

**Understanding anuran responses to rainforest  
fragmentation and oil palm agriculture in the  
Lower Kinabatangan Wildlife Sanctuary, Sabah,  
Malaysia.**

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## SUMMARY

Habitat fragmentation is one of the first causes of biodiversity loss, but there is no consistent pattern describing how species react to it. Tropical forest has been lost due to timber extraction and agriculture. Large areas of protected continuous forest are now limited. It is vital to determine the biodiversity value of these fragmented secondary forests, especially in southeast Asia where the expansion of oil palm plantations has become a major threat for rainforest biodiversity. This study explores the effects of habitat fragmentation and oil palm plantations for Anuran communities of the Lower Kinabatangan Wildlife Sanctuary (LKWS). This thesis provides the first genetic amphibian study for the LKWS and Kabili-Sepilok Forest Reserve (KSFR). Higher species richness in primary (KSFR) and secondary (LKWS) forest habitats were found compared with oil palm plantations. Plantations surrounding the LKWS provide little overall benefit to frog conservation. Inside oil palm plantations lower species richness was found in interior plantations compared with plantation edges. The genetic diversity, genetic structure and migration rates of three species of Bornean frogs were examined using new species-specific microsatellites. Genetic analysis revealed the importance of fragment connectivity and the high conservation value of the study areas inside the LKWS. Phylogenetic diversity results showed that LKWS secondary forest could not be replaced without greater losses of diversity.

The results of this study can be used as a baseline for future conservation and management measures for the amphibians of the LKWS and KSFR.

# **Chapter 1: General Introduction**

## **1.1 FOREWORD AND JUSTIFICATION**

Since the *Acanthostega*, one of the earliest and most primitive known amphibians, “walked” this world around 368 million years ago, amphibians have never faced a decline as significant and alarming as that of today. Currently, along with the spread of the chytridiomycosis fungus, habitat loss and fragmentation are among the major causes of biodiversity loss across the amphibians. Much of this loss has occurred in tropical forest, where amphibians are the most diverse and feature high levels of endemism. Nowadays, large areas of protected continuous forest are limited, especially in southeast Asia where the expansion of agriculture, mostly oil palm plantations, has become a major threat for rainforest biodiversity and has been increasing dramatically since 1960.

In this thesis I aim to assess secondary and primary forest value for amphibians and how habitat fragmentation and land conversion have affected this value. The study was carried out in the Lower Kinabatangan Wildlife Sanctuary (LKWS) and at the Kabili-Sepilok Forest Reserve (KSFR) in Sabah, Malaysia. During 11 months in the field, I surveyed five lots of the LKWS and five areas of KSFR. I collected 600 tissue samples and buccal swabs from 18 different frog species in the LKWS, and 85 tissue samples and buccal swabs from 15 different frog species in KSFR. Abundance and richness, as well as 11 different habitat parameters, were measured in three different habitats (forest, forest edge, plantation and plantation edge) in the LKWS and KSFR. Species richness was higher in forested habitats compared with oil palm plantations, forest edge and plantation edge. Next Generation Sequencing was used to develop microsatellites (SSRs) for four frog species (two forest specialists, one generalist and one plantation specialist). A total of 26 SSRs from three species were fully standardised and used to evaluate the population structure of the three species. Our results revealed the LKWS (five lots) as a key area for conservation, especially lots 6 and 7, acting as a source for introducing additional genetic variation into



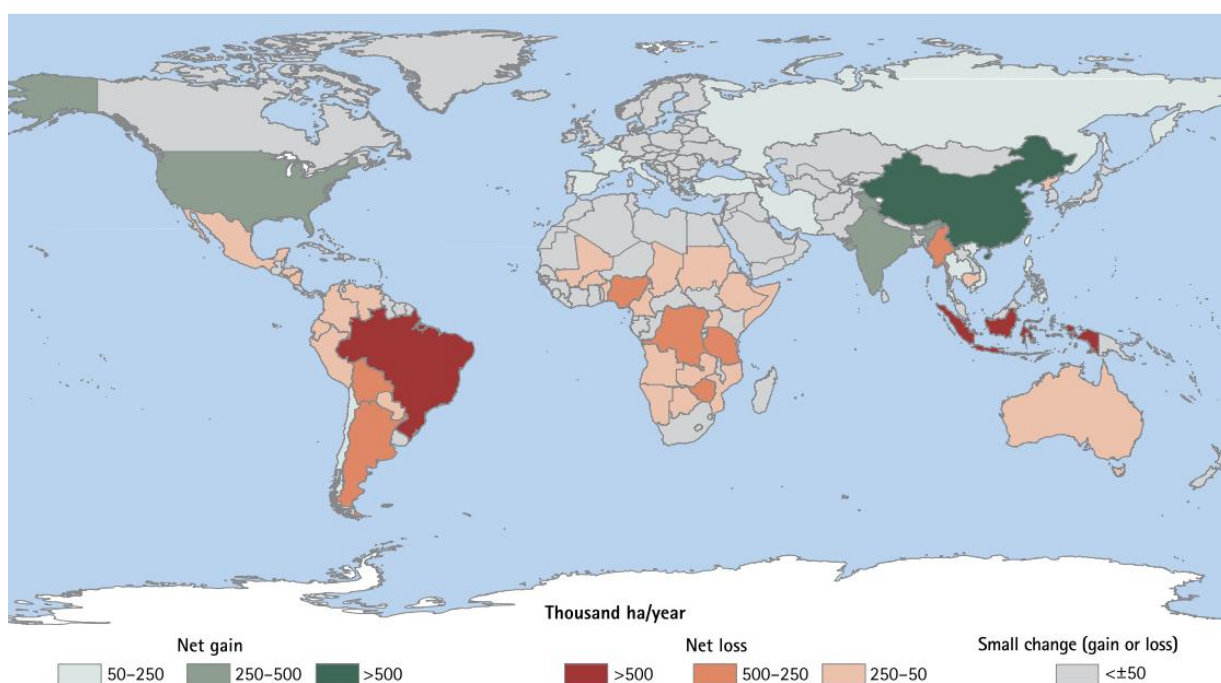
the other areas for our forest specialist species. Our results suggest that in the recent past, *Rhacophorus appendiculatus* and possibly *Hylarana megalonesa*, constituted a single large population with some amount of genetic flow across all the LKWS from Lots 5 to 8.

Mitochondrial 16S ribosomal RNA and cytochrome oxidase I (COI) sequences were used for DNA barcoding and phylogenetic analysis to enable phylogenetic diversity to be estimated at a number of spatial scales. The 16S marker alone could be used to identify 100% of the samples to species level when aligning the sequences in Genbank, whereas a lack of data for COI prevented the identification of most samples. Phylogenetic analysis using 16S sequences showed greater resolution than with COI, with three of four strongly supported clades. However, both markers showed low nodal support for deeper branching events. Phylogenetic diversity (PD) along with other three phylogenetic values suggested the importance of the LKWS for maintaining species diversity. KSFR showed a broader representation of clades than the LKWS but this last one conserve similar levels of evolutionary history than KSFR. There is a need for more intense surveys at KSFR in order to confirm these results. In contrast, different amphibian clades are less likely to survive in oil palm plantations compared to the LKWS. Our results show the importance of preserving secondary forest fragments within agricultural landscapes such as oil palm.

## **1.2 GLOBAL DEFORESTATION AND DEGRADATION OF TROPICAL FOREST**

The world is facing considerable intensification of agricultural activities, agricultural and forestry sectors combined have caused almost 60% of the total reduction in terrestrial biodiversity by 2010 (Kok et al., 2018). Timber extraction are currently responsible for about 52% of forest loss (Kissinger et al., 2012). In the last decade, around 13 million hectares of forest have been

converted to other uses each year or have been lost through natural causes (Figure 1.1) (OECD/FAO, 2019) Destruction of habitat and the elimination or interruption of wildlife corridors have a major impact on plant and animal species with many populations having disappeared already, while many others are increasingly threatened (Goossens et al., 2005; Pounds et al., 2006; Dinerstein et al., 2007).



**Figure 1.1** Annual change in forest area by country, 2005-2015. Red, orange and pink shades represent a net loss of forest area, while green shades represent a net gain (taken from FAO 2015)

Across the globe, forest harvesting and shifting cultivation practices have degraded and fragmented forest on a massive scale (Haddad et al., 2015). Commercial logging and agriculture, in particular for oil palm (*Elaeis guineensis*), are now the major causes of forest and biodiversity loss (Shevade and Loboda, 2019). To meet increasing demand, the area dedicated to palm oil production in producer countries in Southeast Asia such as Malaysia has increased between 2008 and 2014 (Azhar et al., 2017). Between 1990 and 1997 almost 7 million ha of tropical forest were

lost annually, with a further 2.3 ( $\pm$  0.7) million ha degraded (Achard *et al.*, 2002; Edwards *et al.*, 2014). Deforestation due to oil palm, driven by its global market continues to occur despite the efforts of conservationists (Koh and Wilcove, 2008). One of the reasons for the continued expansion of palm oil agriculture are the aggressive campaigns undertaken by the industry, promoting public acceptance of palm oil while dismissing the concerns of conservationists, to the point of claiming palm oil as beneficial to biodiversity and going so far as calling it “planted forest” instead of plantation (Koh and Wilcove, 2008). However, it is beyond dispute that oil palm plantations harbour far fewer forest-dwelling species than primary or even logged forest, and that palm oil expansion occurs at the expense of primary or secondary forest (Fitzherbert *et al.*, 2008; Koh and Wilcove, 2008; Gillespie *et al.*, 2012; Wich *et al.*, 2014; Scriven *et al.*, 2018).

### **1.2.1 OIL PALM AGRICULTURE**

Oil palm is cultivated across more than 13.5 million ha of tropical, high-rainfall, low-lying areas, naturally occupied by tropical forest (Fitzherbert *et al.*, 2008). During the past 30 years, oil palm has become one of the most rapidly expanding crops in the world (Fitzherbert *et al.*, 2008; Koh and Wilcove, 2008; Laurance *et al.*, 2010). Between 1980 and 2000, the annual global production of palm oil increased from 4.5 million to 20.9 million tonnes and was at 30.4 million tonnes by 2010 (Koh and Wilcove, 2007). Palm oil is one of the most extensive tropical crops in the world and has driven the conversion of more than 10 million hectares of forest over the past two decades (Dijkstra, 2016; Spear *et al.*, 2018). Oil palm production has doubled over the last 20 years, now exceeding 35% of total soya oil production (OECD/FAO, 2019).

Currently, 43 countries around the world grow oil palm. Indonesia and Malaysia (Southeast Asia) are the two largest producers in the world, with

the highest total oil palm harvested area in 2013 (Table 1.1) (Vijay et al., 2016). Malaysia and Indonesia are world leader in palm oil trade, together providing 85% of the global supply of 62 Mt in 2016 (Meijaard et al., 2018). Both countries hold more than 80% of Southeast Asia's remaining primary forest, where many endemic species are threatened with extinction by some of the highest global rates of deforestation (Fitzherbert et al., 2008; Turner et al., 2008). Evidence shows that palm oil production has had a substantial negative impact on most species, mainly through the clearing of natural forests on mineral and peat soils (which also requires drainage) to make way for plantations (Meijaard et al., 2018).

Determining which taxa are incapable of finding refuge in oil palm plantations will not only establish species groups of high conservation priority in these regions, but also help enforce effective plantation management and maintenance of overall biodiversity throughout Southeast Asia (Sodhi *et al.*, 2010). Oil palm agriculture have detrimental effects on biodiversity. Paoletti et al. (2018) showed that even though oil palm plantations across Indonesia were the most populated areas hosting high abundance of herpetofauna, still the communities were composed of a few common species. This was also demonstrated by Scriven et al. (2018) and we found similar results in our own study (see Chapter 4). Rare amphibians were more abundant in forested areas and common amphibians were more prevalent in plantations.

**Table 1.1** Percent of FAO reported total global oil palm harvested area in 2013 (Vijay et al., 2016).

Producer Country	FAO Total Oil Palm Harvested Area 2013 (km <sup>2</sup> )	Sample Area (km <sup>2</sup> )	Percent FAO Sampled (2013)
Indonesia	70,800	2,258.5	3.2
Malaysia	45,500	2,289.9	5.0
Nigeria	20,000	609.8	3.0
Thailand	6,264	203.6	3.3
Ghana	3,600	140.1	3.9
Ivory Coast	2,700	315.3	11.7
Colombia	2,500	766.5	30.7
Ecuador	2,188	189.1	8.6
Dem. Rep. of Congo	2,100	105.2	5.0
Papua New Guinea	1,500	162.5	10.8
Cameroon	1,350	161.3	11.9
Honduras	1,250	243.9	19.5
Brazil	1,220	513.2	42.1
Costa Rica	745	166.8	22.4
Guatemala	650	137.9	21.2
Philippines	500	70.9	14.2
Peru	475	280.2	59.0
Mexico	461	25.1	5.5
Venezuela	270	58.3	21.6
Dominican Republic	170	78.1	46.0

## **1.2.2 DEFORESTATION IN SOUTHEAST ASIA**

Among the world's tropical regions, Southeast Asia has experienced the highest rates of deforestation and forest degradation due to logging, agricultural expansion, habitat fragmentation and urbanization (Koh and Sodhi, 2010; Edwards et al., 2011). The rate of deforestation exceeds that of other tropical regions, as does the rate of timber extraction, with most of the remaining forests being classified as production forests and therefore open to logging (Edwards et al., 2011). The forest that remains after logging is vulnerable to conversion to oil palm, now the principal factor driving the loss of lowland forest in Southeast Asia (Edwards et al., 2011). Predictions suggest that Southeast Asia may lose up to 75% of its original forest cover by 2100, and up to 85% of its remaining biodiversity if current rates of deforestation continue. This could represent global extinction for at least 50% of Southeast Asian species (Sodhi et al., 2004; Sodhi et al., 2010).

Indonesia, Malaysia and Brunei comprise the Western Sunda region, which is considered as an important hotspot of biodiversity, due to the high concentration of endemic species found in these regions (Koh and Wilcove, 2007). For example, there are 89 species of amphibians in Malaysia and 17,500 species of vascular plants in Indonesia that do not occur anywhere else in the world. In total, between 1990 and 2010, Malaysia lost 8.6% of its forest cover, or around 1,920,000 ha (Wilcove and Pin, 2010). Compared with the estimated extent of primary forest 8,000 years ago, before large-scale human disturbance, relatively little remains intact in Indonesia (25.6%) and Malaysia (11.6%) (Koh and Wilcove, 2007).

### 1.3 BORNEO

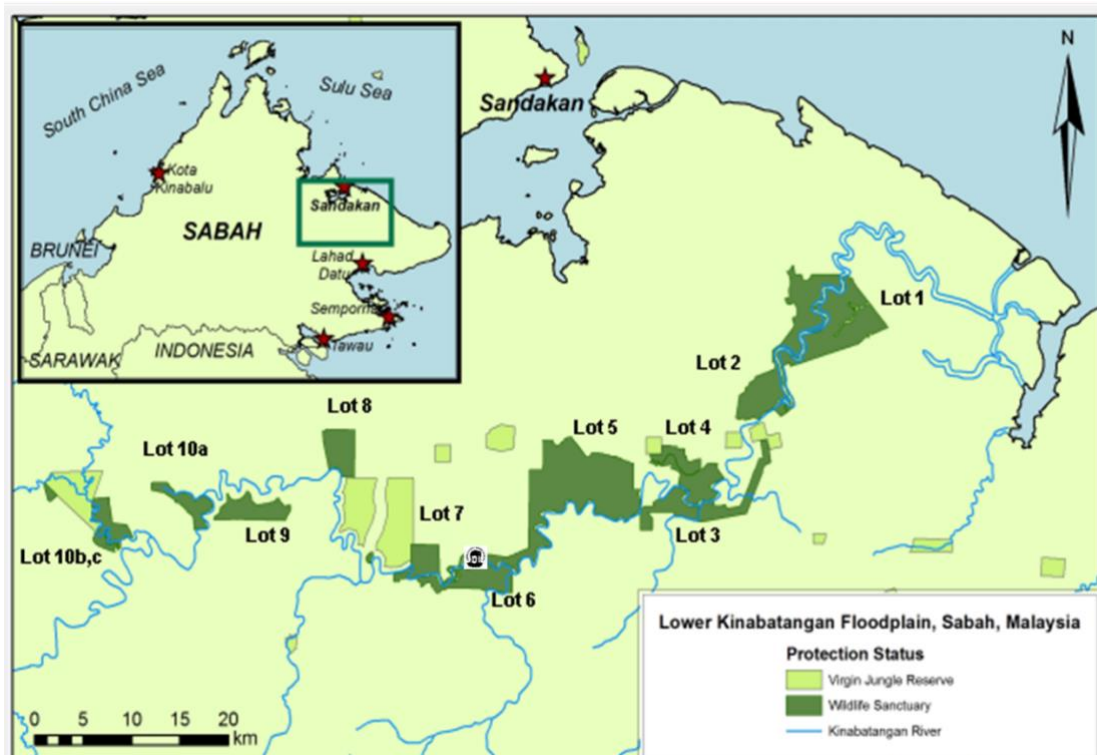
Borneo is one of the most biologically diverse regions on the planet and contains some of the highest species richness throughout the Sunda Shelf with as many as 1,175 tree species, as much as the entire temperate forests of the northern hemisphere (Corlett, 2014). Faunal diversity is estimated to include 260 bird species, 150 frog species and over 50 reptiles (Inger & Stuebing, 2005). Approximately 75 mammals occur (Garbutt & Prudente 2006), including 13 species of primates (Meijaard & Nijman, 2003), the bearded pig (*Sus barbatus*), the Bornean elephant (*Elephas maximus borneensis*), and one critically megaherbivores, the banteng or tembadau (*Bos javanicus lowi*) (Payne et al., 1985).

Borneo is situated on the edge of Wallace's line, which represents a biogeographic boundary dividing Asia from the Melanesian archipelago and Australia, featuring deep sea trenches that have prohibited the dispersal of many terrestrial species (Gardner, 2014). The island straddles the equator and is characterised by tropical climates year-round with humidity exceeding 95%. Borneo's position on the Eurasian plate means it does not experience severe tectonic activity, unlike the neighbouring Indonesian archipelago. The island comprises three countries: Malaysia (states of Sarawak and Sabah), Indonesia (Kalimantan) and Brunei. This thesis is centred on a region defined by the longest river in Sabah, the Kinabatangan and its floodplain, which is of major importance as a wildlife conservation site.

#### 1.3.1 THE LOWER KINABATANGAN WILDLIFE SANCTUARY

The Lower Kinabatangan Wildlife Sanctuary (LKWS) is located along the Kinabatangan river in east Sabah, Malaysia (N5° 28' – N5° 21'; E117° 56' – E118° 09'). The river is mostly flat and low (10-20m ASL) and its associated

forest is classified as extreme lowland forest. Temperatures can fluctuate from 21°C to 34°C and the mean annual precipitation is between 2,500 and 3,500mm (Malaysian Meteorological Services Department) (Ancrenaz et al., 2004). The Lower Kinabatangan Wildlife Sanctuary is comprised of 10 Lots of secondary forest (Figure 1.2). The region includes a variety of habitats, including riverine, seasonally flooded, swamp and dry dipterocarp forests, nipa palm and mangrove (Azmi, 1998).



**Figure 1.2.** Map of the Lower Kinabatangan River Floodplain, comprising of forest lots 1-10 of the Lower Kinabatangan Wildlife Sanctuary and several Virgin Jungle Forest Reserves. Map courtesy of Danika Stark.

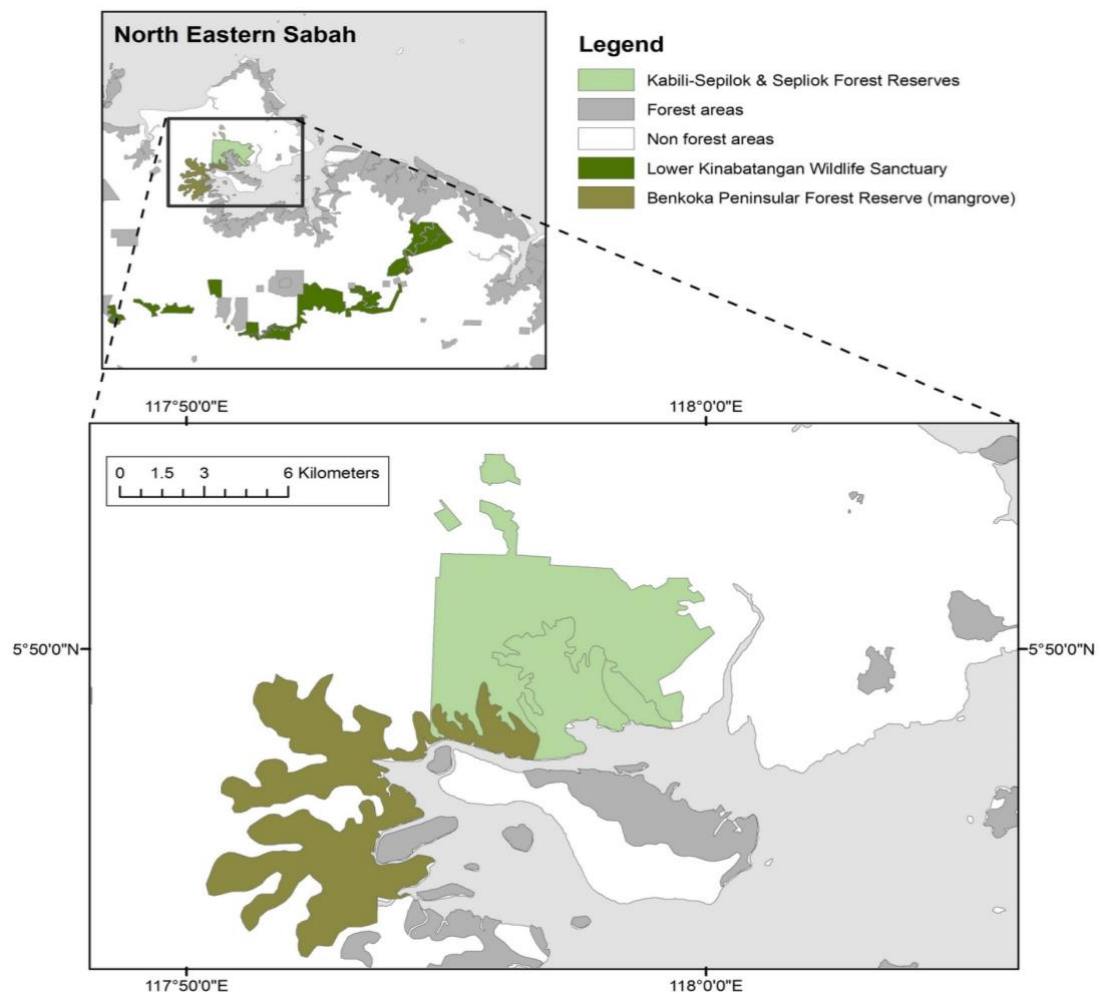
### 1.3.2 KABILI-SEPILOK FOREST RESERVE

Kabili-Sepilok Forest Reserve (KSFR, 58549 N, 1188049 E) is a lowland rainforest reserve of 42.9 km<sup>2</sup> situated 24 km west of Sandakan on the east coast of Sabah (Figure 1.3). KSFR is dominated by a mixed dipterocarp



## *Chapter 1: General introduction*

lowland forest of *Parashorea tomentella* and *Eusideroxylon zwagleri* types, with interspersed keranga forest on sandstone ridges (Bruhl et al., 2003). The reserve is surrounded by various plantations (fruit trees, old rubber and oil palm plantations) to the east, north and west and by mangrove forests and the sea to the south (Bruhl et al., 2003). The area it is exposed to heavy rains from the north-east in December and January. The total annual rainfall is about 3,000 mm. Mean daily temperature is 30°C. There is little information on the amphibians and reptiles in the area, but its biodiversity includes 200 species of birds, over 90 species of mammals and 70 species of butterflies. The reserve is under the management of the Sabah Forestry Department.



**Figure 1.3** Map of the Sepilok region and surrounding area. Hearn A (2011, April), adapted from <http://borneanwildcat.blogspot.com/2011/04/next-up-kabili-sepilok-forest-reserve.html>.

#### 1.4 AN INTRODUCTION TO THE AMPHIBIANS

Caecilians, frogs and salamanders are members of the tetrapod vertebrate Class Amphibia. They currently include more than 7000 recognized species with representatives found in virtually all terrestrial and freshwater habitats, but are absent from the coldest and driest regions or the most remote oceanic islands. The number of recognized species of amphibians has grown

enormously in recent years, with a nearly 50% increase between 1985 and 2004 and an increase in species numbers of 25% in the years between 1992 and 2003 (Stuart, 2008).

The Order Anura is comprised of the frogs (and their subgroup, the toads), and is by far the largest Order, with 5,208 living species currently recognized. Anurans are globally distributed, being found on every continent with the exception of Antarctica (Figure 1). While tropical habitats are richest in anuran diversity, frogs and toads may be encountered in many different environments ranging from dry deserts, through tropical and temperate regions to areas as far north as the Arctic Circle and as far south as Tierra del Fuego at the tip of South America (Stuart et al., 2013). The genetic analyses of the present thesis focussed on three species of frogs that were selected because they represent species that inhabit primary and old secondary forest (*R. appendiculatus*), palm oil plantation (*H. glandulosa*) and both types of habitat (*H. megalonesa*) inside LKWS and the oil palm plantations surrounding the sanctuary as well as primary forest (KSFR). *R. appendiculatus* is an arboreal species that lives in primary and old secondary forests (Diesmos et al., 2004; Inger & Stuebing, 2005).

## 1.5 FRAGMENTATION EFFECTS ON AMPHIBIANS

Habitat fragmentation leads to an increase in patch isolation, and variation in the degree of connectivity among patches (Saunders et al., 1991). After fragmentation, the remaining populations may reduce in size and experience demographic stochasticity and declines in genetic diversity that may lead to increased inbreeding, lower evolutionary potential and a higher risk of extinction (Andersen and Damgaard, 2004; Dixo et al., 2009). Dispersal barriers caused by habitat fragmentation may result in partitioning of genetic variation and increased population differentiation (Lesbarrères et al., 2006).

Frogs are vulnerable to habitat fragmentation, their narrow environmental tolerance and generally low dispersal capacities exacerbate the negative effects of degradation and loss of population connectivity, making them extremely sensitive to demographic isolation (Canestrelli et al., 2008; Dixo et al., 2009). Angelone & Holderegger (2009) studied the European tree frog (*Hyla arborea*) in Switzerland and showed the importance of connectivity for tree frog dispersal and highlighted the impacts of barriers such as the river Reuss to movement. Genetic analyses confirmed that *H. arborea* quickly colonized new ponds within distances up to 4km, provided those ponds were connected in the habitat network (Angelone and Holderegger, 2009). In the last two decades there have been numerous studies that show dispersal barriers may lead to population differentiation in anurans (Marsh et al., 2005; Lesbarrères et al., 2006; Murphy et al., 2010; Arntzen et al., 2017; Cox et al., 2017; Lenhardt et al., 2017).

Southeast Asia is one of the world's biodiversity hotspots for amphibians (Table 1.2), with over 700 species occurring in the region, of which at least 267 species occur in Malaysia (Sodhi et al., 2010; Ahmad, 2017). The state of Sabah holds 109 species of Anura (frogs and toads), representing more than 60% of the total species diversity in Borneo, with a degree of endemism of approximately 17% (Hee and Mohamed, 2008). Most of our current knowledge of Bornean amphibians is derived from the Malaysian states of Sabah and Sarawak, and Brunei Darussalam (Scriven et al., 2018; Inger & Stuebing, 2005), with very few published accounts from the larger Indonesian region of Kalimantan (Sodhi et al., 2010). However, to date there have been no studies related to the population genetic implications of fragmentation on amphibians in the area of the Kinabatangan river where fragmentation due to oil palm plantations has occurred during the last 40 years.

**Table 1.2** Number of species of Anura (frogs and toads) and amphibians (Anura and caecilians) in Malaysia (Ahmad 2017).

Family	Malaysia	Peninsular Malaysia	Borneo Malaysia	Overlap species between Peninsular Malaysia and Malaysia Borneo
Bufonidae	44	19	32	7
Cerotobatrachidae	2		2	0
Dicroglossidae	32	20	18	6
Megophryidae	38	12	29	3
Microhylidae	43	21	25	3
Ranidae	38	18	29	9
Rhacophoridae	57	21	47	11
Ichthyopiidae	13			
Total species of Anura	254	111	182	39
Total species of Amphibian	267			

### 1.5.1 AMPHIBIAN DECLINES

With more than 7,000 species described by the IUCN and more than 40% threatened by habitat loss, amphibians belong to the most threatened class of vertebrates (Bishop et al., 2012). Currently they are losing biodiversity at unprecedented rates (Baillie et al., 2004; Stuart et al., 2004; Hof et al., 2011). Modern amphibians are true survivors, as they have been living on Earth for well over 100 million years (Stuart et al., 2008) but the current estimated extinction rate is over 200 times that of the background extinction rate derived from the fossil record. To date more than 40% of the world amphibian species are in decline (Allentoft and O'Brien, 2010) due to habitat loss and degradation, climate change and chytridiomycosis (Almeida-gomes et al., 2016; Berger et al., 2016; Hudson et al., 2016). Despite this, there remains a lack of data for many species which are potentially threatened (Baillie et al., 2004).

Habitat fragmentation has been suggested as a major cause of recorded declines in global amphibian populations (Pineda and Halffter, 2004) and habitat loss remains the principal threat to amphibians worldwide, and is the primary cause of amphibian extinction (Gascon et al., 2005). Tropical rainforests are becoming increasingly fragmented due to anthropogenic land conversion, and fragmentation is becoming an increasing threat for amphibians in this ecosystem (Funk et al., 2005; Dixo et al., 2009; Gillespie et al., 2012; Riemann et al., 2015; Thompson et al., 2015). Changes in natural habitats due to fragmentation affect anurans in a variety of ways especially via decreases in habitat availability and changes in the spatial configuration and quality of forest fragments (Cushman, 2006). Surprisingly, there are relatively few studies on the relationship between landscape fragmentation and amphibian diversity, especially at tropical latitudes (Pineda and Halffter, 2004).

Martinez-Solano et al (2008) showed how terrestrial environment alteration could affect population characteristic such as dispersal rates and increased the risk of predation in the common toad (*Bufo bufo*). Funk et al. (2005) carried out a study on *Rana luteiventris*, quantifying amphibian dispersal using capture and recapture methodology in combination with genetic analysis, suggesting that dispersal plays an important role in the population dynamics on some amphibians and isolation due to fragmentation may increase local extinction rates. Studies such as those by Hillers et al. 2008 and Gillespie et al. 2012 show how conservation value of different habitat types (secondary forest, oil palm plantations) and quality (availability of aquatic sites for breeding, vegetation structure, and leaf-litter cover) alters the dynamics and composition of anuran assemblages in fragmented landscapes. Similar studies have confirmed that fragmentation is a real problem for the future of amphibians reducing genetic diversity and connectivity (Johansson et al., 2005; Dixo et al., 2009; Allentoft and O'Brien, 2010). Therefore, the maintenance of habitat connectivity should be of high priority for amphibian conservation (Funk et al., 2005) and data from

genetics, on ecological plasticity and sensitivity are essential for effective amphibian conservation planning.

## **1.6 PHYLOGENETIC DIVERSITY**

Phylogenetic diversity (PD) is a measure of the amount of evolutionary history present in a community, is a good indicator of underlying functional diversity and provides an insight into how evolutionary processes have shaped contemporary patterns of species richness (Faith, 1992; Fritz and Rahbek, 2012; Prescott et al., 2016). Incorporating measurements of evolutionary differentiation into conservation planning and an accurate assessment of the diversity and distribution are needed to mitigate extinction of evolutionary lineages, preserving as much as we can of the tree of life (Stuart et al., 2006; Prescott et al., 2016). For amphibians the utility of combining phylogenetic and population genetic markers helps not only to understand the evolutionary history and genetic diversity of taxonomically complex groups of species, but contributes to a more objective assessment of amphibian conservation priorities in tropical areas (Fouquet et al., 2007).

Amphibian studies along the Kinabatangan River have to date focused on patterns of richness and abundance (Gillespie et al., 2012; Scriven et al., 2018), but there is a critical need to understand the population-level effects of land use on species assemblages and the mechanisms that underline anuran responses especially in Southeast Asia where there are currently no reports of chytridiomycosis. A biodiversity assessment of frog communities will be crucial to future conservation strategies, by providing a case study in an unaffected region of the globe. Moreover, in case of a future outbreak we will be able to identify areas with high biodiversity and more stable frog communities to focus efforts in conservation. Understanding the impacts of oil palm expansion on other taxa is vital given the projected expansion of oil palm agriculture (Turner et al., 2008).

### **1.6.1 PHYLOGENETIC DIVERSITY AND CONSERVATION**

Tropical forest are the habitats with the highest biodiversity (Mittermeier et al., 2004), and its conversion to agricultural lands is a major source of biodiversity loss (Laurance et al., 2014). If biodiversity decline continues, we face an extinction crisis compared with some of the biggest mass extinction events in the history of life on Earth (Barnosky et al., 2011). It is critical to find ways to cope with this crisis, not only to protect species for their intrinsic value, but for the importance of the ecosystem functions they may provide (Haddad et al., 2015; Prescott et al., 2016; Riemann et al., 2017). In order to conserve as much as the tree of life as possible it is necessary to incorporate measurements of evolutionary distinctiveness into conservation planning (Redding and Mooers, 2006). Placing particular emphasis on the conservation of evolutionarily distinct species will mean that a greater proportion of evolutionary history is preserved, decreasing the chance of unique phenotypic and ecological traits being lost forever (Prescott et al., 2016).

Phylogenetic diversity (PD), as the amount of evolutionary history present, is increasingly recognized as valuable conservation currency (Davies and Buckley, 2011). PD is rapidly becoming an important component of community ecology, macro-ecology and biodiversity conservation (Winter and Schweiger, 2013; Matos et al., 2017). Phylogenetic diversity measures provide insights into patterns of community assembly, and high levels of PD are associated with higher levels of ecosystem function (Tucker et al., 2017). High levels of phylogenetic diversity may enhance the resilience and evolutionary potential of communities in an era of rapid environmental change (Prescott et al., 2016). Fritz and Rahbek (2012) used PD to provide an inside on how evolutionary processes may have shaped contemporary patterns of species richness on amphibians. Martins et al. (2015) assessed the phylogenetic structure in order to investigate phylogenetic patterns at regional and local scale to understand the influences of seasonal processes



is shaping the structure of anuran communities in Brazil. Amphibians are one of the most threatened high level taxa and understanding the evolutionary processes it is important for their conservation

## **1.7 FRAGMENTATION EFFECTS IN TROPICAL FOREST ON GENETIC DIVERSITY**

Tropical forests are not only one of the most diverse habitats in the world but they also suffer most in terms of habitat destruction (Hillers et al., 2008). Remaining forests are generally fragmented or highly logged (Giam et al., 2011) and forest fragmentation is widely considered to be a primary cause of the current biodiversity crisis (Arroyo-Rodriguez et al., 2017). Habitat fragmentation of the tropics is one of the most critical signs of anthropogenic ecosystem degradation (Laurance, 1999; Peres et al., 2006; Radespiel and Bruford, 2014). The negative effects that can come with fragmentation include population declines, demographic isolation, constrained resource availability, and how fragmentation might alter species potential to respond to large-scale global changes such as climate change (Radespiel and Bruford, 2014). The degree of degradation following fragmentation due to anthropogenic reasons may vary depending on the time since a fragment was isolated and the quality of the surrounding landscape (Hillers et al., 2008). Remnant forests are likely to endure from being smaller, more isolated, and with a greater area located near the edge of the forest (Haddad et al., 2015). However, fragmentation can occur naturally, apart from the direct impacts of forest loss and expanding anthropogenic land cover, fragmentation can occur at the edge of large tracts of forested landscape (e.g. ecotones; Smith et al., 1997). Northern Madagascar forest is a good example on how long-term fragmentation may have existed in forest previously thought to have been continuous (Quéméré et al., 2012). The need to separate natural fragmentation from recent anthropogenic effects carry a methodological challenge in the field of conservation genetics. Radespiel & Bruford (2014) carried on a study to examine the current state of

knowledge on the genetic consequences of rainforest fragmentation for animal species. From a total of 57 studies in the last 10 years they found that only 4.1% were related to amphibians.

