Chapter 1:

General introduction

1.1 Forensic entomology (FE)

Entomology is a word derived from Greek, combining the words *entomon* (meaning insect) and *logos* (meaning science). Therefore, the word entomology conveys the meaning of the study of insects (Gunn, 2009). Forensic entomology denotes the application of insect knowledge or science in legal issues (Hall, 2001). Forensic entomology is a multidisciplinary study which involves taxonomy, ecology, biology, toxicology and physiology.

Forensic entomology is subdivided into 3 principle areas: (i) urban entomology – the area concerning pest infestations (e.g. termites) related to human environments such as buildings; (ii) stored products entomology – the area concerning insect pest infestations or contamination of commercial products such as food, and (iii) medicolegal entomology – the area concerning insects involved in forensic medical case such as murder, suicide and rape. Medicolegal entomology is also known as medicocriminal or forensic medical entomology (Hall, 2001) and it employs the knowledge of insect found at a crime scene to solve the crime (Hall, 1990). In this thesis, the main focus is to study various fly species that are involved in medicolegal entomology.

1.2 Brief history of medicolegal entomology

Medicolegal entomology was first started in China where the first documented forensic entomology case was carried out by a Chinese Commissioner of Justice named Sung Tźu in 13th century. Sung Tźu examined the body and found there were many wounds but all the valuables on the body were left untouched. He suspected that the murderer was a rival and asked all the villagers to take out their sickle for examination. Among the 70-80 sickles, one of them was found to be colonised by flies and that brought the owner to confess to the murder. Sung Tźu solved this crime with the “help”

of flies, which were drawn to the invisible blood stains on the murder weapon (Benecke, 2001, translated from Figure 1).

In 1885, Bergeret d'Arbois, a French doctor, was the first to apply the knowledge of forensic entomology to determining the post-mortem interval (PMI). The case concerned the mummified remains of a child found behind a mantelpiece with fly pupae and larval moths. In his report, he used the entomological specimens as a tool to estimate the PMI of two year (Benecke, 2001). In this context, forensic entomology is not a new science and it has gained a lot of attention and interest by researchers in recent years (Anderson, 2005).

1.3 The use of forensic entomology

When dealing with criminal cases, medicolegal entomology is useful in determining – (i) the time, (ii) the location, and (iii) the cause of death. Entomological evidence is especially relevant after 72 hours of death, as it can provide a more precise estimation of time of death compared to other medical parameters, such as *rigor mortis* (Greenberg & Kunich, 2002) as insects colonise a dead body almost immediately after death (Anderson, 2005). For successful and accurate inference in investigations using entomological evidence, knowledge of insect anatomy or taxonomy, development, behaviour and ecology must first be at hand. In the subsequent chapters, various aspects of entomological studies important for successful use of insect evidence will be described.

1.3.1 Determination of post-mortem interval (PMI)

When a dead body is found, determining the time of death or post-mortem interval (PMI) is very important for investigation purposes. The lapsed time since death can be estimated from entomological evidence based on the observation that different

insect species colonise a corpse immediately after death and in the course of decomposition. This is because the tissue of animals (and humans included) upon death, are attractive to insects as a source of food (Smith, 1986). However, the determination of time of death needs to be complemented with knowledge of an insect's life cycle, growth rate of the insect immature stages and insect species succession associated with various stages of decomposition. All this information, concerning the insect's life cycle, growth rate and succession pattern, often occurs in a predictable manner (Anderson, 2001). Knowledge of either one of these parameters may be more suitable than the other in some of the cases with different decomposition stages. However, the complete information about the insect's growth rate and succession pattern need to be incorporated in order to ensure an accurate estimate of the PMI (Tabor *et al*., 2004). For example, in the early stages of decomposition the use of larval growth development data are more applicable to determine the minimum PMI, while insect succession data are more applicable during the later stages of decomposition (Goff & Flynn, 1991).

Therefore, good experimental designs to acquire this information (e.g. the insect's life cycle, growth rate and succession pattern) are essential to obtain accurate data for precise estimation. The principles of the studies for fly succession will be discussed in Chapter 4.

1.3.2 Inferring the place of death

Insects found on a dead body can be used to determine whether the site, where it is found, is the primary (the place where the crime took place) or secondary (the place where the body was placed, after death) crime scene. Some insect species found on the body do not belong to that area or location, which may suggest that the body had been moved (Gennard, 2007). However, this kind of deduction requires baseline data for species distribution of a particular geographical region to be established. In this thesis,

chapter 3 describes preliminary ecological distribution of Malaysian fly species, which could serve as a reference in future forensic entomological studies.

1.3.3 Estimating the cause of death

Another aspect of forensic analysis is the use of insects to determine whether the victim's death was due to poisoning. If the victim was indeed poisoned, the flesh may contain the toxin which can be recovered by analysing the maggots found on the body (Gennard, 2007). The study of detecting drugs or toxin from the maggots that inhabit corpses is termed 'entomotoxicology'. It is a relatively new branch of forensic entomology (Introna *et al*., 2001). The presence and type of drugs in the body would have an impact on the growth and time interval between larval stages (Goff *et al*., 1989 and 1991; Musvasva *et al*., 2001). This, in turn, may influence the accuracy in the calculation of PMI (Bourel *et al*., 1999).

Insects found on a dead body can also provide information in determining the lethal wound, setting it apart from additional wounds present on the body. This is because flies tend to lay their eggs at natural openings or orifices. Any unnatural openings will point to the additional wound, thereby providing clues to the cause of death. However, it is important to differentiate whether the wound is perimortem or postmortem as failure to do so may lead to a wrong deduction.

1.4 Forensically important insect families

Insects with forensic importance cover a wide range of species from the orders of Diptera, Coleoptera, Hymenoptera, Lepidoptera, Trichoptera, Blattodea, Dermaptera, Isoptera, Hemiptera and Phthiraptera (Smith, 1986). However, the first to arrive and present in abundance are always the ones from the order Diptera.

Among the families of Diptera, three of them are of major importance in forensic entomology, namely Calliphoridae (blow fly), Sarcophagidae (flesh fly) and Muscidae (house flies) (Anderson, 2001; Watson & Carlton, 2003; Byrd & Castner, 2010).

1.4.1 Calliphoridae – blow flies

Smith (1986) stated that the family Calliphoridae includes the genera of greatest importance in forensic entomology, particularly in estimating the post-mortem interval (PMI) of human cadavers. This family comprises over a thousand described species in all zoogeographical regions (Byrd & Castner, 2010). Some of them are known to be synanthropic, or having a close relation with human activities, such as *Chrysomya megacephala*.

In Malaysia, Calliphoridae consist of several forensically important genera such as the *Chrysomya*, *Lucilia*, *Hemipyrellia*, *Calliphora* and *Hypopygiopsis*. Apart from forensic importance, they are also essential in recycling of nutrients and removal or breakdown of vertebrate carcasses (Byrd & Castner, 2001). Some of them are medically important because they are involved in myiasis of human or animal, e.g. *Chrysomya bezziana* and *Lucilia* species.

Calliphorids are among the first to arrive and colonise a corpse (Byrd & Castner, 2010). From some of the experiments conducted, they are reported to arrive within minutes after death (Goff, 2000).

1.4.2 Sarcophagidae – flesh flies

Species from this family are found throughout the world, mostly in tropical and warm temperate countries (Byrd & Castner, 2001). Apart from being forensically

important, species of Sarcophagidae also serve as pollinators as they are always found on flowers, being attracted to the nectar (Bänziger & Pape, 2002).

Sarcophagidae contains three subfamilies: (i) Sarcophaginae (ii) Miltogramminae, and (iii) Paramacronychiinae (Pape, 1996). However, only the subfamily Sarcophaginae is forensically important (Pape, 1996). The species of subfamily Miltogramminae are mainly kleptoparasites of solitary bees or wasps while the species of subfamily Paramacronychiinae are predators or parasitoids of bees, grasshoppers and cicadas (Pape, 1996).

Sarcophagidae are viviparous and sometimes, albeit rarely, ovoviviparous (Shewell, 1986; Colless & McAlpine, 1991). They deposit the mature first-instar larvae to decomposing matter (Smith, 1986), and as such, eggs obtained from a dead body should not be concluded to originate from the Sarcophagidae. In addition, the sarcophagid's larvae obtained from corpses must include this fact into consideration, and the time for egg hatching must be eliminated for accurate PMI estimations (Byrd & Castner, 2001).

1.5 Morphology-based identification

Species identification of entomological specimens is a critical step in forensic entomology investigation. This is because accurate identification is vital in determining accurate investigation outcomes (Smith, 1986). However, this also represents the main challenge for forensic entomologist (Greenberg & Kunich, 2002). There are several limitations in using morphology-based identification. Firstly, the specimens obtained from the crime scene are not necessarily intact or may be damaged, and this may lead to a lack of characters for identification. Secondly, most specimens collected are in immature stages and there is currently a lack of comprehensive taxonomic literature for immature stages, particularly the family Sarcophagidae (Smith, 1996; Byrd and Castner

2010). Finally, immature specimens often need to be reared until adulthood to facilitate the species identification. This step is time consuming and without proper laboratory skills and conditions the specimens often do not survive until maturity. In addition to all the reasons mentioned, comprehensive identification keys of forensically important fly species for regional usage are difficult to obtain due to different species distributions between locations (Greenberg & Kunich, 2002). Therefore, a localised distribution study of forensically important species is essential to establish entomological data, to be useful as a reference for forensic investigation.

Currently, morphology-based identification is facing another critical global problem in which there are a dwindling number of skilled and experienced taxonomists (Anderson, 2005). As a result, alternative methods are proposed to circumvent the limitation of morphology-based identification. One of the more popular and consistent alternatives is the use of DNA-based identification which is currently favoured by most researchers (Wallman & Donnellan, 2001; Well *et al*., 2001; Schroeder *et al*., 2003a; Wells & Williams, 2005; Nelson *et al*., 2007 & 2008; Tan *et al*., 2009; Singh *et al*. 2010).

