MOLECULAR SYSTEMATICS AND GUT MICROBIOME OF ENDANGERED MALAYAN SIAMANG (*Symphalangus syndactylus)* IN MALAYSIA

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ABSTRACT

The siamang (*Symphalangus syndactylus*) is native to Peninsular Malaysia, Sumatra in Indonesia, and Southern Thailand. Morphologically, two subspecies have been proposed: *Symphalangus syndactylus syndactylus* (Sumatran population) and *Symphalangus syndactylus continentis* (Peninsular Malaysia population). However, the current publication identified siamang as single species due to a lack of study for subspecies distinction especially through the molecular approach. Microbial diversity studies have been recorded in major primate, yet Malaysia has a large deficit in research on primate gut microbiome and no study has been carried out on the gut microbiota of the Hylobatidae species, including *S. syndactylus*. Thus, this study aims to clarify the siamang subspecies' status based on mtDNA *D-loop* sequences and identify the gut microbiome composition of Malaysian *S. syndactylus*. There are 16 siamang fecal samples have been collected from different localities in Peninsular Malaysia. DNA sequence from *D-loop* locus with 600 bp size was amplified and analysed along with 35 GenBank sequences to resolve the taxonomic issue. Gut microbiome identification was conducted by targeting the 16s rRNA region through Next Generation Sequencing (NGS). Phylogenetic analysis results in two distinct clades formed by *S. s. syndactylus* and *S. s. continentis*, which supported by a 100% bootstrap value for NJ, 99% bootstrap value for both MP and ML and a 1.00 posterior probability value for BI analysis. Peninsular Malaysia's population diverge early (~1.92MYA) than the Sumatran population (~1.85MYA) through southward expansion. For gut microbiome analysis, a total of 1271030 operational taxonomic units (OTUs) reads were assigned to 110 families and 173 genera of microbes dominated by Lachnospiraceae family and genus *RFN20*. In conclusion, siamang from Peninsular Malaysia and Sumatra are different subspecies based on molecular approach and Malayan siamang has high diversity and abundance of gut microbiome composition with eleven pathogenic bacteria identified in Malayan siamang gut.

ABSTRAK

Siamang (*Symphalangus syndactylus*) bertaburan di Semenanjung Malaysia, Sumatra dan Selatan Thailand. Dari aspek morfologi, dua subspesies telah dikelaskan iaitu *Symphalangus syndactylus syndactylus* (Populasi Sumatra) dan *Symphalangus syndactylus continentis* (Populasi Semenanjung Malaysia). Walaubagaimanapun siamang telah diklasifikasikan sebagai satu spesies kerana kekurangan kajian untuk membezakan subspesies terutamanya melalui pendekatan molekul. Kajian kepelbagaian mikrob telah direkodkan dalam keluarga primat, namun terdapat jurang yang besar dalam penyelidikan mikrobiom usus primat di Malaysia dan belum wujud kajian dijalankan terhadap mikrobiom usus spesies Hylobatidae di Malaysia termasuklah *S. syndactylus*. Oleh itu, kajian ini bertujuan menentukan status subspesies siamang berdasarkan jujukan mtDNA D-*loop* dan mengenal pasti mikrobiom usus *S. syndactylus* Malaysia. Terdapat 16 sampel tinja siamang telah dikumpul daripada pelbagai lokasi di Semenanjung Malaysia. Jujukan DNA daripada lokus D-*loop* sepanjang 600-*bp* telah diamplifikasi dan dianalisis bersama 35 jujukan daripada GenBank bagi menyelesaikan isu taksonomi. Mikrobiom usus dikenalpasti dengan menyasarkan lokus 16sSrRNA melalui *Next Generation Sequencing* (NGS). Analisis filogeni menghasilkan dua klad berbeza iaitu klad *S. s. syndactylus* dan *S. s. continentis* yang disokong oleh 100% nilai bootstrap untuk NJ, 99% nilai bootstrap untuk kedua-dua MP dan ML dan 1.00 nilai kebarangkalian posterior untuk analisis BI. Populasi Semenanjung Malaysia mencapah awal (~1.92MYA) daripada populasi Sumatra (~1.85MYA) melalui arah selatan. Untuk analisis mikrob usus, sejumlah 1271030 bacaan unit taksonomi operasi (OTU) merangkumi 110 keluarga dan 173 genera mikrob didominasi keluarga Lachnospiraceae dan genus *RFN20*. Kesimpulannya, siamang Malaysia dan Sumatra merupakan subspesies yang berbeza melalui pendekatan molekul, dan siamang Malaysia mempunyai mikrobiom usus dengan kepelbagaian yang tinggi dengan 11 bakteria pathogen yang berpotensi telah dikenal pasti dalam usus siamang Malaysia.

