



Faculty of Resource Science and Technology

**VARIABILITY OF DOMINANT ENDOPHYTIC FUNGI  
ASSOCIATED WITH *Nypa fruticans* AND SELECTED  
WATER PARAMETERS IN KUCHING WETLAND  
NATIONAL PARK (KWNP), SARAWAK**

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**UNIVERSITI MALAYSIA SARAWAK**  
**FACULTY OF RESOURCES SCIENCE AND TECHNOLOGY (FRST)**

**SELECTED WATER PARAMETERS OF KUCHING WETLAND NATIONAL PARK  
(KWNP)**

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Bachelor of Science with Honours  
Aquatic Resource Science and Management

## DECLARATION

I hereby declare that no portion of the work referred to in this dissertation has been submitted of an application for another degree of qualification or any other university or institution of higher learning.



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## **List of Abbreviations**

<b>APHA</b>	American Public Health Association
<b>DO</b>	Dissolved oxygen
<b>ITS</b>	Internal transcribe spacer
<b>KWNP</b>	Kuching Wetlands National Park
<b>PCR</b>	Polymerase Chain Reaction
<b>PDA</b>	Potato dextrose agar
<b>TSS</b>	Total suspended solids
<b>USEPA</b>	United States Environmental Protection Agency
<b>USGS</b>	United States Geological Survey
<b>WWF</b>	World Wildlife Fund

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# Variability of Dominant Endophytic Fungi Associated with *Nypa fruticans* and Selected Water Parameters in Kuching Wetland National Park (KWNP), Sarawak

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## ABSTRACT

A research was done in Kuching Wetland National Park (KWNP), Sarawak to analyse the basic physico-chemical water properties of KWNP, the dominance of endophytic fungi that associated with *Nypa fruticans* as well as to relate the dominant endophytic fungi with respects to the variability of the water parameters. The present study involved the combination of water parameters assessments, fungal screening in heavy metals and molecular techniques. Sampling was carried out on September 2013 and selected water parameters; pH, temperature, salinity, turbidity, total suspended solids and nutrients were analysed. A sample of *Nypa fruticans* from each sampling stations were taken for endophytic fungi works. The range of environmental conditions; pH, temperature, salinity, turbidity and total suspended solids are 8.0 to 8.6, 28.29 to 29.49 °C, 7 to 22 PSU, 833.7 to 1561.07 NTU and 0.017 to 0.123 mg/L, respectively. Based on the result of water parameters, five representative sites were chosen. Endophytic fungal isolates from these five stations were screened in two concentrations (500 ppm and 1000 ppm) of four heavy metals; Zinc (Zn), Lead (Pb), Copper (Cu) and Chromium (Cr) to select the dominant fungal isolates. A total of 8 isolates were able to grow; three isolates can resist in Zn, Pb and Cu while the other five isolates can resist in Zn, Pb and Cr. Molecular techniques done using Internal Transcribed Spacer (ITS), ITS 1 and ITS4 as primers. Phylogenetic tree constructed supported the morphological identification of Isolate 2, 3, 4, 6, 7 and 8, which is *Pestalotiopsis* sp. *Pestalotiopsis* sp were identified to have high tolerance on wide environmental conditions. Future research on morphology should be done using Scanning Electron Microscope or Transmission Electron Microscope to further distinguish and correctly defined the species of endophytic fungi.

