PHYLOGENY AND PREVALENCE OF FILARIAL PARASITES (NEMATODA: ONCHOCERCIDAE) FROM THE COMMON TREESHREW **(*Tupaia glis*)** IN PENINSULAR MALAYSIA

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PHYLOGENY AND PREVALENCE OF FILARIAL PARASITES (NEMATODA: ONCHOCERCIDAE) FROM THE COMMON TREESHREW (*Tupaia glis*) IN PENINSULAR MALAYSIA

ABSTRACT

Filarial nematodes cause lymphatic filariosis and zoonotic diseases in humans. However, the origins and evolution of these group of parasites is still unclear because the ancestral species has not yet been discovered. This study reviews the taxonomic and phylogenetic relationship of filarial parasites from common treeshrews, *Tupaia glis*. The study also intends to determine the prevalence of filarial parasites in relation to the distribution of hosts in Peninsular Malaysia. Cage trapping techniques were used to capture host animals in selected sites throughout Peninsular Malaysia. A total of 98 common treeshrews were captured and examined. Two adult species of filarial parasites, *Malayfilaria sofiani* and *Mansonella dunni* were identified, and data on their distribution and prevalence was recorded. The parasites were subjected to details morphological and molecular analyses. Polymerase chain reaction (PCR) was performed using *cox*1 and 12S rRNA genes. Phylogenetic analysis indicated that *M. dunni* have a close affinity with *Mansonella ozzardi* which is a human parasite in South America, but their genetic distance was substantially large (p-distance 6.1-6.4%). *M. sofiani* appears to be closely related to *Wuchereria* spp. and *Brugia* spp. but differs based on several morphological characteristics. The Kimura 2-parameter distance between the *cox*1 gene sequences of *M. sofiani* and *W. bancrofti* was 11.8%. Molecular analyses indicated that *M. sofiani* differs from both *W. bancrofti* and *Brugia* spp. at the genus level. The total percentage of prevalence was 31% with 30 individuals of common treeshrews were infected. *M. sofiani* adult is only restricted to secondary forest of Jeram Pasu, Kelantan with 6.1% prevalence whereas *M. dunni* have a 23.4% prevalence and widely distributed in primary and secondary forest of Peninsular Malaysia. No adult *Brugia tupaiae* was found in this study, however one common treeshews from Gemas, Negeri Sembilan was infected with microfilaria of *Brugia tupaiae*. This study concluded that *Malayfilaria sofiani* appears to be a new genus and new species. while *Mansonella dunni* was closely related to human parasite, *M. ozzardi* and *M. perstans.* Only one common treeshews from Gemas, Negeri Sembilan was infected with *B. tupaiae*. Contrary to previous studies, there is a significant decrease in prevalence of *B. tupaiae.* Fortunately, there was no filariasis cases associated with filarial parasites of common treeshrews was reported recently. Filarial parasites are one of the agents of emerging zoonotic diseases. This study serves as a guide for medical

practitioners and other authority to pinpoint the distribution of treeshrews filarial parasites. The present study provides molecular and morphological data that can be used to identify the filarial parasites, if zoonotic cases involving human occurs.

Keywords: Filarial nematodes, Molecular identification, Onchocercidae, Scandentia

FILOGENI DAN PREVALEN PARASIT FILARIA (NEMATODA: ONCHOCERCIDAE) DARI TUPAI MUNCONG BESAR (*Tupaia glis*) DI SEMENANJUNG MALAYSIA

ABSTRAK

Filarial nematod menyebabkan filariosis limfatik dan penyakit zoonotik pada manusia. Walau bagaimanapun, asal dan evolusi kumpulan parasit ini masih tidak jelas kerana spesies leluhur belum dikenalpasti. Kajian ini bertujuan untuk mengkaji hubungan taksonomi dan filogenetik parasit filarial dari tupai muncong besar, *Tupaia glis*. Kajian ini juga bertujuan untuk menentukan kebarangkalian parasit filarial berhubung kait taburan haiwan perumah di Semenanjung Malaysia. Teknik penangkapan menggunakan sangkar digunakan untuk menangkap haiwan perumah di tapak kajian terpilih di seluruh Semenanjung Malaysia. Seramai 98 ekor tupai muncong besar ditangkap dan dikaji. Dua spesies parasit filarial, *Malayfilaria sofiani* dan *Mansonella dunni* telah dikenal pasti dan data mengenai taburan dan prevalennya telah direkodkan. Analisis morfologi dan molekul yang terperinci telah dijalankan ke atas parasit. Tindakbalas Rantaian Polimerase (PCR) telah dilakukan dengan menggunakan gen *cox*1 dan 12S rRNA. Analisis filogenetik menunjukkan bahawa *M. dunni* mempunyai hubungan rapat dengan *Mansonella ozzardi* yang merupakan parasit manusia di Amerika Selatan tetapi jarak genetik mereka adalah besar (p-distance 6.1-6.4%). *M. sofiani* nampaknya berkait rapat dengan *Wuchereria* spp. dan *Brugia* spp. tetapi berbeza berdasarkan beberapa ciri morfologi. Jarak Kimura 2-parameter antara urutan *cox*1 gen *M. sofiani* dan *W. bancrofti* adalah 11.8%. Analisis molekul menunjukkan bahawa *M. sofiani* berbeza daripada kedua-dua *W. bancrofti* dan *Brugia* spp. di peringkat genus. Jumlah peratusan prevalen adalah 31% dengan 30 individu tupai muncong besar dijangkiti. Cacing dewasa *M. sofiani* hanya terhad kepada hutan sekunder di Jeram Pasu, Kelantan dengan prevalen 6.1% manakala *M. dunni* mempunyai prevalen 23.4% dan bertaburan secara meluas di hutan primer dan sekunder di Semenanjung Malaysia. Tiada *Brugia tupaiae* dewasa ditemui dalam kajian ini, namun satu individu tupai muncong besar dari Gemas, Negeri Sembilan telah dijangkiti oleh mikrofilaria *Brugia tupaiae*. Kajian ini menyimpulkan bahawa *Malayfilaria sofiani* merupakan genus baru dan spesies baru. manakala *Mansonella dunni* berkait rapat dengan parasit manusia, *M. ozzardi* dan *M. perstans*. Hanya satu individu tupai muncong besar dari Gemas, Negeri Sembilan yang dijangkiti *B. tupaiae*. Berbanding dengan kajian terdahulu, terdapat penurunan ketara prevalen untuk *B. tupaiae*. Tiada kes filariasis yang berkaitan dengan parasit filarial daripada tupai muncong

besar dilaporkan baru-baru ini. Filarial parasit adalah salah satu daripada agen penyakit zoonotik yang muncul. Kajian ini berfungsi sebagai panduan untuk pengamal perubatan dan pihak berkuasa lain untuk menentukan taburan parasit filarial tupai muncong besar. Kajian ini menyediakan data molekul dan morfologi yang boleh digunakan untuk mengenal pasti parasit filarial, sekiranya kes zoonotik melibatkan manusia berlaku.

Kata kunci: Filarial nematod, Identifikasi molekular, Onchocercidae, Scandentia

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

℃	:	Degree Celsius
µm	:	Micrometer
mm	:	Millimeter
%	:	Percentage
PBS	:	Phosphate-buffered saline

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Filarioid nematodes are endoparasites with a predilection for the tissue of terrestrial vertebrates, including humans. Filarioids belong to the superfamily Filarioidea, which is further divided into two families, i.e Filariidae and Onchocercidae. Filarioids from the family Onchocercidae has a specialised life cycle with infective larvae in the arthropods (in the form of microfilariae) which can be found in their definitive host. This stage of their life cycle is an essential factor leading to the transmission of adult filarioids throughout their definitive host's organ systems and tissues (Anderson, 2000). Larvae were transmitted strictly by a vector, haematophagous arthropods.

Several filarioid species from the subfamily Onchocercinae are agents of parasitic diseases (Morales-Hojas, 2009). Lymphatic filarioisis which can lead to a permanent disability in humans, is caused by *Brugia malayi*, *B. timori* and *Wuchereria bancrofti* (Al-Abd et al., 2014). On the other hand, *Onchocerca volvulus* has become the primary agent of onchocercosis, which at most can cause blindness. Furthermore, some species from genus *Mansonella* is an agent of mansonellosis (Bain, 2015) even though the disease is usually asymptomatic. In cases of *Mansonella perstans* infection, it can induce severe clinical features such as angioedema, swelling like 'Calabar swellings', pruritus, fever, headache and pain in bursae and/ joint (Bregani et al., 2006).

Apart from that, it is also an agent of an emerging infectious disease called zoonotic filariosis. Zoonotic filariosis is a medical case of human infection with filarioids from domestic and wild animals (Orihel, 1998). In Malaysia, zoonotic cases involving *B. pahangi* was recorded in 2010 and 2011 from a rural village in Selangor and suburban Kuala Lumpur (Tan et al., 2011; Muslim et al., 2013a; Muslim et al., 2013b). Human dirofilariosis caused by *Dirofilaria repens* was recorded in Kuching, Sarawak (Rohela et al., 2009).

Both medically important genera *Brugia* and *Mansonella* have been found from common treeshrews (Orihel, 1966; Mullin & Orihel, 1972). The other reasons why common treeshrew was chosen as the host and experimental animal for this research are due to its vast geographic range, diverse habitats (including near human settlement) and their close affinity to primates.

Internationally, the study on the filarioids focuses more on the group of species that cause direct diseases and having zoonoses potential to humans (Ta- Tang et al., 2018; Ta-Tang et al., 2021). Nevertheless, they are a group of researchers who studied the phylogeny of filarioids (Uni et al., 2020). Unfortunately, due to its parasitic nature and soft-bodied physiology, there is no fossilized sample. Hence, the study of their phylogeny and evolutionary history has been limited to only morphology and molecular techniques. Lack of biological samples hampered the progress of delineating the phylogeny of the filarioids, especially the subfamily Onchocercinae (Lefoulon et al., 2015).

Surprisingly, the genus that could cause the disease to humans have been recorded in Malaysia. However, despite its fundamental importance in medical and public health, this group of nematodes were poorly sampled and studied in this country.

This study is an attempt to fill in the gap of knowledge regarding filarioid nematodes in Peninsular Malaysia. A major part of this study focusing on filarioids parasitic on a single host species, common treeshrew (*Tupaia glis*). The phylogeny of filarial parasites parasitic on common treeshrews and other findings such as new species, new locality and prevalence was discussed. This study also generally reviewed the whole diversity of filarioids in Peninsular Malaysia.

Prevalence study on this filarioids focusing on identifying "refugium" of filarioids was done by conducting sampling on different habitats (primary forest, secondary forest and sub-urban) throughout Peninsular Malaysia. In addition, a closer look at the taxonomic relationship between filarioids of common treeshrews and other Onchocercinae was conducted by using integrated taxonomy methods (Ferri et al., 2009).

1.2 Objectives of research

This study focuses on the prevalence and systematics of filarioids from common treeshrews (*Tupaia glis*) while also looking at the diversity of filarioids in Peninsular Malaysia. Mainly this study intends,

a. To assess the taxonomic relationship within subfamily Onchocercinae, emphasising species parasitise on common treeshrews using morphological and molecular data.

b. To determine the prevalence of filarial parasites in relation to the distribution of host, common treeshrews (*Tupaia glis*) from different habitats in Peninsular Malaysia.

1.3 Research questions

a. Is current taxonomic classification using morphological characteristics of Onchocercidae in congruence with the molecular characters?

b. Are filarioids from common treeshrews more prevalent in certain types of habitats?

CHAPTER 2: LITERATURE REVIEW

2.1 Filarial parasites

Anderson (1976) in CIH Keys to the Nematode Parasite of the Vertebrate depicted filarial parasites as endoparasites found in tissues surface and between tissue spaces of all classes of terrestrial vertebrates other than fishes. On the other perspective, Yen (1983) define a filarial parasite as 'thread-like arthropods transmitted parasites of various tissues and organs in vertebrates from lower-level amphibians upwards.

Filarial parasites, also known as filarioids are unique as it is fully dependent on haematophagous arthropods as vector to transmit their larvae from one host to another and further complete their life cycle. It also has been distinguished from other nematodes due to a unique stage in their life cycle (Figure 2.1), the pre-larval microfilariae stage.

Demarquay (1863) first scientifically documented microfilariae that were extracted from hydrocoele fluid of a man who originated from Havana, Cuba. Later, Manson (1878) managed to develop the larvae inside a female mosquito. However, he was wrongly concluded that the mode of transmission of the larvae to the human host was through the consumption of water infected by the parasite's larvae. It was not until 1900, that Low had suggested the correct transmission of larvae by blood meal.

The first adult filarial parasites documented throughout history was in 1877 when Bancroft recovered an adult female worm from a patient in Australia and initially was described as *Filaria bancrofti* (Cobbold, 1877). Then, in 1927, new microfilaria describes as *Filaria malayi* was collected from a man in the Malay Archipelago (Brug, 1927). Then, 13 years later in India, Rao and Maplestone recovered the adult worms. These filarial parasites, today recognised scientifically as *Wuchereria bancrofti* and *Brugia malayi*, are the primary cause of lymphatic filariosis.

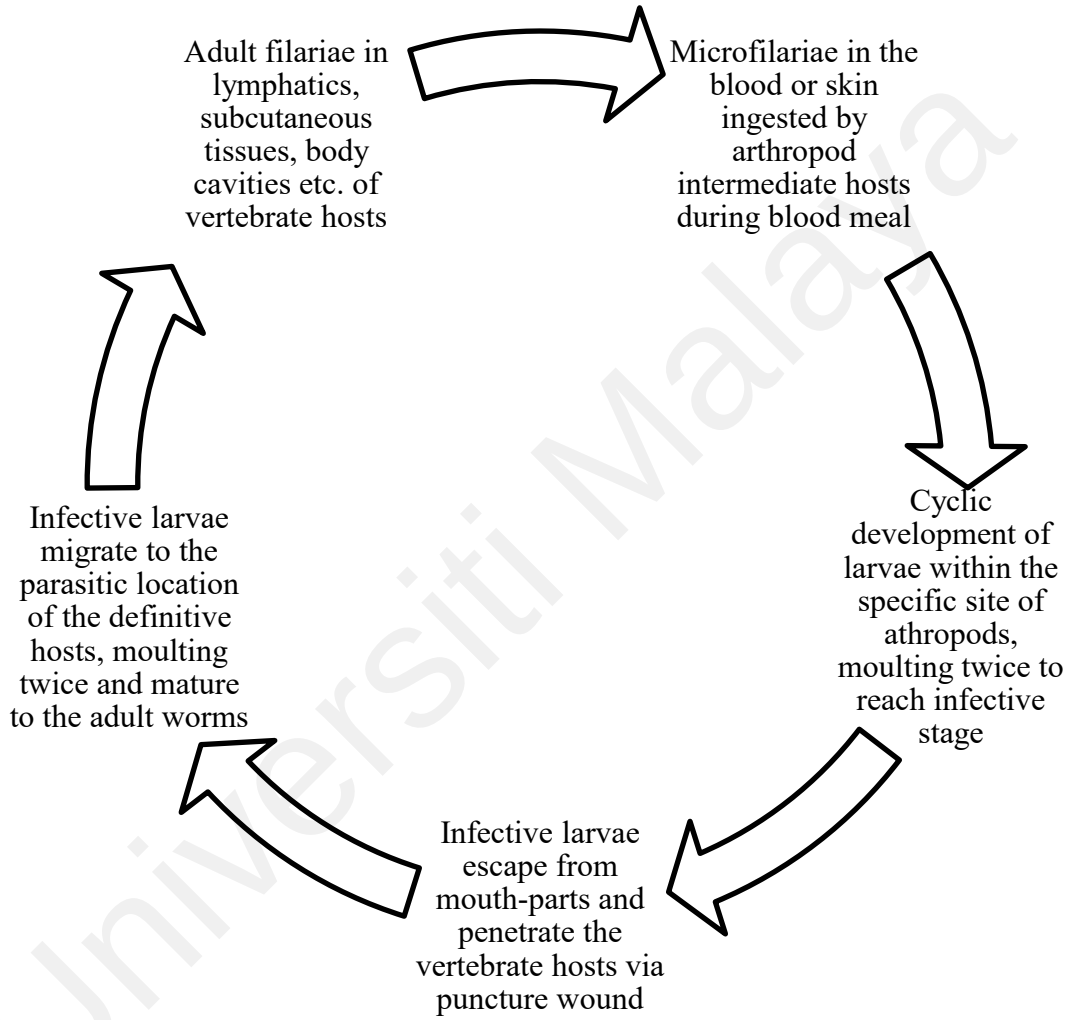

Figure 2.1. Generalised life cycle of filarial parasites (After Yen, 1983)

2.1.1 Diagnostic characteristics of filarial parasites

No single morphological feature can be used to distinguish filarioids up to generic levels. However, a combination of detailed morphological characteristics of microfilariae and adult filarioids can be used to distinguishes between genera. The essential diagnostic characters in classifying filarioids from superfamily Filarioidea and family Onchocercidae are listed below.