## 1.8 PROJECT AIMS AND HYPOTHESES

The main objective of this project was to study the genetic and ecological effects of fragmentation in amphibians associated with oil palm agriculture and in continuous landscape. The Aims were as follows:

- To develop and characterize new microsatellite markers for four species of Bornean frogs.
- To identify population structure in the LKWS and surrounding oil palm plantations.
- To understand the genetic consequences of fragmentation in the LKWS using anuran species with different habitat affinities as a model.
- To analyse patterns of genetic diversity within populations of frogs commonly found in forest (*Rhacophorus appendiculatus* and potentially *Rhacophorus dulitensis*), oil palm plantations (*Hylarana megalonesa*) and in both habitats (*Hylarana glandulosa*), and to compare these results with a relatively undisturbed forest block at KSFR.
- To assess the value of LKWS secondary forest for anuran species richness and composition.
- To analyse spatial patterns of phylogenetic biodiversity (PD) for frog species within the LKWS and KSFR.
- To identify whether habitat fragmentation leads to altered assemblage composition for Anurans.

The hypotheses were as follows:

1. LKWS secondary forest features lower levels of Anuran genetic diversity than KSFR primary forest.
2. Lower levels of intra-specific genetic diversity would be found in recently established *H. glandulosa* populations inhabiting oil palm plantations.
3. Fragmentation due to oil palm agriculture reduces population genetic diversity for the forest specialist *R. appendiculatus* and the generalist *H. megalonesa*.
4. Habitat fragmentation increases intra-specific population genetic differentiation among all frog species (*R. appendiculatus*, *Hylarana megalonesa* and *H. glandulosa*)
5. LKWS secondary forest fragments support higher species richness and endemic species than surrounding oil palm plantations.
6. Habitat heterogeneity influences anuran community composition within forested (LKWS and KSFR) and oil palm plantation habitat types.
7. KSFR primary forest and LKWS secondary forest would hold higher anuran phylogenetic diversity than plantations.

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## **Chapter 2:**

# ***De novo* nuclear marker development from Illumina paired-end data from four species of Bornean frogs**

## 2.1. INTRODUCTION

As mentioned in Chapter 1, oil palm agriculture has been responsible for an average of 270,000 ha of forest conversion annually from 2000–2011 in major palm oil exporting countries (Henders et al., 2015). Using land-use data collected by FAO, Koh and Wilcove (2008) found that >50% of Indonesian and Malaysian oil palm plantations in 2005 were situated on land that was forest in 1990. Understanding the capacity of this new habitat to maintain biodiversity is something that needs to be assessed and is driving a major global research effort (Arntzen et al., 2017; Lenhardt et al., 2017). Organisms react in different ways to habitat change, and as such species-specific tools are needed to measure the influence of this change. One indirect method to assess the effects of such changes is the use of population genetics to infer dispersal and connectivity within and among populations (Goossens et al., 2005). Population genetics requires the use of molecular markers, the most commonly applied to a wide range of biological studies are microsatellites (otherwise known as Simple Sequence Repeats or Short Tandem Repeats) (Hung et al., 2016).

Microsatellites are one of the most widely used tools in conservation genetics (Jarne & Lagoda, 1996; Vieira et al., 2016). They are widely used because of certain desirable characteristics, such as high mutation rates, abundant distribution throughout the genome, ease of use, codominance, high polymorphism and reproducibility (Bruford & Wayne, 1993; Murray et al., 2018). These useful characteristics, however, come with a cost, and microsatellites marker discovery and validation used to be expensive and time consuming (Jehle & Arntzen, 2002). Since their initial assessment as population genetic tools (Bruford & Wayne, 1993), there has been a search for cost effective and time efficient methods for the *de novo* isolation of microsatellites markers, and recently this has involved the use of next generation sequencing (Davey et al., 2011). Next generation sequencing (NGS) has the ability to allow the sequencing of DNA and RNA both quickly

and cheaply, capable of producing an entire human genome within a single day (Grada & Weinbrecht, 2013), whereas initially it took over a decade to deliver a final draft of the first human genome (International Human Genome Sequencing Consortium, 2001). NGS has revolutionised the study of genomics and molecular biology and can be applied in a variety of contexts, ranging from rapidly resequencing whole genomes to the investigation of complex diseases and traits (Naidoo et al., 2011). There are many NGS technologies available today, including Nanopore, PacBio, 10x and Solexa Technology (used in the Illumina genome analyser). Currently, Illumina paired-end sequencing platform is the most commonly used for microsatellite isolation on amphibians (Adamson et al., 2016; Fusinatto et al., 2013; Lewis et al., 2014; Xia et al., 2018).

In order to generate population genetic resources for this forest/agricultural landscape and to obtain a better understanding of population genetic structure for key anurans, *de novo* nuclear markers were developed using next generation sequencing (NGS). This was implemented for four frog species (all categorized as Least Concern by the IUCN) that were found in forest, oil palm plantations and both habitats. *R. appendiculatus* (Frost, 2014) was chosen as it is a forest specialist. *H. glandulosa* (Frost, 2014) was chosen as a plantation specialist, *H. megalonesa* (Frost, 2014) was chosen as it is a generalist, found in both types of habitat and finally we included *R. dulitensis*, the jade tree frog (Frost, 2014), which is also a forest specialist, all four species are currently categorized as Least Concern on the IUCN red list.

## **2.2. MATERIALS AND METHODS**

### **2.2.1 SAMPLE COLLECTION AND DNA EXTRACTION**



In order to develop microsatellite markers for *R. appendiculatus*, *H. megalonesa* and *H. glandulosa*, a single individual for the species was euthanized by whole body cooling following by freezing as the most human way (Lillywhite et al., 2017) to produce sufficient DNA for Next Generation Sequencing (NGS). *R. dulitensis* was categorized as Near Threatened on the IUCN red list by the time of sample collection, so we decided to collect samples using standard toe-clipping as a less invasive source of material for genetic analysis. Samples were collected across four Lots (Lot 8, Pin Supu, Lot 6 and Lot 5) and one plantation (P 1) from the LKWS (Figure 2.1). In order to accurately evaluate the new markers, samples were collected for *R. appendiculatus* (n=49), 34 for *H. megalonesa* and 15 for *H. glandulosa* (Table 2.1). For *R. dulitensis* only five individuals were found so we developed microsatellites *in silico* for this species (Appendix one).

**Table 2.1** Sample list per species in four areas of the LKWS and one plantation.

Species	Number	Population	Area	Habitat
<i>R. appendiculatus</i>	17	LKWS	Lot8	Secondary forest
	9	LKWS	Pin Supu	Secondary forest
	13	LKWS	Lot6	Secondary forest
	10	LKWS	Lot5	Secondary forest
Total	49			

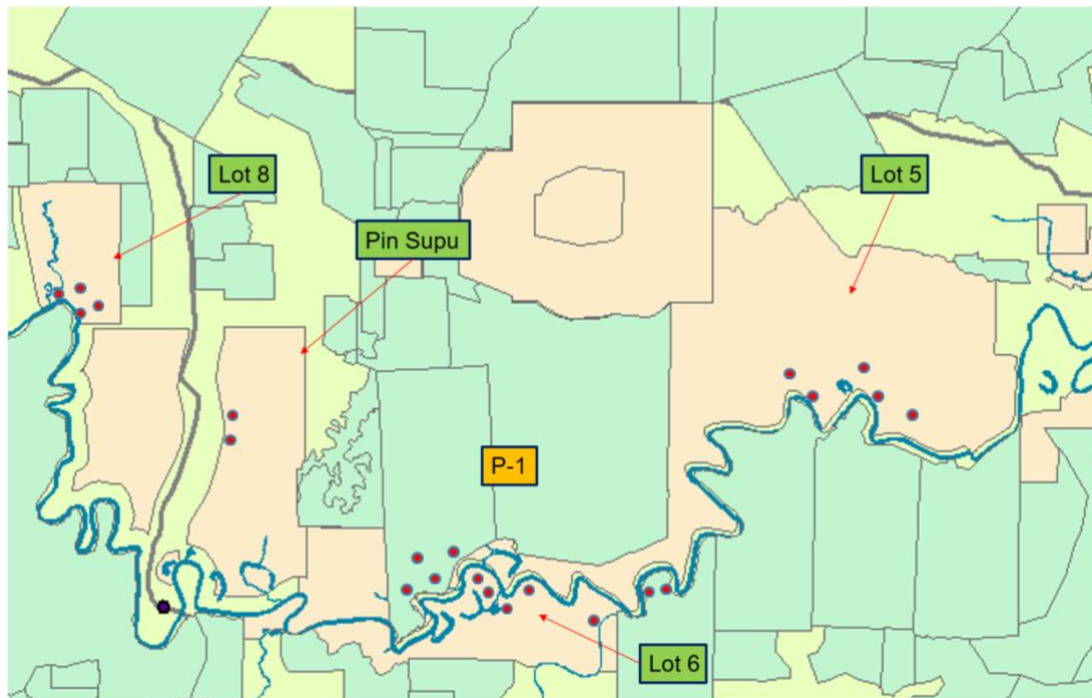
Species	Number	Population	Area	Habitat
<i>H. megalonesa</i>	19	LKWS	Lot8	Secondary forest
	6	LKWS	Lot6	Secondary forest
	9	LKWS	Lot5	Secondary forest
Total	34			

Species	Number	Population	Area	Habitat
<i>H. glandulosa</i>	15	P-1	LKWS	Plantation
Total	15			

All frogs were handled and sampled as described in Martin & Hong (1991). A 1mm square piece from the fourth toe tip of the right hind leg was taken in

order to obtain tissue samples. This methodology allows individual marking and has minimal effects on survival in many anuran species (Grafe et al., 2019). Samples were collected in absolute ethanol and stored at -20°C.

Genomic DNA for NGS was extracted from the liver of each whole individual using the Qiagen DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The same extraction protocol was used for toe-clip samples that were genotyped to obtain population-level polymorphism data. To assess the quality of the DNA, an agarose gel 1% was run in TBE1x, 120V for 30 min, and Qubit-Fluorometric quantification (Invitrogen) was used to quantify the DNA.



**Figure 2.1.** Map of the LKWS. Red points show sampling locations at the different lots and plantation.

## 2.2.2 LIBRARY PREPARATION

Library preparation and Illumina HiSeq (PE300) sequencing was performed by Novogene Bioinformatics Technology Co., Ltd., Beijing, China

([www.novogene.cn](http://www.novogene.cn)). Briefly, a total of 1.0 µg of DNA was used for DNA sample preparation. Sequencing libraries were generated using the Truseq Nano DNA HT Sample preparation Kit (Illumina USA) following the manufacturer's recommendations and index codes were added to attribute each sequence to each sample. Genomic DNA was randomly fragmented to a mean size of 350bp by Covaris cracker, DNA fragments were end polished, A-tailed, and ligated with the full-length adapter for Illumina sequencing with further PCR amplification. Finally, PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent 2100 Bioanalyzer and quantified using real time PCR. The qualified libraries were fed into a HiSeq X system sequencer after pooling according to its effective concentration and data volume.

### **2.2.3 CONTIG ASSEMBLY AND MICROSATELLITE DETECTION**

A total of 20.81Gb of raw data was analysed for the four species. Data quality is shown in table 2.2. Trimmomatic (Bolger et al., 2014) was used to remove the adapters of the raw data along with MUSKET as a next generation sequencing read error correction algorithm for Illumina sequences (Yongchao et al., 2013). *De-Novo* assembly of the cleaned reads was implemented using SOAPdenovo2 (Ruibang et al., 2012). Read processing and assembly parameters followed the program guidelines. For the assembly, three k-mer sizes between 41 to 61 were used. The best assembly was chosen using the total number of contigs produced and the N50 size (Table 2.3). All samples were run through the pipeline individually using the High Throughput Platform YSGO at Cardiff University.

**Table 2.2** Data quality summary

Species	Raw reads	Raw data (G)	Q20(%)	Q30(%)	GC(%)
<i>R. appendiculatus</i>	21381955	6.41	94.4	88.4	43.8
<i>H. megalonesa</i>	17475504	5.24	93.9	87.6	43.4
<i>H. glandulosa</i>	18190783	5.46	94.1	87.8	43.5
<i>R. dulitensis</i>	12325532	3.7	94.7	88.8	43.7

Q20, Q30: Phred quality scores

Detection of microsatellite markers from the assembly data was performed using Primer 3 and MISA software (Rozen & Skaletsky, 2000, Thiel et al, 2003). We searched for complex, mono-, di-, tetra-, penta-, and hexanucleotides. Primer 3 parameters for amplifications size range was between 150 and 500 bp. The primer annealing temperature was restricted to 56-62°C, CG Clamp was set as a minimum of 2 and primer length was 18-22 bp, other settings were as default.

#### 2.2.4 AMPLIFICATION CONDITIONS AND VALIDATION OF PRIMERS

Primers were synthesized with a target product ranging from 100- 300 bp for amplification. Seven samples for each species were used for testing of amplification success rate. All primers were tested to evaluate polymorphism on a 3 % agarose gel. Those loci that could not be successfully amplified, produced faint, unspecific or multiple bands were discarded. Microsatellites that showed polymorphism were fluorescently labelled (5'- FAM, HEX, or NED) and assembled in 3 multiplexes for each species for further testing. To test our microsatellites for polymorphism and stability, 49 samples from *R. appendiculatus*, 34 from *H. megalonesa* and 15 samples from *H. glandulosa* were used. PCR amplification was performed in a final volume of 15 µL comprising 7.5 µL 1X master Mix (QIAGEN, Hilden, Germany, Multiplex Kit), 0.6 µL of each primer (10µM/µL), 1.2L ddH<sub>2</sub>O and approximately 1.5µL diluted genomic DNA (20-100ng/µL). We used the following PCR programme

on an Applied Biosystem Veriti Thermal Cycler: 15 min at 95°C in order to activate HotTaq Polymerase from Qiagen Multiplex PCR kit , 35 cycles at 95°C for 1 min, 58-60°C for 45s, 72°C for 1min, followed by a final extension step for 20 min at 72°C. For genotyping, PCR products were sent for Fragment Analysis (capillary electrophoresis) to MRC PPU DNA Sequencing services from University of DUNDEE Scotland, UK.

## 2.2.5 DATA ANALYSIS

Standard population genetic statistics needed to measure genetic diversity (Guo & Thompson 1992), namely observed and Hardy-Weinberg Equilibrium expected heterozygosity, null alleles, Polymorphic Information Content (PIC) and number of alleles per locus, were estimated using CERVUS V.3.0.7 (Table 2.6). Hardy-Weinberg Equilibrium was assessed along with linkage disequilibrium using GENEPOP V.4.2 (Raymond & Rousset, 1995; Rousset, 2008) and CERVUS v.3.0.7, using the default values of the Markov chain parameters. MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004) was used in order to check for potential scoring errors, large allelic dropout and the presence of null alleles.

## 2.3 RESULTS

### 2.3.1 SEQUENCE ASSEMBLY

The genome assembly with SOAPdenovo resulted in a low scaffold count due to the low sequencing coverage (or depth) of 5X for all four species. A Kmer of 51 was the best option for all genome assembly for all four species (see Table 2.3). *R. appendiculatus* fragment lengths varied from 100 to 10,212 bp with an average of 240bp and a CG content of 41.94% and a N50 value of 259bp. The number of scaffolds larger than 1K bp was 1,790, the

majority of scaffolds were in the range of 100 to 500 bp (1,959,759) with 79,452 scaffolds larger than 500bp. For *H. megalonesa* fragments varied from 100 to 9,198 bp with an average of 205 bp, a CG content of 40.91% and a N50 value of 223 bp. The number of scaffolds larger than 1K bp was 844, with the majority of scaffolds in the range of 100 to 500 bp (883,001) and 13,605 scaffolds larger than 500pb. For *H. glandulosa* fragments varied from 100 to 5828 bp with an average of 203 bp, a CG content of 41.33% and a N50 value of 223 bp. The number of scaffolds larger than 1K bp was 1,051, with the majority of scaffolds from 100 to 500 bp (890,058) and 12,153 scaffolds larger than 500bp. Finally, for, *R. dulitensis* fragments varied from 100 to 4,561 bp with an average of 235 bp, a CG content of 41.17% and a N50 value of 248 bp. The number of scaffolds larger than 1K bp was 691, with the majority of scaffolds from 100 to 500 bp (948,221) with 25,746 larger than 500bp.

**Table 2.3.** Comparison of choosing different Kmer size on the resulting de-novo genome assembly for three species of Bornean frogs.

***R. appendiculatus***

Contigs						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	5321586	103470	1636	181	194	1037811605
51	2034123	55128	676	246	230	478823573
61	1456569	27882	386	248	234	343835930

Scaffolds						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	5160640	147158	3535	196	202	1046136203
51	1959759	79452	1790	259	240	480979437
61	1421573	42526	1154	255	240	344322175

***H. megalonesa***

Contigs						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	5619449	14848	163	150	166	937228022
51	908564	7324	260	217	199	187767310
61	657591	5073	300	218	196	130587868

Scaffolds						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	5525601	27409	543	150	170	941588976
51	883001	13605	844	223	205	188319404
61	645107	9007	946	220	200	130653356

***H. glandulosa***

<b>Contigs</b>						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	6000000	12100	205	150	165	989698541
51	909722	7321	376	219	198	186762029
61	687140	5584	480	219	193	134821642

<b>Scaffolds</b>						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	6000000	20526	585	150	167	993281541
51	890058	12153	1051	223	203	187263868
61	677328	8816	1100	220	196	134916069

***R. dulitensis***

<b>Contigs</b>						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	4320447	39402	375	150	180	779953285
51	982476	16167	216		226	226508498
61	658655	7373	182	238	227	150599438

<b>Scaffolds</b>						
Kmer	>100	>500	>1K	N50	Average length	Total nucleotide length(bp)
41	4210110	62283	982	150	186	785001423
51	948221	25746	691	248	235	227376585
61	644324	12391	543	242	232	150734557



### 2.3.2 MICROSATELLITE SCREENING AND QUALITY EVALUATION

After genome assembly, a total of 1,228 sequences contained microsatellite loci (Complex, di-, tri-, tetra and penta-nucleotide) were found across the four frog species (Table 2.4), using the bioinformatic tool MISA. 718 putative SSR's were found for *R. appendiculatus*, 121 for *H. megalonesa*, 118 for *H. glandulosa* and 271 for *R. dulitensis*. Sixty-one were selected on the basis of the motifs contained and the number of repeats seen. Nine microsatellite markers for *R. dulitensis* were only developed *in-silico* due to the low number of samples obtained in the field (Table 2.4).

**Table 2.4** Number of SSR's developed In-silico and success rate for three species of Bornean frogs. NT not tested.

	R.	H.	H.	R.	Total
	appendiculatus	megalonesa	glandulosa	dulitensis	
MISA	718	121	118	271	1228
In-Silico	12	20	19	9	60
SSR's	10	6	10	NT	26
Success rate (%)	83	30	47	NT	...

After testing by multiplex PCR as describe above, 26 polymorphic loci were successfully developed for the three frog species. These loci were used to genotype 98 individuals for the three different species from five different areas in secondary forest and one plantation (Table 2.5). Ten loci were developed and tested for *R. appendiculatus* ten for *H. glandulosa* and six for *H. megalonesa*. Cross-species amplification of one *H. glandulosa* (Hg1jm) microsatellite was used in *H. megalonesa*. In total, seven microsatellites were tested in *H. megalonesa*. Marker Hg1jm that was design for *H. glandulosa* was found to have more alleles and a higher PIC when were used in *H. megalonesa* (see Tables 2.5 and 2.6).

**Table 2.5.** List of 26 microsatellites (SSR's) belonging to three frog species. *Rhacophorus appendiculatus* (Rajm), *Hylarana megalonesa* (Hmjm), *Hylarana glandulosa* (Hgj).m).

Primer	Species	Primer sequence	Motif	Ta (C°)	Product size range (bp)	Na	PIC
Ra2jm	<i>R. appendiculatus</i>	F:GAGACGCTCCTAATAGTACAG R:TCTATATGCTGGCAACATGG	(AT) <sub>12</sub>	60	191-209	4	0.432
Ra3jm	<i>R. appendiculatus</i>	F:GCTTTGCCTCTGCTACAAGC R:TGAGGAGAACACAGGACAGC	(TG) <sub>12</sub>	60	242-284	19	0.821
Ra4jm	<i>R. appendiculatus</i>	F:ACGGAACAGAGCAACAGACG R:AGTGGCAGCTAAGAGGATGC	(GT) <sub>8</sub>	60	221-229	4	0.331
Ra6jm	<i>R. appendiculatus</i>	F:TGATTATCGACCAGTGAATGG R:CCCGAGAAATCAAATTTAGGC	(TA) <sub>16</sub>	60	265-301	15	0.890
Ra7jm	<i>R. appendiculatus</i>	F:CACAGGTGCAGAAGTCATGG R:CAGTGAGCAGGTATGCAAGC	(GA) <sub>6</sub>	60	134-144	4	0.520
Ra8jm	<i>R. appendiculatus</i>	F:TGTTGATGTACAGTCATTGG R:AAGTGAAATGTATCCACAGG	(AT) <sub>10</sub>	60	170-173	3	0.347
Ra9jm	<i>R. appendiculatus</i>	F:CTGCCGAGTTAAAGTTAGAGG R:CGTTAAAGGACTCAACACTCC	(TG) <sub>10</sub>	60	150-184	12	0.865
Ra10jm	<i>R. appendiculatus</i>	F:TTTGATTGCTCATTGTCTGG R:ATTAACATGCACTGGTCTGC	(AT) <sub>9</sub>	60	173-193	6	0.464
Ra11jm	<i>R. appendiculatus</i>	F:ATGGAGATGGATGCACATGG R:ACGTCATCGTCCATTTGTCC	(AC) <sub>7</sub>	60	206-264	16	0.649
Ra12jm	<i>R. appendiculatus</i>	F:ACGTCATCGTCCATTTGTCC R:GATCCTTTTCATCTCTTACCTCTGC	(TA) <sub>7</sub>	60	176-180	3	0.292
Hm1jm	<i>H. megalonesa</i>	F:GAAAGCCAGCAGTGCATATAG R:CTAGTAGGTCACCTCCAAGG	(AT) <sub>18</sub>	60	262-271	3	0.524

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Hm3jm	<i>H. megalonesa</i>	F:ACAACATAAGGTCTGACAACG R:GCCAAGTACATCAACATACC	(GA) <sub>9</sub>	60	291-299	3	0.308
Hm4jm	<i>H. megalonesa</i>	F:CCAAATCTCCAACACACACG R:TCAATCTATAGGCTGCTTCAG	(TG) <sub>9</sub>	60	96-102	4	0.445
Hm7jm	<i>H. megalonesa</i>	F:AATTATGGTTGGACGACAGC R:TCAGACAATGGCTTATTGGC	(GT) <sub>7</sub>	60	94-104	5	0.539
Hm11jm	<i>H. megalonesa</i>	F:TCACCAGATGTCTTCTTCGC R:CCCAGAATATTCCATGGATC	(ATA) <sub>8</sub>	60	221-228	3	0.584
Hm14jm	<i>H. megalonesa</i>	F:CAGATAAGAGTGAGATTTGC R:CTGCATAGACAGGAGAGC	(CT) <sub>7</sub>	60	201-215	2	0.431
Hg1jm	<i>H. glandulosa</i>	F:CAGACACAACAAACCATCACC R:GTGTTTTTCTGCCTGGTTGC	(ATA) <sub>7</sub>	62	208-238	7	0.695
Hg1jm	<i>H. glandulosa</i>	F:CAGACACAACAAACCATCACC R:GTGTTTTTCTGCCTGGTTGC	(ATA) <sub>7</sub>	60	237-239	2	0.375
Hg2jm	<i>H. glandulosa</i>	F:TGCAGGAGACATGAATGTGG R:GAGCATGAGAAAAGTTCAGATAGC	(TA) <sub>12</sub>	60	492-494	2	0.523
Hg5jm	<i>H. glandulosa</i>	F:TCGAACCTCAACTACTGATCG R:TCCTCTAATCTTGGCCATCC	(TA) <sub>14</sub>	60	242-242	1	0.204
Hg6jm	<i>H. glandulosa</i>	F:TTGGTCACATGCTTGATTGC R:GCACCCTAATTCCTGTTGC	(TG) <sub>7</sub>	60	158-170	4	0.441
Hg7jm	<i>H. glandulosa</i>	F:CTGTAGGGTGATTTAAGAAACG R:AGGATGGAATCAAGCAAACC	(ATT) <sub>11</sub>	60	139-140	2	0.374
Hg8jm	<i>H. glandulosa</i>	F:ATGGGTTGAACGTTGACTGG R:GGGGCTCTGTAGTGATAGGC	(TA) <sub>6</sub>	62	296-297	2	0.440
Hg9jm	<i>H. glandulosa</i>	F:GTTCCATTCAAACTAGCC R:AGATGGACAGAACGTTTAGC	(GA) <sub>8</sub>	62	198-202	3	0.533

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Hg11jm	<i>H. glandulosa</i>	F:TCTGGAATATTGATGCACTCC R:GTTCAATTGCCAAACCATGC	(AT) <sub>7</sub>	62	202-208	4	0.720
Hg13jm	<i>H. glandulosa</i>	F:TATGAACACCATGGCCTCTG R:ATGGTAGTGCGTTGTTGTCC	(AT) <sub>7</sub>	60	205-207	2	0.461

*Ta*-annealing temperature; *Na*-number of alleles; *PIC*-Polymorphic information content.

Allelic number and product size ranges are included in Table 2.5, along with other details for the 26 loci. In general, Hardy Weinberg disequilibrium was found for all three species (Table 2.5). However, MICROCHECKER v 2.2.3 results showed no evidence of scoring error due to stuttering or allelic dropout in any of *R. appendiculatus* markers. Nevertheless, there was evidence of null alleles for 6 of the SSR's (Ra6jm, Ra10jm, Ra11jm, Ra2jm, Ra3jm and Ra9jm) due to a general excess of homozygotes for most alleles size classes. For *H. megalonesa* and *H. glandulosa* there was no evidence of scoring error, allelic dropout or null alleles in any of the markers except for Hg7jm due to a general excess of homozygotes.

**Table 2.6.** Primers pairs showing observed (H.obs), expected heterozygosity (H.exp) and Hardy-Weinberg equilibrium (two-sided p-value) for twenty-six SSR's markers. a) markers for *R. appendiculatus*, b) *H. glandulosa* and c) *H. megalonesa*.

a	Primer	H. exp.	H. obs.	HW
	Ra2jm	0.507	0.220	***
	Ra3jm	0.845	0.535	***
	Ra4jm	0.361	0.267	***
	Ra6jm	0.908	0.681	***
	Ra7jm	0.598	0.915	***
	Ra8jm	0.411	0.289	<b>0.087</b>
	Ra9jm	0.896	0.500	***
	Ra10jm	0.504	0.122	***
	Ra11jm	0.669	0.304	***
	Ra12jm	0.320	0.348	<b>1</b>

b	Primer	H. exp.	H. obs.	HW
	Hg1jm	0.517	1.000	<b>0.333</b>
	Hg2jm	0.628	0.867	ND
	Hg5jm	0.239	0.000	***
	Hg6jm	0.492	0.400	*
	Hg7jm	0.515	0.000	****
	Hg8jm	0.543	0.533	*
	Hg9jm	0.628	0.333	**
	Hg11jm	0.786	0.933	ND
	Hg13jm	0.579	0.933	ND
	Hg3jm	0.129	0.000	

c	Primer	H. exp.	H. obs.	HW
	Hm1jm	0.602	0.639	***
	Hm3jm	0.349	0.083	ND
	Hm4jm	0.496	0.611	<b>0.633</b>
	Hm7jm	0.585	0.528	***
	Hm11jm	0.64	0.472	***
	Hm14jm	0.527	0.639	***
	Hg1jm	0.732	0.389	***

HW -probability of Hardy-Weinberg equilibrium (2-tailed test; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , ND indicates that not done).

The number of alleles per locus for *R. appendiculatus* varied from 3 to 19, PIC ranged from 0.292 to 0.915 and expected heterozygosity ranged from 0.320 to 0.908. For *H. megalonesa*, the number of alleles varied from 4 to 7, PIC ranged from 0.308 to 0.639 and expected heterozygosity varied from 0.349 to 0.602. For *H. glandulosa*, the number of alleles varied from 2 to 4, PIC was between 0.117 and 0.720 and expected heterozygosity ranged from 0.129 to 0.786. Expected and observed heterozygosity as and polymorphic index content (PIC) for all markers are detailed in Table 2.6.

## 2.4 DISCUSSION AND CONCLUSIONS

Even though nuclear microsatellite markers are one of the most popular types of molecular markers in population genetics, their use has been impeded by the lack of available sequences (Yu et al., 2011). In the present study, we successfully developed microsatellite markers for four species of Bornean frogs from the Lower Kinabatangan Wildlife Sanctuary using NGS Illumina pair end data. Even though NGS results from NOVOGENE showed good raw data quality (see Table 2.1), usually, Illumina raw reads are too short to cover the entire microsatellites or to possess enough flanking sequences for primer design (Jennings et al., 2011). Illumina paired-end

sequencing is capable of cost-effectively identifying large numbers of potentially PCR-amplifiable microsatellite loci (Castoe et al., 2012). Nevertheless, a *de novo* assembly was carried out for the four genomes in order to increase the number and length of isolated microsatellites markers (Yu et al., 2016). Even with an average sequencing coverage of 5x, the genome of all four species still provided 1,228 primers, which is sufficient for most of the biological questions.

Our microsatellite data exhibited departures from Hardy Weinberg equilibrium (HWE) in all three species (Table 2.6). Our results suggest a possible Wahlund effect (Newman, 2001; Sinnock, 1975), because we performed our analysis over the entire dataset assuming one population, which could have affected the analyses due to underlying population structure. Deviation from HWE suggests that the allele frequencies are changing within the population, potentially indicating substructure at this geographical level. This might be due to several reasons that include genetic drift, non-random mating, selection or a combination of these processes (Fusinatto et al., 2013; Pardo et al., 2014). Amphibians tend to live in metapopulations, so there could be some level of admixture between subpopulations inside the LKWS and P-1 (Lenhardt et al., 2017; Newman, 2001). Due to the large number of samples analysed, and the fact that we analysed several areas from different habitats, some population structuring inside the LKWS could occur (explored fully in Chapter 3). Other possible reasons for the observed deviation from HWE may be the presence of null alleles or a large allelic dropout (Dakin & Avise, 2004; McKee et al., 2017). However, MICROCHECKER revealed no evidence of scoring error or allelic dropout, implying that our microsatellites are suitable for population genetics analysis. The 26 microsatellites developed in this study will provide new resources to better understand the possible effects of fragmentation due to deforestation and palm oil agriculture in Borneo, enabling us to better understand the factors that are affecting frog communities in a forested/agricultural landscape.

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## **Chapter 3:**

# **Amphibian population genetics in a fragmented landscape: Do oil palm plantations drive population structure?**

### **3.1 INTRODUCTION**

Tropical ecosystems are one of the most diverse habitats on Earth, providing products and services to communities and playing key roles in carbon and hydrological cycles (Laurance et al., 2014). However, large scale land-use change including agriculture is rapidly degrading tropical ecosystems (Fitzherbert et al., 2008). Currently, about one third of the world's land surface is under agricultural cultivation, while a further 30% has been affected by agriculture to some degree (Ellis et al., 2010). Industrial agriculture is associated with a deterioration in soil, air and water quality, precipitating biodiversity declines across the entire taxonomic spectrum (Collins & Fahrig, 2017). Such changes are causing severe difficulties for the maintenance of biodiversity and are an important source of climate change (Bengtsson et al., 2005; Lamb et al., 2016). Large areas of pristine rainforest, grasslands and peatlands are being replaced by agriculture and this trend is continuing (Parish et al., 2008). Studies have shown that the replacement of forest, grasslands, traditional agricultural systems or even fallow lands can lead to losses in ecosystem functionality (Edwards et al., 2010). Traditional smallholder agriculture systems have turned into intensified monoculture of cash crop plantations, and of these, oil palm is the standout example in the tropics (Dislich et al., 2018)

Oil palm is one of the most extensive crops in the world and has driven the conversion of more than 10 million hectares of tropical forest over the past two decades (Dijkstra, 2016; Spear et al., 2018). Oil palm production has doubled over the last 20 years, now exceeding 35% of total soya oil production (OECD/FAO, 2019). The main reasons that its production has boomed include the substantially higher oil yield from oil palm compared to other oilseeds (four and seven times greater than rapeseed and soy, respectively) and its lower price compared with similar crops (Corley & Tinker, 2016). It has become the primary cooking oil for most communities in Africa, the Middle East and Asia (Pirker et al., 2016).

Particularly alarming, conversion of forest to oil palm agriculture in Southeast Asia has been very rapid, with millions of hectares of oil palm plantations replacing forest over the last two decades (Edwards et al., 2013). Consequently, Southeast Asia has the highest rates of deforestation in the tropics (Sodhi, et al., 2010b). An average of 270,000 ha of forest was lost annually due to oil palm agriculture from 2000 to 2011 (Henders & Kastner, 2015). Malaysia and Indonesia hold more than 80% of Southeast Asia's remaining primary forest, while producing more than 80% of the world's palm oil (Fitzherbert et al., 2008). Using land-use data collected by the United Nations Food and Agriculture Organisation (FAO), Koh and Wilcove (2008) found that >50% of Indonesian and Malaysian oil palm plantations in 2005 were located on land that was forest in 1990. Due to this rapid rate of deforestation both countries are facing the extinction of many endemic species (Sodhi et al., 2004).