Key words: *Pestalotiopsis*; extensive hyphae; heavy metals

## ABSTRAK

Satu kajian telah dijalankan di Taman Negara Tanah Lembap Kuching (KWNP), Sarawak untuk menganalisa fisikokimia asas kualiti air KWNP, kulat dalaman utama yang hidup bersama dengan *Nypa fruticans* dan untuk menghubungkaitkan kulat dalaman utama dengan kepelbagaian parameter air. Kajian semasa ini merangkumi kombinasi kajian parameter air, tapisan kulat dalam logam berat dan teknik molekular. Kajian telah dilakukan pada September 2013 dan parameter air yang ditetapkan seperti pH, suhu, kegaraman, kekeruhan, jumlah bahan terampai dan nutrisi telah dianalisa. Sampel *Nypa fruticans* daripada setiap lokasi kajian telah diambil untuk kerja-kerja kulat dalaman. Julat tentang keadaan alam sekitar seperti pH, suhu, kegaraman, kekeruhan, dan jumlah bahan terampai ialah 8.0 hingga 8.6, 28.29 hingga 29.49 °C, 7 hingga 22 PSU, 833.7 hingga 1561.07 NTU dan 0.017 hingga 0.123 mg/L. Berdasarkan keputusan parameter air, lima wakil lokasi kajian telah dipilih. Kulat dalaman terasing daripada lima wakil tempat kajian ini akan ditapis dalam dua kepekatan (500 ppm dan 1000 ppm) dalam empat logam berat iaitu zink (Zn), plumbum (Pb), tembaga (Cu) dan kromium (Cr) untuk memilih kulat utama terasing. Sebanyak 8 kulat terasing dapat hidup; tiga kulat hidup dalam Zn, Pb, dan Cu manakala lima lagi dalam Zn, Pb, dan Cr. Teknik molekular menggunakan Ruang Salin Dalaman (ITS), ITS 1 dan ITS 4 sebagai permulaan. Susur galur genetik mengukuhkan pengelasan berdasarkan morfologi kulat terasing 2, 3, 4, 6, 7, dan 8 iaitu *Pestalotiopsis* sp. *Pestalotiopsis* sp. diketahui mempunyai tahap toleransi yang tinggi pada pelbagai keadaan alam sekitar. Kajian pada masa hadapan tentang sifat kulat patut dilakukan menggunakan mikroskop eletron imbasan atau mikroskop electron penghantaran untuk membezakan dan menamakan spesies kulat dengan lebih tepat.

Kata kunci: *Pestalotiopsis*; rangkaian hypha; logam berat

## 1.0 Introduction

Wetlands cover about 6 % of the world's land surface (WWF, 2004). The sources of water are majority from precipitation, groundwater, and surface water resources. In Malaysia, here relish hot and humid climate over the year and bless with ample mass rainfall to fulfil human water needs. On the whole, wetland plays an important role in hydrological cycles (Ramsar Convention Secretariat, 2010) and serves as a water catchment areas that eventually beneficial to human-being and world ecosystem as sites for "water purification and wastes detoxification, climate regulation, mitigation of climate change, cultural and hydrological services, and production and information functions" (Millennium Ecosystem Assessment, 2005; WWF, 2004).

However, according to recent research made by Prigent *et al.* (2012), observation based on remote sensing application shows a shocking decline of wetland areas at global scale by 6 % during these fifteen years. The reduction of inundated area is at concerning stake; it is scarce water resources for human being. Despite exists as a reservoir, the results demonstrated changes of hydrogeomorphic settings which affect its water quality.

Over decades ago, our wetlands had been threatened critically by overexploitation and urbanization to fulfil housing demands and customer needs. Agriculture field, industrialization and increase in global temperature intense the impact as stated by Australia Department of Environment (2009). Nutrients effluent and rapid growth of aquaculture sectors pressurizes the wetland with the hypernutrient conditions. Thus, what will happens if the environmental parameters such as pH, temperature, nutrients and heavy metal are high in wetland? Therefore, ecologist and biotechnologist come with new eco-biotechnological approaches where decomposer role may resolve these issues (Verhoeven *et al.*, 2006; Azam *et al.*, 1983).

Predominantly, there are numerous wetlands ecosystem exists in Malaysia. In Sarawak, specifically, Kuching Wetland National Park (KWNP) was come into force as a national park since it is the only large resort of intact mangrove ecosystem where mangrove flora and fauna can flourish. They retain complex species communities beneficial to the ecosystem as for example decomposer. Decomposer such as bacteria and fungi are proven to have high detoxification and phytoremediation potential. Many previous studies (Calheiros *et al.*, 2010; Li *et al.*, 2010) had conducted for endophytic bacteria but the research on endophytic fungi are still lacking. Hence, it is vital and judicious to follow-up the environmental conditions of KWNP and endophytic fungal dominance to ensure the ecosystem stability and its serviceable role that eventually uphold conservation worthy.

In this study, the targeted wetland (KWNP) is estuarine ecosystem which is very vibrant and energetic areas. Therefore, most of this paper will discuss about estuary. It is vital to analyse selected environmental parameters since the water flow is always variable and affects much to the physico-chemical of this wetland.

This study was focused on the water parameters at KWNP and dominance of endophytic fungi associated with *Nypa fruticans*. The physical environmental parameters were recorded *in-situ* and the water and palm samples were taken for further chemical analysis and molecular work in the laboratory. The water parameters of KWNP were correlated with the dominant endophytic fungi in the *N. fruticans*.