Cephalic morphology. This feature focuses on the shape and various cuticular structures of the anterior extremity of adult worms. Cephalic structures are usually simple and absent of pseudolabia except for filarioids from subfamily Setariinae with complex cephalic structures in median or lateral cuticular elevations and their spines. The anterior extremity might be dilated and bulbous as in *Wuchereria bancrofti* and *Brugia* spp. The cuticular structures at cephalic areas may be in the form of chitinous spines around the mouth such as *Stephanofilaria* or as a peribuccal (cephalic) shield in the form of cuticular ridges such as in some *Dipetalonema* spp.

Cuticular ornamentations of the body. Cuticular structures of filarioids include annulations or striations of the body. Alae (longitudinally oriented expansions), refractile bosses and transverse ridges are used to classified specific genera or species. For example, transverse ridges are common characteristics of *Onchocerca*, while lateral and caudal alae are characteristics of *Waltonella*.

Spicules. One of the most important features in filariae is the morphology and the size of the copulatory spicules. With the exclusion of *Dunnifilaria* the filarial parasites have dissimilar and generally unequal spicules. The spicular ratio, which is the relative length of the left to right spicule, is frequently used in differentiating between species in genera.

Vulva. The relative position of the female worm's external genitals is used in classifying between family and subfamily. The vulva in Oswaldofilariinae is at the posterior or middle region of the body.

Oesophagus. This is the diagnostic feature in identifying specific subfamilies and genera. Differerent from *Stephanofilaria*, *Brugia* spp. usually have divided oesophagus: glandular and muscular oesophagus. Genera *Pelecitus* (Dirofilarinae) and *Litomosa* (Onchocercinae) have a short and undivided oesophagus. *Edesonfilaria* has a long and sacculate glandular oesophagus.

Caudal morphology. The morphology of the posterior parts of the worms, such as the structure, arrangement and numbers of the caudal papillae around the anus; size, shape, and the tail appendages, are frequently used for classifying genera or species. *Oswaldofilaria* spp. have a rounded tail with a terminal protuberance and two subterminal, lateroventral tongue-like structures. *Setaria javanensis* Vevera, 1923 from subfamily Setariinae has a rounded tail with a sharp point and two ventrolateral, knob-like papillae. Male worms from the subfamily Dirofilariinae have highly developed large and pedunculated caudal alae, distinguishing them from most other members of Onchocercidae. Highly related genera *Brugia* and *Wuchereria* are distinguished based on the number of papillae. *Brugia* spp. have about 11 papillae while *Wuchereria* spp. have about 24. The arrangement of papillae of *Gonofilaria* is in a circle around the anus while those of *Setaria* are in sub-ventral rows. Tail is short in *Dirofilaria*, while it is long in most of the members of Onchocercinae.

2.1.2 Family Onchocercidae and diversity in Malaysia

. Family Onchocercidae consists of a diverse array of filarioids from about 70-80 genera in eight subfamilies. Onchocercidae can be found in almost all vertebrates, including humans (Buckley & Edeson, 1956). Filarioids have been found in primates (Peel & Chardome, 1947; Esslinger, 1979), carnivores (Buckley & Edeson, 1956; Uni, 1983), ungulates (Uni *et al.*, 2004), rodents (Eberhard et al., 1984) and Scandentia (Mullin & Orihel, 1972). From family Onchocercidae it is further divided into seven subfamilies which are Oswaldofilariinae, Waltonellinae, Icosiellinae, Setariinae, Splendidofilariinae, Dirofilariinae, Lemdaniinae and Onchocercinae. All of Onchocercidae comprises of 88 species.

To date, Malaysia's diversity of filarioids comprises of 36 species from 22 genera (Yen, 1983; Uni et al., 2020). Surprisingly, all genus that have capability of transmitting the disease to humans as mentioned above have been recorded in Malaysia. However, no in-depth study, except morphological description was conducted on Malaysian filarioids, particularly those parasitising on wild animals.

2.1.3 Current phylogeny status of the subfamily Onchocercinae

Evolutionary history and the phylogenetic relationship of filarial parasites were not well resolved and remained as speculations. This is partly due to the lack of taxonomic sampling, the rarity of the specimens in the field and a deeper phylogenetic relationship that has not been fully resolved.

Attempt to resolve the phylogeny dated since 1935 by Wher in which he first coined the subfamily Dirofilariinae. Then the classification under the family continues under different researchers (Chabaud & Choquet, 1953; Chabaud & Anderson, 1959). Then, the most comprehensive analysis of the phylogeny based mainly using morphological characteristics was made by Anderson & Bain (1976).

Molecular phylogenetic analysis was mainly based on 12S rDNA and *cox*1 gene sequences (Casiraghi et al., 2001; Casiraghi et al., 2004, Ferri et al., 2011) and also integrated method using both morphological characteristics and molecular analysis in particularly DNA barcoding (Ferri et al., 2009). Recently, by using many multi-gene dataset analyses, Lefoulon et al. (2015) have investigated the phylogeny of the family Onchocercidae. Their results have supported the current taxonomic classification that the Oswaldofilariinae, Waltonellinae and Icosiellinae separated early from their common ancestor compared to other subfamilies. Whereas Splendidofilarinae, Dirofilarinae, and Onchocercinae formed a polyphyletic group, thus their taxonomic classification needs to be revised.

2.1.4 Prevalence and habitat associations of filarioids

Previously, no study had focuses in-depth on the prevalence of filarial parasites parasitizing wild animals in natural environments. Descriptions of the new species of filarial parasites mainly mentioned the number of adult filarioids or microfilariae obtained. Prevalence also considers the distribution of adult filarioids and the microfilariae found in the host animal body parts (Uni et al., 2015; Uni et al., 2020). The microfilariae found was either from the skin snips or the blood of the host animals (Uni et al., 2002).

Case report of *Brugia pahangi* infection to humans in Kuala Lumpur written by Tan et al. (2011) is the first recorded case in the natural environment. It also records the first ever habitats associations of filarioids from subfamily Onchocercinae in Malaysia suburbia. Nevertheless, the prevalence and infection is closely related to the distribution of vectors and may differ from other Onchocercinae species. *Brugia pahangi* in the above zoonotic cases was transmitted from the facultative host (i.e. cats) to the humans by the suburban vector mosquitoes *Armigeres subalbatus* (Muslim et al., 2013).

2.2 Common treeshrew, *Tupaia glis* Diard and Duvaucel, 1820

Taxonomic summary of Common treeshrews, *Tupaia glis.*

Class Mammalia Linnaeus, 1758

Family Scandentia Wagner, 1855

Subfamily Tupaiidae Gray, 1825

Tupaia glis Diard and Duvaucel, 1820

Treeshrews are comprised of a single family in the order of Scandentia and further divided into two families, Ptilocercidae and Tupaiidae. Common treeshrews, *Tupaia glis* or locally known in Malaysia as Tupai muncong besar derived from the family Tupaiidae with the most extant diversity of 19 species (Roberts et al., 2011).

Tupaia glis was commonly found throughout different types of habitats and geographic ranges as depicted by its generic name. This native species of Malaysia, Thailand and Indonesia can be found abundantly in lowland and hill dipterocarp forests. They can also be found thriving in secondary forest, plantation and sub-urban areas. Their natural geographic distribution is in Southeast Asia, south of 10° N latitude, from Hat-Yai in southern Thailand to Singapore and Indonesia on the following islands: Siberut, Batu, Sumatra, Java, Bangka, Riau, Lingga, and Anambas.

Common treeshrews are listed as Least Concern by International Union for Conservation of Nature (IUCN) due to their ability to adapt to ongoing anthropogenic activities by the human that lead to their habitat loss. However, the status of this species requires regular monitoring due to the significant rates of forest degradation in this region. Habitat loss due to deforestation, land conversion for agriculture and plantations, and commercial logging are the biggest threats to this species. In addition, due to their active time and home range that coincides with humans, Mariana et al. (2010) believed that *Tupaia glis* have the potential as a vector to zoonotic diseases.

CHAPTER 3: METHODOLOGY

3.1 Animal host sampling

3.1.1 Sampling site

This research focuses on Peninsular Malaysia. Common treeshrews were captured from 14 localities (Figure. 3.1) at Perlis State Park, Perlis (1), Pulau Langkawi, Kedah (2), Jeram Pasu, Kelantan (3), Jeram Linang, Kelantan (4), Kampung Pantai Batu Burok, Terengganu (5), Kelantan National park, Kelantan (6), Bukit Kanthan, Perak (7), Ulu Kenas, Perak (8) Genting Awana, Pahang (9), Ulu Gombak Forest Reserve, Selangor (10), University of Malaya, Kuala Lumpur (11), Gemas, Negeri Sembilan (12), Krubong, Melaka (13) and Gunung Belumut, Johor (14).

3.1.2 Sampling period

Field sampling to collect the host animal was conducted from October 2013 until November 2016. A total of 24 trapping sessions with a minimum number of two visits were performed on each sampling site. Each sampling session lasted for three consecutive nights.

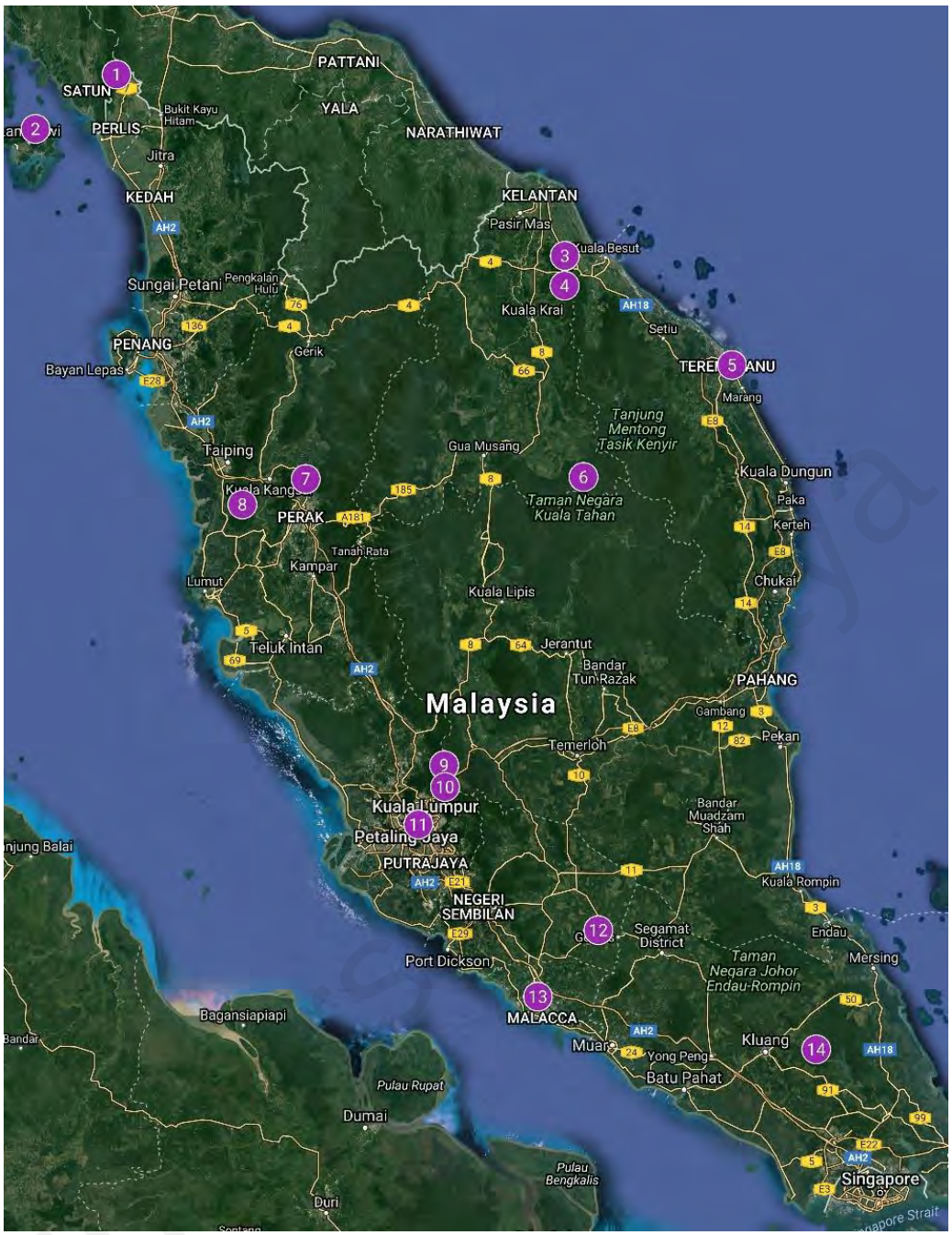

Figure 3.1: Map showing sampling sites of the host animal.

3.1.3 Research permission

Prior to commencing the study on common treeshrews, a special permit to conduct a study was sought from the Department of Wildlife and National Park Malaysia (DWNP) and given under permit number W-00660-16-16. Permission to enter and conduct scientific study in the forest reserve areas was granted by Forestry Department of Peninsular Malaysia. The common treeshrews were handled, anaesthetised and sacrificed in accordance with the protocols set by by the Animal Use and Care Committee, Universiti Malaya, Kuala Lumpur, Malaysia.

3.1.4 Sampling techniques

Treeshrews were captured using locally made mesh wire cage traps, measuring 50 x 15 x 15 cm. A total of 50 mesh wire cage traps, each baited with 'pisang emas', *Musa acuminata* or palm oil fruit, *Elaeis guineensis* were set up in predetermined study sites in the early morning during the period of highest foraging activity of treeshrews. The traps were placed along the trails and positioned on the forest floor, trees and fallen logs for primary and secondary forest habitats. The traps were randomly placed for sub-urban habitats, but each trap was positioned at least 10 meters apart. The traps were checked twice every day, at 0900 hours and 1800 hours, for trapped treeshrews. Other captured animals were examined, but the data obtained was not presented here.

Any trapped treeshrew or other small mammals were identified based on keys and illustrations developed by Medway (1983) and Payne et al. (1985). For undetermined species, morphological measurements were taken based on criteria such as head and body length (HB), ear length (E), hindfoot, tail (T), and body weight. The animal was photographed for future analysis.