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LIST OF SYMBOLS AND ABREVIATIONS

°C	-	Degree Celsius
%	-	Percentage
µg	-	Unit for micro gram
µl	-	Unit for micro per litre
µM	-	Unit for micro per molar concentration
kb	-	Unit for kilobyte
nm	-	Unit for nano meter
Gb	-	Unit for Gigabyte
bp	-	Basepair
ATP	-	Adenosine Tri-Phosphate
BI	-	Bayesian Inference
DNA	-	Deoxyribose Nucleotide Acid
NJ	-	Neighbour Joining
NHP	-	Non-Human Primate
ML	-	Maximum Likelihood
MP	-	Maximum Parsimony
OTU	-	Operational Taxonomical Unit
PCR	-	Polymerase Chain Reaction
PCoA	-	Principal coordinate analysis
PP	-	Posterior Probability
QC	-	Quality Control
qPCR	-	Quantitative Polymerase Chain Reaction (qPCR)
rbcL	-	Ribulose Bisphosphate Carboxylase Large
rRNA	-	ribosomal Ribonucleic Acid
UV	-	Ultra-violet
V	-	Volt

CHAPTER 1

INTRODUCTION

1.1 Background of study

Malaysia is one of the 17 mega-diverse countries (UNDP, 2018) and has at least 300 mammal species that represent 6.8% of mammals in the world (IUCN, 2014). More than 25 species of primates have been recorded in Malaysia (Roos *et al.*, 2014). Generally, the primate was divided into two suborders, Strepsirhini and Haplorhini. Strepsirrhini consists primarily of African and Asian lemurs, galagos and lorises, while Haplorhines are mainly composed of tarsiers and anthropoids (Perelman *et al*., 2011; Fleagle, 2013). Anthropoids further diverge, forming two groups named old world monkey (Cattarrhini) and new world monkey (Platyrrhini) (Grooves, 2001). Hominoidae is one of the families classified under old world monkeys represent lesser apes, gibbons and siamang of eastern Asia and the great ape, chimpanzees and gorillas of Africa and orang utan in southeast Asia (Grooves, 2001; Brandon-Jones *et al*., 2004).

In Malaysia, there are five species from the Hylobatidae family recorded which are *Symphalangus syndactylus* (siamang), *Hylobates lar* (White Handed gibbon), *Hylobates agilis* (Black Handed gibbon), *Hylobates funereus* (Eastern Grey gibbon) and *Hylobates abbotti* (Western Grey gibbon) (Md-Zain, 2019). Siamang identifies as endangered species on Union for Nature Conservation species (IUCN) Red List (IUCN, 2020) and listed in Appendix I by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (O'Brien *et al*., 2003). Among the Hylobatidae member, *S. syndactylus* is the largest species, have a very distinct sound duet with screams and deep booming sounds, and the pelage colour consists of only black hair rather than gibbon, which vary in pelage colours ranging from brown to black (Haimoff, 1981; Nongkaew *et al*., 2018). This species is widely distributed in Peninsular Malaysia and Sumatra, Indonesia (Brandon-Jones, 2004) and Southern Thailand (Thong -Aree, 2000). Thomas (1908) proposed two subspecies of siamang: *S. s. syndactylus* and *S. s. continentis* yet the subspecies classification has not been clarified through molecular study. Generally, siamang's diet comprises mostly of leaves and fruits depending on the season. The food availability may influence the compositions of siamang's gut microbiome as more bacteria that digest fiber are required when fiber-based food such as leaves were consumed the most by the siamang.