1.6 DNA-based identification

Morphology-based species identification for some of the young instar larvae can be difficult or impossible (Gennard, 2007). In recent years, there has been an increase in the use of DNA sequence data in the studies of carrion flies as an aid to accurately identify insect species and also to overcome the limitations of morphology-based identification when dealing with immature specimens (Wallman & Donnellan, 2001; Well *et al*., 2001; Schroeder *et al*., 2003a; Wells & Williams, 2005).

Molecular techniques have proven to be advantageous as they are applicable to any life stages, as long as sufficient amount of specimen's DNA is obtained (Harvey *et*

al., 2003; Tan *et al*., 2009). They also offer the potential to distinguish morphologically similar species or even genera, such as in the case of the species from the family Sarcophagidae (Wells & Sperling, 2001; Zehner *et al*., 2004; Meiklejohn *et al*., 2009). DNA-based identification techniques are also easily transferable between laboratories and are not limited to the requirements of specific taxonomical expertise (Wells & Williams, 2005).

1.6.1 DNA-based identification methods

There are several methods currently available which are preferred by most researchers in this field, such as direct DNA sequencing (Wallman & Donnellan, 2001; Well *et al*., 2001; Wells & Williams, 2005; Nelson *et al*., 2007; Tan *et al*., 2009; Singh *et al*. 2010) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Chen & Shih, 2003; Schroeder *et al*., 2003a).

The method known as PCR-RFLP is both rapid and inexpensive (Gennard, 2007). This method has also demonstrated convincing results in species identification. However, a slight disadvantage of this method lies in the possible presence of intra-specific variation and polymorphism in individuals, which may confuse results and interpretations.

Several PCR-RFLP studies had been carried out to differentiate various species of Calliphoridae, Sarcophagidae and Muscidae (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Vicent *et al*. 2000; Chen & Shih, 2003; Schroeder *et al*., 2003a; Ratcliffe *et al*., 2003; Ames *et al*., 2006). Most of these studies were done using the *cytochrome c oxidase* subunit I gene of the mitochondrial DNA, whilst a few others utilised the variations residing within the internal transcribed spacers (ITS) between the ribosomal DNA.

DNA sequencing has been recently widely used in forensic entomology to permit ease of species identification. Its usefulness has been shown in many published studies in forensic entomology. Sperling *et al*. (1994) were the first to utilise this method in species identification for post-mortem interval estimation. Since then, many researchers employed this method for species identification of forensically important flies such as Calliphoridae, Sarcophagidae and Muscidae (Well *et al*., 2001).

1.6.2 Genetic markers

At present, there are many published studies on the use of DNA-based identification method of forensically important insect species. There are two major DNA regions implicated in species identification: nuclear DNA (nuDNA) and mitochondrial DNA (mtDNA). Nuclear DNA was reported to be used earlier than mtDNA in forensic entomological application (Benecke & Wells, 2001). Nuclear DNA regions which are frequently used are ribosomal DNA genes, such as the 28S rDNA gene (Stevens & Wall, 2001) and its spacer like internal transcribed spacer (ITS) (Ratcliffe *et al*., Nelson *et al*., 2008; Song *et al*., 2008).

However, at present the utility of mtDNA is practiced intensively by researchers and has been proposed for insect species identification in forensic investigations (Benecke & Wells, 2001). This is because mtDNA are: (i) highly conserved among phyla, (ii) maternally inherited with no recombinantion event, (iii) present in high copy numbers and easy to amplify, (iv) evolving faster than nuclear DNA, and (v) lacking non-coding regions (e.g. introns) compared to nuclear gene (Moriyama & Powell, 1997). In addition, universal primers had been published and widely used in many animal systematic studies (Simon *et al*., 1994).

Insect mtDNA comprises approximately 37 genes which include 22 transfer RNAs, two ribosomal RNAs and 13 peptides (Gennard, 2007). In documented studies,

particularly in forensic entomology, regions like *NADH dehydrogenase* gene subunits 4 and 5 (ND4 and ND5), and *cytochrome c oxidase* gene subunits I and II (COI and COII) are among the prominent targets for species identification (Wells & Sperling, 1999; Harvey *et al*., 2003; Zehner *et al*., 2004; Wallman *et al*., 2005; Tan *et al*., 2009).

1.6.2.1 mtDNA *cytochrome c oxidase* gene

The *cytochrome c oxidase* gene encodes a protein that serves as an enzyme that transfer electrons from cytochrome to oxygen and converts it to water. There are 13 subunits in *cytochrome c oxidase*, and three of them, namely subunits I, II and III are encoded by mtDNA (Gennard, 2007)

The *cytochrome c oxidase* gene in mtDNA is the marker of choice for most researchers to serve as genetic marker in species identification because these DNA regions show high variability among the taxa but is relatively conserved within the taxon (Clary & Wolstenholme, 1985).

Cytochrome c oxidase gene subunit I is the biggest subunit amongst the three subunits. Since 2004, this region has been recognised as the marker of choice for DNA-barcoding in identifying and distinguishing various groups of organisms (Hebert *et al*., 2003; Hebert & Gregory; 2005; Meiklejohn *et al*., 2009). The complete or partial sequences of COI and COII are frequently utilised by the researchers for forensically important fly species identification (Sperling *et al*., 1994; Malgorn & Coquoa, 1999; Harvey *et al*., 2003; Nelson *et al*., 2007; Tan *et al*., 2010a & b).

1.6.2.2 nuDNA 28S rRNA gene analysis

Other than mtDNA, the nuDNA is also widely used by researchers for species identification. In such cases, the ribosomal RNA (rRNA) genes are one of the preferred DNA regions, in particular, the 28S ribosomal DNA (rDNA) (Stevens & Wall, 2001).

This is largely due to the fact that the nuclear genes encoding rRNA are present in multiple copies, making them easy to be amplified and subsequently analysed by standard sequencing methods (Byrd & Castner, 2010). These ribosomal coding genes evolve slowly and this makes them suitable for evolutionary studies although their DNA sequences are also useful for species identification (Stevens & Wall, 2001).

1.7 Objectives

The objectives of this study are:

1. To establish baseline data for forensically important fly species with regard to:
    a. Morphological taxonomic study of adult sarcophagid flies (Chapter 2)
    b. Species distribution on different ecological types (Chapter 3)
    c. Dipteran successional pattern on decomposing carcass in Borneo rainforest (Chapter 4)
2. To use DNA-based methods (i.e. DNA sequencing and PCR-RFLP) to characterise forensically important fly species in Malaysia, particularly for species in families:
    a. Calliphoridae (Chapter 5)
    b. Sarcophagidae (Chapter 6)

Chapter 2:

Taxonomical study of Sarcophagidae

2.0 Abstract

In Malaysia, Sarcophagidae have similar forensic importance as Calliphoridae due to their involvement in forensic cases. However, their identification was mostly achieved to the genus level only. Current morphological identification of Sarcophagidae is hindered by its high degree of resemblance in anatomical characteristics and the lack of expertise and experienced taxonomist in Malaysia. It is difficult to distinguish Sarcophagidae to the species level as well as its genus, as both the larvae and adults have high similarities in external appearance. In this study, a detailed description of external taxonomic characters of Malaysian Sarcophagidae was made using *Boettcherisca karnyi* as the reference species. Examination and comparison of 28 Malaysian sarcophagine species were performed to identify which morphological characters may be important and potentially useful for constructing a taxonomic key. Eight major taxonomic characters were examined and evaluated; (i) thoracic chaetotaxy, (ii) hairiness on propleuron, (iii) hairiness on male sternite 4, (iv) pollinosity on abdomen, (v) colouration of genitalia segments, (vi) colouration of antennal segments and palpi, (vii) pattern on wing vein, R1, and (viii) shape of ventralia. The number of postsutural dorsocentral britles (*dc*) is useful for initial segregation of genera in the sarcophagine subfamily while sternite 4 bearing tuft of hairs and shape of ventralia are apomorphic characters, which are useful for distinguishing the genus. In addition, whether the R1 wing vein is setulose is particularly useful for distinguishing the species. With the insight of this study, together with additional works of the male genitalia analysis, a taxonomy key for Sarcophagidae could be established in the near future for Malaysia.

2.1 Introduction

Sarcophagidae are one of the important insect families in the field of forensic entomology (Smith, 1986; Greenberg, 1991; Lee, 1996; Byrd & Castner, 2001; Carvalho & Linhares 2001; Lee *et al*., 2004; Sukontason *et al*., 2001, 2007). They are often the first insects to colonise a corpse (Byrd & Castner, 2001). The Sarcophagidae are also the predominant species recovered from indoor human cadavers in the southeastern part of the United States of America during summer (Smith, 1986; Shewell, 1987). Apart from that, they are very useful in determining the post-mortem interval for a dead body which is physically isolated or covered by garbage, as they may be the only carrion-fly larvae found under that circumstance (Wells *et al*., 2001).

Species identification of Sarcophagidae is therefore crucial in forensic cases as they are important forensic indicators. However, due to lack of expertise and experience in Malaysia, their identification in forensic cases was mostly limited to the genus level (Lee *et al*., 2004; Mohd. Salleh *et al*., 2007). Current morphological species identification of Sarcophagidae is hindered by its high levels of resemblance in anatomical characteristics. It is not easy to distinguish Sarcophagidae to the species level, or even to its genus level, as both larvae and adults have high similarities in external appearance (Smith, 1986; Wells *et al*., 2001; Byrd & Castner, 2010). To date, only the identification of adult male flies can be satisfactorily confirmed, in which the different characteristics of the genitalia are used for species identification (Rohdendorf, 1937; Sugiyama & Kano, 1984; Wells *et al*., 2001; Kurahashi, 2002; Carvalho & Mello-Patiu, 2008; Chaiwong *et al*., 2009). The identification of female flies however remains unresolved due to lack of diagnostic characters even in genitalia. Larval species identification based on morphological characteristics for Sarcophagidae is also notoriously difficult or perhaps still impossible for most of the larval stages (Byrd & Castner, 2010), which warrant the larvae to be reared to adult stage, in order to be

identified using adult characteristics. Identification is still hampered as previously stated, because only the male fly can be unambiguously identified.