3.2 Parasite morphological analysis

Blood samples were taken from the common treeshrews and smears onto a clear microscope glass slide to examine the microfilariae. These thick blood smears were dried overnight, dehemoglobinized in distilled water, then fixed in 95% ethanol for 3 minutes and stained with 3% Giemsa's solution (pH 7.4) for 40 minutes. The stained microfilariae were then examined under a compound microscope.

Skin snips were taken from the face, ears, dorsal side and abdomen of the common treeshrews following the procedure by Uni et al. (2002). Then, the skin was placed in a centrifuge tube (Eiken Chemical, Tokyo, Japan) containing 5 ml saline solution and left overnight at room temperature. The skin snip was removed, and the tubes were centrifuged at 600 x g for 10 min. The supernatant was replaced with 2 % formalin in saline solution. The tubes were centrifuged for 3 min. The supernatant obtained was examined under a compound microscope to look for microfilariae. Microfilariae were also taken from the uteri of adult worms. Length and the width of microfilariae obtained were recorded. Specific morphological details such as body shape, terminal nucleus and sheath have also been observed.

To collect adult filariae, the lymphatic tissue, subcutaneous tissues and peritoneal cavity of common treeshrews was dissected under a stereomicroscope. Standard necropsy procedure is exercised.

The adult worms recovered were placed in three different solutions, 80% ethanol, 70% ethanol and phosphate-buffered saline (PBS). Fragmented female worms were transferred into 80% ethanol for molecular study. The specimens were fixed in 70% ethanol for morphological identification, then cleared with lactophenol solution (R & M Chemicals, Essex, UK) and observed under a compound microscope equipped with a camera lucida (Olympus U-DA, Olympus, Tokyo, Japan). Adult filariae that neither cleared nor fixed were drawn using the same apparatus mentioned above. Finally, pictures of the parasites were taken by using a compact digital camera (Olympus Stylus XZ-2, Olympus, Tokyo, Japan) with an adapter (NY-XZ-1).

Detailed descriptions and measurements of morphological data of adult filariae were recorded using a microscope equipped with a micrometre reticle eyepiece. The characters include body length, body width, midbody ornamentation, the distance between the anterior end and vulva, left and right spicules and length of the oesophagus, the arrangement of caudal papillae and tail. The measured data was presented as the range. The measurement of morphological characters was in micrometres unless stated differently.

3.3 Molecular analysis

3.3.1 DNA extraction

Deoxyribonucleic acid (DNA) was extracted from female adult filariae using iNtRON G-spin^{TM} Total DNA Extraction kit (iNtRON Biotechnology, South Korea). The female adult filariae were chosen for molecular study because their size is relatively bigger, and the number of individuals extracted was higher than male filariae. DNA extraction was done according to manufacturer standard protocol with minor modifications. First, the tissue sample of the filariae was transferred into a 1.5 ml tube using forceps. Then, 200 µl Buffer CL, 20 µl Proteinase K and 5 µl RNase solution were added into the sample tube and vigorously mixed by vortexing. Finally, the sample tubes were placed into a preheated water bath at 56 ºC for 3 hours.

After the tissue samples were lysed entirely, 200 µl of Buffer BL was added into the upper sample tubes and thoroughly mixed. Then the mixture was incubated at 70 ºC for 5 min. Next, to removed un-lysed tissue particles and prevents column clogging, the sample tubes were centrifuged at 13,00 rpm for 5 min. Finally, 400 µl of supernatant were obtained by carefully transferred into a new 1.5 ml tube.

The supernatant was transferred into a Spin Column (2 ml collection tube) and centrifuged at 13,00 rpm for 1 min. The filtrate was discarded, and the Spin Column was reuse for the next step. 700 µl of Buffer WA was added to the Spin Column and centrifuged again at 13,000 rpm for 1 min. The flow-through was discarded, and the former step was repeated two times. Finally, the Spin Column was placed into a new 1.5 ml tube, and 50 µl of Buffer CE was added directly onto the middle section of the membrane. The tube was incubated at room temperature for 1 min and then centrifuged for 1 min at 13,000 rpm to let it elute.

3.3.2 PCR amplification

Premix used as a reagent for Polymerase chain reaction (PCR) was NEXpro^{TM} PCR 2x Master Mix (Genes Labs, Gyeonggi-do, South Korea). The composition component of the master mix reaction was modified based on the protocol by the manufacturer (Table 3.1). Mitochondrial genes, *cox*1 and 12S rRNA and was amplified using primer pairs as described by Agatsuma et al. (2005) and Lefoulon et al. (2015) (Table 3.2).

To check the quality of amplified DNA, the PCR products were electrophoresed on a 1% agarose gel and visualised using nucleic acid staining, RedSafe^{TM} (iNtRON Biotechnology, South Korea). Amplicons were purified and sequenced by a commercial laboratory First Base Co., Selangor, Malaysia.

Table 3.1: PCR master mix reaction composition component.

Component	50 µl reaction
NEXpro^{TM} PCR 2x master mix	25 µl
10 µl forward primer	1 µl
10 µl reverse primer	1 µl
Template DNA	1 µl
Water, RNase- free	22 µl

Table 3.2: Reverse forward primer 5'- 3' sequence and PCR amplification thermal profile. Abbreviations: T: temperature (ºC), D: duration (sec).

Gene	Primers			Thermal profile						Number of cycles
				denaturation		annealing		elongation		
	Designation	Sequence (5' – 3')	Product size (bp)	T	D	T	D	T	D	
cox1	COIintF/ COIintR	TGA TTG GTG GTT TTG GTA A ATA AGT ACG AGT ATC AAT ATC	~650	95	30	52	45	72	90	40
12S rRNA	12SdegF2/ 12SnemR2*	ATTACYTATTYTTAGTTTA CTACCATACTACAACTTACGC	~600	95	30	50	30	72	90	35

3.3.3 Phylogenetics analysis

Sequence editing was conducted by using Chromas Pro (MacCarthy, 1996). Multiple sequence alignment was performed by using the alignment programme, BioEdit. Both procedures above were also done by using Molecular Genetic Analysis (MEGA) Version 7.0.

Maximum likelihood (ML) phylogenetic trees of the single and concatenated gene sequence were constructed by MEGA7 (Saitou & Nei, 1987; Tamura et al., 2013) at 1000 bootstrap replicates. This character-based evolutionary tree buildings method was chosen over maximum parsimony (MP) because it has a higher probability to confer the resolve of the tip species (Patwardhan et al., 2014). Then the tree validity was evaluated using bootstrapping methods. GTR +G DNA substitution model was chosen as the most suitable for this analysis.

Kimura 2- parameter model was used to estimate the differences in the accumulated number of nucleotide substitutions per site. This method is chosen to look at transition and transversion mutation rates at a more realistic level (Patwardhan et al., 2014). This genetic distance calculation was analysed by using MEGA7 (Tamura et al., 2013).

3.4 Prevalence

Prevalence was taken based on the presence or absence of microfilariae and adult filariae from data in sub-chapter 3.2. In addition, the number of infected and non-infected common treeshrews, their locality and distribution were recorded.

$$Percentage\ of\ prevalence = \frac{Total\ individual\ infected}{Total\ individual\ examined} \text{ X } 100 \quad (3.1)$$

Prevalence was calculated based on the above formula.

CHAPTER 4: RESULTS

4.1 Prevalence and distribution of filarial parasites from common treeshrews

Ninety-eight individuals of common treeshrews from 14 areas of nine states and the Federal Territory, Malaysia, were inspected for filarial parasites. Table 4.1 shows the distributions of filarial parasites of common treeshrews in Peninsular Malaysia and their prevalence percentage (%). A total of 30 individuals of common treeshrews were infected with 31 % of prevalence.

Microfilariae of *Malayfilaria sofiani* in the blood of six individuals (6/98, 6.1%) collected from the second-growth forest of Jeram Pasu, Kelantan and Gemas, Negeri Sembilan. Adult worms of this species were found in two treeshrews caught in Jeram Pasu, Kelantan. Only a tiny number of microfilariae were found in skin snips, whereas a large number were found in blood smears. Further observation concludes that the microfilariae found in the skin snips were involved in the blood contamination.

Mansonella dunni was found infected 23 common treeshrews (23/98, 23.4%) from primary and secondary forests visited in this study. The same result has been shown with a previous study by Mullin and Orihel (1972) with a 24% prevalence.

Only one individual was infected with microfilaria of *Brugia tupaiae,* which is common treeshrews from the secondary forest of Gemas, Negeri Sembilan. The current study showed a sharp decline from Orihel (1966), six individuals infected out of 41(14%).

Table 4.1: Percentage of prevalence of filarial parasites in common treeshrews. The distribution based on locality and type of habitat of the host common treeshrews caught in Peninsular Malaysia.

Filarial parasite species	Number of individuals infected	Percentage of prevalence		Type of habitat	Sampling locality with infected host
		Current study	Previous study		
Malayfilaria sofiani	6	6.1 %	-	Secondary forest	1. Jeram Pasu, Kelantan 2. Gemas, Negeri Sembilan
Mansonella dunni	23	23.4 %	24 % Mullin & Orihel (1972)	Primary & secondary forest	1. Perlis State Park, Perlis 2. Jeram Linang, Kelantan 3. Kelantan National Park, Kelantan 4. Ulu Kenas, Perak 5. Ulu Gombak Forest Reserve, Selangor 6. Gunung Belumut, Johor
Brugia tupaiae	1	1 %	14 % Orihel (1966)	Secondary forest	1. Gemas, Negeri Sembilan

4.2 Taxonomic and morphological data

4.2.1 Taxonomic and morphological data of *Malayfilaria sofiani* Uni, Mat Udin & Takaoka, 2017

Taxonomic summary of *Malayfilaria sofiani*

Type host: *Tupaia glis* Diard & Duvaucel, common treeshrew

Type locality: Jeram Pasu, Kelantan, Malaysia (5°48.9272'N, 102°20.8985'E)

Parasitic location in host: Adult filariae in the pericapsular lymphatic tissue of the neck, microfilariae in the blood.

Class Secernentea Von Linstow, 1905

Family Onchocercidae Leiper, 1911

Subfamily Onchocercinae Leiper, 1911

Malayfilaria sofiani Uni, Mat Udin & Takaoka n.g., n.sp. 2017

Type- material specimen storage

For *Malayfilaria sofiani*, the holotype female and allotype male were deposited in the Muséum National d'Histoire Naturelle, Paris, France. The paratype which is consist of six females and nine males were deposited in the Institute of Biological Sciences, University of Malaya, Malaysia (Table 4.1).

Table 4.2: *Malayfilaria sofiani* type – material accession numbers.

Type- Material	Number of specimens	Accession numbers
Holotype female	1	MNHN 95YT
Allotype male	1	MNHN 96YT
Paratype female	6	Ms-B2, Ms-P2, Ms-KE2, Ms-KE3, Ms-KE4, Ms-KE5
Paratype Male	9	Ms-B1, Ms-P1, Ms-P3-5, Ms-KEM2-5

General description of new genus *Malayfilaria*

Anterior extremity of adult slightly expanded into a head bulb (Figure 4.1 A). Present of the narrow buccal cavity. Thin buccal capsule with buccal ring located between the head cuticle and oesophagus apex (Figure 4.1 B). Labial and cephalic papillae are arranged in two circles of four. Present of amphids at the lateral side (Figure 4.1 B). Oesophagus is divided into two parts; muscular and glandular (Figure 4.1 A). The vulva is located at the anterior part of the glandular oesophagus—Midbody with salient annules in the cuticle (Figure 4.1 D).

Female with two lappets located at the tail end (Figure 4.1 E-G). Sheathed microfilaria with one terminal nucleus at the tail end (Figure 4.2 H-J). Males with area rugosa at posterior part (Figure 4.3 O). Spicules with dissimilar in shape and unequal length (Figure 4.3 P). Present of gubernaculum. Present of two lappets and caudal papillae on the tail end (Figure 4.3 Q-R). Filarial parasites of the pericapsular lymphatic tissue region of common treeshrews (Figure 4.4 A).

Description of new species *Malayfilaria sofiani*

Female: Head bulbous, 113 -128 µm wide and 111- 125 µm long (Figure 4.1 A). Pre-oesophageal cuticular ring (buccal capsule), 18- 19 µm wide and 3 µm high (Figure 4.1 B, arrow). Vagina, 219 µm long and 78 µm wide (Figure 4.1 C). Opisthodelphic uteri and ovejector position parallel to the oesophagus. Annules comprise of several striations in the midbody region (Figure 4.1 D, arrow). Pair of ventrolateral lappets at the posterior end of body (Figure 4.1 F-G). Phasmids at base of lappets area (Figure 4.1 G).

Male: Head bulbous, 109 µm wide and 103-113 µm long (Figure 4.3 K and 4.4 D). Pre-oesophageal cuticular ring (buccal capsule), 13-15 µm wide and 4 µm high (Figure 4.3 L and 4.4 D). Oesophagus consisting of muscular and glandular parts (Figure 4.3 K). The apex of the testis (6.4 mm from head, arrow) posterior to oesophagointestinal junction (Figure 4.3 M, arrow). Annules in midbody region (Figure 4.3 N arrow and 4.4 E). Area rugosa (Figure 4.3 O–P) consisting of 181- 443 raised transverse bands, 2 µm high and 4–6 µm apart, located from 123 µm to 853 µm from the tail end. Spicules: left spicule composed of thick-walled proximal part, twisted midsection, and long distal part; right spicule divided into the thick-walled proximal part and thinner-walled distal part, with distal bulb encircled by 8–9 transverse ridges (Figure 4.3 P). Spicule ratio 3.1–3.8:1. Gubernaculum crescent-shaped, 3 µm thick in lateral view (Figure 4.3 P), and horseshoe-shaped, 43 µm long and 3 µm thick in median view. Tail slightly bulbous (Figure 4.3 Q). Caudal papillae: three pairs of precloacal papillae, one precloacal central papilla, one pair of adcloacal papillae, one pair in midline posterior to the cloaca, one pair of large papillae posterior to the cloaca, and one pair of ventrolateral lappets on the tail end; one single large knob on the subterminal, left side (Figure 4.3 Q–R).

Microfilaria: Sheathed microfilariae without fixation (n = 10), taken from uteri of a worm, 205–245 µm long and 5 µm wide (Figure 4.2). Sheathed microfilariae (n = 10) in thick blood films from a common treeshrew: 183–240 µm long and 5–6 µm wide, cephalic space 4–8 µm (2–4% of body length), anterior end to nerve ring 37–50 µm (22–27%) (Figure 4.2H and 4.4 F), excretory pore 59–70 µm (32–38%), anal pore 143–170 µm (77–87%) (Figure 4.4 G), tail 25–43 µm (13–23%), and nucleus at the tail end (Figures 4.2 J and 4.4 G). Small numbers are found in skin snips of a common treeshrew. Sheathed microfilariae without fixation (n = 10) in a skin snip: 190–233 µm long and 5 µm wide.