The gut microbiome is the collective genome of bacteria, archaea, and fungi in the intestine that can be both symbiotic and pathogenic to the host (Taneja, 2017). Microbial diversity studies have been recorded in major primate families, including Cercopithecidae (Cui *et al*., 2019) and Hylobatidae (Jia *et al*., 2018) fecal specimens. Previous studies have shown that many factors such as climate, diet, biogeography, behaviour, phylogeny and diseases have affected the composition and diversity of the gut microbiome in primates (Shokralla *et al.*, 2012; Amato *et al*., 2016; Taneja, 2017; Hale *et al*., 2019). Earlier time, two traditional analytical approaches have been commonly used in microbial study, which is culture-dependent and culture-independent, but the methods are not efficient to recognise the microbes at all level of taxa (Clayton *et al*., 2018). Metabarcoding techniques in Next Generation Sequencing (NGS) have provided new insights into microbiological research and the wide genome sequencing of primates (Amato *et al*., 2016, Malla *et al*., 2019). Hence, the gut microbiome component in siamang could be efficiently identified through microbial metabarcoding to monitor siamang’s health.

1.2 Problem statement

The current taxonomic classification of siamang has sixteen gap years, with the latest update published by Mootnick (2006) indicating that siamang subspecies postulation has been abandoned for more than a decade. This is contributed by flux genetic data gained from extensive primate studies in Malaysia compared to ecological and behavioural data (Abdul-Latiff *et al*., 2014). Besides, prior taxonomic studies focus on *Symphalangus* at the genus level to represent siamang in resolving the complicated phylogenetic relations between Hylobatidae genera. *Symphalangus* is recognised as a single species (Raffles, 1826; Thomas, 1908; Hooijer, 1960; Frisch, 1967; Grooves, 2001; Brandon-Jones, 2004, Thinh *et al*., 2010; Roos *et al*., 2016), yet the debate over the number of subspecies exists within *S. syndactylus* is inconclusive whether *S. s. syndactylus* from Sumatra and *S. s. continentis* from Peninsular Malaysia should be declared as different subspecies. The naming was solely made by relying on the morphology of siamang (Thomas, 1908; Hooijer, 1960; Frisch, 1967; Mootnick, 2006), unable to validate subspecies issues since physical appearance between individuals across generations was varied. Since no sample from Malaysia was involved in earlier molecular research, none of the siamang molecular studies to determine the existence of two separate subspecies have been conducted. There are still no comprehensive studies on the taxonomy and phylogenetic relationship at the subspecies and population level of *S. syndactylus*, especially in Malaysia. Thus, the ambiguity of subspecies and phylogenetic relationships among populations of siamang in Malaysia will be resolved in this study.

Current studies on primate gut microbiome seem likely to be conducted on the Colobinae family since most captive Colobinae species are suffering from mortality and morbidity caused by gastrointestinal distress (Amato *et al*., 2016). A wide gap in research centres on primate gut microbiome in Malaysia especially throughout molecular based method was found as compared to other countries. Besides, Next-generation sequencing (NGS) technologies have provided billions of sequence readings of microorganisms in various environments across different level of taxa (Rees *et al*., 2014). The metabarcoding method could diagnose animal health by assessing the gut microbiome. The study adopting 16S rRNA as a marker for gut microbiome detection in the Hylobatidae family has involved *Nomascus leucogenys* (Jia *et al*., 2018) with absence data available for other genera. While in Malaysia, gut microbiome studies on proboscis monkeys (*Nasalis larvatus*) have been conducted that inferred 16S rRNA as a gene marker (Hayakawa *et al*., 2018). Nevertheless, the precise composition, structure, and role of the gastrointestinal microbiota in the Hylobatidae family remain unclear until now (Jia *et al*., 2018). No study has yet been carried out on the gut microbiota of the Hylobatidae in Malaysia exclusively for siamang. Since microbial discovery is essential to understanding host digestion and immune system (Taneja *et al*., 2017), thus it is important to distinguish the microbes community which can affect the host health of siamang.

1.3 Objectives study

The study aims to:

a) To evaluate the phylogenetic relationship of *S. syndactylus* in Malaysia using the D-*loop* region of mitochondrial DNA (mtDNA).
b) To characterize the gastrointestinal microbiome diversity of *S. syndactylus* from fecal samples using the 16S rRNA region.