Although several reliable taxonomy keys have been published, these keys are restricted to a selected genus or subgenus such as *Boettcherisca* (Lopes, 1961), *Sarcorohdendorfia* (Lopes *et al*., 1979) and *Heteronychia* (Whitmore, 2009); or geographical regions such as Hawaii (Lopes, 1961), Japan (Kano *et al*., 1967), Africa (Zumpt, 1972), Nepal (Kano & Shinonaga, 1994) and South America (Carvalho & Mello-Patiu, 2008).

In Thailand, main contributors of Sarcophagidae taxonomy were Tumrasvin & Kano (1979). Reports of new species or new records of Thailand Sarcophagidae were also made by Pape & Bänziger (2000 & 2003), Kurahashi & Sukontason (2004) and Chawong *et al*. (2009). However, these reports focused mainly on localised diversity and therefore are not suitable or insufficient for application in Malaysia.

In contrast to Thailand, Malaysian Sarcophagidae are poorly studied. Fragmented records and patchy information on Malaysian Sarcophagidae can be found in studies of Kano & Lopes (1969), Lopes *et al*. (1977), Shinonaga & Beaver (1979), Sugiyama *et al*. (1990) and Kurahashi & Leh (2007). Although a larger collection of documented Sarcophagidae with a taxonomic key for oriental Sarcophagidae was published by Kurahashi (2002), half of the number of sarcophagid species described therein does not belong to Malaysia.

The classification of Sarcophagidae has become more challenging as the classification and the nomenclature of this family are still at a research stage, particularly the divisions of genus level which remained inconsistent due to conflicting opinions from different authorities. To date, there are three different systems of classifying Sarcophagidae available (Downes, 1965; Lopes, 1969 & 1989; Lopes *et al*., 1977; Dear, 1980; Verves, 1986; Verves, 1986; Shewell, 1987; Rohdendorf, 1937 &

1965; Pape, 1996). One of the classification systems used for alleged common flesh flies is *Sarcophaga* sensu lato in the broadest sense as proposed by Pape (1996). As opposed to this system of classification, the second classification system is more traditional and uses the original generic position, proposed by authorities such as Lopes (1969 & 1989), Lopes *et al*. (1977), Rohdendorf (1937 & 1965), Shewell (1987) and Verves (1986). The third classification system is an intermediate between these two systems (Downes, 1965; Dear, 1980).

With the combination of all the problems faced by classification and nomenclature of Sarcophagidae, the taxonomy of Malaysian Sarcophagidae is significantly hampered even for the most common species, and has become the main problem for species identification of Sarcophagidae (Carvalho & Mello-Patiu, 2008). Therefore, this study aims to provide a detailed study of taxonomy, identification and classification, which would be particularly useful for Malaysian Sarcophagidae.

2.2 Objectives

The objectives of this study are:

1. To establish a detailed description of external taxonomic characters of Malaysian Sarcophagidae, using *Boettcherisca karnyi* as the reference species.
2. To identify and evaluate morphological characters that are important and useful for establishing a taxonomic identification key, for commonly found sarcophagine species.

2.3 Materials and methods

2.3.1 Collection of fly specimens

The Sarcophagidae flies were collected using rotten meat as bait at various types of habitat throughout Malaysia to cover a wide range of geographical and ecological

environments (for details, refer to chapter 3, Table 3.1). The baits used in the studies were mostly beef and occasionally chicken. A standard of 0.5 kg weight of bait was used each time. The bait was left to decompose for at least a day at room temperature prior to use. At each collection, the bait was exposed to the air. Flies were caught with a hand net using a sweeping method, and this was carried out for duration of one to two hours in the morning at each collecting site. Collected flies were killed using in a killing jar containing cotton wetted with ethyl acetate (to produce vapour).

After the flies were killed and pinned, the legs as well as the proboscis belonging to the specimens were pulled downwards while the wings were properly arranged to allow easy examination under a microscope. Genitalia of male specimen were extended out, as it is widely used as distinctive taxonomic characters for species identification. Extension of male genitalia was carried out under the microscope to ensure complete extension. This step had to be performed with care, to avoid damage to the specimen. A small piece of hard paper was inserted in between the genitalia and fifth sternite to prevent the genitalia from contracting back into the abdomen (Figure 2.1). This step was necessary to facilitate a definite identification for Sarcophagidae (Pape, 1987).

Fly specimens were then identified to species using morphological keys from Kurahashi (pers. comm.) for Sarcophagidae. The classification of sarcophagid species was achieved according to the system described by Lopes (1969 & 1989), Lopes *et al*. (1977), Verves (1986), Shewell (1987) and Rohdendorf (1937 & 1965). The identified flies were preserved in an insect storage box as voucher specimens and kept in the Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya for future reference.

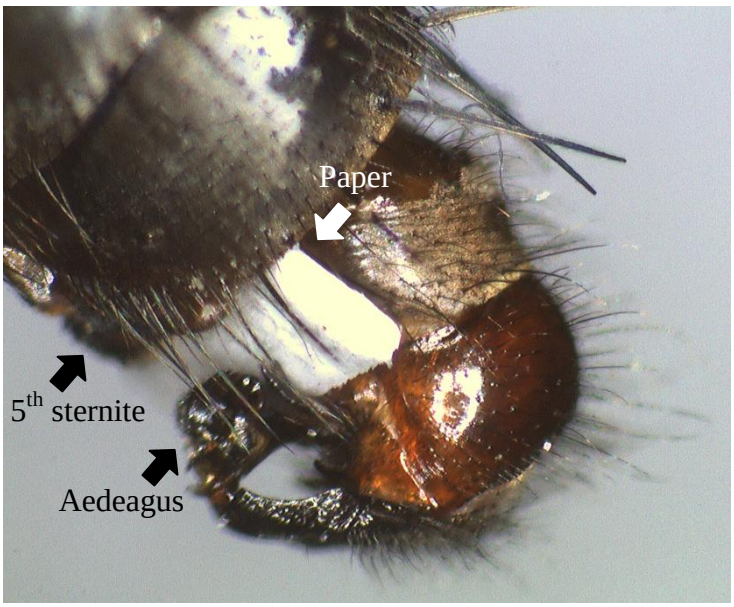


Figure 2.1: A small piece of hard paper was inserted between the fifth sternite and aedeagus of *Boettcherisca karnyi* (Hardy, 1927).

2.3.2 Study of basic external taxonomic characters

Detailed analysis of basic external taxonomic characters of adult sarcophagines was performed on male and female flies of a common Malaysian species, *B. karnyi* (Hardy, 1927). The male and female specimens were examined with detail under the microscope. Body parts and bristles from head, thorax and abdomen were examined and named. Anterior, posterior, lateral, dorsal and ventral views of the head, thorax and abdomen were examined. Subsequently, illustrations were drawn with detailed labeling. Most morphological terminology terms used in illustrations were in accordance with those of Senior-White (1940). Names of bristles were labeled in abbreviation and italicised while body parts were labeled in standard text. Wing venation terminology was identified according to McAlpine *et al*. (1981).

When the need arises to achieve greater detail of genitalic morphological characteristics, postabdomen sections were dissected. Membranes connecting tergites 3 and 4 were cut using forceps. A drop of absolute ethanol was applied to the dissected postabdomen before it was immersed in 10% potassium hydroxide overnight to clear

the integument. Following overnight immersion, tergites 4 and 5 were separated from genitalic segment GS_1 for male and from tergite 6 from female. After cleaning the genitalia with water, the genitalia were studied and examined under a microscope in glycerol. Morphological details of genitalia were illustrated.

2.3.3 Study of important taxonomic characters

Twenty eight species of Malaysian sarcophagine were used in this study (Table 2.1). Several important taxonomic characters were examined carefully in each of the species and distinctive characters specific to a particular species were recorded. Eight important taxonomic characters were then chosen based on traits that potentially showing high variation among species. These characters were evaluated for its significance in taxonomic level differentiation.

2.3.4 Taxonomical classification of Malaysian Sarcophagidae

Classification of Malaysian Sarcophagidae was studied. Subfamilies and tribes of Malaysian Sarcophagidae were identified. Taxonomic keys to separating these subfamilies and tribes of Malaysian Sarcophagidae were constructed with the modifications and alteration of three available taxonomic keys from Shewell (1987), Colless & McAlpine (1991) and Nandi (2002). The taxonomic key constructed was confirmed by the examination of selected Sarcophagidae species from the National Museum of Nature and Science, Tokyo, Japan. The nomenclature of Sarcophagidae was assessed according to International Code of Zoological Nomenclature.

2.4 Results

2.4.1 Fly specimens

A total of 42 sarcophagine fly species were collected from habitats covering a large range of environmental conditions, which included the mangrove swamp, coastal area, highland area and different types of forest. In this study, only 28 species were chosen for the taxonomical study, as they are the easily found species (Table 2.1).

Table 2.1: List of sarcophagine fly species collected in this study. Asterisk indicates the species used in the study of important taxonomic characters.