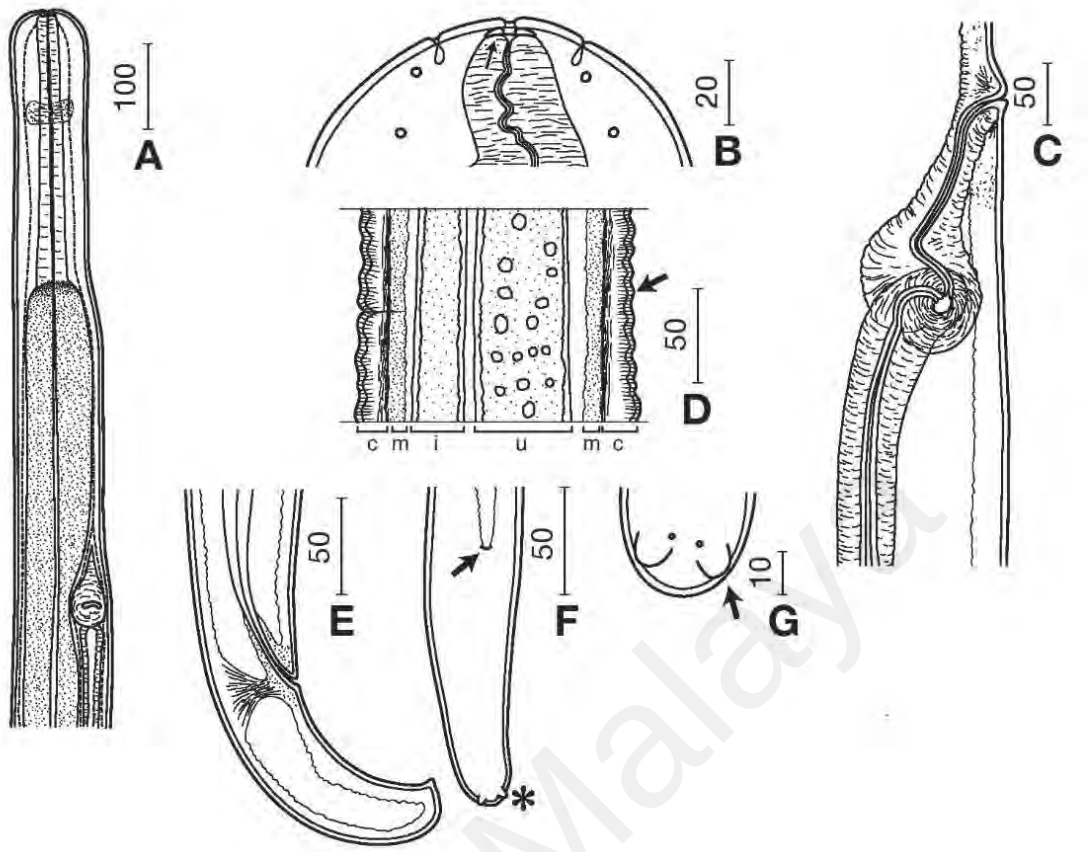

Figure 4.1: Females *Malayfilaria sofiani* (**A-G**), **A)** Anterior part, right lateral view. **B)** Head, dorsoventral view, showing pre-oesophageal cuticular ring (arrow). **C)** Vagina, right lateral view. **D)** Annules (arrow) in midbody; *Abbreviations*: c, cuticle; m, muscle; i, intestine, u, uterus. **E)** Posterior part, right lateral view. **F)** Posterior part, ventral view, showing anus (arrow) and lappets (*). **G)** Lappets (arrow) with phasmidial pore at the posterior end. Ventral view. Unit of bars in µm.

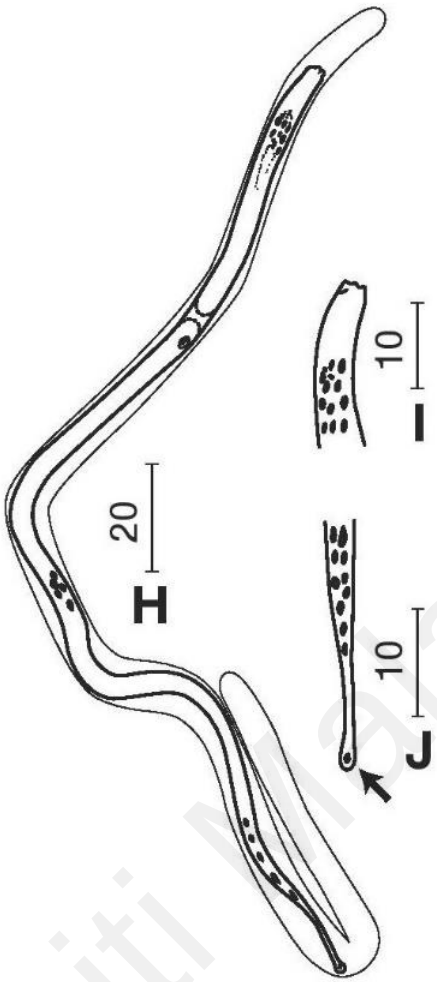

Figure 4.2: *Malayfilaria sofiani* micofilaria (**H-J**), **H)** Microfilaria with sheath. **I)** Head, dorsoventral view. **J)** Tail tip with terminal nucleus (arrow). Unit of bars in µm.

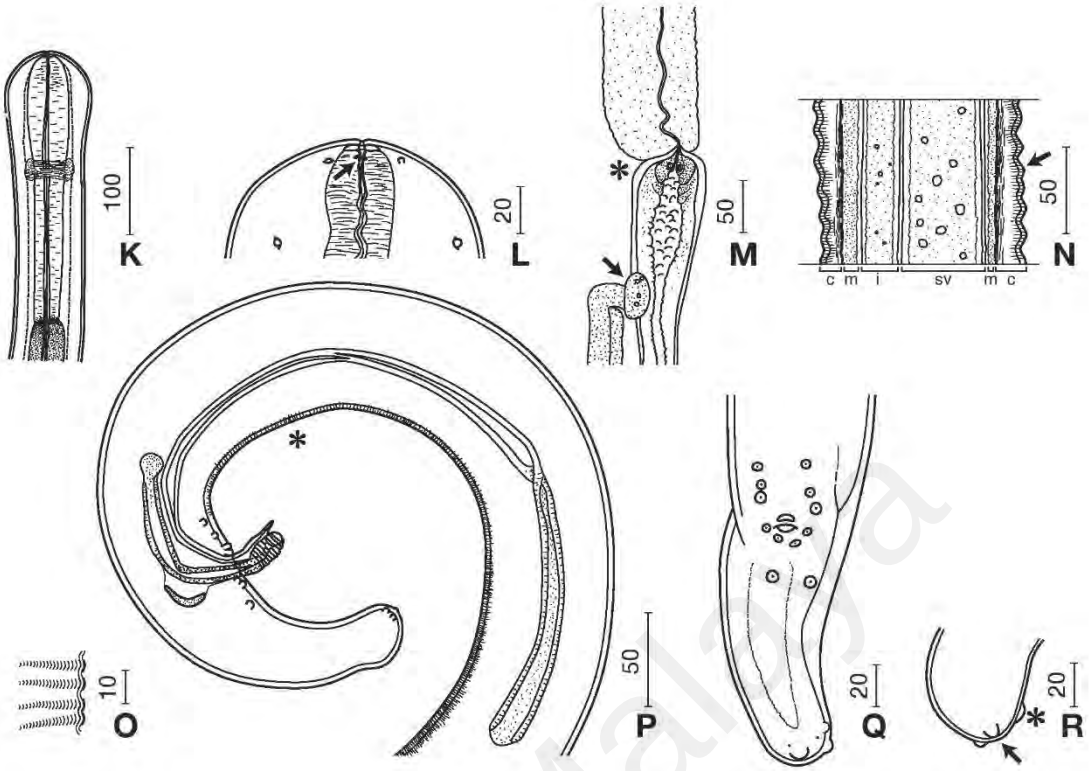

Figure 4.3: Males *Malayfilaria sofiani* (**K-R**), **K)** Anterior part, lateral view. **L)** Head with amphid (arrow), lateral view. **M)** Oesophago-intestinal junction (*) and apex of the testis (arrow). **N)** Annules (arrow) in midbody: c, cuticle; m, muscle; i, intestine; sv, seminal vesicle. **O)** Area rugosa, lateral view. **P)** Posterior part, right lateral view showing area rugosa (*). **Q)** Posterior part, ventral view. **R)** Tail tip with knob (*) and lappets (arrow). Lateral view. Unit of bars in µm.

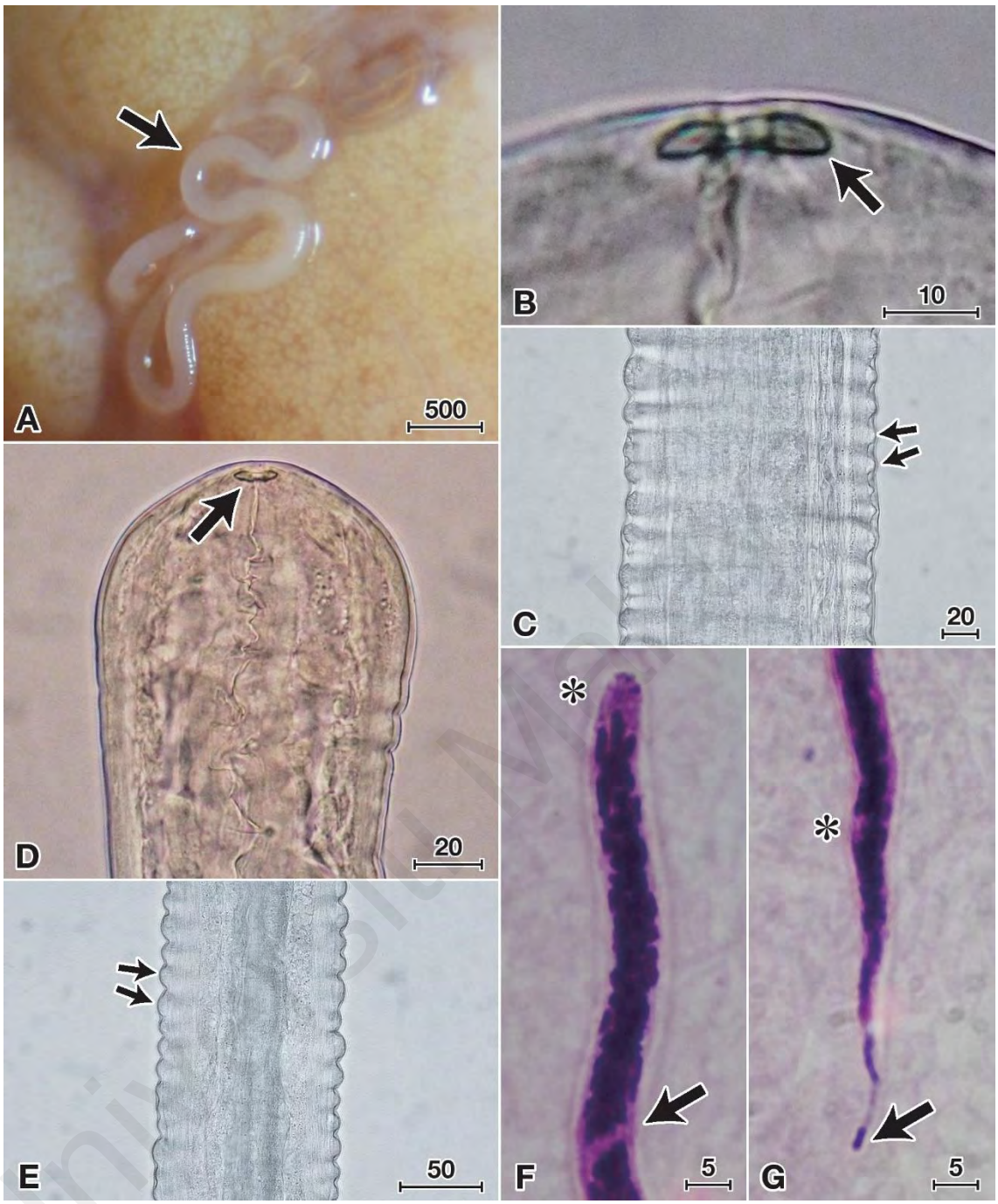

Figure 4.4: Adult females **(A–C),** males **(D–E),** and microfilariae **(F–G)** of *Malayfilaria sofiani***. A)** Adult female (arrow) in pericapsular lymphatic tissues of neck of treeshrew (*Tupaia glis*). **B)** Pre-esophageal cuticular ring (arrow). **C)** Annules (arrows) in midbody. **D)** Bulbous head with pre-oesophagal cuticular ring (arrow). **E)** Annules (arrows) in midbody. **F)** Anterior part with cephalic space (*) and nerve ring (arrow). Giemsa staining. **G)** Posterior part with anal pore (*) and terminal nucleus (arrow). Giemsa staining. Unit of bars in µm.

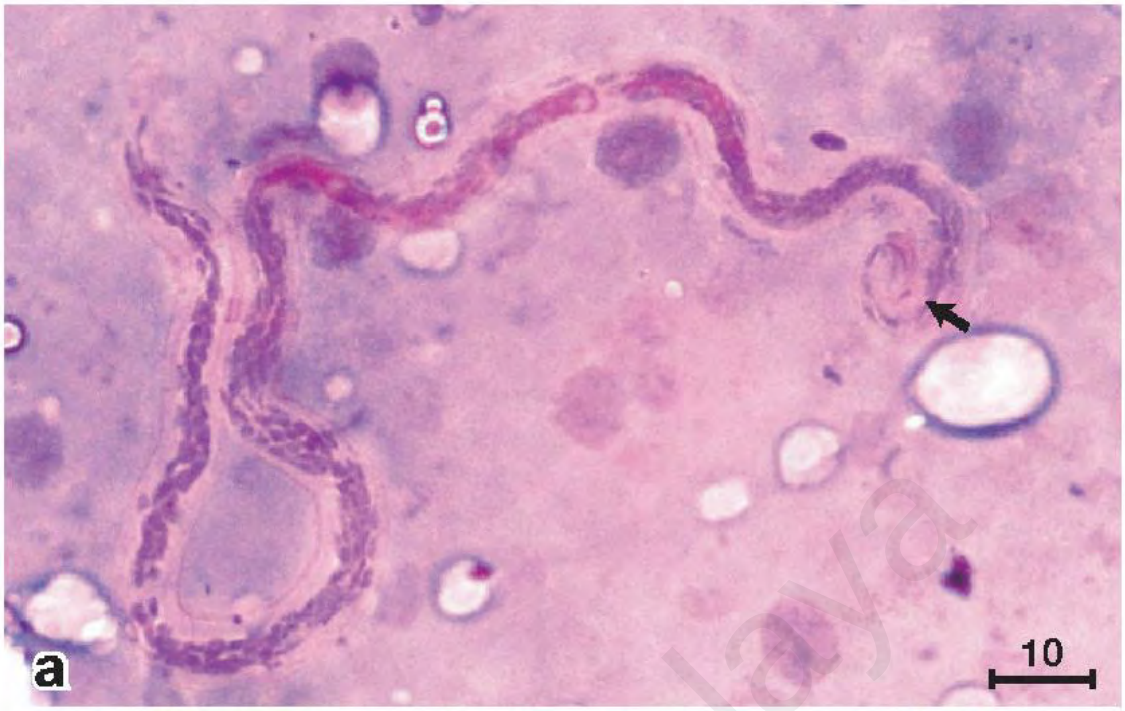

Figure 4.5: Microfilaria of *Malayfilaria sofiani* from common treeshrew caught in Gemas, Negeri Sembilan. Arrow, terminal nucleus. Unit of bars in µm.

4.2.2 Taxonomic and morphological data of *Mansonella* (*Tupainema*) *dunni*

Taxonomic classification of *Mansonella* (*Tupainema*) *dunni* Mullin & Orihel, 1972

Type host: *Tupaia glis* Diard & Duvaucel, common treeshrew

Type locality: Ampang, Selangor, Malaysia

Parasitic location in host: Adult filariae in subcutaneous tissue, microfilariae in the blood.

