellulase, β-glucosidase, and xylan 1,4-β-xylosidase which is known for lignin degradation in the guts of giant pandas through metagenomic analysis (Fang *et al*., 2012).

According to Fang *et al*. (2012), seven out of the 13 operational taxonomic units (OTUs) were unique to pandas compared with other mammals and the high proportion of *Firmicutes* OTUs in gut of giant pandas is the same as to other herbivores. Apart from that, the enzymatic processes as well as the role of intestinal guts in lignin-related degradation in the guts of panda remain unclear. Additionally, the seasonal variation of the gut microbes in panda (Figure 2.2) has developed the possibilities that microorganisms in the bamboo are the one that actually responsible for the digestion of cellulose compounds.

Figure 2.1 *Ailuropoda melanoleuca* (Giant Panda)

(Source: Jennifer Becerra, 13 May 2016)

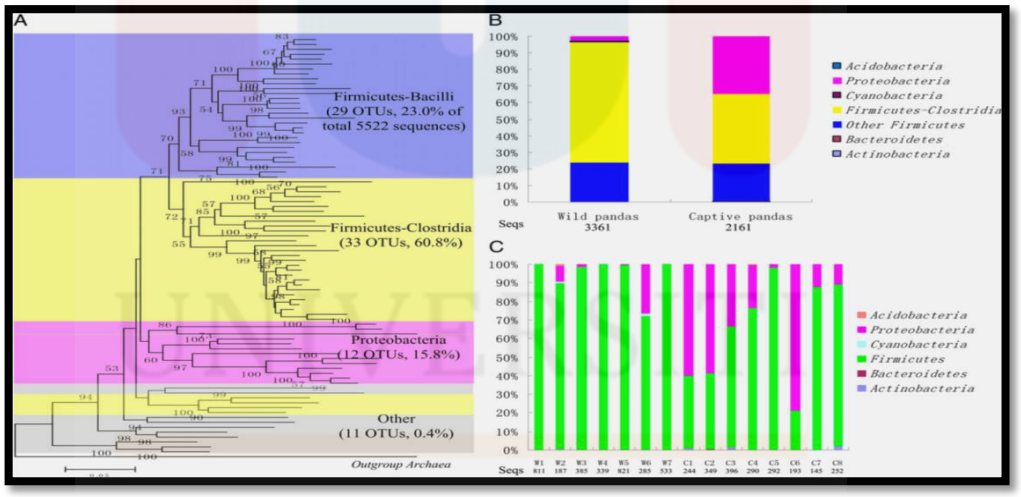


Figure 2.2 Microbial flora of giant panda

(Source: Lifeng et al., 2011)

2.3 The feeding habit of giant panda

Plant Bambusoidea which is commonly known as bamboo belongs to one of the 12 sub-family of Poaceacae (grass family) with a distribution of over 1439 species worldwide and it is predominantly found in Southeast Asia. Bamboo plant which is made up of leaves, shoot, culm sheath, culm and roots are widely used as food source and also for medicine benefits. Several studies have indicated that the lignin component is the major barrier during the enzymatic hydrolysis of cellulose (Wei *et al*., 2014). Bamboo constitutes the primary source of nutrition for wild giant panda with 99% of its diet is composed of different parts of bamboo plant. According to David *et al*. (2006), the nutrient concentration of bamboo plant varies between the species of the bamboo apart from the age and differences in the parts of the plant. David *et al*., (2006), have also argued that the giant pandas have higher preference for younger tissues in relation to greater concentration of N-P-Ca minerals in younger tissues compared to higher concentration of structural components such as cellulose, hemicelluloses and lignin with diluted nutrients in older tissues. Similarly, the cell walls of fibres were thicker in the older shoot.

As for panda in captivity, a wide diversity of non-bamboo food is prepared to supplement a variety of proteins, vitamins and minerals which are needed to support their growth. This includes wide range of grains, meat, eggs, carrots, yams, apples, sweet potatoes as well as bamboo cakes that imitates the structural carbohydrate content of bamboo leaves. The introduction of different types of food to captive panda as a

substituent to bamboo is one of the alternative approach to overcome problems related to the shortage of bamboo supply. This is because the natural phenomenon known as bamboo flowering (Figure 2.3) reduces the food availability for giant pandas for a long period once the bamboo flowers and dies off (Wei *et al*., 2014). It is also believed that giant pandas might consume the inner part of bamboo which is high in extractive and soluble substances primarily the fat, resin and water soluble substances rather than the thicker cell wall fibres which is thickering the older outer shoot which is relatively harder to be digested by the giant panda (Christina & Richard, 2004).

Figure 2.3: Bamboo flowering

(Source: Stephane Schroder, 18 December 2011)

2.4 *Dendrocalamus asper* (Buluh Betung)

Plant microbiota which refers to the microorganisms that live in any plant tissues in the existing environment is the least explored area of metagenomic research compared to soil, human and water metagenome (Wang, 2008). By referring to that, it is believed that the colonization of different kinds of microbes at distinctive parts of the plant for instance bamboo is yet to be identified. Hence, the research on the potential contribution of the microorganism found in *Dendrocalamus asper* (Figure 2.4)in aiding the digestion of lignin related compound in the guts giant pandas is only possible by first discovering the type of microorganism that present in that bamboo species.

Dendrocalamus asper or known as the giant bamboo other than rough bamboo is a bamboo species which is native to Southeast Asia (Figure 2.7). This bamboo originates from Malaysia with lance-shaped leaf blades and many clustered branches with one central dominant branch. Besides that, this plant is able to survive in various soil types and the best growth is shown in well drained heavy soils. This timber bamboo is also well-known for its functions as raw material for fiber board production and as a building material for heavy construction (David *et al*., 2006).

Figure 2.4: *Dendrocalamus asper* (Buluh Betung)

(Source: Palaska, 25 January 2010)

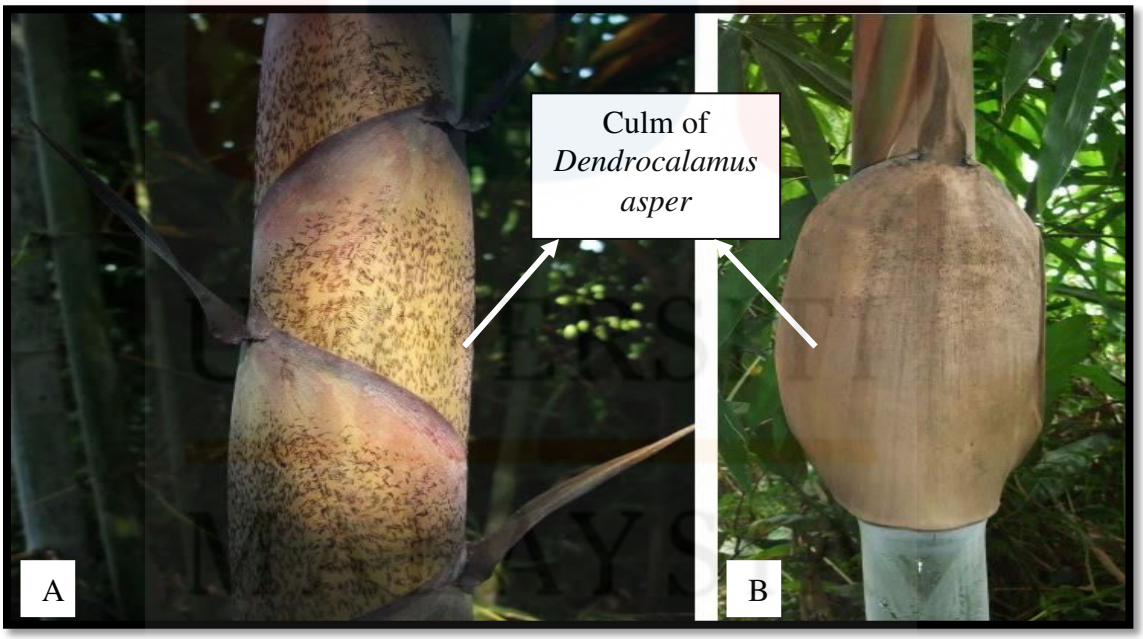
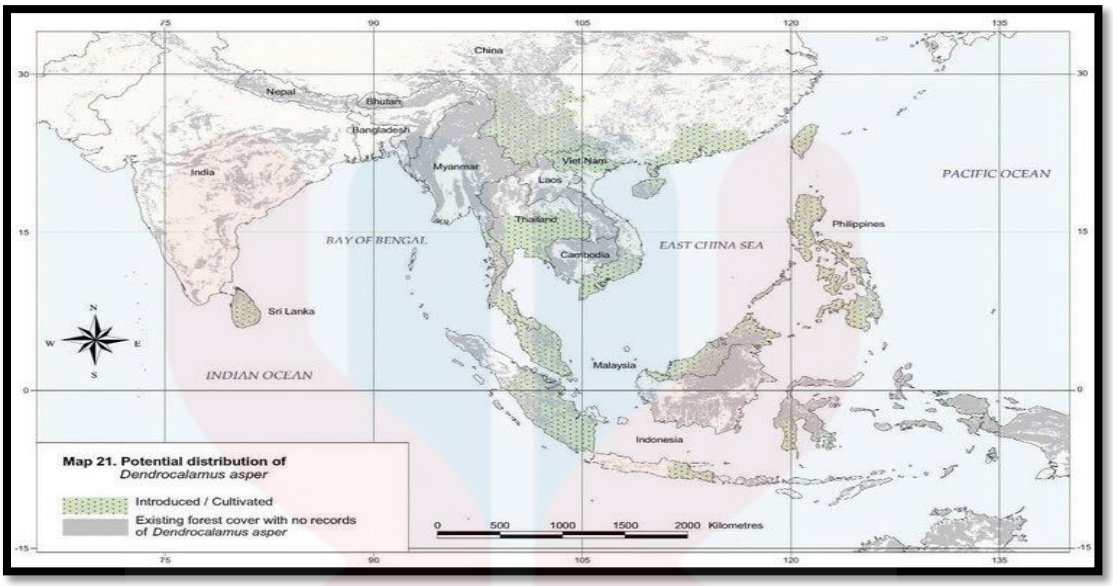


Figure 2.6: Photo A and B shows the culm of *Dendrocalamus asper* (Buluh Betung)

(Source: Somjit, 25 May 2009)



(Figure 2.7 Distribution of *Dendrocalamus asper* in South East Asia)

(Source: Stephane Schroder, 25 January 2010)

2.5 Gut Microbiota

Gastrointestinal microbiota refers to the dense population of microorganisms such as bacteria, Archaea, yeast, fungi and protozoa that resides in the gastrointestinal tract of animals (Linda *et al*., 2014). The homeostatic symbiosis of mammals such as giant panda with their gastrointestinal microbiota is relatively important because microbes provide nutritional contribution to the host whereas the host provides microorganisms with a stable environment together with nutrients to support their livings. Besides that, a research on microbiota has revealed that animals usually acquire most of their gut microbiota from the environment after they are born and the microorganisms will undergo succession until a stable microbiota is established (Linda *et al*., 2014).