Amphibians are one of the most threatened vertebrate groups in the world, even more than birds or mammals (Hoffmann et al., 2010). Even though the number of amphibian species described by 2013 was 7,215, approximately 41 percent of amphibians in the world are still at risk of extinction (Baillie et al., 2004; Peloso et al., 2010; Pratihar et al., 2014). Amphibians are threatened by pollution, climate change, introduced species, road mortality, overharvesting for the pet and food trades, and diseases such as Chytrid fungus (Andrews, et al., 2008; Baillie et al., 2004; Pratihar et al., 2014). Nevertheless, habitat loss and fragmentation remain the primary cause of amphibian population declines (Bishop, Mainguy, et al., 2012; Hero & Kriger, 2015). Globally, an estimated of 63% of all amphibian species have been affected by habitat loss (Stuart et al., 2008).

Amphibians are an integral part of the food web, serving as prey for many species of birds, snakes, fish and others (Kleber del Claro, 2009). Tadpoles contribute to water quality maintenance by feeding on algae and adults can consume large quantities of invertebrates, including disease vectors such as

mosquitoes (Mokay, 2007). Furthermore, amphibians are considered sensitive indicators of environmental stress due to their porous skins, and their health as a taxon is considered to be indicative of the health of the biosphere as a whole (Hero & Kriger, 2015).

Southeast Asia is one of the world's biodiversity hotspots for amphibians, with over 700 species occurring in the region. There are at least 267 species of amphibians in Malaysia (Ahmad & Kemeterian, 2017; Sodhi et al., 2010a). Since the monogram by Inger (1966) the number of Bornean amphibian listed has increased by 50%. The known anuran fauna of Borneo includes 182 species (Ahmad & Kemeterian, 2017). Amphibian distribution on Borneo is not uniform, the East-Malaysian frog fauna counts 31 toad species in 8 genera, 3 species of the genus *Ingerana* (Ceratobatrachidae), 17 dicroglossid species in 4 genera, 28 ranid species in 5 genera, and the family Megophryidae contains 22 species in 6 genera. The latter constitutes approximately 15% of all megophryid species known. The family Microhylidae currently comprises 25 species in 7 genera on Malaysian Borneo, and the Rhacophoridae 40 species in 6 genera. Some species have very restricted ranges, such as *Philautus saueri*, which is only found at 5 locations in Sabah. One of the world's top-10 most wanted "lost" anurans, *Ansonia latidisca* Inger, 1966 was rediscovered on Gunung Penrissen, Western Sarawak (Pratihar et al., 2014).

Compared to other vertebrates in Borneo, relatively few studies have been conducted on frogs, and there are even fewer studies on frog population dynamics and genetics (Emerson et al., 2000; Hertwig et al., 2012; Matsui et al., 2015). Currently, there are large gaps in information on the basic structure of frog communities within fragmented lowland secondary forest in the region. This information is needed in order to manage anuran species sustainably and to ensure that decisions on protected area management include actions for anurans. The consequences of habitat loss and fragmentation due to oil palm agriculture for amphibian populations are still



poorly understood, due to a lack of studies of basic organismal biology and population ecology, the absence of long-term monitoring programs and the fact that population genetic studies have been rarely undertaken.

An indirect method to assess the effects of fragmentation on amphibian populations is by using population genetic tools with neutral molecular markers, such as microsatellites, which are one of the most widely used tools in population genetics and conservation biology (Jarne & Lagoda, 1996; Vieira et al., 2016). Microsatellites are widely used because they have certain desirable characteristics, such as high mutation rates and thus high polymorphism, an abundant distribution throughout the genome, relative ease of genotyping and automation, codominant inheritance and relatively good reproducibility (Murray et al., 2018). The combination of multiple microsatellites allows an accurate estimation of genetic differentiation among adjacent populations (Beebee, 2005). Many amphibians feature classical metapopulation dynamics (Heard et al., 2012; Smith & Green, 2005). In metapopulations, dispersal is the key for long-term viability of subpopulations, requiring the successful breeding of individuals at a location other than where they were born (Lee & Strauss, 2017). Most of amphibians produce a large number of offspring and after larval development and metamorphosis some juveniles will attempt to disperse, a process that can take several years (Semlitsch, 2008). Amphibian dispersal success is conditional on different factors such as water availability and the characteristics of the intervening landscape (Arntzen et al., 2017). A poor quality and fragmented habitat matrix is expected to reduce the ability of animals to travel between suitable habitat patches, increasing the probability of local population extinction (Niebuhr et al., 2015). Linear barriers, such as roads may cause significant increases in genetic differentiation among amphibian populations (Arens et al., 2007; Marsh et al., 2005). If agricultural fields function similarly as migration barriers or sink habitats, increased population differentiation within a meta-population is expected (Arntzen et al., 2017; Lenhardt et al., 2017).

The present study was conducted in the Lower Kinabatangan Wildlife Sanctuary (LKWS) and Kabili-Sepilok Forest Reserve (KSFR), Sabah, Malaysia. Five populations of *Rhacophorus appendiculatus*, *Hylarana megalonesa* and *Hylarana glandulosa* were sampled from secondary forest and oil palm plantation landscapes. The three species in this study were chosen as representatives of species commonly found in forest, in oil palm plantation and in both types of habitats, respectively. In order to clarify the effects of habitat fragmentation on the genetic structure of these populations over the last 40 years, I analysed five populations of the three species from the LKWS and three populations of *H. glandulosa* from oil palm plantations surrounding the sanctuary. Three additional populations of *R. appendiculatus* and *H. megalonesa* were sampled from KSFR and were analysed as a control, from a population featuring uninterrupted gene flow in a primary forest habitat.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 LOWER KINABATANGAN WILDLIFE SANCTUARY (LKWS)**

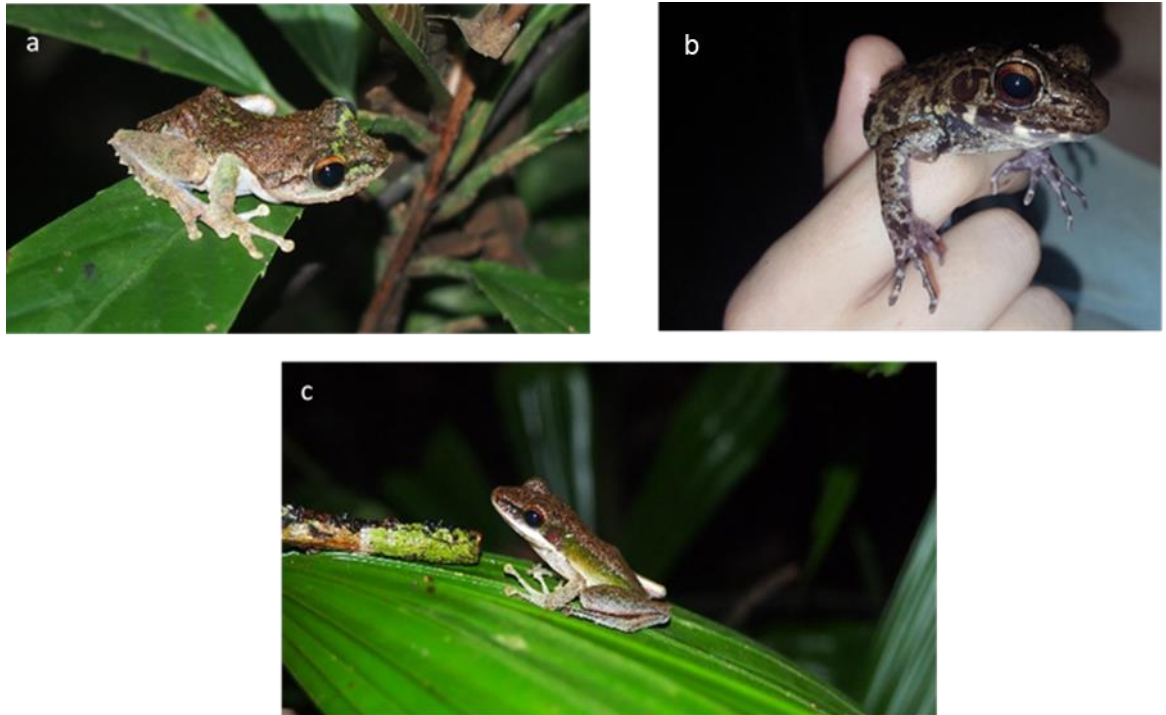
The Lower Kinabatangan Wildlife Sanctuary (LKWS) is located along the Lower Kinabatangan river in east Sabah, Malaysia (N5° 28' – N5° 21'; E117° 56' – E118° 09'; Figure 3). The river is mostly flat and low (10-20m ASL), classified as extreme lowland forest. Temperatures can fluctuate from 21 to 34°C and the mean annual precipitation is between 2500 and 3500mm (Malaysian Meteorological Services Department) (Ancrenaz et al., 2004). The Lower Kinabatangan Wildlife Sanctuary is comprised of 10 lots of secondary forest (Figure 1.2 from Chapter 1) and several Virgin Jungle Forest Reserves. The region includes a variety of habitats, including riverine, seasonally flooded, swamp and dry dipterocarp forests, nipa palm and mangrove.

### 3.2.2 KABILI-SEPILOK FOREST RESERVE

Kabili-Sepilok Forest Reserve (KSFR, N5° 54', E118° 04') is a lowland rainforest reserve of 42.9 km<sup>2</sup> situated 24 km west of Sandakan on the east coast of Sabah (Figure 1.3 refer to Chapter 1). The area surrounding the LKWS has been logged, fragmented and converted since 1959. Post-logging land conversion in the last 30 years has been predominantly to oil palm plantation (Goossens et al., 2005). At the present the KSFR is classified as class VI virgin jungle reserves.

### 3.2.3 SAMPLING AND DNA EXTRACTION

Three model species were used in this study: *R. appendiculatus*, *H. glandulosa* and *H. megalonesa* (Figure 3.1). A total of five areas (known as lots) were sampled for *R. appendiculatus* and *H. megalonesa* within the LKWS (Lots 5-8 and Pin Supu Forest Reserve) and three areas within oil palm plantations (P-1, P-2 and P-3) only for *H. megalonesa*. Additionally, two areas inside oil palm plantations were sampled for *H. glandulosa* (P-1 and P-2). Finally, 21 samples from two transects of *R. appendiculatus* and 16 samples from two transects of *H. megalonesa* were sampled from Kabili-Sepilok Forest Reserve (Appendix 2).



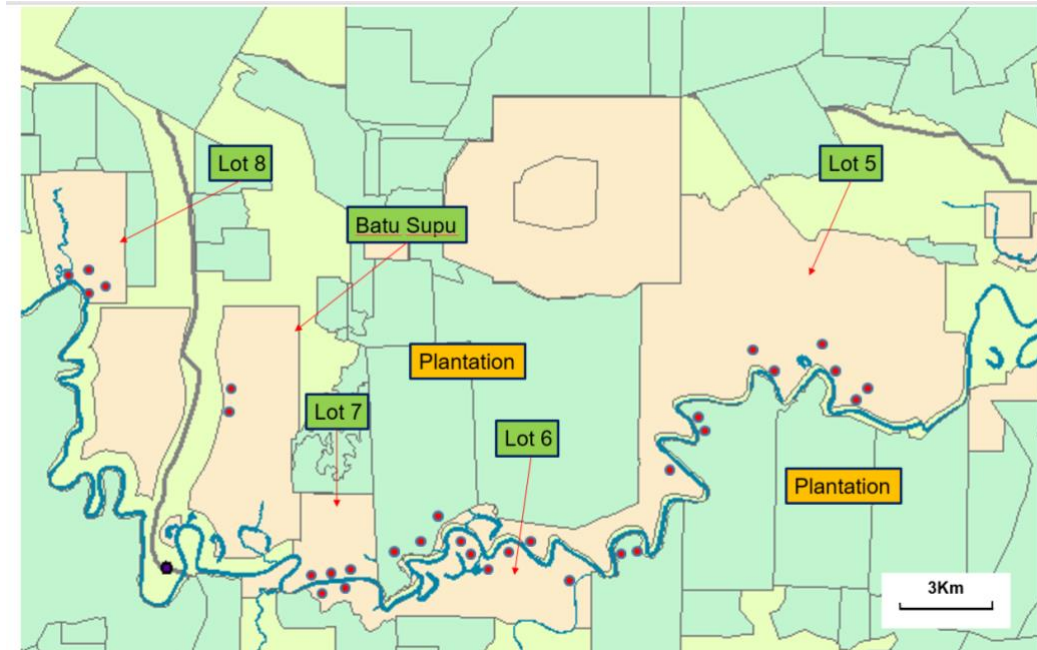
**Figure 3.1** a. typical forest associated species: *R. appendiculatus*, b. a typical oil palm associated species: *H. glandulosa*, c. a generalist species: *H. megalonesa*. (Photos: Juan M. Aguilar- Leon)

Each area/lot was sampled using a transect method. Transect methods are used to determine intraspecific and interspecific changes in amphibian populations (within sites and across changing environmental features). Transects of 200m in length were established across the five areas of the LKWS in order to evaluate the genetic structure of frog communities (Figure 3.2). Transects were sampled for frogs after dusk, between 1830 and 2100 h, which is the period of maximum frog activity (Wells, 2010).

A total of 35 transects were established; 24 transects were established in secondary forest, four in primary forest and seven in plantation. The transects were at minimum 400m apart of each other to increase statistical independence and to minimize problems with pseudo-replication.

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Only 24 transects were used for the genetic analysis; thirteen in secondary forest, 4 in primary forest and 7 in plantations (Appendix 2).



**Figure 3.2** Map with transect positions. Red dots show the 31 transects analysed in this project.

Tissue samples (toe clips) and buccal swabs were collected as a source of DNA (Chapter 2). A total of 107 adult *R. appendiculatus* (70 toe clips and 38 buccal swabs); 111 adult *H. megalonesa* (72 toe clips and 39 buccal swabs) and 45 adult *H. glandulosa* (15 toe clips and 30 buccal swabs) were analysed (Table 3.1). Buccal swabs were taken by opening the animal's mouth and swabbing the surface of the buccal cavity with a sterile cotton bud. Samples were stored in 100% ethanol and at -18°C prior to DNA extraction.

DNA from buccal swabs and toe clips were extracted at the School of Biosciences, Cardiff University. DNA from toe clip was extracted using the QIAgen DNeasy tissue kit (Qiagen, Hilden, Germany, Cat No./ID: 69506) following the manufacturer's protocol. Buccal swabs DNA extraction was

made using the same kit but with a few additional steps: incubation for 5 hours at 56°C with 12 mAU/ml of proteinase K. DNA was eluted in 150 µl TE buffer and stored at -18 °C (Broquet, 2007). Good quality and quantity DNA were obtained using both sources. Quality and quantity were measured visually using agarose gels at 3%. As expected, buccal swabs produced lower DNA concentrations than toe clips. Nevertheless, both DNA sources were suitable for PCR amplification.

**Table 3.1** Summary of samples collected from three species of Bornean frogs.

Species	Sample size	Population	Area	Habitat	Tissue	Swabs
<i>R. appendiculatus</i>	17	LKWS	Lot 8 Pin	Secondary forest	17	0
	9	LKWS	Supu	Secondary forest	9	0
	20	LKWS	Lot 7	Secondary forest	0	20
	20	LKWS	Lot 6	Secondary forest	13	7
	20	LKWS	Lot 5	Secondary forest	10	10
	21	KSFR	KSFR	Primary forest	21	0
Total					70	37

Species	Sample size	Population	Area	Habitat	Tissue	Swabs
<i>H. megalonesa</i>	19	LKWS	Lot 8	Secondary forest	19	0
	9	LKWS	Lot 7	Secondary forest	0	9
	24	LKWS	Lot 6	Secondary forest	6	18
	9	LKWS	Lot 5	Secondary forest	9	0
	14	Plantation	P-1	Plantation	14	0
	12	Plantation	P-2	Plantation	0	12
	8	Plantation	P-3	Plantation	8	0
	16	KSFR	KSFR	Primary forest	16	0
Total					72	39

Species	Sample size	Population	Area	Habitat	Tissue	Swabs
<i>H. glandulosa</i>	28	P-1	LKWS	Plantation	15	13
	17	P-2	LKWS	Plantation	0	17
Total					15	30

### **3.2.4 MICROSATELLITE GENOTYPING AND POPULATION GENETICS PARAMETERS**

Individuals were genotyped using species specific designed microsatellites (see Chapter 2): 10 SSRs designed for *R. appendiculatus*, ten SSRs designed for *H. glandulosa* and six SSRs designed for *H. megalonesa*. A cross-species microsatellite from *H. glandulosa* was used for *H. megalonesa* (Hg1). Allele diversity, Polymorphic Information Content (PIC) and product size range are included in table 3.2, along with other details for the 25 loci.

**Table 3.2.** List of 26 microsatellites (SSR's) developed for three frog species. *Rhacophorus appendiculatus* (Rajm), *Hylarana megalonesa* (Hmjm), *Hylarana glandulosa* (Hgj).m).

Primer	Species	Primer sequence 5'→3'	Motif	H. exp	H. obs	HW	PIC	N	Prod. size range (bp)	Na
Ra2jm	<i>R. appendiculatus</i>	F:GAGACGCTCCTAATAGTACAG R:TCTATATGCTGGCAACATGG	(AT) <sub>12</sub>	0.598	0.345	*	0.524	87	191-209	5
Ra3jm	<i>R. appendiculatus</i>	F:GCTTTGCCTCTGCTACAAGC R:TGAGGAGAACACAGGACAGC	(TG) <sub>12</sub>	0.880	0.402	***	0.864	87	242-284	17
Ra4jm	<i>R. appendiculatus</i>	F:ACGGAACAGAGCAACAGACG R:AGTGGCAGCTAAGAGGATGC	(GT) <sub>8</sub>	0.383	0.207	ND	0.361	87	221-229	5
Ra6jm	<i>R. appendiculatus</i>	F:TGATTATCGACCAGTGAATGG R:CCCGAGAAATCAAATTTAGGC	(TA) <sub>16</sub>	0.910	0.575	ND	0.897	87	265-297	14
Ra7jm	<i>R. appendiculatus</i>	F:CACAGGTGCAGAAGTCATGG R:CAGTGAGCAGGTATGCAAGC	(GA) <sub>6</sub>	0.624	0.862	***	0.557	87	134-144	4
Ra8jm	<i>R. appendiculatus</i>	F:TGTTGATGTACAGTCATTGG R:AAGTGAAATGTATCCACAGG	(AT) <sub>10</sub>	0.499	0.172	***	0.442	87	170-173	4
Ra9jm	<i>R. appendiculatus</i>	F:CTGCCGAGTTAAAGTTAGAGG R:CGTTAAAGGACTCAACACTCC	(TG) <sub>10</sub>	0.750	0.287	***	0.731	87	150-170	12
Ra10jm	<i>R. appendiculatus</i>	F:TTTGATTGCTCATTGTCTGG R:ATTAACATGCACTGGTCTGC	(AT) <sub>9</sub>	0.680	0.115	***	0.644	87	173-193	10



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Ra11jm	<i>R. appendiculatus</i>	F:ATGGAGATGGATGCACATGG R:ACGTCATCGTCCATTTGTCC	(AC) <sub>7</sub>	0.709	0.253	***	0.684	87	206-275	17
Ra12jm	<i>R. appendiculatus</i>	F:ACGTCATCGTCCATTTGTCC R:GATCCTTTCATCTCTTACCTCTGC	(TA) <sub>7</sub>	0.450	0.379	NS	0.418	87	176-180	4
Hm1jm	<i>H. megalonesa</i>	F:GAAAGCCAGCAGTGCATATAG R:CTAGTAGGTCACTTCCAAGG	(AT) <sub>18</sub>	0.631	0.495	NS	0.568	97	262-271	5
Hm3jm	<i>H. megalonesa</i>	F:ACAACATAAGGTCTGACAACG R:GCCAAGTACATCAACATACC	(GA) <sub>9</sub>	0.336	0.113	ND	0.310	97	291-299	9
Hm4jm	<i>H. megalonesa</i>	F:CCAAATCTCCAACACACACG R:TCAATCTATAGGCTGCTTCAG	(TG) <sub>9</sub>	0.608	0.698	*	0.549	97	96-100	5
Hm7jm	<i>H. megalonesa</i>	F:AATTATGGTTGGACGACAGC R:TCAGACAATGGCTTATTGGC	(GT) <sub>7</sub>	0.663	0.588	NS	0.620	97	90-104	8
Hm11jm	<i>H. megalonesa</i>	F:TCACCAGATGTCTTCTTCGC R:CCCAGAATATTCCATGGATC	(ATA) <sub>8</sub>	0.668	0.495	NS	0.614	97	221-228	7
Hm14jm	<i>H. megalonesa</i>	F:CAGATAAGAGTGAGATTTGC R:CTGCATAGACAGGAGAGC	(CT) <sub>7</sub>	0.622	0.577	NS	0.543	97	201-215	4
Hg1jm	<i>H. megalonesa</i>	F:CAGACACAACAAACCATCACC R:GTGTTTTTCTGCCTGGTTGC	(ATA) <sub>7</sub>	0.703	0.392	***	0.703	97	212-238	12
Hg1jm	<i>H. glandulosa</i>	F:CAGACACAACAAACCATCACC R:GTGTTTTTCTGCCTGGTTGC	(ATA) <sub>7</sub>	0.507	0.923	***	0.374	26	237-239	2
Hg2jm	<i>H. glandulosa</i>	F:TGCAGGAGACATGAATGTGG R:GAGCATGAGAAAAGTTCAGATAGC	(TA) <sub>12</sub>	0.452	0.423	ND	0.391	26	492-494	3

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Hg3jm	<i>H. glandulosa</i>	F:TGCATACACTGCATTAAACG R:GTACAGTTTGGCTACGAAGG	(AT) <sub>10</sub>	0.075	0.000	ND	0.071	26	279	1
Hg5jm	<i>H. glandulosa</i>	F:TCGAACCTCAACTACTGATCG R:TCCTCTAATCTTGGCCATCC	(TA) <sub>14</sub>	0.539	0.423	ND	0.472	26	242-247	3
Hg6jm	<i>H. glandulosa</i>	F:TTGGTCACATGCTTGATTGC R:GCACCCTAATTTCTGTTGC	(TG) <sub>7</sub>	0.501	0.538	ND	0.440	26	158-170	6
Hg7jm	<i>H. glandulosa</i>	F:CTGTAGGGTGATTTAAGAAACG R:AGGATGGAATCAAGCAAACC	(ATT) <sub>11</sub>	0.491	0.038	ND	0.366	26	139-140	2
Hg8jm	<i>H. glandulosa</i>	F:ATGGGTTGAACGTTGACTGG R:GGGGCTCTGTAGTGATAGGC	(TA) <sub>6</sub>	0.572	0.577	NS	0.468	26	296-297	3
Hg9jm	<i>H. glandulosa</i>	F:GTTCCATTCACAACTAGCC R:AGATGGACAGAACGTTTAGC	(GA) <sub>8</sub>	0.642	0.423	NS	0.557	26	198-202	4
Hg11jm	<i>H. glandulosa</i>	F:TCTGGAATATTGATGCACTCC R:GTTCAATTGCCAAACCATGC	(AT) <sub>7</sub>	0.790	0.923	ND	0.738	26	202-208	5
Hg13jm	<i>H. glandulosa</i>	F:TATGAACACCATGGCCTCTG R:ATGGTAGTGCGTTGTTGTCC	(AT) <sub>7</sub>	0.544	0.885	**	0.426	26	205-207	3

*H. exp*-Expected Heterozygosity; *H. obs*-Observed Heterozygosity; *HW* -probability of Hardy-Weinberg equilibrium (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001, ND indicates that not done) *PIC*-Polymorphic Information Content; *Ta*-annealing temperature; *Na*-number of alleles.

Microsatellite loci were separated into three groups per species and amplified using the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany, Cat No./ID: 206143). The loci were chosen due to their amplification success and polymorphism in each species, as described in Chapter 2. Primer groups are shown in Table 3.3 as well as allele sizes and annealing temperatures for each primer combination for each of the three species studied.

**Table 3.3** SSR's groups along with allele range sizes and annealing temperatures.

*Rhacophorus appendiculatus*

Locus	Groups	Alleles range	Ta
Ra2jm	1	191-209	60°C
Ra6jm		263-301	
Ra10jm		173-195	
Ra11jm		206-275	
Ra3jm	2	240-284	60°C
Ra7jm		134-160	
Ra9jm		150-184	
Ra12jm		174-196	
Ra4jm	3	221-229	60°C
Ra8jm		170-173	

*Hylarana megalonesa*

Locus	Groups	Alleles range	Ta
Hm1jm	1	232-271	60°C
Hm3jm		285-301	
Hm4jm		96-102	
Hm11jm		215-231	
Hm14jm	2	201-215	60°C
Hm7jm		89-104	
Hg1		206-238	

*Hylarana glandulosa*

Locus	Groups	Alleles range	Ta
Hg8	1	296-297	62°C
Hg9		198-202	
Hg11		202-208	
Hg1	2	237-239	60°C
Hg7		139-140	
Hg13		205-207	
Hg2		492-494	

Hg5	3	242-247	60°C
Hg6		158-170	

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Fragment analysis was carried out using the software GeneMarker 1.95 (SoftGenetics, State College, Pennsylvania, USA) and the genotypes were sorted by species, population, individual and DNA source (toe clips and buccal swabs) for downstream analysis of genetic diversity. MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004) was used in order to check for potential scoring errors, large allelic dropout and the presence of null alleles. GENEPOP version 4.2.1 (Rousset, 2004) was used to calculate deviations from Hardy-Weinberg equilibrium for each population and locus, and to calculate pairwise linkage disequilibrium between loci. Genetic diversity for each population was estimated using observed heterozygosity and that expected under conditions of Hardy-Weinberg equilibrium and inbreeding coefficients ( $F_{is}$ ) were calculated using GENETIX v4.05.2 (Belkhir et al., 2004). Allelic richness (corrected for sample size by rarefaction) was calculated using Fstat v 2.9.3 (Goudet, 2009). The number of private alleles and Shannon's Diversity Index were calculated using GenALEx v 6.5 (Peakall & Smouse, 2012)

In order to compare the genetic diversity of *R. appendiculatus* and *H. megalonesa* from the LKWS in a forested habitat, we sampled four areas for the same species at KSFR. The genetic diversity (differences in heterozygosity estimates and numbers of alleles per locus) between *R. appendiculatus* and *H. megalonesa* populations and the four areas from KSFR were tested pairwise using a nonparametric Wilcoxon signed rank test, with Bonferroni's correction for multiple comparisons.

### 3.2.5 POPULATION STRUCTURE ANALYSIS

STRUCTURE version 2.3.4 (Pritchard et al., 2000) was used to examine the population structure of the three species. *R. appendiculatus* was analysed in secondary forest (LKWS) and primary forest (KSFR) habitats, *H. glandulosa* in oil palm plantation surrounding the LKWS and *H. megalonesa* in both types of habitat. Bayesian clustering, as implemented in STRUCTURE, assigns individuals to clusters without using prior information about their localities of origins. I used an admixture model with correlated allele frequencies, and the number of inferred clusters (K) tested ranged from one (total panmixia) to the number of study locations plus one in each species. STRUCTURE was run for each value of K ten times, with 500,000 Markov Chain Monte Carlo (MCMC) iterations, discarding the first 50,000 MCMC steps as a burn-in phase. The optimal number of clusters was inferred using Evanno et al. (2005)  $\Delta K$  method, as implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). STRUCTURE was also run with the same parameters individually for Lots 5, 6, 7, 8, Pin Supu Forest Reserve (FR) and KSFR to check for possible genetic substructure within areas.

We used GENETIX version 4.05.2 (Belkhir et al., 2004) to perform a model independent factorial correspondence analysis (FCA) on the allelic frequencies from two populations of *R. appendiculatus* (LKWS and KSFR), five populations of *H. megalonesa* (LKWS, KSFR and three plantations) and two populations of *H. glandulosa* (Table 3.1). This analysis was performed across the distribution of the three species inside LKWS, oil palm plantations and SKFR. To examine substructure inside LKWS, the analysis was run for each area (Lots 5-8 and Pin Supu FR) separately. Genetic distances were calculated as  $F_{ST} / (1 - F_{ST})$ , and the significance of matrix correlation coefficients was estimated with 10,000 permutations in GENETIX.

Genetic relationships among populations was also examined by applying discriminant analysis of principal components (DAPC) (Jombart et al. 2010) using the “adegenet 2.1.3” package (Jombart 2008) in R 3.2.2 (R development Core team 2015). DAPC is a multivariate, model-free approach designed to cluster samples based on prior population information (Jombart et al. 2010). DAPC allowed us to analyze the population structure by assessing how well the samples can be reassigned into previously defined populations (Li et al., 2016). The number of retained principal components (PCs) for DAPC was chosen to optimize the alpha score (a measure of trade-off between the power of discrimination and over-fitting of the data to the prior population designations).

### **3.2.6 STRUCTURE ANALYSIS OF LKWS LOTS AND INFERRED GENE-FLOW**

GENETIX v4.05.2 was used to estimate the effective number of migrants per generation ( $N_m$ ) between areas/lots inside LKWS and between cluster made by STRUCTURE, using a private alleles method (Barton & Slatkint, 1986), which is the most reliable method to detect very recent migration due to the rare nature of private alleles (Yamamichi & Innan, 2012). BayesAss v3.0 (Wilson & Rannala, 2003) was also used in order to measure pairwise directional gene flow between areas/lots inside the LKWS and between clusters generated by STRUCTURE. This approach also provides estimates of recent gene flow (last two generations). Here, the probability of finding a particular genotype in a given population can be expressed as a function of allele frequency, immigration rate, inbreeding levels in each population and the time at which the immigration event took place (Goossens et al., 2005).

### 3.3 RESULTS

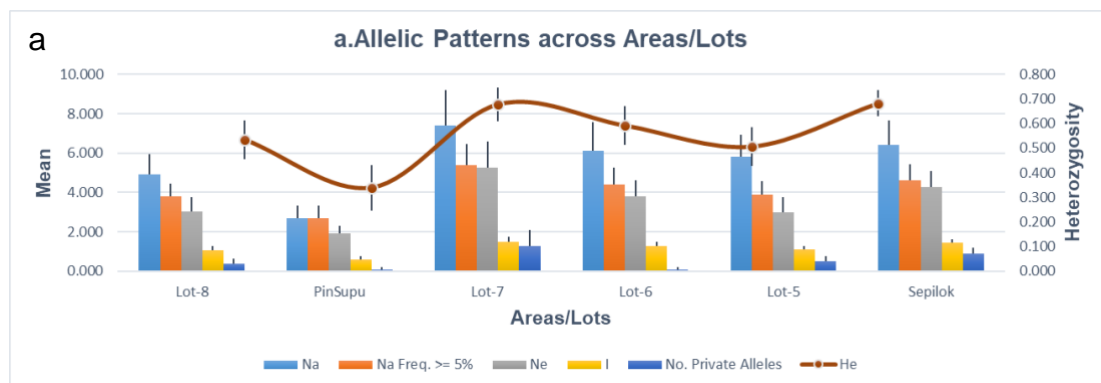
#### 3.3.1 GENETIC DIVERSITY

All loci for all three frog species were polymorphic except for Hg 3 in *H. glandulosa*. *R. appendiculatus* showed the highest number of alleles per locus with 27 (Ra11jm) while plantation specialist *H. glandulosa* had the lowest number of alleles with only one and two for each locus (Hg3jm and Hg7jm, respectively). The observed number of alleles per locus for *R. appendiculatus* ranged from four to 27, with a mean of 5.38, and the mean number of alleles in Clusters LKWS and KSFR were 10.5 and 6.4, respectively. There was no sign of stuttering that might have resulted in scoring errors except for Ra2jm. Samples were re-analysed without locus Ra2jm with no appreciable differences in the final results, therefore the final analysis was carried out including this marker. In general, loci showed a deficit in heterozygotes except for Ra7jm, Ra4jm and Ra12jm but there was no evidence of large allelic dropout for any of the 10 loci. Linkage disequilibrium ( $p < 0.01$ ) was found in 24 out of 45 loci pairs inside LKWS (Appendix 3) but there was no linkage disequilibrium in KSFR. All areas inside LKWS and KSFR showed a deficit in heterozygotes, except for Pin Supu FR. Null alleles for *R. appendiculatus* were inferred when analysing LKWS for seven of the 10 loci (Rajm2, Rajm3, Rajm4, Rajm6, Rajm9, Rajm10, Rajm11). Evidence for null alleles was also found for the KSFR population (Rajm2, Rajm3, Rajm6, Rajm9, Rajm10).

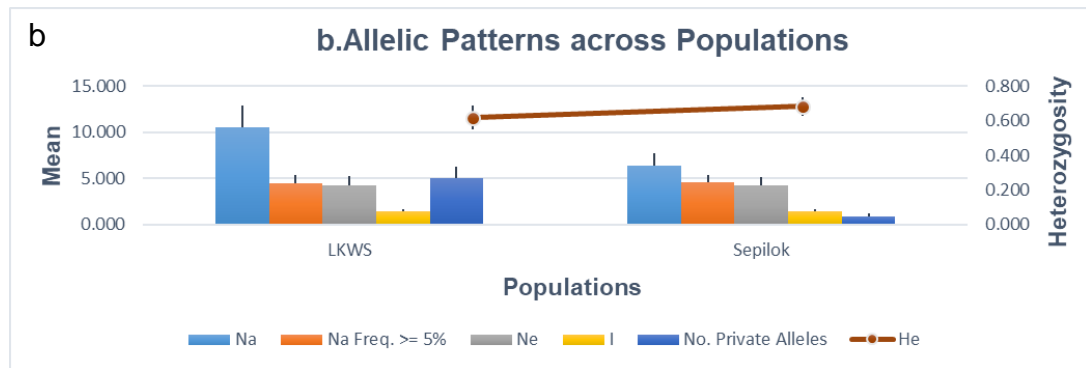
The observed number of alleles per locus for *H. glandulosa* ranged from two to 12 with a mean of 2.2. The mean number of alleles in plantations P-1 and P-2 was 2.7 and 2.4, respectively. There was no evidence of scoring errors due to stuttering, large allelic dropout or null alleles for any of the loci at any of the populations. There was no evidence of linkage disequilibrium or deficit of heterozygotes at plantations P-1 and P-2.

The observed number of alleles for *H. megalonesa* per locus ranged from four to nine, and the mean number of alleles in LKWS, KSFR and plantations were 5.57, 3.71 and 4.29, respectively. Data for all the species are shown in detail in Table 3.4. There was no evidence of scoring errors due to stuttering, large allelic dropout or null alleles for any of the loci at any of the populations.

Genetic diversity for the three species and the clusters obtained from the genetic structure analysis are shown in Table 3.4. High levels of genetic diversity were found for *R. appendiculatus* in LKWS ( $H_e=0.62$ ) and KSFR ( $H_e=0.68$ ). Genetic diversity for each area in LKWS ranged from 0.68 at Lot 7 to 0.34 at Pin Supu FR.  $F_{is}$  values were higher for LKWS (0.36) than KSFR (0.26). In general, genetic diversity was similar for all five LKWS areas. However, the total number of alleles per locus per area and allelic richness were higher at Lots 6 and 7 compared with the other areas inside LKWS. Figure 3.3 compile the allelic patterns and the fluctuation of the GD ( $H_e$ ) across areas/lots and populations for *R. appendiculatus*.