Class Secernentea Von Linstow, 1905

Family Onchocercidae Leiper, 1911

Subfamily Onchocercinae Leiper, 1911

Genus *Mansonella* Faust, 1929

Subgenus *Tupainema* Mullin & Orihel, 1972

Mansonella (*Tupainema*) *dunni* Mullin & Orihel, 1972

Descriptions of *Mansonella* (*Tupainema*) *dunni*

Female: Head bulbous and compressed, 29- 42 mm long and 120 -170 µm wide at midbody (Figure 4.6 A). Pre- oesophageal nerve ring position from the anterior head, 158- 350 µm. Labial and cervical papillae in anterior part (Figure 4.6 A). Vulva opening transverse. Vulva located at or posterior to the oesophagal- intestinal junction, 630- 770 µm. Non- divided oesophagus with a total length of 480- 1190 µm. Four lappets at the posterior end of body (Figure 4.6 B). Tail bent ventrally, 173-288 µm.

Male: filariae worm small and slender. Head bulbous and compressed, 75-80 µm wide and 16-19.5 mm long. Nerve ring position from anterior head, 175-270 µm. Thread-like intestines and non-divided short oesophagus consisting of muscular parts. Total length of oesophagus, 470-670 µm. Body swelling with giant coelomocyte (Figure 4.6 D, *). Area rugosa with tiny points (Figure 4.6 F, *). Spicules: left spicule composed of long and thin distal part, 475-550 µm; right spicule complex, with dilated spoon-shaped distal part (Figure 4.6 G, arrow). Spicule ratio 3.3–3.6. Gubernaculum absent. Tail tapered, 75-96 µm (Figure 4.6 I). Six pairs of caudal papillae around cloaca (Figure 4.6 H). Posterior end with four lappets (Figure 4.6 I, arrows).

Microfilaria: unsheathed slender posteriorly microfilariae in thick blood films from a common treeshrew: 113-207.5 µm long and 2.5–5 µm wide (Figure 4.6 C). Absent of nuclei in the tip of the tail end (Figure 4.6 C, arrow).

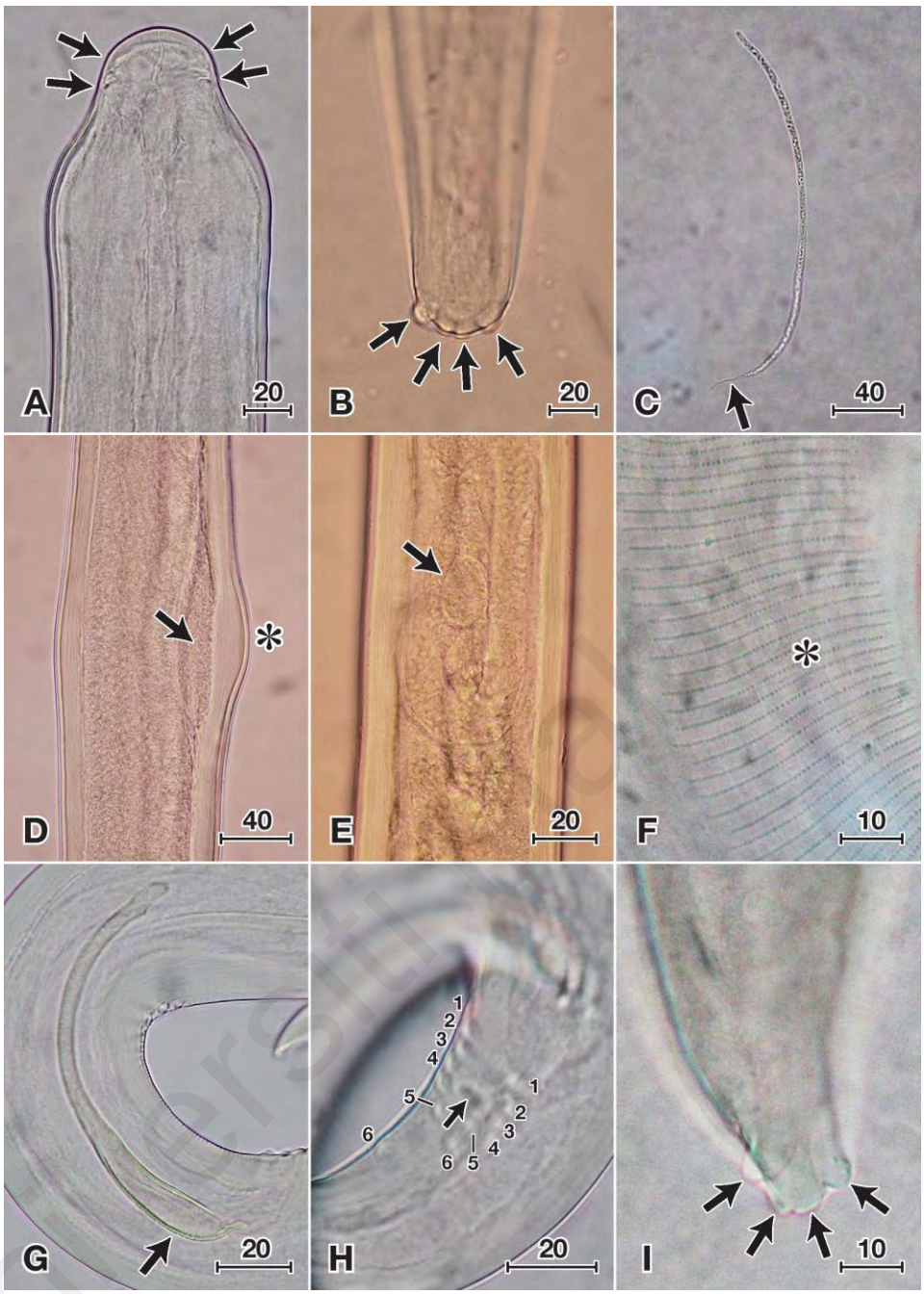

Figure 4.6: *Mansonella (Tupainema) dunni* females (**A-B**), microfilaria from the uterus (**C**), and males (**D-I**). **A**) Anterior part, lateral view; arrows, labial and cervical papillae. **B**) Posterior end with four lappets (arrows), median view. **C)** Microfilaria with a thin tail, lateral view. **D)** Body swelling (*); a giant coelomocyte (arrow), lateral view. **E**) Testis (arrow), lateral view. **F**) Area rugosa with tiny points (*), ventral view. **G**) Right spicule with dilated spoon-shaped distal part (arrow). Area lugosa (*), lateral view. **H**) Six pairs of caudal papillae around cloaca (arrow). **I**) Caudal end with four lappets (arrows)—unit of bars in μm.

4.2.3 Taxonomic and morphological data of *Brugia tupaiae*

Taxonomic classification of *Brugia tupaiae*, Orihel 1966

Type host: *Tupaia glis* Diard & Duvaucel, common treeshrew

Type locality: Johor, Malaysia

Parasitic location in host: Adult filariae in subcutaneous tissue (Orihel, 1966), microfilariae in the blood and the skin (present study).

Class Secernentea Von Linstow, 1905

Family Onchocercidae Leiper, 1911

Subfamily Onchocercinae Leiper, 1911

Genus *Brugia* Brug, 1927

Brugia tupaiae Orihel, 1966

Descriptions of microfilariae of *Brugia tupaiae*

Microfilaria: Sheathed microfilaria in thick blood films (n = 1) from common treeshrews: 275 µm long and 5 µm wide (Figure 4.7). Sheathed microfilaria in the skin snip (n = 1) of common treeshrew: 318 µm long and 8 µm wide. The presence of the terminal nucleus. No adult filarial parasites of *Brugia tupaiae* was detected in this study.

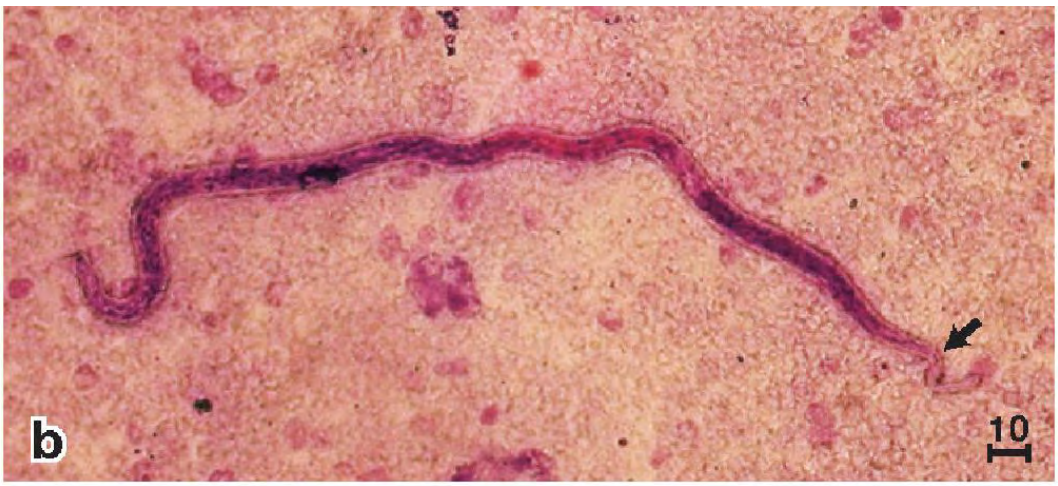

Figure 4.7: Microfilaria of *Brugia tupaiae* in a blood smear. Arrow, terminal nucleus. Unit of bars in µm.

Table 4.3: Comparison of morphological measurements of *Malayfilaria sofiani* from common treeshrews with other closely related filarial species. All measurements are in micrometres (µm) unless stated otherwise.

	Malayfilaria sofiani		***Wuchereria bancrofti* (Cobbold, 1877)**	***Wuchereria kalimantani* Palmieri, Purnomo, Dennis & Marwoto, 1980**	***Brugia malayi* (Brug, 1927)**	***Brugia pahangi* (Buckley & Edeson, 1956)**	***Brugia tupaiae* Orihel, 1966**
References	**Present study**: Specimens with alcohol fixation (group A)	**Present study**: Dead specimens without fixation (group B)	Cobbold, 1897	Bain O. et al., 1985[a]; Palmieri, JR, Purnomo, Dennis DT & Marwoto, HA 1980	**Present study**: supplementary specimens[b]	**Present study**: supplementary specimens[b]	Orihel, TC, 1966
Host animals	*Tupaia glis* Diard & Duvaucel, 1820	*Tupaia glis* Diard & Duvaucel, 1820	*Macaca fascicularis* Raffles, 1821	*Tachypithecus cristatus* (Raffles, 1821)	Man	Cat	*Tupaia glis* Diard & Duvaucel, 1820
Localities	Jeram Pasu, Kelantan	Jeram Pasu, Kelantan	Malaysia	South Kalimantan (Borneo), Indonesia	Pahang, Malaysia	Kuala Lumpur, Malaysia	Malaysia
Female:	H1 (Range, n = 6)	MNHN 95YT (Range, n = 7)			M1b	M3b	

Body length (mm)	34 (32-45)	49 (46-52)	45	61.0-116.3	38	45	19-23
Body width at midbody	150 (150-163)	140 (140-170)	170	200-290	145	60	40-50
Nerve ring from head	163 (163-275)	183 (155-188)	205	174-225	200	188	110-115
Muscular oesopohagus	275 (275–640)	430 (360-430)	390	306-520	350	253	+
Glandular oesophagus	7080 (7080–7625)	7375 (6500-8620)	515	506-1000	620	777	+
Total oesophagus	7355 (7250-7975)	7805 (6920-8970)	905	910-1450	970	1030	1130-1280
Vulva from head	460 (345-638)	650 (650-900)	610	598-1000	690	680	260-478
Tail	111 (111-168)	150 (108-163	160	210-285	193	163	55-80
Microfilaria	Mean (Range, n = 10)	Mean (Range, n = 10)			MS1b	MS2b	
Sheath	+	+	+[a]	+	+	+	+
Body length	212 (183-240)	234 (205-245)	279.5a	154.8-208.5	193-200	225-250	283-322
Body width	5-6	5	8.59 at nerve ring	5.0 (3.9-5.9)	8	7-8	6

Terminal nucleus	+	+	None[a]	None	+	+	+
Male	A1 (Range, n = 6)	MNHN 96YT (Range, n = 5)			M2[b]	M4[b]	
Body length (mm)	29 (26-31)	25 (21-26)	26	36-50	18	16	9.5-11.2
Body width at midbody	120 (120-145)	115 (115-130)	100	115-160	73	60	30-33
Nerve ring from head	168 (138-175)	175 (163-175)	185	201-250	180	Unidentified	110-120
Muscular oesophagus	355 (300–362)	300 (300-380)	350	345-475	238	350	+
Glandular oesophagus	6463 (6050–7275)	6500 (5750-6500)	520	450-810	603	530	+
Total oesophagus	6818 (6375-7575)	6800 (6060-6800)	870	875-1210	841	880	760-1060
Right spicule	148 (131-148)	150 (130-150)	240	160-215	120	83	70-80
Left spicule	477 (426-521)	475 (438-525)	625	683-825	368	195	140-170
Spicule ratio (left spicule/right spicule)	3.2 (3.1–3.8)	3.2 (3.2-3.6)	2.6	3.7-4.8	3.1	2.3	2
Gubernaculum	28 (28–29)	30 (23-34)	No record	27-38	17	16	15

Tail	115 (108-118)	123 (108-123)	185	174-250	95	95	38-47
Parasitic location of adult worms	Tissues surrounding the lymph nodes of the neck		Lymphatic ducts and glands[a]	Inguinal lymph nodes and testis	Lymph vessel	Lymphatic system	Lymphatic tissues

[a] References cited.

[b] specimens were archived at the Department of Parasitology, Faculty of Medicine, Universiti Malaya, Malaysia.

Table 4.4: Comparison of morphological measurements of *Mansonella* (*Tupainema*) *dunni* from current and previous studies. All measurements are in micrometres (µm) unless otherwise stated.

	Mansonella (*Tupainema*) *dunni* Mullin & Orihel, 1972	*Mansonella* (*Tupainema*) *dunni* (Mullin & Orihel, 1972)
References	Present study	Mullin & Orihel. 1972
Host animals	*Tupaia glis* Diard & Duvaucel, 1820	*Tupaia glis* Diard & Duvaucel, 1820, *T. tana Raffles*, 1821
Localities	Perak, Selangor, Kelantan, Johor & Perlis, Malaysia	Perak,Johor, Selangor Perak, Pahang & Sabah, Malaysia
Female:	n = 12	
Body length (mm)	29-42	23.8-46.3
Body width at midbody	120-170	105-175
Nerve ring from head	158-350	230-262
Muscular oesopohagus	+	+

Glandular oesophagus	Not divided	Not divided
Total oesophagus	480-1190	550-850
Vulva from head	630-770	480-730
Tail	173-288	170-202
Microfilaria		
Sheath	None	None
Body length	113-207.5	149
Body width	2.5-5	4
Terminal nucleus	None	None
Male	n = 3	
Body length (mm)	16-19.5	13-19
Body width at midbody	75-80	65-75

Nerve ring from head	175-270	215-225
Muscular oesophagus	+	+
Glandular oesophagus	Not divided	Not divided
Total oesophagus	470-670	440-600
Right spicule	148-150	120-150
Left spicule	475-550	380-410
Spicule ratio (left spicule/right spicule)	3:3-3:6	2.8
Gubernaculum	None	None
Tail	75-96	77-86
Parasitic location of adult worms	Subcutaneous connective tissues	Subcutaneous connective tissues

4.3 Phylogenetics relationship

4.3.1 Accession numbers

The *cox*1 and 12s rDNA nucleotide from *Malayfilaria sofiani* and *Mansonella* (*Tupainema*) *dunni* were sequenced, annotated and deposited in Genbank (Table 4.5).

Table 4.5: List of filarial species used in phylogenetic analyses of this study and their accession numbers.