According to Shamayim *et al*. (2012), the gut microbiota differs from individual to individual because the variation in bacteria composition in the gut may be due to genetic differences apart from responds to age, diet type as well as the type of species. In comparison to animal classification based on diet, herbivores exhibits the largest diversity of gut microbes with plant associated bacteria such as endotypes that are able to survive in acidic stomach digestion even though they originates from plant tissues(Shamayim *et al*., 2012).

Several studies on 16S rRNA sequencing have also discovered that more than 80% of identified phylotypes in the guts of wide range of mammals actually belong to the phyla of *Firmicutes* and *Bacteroidetes.* Research by has also proven that phyla *Firmicutes* dominate the gut microbiota of giant panda. To validate this findings, metagenomic analysis using37-Mbp contig sequences from gut microbes was conducted and 13 operational taxonomic units closely related to *Clostridium* groups I and XIVa from phyla *Firmicutes* including putative genes coding for two cellulose-digesting enzymes, one hemicelluloses digesting enzyme, cellulase, β-glucosidase, and xylan 1,4-β-xylosidase which is known for lignin degradation was detected (Fang *et al*., 2012). In addition, it is believed that all the previously described OTUs were related to phylotypes known to reside in the intestine of rumen but result of study indicated that the bacteria population in the intestine of giant panda is markedly different from herbivores. Apart from that, the elucidation of the intestinal microbial community structure of giant panda is crucial in order to understand the diet paradox.

2.6 Illumina-Next Generation Sequencing

Illumina-Next Generation Sequencing (NGS) is an advanced whole genome sequencing that has been developed to overcome limitations and barriers faced by other existing sequencing approaches such as targeted sequencing, 16S metagenomic sequencing as well as Sanger sequencing. Illumina sequencing uses sequencing by synthesis approach that is involves bridge amplifications on the surface of a flow cell. NGS has shown multiple benefits in microbiology research that not only has revealed exploration on the genome, epigenome and transcriptome but also enables the extraction of genetic information from biological system. Basically, NGS sequences are generated without the need of a conventional vector based cloning procedure which is needed to amplify and separate DNA template (Shadi *et al*., 2012).This sequencing approach is also suitable to be used when the data analysis which is relatively small due to presence of small genome. Besides that, Illumina-next generation sequencing have advantages over other traditional method because it has high data output on the genome of a microorganism apart from its ability to identify changes anywhere in the genome without any knowledge.

In addition, this specific sequencing approach is capable in identifying rare variant that are missed out using CE-based sequencing just in a single run as well as to track microbial adaptation in a very short time using single base resolution. As for the concept of NGS, it involves simple steps whereby the single genomic DNA (gDNA) is fragmented into a library of small segments before it is sequenced in parallel (Shadi *et al*.,

2012). Then, the individual sequenced reads are reassembled by aligning to a reference genome and the whole-genome sequence is derived from the concensus of aligned reads. Apart from that, NGS also prevent cloning bias issues that impact sequencing evenness in sequencing project by performing nucleotide detection one at time

On the other hand, there are some limitations when dealing with NGS. One of it is that NGS have a relative short read length because of optimal signal decay and dephasing (Shadi *et al*., 2012). Not only that, but this drawback limits the application of technologies in situation whereby no reference sequence is available to assign, align and annotate the short sequence that have been generated. The error also increases with a longer sequencing and NGS workflow are time consuming, tedious and require professionals to handle it. Therefore, enhancement of NGS technologies was done to overcome the limitation problem faced by NGS which includes target selection of NGS which is known as sequence capture are applied to eliminate PCR amplications and allow selective analysis of a large number of target sequence.

2.7 MG-RAST Server

Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) is a fully automated, freely available open source annotation service based on SEED framework which is widely used in comparative genomic study. This server compares both protein and nucleotide database using automated functional assignments sequence in

metagenome for complete or nearly complete archaeal and bacterial genome (Meyer *et al*., 2008). It is ideal to use MG-RAST to analyse metagenomic data as it generated to achieve consistency, accuracy and completeness on the use growing library of subsystem that are manually curated. Furthermore, MG-RAST server is also a service that provides a framework for sharing dataset with multiple users at the same it also controls the user access to ensure privacy (Meyer *et al*., 2008). Besides that, all the data on genome is also available for download in a variety of format and all the user have gained full control on their data.

2.9 DNA Extraction using CTAB-based Buffer

Cetylmethly -ammonium bromide or commonly known as CTAB is a salt that act as an effective reducing agent and also as precipitants of nucleic acid and polysaccharides CTAB extraction method with its main function to remove secondary metabolites such as polysaccharides and polyphenols from the plant cell is originally introduced by Doyle & Doyle (Hosseinpour *et al* ., 2013).

Besides that, DNA isolation using CTAB is one of the preferred DNA isolation method especially for plant samples. This is because from CTAB solve the limitation problem of collecting good quality of DNA from mature plant that are composed with high concentration of secondary metabolites. Thus, the extraction of DNA is now possible not only from young plant tissue but also mature tissues which also vital for comparison

studies that uses age factor as an influencing factor for the final result of the studies for instance the identification of the type of microorganisms together with its concentration in bamboo plant at different maturity level. Apart from that, DNA isolation using this method also helps to deal with problems involving DNA contamination, degradation as well as low yield of DNA as a result from irreversible attachment of phenolic compound with DNA molecules from plant samples (Naveed & Saeed ,2011). Not only that, but reproducible and consistent amplification product is also produced using this method which strongly prove the suitability of that DNA for PCR application. DNA extraction using CTAB have also resulted in a very high intact genomic DNA as the DNA is not hydrolysed during the isolation process. According to Hosseinpour *et al*. (2013), this method also is safe to be used unlike phenol-chloroform extraction method that causes serious damage to human health.

Interestingly, a good quality of DNA can only be generated with the action from other chemicals in CTAB buffer. This includes the role of β-mercaptoethanol and polyvinyl pyrrollidone (PVP) to remove phenolic compound. Other than that, chloroform-isoamyl alcohols are important to manage cellular proteins as well as to remove different colouring agents such as dyes, chlorophyll and pigments (Naveed & Saeed, 2011).

CHAPTER 3

MATERIALS AND METHOD

3.1 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* for metagenomic sequencing using different method

3.1.1 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* (Buluh Betung) using SDS-based buffer method

Following the method by Hao *et al*. (2008), the young and matured stem *of Dendrocalamus asper* (Buluh Betung) measuring a length of 30 cm each was collected from the bamboo store in Malaysia National Zoo, Selangor. The both collected bamboo samples were then cut into 15 cm before placing the bamboo in a zip bag containing ice cubes to keep the sample fresh apart from to ease the sample transportation process from Malaysia National Zoo to Prince of Songkla University, Thailand. The bamboo sample was then placed in -20°C freezer before transferring it to -80°C chiller which was located at Biochemistry Laboratory of Prince of Songkla University, Thailand after labeling the zip bag completely with the students details.

The bamboo sample preparation was started by referring to the protocol established by Hao *et al*. (2008), whereby the was defrosted the bamboo sample at room temperature for 15 minutes after it was taken out of the -80°C freezer. After that, one piece of the matured bamboo sample with an average length of 15 cm was taken out of

the zipper bag and it was placed on the working bench that has been sterilized using 70% ethanol. The bamboo sample was then chopped roughly into small pieces using an autoclaved knife.

As for DNA extraction, the extraction was performed based on the modified protocol of Hao *et al*. (2008). Firstly, an approximate of 2 g of the chopped bamboo pieces were transferred into a new sterile 50 ml centrifuge tube and 300 ml of TE buffer was added slowly to the finely chopped bamboo pieces. The bamboo solution was then homogenized using homogenizer at 18 000 r.p.m for 1 minute. After that, the homogenized bamboo solution was filtered using Erlenmeyer flask with 0.03µm autoclaved filter membrane before using the filtrate to be filtered again using a sterilized cloth and followed by filtration using Erlenmeyer flask with 40 µl filter membrane.

After completing the filtration process, the bamboo filtrate was transferred into a new autoclaved 50 ml centrifuge tube and the filtrate was centrifuged at room temperature for seven minutes at 200 g. After the centrifugation process, the formed supernatant was pipette out and transferred into a new autoclaved 50 ml centrifuge tube where 1.79 g of NaCl (at a final concentration of 0.9%) and 1250 µl of 10% of SDS (at a final concentration of 0.063%) were added directly to the supernatant. The bamboo lysate was mixed gently with the buffers before the 50 ml centrifuge tube containing the bamboo lysate was incubated at 4°C for overnight in the refrigerator. The upper phase of the incubated bamboo lysate was carefully transferred a new autoclaved 50 ml centrifuge

tube without disturbing the precipitate. Next, the weight of the centrifuge tube containing the upper phase of the bamboo lysate was measured and a balance with a difference of 0.01 g was prepared for centrifugation. The bamboo lysate was then centrifuged at 5000 g for 12 minutes at 4°C. After the centrifugation process, the pellet which is formed at the bottom of the 50 ml centrifuge tube was collected after discarding the supernatant. The pellet was then resuspended in 50 ml of TE buffer before 0.45 g of NaCl and 315 µL of 10% of SDS were added simultaneously to the pellet. The mixture was mixed well by inverting the 50 ml centrifuge for 50 times and the mixture was placed in the refrigerator to incubate the mixture at 4°C for overnight. Next, the overnight incubated mixture was centrifuged at 5000 g for 10 minutes at 4°C to collect the pellet which is highly rich in microorganisms.