1.4 Scope of study

The study concentrates on a few research scopes in order to achieve the two objectives that have been decided. Firstly, the research emphasises the phylogenetic relationship of siamang at the subspecies level. The fecal samples of siamang from different locations (Genting Highland and Fraser Hill) were collected from two different group named Ramses represent habituated siamang and Socrates represents semi-wild siamang groups. Fecal samples of *H. lar* also have been collected from Johor and Zoo Melaka. Two populations of siamang involved in this study represents Peninsular Malaysia siamang and Sumatran siamang. Sample collections were conducted on December 2019, July 2020 and October 2020. Observation of siamang has been conducted 12 hours per day in order to collect fecal samples which the sampling conducted twice in a month. The samples were extracted using QIAmp Fast DNA Stool Mini Kit following the manufacturer's protocol. Amplification of the targeted D-*loop* region from extracted DNA was conducted through Polymerase Chain Reaction (PCR). 35 sequences were retrieved from Genbank representing Sumatran population and other Hylobatidae members for phylogenetic analysis. The phylogenetic analysis includes reconstruction NJ, ML, MP, BI tree, molecular clock estimation tree and minimum spanning network analysis. High throughput sequencing through NGS method was conducted to obtain 16S rRNA sequences. The analysis involves two levels, DNA sequencing followed by a phylogenetic evaluation to clarify the siamang subspecies status. Then, characterisation of siamang gut microbiome data was analysed using CLC Genomic Workbench software.

1.5 Significance of study

Data extracted from this research will be advantageous for conserving *S. syndactylus* in Malaysia by updating the number of siamang population in Malaysia. Besides, by presenting clear taxonomic nomenclature, this information will be useful in improving the systematic classification of primates in Malaysia. This finding is also important to conservation management, such as during siamang translocation, as subspecies distinction is vital for conserving genetic diversity. Furthermore, the molecular data aids in investigations into illegal pet trade and forensics involving Malayan and Sumatran siamangs. Next, a microbial study in the gut microbiome positively enhances the understanding of siamang health by screening the gut microbiome using the metabarcoding technique. This data will be valuable in the management of *ex-situ* conservation in terms of monitoring the growth and health of siamang, which this method is also applicable to other primate in Malaysia. The data contributes to the computation of more microbe data to prevent and detect any microbes that may cause zoonotic disease in primates. Holistically, the result obtained from this research will be intuitive about this species and becomes the earliest data record that may be advantageous the future reference.

CHAPTER 2

LITERATURE REVIEW

2.1 Primate

According to Ankel-Simons (2010), the terms that delineate the order primate is still not rigid since features that describe primate are not exclusively refer to this order. The definition of primate rather depends on the common combination of traits which also can be found in other mammalian orders. Mayor (1990) is the first researcher who defines primate as placental mammals having orbits encircled with bone; clavicle and flat nails on at least some digits; brain with posterior lobe and culcurine sulsus; large caecum; penis pendulous and scrotal testes (male); two pectoral mammae (female). The cheek teeth appear to be plain and low crowned, usually with surface wrinkle development and upper molar hypocones (Hunter & Jenvel, 1995). Primate are distinct from other mammals through their social groups and diet preferences. Most primates consume a variety of plant species, and they are responsible for dispersal agents (Abdul-Latiff, 2012).

This order has been captivated by its own group diversity compared to other mammals since primate have 61 extant genera with 252 species containing 405 species (Ankel-Simons, 2010) which exploration in future taxonomic studies may reveal more unidentified primate members. The only primates that occupy all seven continents of the planet are human beings, while non-human primates exist on the continents of South America, Africa and Asia across different circumstances ranging from hot, dry savannahs to lush tropical rainforests (Munir, 2018). In Asia, currently, there are 119 species of non-human primates distributed in 22 countries, and 133 of the species have been assessed to be identified as Critically Endangered (17 species). The earliest taxonomist such as Kay & Williams (1994), claimed primates merge into two suborders known as Prossimii (the prosimians or are monkeys) and Anthropoidea (anthropoid, Old and New World monkey). Current classification divides primates into two suborders which are Strepsirhini and Haplorhini (Grooves, 2001; Roos, 2016). Strepsirrhini is composed of main prosimians, including lemurs, galagos and lorises of Africa and Asia, while Haplorhines are composed of tarsiers and anthropoids (*et al*., 2011; Fleagle, 2013 Perelman).