**Fig 3.3** Allelic patterns for codominant data of *R. appendiculatus* for areas/lots (a) and populations (b). Na: mean number of alleles; Na (Freq >= 5%): mean number of different alleles with a Frequency >= 5%; Ne: mean number of effective alleles; I: mean Shannon's Diversity Index; number of private alleles: mean number of alleles unique to a single population; He: expected heterozygosity.

Low levels of genetic diversity were found in *H. glandulosa*. Expected heterozygosity ranged from 0.34 in areas T24 at P-2 to 0.41 in T3 at P-1 (Table 3.4).  $F_{is}$  values were not significantly different from zero for all areas and populations after applying Bonferroni correction.

Moderate levels of genetic diversity were found in our generalist species *H. megalonesa*. Expected heterozygosity was 0.51 at KSFR and 0.50 at LKWS. Genetic diversity within LKWS ranged from 0.41 at Lot 5 to 0.49 at Lots 6 and 7. Genetic diversity within plantation habitats ranged from 0.29 for area T23 at plantation P-2 to 0.48 for area PE-4 at plantation P-3 (Table 3.4). Unpaired T-test result revealed lower levels of expected heterozygosity in plantation populations (P-1 and P-2) compared to forested habitats in LKWS and SKFR (P value = 0.032) for *R. appendiculatus* and *H. glandulosa*. There were no statistical differences between forested habitats and plantations for *H. megalonesa* (P value = 0.107).

**Table 3.4** Estimated genetic parameters for three species of Bornean frog populations and cluster defined by STRUCTURE analysis. (N) number of samples, (A) alleles per area, (Ar) allelic richness, (He) expected heterozygosity, (Ho) observed heterozygosity, (Fis) inbreeding coefficient.

Specie	Population	Area	N	A	Ar	He	Ho	Fis	
<i>K. appendiculatus</i>	LKWS	Lot5	20	5.8	4.79	0.52	0.39	0.29	
	LKWS	Lot6	21	6.1	5.14	0.59	0.43	0.29	
	LKWS	Lot7	20	7.4	6.33	0.68	0.43	0.39	
	LKWS	Pin							
	LKWS	Supu	9	2.7	2.90	0.34	0.38	-0.05	
	LKWS	Lot8	17	4.9	4.59	0.53	0.36	0.36	
	KSFR	KSFR	21	6.4	5.71	0.68	0.52	0.26	
		Clusters							
	A-1	...	41	7.6	5.8	0.53	0.37	0.33	
	A-2	...	46	8.6	6.9	0.66	0.43	0.36	
LKWS	...	87	10.5	7.9	0.62	0.40	0.36		
KSFR	...	21	6.4	5.71	0.68	0.52	0.26		
Specie	Population	Area	N	A	Ar	He	Ho	Fis	
<i>H. glandulosa</i>	P-1	T3	7	2.30	1.60	0.41	0.57	-0.32	
	P-1	T10	7	2.20	1.60	0.40	0.53	-0.27	
	P-1	PE-2	6	2.30	1.45	0.40	0.57	-0.32	
	P-1	PE-1	9	2.00	1.47	0.36	0.51	-0.36	
	P-2	T23	11	2.40	1.58	0.40	0.48	-0.15	
	P-2	T24	6	2.00	1.46	0.34	0.50	-0.38	

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Specie	Population	Area	N	A	Ar	He	Ho	Fis
Clusters								
	B-1	...	29	2.7	...	0.45	0.49	-0.06
	B-2	...	17	2.4	...	0.39	0.47	-0.15
<i>H. megalonesa</i>	LKWS	Lot5	9	2.29	2.59	0.41	0.70	-0.68
	LKWS	Lot6	24	4.29	3.10	0.49	0.68	-0.37
	LKWS	Lot7	9	3.14	2.85	0.49	0.70	-0.39
	LKWS	Lot8	21	3.57	2.82	0.47	0.53	-0.09
	KSFR	KSFR	16	3.71	2.80	0.51	0.72	-0.36
	P-1	PE-1	10	2.86	2.93	0.46	0.63	-0.26
	P-1	P-2	4	2.14	2.71	0.37	0.60	-0.30
	P-2	T23	4	1.86	2.43	0.29	0.40	-0.15
	P-2	T24	8	2.57	2.76	0.37	0.56	-0.31
	P-3	PE-4	3	3.00	3.00	0.48	0.65	-0.16
	P-3	PE-5	4	2.57	2.29	0.42	0.64	-0.39
Clusters								
	LKWS	...	63	5.57	4.58	0.50	0.63	-0.24
	KSFR	...	16	3.71	4.43	0.51	0.72	-0.36

### 3.3.2 POPULATION STRUCTURE

Population differentiation ( $F_{ST}$ ) after Bonferroni adjustment was significant ( $p < 0.05$ ) for four out of 10 areas/lots comparisons inside the LKWS for *R. appendiculatus* (table 3.5).  $F_{ST}$  values between areas within the LKWS and KSFR population for *R. appendiculatus* ranged from 0.235 to 0.032, with Pin Supu FR and Lot 5 the most differentiated from KSFR (0.235 and 0.192 respectively), with  $p < 0.05$  (Table 3.5). Areas that were the most differentiated inside LKWS were Pin Supu FR compared to Lot 7 ( $F_{ST}=0.218$ ;  $p < 0.05$ ) and Lot 7 compared to Lot 5 ( $F_{ST}=0.172$ ;  $p < 0.05$ ). LKWS as a whole was also significantly differentiated from KSFR, albeit with a lower value ( $F_{ST}=0.098$ ;  $p < 0.05$ ).

**Table 3.5**  $F_{ST}$  values for *R. appendiculatus* from LKWS areas and KSFR. P-values are shown below the diagonal. n.s. – non significant p values.

	Lot8	Pin Supu	Lot7	Lot6	Lot5	KSFR
Lot8	<b>0</b>	0.077	0.137	0.030	0.063	0.132
Pin Supu	n.s	<b>0</b>	0.218	0.079	0.055	0.235
Lot7	0.001	0.001	<b>0</b>	0.060	0.172	0.032
Lot6	n.s	n.s	0.001	<b>0</b>	0.040	0.091
Lot5	n.s	n.s	0.001	n.s	<b>0</b>	0.192
KSFR	0.001	0.001	0.001	0.001	0.001	<b>0</b>

There were very low  $F_{ST}$  values for *H. glandulosa* in plantations P-1 and P-2 ( $F_{ST} = 0.056$ ;  $p < 0.05$ ).  $F_{ST}$  values for plantation areas were low to moderate with values ranging from 0.082 to 0. The most differentiated area was T24. Nevertheless, all  $F_{ST}$  values for areas inside P-1 and P-2 were no significant (Appendix 4).

**Table 3.6**  $F_{ST}$  values between the two plantations P1 and P2 for *H. glandulosa* (above diagonal). Corresponding P-values are shown below the diagonal.

	P-1	P-2
P-1	<b>0</b>	0.056
P-2	0.007	<b>0</b>

$F_{ST}$  values did not reveal any evidence of differentiation between forested areas inside the LKWS for *H. megalonesa* (Appendix 5), but there was a significant differentiation between plantations P-2 and P-3 (Table 3.7). *H. megalonesa* showed low genetic differentiation within LKWS as well as when compared with KSFR ( $F_{ST} = 0.013$ ).  $F_{ST}$  values among Lots within the LKWS ranged from 0.118 to 0.012. However, all  $F_{ST}$  values for populations and areas in LKWS and SKFR were non-significant ( $p > 0.05$ ) except for Lot 7 with SKFR ( $F_{ST} = 0.106$ ;  $p$ -value  $< 0.05$ ).

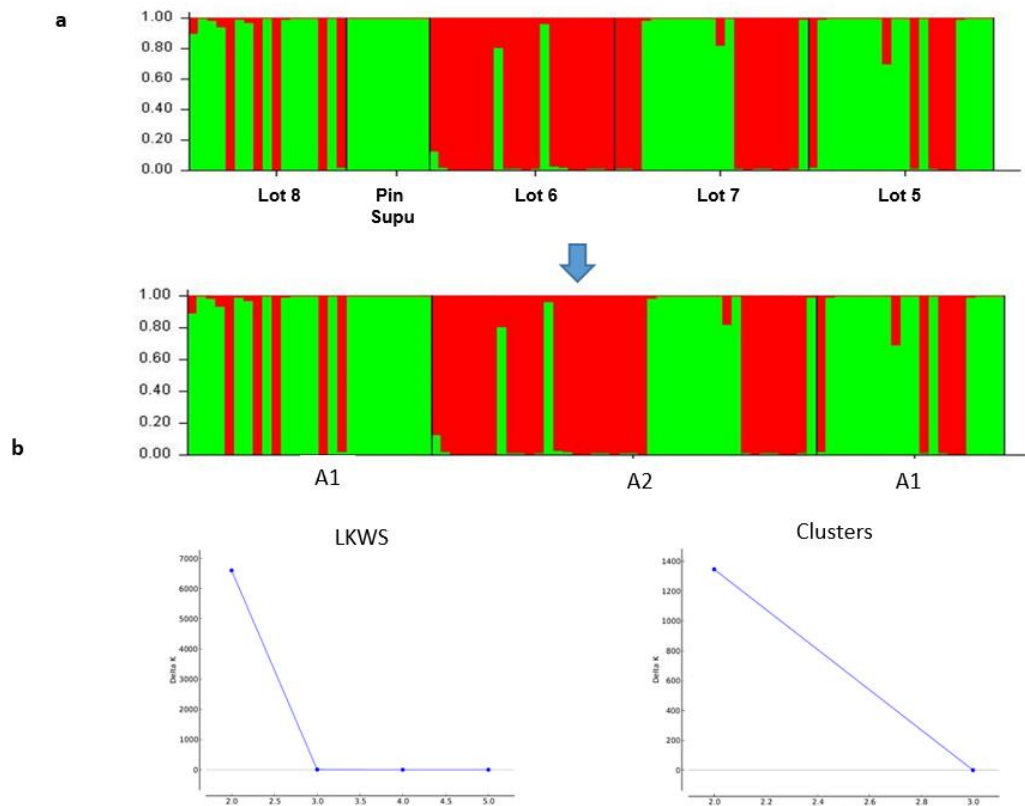
**Table 3.7**  $F_{ST}$  values between the three plantations P-1, P-2 and P-3 for *H. megalonesa* (Above diagonal). P-values are shown below the diagonal.

	P-2	P-3	P-1
P-2	<b>0</b>	0.165	0.079
P-3	0.037	<b>0</b>	0.061
P-1	n.s	n.s	<b>0</b>

n.s. – non significant P-values

STRUCTURE revealed two clusters for *R. appendiculatus* within LKWS (Figure 3.4a). However, there was no clear geographic division between the clusters, even though their existence was supported by the delta K method of Evanno (Evanno et al., 2005) (Figure 3.4b), Factorial Correspondence Analysis (FCA) and  $F_{ST}$  values (0.079) (Figure 3.7a1). The cluster comprising

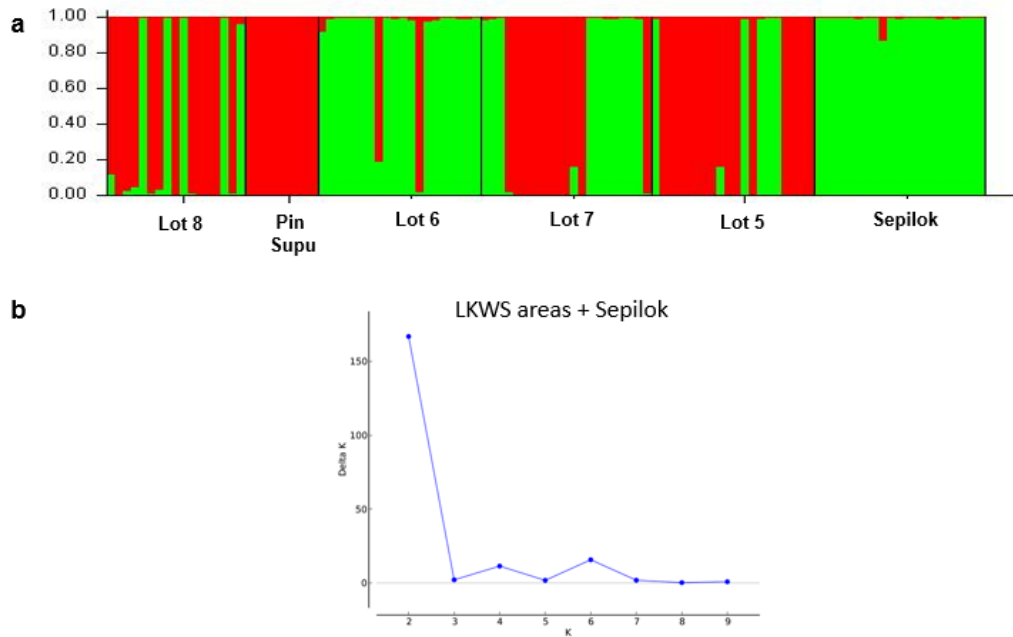
Lot 8, Pin Supu FR and Lot 5 is hereon referred as A1 and cluster with Lot 7 and Lot 6 as A2 (Figure 3.4a).



**Figure 3.4** (a) STRUCTURE analysis plots for *R. appendiculatus*. (b) Inference for the best value of K based on the  $\Delta K$  method among runs for all LKWS areas/Lots and by clusters inside the LKWS.

When analysing LKWS with KSFR using STRUCTURE, based on the Evanno method, the most probable number of clusters was again two ( $k=2$ ) (Figure 3.5a). Additional evidence of substructure is indicated by a second and third (much less pronounced) peaks at  $K=4$  and  $K=6$  (Figure 3.5b). FCA and DAPC analysis supported the existence of these two clusters, but showed some degree of genetic admixture between LKWS and KSFR (Figure 3.7a2 and Appendix 6).

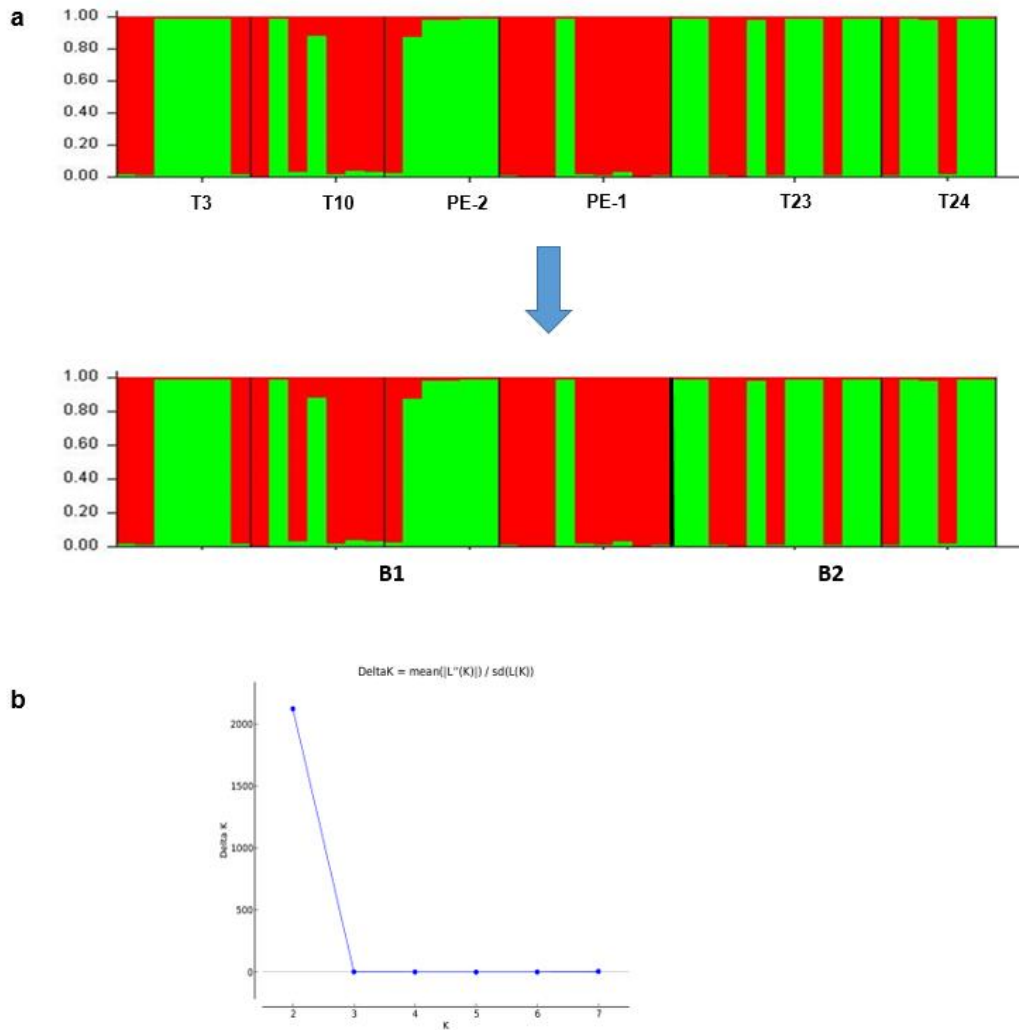
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**Figure 3.5** (a) STRUCTURE analysis for *R. appendiculatus* for LKWS and KSFR. (b) Inference for the best value of K based on the  $\Delta K$  method among runs for all LKWS areas/lots and KSFR population.

STRUCTURE results for *H. glandulosa* within plantations showed two highly distinct clusters (Figure 3.6a) supported by the analysis of  $\Delta K$  values corresponding to  $K = 2$  (Figure 3.6b). These clusters are hereon referred as B1 (1 to 4) and B2 (5 and 6) (Figure 3.6a). FCA analysis showed some degree of separation and admixture between clusters B1 and B2 (Figure 3.7 b1).

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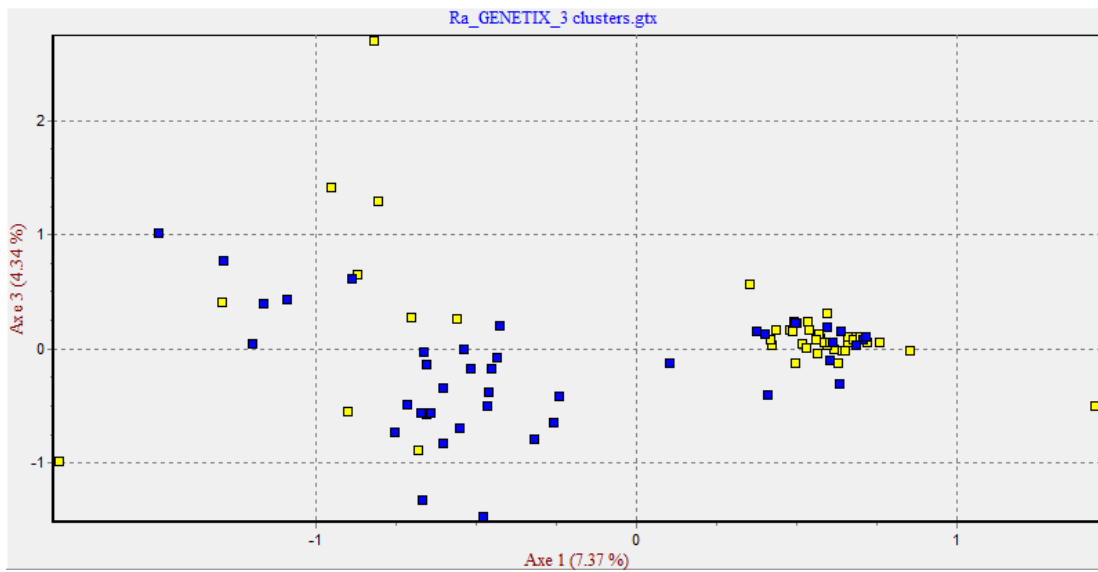
**Figure 3.6** (a) STRUCTURE analysis for *H. glandulosa*. (b) Inference for the best value of K based on the  $\Delta K$  method among runs for all populations and by sector

Four areas/lots within LKWS and three from KSFR were analysed for *H. megalonesa* but no population genetic structure was detected inside LKWS or KSFR ( $K=1$ ). Six areas were analysed from three distinct oil palm plantations (P-1, P-2 and P-3) with no structuring either. FCA confirmed these results showing all populations as a single group (Figure 3.7, c1).

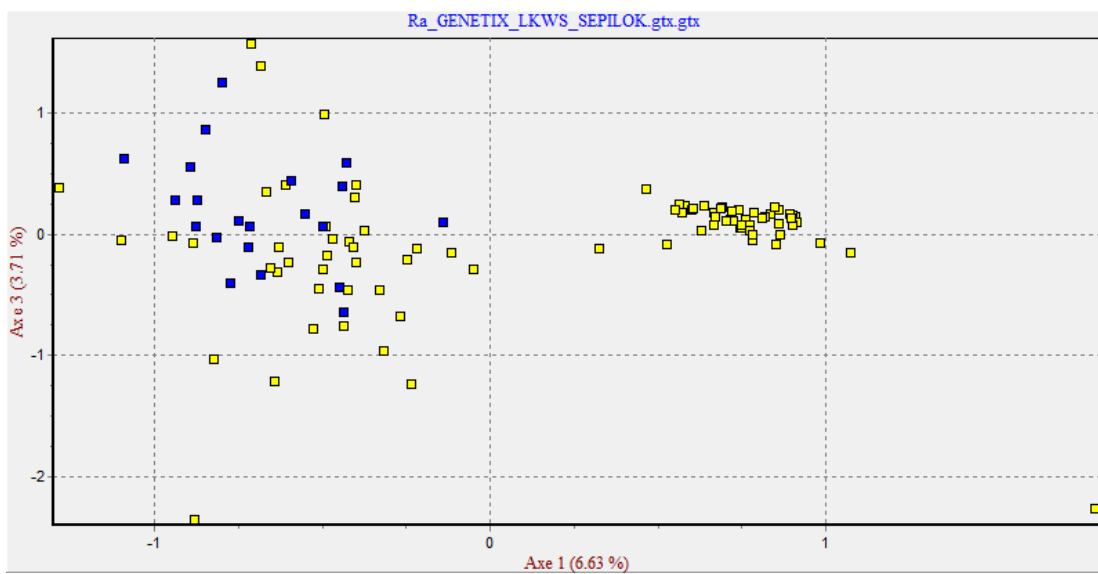


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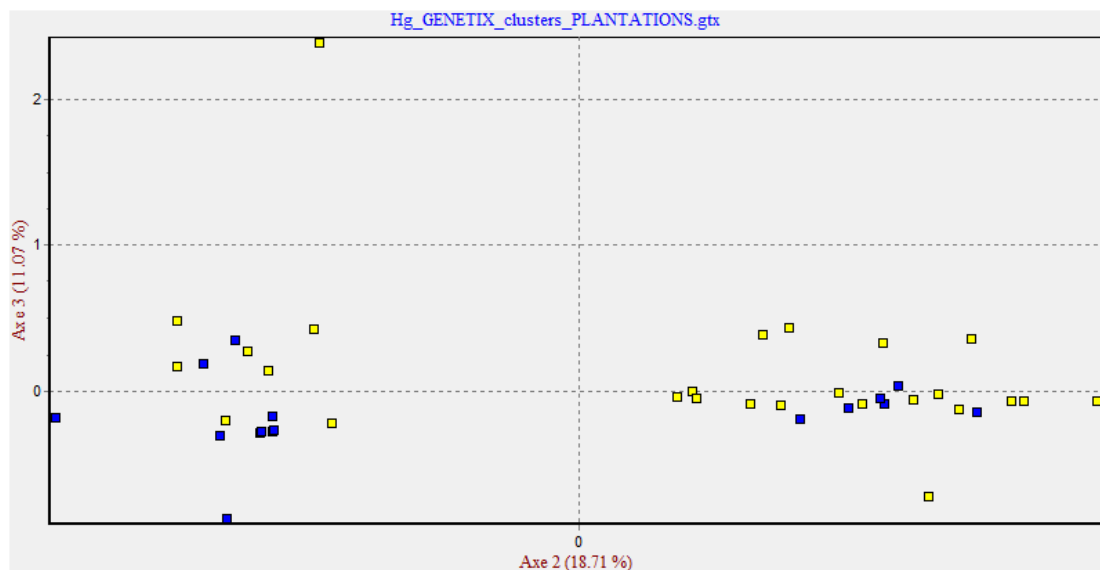
a1



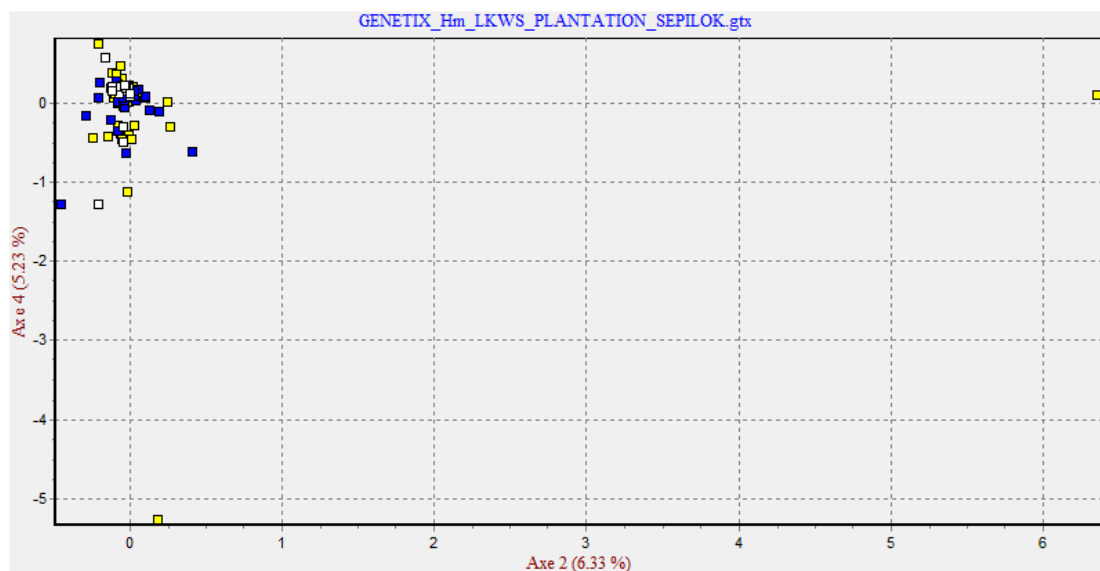
a2



b1



c1

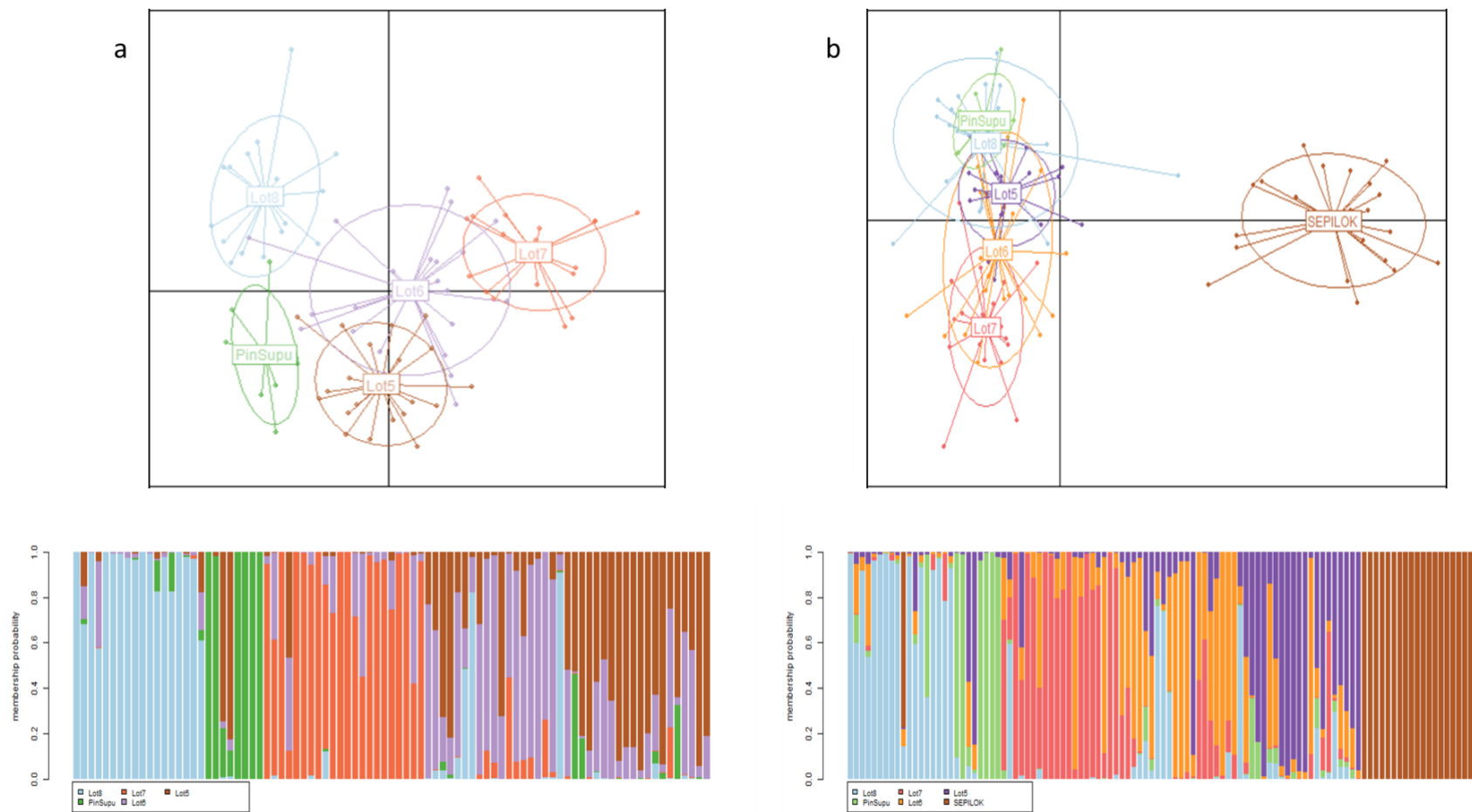


**Figure 3.7** Factorial Correspondence Analysis (FCA) for three species of Bornean frogs. a1: *R. appendiculatus* FCA for STRUCTURE clusters inside LKWS A1 (Yellow) and A2 (Blue). a:2: *R. appendiculatus* FCA for LKWS populations (Yellow) and KSFR (Blue). b1: *H. glandulosa* FCA for plantations P-1 (Yellow) and P-2 (Blue). c1: *H. megalonesa* FCA for LKWS (Yellow), KSFR (White) and plantations (Blue).

DAPC analysis for *R. appendiculatus* inside the LKWS provided a more comprehensive picture of geographic division inside the sanctuary than STRUCTURE. The optimal alpha-score was achieved by retaining 30 principal components. DAPC clustering showed that individuals from Lot 6, Lot 5 and Lot 7 were grouped together, separate from lot8 and Pin Supu FR (Figure 3.8a). However, the ellipses for these areas overlapped, with the exception of Lot 8. There was a 88% successful assignment rate for the whole data set. Lot 8, Lot 7 and Lot 5 all had >90% successful reassignment, indicating clear-cut groups. However, Pin Supu FR and Lot 6 showed <80% successful reassignment, suggesting admixture and poorly supported groups (Figure 3.8a). DAPC results from *R. appendiculatus* between LKWS and KSFR showed a clear separation of KSFR with >90% successful reassignment (Figure 3.8b). However, when LKWS data were analysed alone it revealed substantial overlap with a <80% successful reassignment was found, mirroring the results of STRUCTURE analysis (Appendix 6).

DAPC clustering for *H. glandulosa* showed that individuals for plantations P1 and P2 grouped separately (Appendix 7). Plantation P1 had a successful reassignment of 93%, indicating a clear cut group. Plantation P2 had a successful reassignment of 88% indicating some degree of overlap, similar to results from STRUCTURE and FCA analysis. Finally, DAPC results for *H. megalonesa* did not reveal any substructuring, giving similar results to the STRUCTURE analysis.

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**Figure 3.8** DAPC scatter plots for the LKWS (a) and between the LKWS and KSFR based on 30 PCA components, and (b) Individual membership plots.

### 3.3.3 IMMIGRATION

Number of migrants and directional migration rates were analysed between lots within LKWS for *R. appendiculatus* and *H. megalonesa*, as well as between plantations for *H. megalonesa*. Initially, BayesAss was run for each of the three species of frogs with the default values (0.1) for the three continuous parameters: migration ( $\Delta M$ ), allele frequencies ( $\Delta A$ ) and inbreeding coefficients ( $\Delta F$ ); and acceptance rates were assessed. The three parameters were adjusted until acceptance rates were within accepted boundaries (i.e. between 20-60%). The optimal run parameters at each time point for the two species were  $\Delta M = 0.3$ ,  $\Delta A = 1.0$  and  $\Delta F = 1.0$ . Three runs were performed per time point using different random seeds (starting points) with 10,000,000 MCMC iterations following a burn-in of 1,000,000 MCMC iterations and a sample interval of 5,000 (Table 3.8). BayesAss analysis for *R. appendiculatus* inside the LKWS showed no contemporary migration among the samples within the confidence interval (95%). All population inside the LKWS showed higher levels of native population assignment. Non-immigration values for each Lot ranged from 0.85 to 0.79 (Table 3.8a), and positive immigration estimates ranged from 0.053 (from Lot 8 to Lot 7) to 0.035 (from Lot7 to PinSupu). Higher migration rates were, however, found between clusters A1 and A2, with the higher estimate being in the direction A2 to A1 (0.20) (Table 3.8b). It should be pointed out that as pointed out by Faubet et al. (2007) and Palstra et al. (2007), BayesAss has strong limitations for estimating very recent migration, therefore migration estimates should be interpreted with caution.

The estimated number of migrants per generation ( $N_m$ ) using GENETIX showed concordance with (and are related to) the  $F_{ST}$  results. For *R. appendiculatus* values were higher between Lots 5 and 6 (5.1) and Lots 6 and 7 (4.65). When compared with KSFR, values were also high for Lots 6 and 7 (2.21 and 6.27 respectively) (Appendix 8).

**Table 3.8** Estimates of migration rates inside LKWS, STRUCTURE clusters and plantations using BayesAss 1.3. Bold values along the diagonal are the proportion of frogs that were assigned to the site of capture and are thus non-migrant frogs. Values in brackets represent the 95% confidence limits. *m*, total migration rate into each population.