After centrifugation process was completed, 3 ml of TE buffer was added to 0.17g of the recovered pellet. After the addition of TE buffer, the pellet was also treated with 10 µl of lysozyme (2 mg/ml) for 1 hour and 15 minutes at temperature of 37°C. After that, the mixture was centrifuged at room temperature for 12 minutes at 200 r.p.m and pellet which is formed at the bottom of the 50 ml centrifuge tube was collected by transferring the above supernatant layer into a new 50 ml centrifuge tube. The centrifuge tube with the pellet was labeled as A whereas the centrifuge tube containing the supernatant was labeled as B. the centrifuge tube labeled as B was centrifuged again at 12000 g for 10 minutes at room temperature. After the centrifugation process, the pellet formed in the 50 ml centrifuge tube which is labeled as B was collected after removing the supernatant. Next, 500 µl of proteinase K solution (500 mM Tris, 10 mM NaCl, 20 Mm EDTA, 1%

SDS) was added to the pellet in the 50 ml centrifuge tube that is labeled as B. The mixture was then incubated in dry incubator at 55°C for 6 hours. The mixture was incubated again ata higher temperature of 60°C for 20 minutes in the dry incubator to deactivate proteinase K and lysozyme enzyme. After the incubation process, the mixture was stored in the refrigerator at 4°C for overnight. Next, 500 µl of lysate was pippeted out of the incubated mixture and it was transferred into an autoclaved 1.5 ml eppendorf tube. The lysate in the 15 ml centrifuge tube was extracted twice with 500 µl of chloroform-isoamyl alcohol with a ratio of (24:1) before centrifuging the mixture at 12000 g for 12 minutes at room temperature.

From the centrifugation process, the formed supernatant was transferred into a new autoclaved 1.5 ml eppendorf tube and 750 µl of isopropanol was added to the supernatant before the eppendorf tube was inverted for 50 times. The supernatant with isopropanol in it was incubated for 1 hour 30 minutes at 20°C in the dry incubator before it is centrifuged at 12,000 g for 20 minutes at room temperature. After centrifugation, the supernatant was completely removed while 250 µl of 70% ethanol was added to the pellet and the eppendorf tube was inverted up and down for 20 times. The pellet was then centrifuged at 12,000 g for 12 minutes at room temperature before the pellet formed from the centrifugation process was rinsed with 500 µl of ethanol .The DNA pellet was stored in 10 µl of TE buffer .

3.1.2. Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* (Buluh Betung) without liquid nitrogen for Wizard Genomic DNA Purification Kit

A young and matured stem *of Dendrocalamus asper* (Buluh Betung) measuring a length of 30 cm was collected from the bamboo store in Malaysia National Zoo, Selangor. The collected bamboo sample was then put and sealed in a zip bag containing ice cubes to keep the sample fresh apart from to ease the sample transportation process. The bamboo sample was then placed in -20°C freezer after labeling the zip bag completely in the Post Graduate Laboratory in Science building of Universiti Sains Malaysia, Penang.

In order to carry out microbial genomic DNA extraction from *Dendrocalamus asper*, firstly the bamboo sample preparation must be done. As for DNA extraction using Wizard Genomic DNA Plant Purification Kit, the sample preparation as well as the microbial genomic DNA extraction was carried out at Biotechnology Laboratory which is placed in the Science building of Universiti Sains Malaysia, Penang. The bamboo sample preparation was begun by defrosting the bamboo at room temperature for 30 minutes after it was taken out of the -20° C freezer.

By following the technical manual of Wizard genomic DNA purification kit (2011), the DNA extraction was carried out. One piece of bamboo with an average length of 10 cm was taken out of the zipper bag and it was placed on the working bench that has

been sterilized using 70% ethanol. The exposed inner layer of the bamboo piece was then swabbed randomly in a repeated manner using a cotton bud that has been sterilized using double-distill water**.** Later on, the sterilized cotton bud was immersed in 600 µl of Nucleic lysis buffer which is poured into a new 1.5 ml microcentrifuge tube each and every time the bamboo tissue is swabbed with the cotton bud until the solution appears cloudy. The mixture was vortexed for three seconds to wet the bamboo tissues completely in the nucleic lysis buffer before the 1.5 ml centrifuge tube containing the bamboo mixture was incubated at 65°C for 15 minutes in the water bath. After the incubation process was completed, 3 µl of RNase solution was added to the bamboo lysate in 1.5 ml centrifuge tube and the lysate was mixed well by inverting the 1.5 ml centrifuge tube up and down for 5 times.

Next, the bamboo lysate was incubated at 37°C for 15 minutes in water bath and the bamboo lysate was allowed to cool to room temperature for 5 minutes after the incubation process. Once the bamboo lysate was cooled, 200 µl of Protein Precipitation solution was added to bamboo lysate and it was vortexed vigorously at high speed for 20 seconds. Subsequently, the bamboo lysate in the 1.5 ml centrifuge tube was centrifuged at room temperature for 3 minutes at 13,000 g. After centrifugation was completed, the supernatant which contains the DNA was carefully pipetted out using 1000 µl pipette and it was transferred into a new clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. After the supernatant was mixed well with isopropanol, the bamboo lysate was gently mixed by inversion until a tread like strands of DNA forms a visible mass. The bamboo lysate was then centrifuged at 14,000 g for 1 minute. After

centrifuging, the formed supernatant was carefully decanted and 600 µl of room temperature ethanol was added to the DNA pellet which is attached at the side of the 1.5 ml centrifuge tube. The 1.5 ml centrifuge tube was inverted for 50 times to washout the excess salts on the DNA pellet. The DNA pellet was then centrifuged at room temperature for 1 minute at 14,000g. The supernatant was removed completely after the incubation process and the 1.5 ml centrifuge tube was inverted onto a clean absorbent paper to air dry the DNA pellet for 15 minutes. Next, the air-dried DNA pellet was added to 100 µl of DNA rehydration solution before it was incubated at 65 °C for 1 hour in water bath. The solution was mixed gently by tapping the microcentrifuge tube.

3.1.3 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* (Buluh Betung) using liquid nitrogen and Wizard Genomic DNA Purification Kit

The young and matured stem *of Dendrocalamus asper* (Buluh Betung) measuring a length of 30 cm was collected from the bamboo store in Malaysia National Zoo, Selangor. The collected bamboo sample was then put and sealed in a zip bag containing ice cubes to keep the sample fresh apart from to ease the sample transportation process. The bamboo sample was then placed in -20°C freezer after labeling the zip bag completely in the Post Graduate Laboratory in Science building of Universiti Sains Malaysia, Penang.

In order to carry out microbial genomic DNA extraction from *Dendrocalamus asper*, firstly the bamboo sample preparation must be done. As for DNA extraction using Wizard Genomic DNA Plant Purification Kit, the sample preparation as well as the microbial genomic DNA extraction was carried out at Biotechnology Laboratory which is placed in the Science building of Universiti Sains Malaysia, Penang. The bamboo sample preparation was begun by defrosting the bamboo at room temperature for 30 minutes after it was taken out of the -20° freezer. Next, one piece of bamboo with an average length of 10 cm was taken out of the zipper bag and it was placed on the working bench that has been sterilized using 70% ethanol. Only the inner tissue layer of the bamboo plant was scrapped using a sterilized knife and the collected tissue layer was crushed into fine powder by using liquid nitrogen.

As for the DNA extraction process, the technical manual of Wizard Genomic DNA Purification Kit (2011) was used as a reference to extract genomic DNA using the kit. 2 g of bamboo powder which was formed by crushing the inner layer of the bamboo piece with liquid nitrogen was immersed in 600 µl of nucleic lysis buffer. The mixture was vortexed for three seconds to wet the bamboo tissues completely in the nucleic lysis buffer before the 1.5 ml centrifuge tube containing the bamboo mixture was incubated at 65°C for 15 minutes in the water bath. After the incubation process was completed, 3 µl of RNase solution was added to the bamboo lysate in 1.5 ml centrifuge tube and the lysate was mixed well by inverting the 1.5 ml centrifuge tube up and down for 5 times. Next, the bamboo lysate was incubated at 37°C for 15 minutes in water bath and the bamboo lysate was allowed to cool to room temperature for 5 minutes after the incubation process. Once

the bamboo lysate was cooled, 200 µl of Protein Precipitation solution was added to bamboo lysate and it was vortexed vigorously at high speed for 20 seconds. Subsequently, the bamboo lysate in the 1.5 ml centrifuge tube was centrifuged at room temperature for 3 minutes at 13,000 g. After centrifugation was completed, the supernatant which contains the DNA was carefully pipetted out using 1000 µl pipette and it was transferred into a new clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. After the supernatant was mixed well with isopropanol, the bamboo lysate was gently mixed by inversion until a tread like strands of DNA forms a visible mass.

The bamboo lysate was then centrifuged at 14,000 g for 1 minute. After centrifuging, the formed supernatant was carefully decanted and 600 µl of room temperature ethanol was added to the DNA pellet which is attached at the side of the 1.5 ml centrifuge tube. The 1.5 ml centrifuge tube was inverted for 50 times to washout the excess salts on the DNA pellet. The DNA pellet was then centrifuged at room temperature for 1 minute at 14,000g. The supernatant was removed completely after the incubation process and the 1.5 ml centrifuge tube was inverted onto a clean absorbent paper to air dry the DNA pellet for 15 minutes. Next, the air-dried DNA pellet was added to 100 µl of DNA rehydration solution before it was incubated at 65 °C for 1 hour in water bath. The solution was mixed gently by tapping the microcentrifuge tube.

3.1.4 Extraction of surface microbiota genomic DNA from *Dendrocalamus asper* (Buluh Betung) using CTAB-based buffer method

The stem of *Dendrocalamus asper* (Buluh Betung) measuring a length of 15 cm was cut down from Lojing Highlands, Kelantan using a machete and the bamboo sample was put and sealed in a zip bag containing ice cubes to keep the sample fresh apart from to ease the sample transportation process. The bamboo sample was then placed in -20°C freezer which is located in the Postgraduate Laboratory of University Malaysia Kelantan after labeling the zip bag completely.

The bamboo sample was prepared in the Environmental Laboratory which is located at the second floor of University Malaysia Kelantan. The most crucial step before conducting any molecular work is the preparation of sterilized and antiseptic environment to prevent undesirable contamination on the sample. Therefore, the working bench in laminar flow cabinet in the Environmental Laboratory was sterilized by spraying 70% of ethanol before arranging the cleaned apparatus for sample preparation on the bench. As for the sample preparation, the bamboo was left to defrost for 10 minutes after it was taken out of the -20°C freezer. The bamboo sample was then placed on top of a clean zipper bag which was put on the floor outside of the Environmental Laboratory before the bamboo was cut horizontally into half using a sterile knife. After the bamboo sample was cut open into half, the bamboo was further cut into a smaller piece with an estimated length of 15 cm each. The bamboo that has been cut into smaller pieces was then placed

in a new zipper bag before it is taken to the laminar flow chamber to carry out the next step.