Simiiformes is one of the infraorder classified under Haplorhini and is commonly known as the group of anthropoids (Grooves, 2001). The terms of Anthropoidae proposed by Mizart (1864) are widely used by the researcher who has been against Hoffstetter (1974), who strictly suggest Simiiformes suit the best to portray anthropoids as previous term rules of nomenclature do not apply beyond the family group level. Generally, members of Simiiformess are stipulated as diurnal primates and also claimed as cathemeral animals (Curtis & Rasmussen, 2006; Ankel-Simon & Ramussen, 2008). The infraorder further separates, forming two parvorders which are Catarrhini (Old World monkeys, apes, and humans) and Platyrrhini (New World monkeys), Grooves (2001). The family of Catarrhini represent by Pitheciidae, Atelidae, Aotidae, Cebidae, and Callitrichidae. Two superfamilies are included under Old World monkey, which is Cercopithecoidea and Hominoidea (Pozzi *et al*., 2014). The Hominoid apes include the lesser apes, gibbons and siamang of eastern Asia and the great ape, chimpanzees and gorillas of Africa and orang utan southeast Asia (Grooves, 2001; Brandon-Jones, 2004).

Hominoidae is distinguished from other primates by its greater freedom of movement in the growing of shoulder joints under the influence of brackets (Grehan, 2006). In general usage, the term ape does not include humans and therefore it is not equivalent to the Hominoidea taxon. The term ape precisely refers to all members of the Hominoidea superfamily except human beings (Dixson, 1983). There are two branches that still exist in the Hominoidea superfamily, namely gibbon (small ape) and hominids (large ape). In line with the development of zoology, it is clear that the absence of tails is found in several different species. Besides, the most distinct features among Hominoidae members are body posture and locomotion strategies. As shown in Figure 2.1, there was a difference in the body size and features for five genera under the Hominoidea superfamily.

Usually, apes cannot stand upright, and Pickford (2006) claimed the ape was fully bipedal due to its femora structure. Chimpanzees, gorillas and orang utan are semi-orthograde, and they are limited to standing in upright positions compared to humans, while smaller apes tend to be pronograde. All Hominoids except humans are quadrumanous where the feet were constructed along the line of hands which benefits in arboreal locomotion such as grasping (Thorpe & Crompton, 2006). Roos (2016) noted Hominoidea superfamily diverges into two families categorised as Hylobatidae (small apes) and Hominidae (great apes and humans). Hominoidea further splits into four genera comprising of Pongo (orang utan), Gorilla (gorilla), Homo (human) and Pan (chimpanzee). Hylobatidae includes four genera named *Nomascus* (Crested gibbon), *Hoolock* (White-Browed gibbon), *Hylobates* (Hylobates gibbon) and *Symphalangus* (siamang) (Grooves 2001).