The objectives of this study were to; (1) analyse the basic physico-chemical water properties at KWNP, (2) identify the endophytic fungi presents living within *Nypa fruticans* at KWNP, and (3) relate the dominant endophytic fungi with respects to the variability of water parameters in KWNP.

## **2.0 Literature Review**

### **2.1. Definitions of wetlands**

According to the Ramsar Convention Secretariat (2010), wetlands is defined as “A wide variety of habitats such as marshes, peatlands, floodplains, rivers and lakes, and coastal areas such as salt marshes, mangroves, and sea grass beds, but also coral reefs and other marine areas no deeper than six metres at low-tide, as well as human-made wetlands such as waste-water treatment ponds and reservoirs”. Simply put, wetlands are areas where water flowing through, permanently or temporarily and involves in the hydrological cycle, directly or indirectly.

Since the scope of wetland areas is so wide, wetlands classification is important to clarify and ascribed particular wetlands according to their classes. For certain countries, the classification of wetlands are varied but most ecologists come to decision, the classification are based on “topographic characteristics rather than on vegetational and hydrochemical attributes” (Britton and Crivelli, 1993; Finlayson and Von Oertzen, 1993).

### **2.2. Estuarine ecosystems**

Wetlands comprising estuarine ecosystem is one of the dynamic ecosystem since it receiving both saltwater and freshwater, especially in Malaysia, they experienced the routine twice per daily. Estuary is the intersection between these two water bodies, it receives many severe impacts as a result of anthropogenic activities; majority is eutrophication due to agriculture, sewage and non-point pollution (Verhoeven *et al.*, 2006). The harsh environment within wide salinity gradient and sudden fluxes in temperature, oxygen concentration, nutrient and turbidity limits the biodiversity and survival of certain community (Haedrich, 1992).

### 2.3. Importance of estuarine ecosystem

Saline mangrove ecosystem possesses the ecological role and has high eco-biodiversity value. It provides the conducive habitat for mangal flora and fauna. For instance, mangrove vegetation such as genera *Avicenna sp.* and *Sonneratia sp.* are the food for Proboscis monkey that is endemic to Borneo (Nagelkerken *et al.*, 2008). Plus, the mangrove plant offers physical protection to our shoreline.

Not just for water source, estuary meet population's needs with protein and food stock as well economy value. It promotes favourable breeding and feeding ground for wildlife and aquatic organisms (Nyanti *et al.*, 2012). Besides that, it acts as refuge site for endangered species; *Crocodylus porosus* and *Trachypithecus villosus*. Based on the previous study, estuary is believed can substitutes tertiary treatment of municipal wastewater because it promotes efficient nitrogen removal via denitrification process (Verhoeven *et al.*, 2006; Day Jr. *et al.*, 2003).

### 2.4. Case histories on water quality of estuarine ecosystem

#### 2.4.1. pH

pH is parameter used to indicate the general state of water chemistry; acidity and alkalinity. The pH of neutrality is depends on temperature, but is near pH 7 at biologically relevant temperature (Hutchinson, 1957). The pH of estuarine is around 6.5 to 8.5; pH 7.0 to 7.5 during freshwater flush-out, and 8.0 to 8.6 during saltwater intrusion (US EPA, 2006). Seitzinger's study (1991) proved that with the increased pH above 9.5, the sediment-water phosphate flux increased significantly with the high occurrence of algal blooms indicated the relationship between these three factors.



#### 2.4.2. Salinity (PSU)

Salinity is the parameter to measure “total amount of dissolved material in grams in one kilogram of sea water” (Stewart, 2008). Theoretically, freshwater did not present salt content. However, due to some natural process such as weathering rock beds and input of run-off, it is not possible to get salinity reading. Basically, salinity of seawater is 35-37 PSU. In estuarine, yet, the salinity will be varied according to tidal ranges and capacity of freshwater to dilute saltwater (US EPA, 2006).

#### 2.4.3. Temperature (°C)

Below show the equation of the carbonate-bicarbonate equilibrium.