By following the modified method established by He (2011), DNA extraction was started by taking out only one piece of bamboo with an average length of 15 cm from the zipper bag and the exposed inner layer of the bamboo piece was then swabbed randomly in a repeated manner using sterilized cotton bud in the laminar flow**.** Next, the sterilized cotton bud was immersed in 15 ml of double distilled water (DDH_2O) each and every time the bamboo tissue is swabbed with the cotton bud. After that, only an aliquot of 1ml of the bamboo solution out of 15 ml of previously prepared bamboo solution was transferred into a new 2 ml eppendorf tube before the aliquot in the eppendorf tube was inverted vigorously for 50 times. The well-mixed bamboo solution of 1ml was then centrifuged at 1 000 r.p.m for 10 minutes.

The bamboo solution was separated into two distinct layers after centrifugation which is an aqueous layer that is known as supernatant together with sediment that settle downs at the bottom of the eppendorf tube. By referring to that, the supernatant which was formed from the centrifugation process was pipetted out from the bamboo solution while the sediment which is attached to the bottom of the eppendorf tube was recovered. After recovering the sediment, 560 µl of TE buffer together with 600 µl of CTAB buffer were added simultaneously to the sediment in the eppendorf tube. The eppendorf tube was inverted up and down for 50 times. Next, two types of enzymes which are 5µl of

proteinase K and 5 µl of lysozyme were added directly to the lysate and the lysate was mixed well with the buffer and enzymes when the 2 ml eppendorf tube was inverted up and down for 50 times. Consequently, the lysate was incubated in water bath for 30 minutes at 65 °C. Following the incubation process, an equal volume of lysate was divided into two new 2ml eppendorf tube labelled as sample A and sample B respectively whereby each eppendorf tube containedan estimated of 750 µl of bamboo lysate. This step was then followed by the addition of 300 µl of chloroform into each of the 2 ml eppendorf tube and the lysate in both of the 2 ml eppendorf tube were left aside at room temperature for 5 minutes. After 5 minutes, the both 2 ml eppendorf tube that contains the lysate was centrifuged for 10 minutes at 14 000 r.p.m and the supernatants which formed in both of the 2 ml eppendorf tube during the centrifugation process was recovered as much as possible and it was transferred into a new 2 ml eppendorf.

After that, equal volume of cold ethanol as to the volume of supernatant was added in which 1ml of cold ethanol was added to 1 ml of supernatant. After the addition of cold ethanol to the supernatant, the 2 ml eppendorf tube containing the lysate was inverted up and down for 50 times before the lysate was centrifuged at 14 000 r.p.m for 15 minutes. After the centrifugation process, the lysate was completely removed from the 2 ml eppendorf tube before 1ml of ethanol at room temperature was added to the DNA pellet which attach to the side of the 2 ml of eppendorf tube. Right after the addition of ethanol to the DNA pellet, the DNA pellet was centrifuged at 14,000 r.p.m for 2 minutes. From the centrifugation process, the supernatant was removed completely. The 2 ml eppendorf tube that contains the DNA pellet was air dried for 15 minutes in the laminar

flow cabinet and after that the air-dried DNA pellet was resuspended in 50 µl of TE buffer. The 2 ml effendorf tube containing the extracted DNA pellet was sealed with parafilm before it was stored in -20° C freezer in the Microbes Laboratory of University Malaysia Kelantan.

3.2 DNA quality and quantity checking using Agarose Gel Electrophoresis and Nanodrop Spectrophotometer

Following the method by Yeates et al. (1998), DNA in liquid form was subjected to gel electrophoresis in 1% agarose to test for DNA extraction quality. Next, the DNA was subjected to the NanoDrop™ spectrophotometer to test for purity in which the genomic material is considered free from contaminants if the reading falls between the ratio of 1.8 and 2.0.

3.2.1 Agarose Gel Electrophoresis

Agarose gel of 1.0 % was prepared to run gel electrophoresis for extracted DNA using SDS-based buffer method as well as Wizard Genomic DNA Purification Kit by using the modified method of Paulse *et al*. (2012). 1.0 g of agarose powder was added into 100 ml 1X TAE in a microwavable flask whereas an agarose gel of 0.8% was prepared for the extracted DNA using Cetyl Trimethylammonium Bromide (CTAB) based Extraction Buffer by dissolving 0.4 g of agarose gel into 50 ml of 1X TAE in 250 ml conical flask. The mixture was then placed in microwave for 1 minute to dissolve the

agarose powder into a transparent liquid. The mixture was left at room temperature of 37°C for five minutes to cool down. Then, one µg of ethidium bromide (10mg/ml) was added to the mixture containing DNA extracted using CTAB buffer while DNA extracted using SDS buffer and Wizard Genomic DNA Purification Kit was stained using SYBR green. The agarose gel was poured slowly into the tank and the comb was inserted in the gel. The agarose gel was left to solidify for at least 30 minutes before 1X TAE buffer was poured into the gel tank to submerge the gel from two to five mm depth. The first well was filled with 6 µl of DNA ladder and the other wells are filled with the DNA sample which was mixed with 5 µl of 6X dye.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Importance of DNA extraction protocol in identifying surface microbiota of *Dendrocalamus asper* (Buluh Betung)

Basically, biological diversity which is made up of flora and fauna contributes to richness of life on Earth and it is documented under a discipline known as Taxanomy. Taxanomy is recognized as the science of naming and classifying living organisms according to shared features and species determination through this traditional method is still not efficient and accurate in the context of morphological identification of damaged specimens that have missing body parts apart from very small specimens that cannot even be viewed under microscope. In order to cope with the limitations exhibit by traditional method, a revolutionary approach in taxanomy called DNA metagenome is introduced as advancement in science and technology (Amrita *et al*., 2014).

Principally, American Association for Clinical Chemistry (2015), has defined deoxyribonucleic acid as a long nucleic acid molecule composed of repeating subunits known as nucleotide is usually isolated from the samples using the combination of physical and chemical method in a process known as DNA extraction. According to Chen *et.al.* (2010), DNA extraction is trending now as a routine step in most of the biological

studies including phylogenetic inference, molecular identification as well as in genetics and genomics field due to the fact that the highly conserved stretches of DNA vary at a very minor degree during the evolution within the species which enables a high degree of precision on data established in identifying the already existing or a new breakthrough species even from the availability of limited resources of the specimen such as small, damaged or processed material through metagenome analysis. According to Johana *et al.* (2011), different DNA isolation procedures have multiple effects on the quality and the quantity of DNA. This is because each extraction methods varies in term of types and concentration of denaturant chemicals used apart from distinct purification steps that resulted in different efficiency in isolating a satisfying quantity and quality of DNA from the same sample In the concern of that fact, four distinct methods have been attempted to isolate bacterial DNA from *Dendrocalamus asper* (Buluh Betung), a native giant bamboo which is widely found in South East Asia (David *et al*., 2006) with the aim to establish an ideal microbial DNA extraction technique using woody stem plant which does not only contribute in the optimization of the DNA yield but also functions to minimize DNA degradation apart from being efficient in term of cost, labour and time.

Besides that, this experiment is also aimed to identify the type of microorganisms present in the DNA isolated from the optimized extraction method for *Dendrocalamus asper* through metagenomic sequencing. The identification of microbes in *Dendrocalamus asper* is essential to solve the speculation on the source of origin of the microorganisms that resides in the guts of giant panda. This is because Fang *et al.*, (2012),

has argued that the capability of giant panda to survive with a change in its feeding habit from a meat eater to bamboo lover even though they are genetically deficient in cellulose digesting enzymes as well as lignin-degrading genes in their genome has developed speculation among the scientist that the guts microbes of giant panda is the one responsible in helping the giant panda to digest the bamboo fibres. The doubt is further extend to another probability that the gut microorganism actually comes from the bamboo plant itself as giant panda are highly in contact with bamboo plant compared to any other substances. Hence, this experiment is designated to test the significance of the proposed hypothesis that the microorganisms in the giant panda's gut is actually contributed by the bamboo plant by identifying the colonization of microorganisms in bamboo plant through metagenomic analysis. Not only that, but bamboo plant specifically of *Dendrocalamus asper* species was chosen as the sample to be tested as this experiment is to determine the correlation between the guts microbes of giant panda found in Malaysia National Zoo with the microorganisms found on the its favourite bamboo species which is *Dendrocalamus asper* species because the giant pandas in Malaysia loves to eat *Dendrocalamus asper* compared to any other native bamboo species as its diet.

In accordance to that, the two widely used approaches for DNA extraction which comprises of the conventional method and also commercially available extraction kit method were examined and compared in this experiment to analyze the efficiency and suitability of these different approaches in isolating a good quality and quantity of bacterial DNA from *Dendrocalamus asper* (Chen *et al*., 2010). Thus, two distinct buffer-

based conventional methods which comprises of sodium dodecyl sulphate-based extraction buffer and cetyl trimethylammonium bromide (CTAB) based extraction buffer were selectively used together with a commercially available DNA extraction kit known as Wizard Genomic DNA purification Kit to isolate DNA from inner layer of *Dendrocalamus asper.* Even though the conventional method as well as the DNA extraction kit show variation in terms of sensitivity and purity of the extracted DNA due to the different type and concentration of chemicals used such as SDS as the main chemical for SDS-based extraction buffer while CTAB chemical is the main ingredient in CTAB- based extraction buffer, Hamid *et al*., (2012), emphasized that still each of the protocol applies four same basic steps for DNA isolation in which firstly the cellular structures are disrupted by either using detergent, chaotrophic salts or alkaline denaturant to create a lysate before separating the soluble DNA from plant debris and other insoluble material by either through centrifugation, magnetic clearing or filtration. After that, the DNA of interest is further purified by using multiple restriction enzymes such as lysozyme to digest soluble proteins and other nucleic acid and the DNA pellet is then precipitated out by using chemicals with a low dielectric constant such as alcohol like ethanol (Hamid *et.al*, 2012).

Apart from that, William & Kimberley, (2001) have stated that efficiency of all the methods can be determined in terms of the quantity and quality of the extracted DNA by using the standard molecular biology techniques such as agarose gel electrophoresis and spectrophotometer. Gel electrophoresis is one of the common tool used in analyzing the concentration of DNA in the solution based on the separation of bands in relative to

distances travelled by different size of DNA fragments whereas spectrophotometer is used to provide a n accurate estimation on the purity of a solution of DNA with a comparison of the optical density values of the solution at various wavelength such as A_{230}, A_{26o} and A_{280}.