Number	Sarcophagid Species
1.	* *Alisarcophaga gressitti* (Hall et Bohart, 1948)
2.	* *Boettcherisca javanica* Lopes, 1961
3.	* *Boettcherisca highlandica* Kurahashi & Tan, 2009
4.	* *Boettcherisca karnyi* (Hardy, 1927)
5.	* *Boettcherisca krathonmai* Pape & Bänziger, 2000
6.	* *Boettcherisca peregrina* (Robineau-Desvoidy, 1830)
7.	* *Harpagophalla kempi* (Senior-White, 1924)
8.	* *Hosarcophaga serrata* (Ho, 1938)
9.	* *Iranihindia martellata* (Senior-White, 1924)
10.	* *Leucomyia alba* (Schiner, 1868)
11.	* *Lioproctia pattoni* (Senior-White, 1924)
12.	* *Lioproctia saprianovae* (Pape & Bänziger, 2000)
13.	* *Liopygia ruficornis* (Fabricius, 1794)
14.	* *Liosarcophaga brevicornis* (Ho, 1934)
15.	* *Liosarcophaga dux* (Thomson, 1869)
16.	*Liosarcophaga mimobrevicornis* Sugiyama, 1990
17.	* *Myorhina borneensis* (Shinonaga & Lopes, 1975)
18.	* *Myorhina globovesica* (Ye, 1980)
19.	*Myorhina sp. nov.*
20.	* *Parasarcophaga albiceps (Meigen, 1826)*
21.	*Parasarcophaga javana (Macquart, 1851)*
22.	* *Parasarcophaga lopesi (Verves, 1980)*
23.	* *Parasarcophaga misera (Walker, 1849)*
24.	*Parasarcophaga omari Kurahashi & Leh 2007*
25.	*Parasarcophaga scopariiformis (Senior-White, 1927)*
26.	* *Parasarcophaga taenionota (Wiedemann, 1819)*
27.	* *Rosellea notabilis (Kano & Lopes, 1969)*
28.	*Sarcorohdendorfia antilope (Böttcher, 1913)*
29.	* *Sarcorohdendorfia inextricata (Walker, 1859)*
30.	* *Sarcorohdendorfia seniorwhitei (Walker, 1859)*
31.	*Sarcorohdendorfia sp. nov.*
32.	* *Sarcosolomonia crinita (Parker, 1971)*
33.	* *Sarcosolomonia rohdendorfi Nandi, 1976*
34.	* *Seniorwhitea princeps (Wiedemann, 1830)*
35.	*Seniorwhitea orientalis (Parker, 1917)*

Table 2.1: *(continued)*

Number	Sarcophagid Species
36.	*Sinonipponia bengalensis Nandi 1977*
37.	*Sinonipponia hainanensis (Ho, 1936)*
38.	*Sarcophaga (s. lat.) aquila Sugiyama, 1990*
39.	*Sarcophaga (s. lat.) brachiata Sugiyama, 1990*
40.	** Sarcophaga (s. lat.) longifilia (Salem, 1946)*
41.	*Sarcophaga (s. lat.) quinqueramosa Sugiyama, 1990*
42.	*Sarcophaga (s. lat.) robustispinosa Sugiyama, 1990*

2.4.2 Study of basic external taxonomic characters

A commonly found Malaysian Sarcophagidae species, *B. karnyi* (Hardy, 1927) was chosen as the fly model in the study of external taxonomic characters. Detailed illustrations of head, thorax, abdomen, wings and genitalia of *B. karnyi* are shown in Figures 2.2 to 2.11. Different views of the body parts (e.g. dorsal, ventral and lateral) were examined and illustrated to achieve a greater detail of identification. The abbreviation used for body parts, chaetotaxy and venation in the illustrations to describe the anatomy are explained in Tables 2.2 to 2.4. The anatomical terms used in this study are mostly adopted from McAlpine *et al*. (1981).

Adults of Sarcophagidae are very similar to the Tachinidae (Pape, 1987). They can be easily confused or misidentified by the inexperienced entomologist. Dipterans including sarcophagine are divided into three sections or tagmata: the head, the thorax and the abdomen.

A big portion of a sarcophagid's head is taken up by the compound eyes. The eyes of Sarcophagidae are dichoptic, which means that there is a distance between two eyes in male and female (Fan, 1992). The distance between the eyes in males is smaller compared to females, with a smaller value of width of frons (WF) (Figure 2.3). Therefore, the sex of sarcophagid flies is not easy to identify by looking at their eyes, although this character is usually used to determine the sex of Calliphoridae. The frontal stripe of Sarcophagidae is usually wider or at least the same as the width of the

parafrontal on the head (Figure 2.2b). A pair of antennae is located at the front of the head. Each antenna is divided into three main segments: the first antennal segment (AS1), the second antennal segment (AS2) and the third antennal segment (AS3). On the AS3, the longest segment among the three, a feather-like structure, named the arista can be seen (Figure 2.3a). The proboscis of the fly is below the head, inside the buccal cavity.

The thorax is divided into three segments: prescutum, scutum and scutellum. The scutum bears a pair of wings, and a pair of legs is located on each segment of the thorax. Like other true flies, sarcophagine flies have one pair of functional wings and the hind pair of wings has been modified into halteres (Figure 2.4). Wings are hyaline without any pattern. The legs are named according to their arrangement from the head, namely the fore leg (at the prescutum), the mid leg (at the scutum) and the hind leg (at the scutellum) (Figure 2.7).

The abdomen is divided into five major segments preceding the genitalia. The abdominal structure from the dorsal view consists of tergites while the abdominal structures from the ventral view ate the sternites. The tergites are divided into first to fifth tergites but only four segments are visible, where as the first and second tergites are combined into a syntergite (Figures 2.8a & b). Five segmented sternites are visible from ventral view and they overlap with corresponding tergites (Figures 2.9a & b). The fifth sternite is mostly concealed. The posterior margin of the fifth sternite in males is strongly bilobate, like an inverted Y and usually indented. For females, the fifth sternite is straight or shallowly concave at the posterior margin.

The postabdomen comprises the genital segments after the sixth tergite and sternite, where the genitalia are located. In males of sarcophaginae, the genitalia are usually large and exposed. The sixth tergite is reduced and sometimes modified into a membranous structure. Usually, this structure is not visible unless it is pulled out from

the postabdomen. The sixth sternite is a sclerotised and elongated narrowness bow-liked structure that hides inside the fifth sternite, which forms the marginal frame of the genital pouch. The spiracle of the sixth sternite is situated on the membrane near adjacent segment. Segments seven and eight are combined as systernites, and they are located on dorsal side. Here, the sternites have degenerated and fused with tergites and cannot be separated. It is named as the first genitalic segment (GS_1). The second genitalic segment (GS_2), or the epandrium (ep), is the ninth tergite (Figure 2.10a). Other important structures of male genitalia are the cerci, surstylus, pregonite, postgonite and aedeagus. The aedeagus consists of the theca, harpe, corpus, ventralia, vesica, juxta, juxtal process and stylus (Figure 2.11). In females, tergite 6 is smaller and narrower than tergite 5 while tergite 7 is frequently absent, or, if present, seldom complete dorsally and present only lateral plates. Tergite 8 is always present but reduced to two elongated plates, detached laterally and sometimes incomplete at the centre by membrane (Figure 2.10b). Compared to sternite 5, sternites 6 and 7 are wider, while sternite 8 is smaller and sometimes reduced.

2.4.2.1 Morphological illustrations

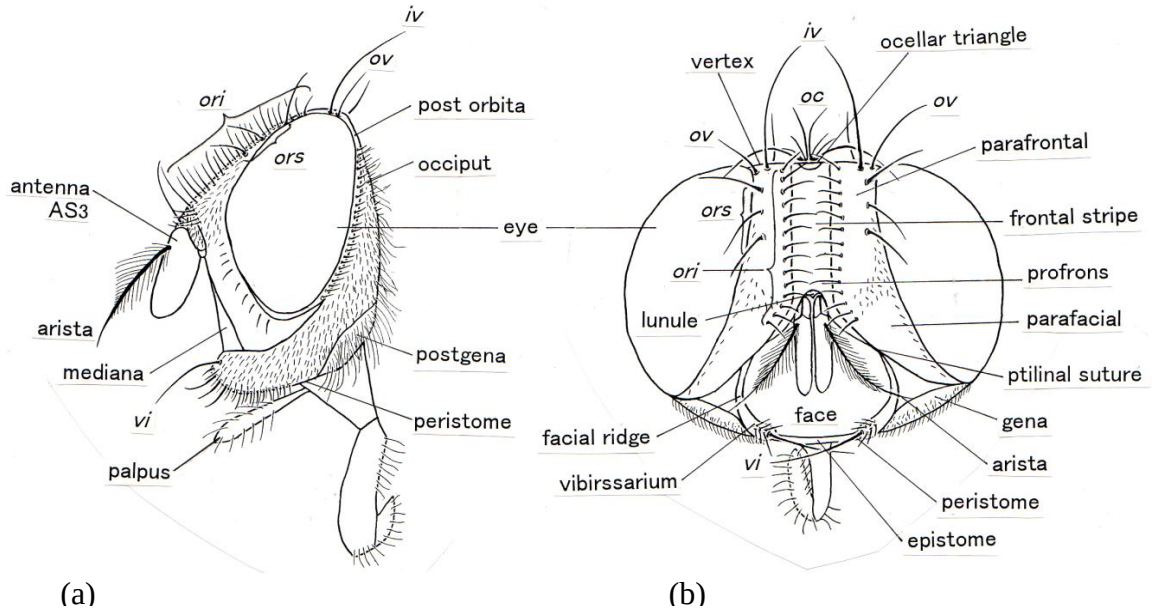


Figure 2.2: Head of *Boettcherisca karnyi*, (a) left lateral view (b) anterior view. See Tables 2.2 & 2.3 for abbreviations.