Figure 1. Carbonate ( $\text{CO}_3^{2-}$ ) – bicarbonate ( $\text{HCO}_3^-$ ) equilibrium

Generally, temperature is closely related with pH and dissolved oxygen. During high temperature (indicates sunlight intensity), photosynthesis is intense. Based on the Figure 1 above, degree of temperature may affect pH and DO. Photosynthesis removes carbon dioxide ( $\text{CO}_2$ ) from water, the carbon equilibrium tends to replace the  $\text{CO}_2$  by combining of bicarbonate ( $\text{HCO}_3^-$ ) with hydrogen ion ( $\text{H}^+$ ) forming carbonic acid ( $\text{H}_2\text{CO}_3$ ) again and dissociates into  $\text{CO}_2$  and water. Capacity of water to hold DO is independent with the water temperature. Hence, hot water will cause high pH (alkaline water) and low DO content. Muhammad Syukri's (2009) study at Merbok's estuary revealed the temperature pattern during high discharge period is about 28.8 to 30.3 °C whereas low discharge is 29.3 to 30.1 °C.

#### **2.4.4. Turbidity (NTU) and Total Suspended Solids (TSS)**

Turbidity is a measure of water clarity/cloudiness by the increases amount of material suspended in water will decreases the passage of light through the water (USEPA, 2012). In other word, turbidity is dependent with the amount of total suspended solids (TSS) and nutrients. As TSS and nutrient increases, turbidity reading will be increases.

#### **2.4.5. Nutrients**

As estuary is a nutrient sinks, the types of nutrient that always been found are nitrogenous compound and phosphate. Process of nitrate removal is called denitrification, and with the absence of oxygen for decomposition, nitrate can be used as an electron acceptor (Verhoeven *et al.*, 2006). Meanwhile, the phosphorus removal is by plant uptake and soil adsorption. Even though estuary acts as nutrient sinks; however if continuous nutrient input to the ecosystem until it is hyper-nutrient (eutrophic), it may give detrimental effect to itself and to the community structure. Nutrients may reflect the health of the plant community; if nutrients are sufficient for them, they will grow well. Study made by Tanaka and Choo (2000) at Matang Mangrove Estuary concluded that the out welling from mangrove's sediment had considerably supplied indirectly to the estuary's nutrient.

#### **2.5. Taxonomy and morphology of endophytic fungi**

The discovery of *Pestalotiopsis* sp was first discussed by Guba (1929) in his monograph; "Monograph of Pestalotia and Monochaetia" then followed further by Steyaert (1949) with some modification that recognizes and differ *Pestalotiopsis* with *Pestalotia*. Steyaert (1949) classified *Pestalotiopsis* into sections on the number of apical appendages basis; Monosetulatae, Bisetulatae, Trisetulatae and Multisetulatae with presence of single,

double, triple or more than triple apical appendages, respectively. Then, he further differentiated them based on difference morphology of conidial, median cells pigmentation, and presence of spatulated appendages. Generally, *Truncatella angustata* was used as an outgroup or reference taxa. According to Jeewon *et al.*, (2003) and Ji and Tong (2004), *Pestalotiopsis* and *Truncatella* are anamorphic members falls under the family Amphisphaeriaceae as displays in Figure 2. *Truncatella* Steyaert was known for bearing 4-celled conidial forms while *Pestalotiopsis* Steyaert for 5-celled forms (Steyaert, 1949).

Kingdom: Fungi

Subkingdom: Dikarya

Division: Ascomycota

Class: Sordariomycetes

Subclass: Xylariomycetidae

Order: Xylariales Nannf., 1932

Family: Amphisphaeriaceae G. Winter, 1885

Genus: *Pestalotiopsis* Steyaert, 1949

Genus: *Truncatella* Steyaert, 1949

Figure 2. The taxonomic hierarchy for *Pestalotiopsis* and *Truncatella* (Eriksson *et al.*, 1998)

## 2.6. Diversity of endophytic fungi associated with *N. fruticans*

In local name, *Nypa fruticans* is called 'Nipah' palm tree, sometimes known as 'plant with thousand uses'. It is landward plant, favours brackish water environment and colonizes upper tidal reaches of rivers (Tsuji *et al.*, 2011). The hypersaline water during high tide may inhibit some plants' growth since excessive salt contents could give destructive effect to the plant's metabolic process by decreasing the fluid content (Calheiros *et al.*, 2010).

According to Hallmann *et al.* (1998), endophytic fungi defined as "fungi that can be isolated from surface-disinfected plant tissues or extracted from within the plant and not harmful to the host". Recent studies had shown that the endophytic fungi can behave as phytoremediation or bioremediation at contaminated sites (Li *et al.*, 2010). Manoharachary *et al.* (2005) mentioned the alteration between freshwater flush and saltwater invasion during pre- and post-monsoon created a favourable condition for growth of freshwater fungi in Western Ghats mangrove, India.