Species	Accession number	
	*cox*1	12s rDNA
Malayfilaria sofiani	F0: KX944563 F1: KX944564 F2: KX944565	F0: KX944561 F1: KX944562 F2: KX944560
Mansonella (*Tupainema*) *dunni*	M1: KY434306 M2: KY434307	M1: KY434310 M2: KY434311
Wuchereria bancrofti	AJ271612	NC_016186
Brugia malayi	AJ271610	AJ544843
Brugia pahangi	AJ271611	AJ544842
Mansonella (*Mansonella*) *ozzardi*	KP760195	JF412321
Mansonella (*Tetrapetalonema*) *atelensis amazonae*	AM749278	AM779823
Mansonella (*Cutifilaria*) *perforata*	AM749265	AM779803
Mansonella (*Esslingeria*) *perstans*	LT623909	LT623913
Onchocerca gibsoni	AJ271616	AY462913
Onchocerca gutturosa	AJ271617	AY462923
Onchocerca ochengi	KC167351	AY46291
Onchocerca volvulus	KC167355	AJ544840
Onchocerca lupi	KP283477	GU365879
Onchocerca skrjabini	AM749269	AM779809
Onchocerca suzukii	AM749277	AM779811
Onchocerca flexuosa	HQ214004	HQ214004
Onchocerca dewittei japonica	AB518691	AM779815
Onchocerca eberhardi	AM749268	AM779810

Onchocerca takaokai	AB972361	AB972364
Onchocerca lienalis	KX853326	AY462924
Onchocerca cervipedis	KX853324	JX075208
Onchocerca boehmi	KX853323	KX853315
Onchocerca armillata	KX853322	KX853314
Onchocerca jakutensis	KT001213.1	DQ523745
Onchocerca ramachandrini	KC167357	KC167341
Cercopithifilaria japonica	AM749262	AM779794
Cercopithifilaria multicauda	AB178849	AM779799
Cercopithifilaria bulboidea	AB178839	AM779780
Cercopithifilaria shohoi	AB178851	AM779797
Acanthocheilonema vitae	AJ272117	HQ186249
Acanthocheilonema odendhali	KP760168	KP760314
Acanthocheilonema reconditum	JF461456	KP898741
Brenlia jittapalongi	KP760170	KP760316
Litomosoides sigmodontis	AJ271615	AJ544848
Litomosoides solaria	KP760193	KP760338
Litomosoides hamletti	AJ544868	AJ544847.1
Litomosoides yutajensis	AJ544869	AJ544846
Litomosoides brasiliensis	AJ544867	AJ544850
Filaria latala	KP760186	KP760332

4.3.2 Phylogenetic analyses

Nucleotide sequences of *cox*1 and 12s rDNA of *Malayfilaria sofiani* and *Mansonella* (*Tupainema*) *dunni* from this study aligned with nucleotide sequences Onchocercidae accessed from Genbank (Table 4.3). Only species with both the *cox*1 and 12s rDNA nucleotide sequences were selected, and each species was not duplicated. The phylogenetic tree was constructed using the maximum-likelihood method (ML) based on combined *cox*1 and 12s rDNA. Maximum likelihood was chosen because it was commonly described and was more concordant with other studies although analyses using other method was also available. The tree model selected was based on GTR + G + I and replicated with 1000 bootstraps.

The present phylogenetic tree has shown that *Malayfilaria sofiani* and *Mansonella* (*Tupainema*) *dunni* have a harmonious relationship with the morphological data. The phylogenetic trees concurred with earlier findings by Uni et al. (2017) and Lefoulon et al. (2015). The present analysis also produced a phylogenetic tree that resolved polyphyly of Onchocercinae as indicated by Eamsobhana et al. (2013); Liu et al. (2013), and McNulty et al. (2012).

The present analysis also fully supports the genus status of *Malayfilaria* and species status of *Malayfilaria sofiani*. Based on molecular characters, *M. sofiani* forms a robust monophyletic clade closely related to *Wuchereria bancrofti* and *Brugia malayi*. *Mansonella* (*Tupainema*) *dunni* formed a sister clade among genus Mansonella. *Mansonella* (*T.*) *dunni* appear to be closely related to *Mansonella ozzardi*, a human parasite first discovered in Haiti (Manson, 1897). Apart from this close relationship in their nucleotide sequences, both species also shared some similarities in their morphological characteristics, which will be described in detail in Chapter 5.

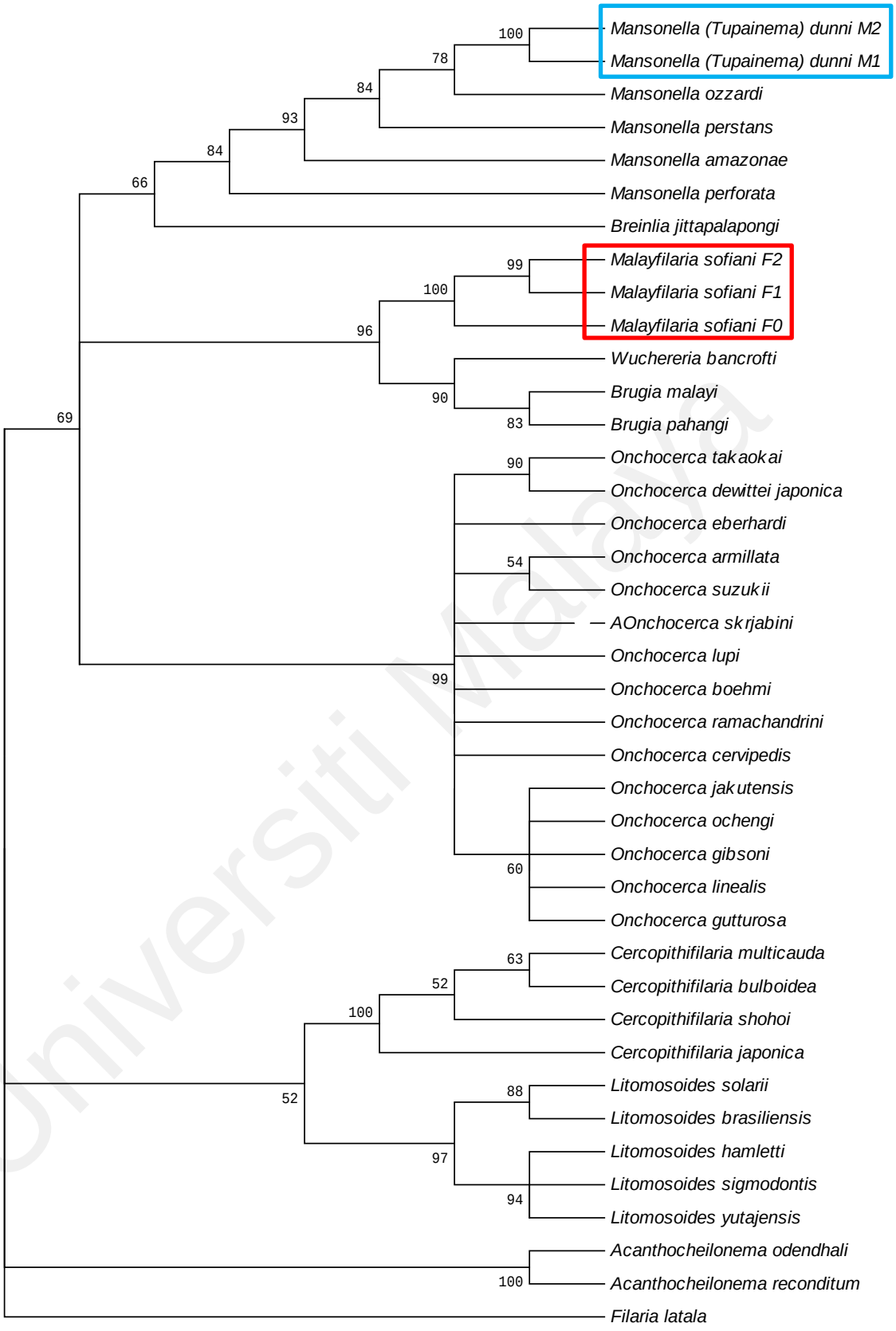

Figure 4.8: Phylogenetic position of *Malayfilaria sofiani* and *Mansonella dunni* inferred using the maximum- likelihood method (ML), based on concatenated 12s rDNA and *cox1* nucleotide sequences.

CHAPTER 5: DISCUSSION

This chapter discuss the findings of this study and how this had changed our current knowledge on subfamily Onchocercinae. The discussion will focus on the prevalence, morphological characteristic, phylogeny, host-vector relationships, and the proposed evolutionary history of *Malayfilaria sofiani*, *Mansonella dunni* and *Brugia tupaiae*. Finally, I will compare between three species of filarial parasites found from *Tupaia glis* with their closely related species.

5.1 Prevalence of *Malayfilaria sofiani, Mansonella* (*Tupainema*) *dunni* and *Brugia tupaiae*

Essential findings for the prevalence of filarioids from the common treeshrews is the significant drop of individuals parasitised by *Brugia tupaiae.* One of the factors leading to these findings may be the drop in the abundance of the host species. However, the host species abundance needs to be ruled out as it does not affect the prevalence of *Mansonella* (*Tupainema*) *dunni* (Table 4.3). Furthermore, the only microfilaria was able to be retrieved from the common treeshrews infected with *Brugia tupaiae.* This lead to my assumptions that methods of dissection under a stereomicroscope to collect the adult filarioids had restricted the number of adult *Brugia tupaiae* in this study. In supporting my assumptions, Orihel (1966) also expressed his concern that only fragments of adult filarioids and microfilaria samples managed to be retrieved from infected treeshrews in Selangor and North Borneo.

Nevertheless, this study record a new distribution of *Brugia tupaiae* at palm oil plantation in Gemas, Negeri Sembilan. Uni et al. (2017) described the *Malayfilaria sofiani* distribution and mentioned that the species might be endemic in the secondary forest of Jeram Pasu, Kelantan. However, upon continuing my sampling throughout Peninsular Malaysia, I also discovered an individual of common treeshrews infected with microfilaria of *Malayfilaria sofiani* in Gemas, Negeri Sembilan.

Even though the prevalence of common treeshrews infected by filarioids is low (31%), many factors will put this particular host-parasites combination as a threat for potential zoonoses agents. First and foremost, the active time and home range of common treeshrews coincides with humans (Mariana et al., 2010). Then, the abundance of vector species and the availability of *Wolbachia* endosymbionts.

5.2 Morphological analysis of *Malayfilaria sofiani, Mansonella* (*Tupainema*) *dunni* and *Brugia tupaiae*

This study strongly proposed that three species of filarial parasites can be found from Common treeshrews *Tupaia glis*. Apart from the previously described species (i.e., *Mansonella* (*Tupainema*) *dunni* and *Brugia tupaiae*), newly finding from this study is the new genus and the new species *Malayfilaria sofiani*.

5.2.1 Morphological analysis of *Malayfilaria sofiani*

Based on its morphological characteristics, *M. sofiani* is closely related to *Wuchereria* spp. and *Brugia* spp. from subfamily Onchocercinae. However, the current species appear morphologically different from these species in having a salient pre-oesophagal cuticular ring, an extremely long glandular oesophagus, annules in the midbody region, and small lappets at the terminal end of the tail. No description of a cuticular ring can be found for any species of *Wuchereria* or *Brugia* except *B. patei* (Buckley et al., 1958); however, in this study, I have identified a small cuticular ring in *B. malayi* and *B. pahangi* based on the sample provided by the Department of Parasitology, Faculty of Medicine, Universiti Malaya. This is a new additional morphological characteristic observed from *B. malayi* and *B. pahangi*.

Apart from the species mentioned, the onchocercid nematodes *Acanthocheilonema* spp. also have a salient pre-oesophagal cuticular ring and a stout glandular oesophagus (Bain et al, 1982; Uni 2013). On the other hand, *Cercopithifilaria* spp., the subcutaneous filarial parasites also have a small pre-oesophagal cuticular ring but no glandular oesophagus (Bain, 2001; Uni, 2001). This correlation leads Chabaud and Bain (1994) to suggest that *Cercopithifilaria* was derived from the *Acanthocheilonema* lineage. In *Onchocerca* spp., a stout glandular oesophagus is strongly believed to be one of the primitive characteristics of filarial parasites (Yagi, 1994). In congruence with the previous findings, I infer that the *Malayfilaria sofiani* species has primitive morphological characteristics compared with its morphologically related species, the *Wuchereria* spp. and *Brugia* spp.

5.2.2 Morphological analysis of *Mansonella* (*Tupainema*) *dunni*

Genus *Mansonella* has been undergone much taxonomic revision due to many factors. Despite the several taxonomic adjustments and the latest by Bain et al. (2015), some of the species remain insufficiently described. This problem is due to the lack of current taxonomic sampling and fossilised samples.

The insufficiently described species concerning their taxonomic, morphological characteristics is their position and number of their head papillae, the morphology of the digestive tract, the vagina structures, caudal extremity, distal extremities of the spicules, absence or presence of annular swelling and area rugosa (Petit et al. 1985; Bain et al. 1985; Uni et al. 2002). Therefore, in addressing the issue above, this study observes all the above-mentioned morphological characteristics and those not described in the original article.

Eberhard and Orihel in 1984 assigned the genus *Mansonella* into four subgenera which are *Mansonella, Tetrapetalonema, Sandnema* and *Tupainema.* This assignment is based only on comparative morphological studies. Then, using the same method, Bain et al. (2015) reassigned the subgenera into *Mansonella, Cutifilaria, Esslingeria, Filyamagutia, Pseudolitomosa, Tetrapetalonema* and *Tupainema.* Even though significant revision occurred, both findings agreed on *Mansonella dunni* as single species in subgenus *Tupainema* and this species is closely related to subgenus *Mansonella.*

Species description based solely on morphological characteristics is not absolute. In most cases, a follow-up study with additional molecular analysis will redescribe the species as synonyms or under different genus. For example, Uni & Bain (2006) describe a new species *Loxodontofilaria caprini,* from Japanese serow, *Capricornis crispus* based only on morphological characteristics of female filarioids. Then, 11 years later Lefoulon et al. (2017) studied the molecular relationship of genus Onchocerca and discovered that *Loxodontofilaria caprini* is closely related to *Onchocerca cervipedis, Onchocerca boehmi, Onchocerca suzukii, Onchocerca armillata* and *Onchocerca dewittei japonica.* Lefoulon et al. (2017) then revised the species as *Onchocerca caprini.*

The single species in the subgenus *Tupainema*, *Mansonella dunni* exhibit morphological characteristics, which is most closely related with subgenus *Mansonella,* specifically *Mansonella ozzardi.* The shared morphological characteristics between the two species is in term of absence of nuclei at the tip of the tail of their microfilariae (Eberhard & Orihel, 1984). On the other hand, the differences are considered as the subgeneric morphological characteristics between the subgenus (Bain et al., 2015). The differences are in the orientation of their cephalic extremities, the position of the vulva, morphology of the male tail, type of host animal and their respective geographical differences.

5.2.3 Morphological analysis of *Brugia tupaiae*

Throughout this study, I did not manage to sample an adult *Brugia tupaiae*. Only microfilariae were observed in one adult of the common treeshrews caught from Gemas, Negeri Sembilan. The microfilaria observed exhibits the same characteristics as described by Orihel (1966), which is sheathed and terminal nucleus presence at the tip of the tail.

5.3 Phylogeny, host-vector relationship and evolutionary history of *Malayfilaria sofiani*

This study supports Ferri et al. (2009) finding that species distinction and identification of filarial parasites in the family Onchocercidae using single molecular markers are accurate. Regarding genetic distance, intraspecific and interspecific distance distances between *Malayfilaria sofiani, Mansonella dunni*, and all the studied species are lower than 2% and higher than 4.5%, respectively (Appendix A). *Malayfilaria sofiani* shown a closer p-distance with its strongly associated species which is *W. bancrofti. Mansonella dunni* also shown a distinct association with *Mansonella ozzardi* with a genetic distance of 6.1- 6.4 % (Appendix B).