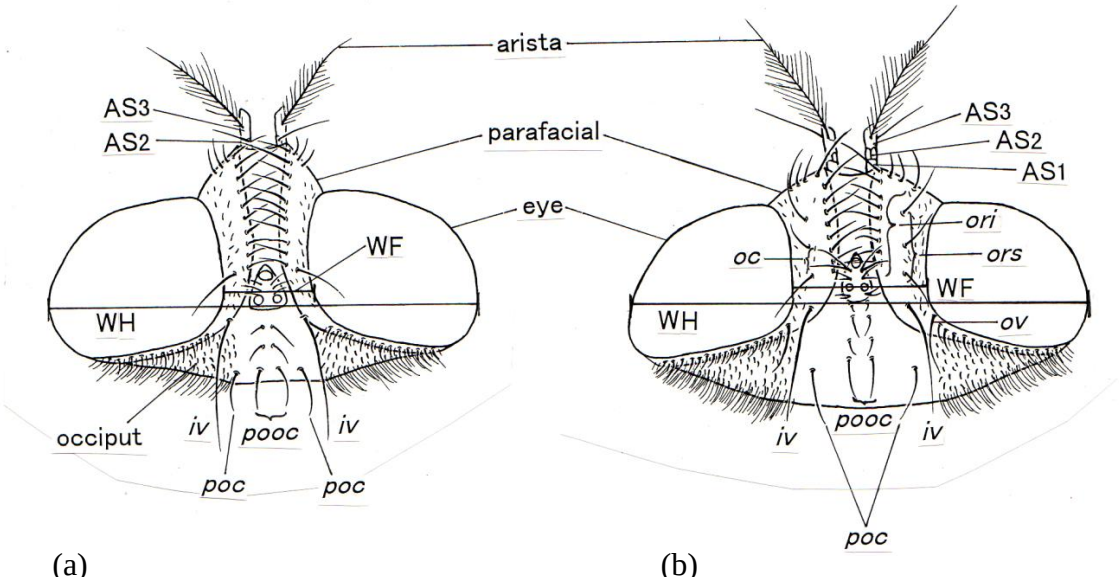


Figure 2.3: Head of *Boettcherisca karnyi*, (a) dorsal view of male (b) dorsal view of female. See Tables 2.2 and 2.3 for abbreviations.

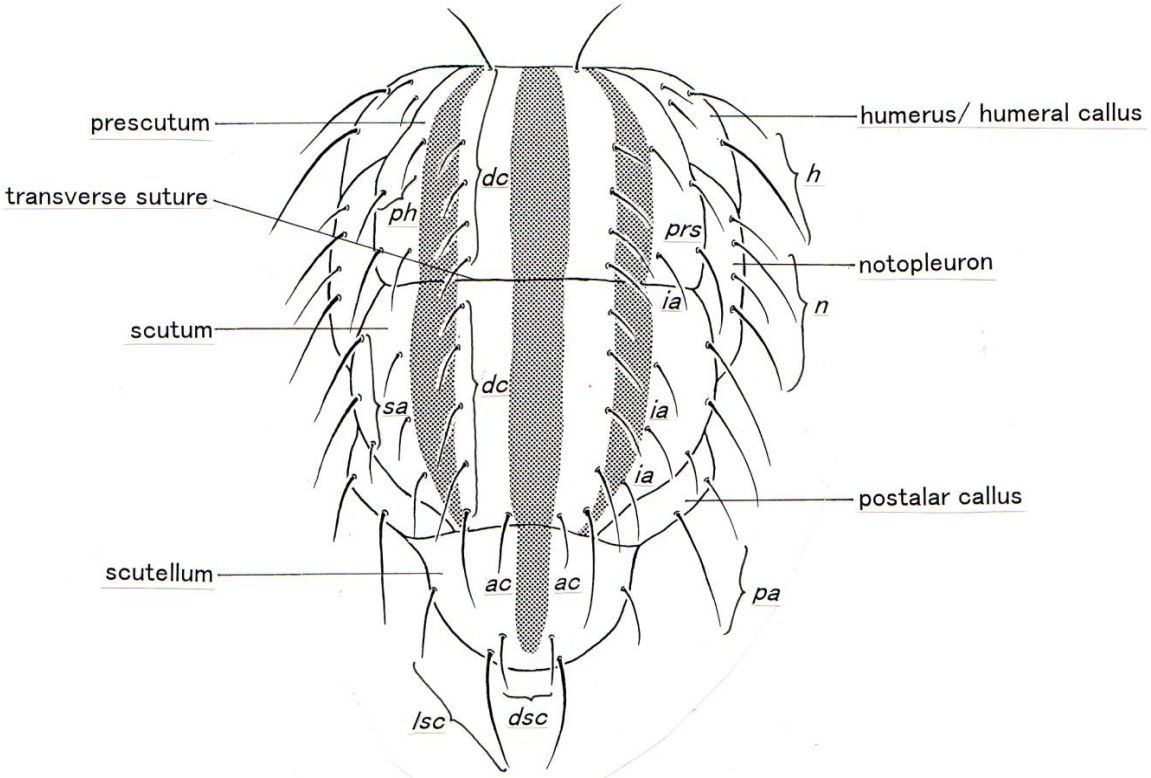


Figure 2.4: Thorax of *Boettcherisca karnyi*, dorsal view. See Table 2.3 for abbreviations.

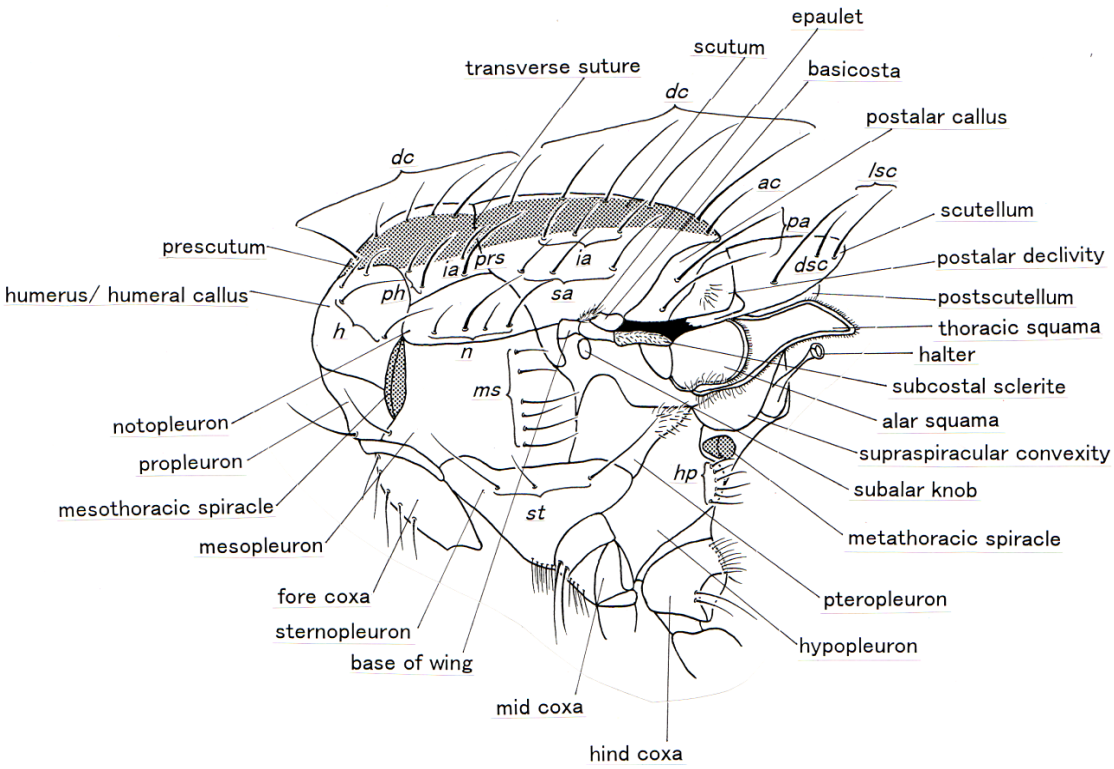


Figure 2.5: Thorax of *Boettcherisca karnyi*, left lateral view. See Table 2.3 for abbreviations.

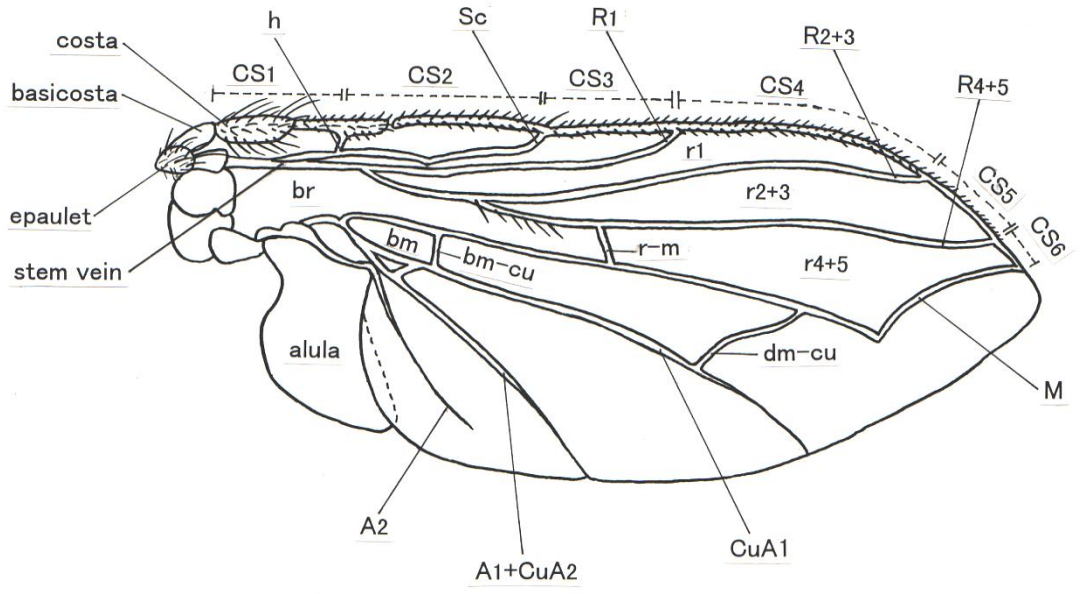


Figure 2.6: Wing of *Boettcherisca karnyi*, dorsal view of right wing. See Table 2.4 for abbreviations.