Table 4.0: Nanodrop Spectrophotometer Reading for Extracted DNA from *Dendrocalamus asper* (Buluh Betung) using three different extraction method

Type of DNA Extraction Method	Concentration of dsDNA (ng/µL)	Reading of A_{260}	Ratio reading of A_{260}/A_{280}
CTAB-based DNA Extraction Buffer (Sample A)	10.84	0.22	1.24
CTAB-based DNA Extraction Buffer (Sample B)	19.66	0.39	1.82
SDS-based DNA Extraction Buffer	8.90	0.18	1.30

4.1.1 Effect of surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using SDS-based extraction method

By using the modified DNA extraction protocol that have been stated in section 3.1.1 of Hao *et. al*, (2008), a poor quality and quantity of DNA was extracted from the inner layer of the bamboo tissue by using SDS- based DNA extraction buffer or also known as an alkaline denaturation method. This is because based on table 4.0, DNA yield of only 8.90 ng/µL with a ratio of 1.30 for the wavelength measurement at A_{260}/A_{280} was

recorded from the Nanodrop spectrophotometer reading on the extracted bamboo sample. On the other hand, the gel image in figure 4.2 shows smearing of the DNA when the extracted DNA from the bamboo sample was subjected to gel electrophoresis. Based on the result from gel electrophoresis, the smeared visual of the DNA instead of a well-separated bands on agarose gel suggest a significant degradation of the genomic DNA from bamboo. One of the justifications for the DNA degradation will be condition and the age of bamboo (Ambika, n.d.). According to Astha *et al*. (2007), DNA extracted from mature bamboo stem as in this case is reported to generate poor quality and low yield due to the presence of high concentrations of polyphenols, tannins, polysaccharides and other secondary metabolites such as salts or phenols that can inhibit enzymatic reaction and cause variation in UV spectrophotometric measurement as well as in gel migration. Furthermore, the improper handling and storage of the plant tissue after harvesting bean also be one of the factor that leads to denaturation of the genomic DNA in bamboo plant (Johanna *et al*., 2011).This is because ideally the plant material must either collected freshly and directly subjected to processing or it must be stored at –20ºC or at –80ºC immediately if the sample is about processed later on and freezing prevents nuclease activity that would otherwise degrade the DNA in thawed tissue (Liese, 1985).

But, in this case, the bamboo sample was only kept in ice cubes of 4°C for two days while transporting the bamboo sample from University Malaysia Kelantan to Prince of Songkla University, Thailand in which the temperature is not ideal to inhibit the nuclease activity thus leading to degradation of DNA. On top of that, a low yield of DNA might have resulted from the insufficient modification in the protocol in terms of the

concentration of chemicals and enzymes used compared to the original protocol by Hao *et al*., (2008). This is because the protocol by Hao *et al*., (2008), is designated to extract DNA from freshly harvested soft plant tissue such as *Mallotus nudiflorus* compared to the old and hardy *Dendrocalamus asper* that was used in this experiment.

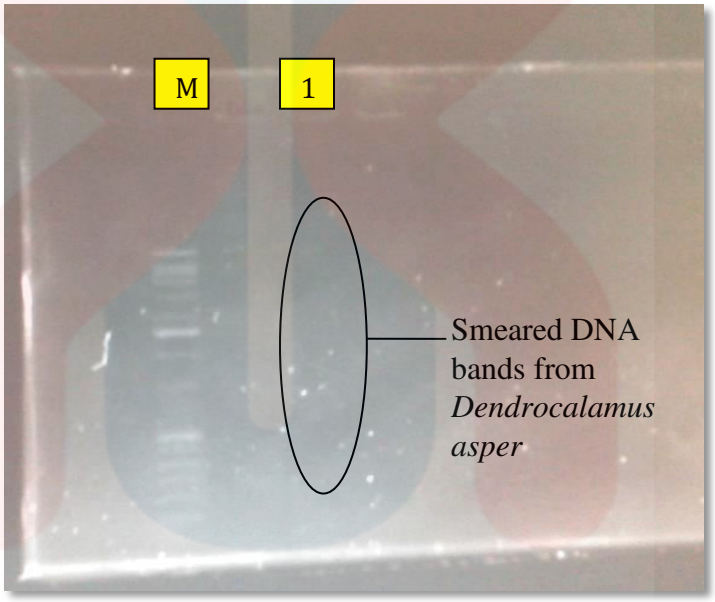


Figure 4.2 : Agarose gel electrophoresis profiles of extracted DNA from inner surface of *Dendrocalamus asper* using SDS-based DNA extraction method(M:1kb DNA ladder, 1: DNA Sample, 100 volts)

This fact is further strengthen by the low DNA purity as the ratio reading of A_{260}/A_{280} for spectrophotometer was only1.30 whereby the ratio reading is lower than the purity range of 1.8 to 2.0 for DNA. According to William & Kimberley, (2001), the strong absorbance at A_{280} will also result in a low A_{260}/A_{280} ratio that indicates presents of contaminating protein or phenol. Based on article by Suzanne (2010), contamination of protein in the

extracted DNA might occur when a large amount of sample was used besides due to some protein that does not completely dissolve in the lysis buffer. By referring to that, the bamboo tissue in this experiment might not be completely dissolved in sodium dodecyl sulphate buffer whereby SDS is an anionic surfactant which is added to the bamboo solution with an idea to disrupt the cell membrane and denature proteins together with lipid components of the plant tissue.

4.1.2 The differences in preparation step for surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using Wizard Genomic DNA Purification Kit and its importances

Next is DNA extraction using the commercially available Wizard Genomic DNA Purification Kit of Promega brand was conducted on the inner layer of the bamboo plant. DNA from the bamboo plant was extracted twice using this protocol with a slight adjustment at the initial part of the DNA extraction which is in the bamboo sample preparation step to analyse the importance of mechanical shearing in obtaining a satisfactory concentration of DNA from the sample. In the first extraction process, the swabbed inner layer of bamboo tissue using the sterilized cotton bud was immersed directly into the nucleic lysis buffer and continued with others steps as in the kits protocol without performing any mechanical disruption such as disruption using mortar and pestle or even disruption using rotor-stator homogenizer on the bamboo tissue.

On the other hand, the inner tissue layer of bamboo plant was firstly ground into powder using liquid nitrogen before placing the fine bamboo powders into the lysis nucleic solution in accordance to the second method. Basically, the commercially available extraction kit was used in this experiment to test for the DNA outcome compared to the conventional method. As stated by Johanna *et al*. (2011), the use of commercialized kits did significantly shorten duration of DNA isolation procedure so that a few replicates of the experiment can be carried out for the same duration of time taken to complete a set of DNA extraction using traditional method. Apart from that, the use of commercialized kit also provides a better reliability as there is no needs to prepare the extraction buffers and chemicals manually which will help to minimize the level of contamination and experimental errors besides providing higher safety reassurance than the conventional DNA isolation procedure because most of the extraction kit does not include the usage of hazardous chemicals such as phenol and chloroform in their protocol.

4.1.3 Effect of surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using liquid nitrogen for Wizard Genomic DNA Purification Kit

By using the DNA extraction method as stated in section 3.1.2, no DNA bands were observed in the gel image upon performing agarose gel electrophoresis indicating absence of DNA fragments in that bamboo sample meanwhile a visible DNA band was observed as in Figure 4.2 for the gel image of the DNA extracted using the method described in section 3.1.3. Based on the gel electrophoresis results in comparison to the

two different sample preparation steps for DNA extraction using the Wizard Genomic DNA purification kit, it is assumed that the usage of liquid nitrogen in the sample preparation step has a significant effect on DNA yield due to the reason that only the bamboo sample which was ground using liquid nitrogen has produced a visible DNA band in the gel image while no bands could be observed for the DNA extracted using the first isolation method. This assumption is then strongly supported by the fact by Siun & Beow (2009), that liquid nitrogen actually helps to produce a higher yield of DNA during the extraction process as in method 3.2.2 because the low temperature of liquid nitrogen does not only contributes in deactivating the activity of harmful chemicals and enzymes such as nuclease that can denature DNA but it also causes the cell wall to be easily break down when the bamboo sample become fragile at a low temperature. Therefore, it is hypothesized that high concentration of DNA fragments are released from the bamboo sample following the efficient cell wall disruption using liquid nitrogen as represented in Figure 4.2 compared to the first extraction method which is lacking in mechanical disruption step.

Besides that, based on facts by Mapula *et al*. (2015), it is reasonable to conclude that the combination of tissue grinding using liquid nitrogen together lysis buffers in the method as described in Section 3.1.3was more efficient in releasing DNA fragments from the sample compared to method 3.1.2 which only follows the lytic procedure available in commercial kits for this experiment as additional mechanical shearing is required to disrupt the thicker cell components of bamboo which is not possible by only using lysis buffer. However, it is also assumed that the bands in Figure 4.2 which was formed by

using n the second extraction method actually represent plant DNA instead of microbial DNA because William & Kimberley, (2001) have stated in their literature review on that liquid nitrogen is known for its efficiency in breaking the cell wall of the plant which will results in the release of high concentration of plant DNA compared to microbial DNA.