Several similar studies done in Brunei (Hyde, 1992) and Thailand (Pinnoi *et al.*, 2006) revealed fungi of group Ascomycota is the most common fungi associated with *Nypa fruticans* in estuary due to their ability to degrade cellulose and other plant polymers of leaf litter and drifted wood. It is more efficient for nutrient utilisation than bacteria since they possess extensive hyphal mechanisms for better nutrient absorption and faster growth rates (Thormann, 2005). Since fungi is "host-specificity and substratum-recurrence" (Hyde *et al.*, 2007), they can be used as bio-indicator, for example, *Sphagnum*, *Drepanocladus*, and *Scorpidium* mosses.

## **2.7. Tolerance of endophytic fungi on heavy metal**

Globally, human practising dilution paradigm where everything they unwanted; waste, chemical, and household will be dump into a water body. Some of the substance may contain heavy metals; some may essential for health of certain species, yet some may have negative effect. Based on Mohd Harun *et al.* (2007) paper that almost similar with present, the most heavy metals that can be found in estuary are Zinc (Zn), Lead (Pb), Copper (Cu), Chromium (Cr) and Cadmium (Cd). According to Carlile *et al.*, (2001) some species of endophytic fungi have high tolerance level on heavy metals and they utilised it for metabolic activities and produces alcoholic, citric acid and certain enzymes as by-product. Endophyte fungi also can be used as pesticides as it produces alkaloids to inhibit herbivores and insects (Zhang *et al.*, 2006).

## **2.8. Molecular works on endophytic fungi**

Ideally, identification of endophytic fungi always had been done using the combination of morphological and molecular methods where the morphological-based identification as comparison with ribosomal DNA (rDNA) gene sequences (Zhang *et al.*, 2006).

Molecular works that involved fungi and bacteria in plant species generally used internal transcribed spacer (ITS) to locate the gene sequences desired for molecular identification. However, Hu *et al.* (2007) mentioned that instead ITS sequences,  $\beta$ -tubulin genes resolves *Pestalotiopsis* better. Yet, in several papers, ITS still can be reliable to achieve the required sequence for fungal identification (Oliveira, 2013; Maharachchikumbura *et al.*, 2011; Guo, 2010). What is ITS? ITS is an internal transcribed spacer of nuclear ribosomal DNA (rDNA) and detected by Sanger sequencing approaches of 450 to 700 bp (Onn, 2010).

In DNA amplification, ITS is added to acts as primer, searching for match gene sequence and binding on the templates. During the process, master mix is prepared to avoid pipetting error. Mass preparation of reagent for a number of reactions is prepared in one microcentrifuge tube for efficient work and reliable amount of chemical to be transferred off.

### 3. Materials and Methods

#### 3.1 Study site

Below is the maps of the sampling site (Kuching Wetland National Park) which situated between Kampung Salak and Telaga Air.