<b><i>a. R. appendiculatus</i></b>			
	Into site:		
	<b>Lot8</b>	<b>Pin Supu</b>	<b>Lot7</b>
<b>Lot8</b>	<b>0.83215</b> (0.67595, 0.99122)	0.03808 (0.00001, 0.20319)	0.05336 (0.00005, 0.22065)
<b>Pin Supu</b>	0.04368 (0.00001, 0.19822)	<b>0.85063</b> (0.67832, 0.99390)	0.04728 (0.00007, 0.20777)
<b>Lot7</b>	0.03839 (0.00002, 0.18916)	0.03517 (0.00001, 0.17458)	<b>0.79820</b> (0.6721, 0.97598)
<b>Lot6</b>	0.04022 (0.00001, 0.18825)	0.03862 (0.00001, 0.19152)	0.05138 (0.00004, 0.22148)
<b>Lot5</b>	0.04553 (0.00001, 0.20723)	0.03747(0.00001, 0.18239)	0.04977 (0.00008, 0.20930)
<i>m</i>	0.08392	0.14934	0.20179
<b><i>b. R. appendiculatus</i></b>			
	Into site:		
	<b>Lot6</b>	<b>Lot5</b>	
<b>Lot8</b>	0.04011 (0.00003, 0.20184)	0.04099 (0.00001, 0.21122)	
<b>Pin Supu</b>	0.04716 (0.00002, 0.22807)	0.04405 (0.00002, 0.19745)	
<b>Lot7</b>	0.04234 (0.00002, 0.19802)	0.04015 (0.00001, 0.18936)	
<b>Lot6</b>	<b>0.82581</b> (0.67352, 0.99139)	0.04293 (0.00001, 0.20180)	
<b>Lot5</b>	0.04456 (0.00001, 0.20628)	<b>0.83185</b> (0.67499, 0.98861)	
<i>m</i>	0.17417	0.16812	

Cluster	A-2	A-1
A-2	<b>0.84372</b> (0.67736, 0.99228)	0.20483 (0.02578, 0.32815)
A-1	0.15627 (0.00771, 0.32263)	<b>0.79516</b> (0.67184, 0.97421)
<i>m</i>	0.15627	0.20483

***c. H. megalonesa***

Plantation	Into site:		
	P-2	P-3	P-1
P-2	<b>0.86859</b> (0.68906, 0.9955)	0.07303 (0.00111, 0.22905)	0.07204 (0.00091, 0.23210)
P-3	0.06618 (0.00080, 0.23137)	<b>0.82272</b> (0.67325, 0.99086)	0.09691 (0.00151, 0.27295)
P-1	0.06522 (0.00072, 0.22635)	0.10424 (0.00216, 0.28199)	<b>0.83104</b> (0.67318, 0.99266)
<i>m</i>	0.1314	0.17727	0.16895

Our results suggest that *R. appendiculatus* in the different Lots inside LKWS have a relatively low probability of being migrants from neighbouring Lots. Instead, it seems most of the gene flow inside the LKWS occurs between the two metapopulations A-1 and A-2 (Table 3.8b)

Migration rates for *H. megalonesa* inside the LKWS could not provide reliable results as the  $F_{ST}$  values between the different lots were non-significant ( $P > 0.05$ ), therefore, results for these pairwise estimates were discarded (Faubet et al., 2007). However, migration appeared to be bidirectional between plantations with the  $F_{ST}$  values implying more gene-flow between P-3 and P-1 (Table 3.8c).

### 3.4 DISCUSSION

Even though the three species in this study have a ‘Least concern’ conservation status in the IUCN red list, all have a decreasing or unknown population trend (Diesmos et al., 2004). They were selected because they represent species that inhabit primary and old secondary forest (*R. appendiculatus*), palm oil plantation (*H. glandulosa*) or both types of habitat (*H. megalonesa*) inside LKWS and the oil palm plantations surrounding the sanctuary as well as primary forest (KSFR). *R. appendiculatus* is an arboreal species that lives in primary and old secondary forests (Diesmos et al., 2004; Inger & Stuebing, 2005). In our study it was found almost exclusively in these two types of habitat, except for two samples where was found at plantation edge. *H. megalonesa*’s habitat and ecology requirements makes it perfect as a generalist species due to its tolerance of disturbance. It has been recorded inhabiting forest as well as oil palm plantations (IUCN SSC Amphibian Specialist Group, 2018). *H. megalonesa* was found almost evenly in the three types of habitat. Even though *H. glandulosa* was found in all three habitats it was much more abundant in oil palm plantations. Due to the lack of research on these three species, this is the first genetic study to feature



them. For all the reasons mentioned above these three species make ideal model species for this study.

Forests in Southeast Asia are threatened by a high level of logging and the expansion of large scale oil palm agriculture (Edwards et al., 2014). This study is among the first assessments of how habitat fragmentation due to oil palm plantation is affecting genetic biodiversity of frogs in Sabah. Large scale logging is usually associated with habitat fragmentation that is typically expected to lead to a decrease in genetic diversity due to stochastic processes (e.g. genetic drift), which will strongly affect small populations (Valbuena-Ureña et al., 2017). My results reveal that some genetic diversity has been lost in *R. appendiculatus* due to fragmentation. Lower levels of genetic diversity and inbreeding were found when compared to a non-fragmented population (KSFR). I also showed that a frog species more commonly associated with a modified habitat – that of oil palm plantations (*H. glandulosa*) shows lower overall genetic diversity than primary and secondary forest species.

### **3.4.1 GENETIC DIVERSITY**

I analysed the genetic differentiation of *R. appendiculatus* and *H. megalonesa* within LKWS and KSFR in order to investigate population genetic differentiation between a secondary forest habitat surrounded by oil palm plantations and a primary forest habitat. Microsatellite data for both species exhibited departures from HWE and LD for 26 of the 45 loci pairs for *R. appendiculatus* and six of the 21 pairs for *H. megalonesa*. However, for each species analyses were performed over the whole dataset as a single metapopulation, thus deviations from HWE and LD are likely to represent population structure, as opposed to problems with the behaviour of the markers themselves. MICROCHECKER revealed no evidence of scoring errors or allelic dropout. In addition, we re-run all analyses using only tissue

samples in order to compare the results obtained in this study. There were no main differences when tissue samples were analysed. We therefore decided to use all 10 and six loci of each species for the analysis.

Accordingly with our first hypothesis (Chapter 1), we found that the forest species *R. appendiculatus* has lower level of genetic diversity (GD) inside LKWS when compared with KSFR, except for Lot 7 (0.68). Although, GD was not as high as in primary forest, *R. appendiculatus* showed high levels of GD ( $H_e > 0.5$ ) in all lots of except for Pin Supu FR (0.34), but this could be biased by the low number of samples analysed for this area (Table 3.2). Typically, habitat fragmentation leads to a decrease in genetic diversity due to stochastic effects that will have the strongest consequences for small populations (Arroyo-Lambaer et al., 2018). Species restricted to small geographic areas may experience a high risk of extinction if populations become fragmented and isolated from each other (Valbuena-Ureña et al., 2017). However, there is evidence that habitat fragmentation *per se* has a weaker effect than habitat loss on biodiversity, and can give rise to neutral or even positive effects (Fahrig, 2003; Templeton et al., 1990; Valbuena-Ureña et al., 2017). Number of private alleles and Shannon Diversity Index (Figure 3.3) revealed that lots 6 and 7 (inside LKWS) are more genetically diverse compared to the other three areas, even than KSFR. Furthermore, lots 6 and 7 showed the highest levels of allelic richness and expected heterozygosity (Table 3.4).

High levels of inbreeding ( $F_{IS}$ ) were found in both types of habitat just for *R. appendiculatus* (see Table 3.4). There are no studies showing adults from *R. appendiculatus* avoiding inbreeding, so it is possible that higher  $F_{IS}$  values could indicate an unusually locally structured breeding system. Chandler et al (2008) observed an inbreeding preference pattern in the spotted salamander (*Ambystoma maculatum*), suggesting that females preferentially used storage sperm from males who will produce offspring with lower heterozygosity. There is therefore a need for more studies of the biological

aspect of the species to understand how its mating system could interact with its genetic diversity and structure. It would also show a possible bottleneck and subsequent inbreeding due to habitat fragmentation as previously showed on the European tree frog (*Hyla arborea*) (Andersen et al., 2004).

The relatively high levels of GD for *R. appendiculatus* could be a reflection of the conditions in the forests of the LKWS (Ancrenaz et al., 2004; Estes et al., 2012; Evans et al., 2016; Scriven et al., 2018). The levels of GD and the amount of private alleles found in the LKWS compared with a non-fragmented primary forest (Table 3.4) highlights the importance of riparian lowland forest fragments in sustaining amphibian genetic diversity and the importance of this area for future amphibian conservation plans with special focus on Lots 6 and 7. It is important, however, to acknowledge that the number of samples analysed as well as the sample effort for LKWS was higher than for KSFR.

Our second and third hypotheses were partially confirmed (refer to Chapter 1). We recorded low levels of GD for *H. glandulosa* in plantation habitats ( $He < 0.5$ ), but with no evidence of inbreeding. It could be the case that even though GD is low, *H. glandulosa* is well adapted to plantations or disturbance generally, and is therefore able to avoid inbreeding due to high levels of dispersal (Austin et al., 2003). On the other hand, the generalist *H. megalonesa*, seems to have low levels of GD in plantations with high levels of inbreeding. However, species that are not as well adapted to this habitat type may be more strongly affected in its dispersal and mating opportunities, consequently struggling to maintain genetic parameters that are important for the survival of the species such as genetic diversity and inbreeding.

### 3.4.2 POPULATION STRUCTURE

Many amphibians in natural environments form meta-populations (Smith & Green, 2005a). Due to the particular ecological habitat requirements for anurans in non-continuous habitat types such as ponds and other small water bodies, dispersal, gene flow and general colonization dynamics will be key factors in maintaining the equilibrium between extinction and colonisation for localised populations (Allentoft & O'Brien, 2010). Accordingly with our fourth hypothesis (refer to Chapter 1), our analysis shows structuring within the LKWS for *R. appendiculatus* indicating metapopulation isolation (Figure 3.4). A study on *Rana dalmatina* populations shows higher genetic population subdivision among fragmented populations compared with non-fragmented (Lesbarrères et al., 2006). Similar results were found with *Acris Blanchardi* (cricket frogs) populations, where functional connectivity was affected by the landscape matrix (highways) (Youngquist et al., 2017). DAPC analysis and  $F_{ST}$  values for *R. appendiculatus* inside the LKWS showed some genetic similarity between Lot 6 and the opposite Lots 5 and 7. There was evidence of gene flow to the remotest Lot (8) (Table 3.5) and some of its genetic diversity is shared between the remaining Lots as revealed by the STRUCTURE results (Figure 3.5). This was confirmed by the FCA analysis (Figure 3.8 a1), separating both areas as one cluster (A2) from the rest (A1), but showing some degree of gene flow between clusters. Apart from the two main clusters inside the LKWS, STRUCTURE and DAPC revealed an additional cluster in KSFR, to be expected given its geographic isolation from the rest of the samples.  $F_{ST}$  values and FCA results showed significant genetic differentiation between LKWS and KSFR, revealing the importance of LKWS and KSFR as independent genetic populations. Nowadays, habitat fragmentation is still one of the greatest threats for amphibian populations (Bishop, Angulo, et al., 2012). When a metapopulation is fragmented (by natural or anthropogenic processes), they could become transformed into isolated demes where genetic drift, inbreeding and selection will act without the buffering effect of gene flow (Marsh & Trenham, 2000). Our results showed the importance of managing and protecting the study the LKWS.

As expected, there were no signs of population structure for *H. megalonesa*, our generalist species, with no significant  $F_{ST}$  values for any areas/lots or populations (LKWS and KSFR). Even though there was significant genetic differentiation between plantations P-2 and P-3 (Table 3.7) STRUCTURE, DAPC and FCA results revealed a lack of it for this specie. The lack of structure for this species could, however, also be explained by the low number of informative molecular markers used in this study.

Our analysis showed structuring within the oil palm plantation populations (P-1 and P-2) for the plantation specialist *H. glandulosa* (Figure 3.8 b1). DAPC and  $F_{ST}$  values showed a low but significant level of genetic differentiation (Table 3.6). It seems clear that this species is adapted to disturbed habitats and their population structure could be explained by a lack of specific habitat requirements and barriers, since its known to be tolerant to habitat disturbances (IUCN, 2018).

### **3.4.3 MIGRATION RATES**

Analysis of migration rates allowed us to determine that recent migration in *R. appendiculatus* has been low and bidirectional inside the LKWS when analysed by lots but when grouping into clusters (A-1 and A-2) they behave as a metapopulation (Table 3.8) with high level of migration. The results showed high gene flow from A-2 (Lots 6 and 7) to A-1 (Lots 5 and 8, and Pin Supu FR) confirming the importance of the two areas on the maintenance of genetic diversity. Even though we observed low rates of migration between lots, it seems more likely that movements happened between adjacent lots. We inferred a slightly lower migration rate from Lot 7 to Pin Supu (0.035) probably due to the fact that these areas are separated from each other by a road. Negative effects of roads have been showed to be an important force for partitioning genetic diversity and engendering genetic structure in amphibians (Marsh et al., 2004; Johansson et al., 2005). There is a particular

case for Lots 7 and 8 and between Lots 7 and 6 where we inferred gene-flow between these areas (Table 3.8), stronger from Lots 8 to 7 and 6 to 7 (0.053 and 0.051 respectively). Despite these two cases, overall, the LKWS showed low migration rates, probably due to a remnant of the ancestral genetic diversity that is shared between these areas before the actual fragmentation happened (almost 40 years ago), this is supported by the high  $F_{ST}$  between these two areas (0.135;  $p < 0.05$ ) and the relatively low  $N_m$  (1.85).

Inside plantations,  $N_m$  values for *H. megalonesa* revealed some degree of bi-directional gene flow between all plantations, but especially between P-1/P-3 (7.33) (Appendix 9). These results were confirmed by the migration rates inferred using BayesAss (Table 3.8). There is a possibility that this is also a remnant of ancestral genetic biodiversity, and is supported by the low  $F_{ST}$  between these two areas (0.061;  $p < 0.05$ ) and the relatively high  $N_m$  (7.33) as expected when historical dispersal rates and gene flow are high (Funk et al., 2005).

#### **3.4.4 CONSERVATION IMPLICATIONS**

The Lower Kinabatangan has been affected by substantial economic activities since 1950. From logging to the development of agriculture crops, mainly paddy fields, coffee, cocoa and tobacco (Vaz & Payne, 1998). Finally, in the 1980's some over-logged forests in the Kinabatangan were re-designated for permanent conversion to agriculture with large scale conversion to oil palm plantations (Vaz & Payne, 1998). The area of the LKWS has been planted with oil palm for over 28 years. To date there have been no studies on amphibian genetics in LKWS. This study helps to better understand how frogs are dispersing in the region. By analysing the genetic diversity and structure of these three species, we can infer that plantations are having an overall negative effect on GD and even though not all lots of the LKWS were analysed in this study it seems that Lots 7 and 6 are key

refugia for GD and are thus important areas for future conservation. It is clear that we cannot extrapolate our results from only one forest specialist but we can say that maintaining the existing integrity of the LKWS is a key factor in order to conserve all amphibians in the area. From a conservation perspective, these results suggest that Lots 6 and 7 may act as an important potential source for introducing additional genetic variation into the other three areas for our forest specialist species, should this become necessary. Additionally, our results suggest that in the recent past *R. appendiculatus* and possibly *H. megalonesa* constituted a single large population with some gene flow across all the LKWS from Lots 5 to 8.

Ongoing loss of genetic diversity is likely to be an important underlying factor in global amphibian declines (Allentoft & Brien, 2010). Populations with low levels of GD have a higher risk of extinction by having lower fitness (Shaffer, 1990). Understanding the factors that influence gene flow among populations is important, because population connectivity is critical to issues such as the recolonization of habitat patches subject to local extinction, the spread of GD, disease transmission, and the degree of local adaptation (Reed et al., 2011). Our study emphasises the need to understand population genetic structure as well as the gene flow between fragmented areas and will contribute with valuable information to future management and conservation programs. A clear definition of conservation units are crucial for maintaining the distinct evolutionary lineages and the species evolutionary potential (Valbuena-Ureña et al., 2017). In *R. appendiculatus* the evolutionary potential is manifested within the species as a whole as well as within each lot. To ensure that the evolutionary potential and the genetic diversity within the distinct areas/lots is not lost conservation strategies should be adopted. Therefore, such strategies should focus on habitat preservation and restoration of each sector, with the aim of maintaining the strong population structure highlighted by this study.

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## **Chapter 4:**

# **The value of secondary forest for tropical Anuran communities: DNA barcoding and Phylogenetic diversity of the Lower Kinabatangan Wildlife Sanctuary and Sepilok Forest Reserve**

## **4.1 INTRODUCTION**

Borneo in Southeast Asia has an immense potential for new discoveries. It harbours large and continuous tracts of virgin forest, much of which remains unexplored (WWF, 2010). The heart of the Borneo hosts multiple hotspots of species richness and endemism for plants and vertebrates (Marchese, 2015). In spite of its mega diversity, over the past 40 years Borneo has experienced rapid conversion of forest into agricultural land, with the percentage of primary forest (including intact and selective logged forest) in the island dropping from 76% in the 1970s to 51% in 2015 (Miettinen et al., 2019). At the same time, the area of industrial oil palm plantations has grown rapidly, with millions of hectares of plantations replacing forest over the last three decades (Wilcove et al., 2013). Evidence suggests that a major wave of extinction has already begun (Alroy, 2017), implying that much of Borneo's undiscovered biodiversity may be lost before its described by science.

Among vertebrates, amphibians are the most threatened in the tropics with a third of currently known species being endangered (Howard et al., 2014), and most of them experiencing population declines (Stuart et al., 2004). Even though amphibians have the highest species discovery rates (Tapley et al., 2018), the same features that make them so diverse could also make them particularly susceptible to anthropogenic threats such as habitat fragmentation, climate change and pathogens (Bishop et al., 2012; Hero & Kriger, 2015). Due to these reasons, there is a need to evaluate the influence of forest loss on amphibian biodiversity. The rapid and accurate description of novel diversity and the assessment of its distribution in secondary and primary forest will help to inform better conservation strategies to reduce the potential negative impacts of further habitat degradation.

DNA barcoding is a molecular methodology that is routinely used for specimen identification and species discovery (Collins et al., 2013). In general, barcoding algorithms for specimen identification compare individual DNA sequences against a reference library of homologous sequences for which identification is supported by curated voucher specimens. If genetic distances between the unknown sample and known species in the reference library are smaller than a pre-established threshold, the unknown specimen likely corresponds to the closest species in the reference collection (Guarnizo et al., 2015). Alternatively, if genetic distances are larger than the threshold, the unknown specimen may correspond to a species not in the reference library or possibly to an as yet undescribed new species (Guarnizo et al., 2015). Even though DNA barcoding's primary application will continue to be the identification of unknown samples; ecologist, evolutionary biologist, and conservationist are already adopting DNA barcodes wholesale as a versatile tool in their respective fields (Joly et al., 2014; Guarnizo et al., 2015). As an example, current conservation research using DNA barcodes can be used for quantifying species richness and evolutionary diversity within and among communities (Kress et al., 2015). The amount of biological diversity present in an environment can be quantified by either analysing the number and distribution of species (using classical diversity indices) or can be augmented by using estimated evolutionary diversity among species for which genetic distances have been calculated. DNA sequence data can therefore provide an evolutionary dimension to diversity estimates by incorporating evolutionary distinctiveness among species, an approach most commonly known as Phylogenetic Diversity (Faith, 1992).

Phylogenetic diversity (PD), is a term originally coined by Richard Vane-Wright et al (1991) as an additional dimension in nature conservation decision making. Faith (1992) defined the PD of a set of species as equal to the sum of the lengths of all those branches on the tree that span the

members of the set. It is possible to use the variance within DNA barcoding sequences to construct a phylogeny, and afterwards calculate the PD of that data set. The larger the total sum of branch lengths, the higher the level of evolutionary divergence between nodes, which translates to a higher PD (Faith, 1992). DNA barcodes can provide a universal marker across species in a community or a region for which phylogenetic diversity (genetic distance), can be calculated within and across ecological communities at varying geographic scales (Chen et al., 2010). When compared with species richness in the same communities, these genetic measures can also be used to evaluate species boundaries, can serve as clues to assist in documenting new species, and can identify targeted habitats for conservation (Kress, et al., 2015).

The majority of tropical amphibian conservation initiatives so far have been focused on the Neo-tropics (Rowley et al., 2010), where species decline and threats posed towards populations have been well documented (e.g. Lips et al., 2008; Pounds & Crump, 1994). Despite harbouring high levels of amphibian diversity (Frost, 2009, cited in: Rowley et al., 2010; Hertwig et al., 2013), current efforts to monitor and protect amphibian species within South East Asia are severely lacking (Rowley et al., 2010; Hertwig et al., 2013). While supporting a disproportionate level of diversity for its size (Myers et al., 2000), this region also suffers from the highest deforestation rate in the world (Sodhi et al., 2010). Negative effects of the conversion of tropical forest to intensive agriculture has negative effects on biodiversity and oil palm agriculture is no exception: large scale land-use change are rapidly depleting tropical ecosystems (Fitzherbert et al., 2008; Wang & Foster, 2015). Malaysia and Indonesia hold more than 80% of Southeast Asia's remaining primary forest, while producing more than 80% of the world's palm oil (Fitzherbert et al., 2008; Miettinen et al., 2019). The expansion of tropical agriculture, such as oil palm, is a major driver of biodiversity loss. A key question is whether biodiversity losses can be minimized by restricting future

expansion to low productivity farmland and retaining forest fragments, especially in a rapidly changing tropical landscape.

South East Asia is one of the world's biodiversity hotspots for amphibians, (Ahmad., 2017; Sodhi et al., 2010). Within Sabah, several amphibian species have shown evidence of population declines (Van Dijk et al., 2004). Furthermore, the Global Amphibian Assessment, described over 20% as vulnerable, endangered or critically endangered (Stuart et al., 2004). Most of our current knowledge of Bornean amphibians is derived from the Malaysian states of Sabah and Sarawak, and Brunei Darussalam (Scriven et al., 2018; Inger & Stuebing, 2005) with very few published accounts from the larger Indonesian region of Kalimantan (Sodhi et al., 2010). There are several gaps on the current knowledge of amphibian biodiversity and patterns of species richness within Malaysian Borneo, which is highlighted by the discovery of three likely undescribed anuran species (Gillespie et al., 2012) and the recent discovery of a new species (Matsui et al., 2017). Without a proper identification of the biodiversity, effective conservation management schemes would be difficult to implement.

In this study, I focused on evaluating the environmental drivers of frog diversity, here examining forest quality. I aimed to contribute to a better understanding of the responses of highly diverse amphibian assemblages to habitat disturbance for the implementation of future conservation strategies with special emphasis on the conservation value of secondary forest surrounded by oil palm plantations. I evaluated the value of secondary and primary forest for anuran communities at the community and PD level and demonstrate the application of DNA barcoding as a tool for anuran diversity assessments. Surveys were implemented during 11 months along the Lower Kinabatangan Wildlife Sanctuary (LKWS) and a one-week survey at Kabili-Sepilok Forest Reserve (KSFR). We sampled 36 transects inside the Sanctuary and four transects in KSFR. Species diversity (Shannon index)

and abundance were calculated for all species as well as phylogenetic diversity.

Forested areas were found to be more diverse in species compared to plantations, where there was a greater abundance of individual frogs but with lower diversity. In order to facilitate species discovery, we used the DNA barcode markers 16S rRNA and Cytochrome Oxidase subunit I (COI). One of the objectives on the study was to demonstrate the application of DNA barcoding and phylogenetic within amphibian diversity assessment. Phylogenetic results were similar for both markers. however, 16S was more reliable for depicting the evolutionary relationships among species. Phylogenetic Diversity (PD), a measure of phylogenetic richness, and the mean pairwise distance (MPD) between species, were higher in forested habitats (Sepilok and LKWS) than in oil palm plantations but did not differ inside the LKWS. In contrast, the mean nearest taxon distance (MNTD), the mean distance separating each species in the community from its closest relative, was higher in oil palm and pasture than in forest. Finally, PD in oil palm and pasture was found to increase with the extent of remnant forest cover.

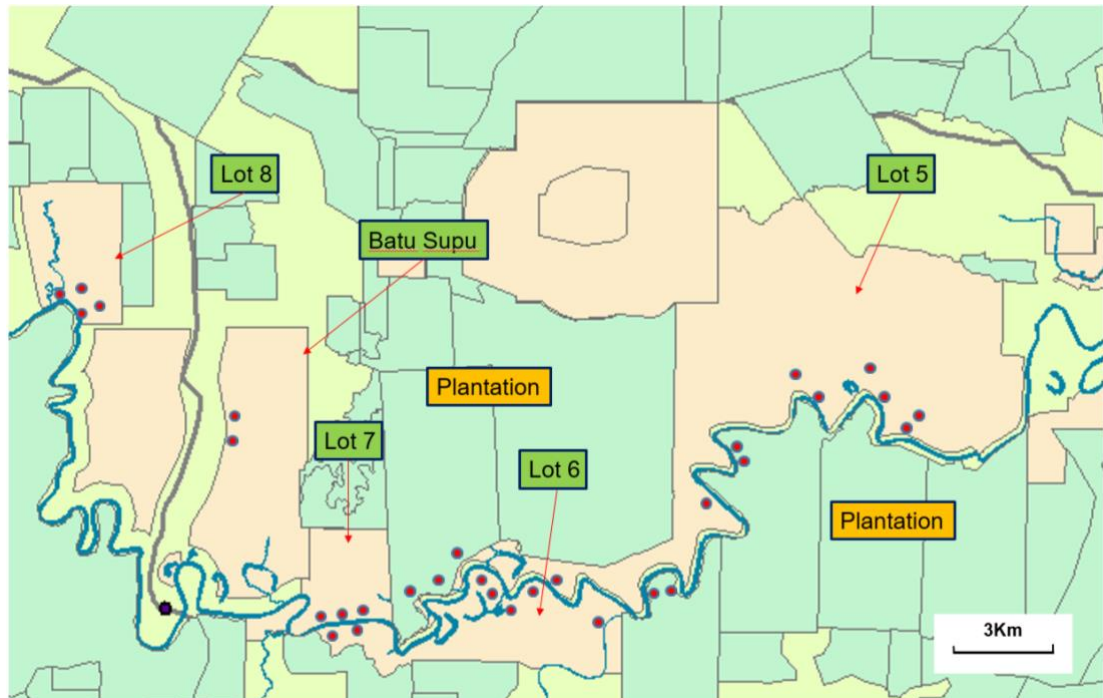
## **4.2 MATERIALS AND METHODS**

### **4.2.1 STUDY AREA**

The field work was mainly conducted in the Lower Kinabatangan Wildlife Sanctuary (LKWS) and during a short campaign at Kabili-Sepilok Forest Reserve (KSFR). The LKWS is located along the Lower Kinabatangan River floodplain in eastern Sabah, Malaysia (Figure 1.2 refer to Chapter 1). The area surrounding the LKWS has been logged, fragmented and converted since 1959 (Refer to Chapter 3).

#### **4.2.2 ANURAN SURVEY AND HABITAT PARAMETERS**

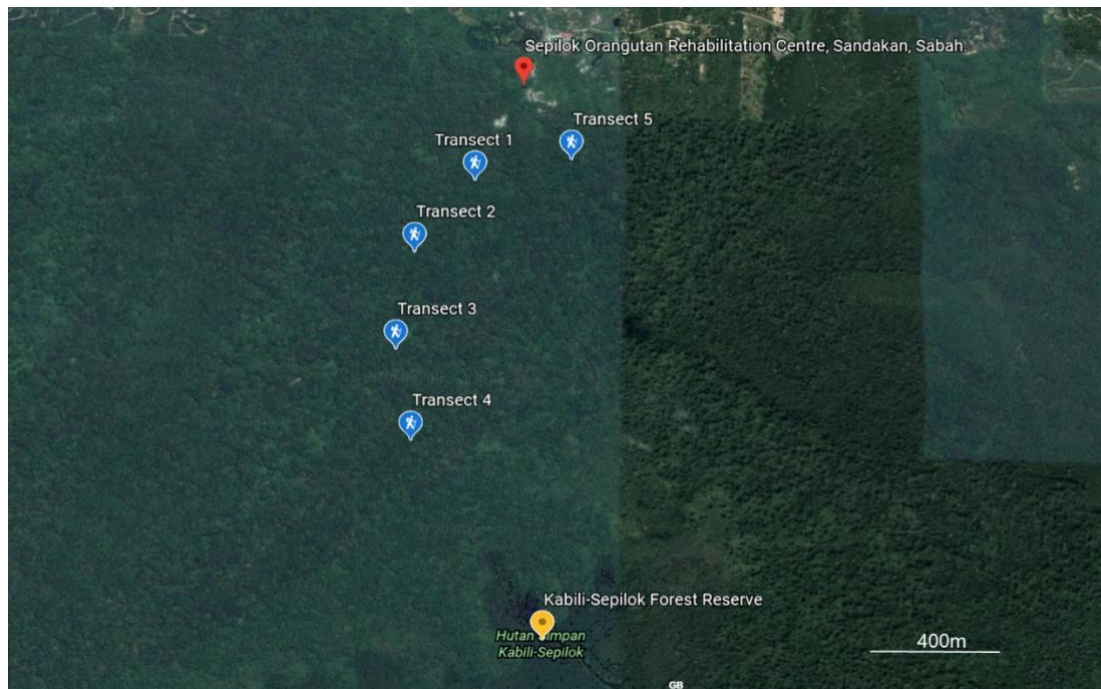
Data were collected in two separated field seasons of six and five month's duration, respectively. The first season was between April and September 2017 and the second season was between September and February 2018, spanning the dry and wet season. Transect methods were used to determine intraspecific and interspecific changes in amphibian populations within sites and across changing environmental features. In a recent study of amphibian biodiversity along the Kinabatangan River, Scriven et al. (2018) selected 74 transects in four habitat types. In this study, a subsample of these transects was used with a fixed length. Thirty-nine transects of 200m in length were established across the LKWS (Figure 4.1) and five more transects at KSFR (Figure 4.2). To reduce seasonal sampling effects, each LKWS transect was sampled three times during the 11 months, with repeated censuses a minimum of 14 days apart.



**Figure 4.1** Map with transect positions at the LKWS. Red points show the 39 transects already analysed in this project.

For KSFR, the whole survey and sampling was carried out during one week. The research plots were located 500 metres from the Sepilok Orangutan Rehabilitation Centre located on the northern edge of the Sepilok Forest Reserve (Figure 4.2). Transects were surveyed using the visual encounter survey method (VES) to measure richness and abundance (Doan 2003; Heyer *et al.* 2004). VES specifically focuses on amphibians active at night in the understory and has been used successfully by a variety of studies (Scriven *et al.*, 2018; Gillespie *et al.* 2012; Ernst & Rödel 2005; Eigenbrod *et al.* 2008).





**Figure. 4.2** Map with transect positions at the KSFR region. Blue dots show the five transects analysed in this project. Image taken from Google Earth (<https://www.google.com/intl/es-419/earth/>).

Transects were surveyed and sampled for frogs after dusk between 1830 and 2100 h, which is the period of maximum frog activity (Wells K.D., 2010), with two people searching visually and acoustically for anurans. Twenty-five transects were established in secondary interior forest (more than 100m from the river bank), nine at the forest edge (less than 50m from the river bank), five in the plantation interior (more than 100m from the plantation boundary), five at the plantation edge (less than 50m from the plantation boundary) and five in primary forest. Adjacent transects were at minimum 400m apart of each other to increase statistical independence and to minimize problems with pseudo-replication.

Ten structural habitat parameters were measured considered potentially relevant on tropical anuran diversity (Scriven et al., 2018) in all transects. Parameters that were indicative of habitat variability in canopy, mid strata,

understory and forest floor were chosen for both forested habitats (LKWS and KSFR) and plantation habitats. Tree circumference and canopy cover are indicative of relative disturbance levels in tropical forest habitats and influence ectotherm species composition (Vitt et al., 1998; Whitfield and Pierce, 2005); understory vegetation density contributes to habitat structural complexity, which in turn has been shown to influence anuran diversity (Wanger et al., 2009; Gillespie et al., 2015); and fallen logs and leaf litter contribute to heterogeneity of forest floor microhabitats, which can also influence amphibian communities (Gardner et al., 2007b; Wanger et al., 2009). All habitat parameters: the number of trees and their mean circumference and variance (at 1.5 m high), the number and mean diameter of logs and fallen trees (diameter  $\geq 0.1$  m), canopy density, mean canopy density, variance of canopy density, understory vegetation density, forest litter cover (leaves, twigs), were measuring following Scriven et al (2018) methodology.

### **4.2.3 DNA SAMPLING AND EXTRACTION**

DNA samples were taken from buccal swabs, by swabbing the surface of the buccal cavity with a sterile cotton bud (Figure 4.3). Buccal swabs were used as a less invasive approach than others regularly used in the field (e.g. toe clipping). Martin & Hong's (1991) methodology was used to handle frogs. The following habitats were surveyed: tree trunks, forest floor branches, leaf litter, rocks in streams and understory vegetation. A total of 72 animals were swabbed. Samples were stored in 100% ethanol and at  $-18^{\circ}\text{C}$  prior to DNA extraction.



**Figure 4.3** Buccal swab sampling from *Polypedates macrotis*. (Photo: Juan M. Aguilar-León)

DNA from buccal swabs were extracted at the School of Biosciences, Cardiff University (Export Permit Number: JHL(PB)600-3/18/1/1 Jdl.23). DNA was extracted using the QIAgen DNeasy tissue kit (Qiagen, Hilden, Germany, Cat No./ID: 69506) following the manufacturer protocol with a few additional steps: incubation for 5 hours at 56°C with 12 mAU/ml of proteinase K. DNA was eluted in 150 µl TE buffer and stored at -18 °C (Broquet et al., 2007). Quality and quantity were measure visually using agarose gels at 3%.

#### **4.2.4 DNA BARCODING**

##### **4.2.4.1 PRIMERS**

A genetic marker suitable for DNA barcoding should meet a number of criteria. First, it needs to have enough variability to discriminate among most species, but at the same time is sufficiently conserved to be substantially less variable within than among species (Valentini et al., 2008; Murphy et al., 2013; Coissac et al., 2016). Second, priming sites need to be conserved enough between species to allow reliable differentiation among them. Third,

the gene fragment needs to carry sufficient phylogenetic information to assign species to major taxa using a simple phenetic approach (e.g. Maximum likelihood; neighbour-joining). Finally sequence alignment should be straight forward, including between distantly related taxa (Vences, et al., 2005; Valentini et al. 2008). The first barcoding marker used in this study was a partial fragment of the mitochondrial DNA Cytochrome Oxidase subunit (CO1, maximum length 650pb) (Murphy et al., 2013). The CO1 primers used were: Chmf4, 5' - TYT CWA CWA AYC AYA AAG AYA TCG G – 3';Chmr4, 5' - ACY TCR GGR TGR CCR AAR AAT CA - 3'. The CO1 is the standardized universal barcoding marker across most vertebrate taxa and has demonstrated high amplification success rates within amphibian orders (Che et al., 2012; Murphy et al., 2013).

The second barcoding marker used was a fragment from the 16S ribosomal RNA gene (Vences et al 2007). The 16S marker is often used as a secondary universal barcoding marker for amphibians due to its high amplification success rate and efficiency for species identification (Vences, et al., 2005). Furthermore, this gene has been identified as one of the standard fragments used for amphibian phylogenetic reconstruction (Vences et al., 2005). The following universal primers (Palumbi et al., 1991) have been used frequently in many other amphibian studies (Vences et al., 2012) and were selected to amplify a 600bp region of the 16S fragment: 16SA-L, 5' - CGC CTG TTT ATC AAA AAC AT - 3'; 16SB-H, 5' - CCG GTC TGA ACT CAG ATC ACG T - 3'.