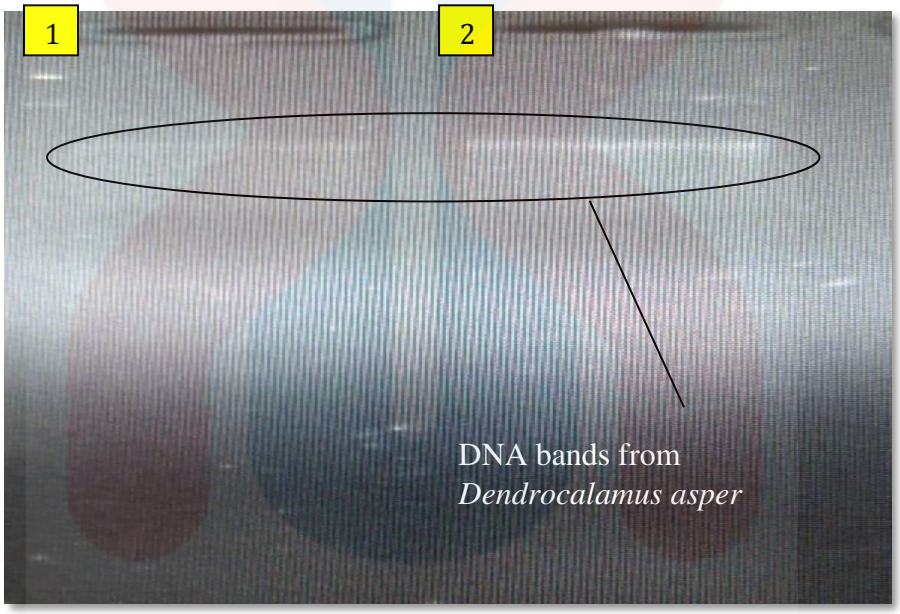


Figure 4.2 : Agarose gel electrophoresis profiles of extracted DNA from inner surface of *Dendrocalamus asper* using Wizard Genomic DNA Purification Kit method (1-2: DNA Samples, 100 volt)

4.1.4 Effect of surface microbiota genomic DNA extraction from *Dendrocalamus asper* (Buluh Betung) using CTAB-based extraction buffer

Based on the modified CTAB-based DNA extraction buffer method as stated in section 3.1.4, two replicates of the bamboo sample namely sample A and sample B were generated whereby the DNA yield for sample A was 10.84 ng/µL whereas a DNA concentration of 19.66 ng/µL was tabulated as in table 4.0 for sample B after the DNA samples are subjected to spectrophotometer Nanodrop analysis. As for the average reading for the wavelength at A_{260}/A_{280}, a reading of 1.24 was recorded for sample A meanwhile a value of 1.82 was obtained for sample B. As for the result from agarose gel electrophoresis as shown in Figure 4.3, a clear DNA is visible in lane 3 of the gel image indicating the presence of DNA in the bamboo sample. It can be seen clearly that sample A generates a better quality and quantity of DNA as it falls in the range for pure DNA which is 1.8 compared to sample B that have a lower ratio for wavelength at A_{260}/A_{280} which point out that there is protein contamination in the sample Astha *et al*., (2007), that may result from the uneven distribution of the DNA fragments while the sample was divided into equal quantity into both of the eppendorf tube. As for the agarose gel electrophoresis result, a clear DNA bands was seen in lane 3 on the gel image as shown in Figure 4.3.

Basically, it can be concluded that the modified CTAB-based extraction buffer is efficient in isolating DNA from *Dendrocalamus asper* and this is because Chen *et al.*

(2010), has proven that DNA extraction using CTAB extraction buffer are able to generate a good quality and quantity of DNA especially from plant as CTAB is a cationic detergent which is widely used when purifying DNA originating from plant tissues. In addition, the correct DNA extraction techniques including the usage of sterilized apparatus together with a simpler DNA extraction method has reduce the contamination in the bamboo sample and resulted in a better quality of DNA as in table 4.0. Besides that, it can be assumed that the type of denaturant chemical in the lysis buffer such CTAB and EDTA together with the amount of chemicals used is sufficient enough to disrupt and release a high concentration of DNA from the woody bamboo plant.

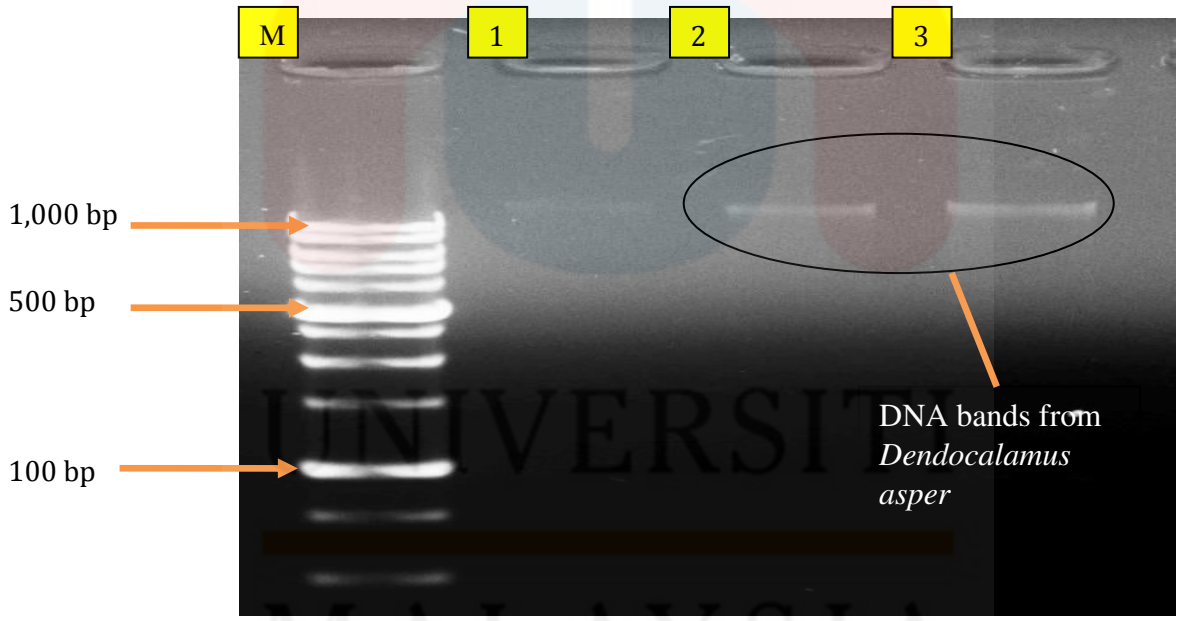


Figure 4.3 : Agarose gel electrophoresis profiles of extracted DNA from inner surface of *Dendrocalamus asper* using CTAB-based DNA extraction method (M:1kb DNA ladder, 1-3: DNA Sample, 100 volts)

4.2 Comparisons of the gel electrophoresis and spectrophotometer Nanodrop reading for different DNA extraction method for *Dendrocalamus asper* (Buluh Betung)

In comparison with the results on DNA concentration as well as the quality of DNA extracted using different extraction method which includes SDS-based extraction buffer, Wizard Genomic DNA purification kit and SDS-based extraction buffer, the genomic DNA extracted using CTAB-based buffer has proven to produce a higher yield of DNA as shown in Figure 4.5 apart from two clear DNA bands of high intensity with slight RNA contaminations were observed in gel image compared to that of DNA extracted using Wizard Genomic DNA purification Kit as well as SDS-based extraction buffer. Besides that, the ratio reading of wavelength at A_{260}/A_{280} also showed a higher reading of 1.8 for sample B compared to a value of 0.32 for DNA extracted using SDS method with a difference of 0.52 for both of the reading as tabulated in table 4.0.From this results, it can be concluded that CTAB-based buffer is the best method to isolate DNA of a good quality as well quantity from the woody plant of *Dendrocalamus asper*. Basically the effectiveness of CTAB-based buffer in DNA extraction has resulted from the sufficient concentration of chemicals and denaturing enzymes used in this protocol apart from the correct molecular techniques that have been mastered especially in handling DNA as stated by Mapula *et al.*, (2015). This is actually aided by the presence chemicals and salt such as NaCl salt, mercaptoethanol, Tris-HCL, EDTA and CTAB in the CTAB buffer plays a significant role in DNA isolation. The ultimate function of CTAB buffer in DNA extraction is to solubilise plant cell wall and lipid membranes of

internal organelles apart from to denature plant proteins that might contaminate the extraction of microbial DNA. This function of CTAB buffer is supported by the chemicals present in it through which B-mercaptoethanol helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and also to remove polyphenols present in plant cell (Astha *et al*., 2007).

Meanwhile CTAB is responsible to disrupt the cell membrane and to solubilise plant cell wall so that the genomic DNA is released out of the cell whereas Tris-HCL at pH 8 together with sodium chloride salt which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together. Besides that, the addition of two types enzymes namely lysozyme and proteinase K to the bamboo mixture together with CTAB buffer and TE buffer in this method has an effect on the satisfying DNA quantity in this experiment on the point that lysozyme function to degrade the bacterial cell wall by cleaving the polysaccharide sugar residues in the cell walls to release the genomic DNA from bacterial cytoplasm whereas proteinase K which is a broad spectrum serine protease is used to digest contaminating proteins which is present during DNA extraction (William & Kimberley, 2001).

In addition, William & Kimberley, (2001) have stated that proteinase K also helps to degrade nucleases and protect the nucleic acids from nuclease attack. Inclusion of chloroform in this reaction is to solubilize lipids apart from separating proteins and

polysaccharides from the nucleic acid in the bamboo mixture by binding up the complexed protein and polysaccharides together while chilled ethanol is added in this DNA extraction procedure with the aim to precipitate DNA out of the water-based bamboo mixture because DNA becomes less hydrophilic and its solubility becomes markedly low in the presence of lower dielectric constant solvents such as ethanol. Besides that, the addition of alcohol especially ethanol is one of the simple way to purify DNA and the reason for the use of cold ethanol instead of ethanol at room temperature is due to the fact that lower temperature will slows down the enzyme reaction that might degrade the DNA. The addition of 70% ethanol at room temperature is also needed to wash away any residual salt including sodium chloride salt from the pelleted DNA so a pure DNA is obtained at the end of this experiment the DNA pellet must be resuspended in 50 µlof TE buffer because DNA must be stored in alkaline buffer to prevent it from being degraded.

Besides that, Chen *et al*. (2010), also argued that phenol extractions when coupled with SDS tends to produce a low yield of DNA for plants that have a high content of polyphenolic compound such as bamboo plant which made CTAB-based buffer extraction a better option for DNA extraction from bamboo plant. Not only that, but CTAB-based extraction buffer still showed a better performance in comparison with the Wizard genomic DNA extraction kit because a higher yield of DNA is retrieved from the conventional SDS or CTAB-based protocol compared to the commercial kit protocol as previously observed in bamboo plant. The two different conventional methods were

practiced to isolate DNA from the inner part of bamboo due to its ability to generate DNA of a higher purity as a result from pre-treatment with a few types of enzymes such as lysozyme and proteinase K apart from strong chemical named chloroform which is needed for deproteinization of cell components in the bamboo which is usually absent in commercialized extraction kit. Not only that, but results obtained from the extraction kit can be inefficient at times especially when handling plants with polysaccharides or high polyphenolic content like bamboo plant and classical protocols enabled access to more diverse endophytic bacterial communities than commercial kits (Astha *et.al* , 2007).