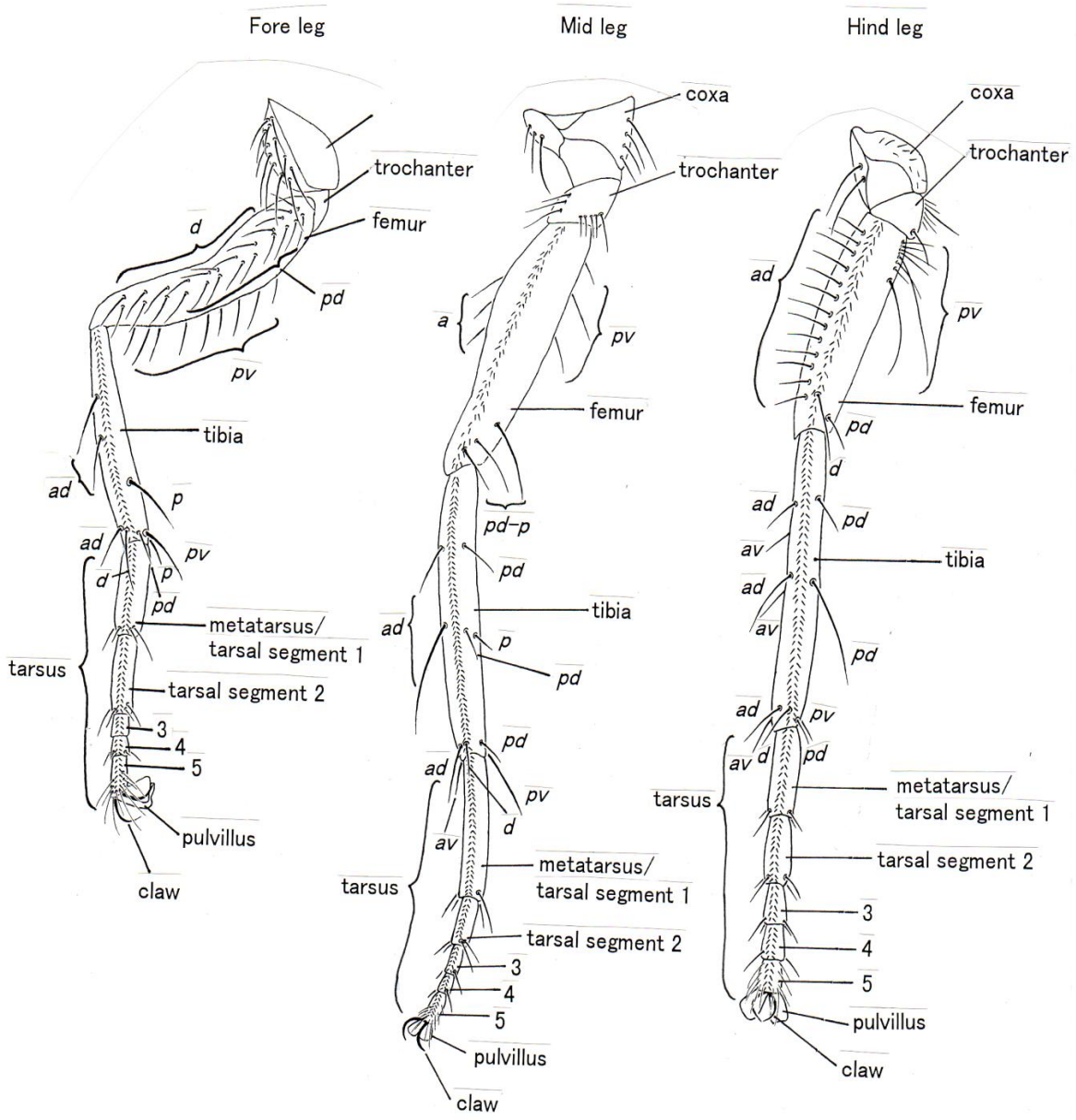


Figure 2.7: Legs of *Boettcherisca karnyi*, (a) dorsal view of left fore leg (b) dorsal view of left mid leg (c) dorsal view of left hind leg. See Table 2.3 for abbreviations.

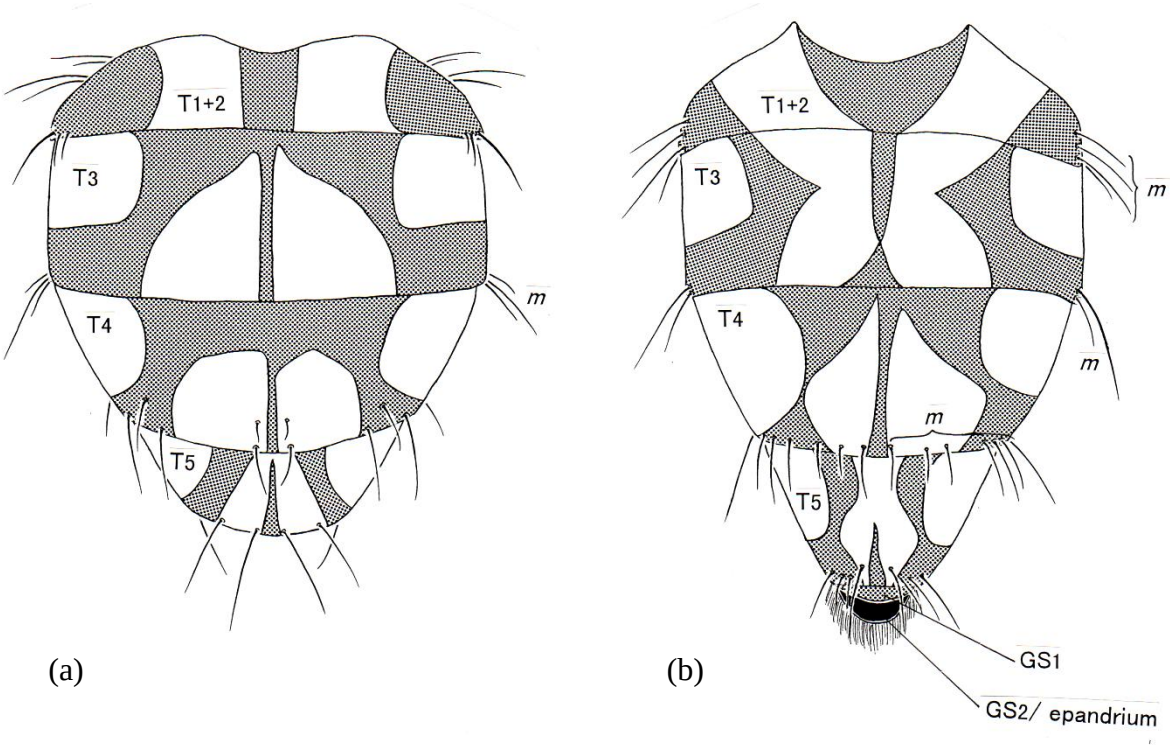


Figure 2.8: Abdomen of *Boettcherisca karnyi*, (a) dorsal view of female (b) dorsal view of male. See Tables 2.2 & 2.3 for abbreviations.

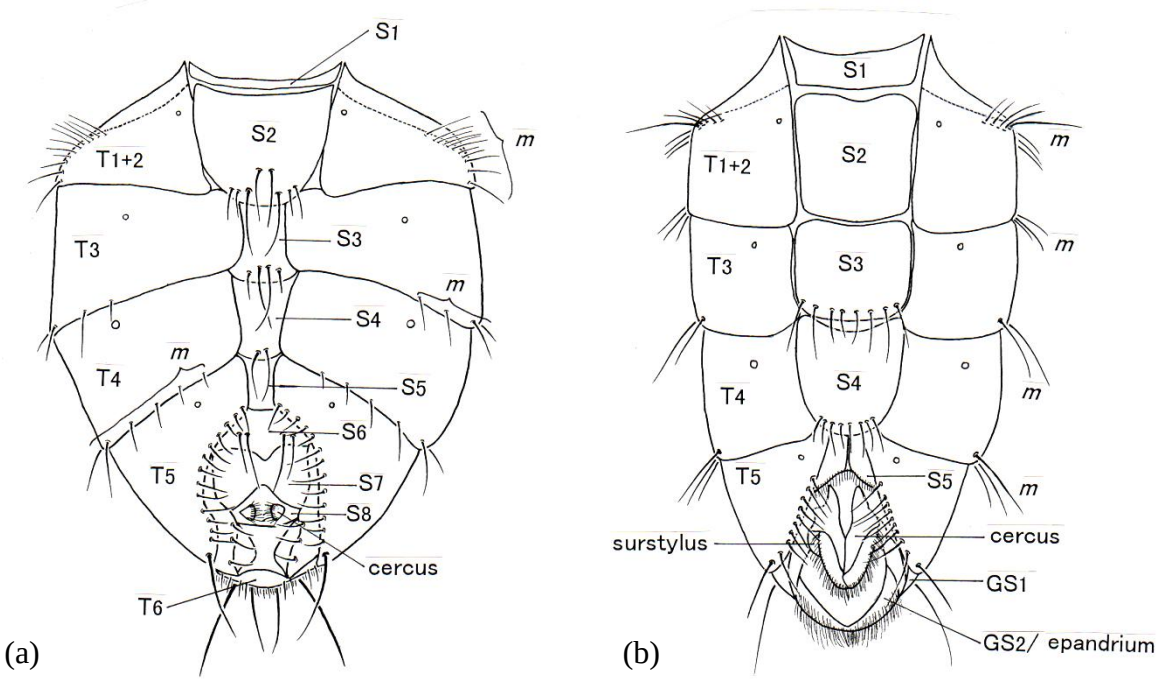


Figure 2.9: Abdomen of *Boettcherisca karnyi*, (a) ventral view of female (b) ventral view of male. See Tables 2.2 & 2.3 for abbreviations.