#### **4.2.4.2 DNA AMPLIFICATION**

PCR amplification for 16S and CO1 mtDNA fragments were performed to produce a final reaction volume of 15ul comprising 7.5 µL 1X master Mix (QIAGEN, Hilden, Germany Multiplex Kit), 0.6 µL of each primer (10µM/µL),

1.2µL ddH<sub>2</sub>O and approximately 1µL diluted genomic DNA. Following optimisation using temperature gradients, the annealing temperature was increased from 46°C to 56°C. The final PCR conditions for both genetic markers consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing temperature at 56°C for 1 minute and an extension for 1 minute at 72°C, finally an elongation period for 10 minutes at 72°C. Amplification success was determined following the same procedure as described for DNA extractions. The brightness of each band was used to qualitatively determine the dilution of the PCR product before sequencing. The PCR process for samples with very weak bands was repeated until brighter bands were observed, if no improvement was made, PCR products remained undiluted.

#### **4.2.4.3 SEQUENCE ANALYSIS**

The 16S and COI gene fragments were amplified and sent for sequencing in both directions to Eurofins UK. Sequence results were initially analysed in Geneious v.4.8.5 (Drummond et al., 2009) and optimised by visual inspection of chromatograms, resulting in the creation of a single contig for each sample. If sequences of either direction were of low quality, or resulted in a sequence shorter than 550bp, the sample was re-amplified, and sequencing was repeated. If the length of the sequence was not improved, the longest direction was used in further sequence analysis. All sequences were aligned using ClustalX v2.0 (Larkin et al., 2007) using the multiple alignment option, and subsequently optimised by eye. We generated consensus DNA sequences data for 61 sampled individuals (Table 4.1), consisting of ~600bp for both markers (58 sequences for 16S and 39 sequences for COI). BLAST searches of each sample fragment were performed to ensure the correct fragments had been amplified and that no foreign DNA contaminant (e.g. human) had been sequenced.

#### 4.2.4.4 PHYLOGENY RECONSTRUCTION

Inferred phylogenetic relationships among frog samples were based in two different datasets. The first set consisted of the 16S fragments sequences (~600pb in length) and the second consisted of the COI fragments sequences (~600pb in length). A third dataset was tested using the concatenated COI and 16S sequences (1200bp in length) but ended up representing fewer species (Appendix 9). We chose *Megophrys nasuta* as an outgroup due to be closely related with our in-group and help us to determine the lineages of the tree that are the oldest and which characters states are ancestral. A pairwise distance matrix was estimated for both markers using PAUP v4.0 in order to choose the best fitting evolutionary model (Swofford, 2003). Evolutionary history was inferred for both markers using the Maximum Likelihood method based on the General Time Reversible model (GTR + G + I). The tree with the highest log likelihood is shown in figure 4.1 for 16S and 4.2 for COI. The percentage of replicates in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then we selected the topology with the best log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites, 4 categories (+G, parameter = 0.6593 for 16S and 0.6064 for COI). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 21.48% sites for 16S and 26.28% for COI). The tree is drawn to scale, with branch lengths proportional to the number of substitutions. The analysis involved 59 nucleotide sequences for 16S and 41 for COI. All positions containing gaps and missing data were eliminated. There was a total of 440 positions in the final dataset for 16S and 527 for COI. We used Geneious v.4.8.5 to perform all the alignments of the forward and reverse sequences as well as the consensus sequences.

#### **4.2.5 SPECIES DIVERSITY AND COMMUNITY COMPOSITION**

Local species diversity was calculated using the Shannon Diversity Index (Spellerberg & Fedor, 2003) and relative abundance (number of frog individuals of all species) per transect (MacArthur, 1960) between lots inside the LKWS (Figure 4.3), four different habitat types (secondary forest, secondary forest edge, oil palm plantation, oil palm plantation edge) and inside KSFR (primary forest). In order to show total species richness in relation to sampling effort (i.e., number of transects), sample-based rarefaction curves were calculated. The Biodiversity Pro 2.0 software (McAleece et al., 1997) was used to estimate diversity indices for the four main habitat types.

We further compared patterns of species composition between secondary forest (LKWS), plantation and primary forest (Sepilok) habitats. All statistical analyses were implemented using R statistical software version 3.4.2. Patterns of variation in anuran community composition across forested and plantation habitats were explored using nonmetric multidimensional scaling (NMDS) ordination using the package Vegan (Oksanen et al., 2011), with Jaccard's distance measure for binary (presence/absence) data and the subsequent dissimilarity matrix of pairwise dissimilarities between sampling sites (Oksanen et al., 2013). Twenty random starting configurations were used, and the final configuration had the lowest residual stress. In order to reduce residual stress, we used a two-dimensional

NMDS plot, and residual stress of the final ordination plot for LKWS secondary forest vs Sepilok was 0.178 and for LKWS and plantations was 0.169. We used the function 'envfit' in the R package vegan (Oksanen et al., 2013) to overlay environmental parameters ( $P > 0.05$ ) onto the NMDS plots, without disrupting the original ordinations. Overlaying environmental

parameters onto NMDS ordinations using envfit generates correlation coefficients (represented as linear vectors on the ordination plots), R<sup>2</sup> values and significance values based on the probability that 999 random permutations of environmental parameters would give a better fit than the true environmental parameters. We overlaid 10 structural habitat parameters onto the NMDS ordination.

#### **4.2.6 PHYLOGENETIC DIVERSITY**

We calculated four measures of phylogenetic diversity using the 16S data for four different habitat types inside the LKWS (secondary forest, secondary forest edge, plantation and plantation edge) as well as between lots (Lot5, Lot6, lot 7 Lot8 and Pin Supu) and for KSFR:

- A. Phylogenetic diversity adjusted for species richness (sesPD) – PD is positively correlated with species richness (Swenson 2014). These variables can be assessed by comparing the PD values of the observed community with that of communities of equal species richness created by null models which randomly draw species from the regional species pool. Communities with greater PD than expected given the species richness have positive values of sesPD, and those with less than expected have negative values;

- B. sesMPD (mean pairwise distance) – MPD is the average phylogenetic distance between individuals in a community. This is influenced by relationships in deep evolutionary time. Higher values suggest that

species are distributed across a wide range of clades, and low values suggest phylogenetic clustering. Communities with greater MPD than



expected given the species richness have positive values, and those with less than expected have negative values;

**C.** sesMNTD (mean nearest taxon distance) – MNTD is the average distance between an individual and the most closely related (non-conspecific) individual. High values of MNTD suggest that closely related individuals do not co-occur in the community, and low levels suggest that they do. MNTD can be adjusted for species richness. Communities with greater MNTD than expected given the species richness have positive values, and those with less than expected have negative values;

**D.** Phylogenetic beta diversity (phylobetadiversity) – this measure uses MPD between pairs of communities and uses these phylogenetic distances to cluster communities based on their phylogenetic similarity. Measures the phylogenetic distance among communities and as such allows us to connect local processes, such as biotic interactions and environmental filtering.

Metrics were calculated using the *picante* package in R version 3.03 (R Core Team 2014) (Kembel et al., 2010). To calculate SES, we used null models with an independent swap algorithm that constrains species richness at each point but randomly draws species from the regional species pool to generate 999 null communities against which to compare the observed community. We did this for each metric (PD, MPD and MNTD).

## 4.3 RESULTS

### 4.3.1 DNA BARCODING

Both loci fulfilled the requirements of barcoding markers. A total of 24 species were successfully identified (Table 4.1). Nineteen species were found in the LKWS and 16 were from the KSFR. The lack of data in GeneBank and the low amount of DNA amplification product for COI marker made it difficult to confirm sample identity. We were able to identify 100% of sequenced samples using the 16S fragment, whereas only 83% of the sequences could be unambiguously identified using COI. Our findings therefore suggest that 16S fulfils the requirements for a universal DNA barcoding sequence for Bornean frogs (Vences, et al., 2005).

**Table 4.1** Samples of anuran DNA with their species, phylogenetic tree code and the location where the specimen was found. This table displays only the corrected species name based on genetic analysis.

Sample	Specie	Code
1	<i>Rhacophorus dulitensis</i>	RdS50
2	<i>Rhacophorus dulitensis</i>	Rd2-69
3	<i>Rhacophorus dulitensis</i>	RdS72
4	<i>Rhacophorus dulitensis</i>	RdS255
5	<i>Rhacophorus dulitensis</i>	Rd1-300
6	<i>Rhacophorus dulitensis</i>	Rd1-357
7	<i>Rhacophorus dulitensis</i>	RdS106
8	<i>Rhacophorus pardalis</i>	RpS106
9	<i>Rhacophorus pardalis</i>	RpS218
10	<i>Rhacophorus pardalis</i>	RpS240

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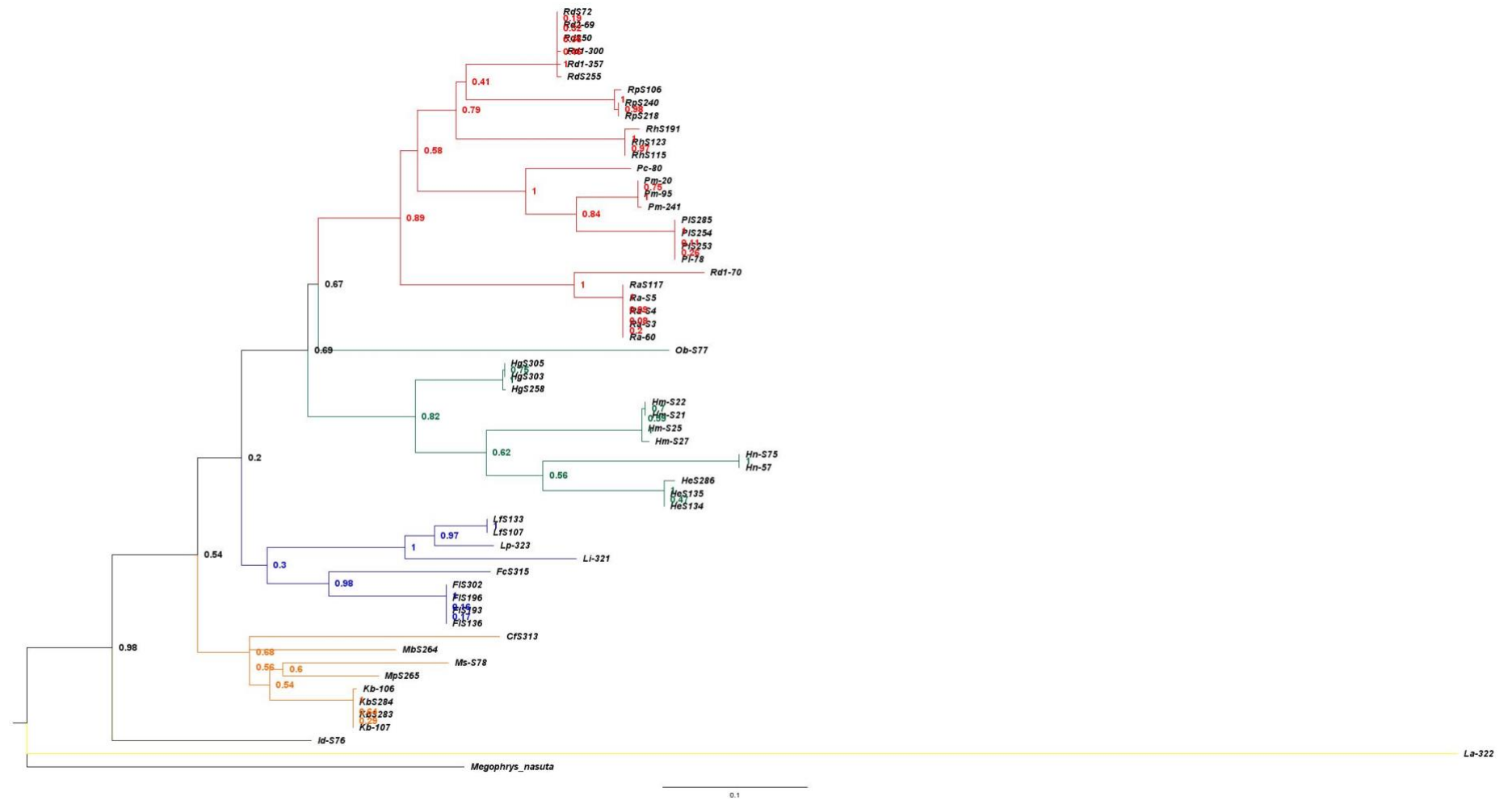
11	<i>Rhacophorus pardalis</i>	RpS114
12	<i>Rhacophorus harrissoni</i>	RhS115
13	<i>Rhacophorus harrissoni</i>	RhS123
14	<i>Rhacophorus harrissoni</i>	RhS191
15	<i>Rhacophorus harrissoni</i>	RhS197
16	<i>Polypedates macrotis</i>	Pm-20
17	<i>Polypedates macrotis</i>	Pm-95
18	<i>Polypedates macrotis</i>	Pm-241
19	<i>Polypedates colletti</i>	Pc-80
20	<i>Polypedates leucomytax</i>	Pl-78
21	<i>Polypedates leucomytax</i>	PIS253
22	<i>Polypedates leucomytax</i>	PIS254
23	<i>Polypedates leucomytax</i>	PIS285
24	<i>Rhacophorus appendiculatus</i>	Ra-S3
25	<i>Rhacophorus appendiculatus</i>	Ra-S4
26	<i>Rhacophorus appendiculatus</i>	Ra-S5
27	<i>Rhacophorus appendiculatus</i>	Ra-60
28	<i>Rhacophorus appendiculatus</i>	RaS117
29	<i>Rhacophorus appendiculatus</i>	RaS56
30	<i>Rhacophorus appendiculatus</i>	Rd-1-70
31	<i>Occidozyga baluensis</i>	Ob-S77
32	<i>Hylarana glandulosa</i>	HgS305
33	<i>Hylarana glandulosa</i>	HgS303
34	<i>Hylarana glandulosa</i>	HgS258
35	<i>Hylarana megalonesa</i>	Hm-S21
36	<i>Hylarana megalonesa</i>	Hm-S22
37	<i>Hylarana megalonesa</i>	Hm-S25
38	<i>Hylarana megalonesa</i>	Hm-S27
39	<i>Hylarana nicobariensis</i>	Hn-57
40	<i>Hylarana nicobariensis</i>	Hn-S75
41	<i>Hylarana erythraea</i>	HeS134
42	<i>Hylarana erythraea</i>	HeS135

43	<i>Hylarana erythraea</i>	HeS286
44	<i>Limnonectes finchi</i>	LfS107
45	<i>Limnonectes finchi</i>	LfS133
46	<i>Limnonectes palavanensis</i>	Lp-323
47	<i>Limnonectes ingeri</i>	Li-321
48	<i>Fejervarya cancrivora</i>	FcS315
49	<i>Fejervarya limnocharis</i>	FIS136
50	<i>Fejervarya limnocharis</i>	FIS193
51	<i>Fejervarya limnocharis</i>	FIS196
52	<i>Fejervarya limnocharis</i>	FIS302
53	<i>Chaperina fusca</i>	CfS313
54	<i>Microhyla borneensis</i>	MbS264
55	<i>Metaphrynella Sundana</i>	Ms-S78
56	<i>Microhyla perparva</i>	MpS265
57	<i>Kaluola baleata</i>	Kb-106
58	<i>Kaluola baleata</i>	Kb-107
59	<i>Kaluola baleata</i>	KbS283
60	<i>Kaluola baleata</i>	KbS284
61	<i>Ingerophrynus divergens</i>	Id-S76
62	<i>Leptobranchium abbotti</i>	La-322

Species identification errors for both markers were detected by erroneous morphological identification in the field due to human error, but in the case of COI the lack of nucleotide data also was a consideration. One misidentification was due to similarities in the appearance of *M. borneensis* and *M. perparva*. Additionally, labelling error in the field was made during sampling. One example of *C. fusca* was highlighted as *O. leavis* by both barcoding markers and one example of *R. pardalis* was highlighted as *H. glandulosa* as well as one *R. appendiculatus* was mislabel as *R. dulitensis*. These findings are supported by conclusions of Shen *et al.* (2013) and Guarnizo *et al.* (2015) showing that DNA barcoding can be an essential tool for data quality control, often outperforming morphological identifications.

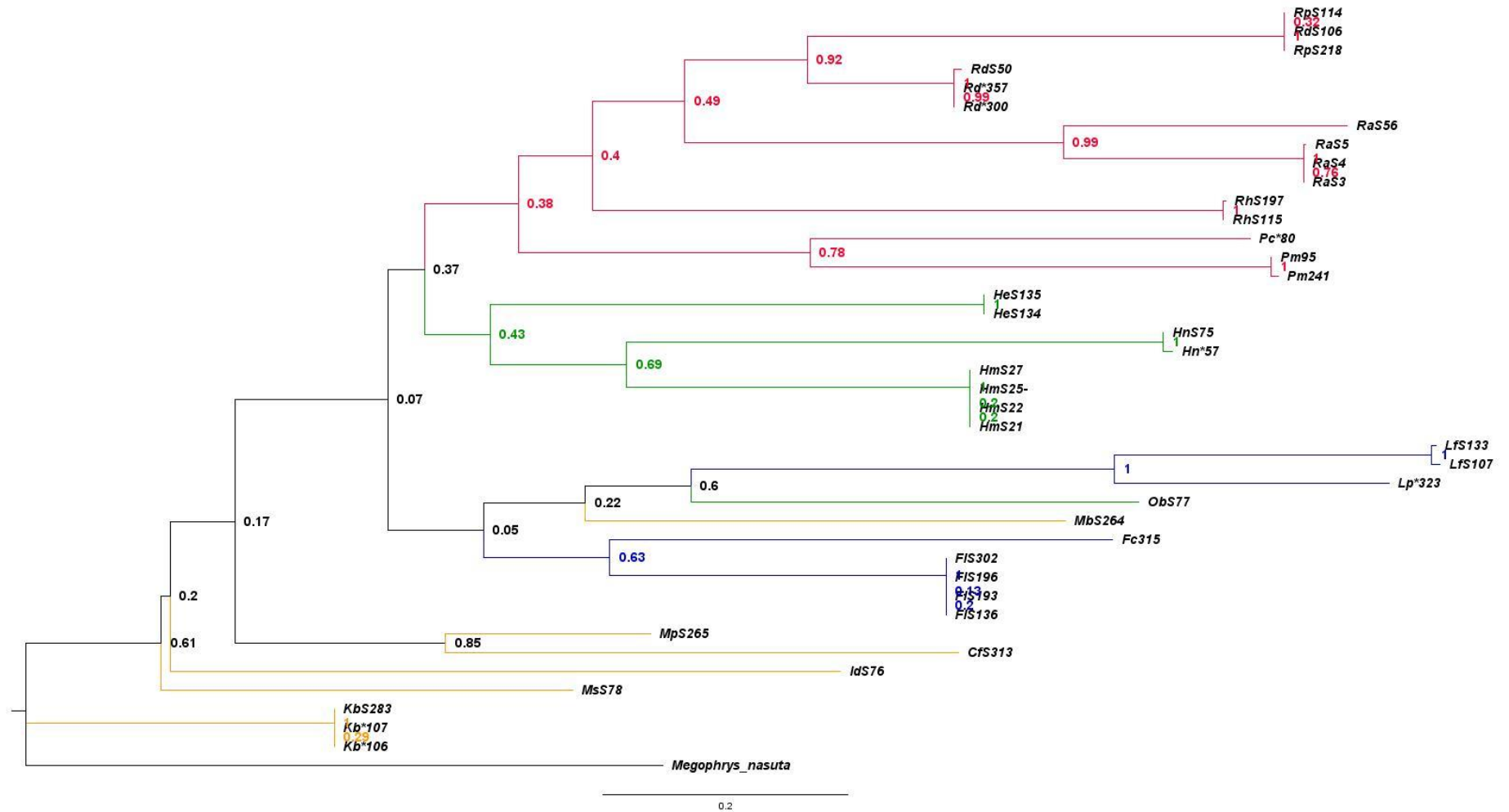
### 4.3.2 PHYLOGENETIC ANALYSIS

Although both markers failed to obtain higher nodal support for deeper clades, the 16S marker was able to separate the samples in seven major clades (Figure 4.4). Four strongly supported clades were identified within the 16S Maximum Likelihood (ML) phylogeny. The first is formed by the samples collected from species within the *Rhacophoridae* family with a posterior probability of 0.86. Within this clade, all seven species (*R. dulitensis*, *R. pardalis*, *R. harrissoni*, *R. appendiculatus*, *P. leucomitax*, *P. colletti*, and *P. macrotis*) segregated independently such that the phylogenetic relationships among the species within the genus *Rhacophorus* was clear when using the 16S marker. Nevertheless, sample Rd1-7 showed a very long branch that separated it from the other Ra samples. The second clade represents the only genus of the Ranidae family sampled, *Hylarana*, which is a monophyletic clade with a posterior probability of 0.82. All species also segregated independently. The third clade is formed by the three *Limnonectes* species sampled. This clade is also monophyletic with a posterior probability of 1. Similarly, the fourth clade, formed from the two *Fejervarya* species is monophyletic with a posterior probability of 0.98. *Limnonectes* and *Fejervarya* are both placed within the Dicroglossidae family, however, as these clades remain unconnected by a strong supported node, it is not possible to infer the taxonomic relationships between the two genera using this approach alone. There was a possible fifth clade identified from the five species from the *Microhylidae* family, however, as these clades remain unconnected (node support = 0.68), it is not possible to confirm their taxonomic relationships. The clades for Megophryidae (*Leptobrachium abbotti*) and Bufonidae (*Ingerophrynus divergens*) families were represented by just one sample of each species.



**Figure 4.4** Maximum likelihood tree of anuran samples produced using the 16S dataset. Species first letters are presented adjacent to the samples. Clades (Red: Rhacophoridae, Green: Ranidae, Blue: Dicroglossidae, Brown: Microhylidae, black: Bufonidae and yellow: Megophryidae).

The COI marker resolved all species IDs but the assignment of samples to higher taxonomic levels (Families, genera) was unsuccessful and there was no clear clade separation. The ML phylogeny showed poor nodal support not just for deep clades and could not be reliably interpreted (Figure 4.5). The lack of resolution by the COI fragment indicates a lower resolving power of this marker for anuran phylogenetic studies, rather than evidence for an undescribed phylogenetic relationship between these taxa. The COI ML phylogeny was able to separate species in some cases, but in general terms was found to mix species and families (Figure 4.5). The concatenated dataset produced a very similar phylogeny to the 16S alone, despite the lack of samples (24 samples) and can be viewed as a good representation of the phylogenetic diversity with 13 species of the 15 reported in this study and three of the four clades, featuring high bootstrap support.



**Figure 4.5** Maximum likelihood tree of anuran samples produced using the COI dataset. Species first letters are presented adjacent to the samples (Red: Rhacophoridae, Green: Ranidae, Brown: Microhylidae and Blue: Dicoglossidae).



It is important note that for both markers *R. appendiculatus* was divided into two strongly supported clades in all phylogenetic trees (samples Rd1-70 for 16S and RaS56 for COI). The results of this study for the DNA barcoding analysis implies that the COI fragment is inferior to 16S in terms of anuran phylogenetic reconstruction. There are several studies that support these findings (Vences et al., 2005; Smith et al., 2008; Che et al., 2012; Guarnizo et al 2015). Possible explanations, that have to be explored in future studies, include a high saturation of the fragments due to rapid rates of evolution in COI, or a low number of variable sites (Che et al., 2012).

#### **4.3.3 ABUNDANCE AND SPECIES DIVERSITY**

The visual encounter survey sampling (on 44 transects) allowed us to detected a total of 25 species belonging to six different families (*Rhacophoridae*, *Ranidae*, *Dicroglossidae*, *Microhylidae*, *Megophryidae* and *Bufo**nidae*) over all three habitats (secondary forest, primary forest, plantation) with 15 of them showing a decreasing population trend according to the IUCN red list (Table 4.2).

**Table 4.2** List of species found in LKWS and Sepilok Reserve and its population status. Primary Forest (PF), interior secondary forest (ISF), secondary forest edge forest (SFE), interior plantation (IP) and plantation edge (PE).

SPECIES	KSFR	ISF	SFE	IP	PE	Endemic	Abbreviation	IUCN Pop. Trend.	IUCN Threat
<b>Rhacophoridae</b>									
<i>Rhacophorus appendiculatus</i>	82	181	40	0	2		Ra	Decreasing	LC
<i>Rhacophorus pardalis</i>	2	29	4	0	0		Rp	Decreasing	LC
<i>Rhacophorus harrissoni</i>	0	1	0	0	0	Y	Rh	Decreasing	LC
<i>Rhacophorus dulitensis</i>	0	5	3	0	1	Y	Rd	Decreasing	LC
<i>Polypedates macrotis</i>	0	14	3	0	0		Pm	Unknown	LC
<i>Polypedates leucomystax</i>	0	9	0	0	7		Pl	Stable	LC
<i>Polypedates colletti</i>	30	8	15	0	0		Pc	Decreasing	LC
<i>Nyctixalus pictus</i>	1	0	0	0	0		Np	Decreasing	NT
<b>Ranidae</b>									
<i>Hylarana megalonesa</i>	18	64	6	4	68	Y	Hm	Unknow	LC
<i>Hylarana glandulosa</i>	2	75	9	39	47		Hg	Unknow	LC
<i>Hylarana erythraea</i>	0	12	0	4	7		He	Stable	LC
<i>Hylarana nicobariensis</i>	2	9	0	0	1		Hn	Stable	LC
<b>Dicroglosidae</b>									
<i>Fejervarya cancrivora</i>	0	0	1	0	0		Fc	Increasing	LC
<i>Fejervarya limnocharis</i>	0	0	1	0	21		Fl	Stable	LC
<i>Occidozyga laevis</i>	0	0	10	0	0		OI	Stable	LC
<i>Occidozyga baluensis</i>	1	0	0	0	0	Y	Ob	Decreasing	LC
<i>Limnonectes finchi</i>	13	1	0	0	0	Y	Lf	Decreasing	LC
<i>Limnonectes ingeri</i>	18	0	0	0	0	Y	Li	Unknow	LC

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<i>Limnonectes leporinus</i>	37	0	0	0	0	Y	LI	Decreasing	LC
<i>Limnonectes palavanensis</i>	1	0	0	0	0		Lp	Decreasing	LC
<b>Microhylidae</b>									
<i>Microhyla borneensis</i>	3	3	3	0	3	Y	Mb	Decreasing	LC
<i>Microhyla perparva</i>	14	11	30	0	1	Y	Mp	Decreasing	LC
<i>Kaloula baleata</i>	0	1	1	0	1		Kb	Stable	LC
<i>Metaphrynella Sundana</i>	0	0	5	0	13		Ms	Decreasing	LC
<b>Megophryidae</b>									
<i>Leptobrachium abbotti</i>	9	0	0	0	0	Y	La	Decreasing	LC
<b>Bufonidae</b>									
<i>Ingerophrynus divergens</i>	37	0	0	0	0		Id	Decreasing	LC

Biodiversity Pro v2.0 (McAleece., et al 1997) was used to assess the richness of species using Shannon’s Diversity Index (SI). We found 19 frog species on 33 transects in the LKWS (secondary forest), 12 frog species were found on nine transects in the surrounding plantations and 15 species on five transects were found at KSFR (Class VI Forest Reserve). High values of Shannon Diversity (SI=1.114) were found at the LKWS secondary forest edge, while the plantations showed the lowest index (SI=0.477) (Table 4.3a). These results are comparable with those found by other authors at the LKWS (Scriven et al., 2018; Gillespie et al., 2012; Barnett J et al., 2013; Riemann, et al., 2015). Relative number of species and species richness (SI in each habitat) was higher in interior secondary forest (mean = 17.32 ; SI = 1.204), followed by primary forest (mean = 9.32; SI = 1.176), secondary forest edge (mean = 4.84; SI = 1.114 ), plantation edge (mean = 6.84; Shannon index = 1.041) and finally plantation (mean = 1.88; SI = 0.477) (Table 4.3a). Inside the LKWS we found higher number of species and values of SI in Lot 5 (mean = 6.89; SI = 1.114) compared with the other lots, followed by Lot 8, Pin Supu, Lot7 and Lot6 (Table 4.3b)

**Table 4.3.** Shannon Index of (a) four major habitat types inside the LKWS and (b) for each lot of the LKWS.

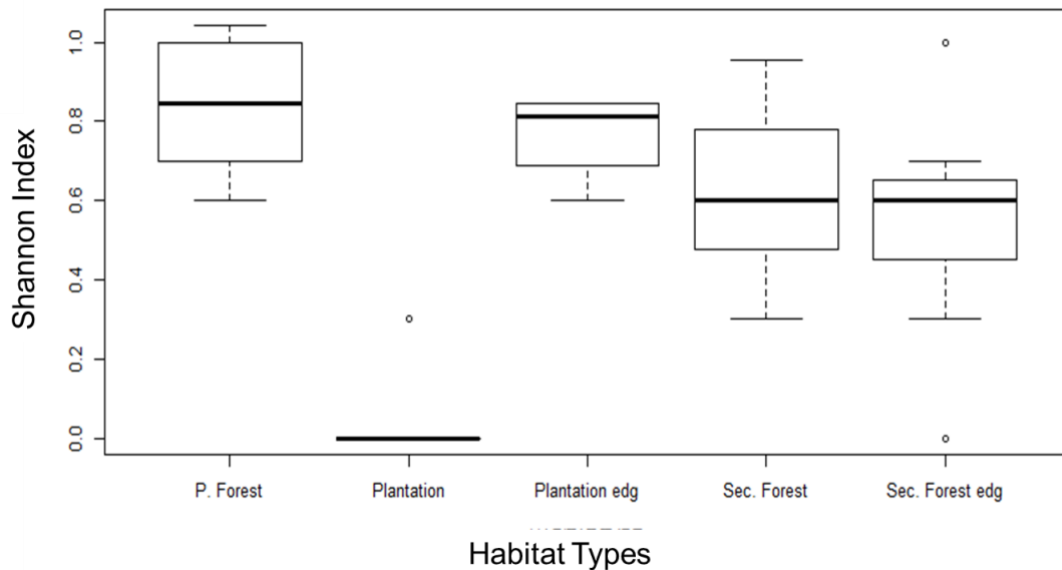
a. Index	SF	SFE	P	PE	KSFR
Shannon Hmax Log Base 10	1.204	1.114	0.477	1.041	1.176

b. Index	Lot 5	Lot 6	Lot 7	Pin Supu	Lot 8	KSFR
Shannon Hmax Log Base 10	1.114	0.903	1.041	0.778	0.903	1.176

\*FE: secondary forest edge; F: secondary forest; P: plantation; PE: plantation edge.

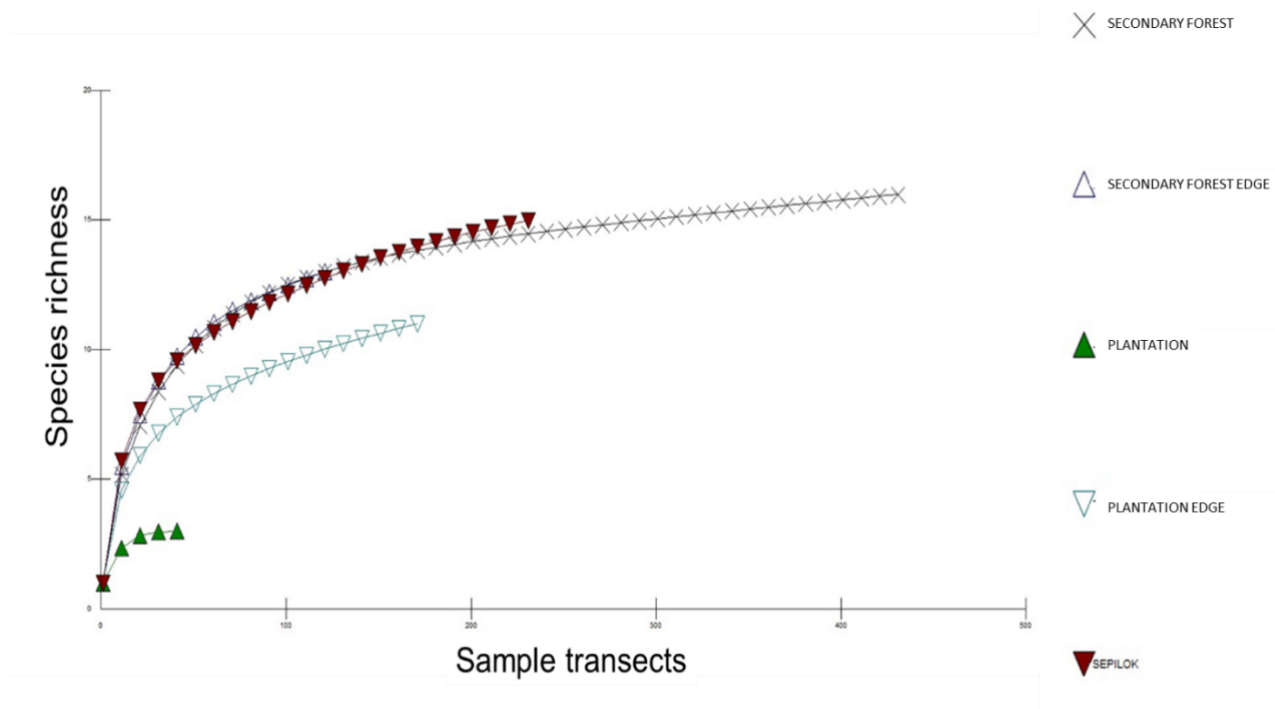
Local species richness, (Shannon Index) encountered on a transect during the field sampling (Figure 4.6), only differed significantly between three pair

of habitats (Appendix two). Species richness was significantly higher in primary forest compared with interior plantations ( $p < 0.001$ ), plantation edge have higher levels of richness than interior plantations ( $p < 0.05$ ) and secondary forest compared with plantation habitats ( $p < 0.05$ ).