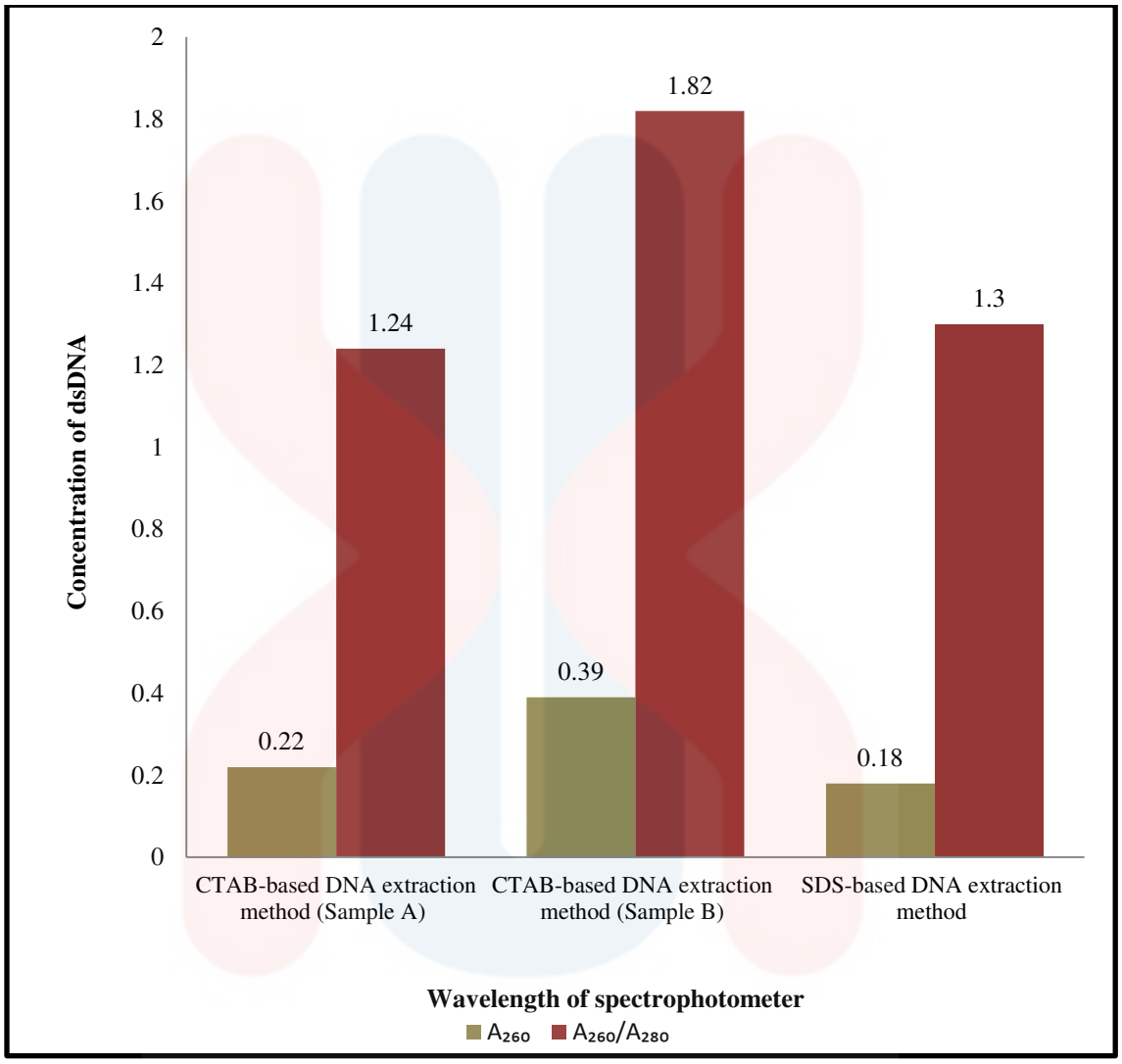


Figure 4.4: Graph on the Nanodrop Spectrophotometer reading for wavelength at A_{260}/A_{280} and A_{260} of extracted DNA from *Dendrocalamus asper* (Buluh Betung) using three different extraction method

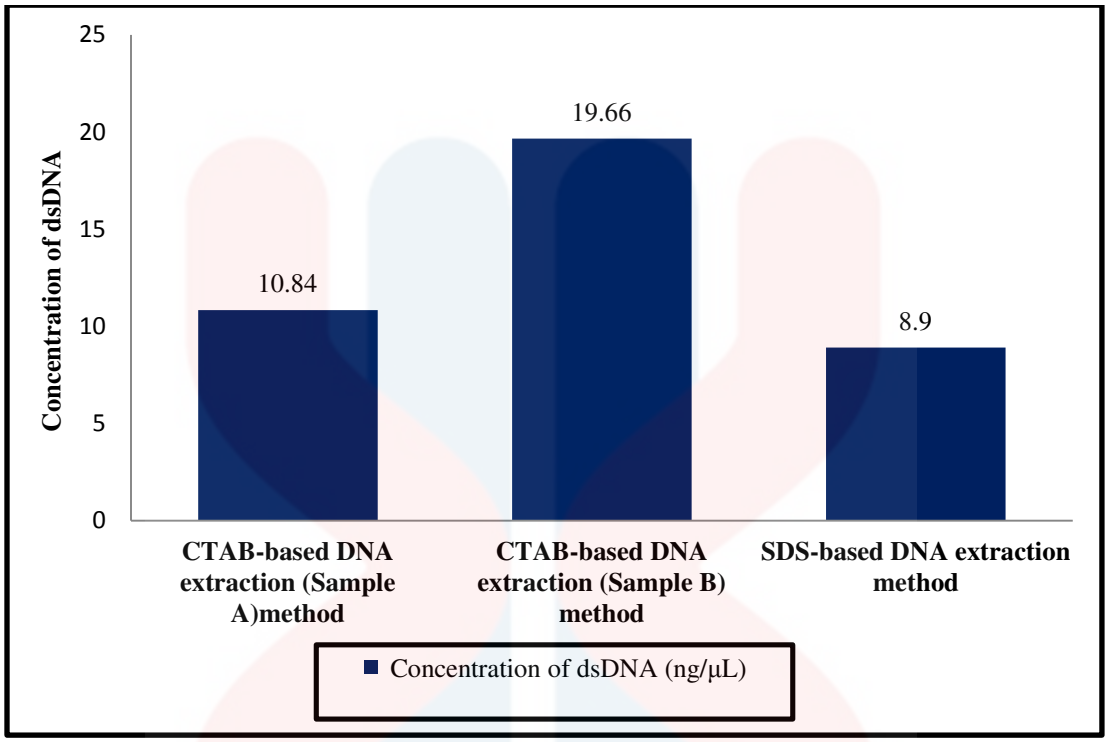


Figure 4.5: Graph on the Nanodrop Spectrophotometer reading for dsDNA concentration (ng/µL) of extracted DNA from *Dendrocalamus asper*(Buluh Betung) using three different extraction method

CONCLUSION AND SUGGESTIONS

5.1 Conclusion

In a nutshell, the objective of establishing an ideal genomic DNA extraction technique from *Dendrocalamus asper* (Buluh Betung) was achieved in the end of this study with the modified CTAB-based buffer was proven to be an ideal method to extract a good quality and quantity of microbes DNA from *Dendrocalamus asper* compared to the SDS-based extraction buffer together with Wizard genomic DNA extraction kit. This is because even though there were many presented protocols for nucleic acids extraction, the optimized CTAB protocol is efficient in eliminating the contamination that results from the specific characteristics of plants like the presence of rigid polysaccharide, cell wall, pigments, chemical heterogeneity of secondary metabolites as the cell lysis procedures of classical protocols were superior to those of commercially available DNA extraction kit. In addition, DNA extraction using CTAB-based buffer also has a great deal of advantages in relative to other methods such as only a small amount of tissue is required for extracting DNA apart from being effective in terms of cost and time. Not only that, but a good yield of DNA is also be able to be achieved by only using fewer chemicals in CTAB protocol compared to the rest.

5.2 Recommendations

As for the recommendation, this study can be improvised by adding RNAse enzyme in the modified CTAB-extraction buffer to remove the contaminated RNA from the extracted DNA as shown in figure 4.3. Besides that, the study can be replicated by using a freshly harvested bamboo in order to determine the effect of age of a sample in isolating a good quantity and quality of DNA. In order to achieve the second objective which is to determine the diversity of microorganism in *Dendrocalamus asper*, a good quality and quantity of extracted DNA using CTAB-based buffer method must be sent for metagenomic sequencing and the sequenced result should be analysed using MR-RAST software.

REFERENCES

20 December 2013. An introduction to illumina next generation sequencing technology for microbiologists. Retrieved from http://www.illumina.com/technology/next-generation-sequencing.html

Ambika, B.,G. (n.d.) DNA quality : Electrophoresis, spectrophotometry and fluorometry. Retrieved on 5 January 2017, from, www.nbpgr.ernet.in/Portals/6/DMX/GENOMIC_RESOURCES/DNA%20quality-spectrophotometry%20and%20electrophoresis.pdf

American Association for Clinical Chemistry(AACC).(2015).Laboratory methods used for medical lab testing .Retrieved on February 6,2016,from http://labtestonline.org/understanding/features/methods/?show_all=1&printpreview=1

Amrita, S., John, C., M,S., Alfried, P,V. & Rudolf, M. (2014). Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (Pygathrix *nemaeus*). *Molecular Ecology Resources*.doi: 10.1111/1755-0998.12302

Andrew, T. & Georgi, M. (2015).DNA structure and function. *The Febs Journal.*doi:10.111/febs.13307 2015

Astha, V., Harish, P. & Neetha, S. (2007). Plant genomic DNA isolation: An art or a science. *Biotechnology Journal*, 2, 386-392. doi: 10.1002/biot.200600195

Bounsong, S., Piyawade, B., Vallayuth, F.& Waranyu, R.(2013).Physical and mechanical properties of some Thai bamboos for house construction.Royal Forest Department. Retrieved on 7 January, 2017, from www.itto.int/files/itto_project_db_input/2585/Technical/Physical%20and%20Mechanical%20Properties%20of%20Thai%20Bamboos%20for%20House%20Construction.pdf

Chen, H., Rangasamy, M., Tan, S, Y. & Siegfried, B, D. (2010). Evaluation of five methods for total DNA extraction from western corn rootworm beetles. *PLoS ONE*, 5(8).doi: 10.137/journal.pone.001963

Christina, S. G. & Richard, J. M. (2004). Ultrastructure of fibre and parenchyma cell walls during early stages of culm development in *Dendrocalamus asper*. *Annals of Botany*, *95*(4), 619-629. doi:10.1093/aob/mci068

David, E. W., Zhang, A., Zhang, H., Donald, L. J. & Susie, E. (2006). Giant pandas. Mexico: Cambridge University Press, New York, 1, 1-541