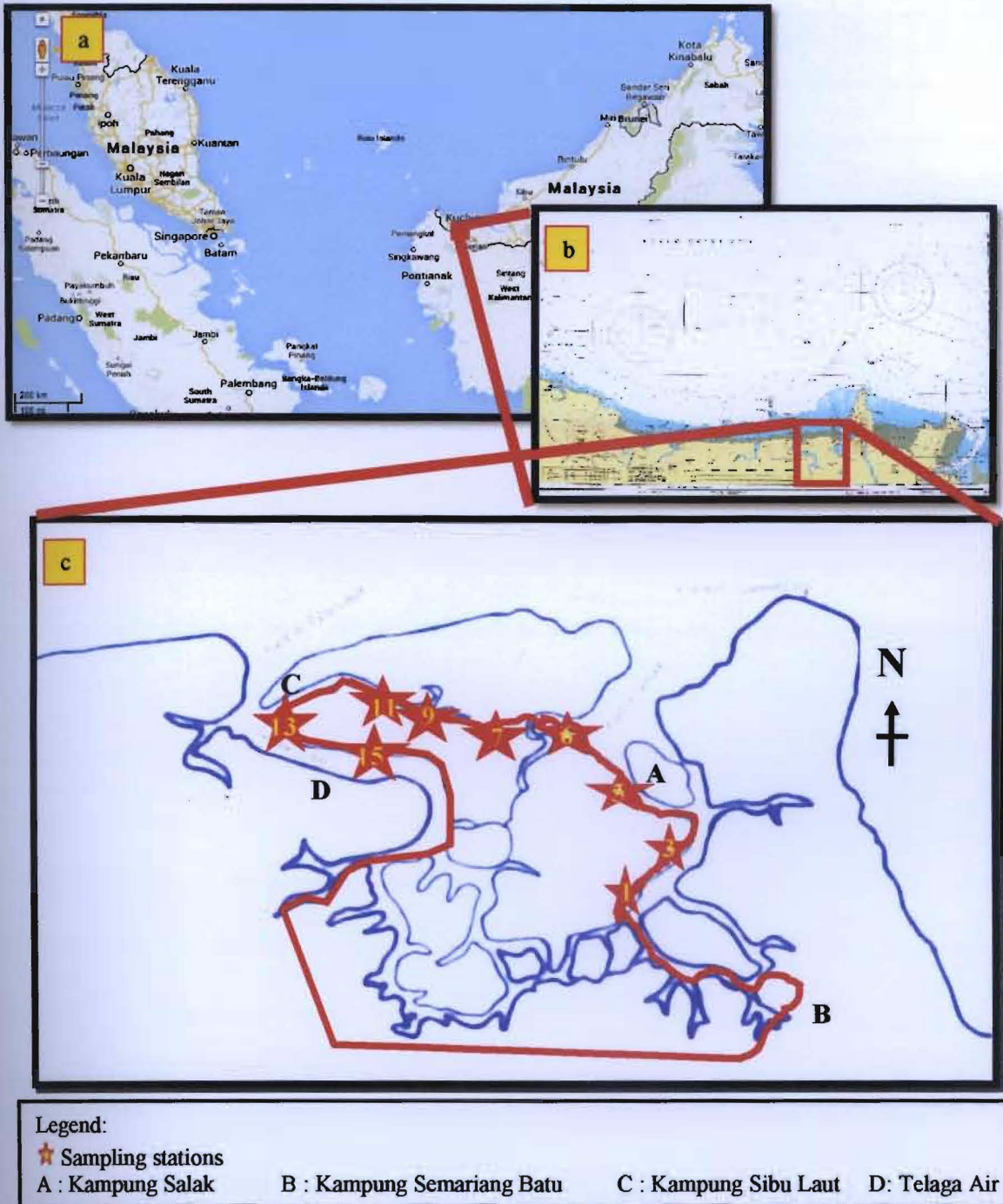


Figure 3. The maps of sampling site at scale 1: 20000 000 (a: Malaysia's map), 1:100 000 (b: Tanjung Po, Sarawak's map) and 1: 50 000 (c: Kuching Wetlands National Park's boundary). Station 1, 3 and 5 are more upstream stations whilst Station 13 and 15 more exposed to saline water. Other stations received more frequent fluctuation of saline and fresh water.

Kuching Wetlands National Park (KWNP) is one of the Ramsar Convention site and are estuarine ecosystem which is located in western Sarawak, 15 km away from Kuching, the capital city of Sarawak. It was implemented as national park on 24<sup>th</sup> July, 2002 (Gazette No.: 3512, date of publication: 10<sup>th</sup> October, 2002). Based on the map provided by the Sarawak Forestry Department (*refer Figure 3*), there are three villages situated outside the site boundary; Kampung Salak (east), Kampung Semariang Batu (southeast), and Kampung Sibulaut (northwest). KWNP were receiving saltwater from South China Sea while freshwater from two major rivers, Sungai Salak and Sungai Sibulaut. In order to get access to the wetlands, one can enter via jetty at Telaga Air, Kampung Semariang Batu or Kampung Santubong.

### **3.2 Time and date of sampling**

The field work was performed along the borderline of KWNP (approximately 30 km) on Thursday, 19 September 2013 via access point at jetty Kampung Semariang Batu during the incoming high tide, accompanied by Sarawak Forestry officers, Encik Muliadi Aden and Encik Ishak Hassim. Total of the stations were 16 where the existence of *Nypa fruticans* populations or locally known as 'Nipah' palm along the KWNP borderline were determined. However, only nine (9) stations were done in this present study due to slight differences from one station to adjacent station, manpower limitation and short timeframe. The tide table was provided by the Sarawak Marine Department and Kuala Santubong had been as a predicted port reference since it is the nearest to the site.