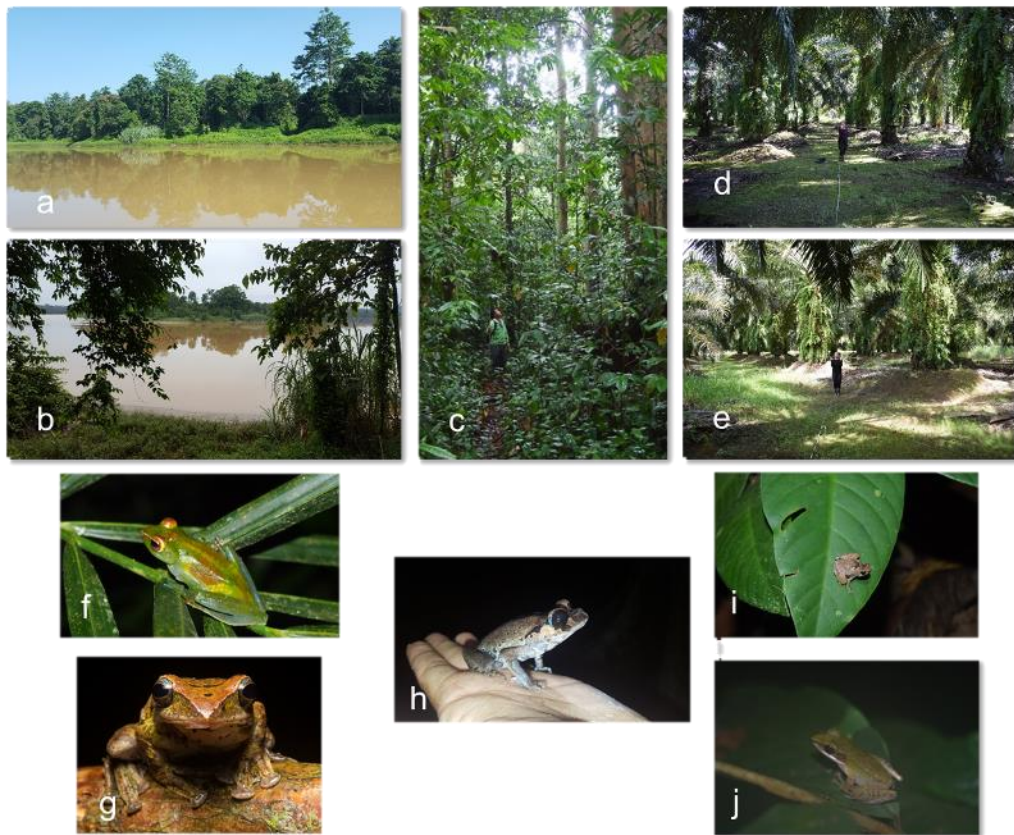
**Figure 4.6** Local species richness (Shannon Index) in each habitat type. Forest (F), forest edge (FE), plantation (P) and plantation edge (PE) inside the LKWS. Open circles refer to outliers due to the differences in values.

There was no obvious difference in total species richness between forested habitats (primary and secondary forest), when visually comparing estimated species numbers in relation to standardized sampling effort. However, in plantation habitats, the total species richness was reduced compared with less-disturbed (primary and secondary forest) habitats. The steep slope in the rarefaction plots for forested habitats indicates that a proportion of the species diversity remains to be discovered (Figure 4.7)



**Figure 4.7** Species richness rarefaction plot for the LKWS in the studied habitat types.

We found 13 species restricted to forested habitats (primary and secondary) and none were restricted to plantation habitats (plantation and plantation edge) (Table 4.2). Ten endemic species from Borneo were detected from which eight were restricted to forested habitats (Figure 4.8). During the entire field work campaign, one individual each of *Microhyla perparva* and *Rhacophorus dulitensis* were detected in plantation edge (Table 4.2).



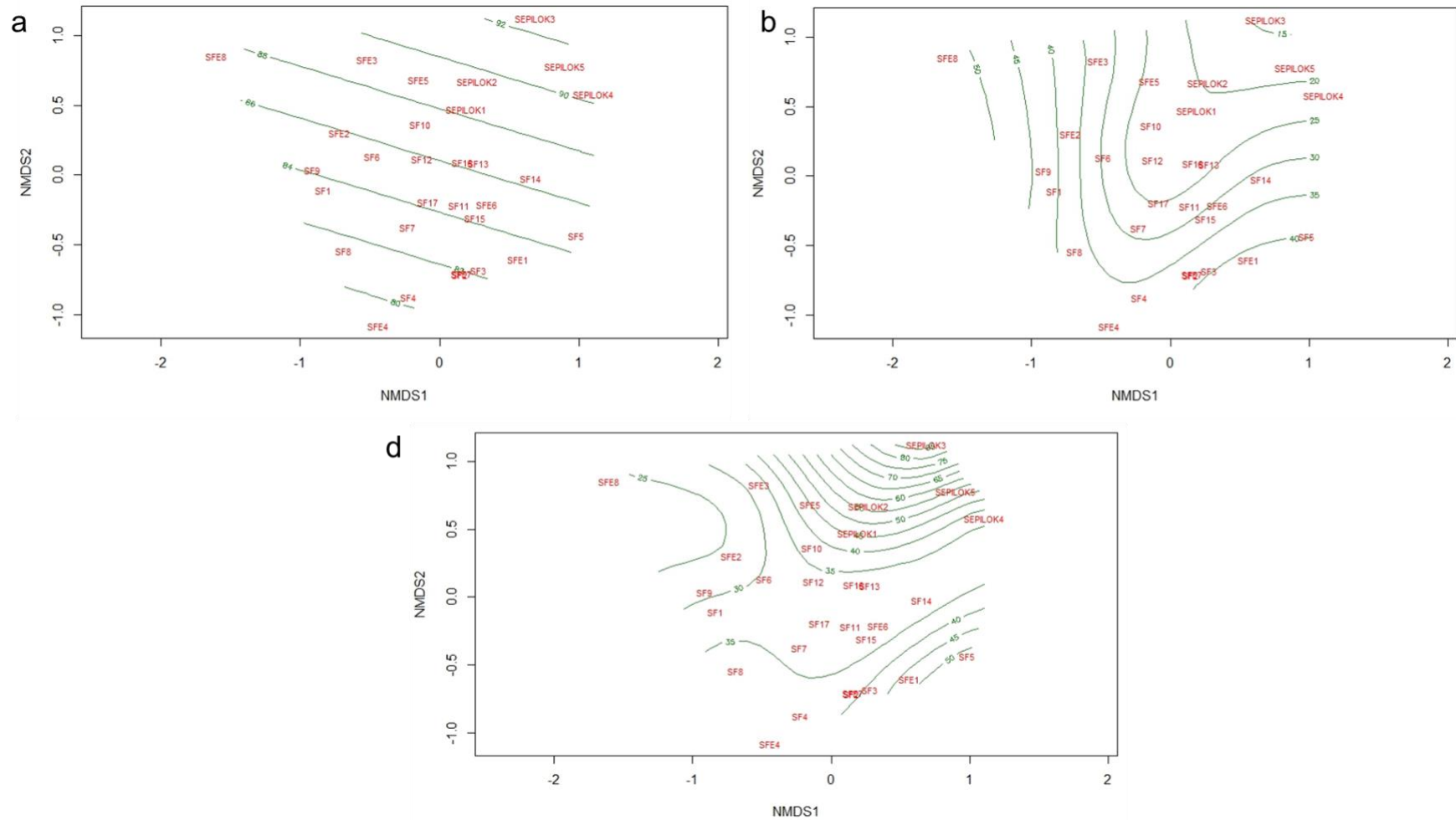
**Figure 4.8** Habitat types and example of endemic species encountered in each habitat type in the LKWS and Sepilok: Secondary forest (a) and *Rhacophorus harrissoni* (g), Secondary forest edge (b) and *Rhacophorus dulitensis* (f), primary forest (c) and *Nyctixalus pictus* (h), plantation (d) and *Microhyla borneensis* (i), plantation edge (e) and *Hylarana megalonesa* (j).

#### **4.3.4 VEGETATION STRUCTURE AND SPECIES COMPOSITION**

LKWS secondary forest sites were differentiated from primary forest ( $p < 0.05$ ) by only three structural habitat parameters from ten that were measured. Differences between these two habitats were most strongly explained by canopy cover density (Appendix three). Differences were explained by canopy cover density, mean diameter of logs and understory vegetation density. Primary forest was characterised by high canopy cover, standard deviation of canopy cover, mean diameter of logs and understory vegetation density, all of which had relatively low secondary forest sites (Figure 4.9).

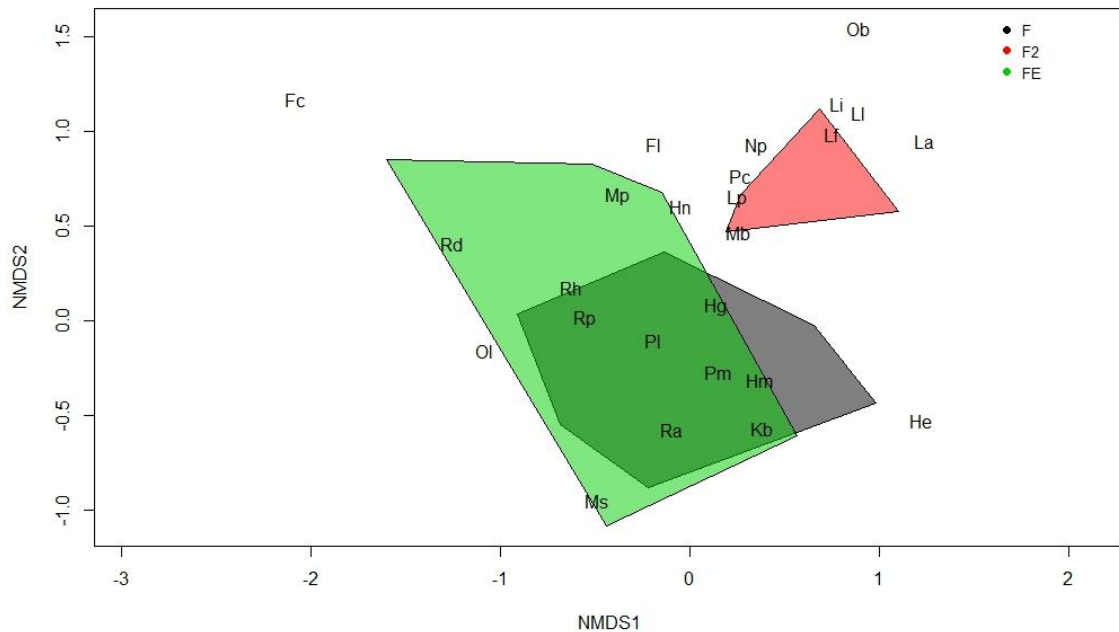


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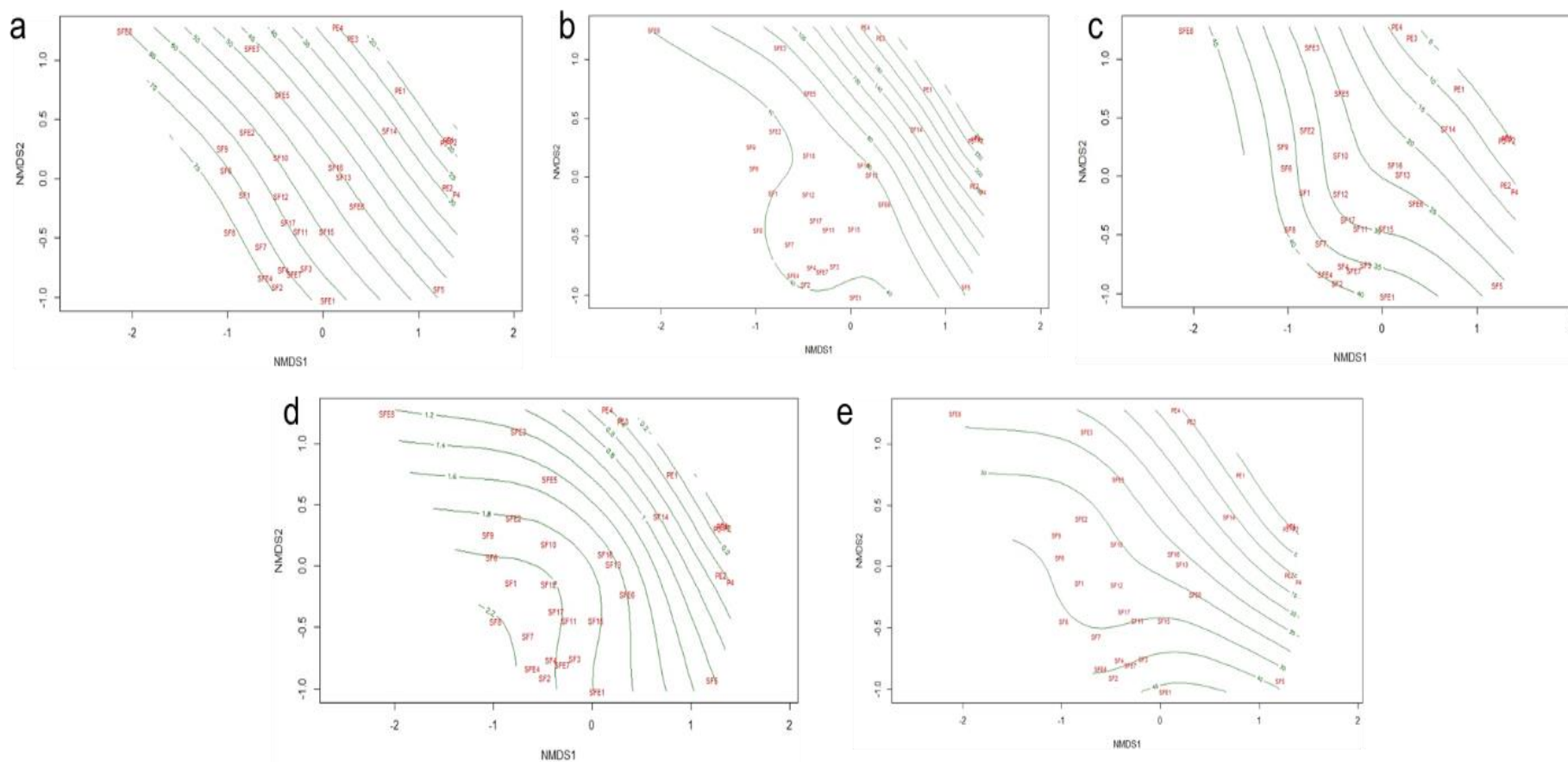
**Figure 4.9** Non-metric multidimensional scaling (NMDS) ordination diagram magnitude (vector length) of significant ( $P < 0.05$ ) fitted environmental parameters overlaid into the ordination space: (a) canopy cover; (b) mean diameter logs and (c) understory vegetation density.

Anuran species varied in their level of affinity with either LKWS secondary forest or primary forest. Species strongly associated with primary forest were comprised mostly of terrestrial species (e.g., *Nyctixalus pictus*, *Occidozyga baluensis* and *Leptobrachium abboti*). Species strongly associated with secondary forest comprised a range of both arboreal (e.g., *Rhacophorus pardalis* and *Rhacophorus dulitensis*) and terrestrial species such as *Microhyla borneensis* and *Kaluola baleata* (Figure. 4.10). Several species, such as *Nyctixalus pictus*, *Ingerophrynus divergens*, *Leptobrachium abboti*, *Limnonectes palavanensis*, *Limnonectes leporinus*, *Limnonectes ingeri* and *Occidozyga baluensis* were restricted to primary forest. Contrary only two species were found at forest edges, namely *Occidozyga laevis* and *Fejervarya cancrivora*. The lack of clustering of anuran species in the centre of the NMDS plot suggests that the occurrence of most species was influenced by these parameters. However, the greatest variability on anuran assemblage composition was in relation to canopy cover and understory vegetation density (Appendix 3).



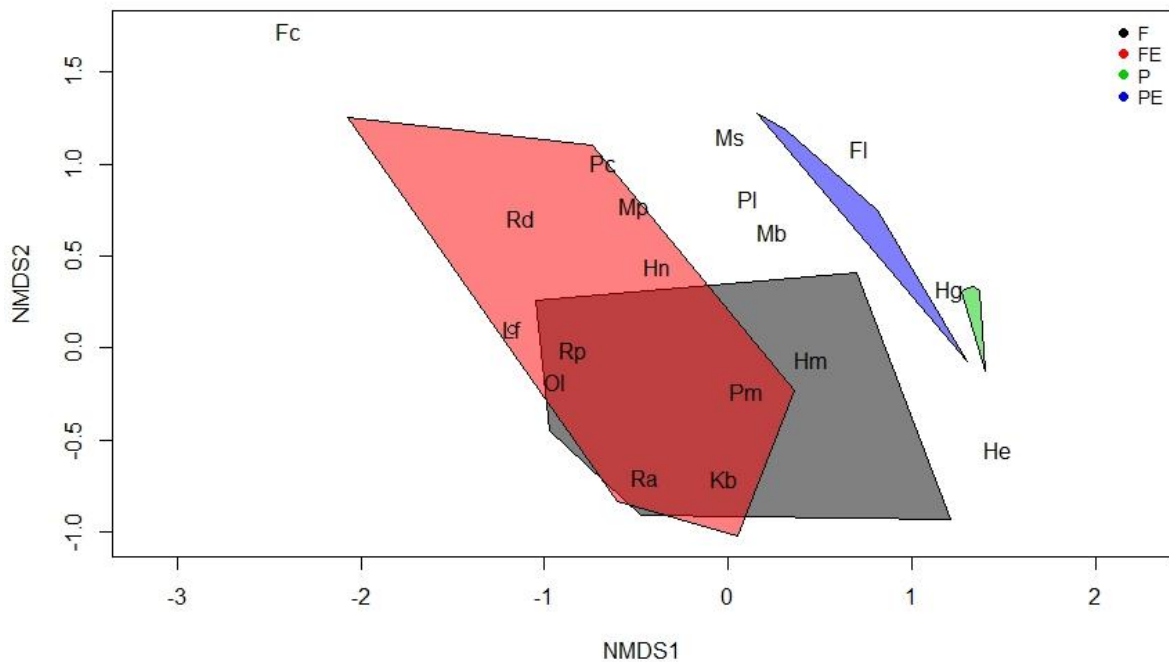
**Figure.4.10.** Dissimilarity of species composition in all different habitat types. Nonmetric multidimensional scaling (NMDS) using Bray- Curtis dissimilarity for binary (i.e., presence-absence) data. Each colour represents one type of habitat; secondary forest (F): gray; primary forest (F2): red; secondary forest edge (FE): green.

LKWS secondary forest sites were strongly differentiated from plantation sites ( $P < 0.01$ ) by five out of ten structural habitat parameters (Appendix four). Differences between LKWS forest and plantations sites were most strongly explained by number of trees, mean tree circumference, mean diameter logs, understory vegetation density and leaf litter. LKWS forest were characterised by high number of trees, mean tree circumference, mean diameter of logs, understory vegetation density and leaf litter, all of which had relatively low at plantation sites (Figure 4.11).



**Figure 4.11** Non-metric multidimensional scaling (NMDS) ordination diagram magnitude (vector length) of significant ( $P < 0.01$ ) fitted environmental parameters overlaid into the ordination space: (a) number of trees; (b) mean tree circumference; (c) mean diameter logs and (d) understory vegetation density and (e) leaf litter.

Anuran species varied considerably in their level of affinity with either LKWS secondary forest or plantation sites. We only found two species strongly associated with plantation (*Fejervarya limnocharis* and *Metaphrynella Sundana*). Species strongly associated with secondary forest comprised a range of both arboreal (e.g., *Rhacophorus appendiculatus* and *Rhacophorus pardalis*) and terrestrial species such as *Occidozyga laevis* and *Limnonectes finchi* (Figure 4.12). There were seven species restricted to secondary forest. In contrast, no species associated exclusively to plantation sites were found. The lack of clustering of anuran species in the centre of the NMDS plot suggests that the occurrence of most species was influenced by these five parameters.



**Fig 4.12** Dissimilarity of species composition in all different habitat types. Nonmetric multidimensional scaling (NMDS) using Bray- Curtis dissimilarity for binary (i.e., presence-absence) data. Each colour represents one type of habitat (secondary forest (F): gray; secondary forest edge (FE): red; oil palm plantation: green; oil palm plantation edge (PE): purple).

### 4.3.5 PHYLOGENETIC DIVERSITY

Values for sesPD, sesMPD and sesMNTD inside the LKWS were relatively similar across all five lots, except for sesPD in Lots 5 and 7, where the values were higher (Table 4.4a). sesPD were high in secondary forest with lower values found at plantation sites. Interestingly, sesMPD values were the same for secondary forest and plantation edge sites, with lower values found only in plantations. sesMNTD values were lower for forested sites in comparison with plantation sites (table 4.4b). Phylogenetic values (sesPD, sesMPD and sesMNTD) were higher when comparing forested habitats (LKWS and KSFR) with plantation sites (table 4.4c) but the PD values were similar when comparing plantations to the LKWS forest lots individually (table 4.4a).

**Table 4.4** Summary of all the phylogenetic values. (a) LKWS lots ; (b) Habitat types and (c) Populations.

<b>a</b>	<b>sesPD</b>	<b>SR</b>	<b>sesMPD</b>	<b>MPD p.val</b>	<b>sesMNTD</b>	<b>MNTD p</b>
Lot 5	1.68	13	0.36	0.02	0.19	0.04
Lot 6	1.19	8	0.36	0.08	0.21	0.07
Lot 7	1.63	11	0.37	0.07	0.23	0.22
Pin						
Supu	0.98	6	0.36	0.13	0.23	0.31
Lot 8	1.11	8	0.36	0.07	0.23	0.02

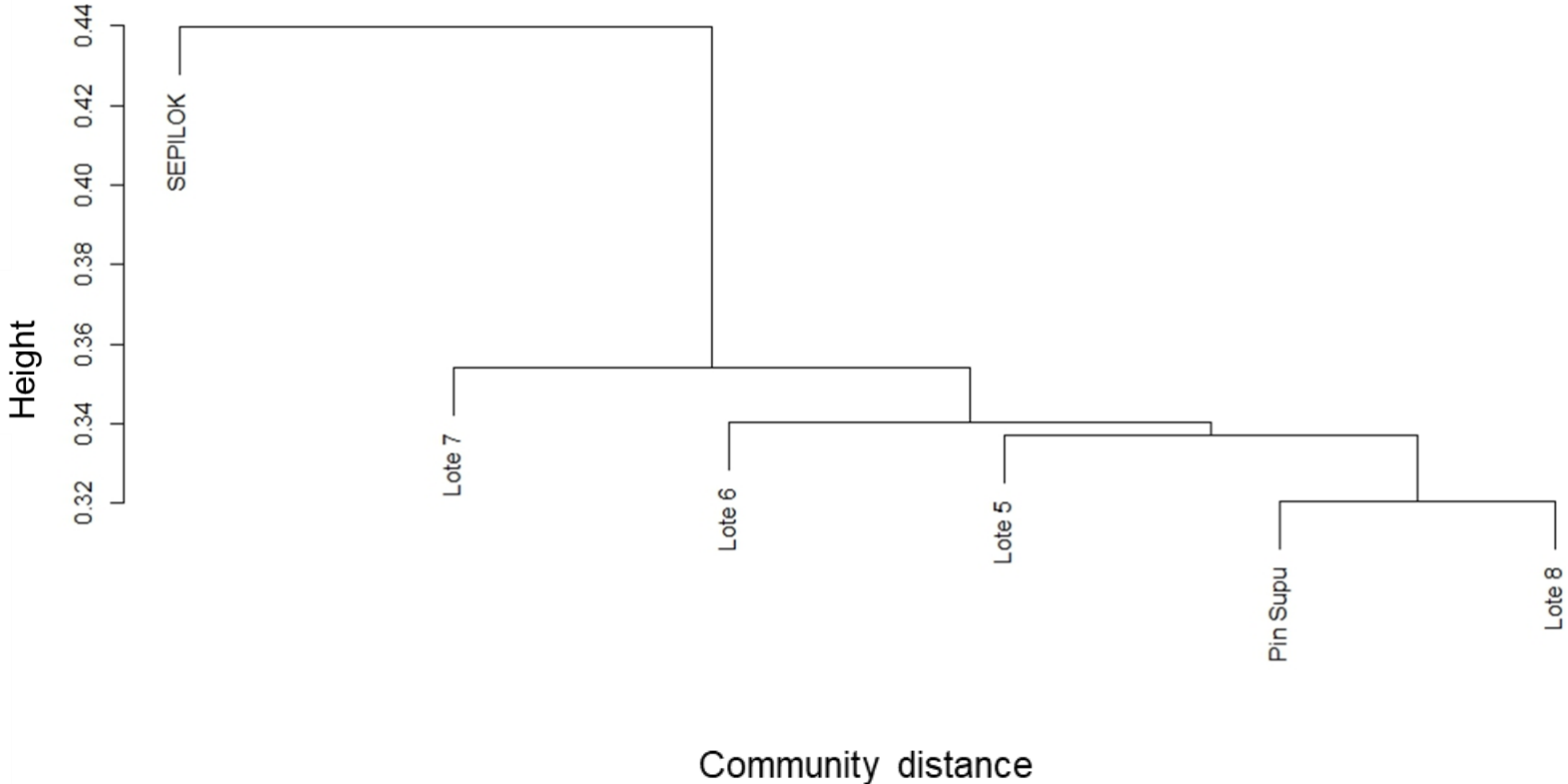
<b>b</b>	<b>sesPD</b>	<b>SR</b>	<b>sesMPD</b>	<b>.obs.p</b>	<b>sesMNTD</b>	<b>mntd.obs.p</b>
SF	2.31	16	0.43	0.10	0.21	0.08
SFE	1.87	13	0.39	0.01	0.21	0.03
P	0.44	3	0.30	0.04	0.27	0.10
PE	1.78	11	0.43	0.29	0.25	0.40

<b>c</b>	<b>sesPD</b>	<b>SR</b>	<b>sesMPD</b>	<b>mpd.obs.p</b>	<b>sesMNTD</b>	<b>mntd.obs.p</b>
LKWS	2.37	19	0.37	0.00	0.18	0.02
KSFR	2.72	13	0.51	0.58	0.29	0.67
Oil Palm	1.61	12	0.37	0.05	0.20	0.09

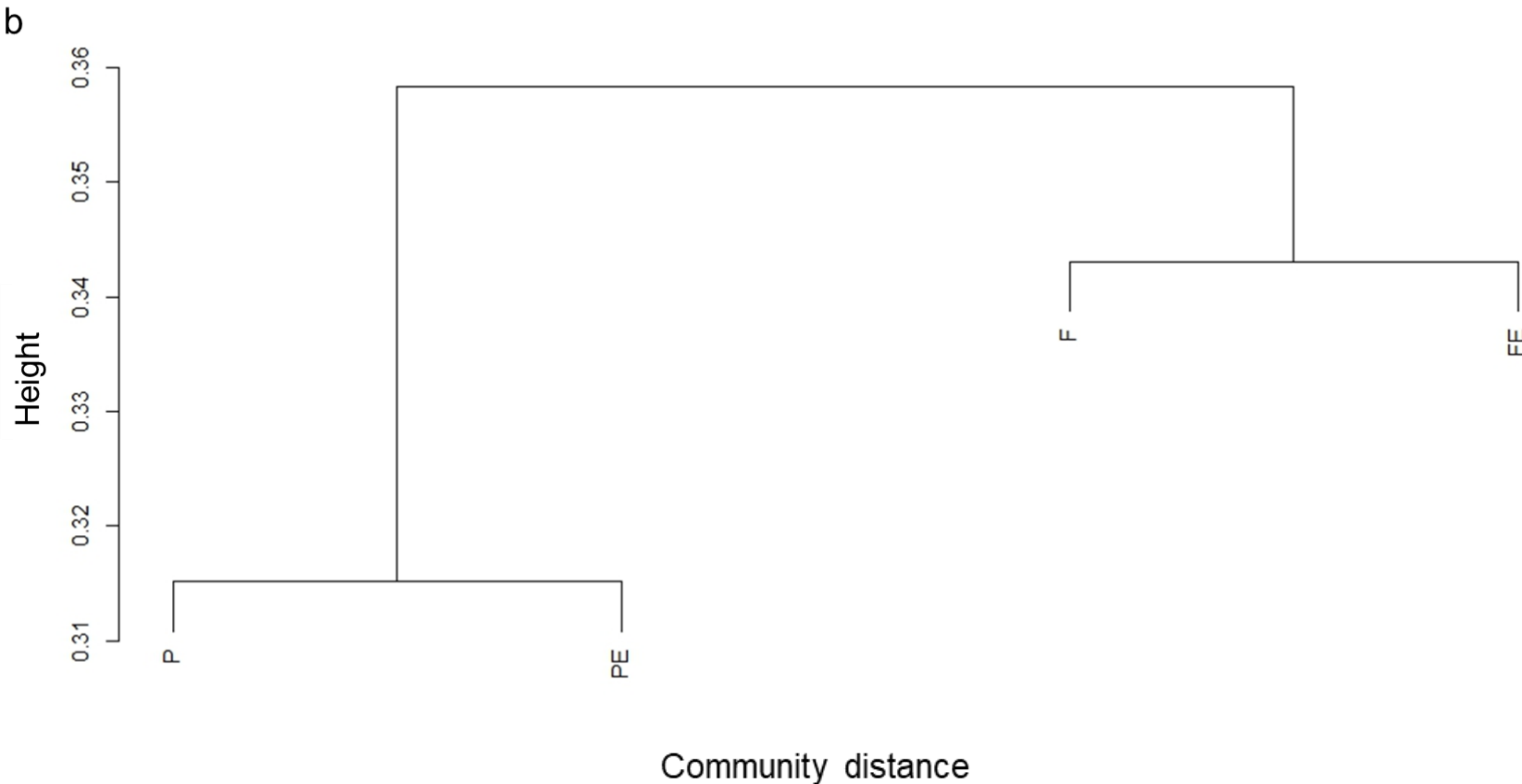
SR: species richness; SF: secondary forest; FE: secondary forest edge; F: primary forest; P: plantation; PE: plantation edge.

Exploring the correlation of phylobetadiversity with the different habitat types (community distance) allowed us to more rigorously quantify connectivity among communities. Variance in beta phylobetadiversity across the different populations and habitat types can be seen in (Figure 4.13a,b). The cluster analysis divided KSFR and LKWS as separate communities based on their evolutionary similarity (height) (figure 4.13a) as well as the forested and matrix habitat types (Figure 4.13b).

a







**Figure 4.13** Cluster dendrogram for the different populations (a) and habitats types inside LKWS (b)

## **4.4 DISCUSSION**

This study represents the first amphibian phylogenetic analysis to take place along the Lower Kinabatangan floodplain and provides a preliminary comparison of the phylodiversity from the LKWS and the different types of habitats inside it. We also compared the phylodiversity of the LKWS a secondary forest with a primary forest such as KSFR. Finally, this study provides with the only COI barcodes for 10 lowland Bornean frog species. Our findings will encourage continuation of amphibian investigations in this region because, despite severe forest disturbance, high amphibian diversity persists (Gillespie et al., 2012; Scriven et al., 2019).

### **4.4.1 DNA BARCODING**

Using the GenBank BLAST tool to confirm the identity of the amplified sequences, 100% of 16S fragment sequences showed sequence similarity to all 25 morphological identified amphibian species. However, COI sequences yielded low query success due to PCR amplification failure and the poor representation of COI sequences of Bornean herpetofauna in global sequence databases. Nevertheless, both markers used in this study (16S and COI) fulfilled the requirements of barcoding markers when combined (Hebert et al., 2004; Mneji et al., 2019) by identifying the species in 100% of samples collected, with the exception of those that failed to amplify or could not be found in the reference library of barcodes on NCBI. Our results suggest that both loci should be used for amphibian studies, but the mitochondrial 16SrRNA gene fulfils the requirements for a universal DNA barcoding marker in amphibians for this area. In terms of priming sites and identification of major clades, our study revealed that the 16S fragment is substantially superior to COI. Even though substitutions are common in some variable regions of the 16S gene, the sequence is a highly conserved

mitochondrial marker (Mneji et al. 2019). Amphibians are a relatively old group and thus substitutions have had a long evolutionary timescale to accumulate and to differentiate among species (Vences et al 2005). The COI primer pair has seen comparatively little use in amphibians since its introduction (Che et al., 2012). Therefore, the fact that we observe a poor representation of COI sequences for Bornean amphibians in the GeneBank allow us to say that the 16S can be consider as truly universal DNA barcode marker. These results illustrate the relevance of molecular data in species identification within Bornean amphibians.

Finally, since the aim of this study was to provide DNA barcodes and to assist in measuring PD, the use of 16S and/or COI fragments alone as suitable markers for amphibian phylogenetic reconstruction is not recommended. If these fragments are going to be use to elucidate genealogical relationships, data from both markers should be combine with other loci fragments, including nuclear DNA sequences such as RAG-1 (Canedo & Haddad, 2012).

#### **4.4.2 PHYLOGENETIC ANALYSIS**

Neighbour-joining trees (NJ) constructed based on COI and 16S datasets for all species sequenced for this study clustered most individuals with other members of their taxonomic lineage. The clustering was in accordance with morphological identification, enabling the efficient differentiation of species. 16S phylogeny grouped samples of the same species together with higher support and showed deeper clades with higher nodal support than COI (Figures 4.5 – 4.6). COI failed to cluster members of the Microhylidae family. The 16S phylogenies displayed a stronger and deeper clade divergence when compared with COI and successfully clustered member of Microhylidae

family and the remaining four families, supporting findings from previous phylogenetic reconstructions (Pyron & Wiens, 2011, Nnij et al., 2019).

Though it is possible to compare results with previous studies that include closely related species to those in this investigation, no publication has produced a phylogeny including a similar assemblage. Therefore, the specific evolutionary relationships between these species remain to be fully resolved and further analysis of the phylogeny of the Kinabatangan anuran species is required using additional markers, and especially nuclear DNA. Further research should include examination of *R. appendiculatus*, which was divided into two strongly supported clades in both phylogenetic trees (sample Rd1-70 for 16S and RaS56 for COI). This may suggest the presence of a cryptic species. This finding may have affected the results of population structure of Chapter 3 by introducing false structure. However, due to the small sample size (six individuals for 16S and four for COI) no definitive conclusion can be made. To determine the significance of this divergence, a multiple gene phylogenetic analysis and morphometric analysis is required on a larger sample size

#### **4.4.3 VALUE OF SECONDARY FOREST FOR AMPHIBIAN DIVERSITY**

Using a combination of visual and acoustic methods for detection and morphological identification, we assessed amphibian species abundance, diversity and community composition in forested (LKWS and KSFR) and palm oil habitats that differed in their structure and their degree of disturbance. Consistent with our fifth hypothesis (refer to Chapter 1), oil palm plantation support lower species richness, fewer endemic species and mostly disturbance-tolerant species compared to secondary forest habitats. Scriven et al (2018) showed similar results confirming that oil palm plantations surrounding the LKWS provide little overall benefit to conservation of

Bornean anuran diversity. Even though *M. perparva* and *R. dulitensis* (forested restricted species) were found at plantation edge, this were an isolated event and could be due to the presence of a stream in between forest edge and plantation edge that could buffer against microclimatic changes allowing one individual to move just temporally away from the forest (Riemann, et al., 2015).

Accordingly with our sixth hypothesis, amphibian species composition was influenced to various degrees by structural habitat parameters. Our results suggest that amphibian species composition in forested habitats (LKWS and KSFR) change strongly with variability in canopy cover density. The fact that amphibian species composition inside LKWS in comparison with KSFR are only strongly affected by canopy cover density suggest that this areas/lots may have an important role in the conservation of anuran biodiversity. It is important to note that the sampling effort done in KSFR was not comparable with the LKWS (one week compared to 11 months). However, we still found evidence that primary forest supports higher number of endemic species compared with secondary forest (Table 4.2). Forest sites were strongly differentiated from plantation sites by six habitat parameters (Appendix four). Results were similar than that the ones from Scriven et al (2018) and revealed the levels of disturbances in oil palm plantations possible due to anthropogenic of anthropogenic disturbance levels by human access into the oil palm plantations

Our study showed evidence that oil palm plantations support lower species richness, fewer endemic species and mostly disturbance-tolerant species compared to rainforest habitats and provides little overall benefit to conservation of Bornean anuran diversity (see also Scriven et al., 2018). This study demonstrated that surprisingly high numbers of primary forest species can be found in areas of secondary forest (Table 4.2) and suggests these habitats can provide important conservation services. However, due to the

markedly differences in sampling effort between these two habitats, there is the need of a larger survey at primary forest. Nevertheless, this is a preliminary study that provides some empirical evidence regarding the unique importance of the LKWS secondary forest, highlighting the need of retaining comprehensive reserve networks such as the LKWS as part of a wider landscape management strategy. Finally, we provide insights regarding the complexities involved in answering simple questions about the biodiversity conservation value of degraded habitats and caution against drawing firm conclusions from studies that focus on a limited number of these.