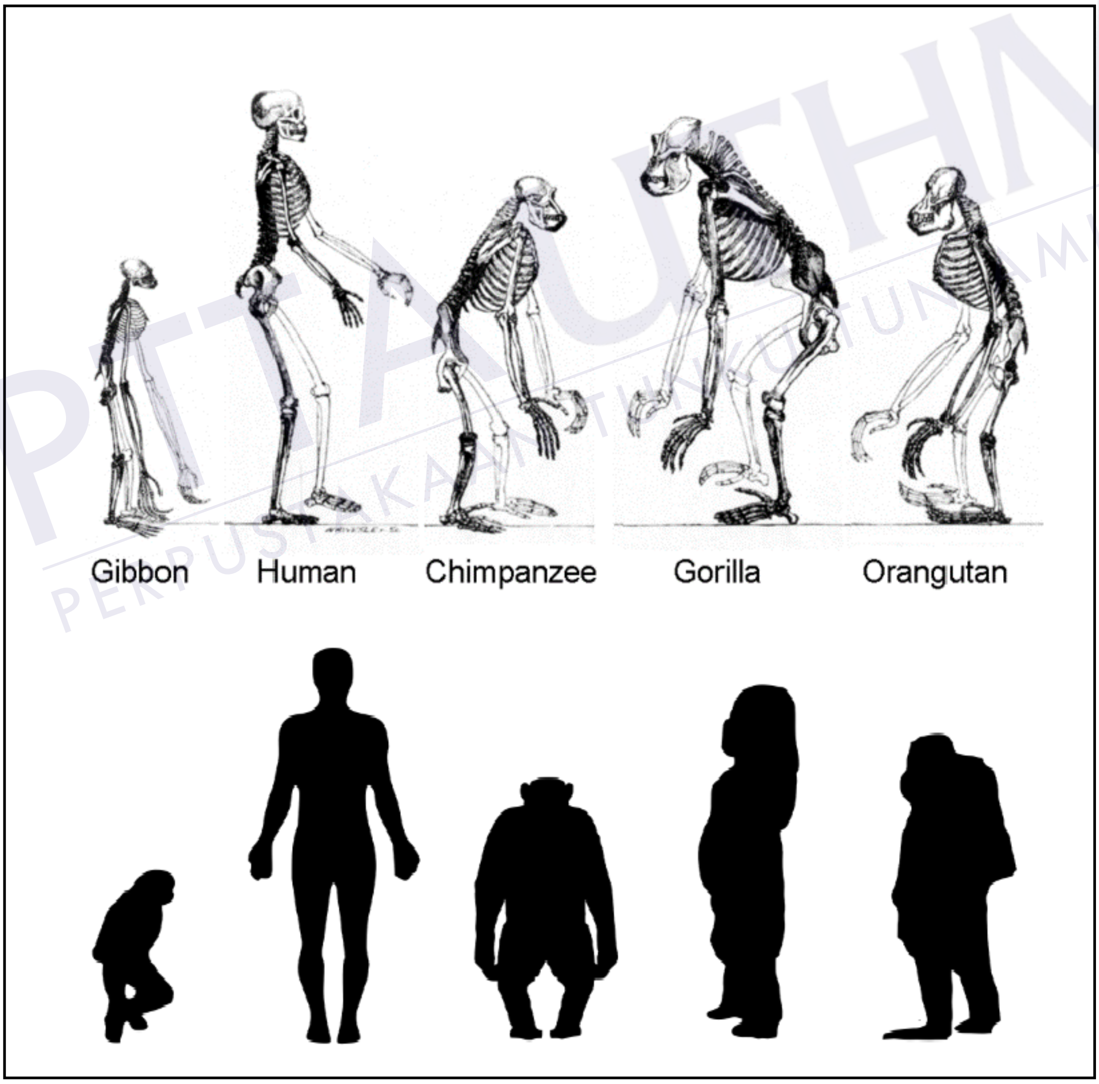

Figure 2.1: Comparison of body posture of gibbon, human, chimpanzee, gorilla and orang utan (from left) (TimVickers, 2007).

2.2 Hylobatidae

Hylobatidae is one of the primate families that consists of a small apes known as gibbon and siamang (Roos, 2016). This family comprises more than 14 species making the family the most diverse group of extant apes (Mootnick, 2006; Thinh *et al*., 2010). Among large-bodied apes, this family have the lightest weight varying from 5.3-11.9 kg (Smith & Jungers, 1997; Nijman & Menken, 2005), whereas the weight of larges apes are usually from 30 kg on average to more than 200 kg (Fleagle, 2013). Hylobatids is the first ape that branch out from Hominoid that diverges from the old world monkey (Fleagle, 2013). This family clade is the sole branch of hominoid evolution that originated in Asia with unknown or suspected conjunction from Europe and the Middle East (Reichard *et al*., 2016). Hylobatidae can be easily distinguished from other Haplorhine as the most significant differences are body size, locomotion strategies, social organisation and the unique way of communication called duet (Reichard *et al*., 2016).