Fang, W., Fang, Z ., Zhou, P., Chang, F., Hong, Y., Zhang, X ., Peng, H. & Xiao, Y. (2012). Evidence for lignin oxidation by the giant panda fecal microbiome. *Plos One*.*7*(11) :e50312. doi:10.1371/journal.pone.0050312

Fu, J., Henry, M., Joao., Yu, C., Artur, C. P., Georg, M. G. & Gibson, S. N. (2011, April 15). Changes in the bacterial community structure and diversity during bamboo retting. *Biotechnology Journal*, 6, pp. 1262–1271.doi : 10.1002/biot.201100105

Hamid, K. & Khosro, G.(2012). DNA purification and isolation of genomic DNA from bacterial species by plasmid purification system. *African Journal of Agriculture Research*, 7(3), 433-442. doi: 10.5897/AJAR11.1370

Hao, X, W., Zhao, L,G ., Ying, Z.& Yue, M,S. (2008). Enriching plant microbiota for a metagenomic library construction. *Environmental Microbiology*,10 (10) ,2684-2691. doi: 10.1111/j.1462-2920.2008.01689.x

He,F. (2011). E.Coli genomic DNA extraction .*Bio-protocol* Bio101:e97.http://www.bio-protocol.org/e97

Hofmann, R. R. (1989). Evolutionary steps of ecophysiological adaptation and diversification of ruminants: A comparative view of their digestive system. *Oecologia*, 78, 443-457. Retrieved from http://www.uky.edu/Ag/AnimalSciences/instruction/asc684/PDF/oecologia78_443.pdf

Hosseinpour, N,A .& Nematadeh, G,H.(2013). Introducing a new method of genomic DNA extraction in dicotyledonous plants. *Journal of Agriculture Science*,2(6), 242-248. Retrieved from http://www.scholarly-journals.com/SJAS

Jennifer, B. (2016). Springtime in Panda Canyon. Retrieved on 13 September2016, from http://zoonooz.sandiegozoo.org/2016/05/13/springtime-in-panda-canyon/

Johanna, M., Catarina, S. & Maria, S. (2011). The currently used commercial DNA extraction methods give different results of clostridial and actinobacterial populations derived from human fecal sample. *FEMS Journal* ,1574-6941.doi: 10.1111/j.1574-6941.2011.01257.x.

Leveau, J. H. (2007, September 21). The magic and menace of metagenomics: Prospects for the of plant growth-promoting rhizobacteria. *Plant Pathology*, 119, 279-300.doi:10.1111/j.1462-2920.2008.01689.

Lewis, M.(n.d). Agarose gel electrophoresis(basic method).Retrieved on March 21,2016,from http://www.methodbook.net/dna/agarogel.html.

Li, M,W., Santha, S.& Lee, Q, P.(2014). An optimised high-salt CTAB protocol for both DNA and RNA isolation from succulent stems of *Hylocereus* sp. *Journal of Medical and Bioengineering*, 3(4).

Li, Y., Guo,W., Han, S., Kong, F., Wang, C., Li, D., Zhang, H., Yang, M., Xu, H., Zeng, B. & Zhao, J. (2015). The evolution of the gut microbiota in the giant and the red pandas. www.nature.com. doi: 10.1038/srep10185

Liese,W.(n.d.). Anatomy and properties of bamboo. 197-263. Retrieved on 5 January 2017,www.inbar.int/wpcontent/uploads/downloads/2012/09/inbar_pr_03_1.pdf

Lifeng, Z., Qi, W., Jiayin, D., Shanning, Z. & Fuwen, W. (2010).Evidence of cellulose metabolism by the giant panda gut microbiome. *PNAS*, 108(43), 17714-17719. Retrieved from www.pnas.org/cgi/doi/10.1073/pnas.1017956108

Linda,V,T., Theo,O.& Kaori, S. (2014). Exploring the influence of gut microbiota and probiotics on health: a symposium report. *British Journal of Nutrition*, 112. doi: 10.1017/s0007114514001275

Mapula, K, A, M., Jean, B, R. & Marla, T. (2015). Impact of metagenomic DNA extraction procedures on the identifiable endophytic bacterial diversity in Sorghum bicolor (*L. Moench*). *Journal of Microbiological Methods*, 112(2015),104-117. Retrieved from journal homepage: www.elsevier.com/ locate/jmicmeth

Meyer, F., Paarmann, D., Souza, M, D., Olson, R., Glass, E. M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., Edwards, R.A. (2008). The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenome. *BioMed Central Bioinformatics*, *386*(9),1471-2105.doi:10.1186/1471-2105-9386

Naveed, M. & Saeed, M. (2011). Biological screening of *viola betonicifolia* smith whole plant. *African Journal of Pharmacy and Pharmacology*, 5(20), 2323.doi:10.5897/AJPP11.641

Palaska. (2010). *Dendrocalamus asper*. Retrieved on 16 February 2016, from, https://www.pinterest.com/pin/372250725423044055/

Paul, D.(2016). Red panda-Photographic expedition. Retrieved on 8 January 2017, from, http://www.touchthewild.co.uk/wild-life-tours/red-panda-photographic-expedition/

Paul, M. (2016) Red Panda – Photographic Expedition.Touch the wild. Retrieved on 7 January 2017, from http://www.touchthewild.co.uk/wild-life-tours/red-panda-photographic-expedition/

Promega Corporation. (2011).Protocol & applications guide. Retrieved on 25 November, 2016,fromhttps://worldwide.promega.com//media/files/resources/paguide/a4/chap1a4.pdf?la=en

Rahul, S, M., Sudipto, S. & Santasabuj, D. (2015). Metagenomic surveys of gut microbiota. *Genomics Proteomics Bioinformatics,* 13, 148-158. Retrieved from http://dx.doi.org/10.1016/j.gpb.2015.02.005

Shadi, S., Jennifer,L., Spall., Joel, F,G. and Mehrda, H. (2012). Next-generation sequencing technologies for environmental DNA research. *Molecular Biology*, 21,1794-1805. doi:10.1111/j.1365-294x

Shamayim, T. R. P., Luis, E. S. G., Berenice , J. M., Luis, M. B., Monica, R., Julio, M., Marco, A. R., Ernesto, O. O. & Esperanza, M. R. (2012). Gut and root microbiota commonalities. *Applied and Environmental Biology* , *79*(1), 2-9. doi: 10.1128/AEM.02553-12

Siun,C,T. & Beow,C,Y. (2009). DNA,RNA and protein extraction: The past and present. *Journal of Biomedicine and Biotechnology*,10.doi:10.1155/2009/574398

Somjit. (2009).Culm of *Dendrocalamus asper*. Retrieved on 11 December 2016, from, https://sites.google.com/site/bamboosthailand/home/-1-native-species/dendrocalamus-sp-n-thailand-sang-mon

Stephane, S. (2011). Bamboo Flowering Habits. Retrieved on 16 February 2016, from, https://www.guaduabamboo.com/identification/bamboo-flowering-habits

Suzanne, K. (2010). How DNA extraction kits works in the lab. *Bitesize Bio*. Retrieved on 4 January, 2017, from,http://bitesizebio.com/13516/how-dna-extraction-rna-miniprep-kits-work/

Takema, F. & Naruo, N. (2000). Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). A*pplied and Environmental Microbiology, 66*(2) , 643-650. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10653730

Tania, A., Nuria, M., Ester, A, S., Carlos, M,D. & Sophie, A,H. (2012). Selective elimination of chloroplastidial DNA for metagenomics of bacteria associated with the green alga *Caulerpa taxifolia* (bryopsidophyceae). *Phycological Society of America*, 48, 483-490. doi:10.1111/i.1529-8817.2012.01124.x

Wang, D. H. (2015, August 9). Low daily energy expenditure enables giant pandas to survive on bamboo, *58*(9), 925-926. doi: 10.1007/s11427-015-4917-1

Wang, H. X., Geng, Z. L., Zeng, Y. & Shen, Y. M. (2008). Enriching plant microbiota for a metagenomic library construction. *Environmental Microbiology*, *10*(10), 2684–2691. doi:10.1111/j.1462-2920.2008.01689.x

Wei, F., Hu, Y., Ya, L., Nie, Y., Wu, Q. & Zhang, Z. (2014). Giant pandas are not an evolutionary cul-de-sac: Evidence from multidisciplinary research. *Molecular Biology*, *32*(1) , 4-12. doi:10.1093/molbev/msu278

William, C. & Kimberley,C. (2001). An introduction to DNA: A spectrophotometry, degradation and the Franken gel experiment. *Proceedings of the 22nd Workshop/Conference of the Association for Biology Laboratory Education*

(ABLE), 81-99. Retrieved on 6 January 2017, from www.ableweb.org/volumes/vol-22/5-clark.pdf

Xue, Z., Zhang, W.,Wang, L., Hou, R., Zhang, M., Fei, L., Zhang, X., Huang, H., Laura C. B., Jiang, Y., Jiang, C., Zhao, L., Pang, X. & Zhang, Z. (2015, May 19). The bamboo-eating giant panda harbors a carnivore-like gut microbiota with excessive seasonal variations. *mBio*, *6*(3). doi:10.1128/mBio.00022-15

Younes, M., Naima,A., Mireille,H. & Francoise,C. (2004).Comparisons of different methods of isolation of DNA of commonly encountered *Candida species* and its quantitation by using a real-time PCR-based assay. *Journal of Clinical Microbiology*, 42(7), 3159-3163. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15243076

Zhao, H., Yang, J. R., Xu, H. & Zhang, J. (2010). Pseudogenization of the umami taste receptor gene Tas1r1 in the giant panda coincided with its dietary switch to bamboo. *Oxford Journals*, *27*(12), 2669–2673. doi : 10.1093/molbev/msq 153

Zhu, L., Wu, Q., Dai, J., Zhang, S. & Wei, F. (2011). Evidence of cellulose metabolism by the giant panda gut microbiome. *PNAS*, *108*(43), 17714–17719. Retrieved from www.pnas.org/cgi/doi/10.1073/pnas.1017956108

APPENDIX

CHEMICALS	FUNCTION
• Sodium dodecyl sulphate	• A detergent that destroys protein configuration
• Tris-EDTA buffer (TE buffer)	• Protects DNA& RNA from degrading
• Lysozyme enzyme	• Breaks down bacterial cell wall
• Proteinase K enzyme	• Inactivates nucleases
• Ethanol	• Forces DNA to precipitate out
• Isopropanol	• To precipitate DNA
• Chloroform-isoamyl alcohol	• To remove protein from nucleic acid