The tree topologies generated based on the concatenated sequence of 12S rDNA and *cox*1 genes in the current study produced a highly supported clade (Figure 4.7). Thus, the genera clade of *Brugia- Wuchereria, Mansonella, Onchocerca, Litomosoides* and *Acanthocheilonema* form monophyly and is in congruence with the previous findings (Morales- Hojas, 2009; Lefoulon et al., 2015; Uni et al., 2017).

Malayfilaria sofiani is placed as a sister taxon to the *Wuchereria- Brugia* clade. *Wuchereria- Brugia* had always formed a robust sister clade which also had been highlighted by previous molecular findings (Xie et al., 1994; Casiraghi et al., 2004; Bain et al.,2008; McNulty et al., 2012). This newly discovered association is also supported by some shared morphological similarities such as having glandular oesophagus, pre – oesophageal cuticular rings and lappets.

For *Wolbachia* endosymbionts; *Malayfilaria sofiani, Brugia timori, Brugia malayi, Brugia pahangi and Wuchereria bancrofti* harboured the same *Wolbachia* from supergroup D (Uni et al., 2020). All the above similarities between *Malayfilaria sofiani* and *Brugia- Wuchereria* species in terms of morphological characteristics, molecular findings, parasitic location, and *Wolbachia* endosymbionts support the hypothesis that they shared the same potential vectors, which is insects belonging to the family Culicidae (Anderson, 2000).

Wuchereria bancrofti and *Wuchereria kalimantani* parasitise humans and silvered leaf monkeys, *Presbytis cristatus* respectively. While *Brugia* spp. parasitise a wide host-spectrum, including human, primates, lagomorphs, carnivores, and treeshrews (Buckley & Edeson, 1956; Buckley et al., 1958; Dissanaike & Paramanathan, 1961; Jayawardene, 1962; Ash & Little, 1964; Orihel, 1966; Mullin & Orihel, 1972; Partono, 1977; Eberhard, 1984). Thus, there are no direct relationships between the host species of the three filarial genera *Malayfilaria*, *Wuchereria*, and *Brugia*.

Among filarial parasites, Onchocercidae was considered a recent nematode due to their highly evolved life cycle comprised of specialised eggs and the microfilariae transmitted by hematophagous arthropods. Bain (2002) speculated that their origin is remote, and the nascent lineages are firstly formed by the ancient genera *Oswaldofilaria,* somewhere in the secondary era (approximately 150 million years ago). However, the major expansion which leads to the formation of genus *Malayfilaria* and *Mansonella* was believed to be during the Paleocene and the Pleistocene (66 to 2.5 million years ago) in synchronous with the supercontinent breakout that led to the diversification of Onchocercidae host animals (Bain & Chabaud, 1994; Bain, 2002; Uni et al., 2017).

Treeshrews are members of the order of the Scandentia, which is more closely connected to Primates than to Rodentia and Lagomorpha in terms of their phylogeny (Springer, 2004). During the Cretaceous (about 90 million years ago), their common ancestor split into Scandentia, Dermoptera, and Primates, while the genus *Tupaia* formed at the end of the Miocene (roughly 10 million years ago) (Janecka, 2010). According to Roberts et al. (2011), treeshrew diversity in Southeast Asia was driven by Miocene tectonics, volcanism, and spatial instability.

Morales-Hojas (2009) stated that co-speciation between hosts and parasites was more likely when it came to filarioids diversification in the *Wuchereria-Brugia* clade because some *Brugia* and *Wuchereria* species parasitise humans and monkeys. However, because *Malayfilaria sofiani* appears to have more primitive morphological and molecular characteristics than *Wuchereria* and *Brugia* spp., I hypothesise that *Malayfilaria sofiani* ancestral lineage in common treeshrews may have been passed down through vectors to Primates, Carnivora, Rodentia, Lagomorpha, and other mammals through host-switching. This speculation is supported by findings using the *cox*1, 12S rRNA, and *ITS1* genes by Uni et al. (2017), which denoted the *Malayfilaria sofiani* as the basal species of the clade. Then the mentioned species diversified into genus *Brugia* and *Wuchereria* rather than evolving via host-parasite co- speciation. Additionally, molecular findings using the *cox*1, 12S rRNA, and *ITS1* genes by Uni et al. (2017) denoted *Malayfilaria sofiani* as the basal species of the clade.

5.4 Phylogeny, host-vector relationship and evolutionary history of *Mansonella dunni*

The phylogenetic tree presented here is the first record of the molecular study conducted on *Mansonella dunni*. *Mansonella dunni* from common treeshrews positioned closely with *Mansonella ozzardi* and *Mansonella perstans.* Both species is a human parasite and known to cause mansoneliasis and perstans filariasis. This finding is interesting as all the three species come from different regions. *Mansonella ozzardi is* originally from South America, *Mansonella perstans* from Tropical Africa and *Mansonella dunni* of Southeast Asia. However, even geographically far apart, they shared some subgeneric morphological characteristics and the same *Wolbachia* endosymbionts from supergroup F (Uni et al., 2020; Uni et al., 2021).

Generally, genus *Mansonella* is transmitted by vectors from Ceratopogonidae and Simuliidae. The Diptera taxonomic category contains all the known insect vectors of mansonellosis. The biting-midge genus *Culicoides* is the known vector of *Mansonella perstans* and *Mansonella streptocerca*. *Culicoides* does not, however, transmit *Mansonella ozzardi* solely. Various non-Culicoides Ceratopogonidae biting midges and blackflies from the genus *Simulium* are previously reported as the vector agent of *Mansonella ozzardi*.

Mansonella dunni had been highlighted by many researchers as the essential species in understanding the evolutionary history of *Mansonella* genus and human parasitism. *Mansonella dunni* is the only monospecific parasites of tupaiids in the genus compared to others with host species comprised of primate, carnivorous, sciurids and ungulates (Bain et al., 2015). The sole natural vertebrate host of *Mansonella ozzardi* is humans. In contrast, *Mansonella perstans* is known to infect both primates and humans.

In studying the origin of human parasitism caused by *Mansonella*, through molecular analysis the researchers (Muller, 2002; Simonsen et al., 2011; Simonsen et al., 2014; Da Silva et al., 2017; Thuy- Huong et al., 2018) discovered that *Mansonella perstans* was transferred relatively recently from Africa to South America, most likely because of the slave trade.

Looking back into the evolutionary history of the genus *Mansonella*, Bain (2002) speculate that this genus is from an Asiatic region. It is believed to have a remote origin spread by their insectivorous, primate and carnivorous host. Bain further speculated that *Mansonella ozzardi* represent a host switching event from carnivores or sciurids. I would like to disagree with this speculation and propose Scandentia as the zoonoses host to *Mansonella ozzardi* transmission to humans and *Mansonella dunni* as the basal species in the genus. This is due to their similarity in terms of their morphology and molecular. Then Scandentia is also found to have an Asiatic origin. Specifically, the Borneo island in which the highest diversity can be found (8 species).

CHAPTER 6: CONCLUSION

From the findings in this study, it is now established that after 55 years of its first discovery by Orihel (1966), *Brugia tupaiae* prevalence has dropped significantly. Unfortunately, I did not manage to sample any adult *Brugia tupaiae* throughout this study.

Mansonella dunni which is the most commonly found species of filarioids from common treeshrews is found to have a close relationship with human parasites *Mansonella perstans* and *Mansonella ozzardi*. This finding also support the speculation that *Mansonella* lineages are originating from the Asiatic region.

In this study I had discovered *Malayfilaria sofiani* which is found in the pericapsular lymphatic tissues of common treeshrews, (*T. glis*) in Peninsular Malaysia, is a new species and a new genus. The adult worms of the described specimens differed from *Wuchereria* spp. and *Brugia* spp. in having a salient pre-oesophagal cuticular ring, long glandular oesophagus, annules in the midbody region, and lappets on the tail end. Molecular analyses indicated that *M. sofiani* differed from *W. bancrofti* by 10.8% in K2P distances between sequences of the *cox1* of *M. sofiani* n. gen., n. sp. and *W. bancrofti*. Based on the phylogenetic trees for the *cox1* and 12S rRNA, genes, *M. sofiani* was basal to *W. bancrofti* and *Brugia* spp. Therefore, it is speculated that the *Wuchereria* spp. and *Brugia* spp. may have evolved from the ancestral lineage of *Malayfilaria sofiani* in common treeshrews in Malaysia.

Findings from this study is in line with the hypothesis that the delineation of filarial parasites species and genera classification using molecular characteristics is in line with the previously described species using morphological characteristics.

Hopefully, this study's discovery will stimulate a revival of interest in filarial parasites research in Malaysia.

REFERENCES

Agatsuma, T., Iwagami, M., Uni, S., Takaoka, H., Katsumi, A., Kimura, E., & Bain, O. (2005). Molecular phylogenetic relationships among seven Japanese species of *Cercopithifilaria*. *Parasitology International*, 54(3), 195-199.

Al-Abd, N. M., Nor, Z. M., Kassim, M., Mansor, M., Al-Adhroey, A. H., Ngui, R., & Sivanandam, S. (2015). Prevalence of filarial parasites in domestic and stray cats in Selangor State, Malaysia. *Asian Pacific Journal of Tropical Medicine*, 8(9), 705-709.

Anderson, R. C. (1976). No. 3. Keys to genera of the Order Spirurida. Part 3. Diplotriaenoidea, Aproctoidea and Filarioidea. *CIH Keys to the Nematode Parasites of Vertebrates*. Wallingford Oxon UK: CAB International, 59-116.

Anderson, R. C. (2000). *Nematode parasites of vertebrates: their development and transmission*. Wallingford Oxon UK: CAB International, p. 1-16.

Ash, L. R., & Little, M. D. (1964). *Brugia beaveri* sp. n.(Nematoda: Filarioidea) from the raccoon (*Procyon lotor*) in Louisiana. *The Journal of Parasitology*, 119-123.

Bain, O. (2002). Evolutionary relationships among filarial nematodes. *The Filaria* . Springer US. p. 21-29.

Bain, O., Casiraghi, M., Martin, C., & Uni, S. (2008). The nematoda Filarioidea: critical analysis linking molecular and traditional approaches. *Parasite*, 15(3), 342-348.

Bain, O., Dissanaike, A.S. Cross, J.H., Harinasuta, C., Sucharit, S.(1985) Morphologie de Wuchereria bancrofti adult et sub-adult. Recherche de caracteres differentiels entre les souches. *Annales de Parasitologie Humaine et Comparee*., 60, 613–30.

Bain, O., Mutafchiev, Y., Junker, K., Guerrero, R., Martin, C., Lefoulon, E., & Uni, S. (2015). Review of the genus *Mansonella* Faust, 1929 sensu lato (Nematoda: Onchocercidae), with descriptions of a new subgenus and a new subspecies. *Zootaxa*, 3918(2), 151-193.

Bain, O., Mutafchiev, Y., Junker, K., Guerrero, R., Martin, C., Lefoulon, E., & Uni, S. (2015). Review of the genus *Mansonella* Faust, 1929 sensu lato (Nematoda: Onchocercidae), with descriptions of a new subgenus and a new subspecies. *Zootaxa*, 3918(2), 151-193.

Bird, M. I., Taylor, D., & Hunt, C. (2005). Palaeoenvironments of insular Southeast Asia during the Last Glacial Period: A savanna corridor in Sundaland?. *Quaternary Science Reviews*, 24(20), 2228-2242.

Bregani, E. R., Rovellini, A., Mbaïdoum, N., & Magnini, M. G. (2006). Comparison of different anthelminthic drug regimens against *Mansonella perstans* filariasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 100(5), 458-463.

Brown, K. R., Ricci, F. M., & Ottesen, E. A. (2000). Ivermectin: Effectiveness in lymphatic filariasis. *Parasitology*, 121(S1), S133-S146.

Buckley, J. J. C., & Edeson, J. F. B. (1956). On the adult morphology of *Wuchereria* sp.(malayi?) from a monkey (*Macaca irus*) and from cats in Malaya, and on *Wuchereria pahangi* n. sp. from a dog and a cat. *Journal of Helminthology*, 30(1), 1-20.

Camerini, J. R. (1993). Evolution, biogeography, and maps: An early history of Wallace's line. *Isis*, 700-727.

Cannon, C. H., Morley, R. J., & Bush, A. B. (2009). The current refugial rainforests of Sundaland are unrepresentative of their biogeographic past and highly vulnerable to disturbance. *Proceedings of the National Academy of Sciences*, 106(27), 11188-11193.

Casiraghi M, Anderson TJ, Bandi C, Bazzocchi C, Genchi C (2001) A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. *Parasitology*, 122(1): 93–103.

Casiraghi M, Bain O, Guerrero R, Martin C, Pocacqua V, et al. (2004) Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. *International Journal for Parasitology,* 34 (2): 191–203.

Catullo, G., Masi, M., Falcucci, A., Maiorano, L., Rondinini, C., & Boitani, L. (2008). A gap analysis of Southeast Asian mammals based on habitat suitability models. *Biological Conservation*, 141(11), 2730-2744.

Chabaud, A. G., & Bain, O. (1994). The evolutionary expansion of the Spirurida. *International Journal for Parasitology*, 24(8), 1179-1201.

Chabaud, A. G., & Anderson, R. C. (1959). Nouvel essai de classification des Filaires (Superfamille des Filarioidea) II. 1959. *Annales de Parasitologie Humaine et Comparee,* 34(1-2), 64-87.

Chabaud, A. G., & Choquet, M. T. (1953). Nouvel essai de classification des Filaires (superfamille des Filarioidea). *Annales de Parasitologie humaine et comparée*, 28(3), 172-192.

Chen, L. Y., Chen, J. M., Gituru, R. W., & Wang, Q. F. (2012). Generic phylogeny, historical biogeography and character evolution of the cosmopolitan aquatic plant family Hydrocharitaceae. *BMC Evolutionary Biology*, 12(1), 30.

Cobbold, T. S. (1877). On *Filaria Bancrofti*. *The Lancet*, 110(2823), 495-496.

Courchamp, F., Berec, L., & Gascoigne, J. (2008). Allee effects in ecology and conservation. *Environmental Conservation*, 36, 80-85.

De Bruyn, M., Nugroho, E., Hossain, M. M., Wilson, J. C., & Mather, P. B. (2005). Phylogeographic evidence for the existence of an ancient biogeographic barrier: the Isthmus of Kra Seaway. *Heredity*, 94(3), 370-378.

Demarquay, J.N. (1863). Notes on a tumor of the scrotal sac containing a milky fluid (Galactocele of Vidal) and enclosing small worm like beings that can be considered as hematoid helminthes in the embryo stage. *Helminthologie. Gazette Medicale de Paris*, 18, 665–667.

Dissanaike, A. S., & Paramanathan, D. (1961). On *Brugia buckleyi* n. sp. from the heart and blood vessels of the Ceylon Hare. *Journal of Helminthology*, 35(9).

Eberhard, M. L. (1984). *Brugia lepori* sp. n.(Filarioidea: Onchocercidae) from rabbits (*Sylvilagus aquaticus, S. floridanus*) in Louisiana. *Journal of Parasitology*, 576-579.

Eberhard, M. L., & Orihel, T. C. (1984). The genus *Mansonella* (syn. *Tetrapetalonema*): A new classification. *Annales de Parasitologie Humaine et Comparée*, 59(5), 483-496.