This family incorporate synapomorphic feature with Hominoids, including having a large thorax, dorsally positioned scapulae, long clavicles, long forelimbs, a reduced lumbar area, and a greater number of sacral vertebrae compared to monkeys and an absence of tail (Fleagle *et al*., 1976; Geissmann, 2003). Both female and male individuals have similar body weights but are diverse in colouration (Reichard *et al*., 2016). Most of the gibbon species portray different colours for the opposite sex, such as lar gibbon; the colour may vary from brownish-black or buff except for certain species (siamang and Javan gibbon) where both sexes pose the same pelage colour (Bartlett, 2011). The groups vary in pelage colour depending on the species and age group and may also be affected by environmental factors such as pregnancy, malnutrition, nursing, staining on light pelage (from urine or sweats) or hair bleaching in dark pelage and exposure to sunlight (Mootnick, 2006).

Hylobatids are presumed to distributed around 10.5 million years ago and radiated southwards where they started to diverge from reputed gibbon ancestors originated in Eastern Indochina recently after speciation occurred (Chatterjee, 2006). These events occurred in conjunction with substantial environmental changes, which poses concerns about the tolerance of these family members. Even though fossil record for hylobatids was scarce to explain this phenomenon, previous historical data supported the evolution of gibbon and siamang, which are adaptable to environmental changes that enable them to modify their spatial distribution (Jablonski & Chaplin 2009). Recent record portrays Hylobatidae members as endemic to Southeast Asia ranging from southern China, north eastern India and Indonesia, including Borneo, Sumatra and Java (Figure 2.2) (Roos, 2016). *Nomascus* is found throughout southern China, Laos, Vietnam, and parts of Cambodia, while *Hoolock* invades southern China, India, eastern Bangladesh and Myanmar. *Symphalangus* strictly exists in Peninsular Malaysia and Sumatra, Indonesia, and lives sympatrically with either *H. agilis* or *H. lar*, while other genera remain allopatric (Barlett & Light, 2016).