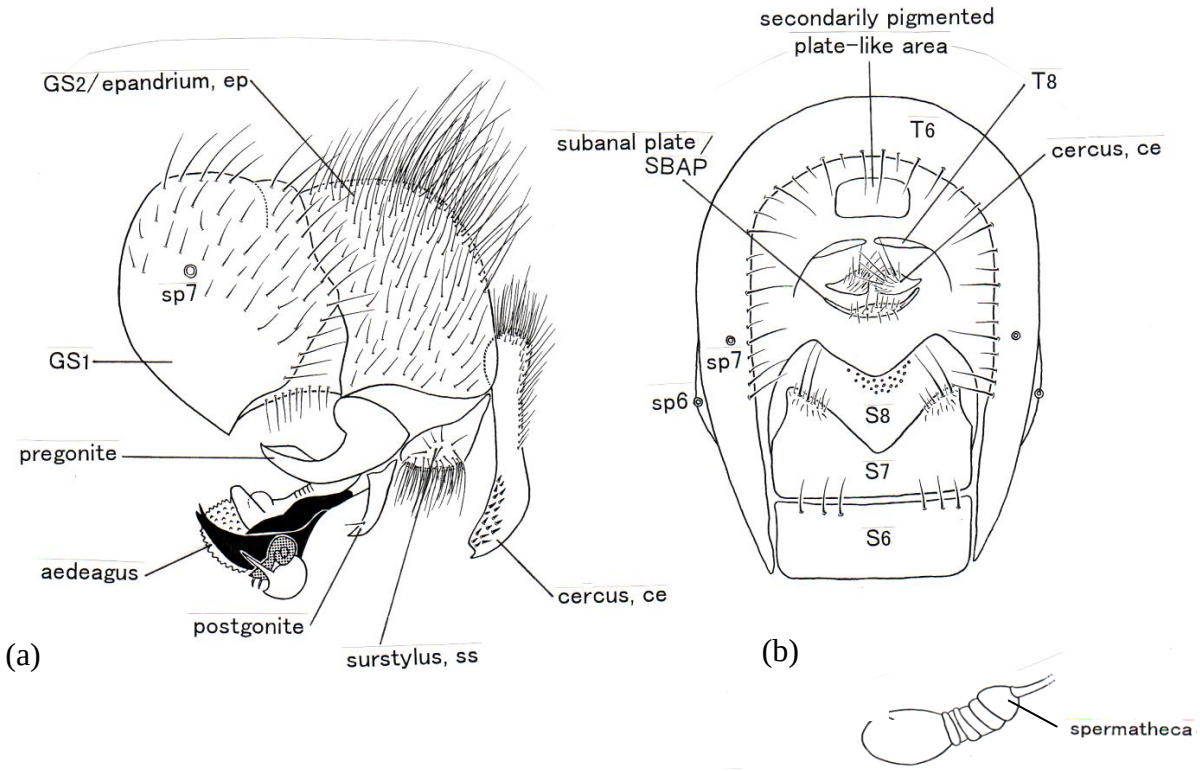


Figure 2.10: Postabdominal section of *Boettcherisca karnyi*, (a) left lateral view of male (b) posterior view of female. Inset: spermatheca. See Table 2.2 for abbreviations.

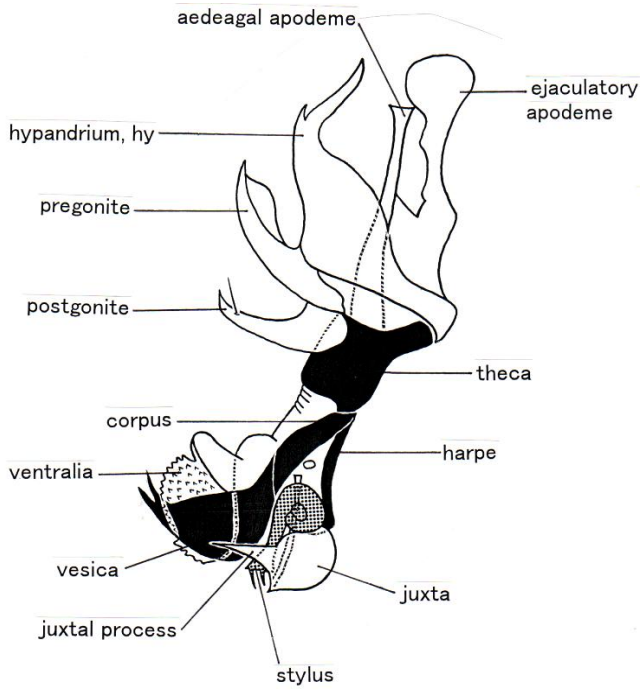


Figure 2.11: Aedeagus of *Boettcherisca karnyi*, left lateral view. Close up of male genitalia from Fig. 5.9(a).

2.4.2.2 Abbreviations used in illustrations

Table 2.2: Abbreviations of terminology for body parts used in study.

Abbreviation	Name in full	Location of body segment
AS1	First antennal segment	Head
AS2	Second antennal segment	Head
AS3	Third antennal segment	Head
WF	Width of frons	Head
WH	Width of head	Head
GS_1	First genitalic segment	Abdomen
GS_2	Second genitalic segment	Abdomen
T_{1+2}	First and second syntergite	Abdomen
T_3	Third tergite	Abdomen
T_4	Fourth tergite	Abdomen
T_5	Fifth tergite	Abdomen
T_6	Sixth tergite	Abdomen
T_8	Eighth tergite	Abdomen
S_1	First sternite	Abdomen
S_2	Second sternite	Abdomen
S_3	Third sternite	Abdomen
S_4	Fourth sternite	Abdomen
S_5	Fifth sternite	Abdomen
S_6	Sixth sternite	Abdomen
S_7	Seventh sternite	Abdomen
S_8	Eighth sternite	Abdomen
sp6	Sixth spiracle	Postabdomen
sp7	Seventh spiracle	Postabdomen

Table 2.3: Abbreviations of chaetotaxy used in this study.

Abbreviation	Name in full	Location of body segment
iv	inner vertical bristles	Head (vertex)
oc	ocellar bristles	Head (vertex)
ori	frontal bristles	Head (frons)
ors	frontal-orbital bristles	Head (frons)
ov	outer vertical bristles	Head (vertex)
poc	postoccipital bristles	Head (vertex)
pooc	postvertical bristles	Head (vertex)
vi	vibrissae	Head (frons)
ac	acrostichal bristles	Thorax (scutum)
dc	dorsocentral britles	Thorax (scutum)
dsc	discal scutellar britles	Thorax (scutellum)
h	humeral britles	Thorax (prescutum)
hp	hypopleural bristles	Thorax (scutum)
ia	intraalar bristles	Thorax (scutum)
lsc	lateral scutellar bristles	Thorax (scutum)
ms	mesopleural bristles	Thorax (scutum)
n	notopleural bristles	Thorax (scutum)
pa	postalar bristles	Thorax (scutum)
ph	posthumeral bristles	Thorax (scutum)
prs	presutural bristles	Thorax
sa	supraalar bristles	Thorax
st	sternopleural bristles	Thorax
a	anterior bristles	Leg
ad	anterodorsal bristles	Leg
av	anteroventral bristles	Leg
d	dorsal bristles	Leg
p	posterior bristles	Leg
pd	posterodorsal bristles	Leg
pd-p	posterodorsal-posterior bristles	Leg
pv	posteroventral bristles	Leg
m	marginal bristles	Abdomen

Table 2.4: Abbreviations of venation used in this study.

Abbreviation	Name in full	Cell / vein
A_1+CuA_2	Fifth longitudinal vein	Vein
A_2	Second anal vein	Vein
bm-cu	Basal mediocubital crossvein	Vein
dm-cu	Discal mediocubital crossvein	Vein
CS_1	First cross section	Vein
CS_2	Second cross section	Vein
CS_3	Third cross section	Vein
CS_4	Fourth cross section	Vein
CS_5	Fifth cross section	Vein
CS_6	Sixth cross section	Vein
CuA_1	Fifth longitudinal	Vein
h	Humeral cross vein	Vein
M	Medial vein	Vein
r-m	Anterior cross vein	Vein
R_1	First longitudinal vein	Vein
R_{2+3}	Second longitudinal vein	Vein
R_{4+5}	Third longitudinal vein	Vein
Sc	Auxiliary	Vein
br	Basal radial cell	Cell
bm	Basal medial cell	Cell
r_1	Marginal	Cell
r_{2+3}	First submarginal	Cell
r_{4+5}	First posterior	Cell

2.4.3 Important morphological characters in Malaysian Sarcophagidae

Examination and comparison of 28 Malaysian sarcophagine species were carried out to identify eight important morphological characters, which may be potentially useful for constructing a taxonomic key. The result of these important morphological characters is shown in Table 2.5 with the comparison among 28 Malaysian sarcophagine species. In general, the number of postsutural dorsocentral britles (*dc*) is an important character to segregate the genera in the sarcophagine subfamily, which ranges from 3, 4 and 5. However, this character is insufficient for generic discrimination and therefore other morphological characters are needed for further identification. In addition, some of the species possess a distinctive character, which is suitable for generic and species identification. These characters include the ventralia bilobed and bearing numerous spines (*Boettcherisca* species), sternite 4 with tuft of hairs (*Sarcorohdenfia* species), colouration of antennal segments and palpi (*L. ruficornis* and *I. martellata*) and R_1 wing vein being setulose (*A. gressitti* and *S. rohdendorfi*).

Table 2.5: Important morphological characters found in 28 Malaysian sarcophagidae species.