#### **4.4.4 PHYLOGENETIC DIVERSITY**

Species richness and branching topology both affect phylogenetic diversity. Adding species to a community increases the sum of branch lengths, and a community comprised of close relatives will have a lower branch length sum than one comprised of an equal number of distantly related species (Prescott et al., 2016). Of relevance to our seventh and eighth hypotheses, results suggest that directing future expansion of oil palm monocultures towards existing secondary forest would carry greater losses of anuran phylogenetic diversity in the LKWS. PD differed between secondary forest habitats and oil palm plantations (Table 4.4c). Similar values of PD were found between LKWS and KSFR, suggesting that on a per species basis, LKWS and Sepilok conserve similar levels of evolutionary history. These results revealed the importance of secondary forest for the maintenance of phylogenetic diversity. On the contrary, some amphibian clades inside oil palm plantations seem less likely to persist. Our results suggest that historic forest loss and fragmentation may have already extirpated the most sensitive forest species from plantations (Irwin et al., 2010) prior to their development. The higher MPD in forested habitats, especially in primary forest, suggests that frog species recorded are distributed across a wider range of clades

than those recorded in oil palm habitats (Table 4.4b,c). It has been showed the importance of humidity environmental variables on PD in amphibians (da Silva et al.,2012). Since oil palm plantation showed high levels of temperatures (Ramdani et al., 2014) this could be a possible explanation of the low levels of PD found in these types of habitat. PD inside LKWS did not show any significant differences between lots, suggesting that each habitat has similar MPD to that expected given the number of species (Table 4.4a).

Similar to other metrics we studied, the mean distance between a species and its closest relative (MNTD) was higher in oil palm habitats compared with secondary forest habitats (Table 4.4b). Secondary forest habitats had lower sesMNTD than oil palm sites, which indicate that forest communities are more phylogenetically clustered in their terminal branches. Together with the finding that oil palm sites have low MPD, this suggests that oil palm plantations represent relatively few clades but contain many species within those clades. Forested habitats have higher MPD and lower MNTD than oil palm (see also Frishkoff et al. 2014), showing that there is a broader representation of clades, but with many closely related species coexisting.

PD and MPD were slightly higher in primary than in secondary forest (Table 4.4a), probably due to the differences in sampling effort. Nevertheless, secondary interior forest PD and MPD values were higher when compared with oil palm plantation habitats (Table 4.4b), suggesting that LKWS secondary forest could not be replaced without significant loss of amphibian phylogenetic diversity. PD was higher in interior secondary forest (Table 4.4b,c), showing the importance of remnant secondary forests for the maintenance of phylogenetic diversity in agricultural landscapes. Finally, beta-phylodiversity plots confirmed the phylogenetic similarities between forest habitats and the segregation with oil palm plantation habitats (Figure 4.10a), as well as primary and secondary forest (Figure 4.10b).

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## **Chapter 5: General discussion**

## **5.1 OVERVIEW**

This study provides the first genetic data for frogs in the Lower Kinabatangan Wildlife Sanctuary (LKWS) and in Kabili-Sepilok Forest Reserve (KSFR). When the study was initiated, limited information was available on Bornean amphibian genetics with most relevant research focusing on evolution and phylogenetic studies, and no studies on amphibian population genetics had been carried out in the area of the Kinabatangan river. In order to address gaps in our knowledge of the genetic and ecological consequences of fragmentation on frog communities in this area, several hypotheses were constructed. An overview of the aims, hypothesis, results and a brief summary of the conservation implications for this study are shown in Table 5.1.

Twenty-six new microsatellite markers were developed and these markers were used to estimate the population genetic structure and genetic diversity, as well as migration rates for three species of Bornean frogs chosen due to their habitat preferences in the area of the LKWS and KSFR. Finally, a combination of non-invasive buccal swab sampling and surveys were used to investigate the value of secondary and primary forest for anuran communities at the community and phylogenetic diversity (PD) level. The results this study, outlined below, make a new contribution to a better understanding of the frog communities in the LKWS and KSFR and provides a suitable methodology for non-destructive sampling (buccal swabs) that can be applied to future work.

**Table 5.1** A brief description of the aims, hypothesis and results in this thesis and their implications for anuran conservation.

Chapter	Aims	Hypothesis	Result	Conservation implications
Chapter 2	Developed and characterization of new microsatellites Markers.	Not applicable.	26 microsatellites for three species of Bornean frogs.  10 in-silico microsatellites for <i>R. dulitensis</i> .	These markers can be used in future studies in the region (also in different species).
Chapter 3	Analyse and identify population genetic structure in three frog species with contrasting habitat associations.	a) Forest populations possess more genetic diversity than those residing in and around oil palm plantations.	Levels of GD were higher in primary and secondary forest. Lowest levels of GD were found in palm oil plantations.	High conservation value of five lots inside the LKWS.
		b) Oil palm plantation frog populations have lower genetic diversity than forest or plantation edge populations.	<i>H. megalonesa</i> showed lower levels of GD and inbreeding than <i>R. appendiculatus</i> .	Require the use of a higher number of microsatellites markers.
			Genetic structure and migration rates was found between fragments.  Evidence suggest two management units for forest species <i>R. appendiculatus</i>	Importance of fragments connectivity for amphibians.  High levels of genetic differentiation split into two management units, which should be better protected.

Asses the value of secondary forest	a) Use of DNA barcoding markers will work as a tool for anuran diversity assessment.	16S fulfil the requirements of a universal DNA barcoding marker.	Relevance of molecular data in species identification for Bornean amphibians.
	b) Secondary forest holds higher levels of species richness than oil palm plantations.	Forested habitats possess higher levels of species richness than oil palm plantations.	Plantations surrounding the LKWS provide little overall benefit to anuran conservation.
	c) Some habitat parameters inside the LKWS and oil palm plantations influence species composition.	Change in canopy cover strongly reflect variability in forested habitats.	Highlight the importance of our study areas on anuran biodiversity conservation.
	d) Forested habitats have higher levels of phylogenetic diversity in forested habitats.	Higher levels of phylogenetic Diversity were found in forested habitats	LKWS secondary forest could not be replaced without significant loss of amphibian phylogenetic diversity.

## 5.2 POPULATION GENETICS IN THE LKWS AND KSFR

Genetic diversity is a key factor for population survival and evolution (Reed & Frankham, 2003) and low genetic diversity can adversely affect populations, limiting their capacity to adapt (Gargano et al., 2015). Genetic variation is also sensitive to habitat disturbance (Vranckx et al., 2011) and currently, anthropogenic habitat disturbance is a major driver of global environmental change (Gonzales et al., 2019; Martínez-solano & González, 2008). The LKWS offers an excellent model environment to test the effects of habitat fragmentation and oil palm agriculture on amphibians; with replicated analysis feasible on both sides of this very large river. Furthermore, the LKWS offers a good understanding of the history of forest clearance and land conversion since 1973. In addition, compared to other vertebrates in Borneo, relatively few studies have been conducted on frog population dynamics and genetics (Emerson et al., 2000; Hertwig et al., 2012; Matsui et al., 2015) whereas multiple studies have now been conducted in fragmentation effects for mammals in the region (Bernard et al., 2014; Brunke, Radespiel, Rita, Michael, & Goossens, 2019; Kieran Love et al., 2017).

Fragmentation affects gene flow among populations of all types of species, from plants and insects (Toczydlowski & Waller, 2019) to small and large mammals (Brunke et al., 2019; Macdonald et al., 2018), constraining the size of a population and increasing its isolation (Couvét, 2002). Most of the literature on habitat disturbance recognizes habitat fragmentation (i.e., subdivision of a continuous area into smaller fragments) that increases fragment isolation and edge effects, and is usually accompanied by habitat loss as a major factor that changes species composition, ecological interactions, gene flow and genetic diversity (Gonzales et al., 2019).

In my study, I found higher levels of GD for the forest adapted species (*R. appendiculatus*) in lots 6 and 7 of the LKWS secondary forest, an area that has been fragmented for almost 40 years due to oil palm agriculture. This may be explained because the secondary forest lots (6 and 7) of the LKWS

are large enough to hold populations that are not yet losing genetic diversity and/or because the area retains favourable habitat for the survival and stability of the population. It is known that secondary forest can sometimes play an important role in the maintenance of biodiversity (Dent & Wright, 2009) and our results revealed high levels of GD for *R. appendiculatus* across four lots inside the LKWS, especially lot 7 that I found has similar levels of GD as KSFR. However, we should treat the results for KSFR with caution due to the differences in sampling effort between these two habitats. Future work for studying fragmentation effects should also include more intense ecological and genetic surveys in KSFR. Even though we found relatively high levels of GD inside the LKWS and KSFR for the forest adapted species, inbreeding was high for both habitats, which could be an early warning (amber flag) of a possible future problem. There is evidence that fragmentation can lead to neutral or positive effects for wildlife populations that play out over a variety of timescales (Fahrig, 2003; Templeton et al., 1990; Valbuena-Ureña et al., 2017), therefore long-term regular surveying and monitoring are necessary.

Oil palm plantations have been known to be detrimental for biodiversity (Fitzherbert et al., 2008). Negative environmental impact includes wildlife declines, negative carbon balance, and the draining and burning of peat lands (Meijaard et al., 2018) but the negative genetic effects on anuran biodiversity have not been fully studied. In line with my hypothesis, negative effects in terms of demographic isolation were found among populations as a result of oil palm plantation effects. Lower levels of GD were found for *H. glandulosa*, the plantation specialist chosen for this study. In addition, surprisingly low levels of local inbreeding suggest that there may be a degree of inbreeding avoidance which may be an important evolutionary factor leading to asymmetries, depending on the relative costs of inbreeding between the sexes (Austin et al., 2003). In addition, *H. glandulosa* showed some degree of genetic structure that could be explained by how well adapted this species is to disturbed habitats, having no special habitat requirement within its habitat range (Inger & Stuebing, 1997; IUCN, 2018).

Estimating genetic differentiation can help to define the extent of connectivity and gene flow between populations (for example, as measured by Wright's  $F_{ST}$ ) (Frankham, 2006). In a fragmented ecosystem, such as the LKWS, it is important, for long-term population viability, to resolve whether there are barriers to gene flow between fragments. Due to the nature of the sanctuary (a floodplain, which is inundated seasonally and flooded during winter), and as a historic natural corridor, gene-flow is highly likely to be ongoing between forest fragments for taxa adapted to aquatic conditions, despite their fragmentation. Amphibians are thought to have generally low dispersal rates (Blaustein et al., 1994) however Funk et al (2005) found that frogs can have high juvenile dispersal rates over long distances (> 5 Km) and our study showed significant levels of gene flow between adjacent lots. In contrast, higher levels of  $F_{ST}$  were found between more distant lots (lot 7 and Pin Supu FR and between lot 7 and lot 5).

Even though there were substantial levels of admixture between study areas, my results suggests that the forest adapted species studied here could be managed as two separate units inside the LKWS: 1) Lots 6, 5 and 7 and 2) Lot 8 and Pin Supu FR. Habitat fragmentation is still one of the greatest threats for amphibians (Bishop et al., 2012), and in a metapopulation, such as the LKWS, this could finally lead to population isolation (Marsh & Trenham, 2000). These findings will help us to develop future conservation plans to manage and protect this forested species and as a baseline for future studies of other forest specialist species.

### **5.3 VALUE OF SECONDARY FOREST FOR ANURANS**

To evaluate the value of secondary forest for anuran communities, DNA barcoding markers were used to facilitate species identification in forested and oil palm plantations habitats. A total of 25 species belonging to seven taxonomic families were identified in the LKWS and KSFR. Our study revealed that the 16S mitochondrial barcoding sequence can be used as a universal marker in amphibians in this region and performs better than the

more traditional barcoding marker COI, in accordance with previous work (eg Vences et al., 2012).

DNA barcodes can also be applied as a tool for addressing fundamental questions in conservation biology such as the most evolutionarily rich habitats, which can subsequently be targeted for protection (Kress et al., 2015). In order to choose one of the markers to study the phylogenetic diversity (PD), both markers were tested on their reliability of producing phylogenetic analysis. Even though both neighbour joining (NJ) trees clustered most of the species, 16S marker was superior, with strongly supported likelihood values and deeper clade divergence accordingly. 16S marker has proven to be the better marker for phylogenetic reconstruction in other studies on amphibians (Darst & Cannatella, 2004; Emerson et al., 2000; Matsui et al., 2016; Wilkinson, Drewes, & Tatum, 2002). Following these results, 16S was used in order to calculate the PD inside the LKWS and KSFR. Supporting with my hypothesis, higher levels of PD were found in forested habitats compared with oil palm plantations, suggesting that the different amphibian clades are less likely to survive in these disturbed habitats. The negative effect of oil palm agriculture on PD in amphibians has not been explored, but our results are comparable with Prescott et al (2016) who focused on PD in birds founding low levels of GD inside oil palm plantations. The low levels of PD found inside plantations may be explained by the loss of habitat and fragmentation that may have eliminated the most sensitive species from plantations. Our results suggested that LKWS secondary forest could not be replaced without a significant loss of amphibian PD.

Finally, using a combination of visual encounter surveys (VES), Shannon Index and community composition, amphibian diversity in different types of habitats were studied. In support of our hypotheses a higher number of species were found at forested habitats compared with oil palm plantations (Fitzherbert et al., 2008). Rare and endemic amphibians were much more



abundant in secondary and primary forest, and common amphibians were more prevalent in oil palm plantations (Paoletti et al., 2018; Scriven et al., 2018). Four significant factors were found to be associated with species richness and composition between LKWS (secondary forest) and KSFR (primary forest). Differences between the LKWS and KSFR were explained by canopy cover density, mean diameter of logs and understory vegetation density. Inside the LKWS habitat types the results showed similarities with the results from Scriven et al. (2018) where the canopy cover, log diameter and vegetation density were higher at interior secondary forest compared with forest edge and plantations.

The future of amphibians in the LKWS depends on the correct management of the sanctuary. Even though the current study was challenging in many aspects, a full answer can be given to the posed hypothesis. This study provides a useful baseline information regarding genetic diversity, gene flow, migration rates, amphibian species richness and phylogenetic diversity of frogs at the LKWS. Furthermore, it identifies a guideline for future research in order to answer questions on genetics and ecology of this frog species. The addition of this data along with future studies will be key in order to have a complete management plan that will help long term survival of amphibians in the LKWS.

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**APPENDIX 1**

Nine In silico microsatellites for *R. dulitensis*.

<b>Rd1jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size (bp)</b>
Forward primer	F:TTCTAAAGGTCACCTTGTGG	55.98	42	249
Reverse primer	R:TCAAGTCTAAGTGCTCACC	55.42	47	
<b>Rd2jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:GTTGCTATGTTCTTTCCCTGC	52.4/59.5	48	139
Reverse primer	R:CATTCACTCACACAGATACC	49.7/56.4	45	
<b>Rd3jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:ACTGTACACCATACTCAAGC	49.7/56.4	45	232
Reverse primer	R:AGCACTCACAGATTATGAAGG	50.5/57.5	43	
<b>Rd4jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:CCAAATGAATATCCAAGAGC	47.7/54.3	40	190
Reverse primer	R:AGATTGCGTACTTGTCTTGC	49.7/56.4	45	
<b>Rd5jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>

Appendices

Forward primer	F:GCTGTTTATTTGGCTCTAGG	54.4	45	192
Reverse primer	R:TTTGCTTGAAAGAGACTTCC	54.7	40	
<b>Rd6jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:TGTCTCTTTCACCAATAGGC	49.7/56.4	45	220
Reverse primer	R:GGTTTCTTAATCCCCAAAGC	49.7/56.4	45	
<b>Rd7jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:ATGCCATAAAGTGTCTGTCC	55	45	212
Reverse primer	R:TTTGTGATGCTCTAGTGACC	53.7	45	
<b>Rd8jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:TATTAGCACCCTGTATCC	55	45	145
Reverse primer	R:TGGACGTAATCTGTTTACCC	54.2	45	
<b>Rd9jm</b>	<b>Sequence (5' . 3')</b>	<b>Tm</b>	<b>%GC</b>	<b>Product size</b>
Forward primer	F:CTCTTGTCACCGTCTACACC	54	45	274
Reverse primer	R:GTACCATGGAGAATGAATGC	55.4	45	

*T<sub>m</sub>*-Melting temperature range

**APPENDIX 2**

Subsample of 24 transects that were used for the genetic analysis.

<b>LOT</b>	<b>Transect</b>	<b><i>H.megalonesa</i></b>	<b><i>H. glandulosa</i></b>	<b><i>R. appendiculatus</i></b>
<b>LOT5</b>	<b>T1*</b>	6	...	10
	<b>T3*</b>	3	...	10
<b>LOT6</b>	<b>T1</b>	6	...	...
	<b>T2</b>	...	...	10
	<b>T5</b>	14	...	...
	<b>T6</b>	...	...	10
	<b>T9</b>	4	...	...
<b>LOT7</b>	<b>T11</b>	5	...	10
	<b>T12</b>	4	...	...
	<b>T15</b>	...	...	10
<b>Pin Supu</b>	<b>T8*</b>	...	...	9
<b>LOT8</b>	<b>T6*</b>	9	...	10
	<b>FE-6</b>	10	...	7
<b>KSFR</b>	<b>ST-1</b>	...	...	10
	<b>ST-2</b>	...	...	11
	<b>ST-3</b>	10	...	...
	<b>ST-4</b>	6	...	...
<b>P1</b>	<b>T3</b>	6	10	...
	<b>T10</b>	...	8	...
	<b>PE-1</b>	8	10	...
<b>P2</b>	<b>T23</b>	6	12	...
	<b>T24</b>	6	5	...
<b>P3</b>	<b>PE-4</b>	5	...	...
	<b>PE-5</b>	3	...	...



**APPENDIX 3**

Linkage disequilibrium for 10 loci of *R. appendiculatus* inside the LKWS.

Population	Locus#1	Locus#2	P-Value	S.E.
LKWS	Ra2a	Ra6a	0.3135	0.03454
LKWS	Ra2a	Ra10a	0.030740*	0.007465
LKWS	Ra6a	Ra10a	0.59603	0.045816
LKWS	Ra2a	Ra11a	0.027010*	0.012202
LKWS	Ra6a	Ra11a	0.45262	0.047862
LKWS	Ra10a	Ra11a	0.000000*	0
LKWS	Ra2a	Ra3a	0.18912	0.029699
LKWS	Ra6a	Ra3a	0.41416	0.047878
LKWS	Ra10a	Ra3a	0.000000*	0
LKWS	Ra11a	Ra3a	0.000000*	0
LKWS	Ra2a	Ra7a	0.016380*	0.002856
LKWS	Ra6a	Ra7a	0.47896	0.033917
LKWS	Ra10a	Ra7a	0.000000*	0
LKWS	Ra11a	Ra7a	0.000000*	0
LKWS	Ra3a	Ra7a	0.000000*	0
LKWS	Ra2a	Ra9a	0.11087	0.022751
LKWS	Ra6a	Ra9a	0.64777	0.046041
LKWS	Ra10a	Ra9a	0.029730*	0.016992
LKWS	Ra11a	Ra9a	0.000000*	0
LKWS	Ra3a	Ra9a	0.000000*	0
LKWS	Ra7a	Ra9a	0.000000*	0
LKWS	Ra2a	Ra12a	0.2907	0.016099
LKWS	Ra6a	Ra12a	0.38954	0.037908
LKWS	Ra10a	Ra12a	0.004520*	0.001773
LKWS	Ra11a	Ra12a	0.000980*	0.00069
LKWS	Ra3a	Ra12a	0.032870*	0.012983
LKWS	Ra7a	Ra12a	0.000250*	0.000192
LKWS	Ra9a	Ra12a	0.000000*	0
LKWS	Ra2a	Ra4a	0.000000*	0
LKWS	Ra6a	Ra4a	0.33004	0.041234
LKWS	Ra10a	Ra4a	0.009390*	0.005359
LKWS	Ra11a	Ra4a	0.003910*	0.00391
LKWS	Ra3a	Ra4a	0.009650*	0.007348
LKWS	Ra7a	Ra4a	0.001390*	0.000648
LKWS	Ra9a	Ra4a	0.029450*	0.014318
LKWS	Ra12a	Ra4a	0.08969	0.013767
LKWS	Ra2a	Ra8a	0.007060*	0.001887
LKWS	Ra6a	Ra8a	0.11352	0.02339
LKWS	Ra10a	Ra8a	0.000810*	0.00081
LKWS	Ra11a	Ra8a	0.002050*	0.00205

*Appendices*

LKWS	Ra3a	Ra8a	0.020710*	0.009864
LKWS	Ra7a	Ra8a	0.000000*	0
LKWS	Ra9a	Ra8a	0.024820*	0.013252
LKWS	Ra12a	Ra8a	0.034730*	0.005662
LKWS	Ra4a	Ra8a	0.000010*	0.00001

**APPENDIX 4**

Fst values *H. megalonesa* inside P-1 and P-2

	T10	PE-2	PE-1	T23	T24
T3	0.0108	0.0276	0.0542	0.0523	0.0848
T10	n.s	0.073	999	0.0001	0.0605
PE-2	n.s	n.s	0.1241	0.0484	0.0843
PE-1	n.s	n.s	n.s	0.0698	0.1133
T23	n.s	n.s	n.s	n.s	999
T24	n.s	n.s	n.s	n.s	n.s

Appendices

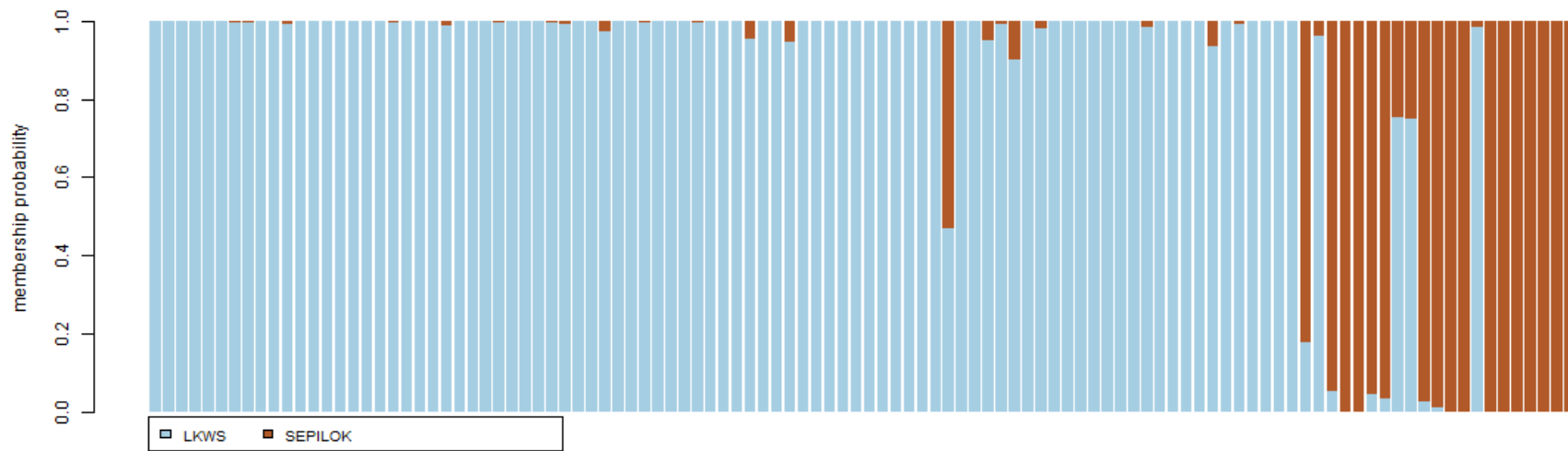
**APPENDIX 5**

F<sub>ST</sub> values between forested areas inside the LKWS for *H. megalonesa*

	Lot5	Lot6	Lot7
Lot8	0.0769	0.0544	0.0422
Lot5	n.s	0.0143	0.1175
Lot6	n.s	n.s	0.0691
Lot7	n.s	n.s	n.s

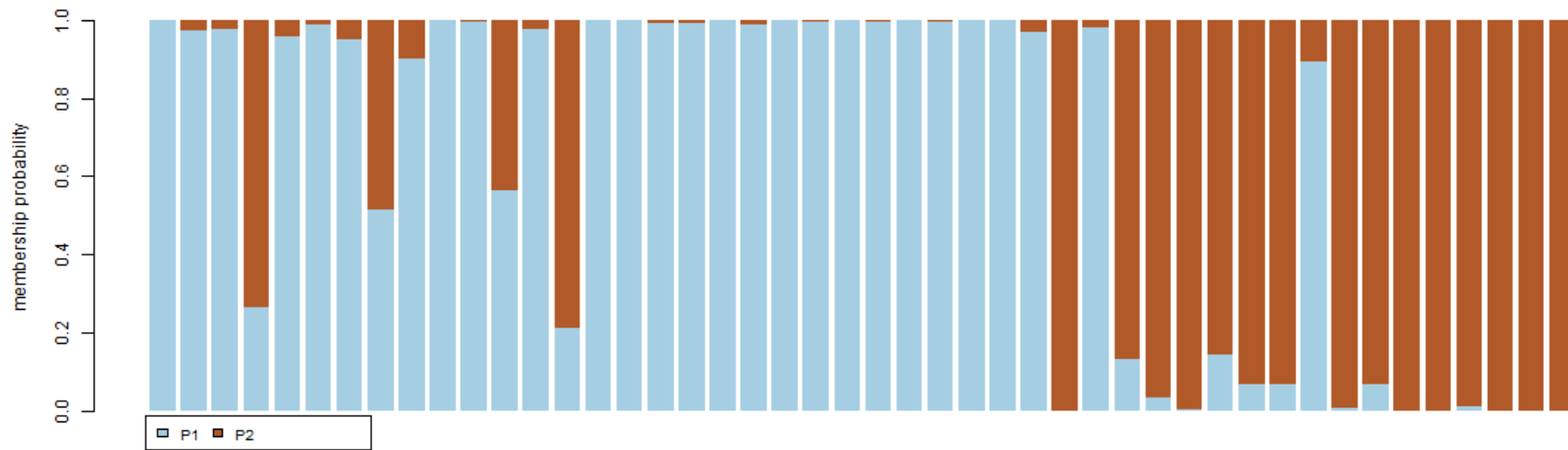
**APPENDIX 6**

DAPC membership probabilities based on the retained 30 PCA components for *R. appendiculatus* at the LKWS and KSFR.



**APPENDIX 7**

DAPC membership probabilities based on the retained 30 PCA components for *H. glandulosa* at P1 and P2 plantations.



Appendices

**APPENDIX 8**

Estimated migrant per generation (Nm) for *R. appendiculatus*

	PinSupu	Lot7	Lot6	Lot5	KSFR
Lot8	2.2	1.85	5.05	2.71	1.62
PinSupu	..	0.84	2.38	4.66	0.71
Lot7	..	..	4.65	1.48	6.27
Lot6	..	..	..	5.1	2.21
Lot5	..	..	..	..	1.14
KSFR	..	..	..	..	..

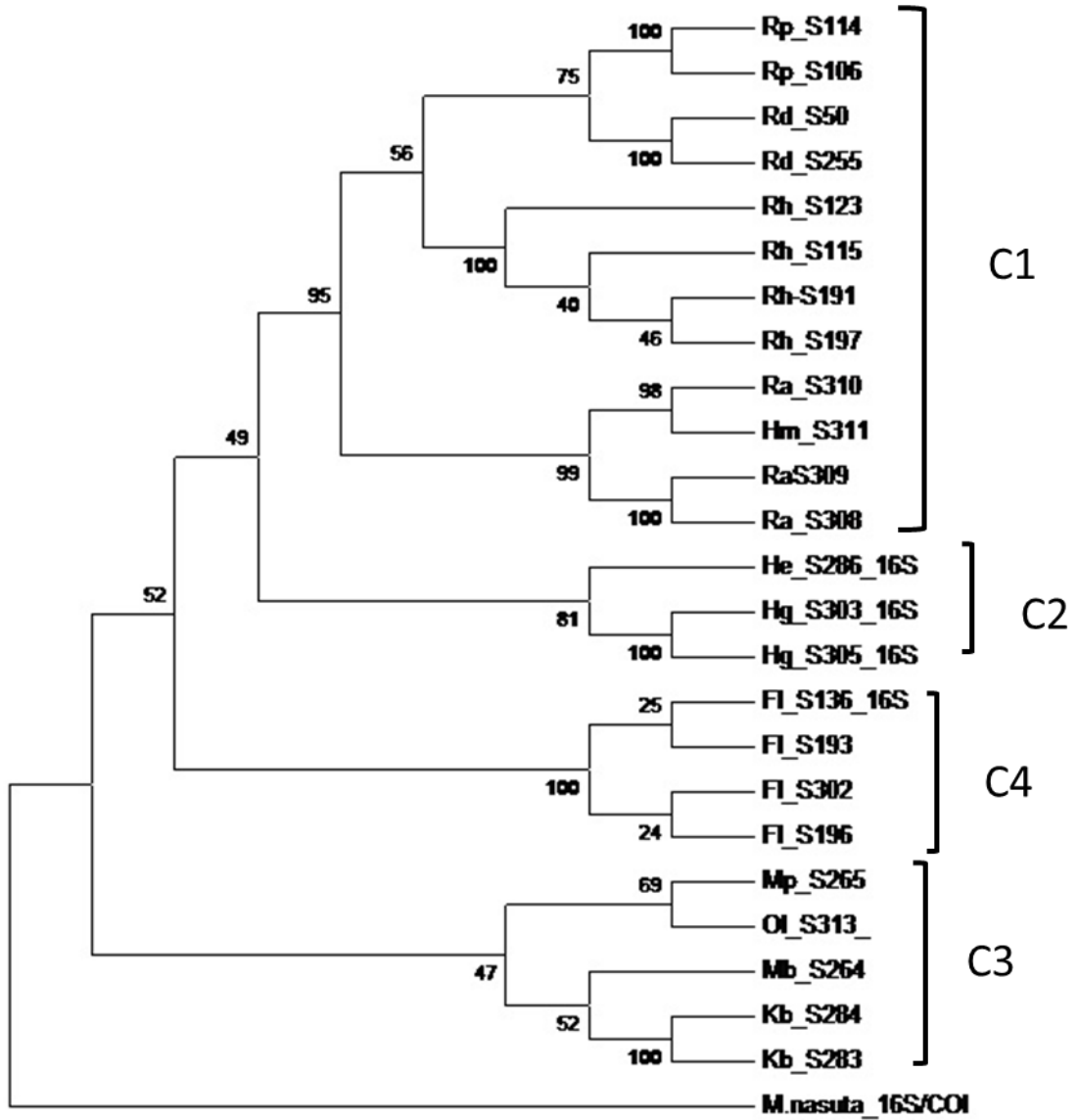
**Appendix 9**

Estimated migrant per generation (Nm) for *H. megalonesa* at plantation sites.

	P-3	P-1
P-2	4.31	4.42
P-3	...	7.33
P-1	...	...

**APPENDIX 10**

Maximum Likelihood tree of anuran samples produced using the concatenated dataset of both the 16S and CO1 gene fragments. Species first letters are presented adjacent to the samples. Clades (C1: Rhacophoridae, C2: Ranidae, C3: Microhylidae and C4: Dicroglossidae).



Appendices

**APPENDIX 11**

Shannon Index P values corresponding to different habitat types combination.

	diff	lwr	upr	P
Sec. Forest - P. Forest	-0.45714407	-1.04814206	0.1338539	0.1936689
Sec. Forest edg - P. Forest	-0.5490869	-1.22929333	0.1311195	0.1615323
Sec. Forest - Plantation	0.81577685	0.22477886	1.4067748	<b>0.0030602</b>
Sec. Forest edg - Plantation	0.72383402	0.04362759	1.4040404	<b>0.0324063</b>
Sec. Forest - Plantation edg	-0.27869028	-0.9242543	0.3668737	0.7254462
Sec. Forest edg - Plantation edg	-0.37063312	-1.09875059	0.3574844	0.5895562
Sec. Forest edg - Sec. Forest	-0.09194284	-0.61363735	0.4297517	0.9859569
Plantation - P. Forest	-1.27292092	-2.00762783 -	0.538214	<b>0.0001714</b>
Plantation edg - P. Forest	-0.17845379	-0.95772814	0.6008206	0.963394
Plantation edg - Plantation	1.09446713	0.31519278	1.8737415	<b>0.0025271</b>

**APPENDIX 12**

NMDS loadings and P values for habitat parameters at forested sites

	NMDS1	NMDS2	r <sub>2</sub>	P
Number of trees (NT)	-0.31843	-0.948	0.069	0.4
Mean tree circumference (MTC)	0.81485	0.5797	0.0598	0.447
Variance tree circumference (VTC)	0.78178	0.6236	0.0301	0.652
Number of logs (NL)	-0.29322	0.9561	0.0352	0.601
Mean log diameter (MLD)	0.86134	0.508	0.2435	<b>0.022</b>
Canopy cover (CC)	0.29071	0.9568	0.4006	<b>0.003</b>
Variance in Canopy Cover (VCC)	-0.47115	-0.8821	0.1047	0.227
Standard deviation Canopy Cover (SDCC)	-0.85042	-0.5261	0.1603	0.085
Understory vegetation density (UVD)	-0.70613	-0.7081	0.2611	<b>0.019</b>
Leaf litter (LL)	0.78106	0.6245	0.0555	0.465



**APPENDIX 13**

NMDS loadings and P values for habitat parameters across forest and plantation sites

	<b>NMDS1</b>	<b>NMDS2</b>	<b>r<sub>2</sub></b>	<b>P</b>
Number of trees (NT)	-0.73105	-0.68232	0.461	<b>0.001</b>
Mean tree circumference (MTC)	0.79046	0.61252	0.6857	<b>0.001</b>
Variance tree circumference (VTC)	-0.17656	-0.98429	0.0061	0.915
Number of logs (NL)	-0.9905	0.13749	0.1441	0.097
Mean log diameter (MLD)	-0.57097	-0.82097	0.6317	<b>0.001</b>
Canopy cover (CC)	-0.57541	0.81786	0.1251	0.127
Variance in Canopy Cover (VCC)	0.99627	-0.08627	0.174	0.054
Standard deviation Canopy Cover (SDCC)	0.96861	0.2486	0.0928	0.23
Understory vegetation density (UVD)	-0.80381	-0.59488	0.4787	<b>0.001</b>
Leaf litter (LL)	-0.71781	-0.69624	0.5846	<b>0.001</b>