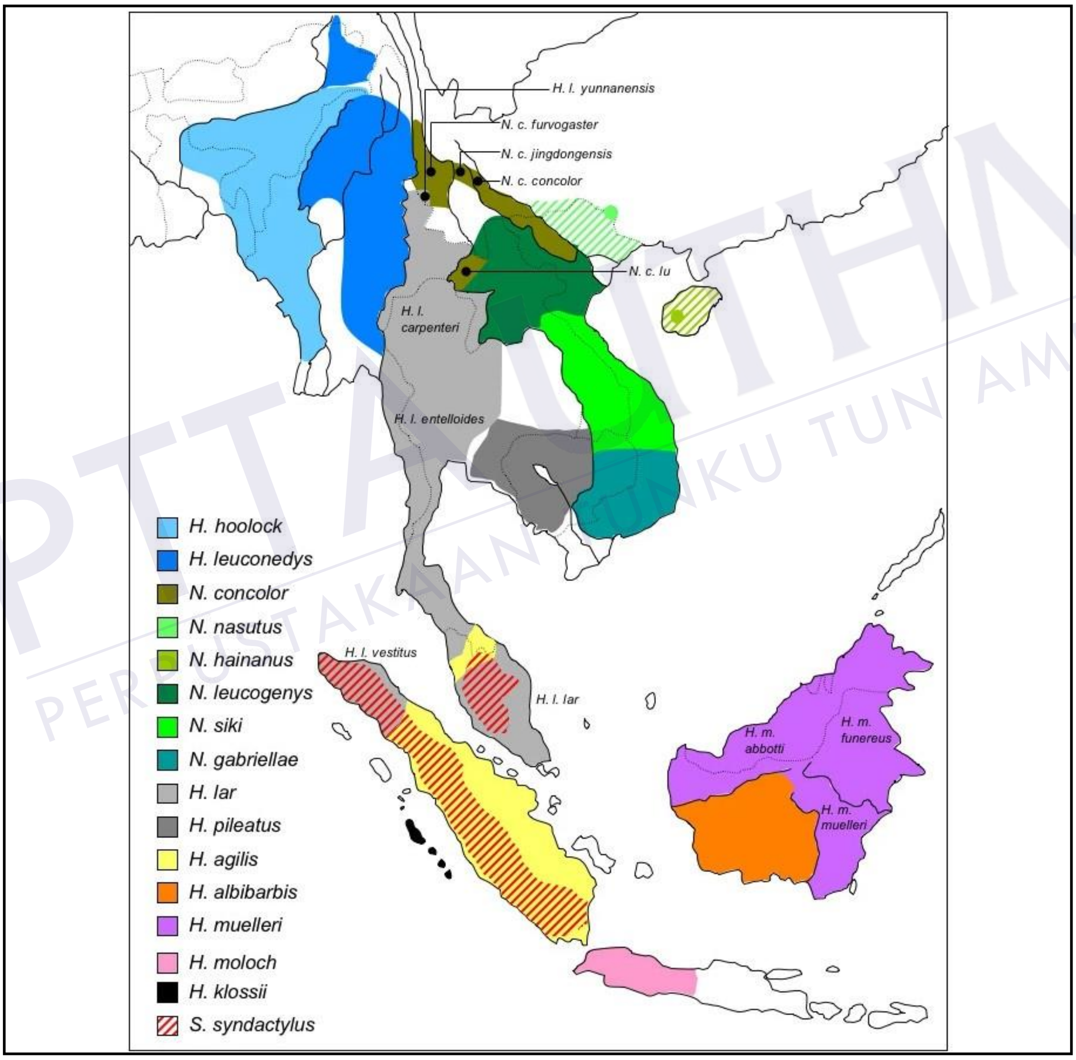

Figure 2.2: Distribution of Hylobatidae members (Thinh *et al.*, 2010)

Pioneer studies suggested vary number of genera in the Hylobatidae family according to different authors (Table 2.1). Currently, there are four genera classified under

Hylobatidae, represented by the genus *Nomascus, Hoolock, Hylobates* and *Symphalangus* represented by various species (Table 2.2). The latest classification was made based on genetic analysis, and the major distinction was found in their diploid chromosome number (*Nomascus*: 2n = 52, *Hoolock*: 2n = 38, *Hylobates*: 2n = 44, *Symphalangus*: 2n = 50) as studied by few researchers (Prouty *et al*., 1983; Liu *et al*., 1987; Wienberg & Stanyon, 1987; Müller *et al*., 2003; Stanyon, 2013). Not only molecular evidence that separates these four genera, but also supported by behavioural data (Marshall & Sugardjito, 1986; Geissmann, 2002) and morphological inference (Groves, 2001; Creel *et al*., 1984). Figure 2.3 shows the illustration for Hylobatidae members. In the taxonomy aspect, it will be difficult for the researcher to identify gibbon species through vocalisation since the vocals are almost indistinguishable and inconsistent. Complex taxonomic classification and phylogenetic relationships have been experienced within gibbon species as hybridisation occurs (Marshall & Sugardjito, 1986; Matsudaira *et al*., 2013), and several radiations split them accordingly (Chan *et al*., 2010; Matsudaira & Ishida 2010; Thinh *et al*., 2010; Israfil *et al*., 2011). This is supported by the fact that genetic studies estimating the divergence period between gibbon species date this radiation to a narrow time gap between 4 and 2 million years ago (Thinh *et al*., 2010; Israfil *et al*., 2011). Subspecies identification is much more challenging as taxonomists create their own terms to define species traits (Mootnick & Grooves, 2005).

The inconvenience methods for gibbon identification at a lower taxonomic level caused an accumulation of studies focusing on the number, name and phylogeny of the genera. In these four genera, 20 species of small Asian apes were recorded (Reichard *et al*., 2016). Between four genera, only *Symphalangus* has only one species. *S. syndactylus* is the only species representing the genus and is known as the largest gibbon (Groves, 2001; Geissmann, 2002, 2007; Thinh *et al*., 2010; Anandam *et al*., 2013). Even though some researchers have made the list of morphological differences among two populations between Sumatra and Peninsular Malaysia and suggested new subspecies (Thomas, 1908; Frsich 1976; Groove 2001; Mootnick, 2006) but lacking molecular proof makes it difficult to reclarify the taxonomic position. Further study is required to clarify the inquiries on separate populations that exist in Sumatra and Malaysian peninsulas as different subspecies.

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