Esslinger, J. H., & Smith, J. L. (1979). *Dipetalonema* (*Acanthocheilonema*) *didelphis* sp. n.(Nematoda: Filarioidea) from opossums, with a redescription of D.(A.) pricei (Vaz and Pereira 1934). *Journal of Parasitology*, 928-933.

Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., Guerrero, R., ... & Casiraghi, M. (2009). Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Frontiers in Zoology*, 6(1).

Ferri E, Bain O, Barbuto M, Martin C, Lo N, et al. (2011) New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. *PLoS One*, 6 (6).

Fong, M. Y., Noordin, R., Lau, Y. L., Cheong, F. W., Yunus, M. H., & Idris, Z. (2013). Comparative analysis of *ITS*1 nucleotide sequence reveals distinct genetic difference between *Brugia malayi* from Northeast Borneo and Thailand. *Parasitology*, 140(01), 39-45.

Francis, C. M., & Barrett, P. (2008). *Guide to the mammals of Southeast Asia*. Princeton University Press.

Fukuda, M., Otsuka, Y., Uni, S., Bain, O., & Takaoka, H. (2010). Genetic evidence for the presence of two species of *Onchocerca* from the wild boar in Japan. *Parasite*, 17(1), 39-45.

Fukuda, M., Otsuka, Y., Uni, S., Bain, O., & Takaoka, H. (2010). Molecular identification of infective larvae of three species of *Onchocerca* found in wild-caught females of *Simulium bidentatum* in Japan. *Parasite*, 17(1), 39-45.

Gaston, K. J. (2000). Global patterns in biodiversity. *Nature*, 405(6783), 220-227.

Giam, X., Ng, T. H., Yap, V. B., & Tan, H. T. (2010). The extent of undiscovered species in Southeast Asia. *Biodiversity and Conservation*, 19(4), 943-954.

Gómez, A., & Nichols, E. (2013). Neglected wild life: Parasitic biodiversity as a conservation target. *International Journal for Parasitology: Parasites and Wildlife*, 2, 222-227.

Janečka, J. E., Miller, W., Pringle, T. H., Wiens, F., Zitzmann, A., Helgen, K. M., ... & Murphy, W. J. (2007). Molecular and genomic data identify the closest living relative of primates. *Science*, 318(5851), 792-794.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16(2), 111-120.

Kochin, B. F., Bull, J. J., & Antia, R. (2010). Parasite evolution and life history theory. *PLoS Biology*, 8(10).

Lafferty, K. D., Allesina, S., Arim, M., Briggs, C. J., De Leo, G., Dobson, A. P., ... & Thieltges, D. W. (2008). Parasites in food webs: The ultimate missing links. *Ecology Letters*, 11(6), 533-546.

Lefoulon, E., Bain, O., Bourret, J., Junker, K., Guerrero, R., Cañizales, I., Kuzmin, Y., T. Satoto, T.B., Cardenas- Callirgos, J. M., Lima, S.Z., Raccurt, C., Mutafchiev, Y., Gavotte, L., & Martin, C. (2015). Shaking the Tree: Multi-locus Sequence Typing Usurps Current Onchocercid (Filarial Nematode) Phylogeny. *PLoS Neglected Tropical Diseases*, 9(11).

Medway, L. (1983). *The wild mammals of Malaya (peninsular Malaysia) and Singapore*. Oxford University Press.

Low, G. C. (1900). A recent observation on *Filaria nocturna* in *Culex*: probable mode of infection of man. *British Medical Journal*, 1(2059), 1456.

Mak, J. W. (1983). Filariasis: an introduction. In Mak, J. W., editor. Filariasis, vol. 19. Malaysia: Institute for Medical Research; 1983. p. 7–10. Bulletin.

Manson, P. (1878). On the Development of *Filaria sanguinis hominis*, and on the Mosquito considered as a Nurse*. *Zoological Journal of the Linnean Society*, 14(75), 304-311.

Mariana, A., Shukor, M. N., Muhd, N. H., Intan, N. B., & Ho, T. M. (2010). Movements and home range of a common species of tree–shrew, *Tupaia glis*, surrounding houses of otoacariasis cases in Kuantan, Pahang, Malaysia. *Asian Pacific Journal of Tropical Medicine*, 3(6), 427-434.

Mat Udin, A. S., Uni, S., Zainuri, N. A., Abdullah Halim, M. R., & Belabut, D. A. (2020). Morphological characteristics of microfilariae in blood smears of the common treeshrew *Tupaia glis* (Mammalia: Scandentia) in Gemas, Negeri Sembilan, *Malaysia. Tropical Biomedicine*, 37(4), 1152–1157.

McInerne, J. O., Littlewood, D. T. J., & Creevey, C. J. (2003). Detecting adaptive molecular evolution: additional tools for the parasitologist. *Advances in Parasitology*, 54, 359-379.

McManus, D. P., & Bowles, J. (1996). Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics.International *Journal for Parasitology*, 26(7), 687-704.

Morales-Hojas, R. (2009). Molecular systematics of filarial parasites, with an emphasis on groups of medical and veterinary importance, and its relevance for epidemiology. *Infection, Genetics and Evolution*, 9(5), 748-759.

Morales-Hojas, R., Cheke, R. A., & Post, R. J. (2006). Molecular systematics of five *Onchocerca* species (Nematoda: Filarioidea) including the human parasite, *O. volvulus*, suggest sympatric speciation. *Journal of Helminthology*,80(03), 281-290.

Mullin, S. W., & Orihel, T. C. (1972). *Tetrapetalonema dunni* sp. n.(Nematoda: Filarioidea) from Malaysian tree shrews. *The Journal of Parasitology*, 1047-1051.

Muslim, A., Fong, M. Y., Mahmud, R., & Sivanandam, S. (2013). Research Note Vector and reservoir host of a case of human *Brugia pahangi* infection in Selangor, peninsular Malaysia. *Tropical Biomedicine*, 30(4), 727-730.

Muslim, A., Fong, M. Y., Mahmud, R., Lau, Y. L., & Sivanandam, S. (2013). *Armigeres subalbatus i*ncriminated as a vector of zoonotic Brugia pahangi filariasis in suburban Kuala Lumpur, Peninsular Malaysia. *Parasites & Vectors*, 6(1), 1-5.

Neiderud, C.-J. (2015). How urbanization affects the epidemiology of emerging infectious diseases. *Infection Ecology & Epidemiology*, 5.

Nowak, R. M., & Walker, E. P. (1999). *Walker's Mammals of the World* (Vol. 1). JHU press.

Nuchprayoon, S., Sangprakarn, S., Junpee, A., Nithiuthai, S., Chungpivat, S., & Poovorawan, Y. (2003). Differentiation of *Brugia malayi* and *Brugia pahangi* by PCR-RFLP of ITS1 and ITS2. *Southeast Asian journal of Tropical Medicine and Public Health*, 34, 67-73.

Orihel, T. C. (1966). *Brugia tupaiae* sp. n.(Nematoda: Filarioidea) in tree shrews (*Tupaia glis*) from Malaysia. The Journal of parasitology, 162-165.

Orihel, T. C., & Eberhard, M. L. (1982). *Mansonella ozzardi*: a redescription with comments on its taxonomic relationships. *The American Journal of Tropical Medicine and Hygiene*, 31(6), 1142-1147.

Orihel, T. C., & Eberhard, M. L. (1998). Zoonotic filariasis. *Clinical Microbiology Reviews*, 11(2), 366-381.

Parnell, J. (2013). The biogeography of the Isthmus of Kra region: A review.*Nordic Journal of Botany*, 31(1), 001-015.

Partono, F., Purnomo, Dennis, D. T., Atmosoedjono, S., Oemijati, S., & Cross, J. H. (1977). *Brugia timori* sp. n.(nematoda: filarioidea) from Flores Island, Indonesia. *The Journal of Parasitology*, 540-546.

Patwardhan, A., Ray, S., & Roy, A. (2014). Molecular markers in phylogenetic studies-a review. *Journal of Phylogenetics & Evolutionary Biology*, 2014.

Payne, J., Francis, C. M., & Phillipps, K. (1985). *Field guide to the mammals of Borneo*. Sabah Society.

Peel, E., & Chardome, M. (1947). Note complementaire sur des filarides de chimpanzes. *Annales De La. Societe Belge De Medecine Tropicale*, 27, 241-250.

Pereira, F. B., Lima, S. S., & Bain, O. (2010). *Oswaldofilaria chabaudi* n. sp.(Nematoda: Onchocercidae) from a South American tropidurid lizard (Squamata: Iguania) with an update on O*swaldofilariinae*. *Parasite*, 17(4), 307-318.

Poulin, R., & Leung, T. L. F. (2010). Taxonomic resolution in parasite community studies: Are things getting worse?. *Parasitology*, 137(13), 1967-1973.

Razzaq, F., Khosa, T., Ahmad, S., Hussain, M., Saeed, Z., Khan, M. A., ... & Iqbal, F. (2015). Prevalence of *Anaplasma phagocytophilum* in horses from Southern Punjab (Pakistan). *Tropical Biomedicine*, 32, 233-239.

Roberts, T. E., Lanier, H. C., Sargis, E. J., & Olson, L. E. (2011). Molecular phylogeny of treeshrews (Mammalia: Scandentia) and the timescale of diversification in Southeast Asia. *Molecular Phylogenetics and Evolution*, 60(3), 358-372.

Rohela, M., Jamaiah, I., Hui, T. T., Mak, J. W., Ithoi, I., & Amirah, A. (2009). *Dirofilaria* causing eye infection in a patient from Malaysia. Southeast *Asian Journal of Tropical Medicine and Public Health*, 40(5), 914-918.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4), 406-425.

Sardarian, K., Maghsood, A. H., Ghiasian, S. A., & Zahirnia, A. H. (2015). Prevalence of zoonotic intestinal parasites in household and stray dogs in rural areas of Hamadan, Western Iran. *Tropical Biomedicine*, 32(2), 240-246.

Smales, L. R. (2002). A cladistic analysis of the tribe Labiostrongylinea Beveridge, 1983 (Nematoda: Cloacinidae) parasitic in macropodoid marsupials (Marsupialia: Macropodoidea), with a redescription of *Parazoniolaimus collaris* Johnston & Mawson, 1939. *Systematic parasitology*, 51(3), 179-197.

Sukhdeo, M. V. (2010). Food webs for parasitologists: A review. *Journal of Parasitology,* 96(2), 273-284.

Sukhdeo, M. V. (2012). Where are the parasites in food webs? *Parasites & Vectors*, 5, 239.

Ta-Tang, T. H., Crainey, J. L., Post, R. J., Luz, S. L., & Rubio, J. M. (2018). Mansonellosis: current perspectives. *Research and reports in tropical medicine*, *9*, 9–24.

Ta-Tang, T. H., Luz, S. L., Crainey, J. L., & Rubio, J. M. (2021). An Overview of the Management of Mansonellosis. *Research and Reports in Tropical Medicine*, 12, 93-105.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725-2729.

Tan, L. H., Fong, M. Y., Mahmud, R., Muslim, A., Lau, Y. L., & Kamarulzaman, A. (2011). Zoonotic *Brugia pahangi* filariasis in a suburbia of Kuala Lumpur City, Malaysia. *Parasitology International*, 60(1), 111-113.

Thompson, R. C. A., Lymbery, A. J., & Smith, A. (2010). Parasites, emerging disease and wildlife conservation. *International Journal for Parasitology*, 40(10), 1163-1170.

Thompson, R. C., Kutz, S. J., & Smith, A. (2009). Parasite zoonoses and wildlife: emerging issues. *International Journal of Environmental Research and Public Health*, 6(2), 678-693.

Uni, S. (1983). Filarial parasites from the black bear of Japan. *Annales De Parasitologie Humaine Et Comparee*, 58(1), 71-84.

Uni, S., Bain, O., Takaoka, H., Katsumi, A., Fujita, H., & Suzuki, Y. (2002). Diversification of *Cercopithifilaria* species (Nematoda: Filarioidea) in Japanese wild ruminants with description of two new species. *Parasite*, *9*(4), 293-304.

Uni, S., Bain, O., & Takaoka, H. (2004). Affinities between *Cutifilaria* (Nematoda: Filarioidea), parasites of deer, and *Mansonella* as seen in a new onchocercid, *M.*(*C.*) *perforata* n. sp., from Japan. *Parasite*, 11(2), 131-140.

Uni, S., Fukuda, M., Agatsuma, T., Bain, O., Otsuka, Y., Nakatani, J., ... & Takaoka, H. (2015). Onchocerca takaokai n. sp.(Nematoda: Filarioidea) in Japanese wild boars *(*Sus scrofa leucomystax*):* Description and molecular identification of intradermal females. *Parasitology International*, 64(6), 493-502.

Uni, S., Bain, O. (2006) New filarial nematode from Japanese serows (*Naemorhedus crispus*: Bobidae) close to parasites from elephants. *Parasite*, 13 (3), 193–200

Uni, S., Udin, A. S. M., Agatsuma, T., Saijuntha, W., Junker, K., Ramli, R., ... & Azirun, M. S. (2017). Morphological and molecular characteristics of *Malayfilaria sofiani* Uni, Mat Udin & Takaoka ng, n. sp.(Nematoda: Filarioidea) from the common treeshrew *Tupaia glis* Diard & Duvaucel (Mammalia: Scandentia) in Peninsular Malaysia. *Parasites & Vectors*, 10(1), 1-14.

Uni, S., Udin, A. S. M., Agatsuma, T., Junker, K., Saijuntha, W., Bunchom, N., ... & Azirun, M. S. (2020). Description, molecular characteristics and *Wolbachia* endosymbionts of *Onchocerca borneensis* Uni, Mat Udin & Takaoka n. sp.(Nematoda: Filarioidea) from the Bornean bearded pig *Sus barbatus* Müller (Cetartiodactyla: Suidae) of Sarawak, Malaysia. *Parasites & Vectors*, *13*(1), 1-16.

Verocai, G. G., Lejeune, M., Beckmen, K. B., Kashivakura, C. K., Veitch, A.M., Popko, R. A., Fuentealba, C., Hoberg, E. P., Kutz, S. J. (2012). Defining parasite biodiversity at high latitudes of NorthAmerica: new host and geographic records for *Onchocerca cervipedis* (Nematoda: Onchocercidae) in moose and caribou. *Parasites & Vectors*. 5 (1), 1-8.

Voris, H. K. (2000). Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. *Journal of Biogeography*, 27(5), 1153-1167.

Wehr, E. E. (1935). A revised classification of the nematode superfamily Filarioidea. *Proceedings of the Helminthological Society of Washington*, 2(2), 84-88

Woodruff, D. S. (2010). Biogeography and conservation in Southeast Asia: how 2.7 million years of repeated environmental fluctuations affect today's patterns and the future of the remaining refugial-phase biodiversity.*Biodiversity and Conservation*, 19(4), 919-941.

Wurster, C. M., Bird, M. I., Bull, I. D., Creed, F., Bryant, C., Dungait, J. A., & Paz, V. (2010). Forest contraction in north equatorial Southeast Asia during the Last Glacial Period. *Proceedings of the National Academy of Sciences*, 107(35), 15508-15511.

Xie, H., Bain, O., & Williams, S. A. (1994). Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. *Parasite*, 1, 141-151.

Yagi, K., Bain, O., & Shoho, C. (1994). Onchocerca suzukii n. sp. O. skrjabini (= O. tarsicola) from a relict bovid, *Capricornis crispus*, in Japan. *Parasite*, 1(4), 349-356.

Yen, P. K. F. (1983). Taxonomy of Malaysian filarial parasites. In: Mak, J. W., editor. Filariasis, 19, Malaysia: Institute for Medical Research; 1983. p. 17–35. Bulletin.