Variable	Characters	Species involved [a]
Colouration of antennal segments and palpi	Yellowish orange / Fuscous black	*L. ruficornis* and *I. martellata* / 26 remaining species
Pollinosity on abdomen	Golden-dusted / Grey-dusted	*B. krathonmai*, *B. highlandica*, *L. saprianovae*, *Myorhina* spp., *R. notabilis* and *S. seniorwhitei* / 21 remaining species
Colouration of genitalic segments (GS_1 and GS_2)	Brown / Fuscous black	*B. javanica, B. highlandica, B. krathonmai, L. ruficornis, L. dux* * / 23 remaining species
Wing vein (R_1)	Setulose / Bare	*A. gressitti* and *S. rohdendorfi* / 26 remaining species
Hairiness on propleuron	Present / Absent	*A. gressitti, Boettcherisca* spp., *H. serrata, Lioproctia* spp., *S.* (s.lat.) *longifilia* and *S. princeps* / 18 remaining species
Hairiness on male sternite 4 (S_4)	With tuft of hairs / Bare	*Sarcorohdendorfia* spp./ 26 remaining species
Thoracic chaetotaxy	Postsutural *dc* 3 / Postsutural *dc* 4 / Postsutural *dc* 5	*Myorhina* spp. and *Sarcosolomonia* spp. / *L. alba, Lioproctia* spp., *R. notabilis, Sarcorohdendorfia* spp., and *Seniorwhitea* spp. / *A. gressitti, Boettcherisca* spp., *H. kempi, H. serrata, I. martellata, Liosarcophaga* spp., *Parasarcophaga* spp., *Seniorwhitea princeps* and *Sarcophaga* (s. lat.) *longifilia*
Shape of ventralia	Bilobed and bearing numerous spines / Other shapes	*Boettcherisca* spp. / 23 remaining species

[a] Species list refers to Table 2.1

* Species with variations, which possesses two opposed characters

2.4.4 Taxonomical classification of Malaysian Sarcophagidae

A taxonomic key to separate the subfamily level for Malaysian Sarcophagidae was constructed with modification from Shewell (1987), Colless & McAlpine (1991) and Nandi (2002). There are only two subfamilies of Sarcophagidae in Malaysia, which can be easily distinguished by the pattern of the arista (refer to section 2.4.4.1).

Most of the forensically important sarcophagid species are from the subfamily Sarcophaginae (Nandi, 2002). Under this subfamily, Protodexiini and Sarcophagini are the only two tribes found in Malaysia. The characteristic of presutral acrostichal bristles (*ac*) on the thorax is the trait able to separate the tribes of Protodexiini and Sarcophagini (refer to section 2.4.4.2).

2.4.4.1 Key to the subfamilies of Malaysian Sarcophagidae

Modified from Shewell (1987), Colless & McAlpine (1991) and Nandi (2002).

1. Arista plumose; hind coxa with fringe of hairs on posterior margin. Four notopleural bristles, two strong primary bristles, two weak subprimary bristles, oriented intervally. Sernites 3 to 4 fully exposed, not concealed with corresponding tergite ……..……. **Subfamily Sarcophaginae**
- Arista bare or pubescent, rarely short plumose; hind coxa bare on posterior margin. Two notopleural bristles. Sternites 3-4 concealed with corresponding tergites … ...…..……..……...… **Subfamily Miltogramminae**

2.4.4.2 Key to the tribes of Malaysian Sarcophaginae

1. Presutral *ac* strong, usually arranged in distinct row ……..…. **Tribe Protodexiini**
- Presutural *ac* absent, at most relatively weakly developed ac present on anterior or posterior prescutelar areas …….……..… **Tribe Sarcophagini**

2.5 Discussion

There are several factors that determine the usefulness of taxonomic keys. One requires a large enough number of specimens representing similar and different species, from different habitats, populations and localities to allow comprehensive morphological observations and meaningful comparisons to be made. Collection of specimens should also be accompanied by good and appropriate specimen preservation techniques to allow an accurate description of morphological characteristics. Only then can one identify distinctive derived characters (i.e. autapomorphic) which can be used as definitive keys for species identification.

As mentioned, proper specimen preservation is essential to allow accurate identification of species. The legs of flies were pulled downward to expose the pleuron of the thorax and the underside of the abdomen to ensure that bristles were exposed clearly to facilitate the examination. If specimens are not preserved in good condition, the important characteristic traits could be lost and would hamper species identification. In the case of sarcophagid identification, the hairiness of the propleuron is important for species discrimination. Therefore, the fore legs of the fly must not block the view of the bristles on the propleuron. In addition, care must be taken when pulling out the male genitalia, as it is a vital character to identify Sarcophagidae species unambiguously. Previously, many of the taxonomy keys were established based on the characters of the male genitalia (Rohdendorf, 1937; Sugiyama & Kano, 1984; Wells *et al*., 2001; Kurahashi, 2002; Carvalho & Mello-Patiu, 2008; Chaiwong *et al*., 2009).

Examination and comparison of specimens from different localities across wider geographical and ecological ranges are necessary to confirm the morphological variations. This is to ensure that the identification takes into account a range of variation in a species.

Important and distinctive morphological characters need to be identified before a taxonomic key can be developed. This is because only distinctive characters would allow one to distinguish the organisms to their species level. Although distinctive characters are very crucial, a taxonomic key cannot be developed without comparing the similarities between species (i.e. synapomorphic characters). A taxonomic key is an identification system with a succession of paired choices leading to the identification of a species. Such a key is always branched in two at each stage and therefore is named a dichotomous key. A dichotomous key may not reflect species relationships; however, closely related species usually share similar morphological characters, and as such would be grouped together.

In this study, eight distinctive and important morphological characters, including one of male genitalia, were identified and compared among 28 Malaysian sarcophagine species (Table 2.5). These characters were described with contrasting traits (e.g. absent and present, with and without), which represents the basic principals of establishing a dichotomous key. These important characters include the chaetotaxy, pollinosity on abdomen, colour of antennae and genitalic segments (Shewell, 1987; Triplehorn & Johnson, 2005). Chaetotaxy is the arrangement of major bristles (e.g. number of bristles, present or absent of bristles), which always provides the diagnostic characters in species identification and the systematics of flies (Senior-White *et al*., 1940; Triplehorn & Johnson, 2005). In this study, the number of postsutural dorsocentral britles (e.g. 3, 4 and 5) is found to be important for genus discrimination. In addition, an apomorphic character (a tuft of hair at forth sternite), possessed by *Sarcorohdendorfia* species, is also found to be useful for genus identification. This observation corroborates with Lopes and Kano (1979) in their study of *Sarcorohdendorfia*.

The classification of Sarcophagidae remains arguable where different classification systems are use by different researchers. In this study, the classification

system described by Lopes (1969 & 1989), Lopes *et al*. (1977), Rohdendorf (1937 & 1965), Shewell (1987) and Verves (1986) is adopted. Pape's (1996) classification system which proposed the placement of most species (approximately 130 genera) into one genus of *Sarcophaga* in the broad sense is not adopted in this study, due to two reasons. Firstly, Pape's classfication system would mean that over 790 species will be placed in one genus. In comparison, other genera in subfamilies Miltogramminae and Paramacronychiinae for example, have not more than 100 species in one genus. It should be noted that the generic and subgeneric concepts are far from stable in Pape's catalogue. For example, Kano and Lopes (1969) has placed *Burmanomyia notabilis* as a member of the genus *Burmanomyia*, while according to Pape (1996), this species belongs to the subgenus *Lioproctia* (species named as *Sarcophaga* (*Lioproctia*) *notabilis*). This apparent discrepancy obviously warrants further investigation and analysis.

The second reason is that there are sufficient morphological characters recognised that may be used to group the *Sarcophaga* sensu lato species into its distinct genus, and division of these fly species into genera or even subgenera and species group can be achieved. For example, the ventralia of all species of *Boettcherisca* is well developed, bilobed and bearing numerous spines (Lopes, 1960; Kurahashi, 2002). These are apomorphic characters which are sufficient to group the species into this distinct genus. One of the new species from Peninsular Malaysia, *Boettcherica highlandica*, has been discovered and placed under this genus due to the pattern of its ventralia which is similar to what is described above (Kurahashi & Tan, 2009).

Classifications are always based on scientific hypotheses, whereby these hypotheses may be changed or rejected as understanding of insect increases through studies (Triplehorn & Johnson, 2005). Changes in those classifications are common and revealed the progress of fundamental scientific research and change of ideas.

Agreement of these changes indicates the advance of understanding of insect diversity and relationship of the species. Therefore, Sarcophagidae studies are still at the early stage of understanding, and as such, the relationship and diversity of the species are far from classification stability.

2.6 Conclusion

The taxonomy, classification and systematics of Sarcophagidae are still in their infancy in which the genus and subgenus level are yet to be resolved. Active studies are currently in progress globally to solve this problem, including Malaysia. High similarity of general external morphology among Sarcophagidae species is an obstruction in establishing their taxonomy keys. To date, with little understanding and sparse information of Sarcophagidae, no suitable taxonomic key has yet been published for identifying Malaysian sarcophagid genera and species. A taxonomy key which covers Malaysian Sarcophagidae needs to be established. With the insight of this study, a taxonomic key of the Sarcophagidae could be established in the near future for Malaysia with analysis of the male genitalia.

Chapter 3:

Species checklist and ecological distribution study of Calliphoridae and Sarcophagidae

3.0 Abstract

One of the initial clues derived from the insects obtained from a corpse is whether the place, where the body is found, is the first crime scene or not. The principle where this information is derived lies with the distribution and ecological knowledge of these species. In this study, a Malaysian forensically important Dipteran species checklist and their geographical and ecological distributions of the Calliphoridae and Sarcophagidae are obtained. Eighteen species of Calliphoridae and 42 species of Sarcophagidae were collected from Peninsular Malaysia and Sarawak. Among these species, seven new records and three new species were discovered. Fly species collected were also categorised into seven ecological habitats according to their occurrences. Some data (e.g. the habitat specific species and endemic species) obtained in this study will facilitate the ecological study of forensically important carrion flies and may provide some useful information for criminal investigation.

3.1 Introduction

Maggots found on a corpse can be used to estimate the PMI. However, the type of fly species that are attracted to a corpse are influenced by environment factors and this information is considered to be vital when interpreting the PMI. Information of the insect species on the dead body can also offer information like whether the corpse has been moved. Insect species collected on a corpse is an indicator of different habitats and geographic regions (Gennard, 2007). For this to be successful, however, it is important to acquire the knowledge of the species' diversity and their geographic as well as ecological distribution, before any meaningful deductions in relation to forensics can be made (Barbosa *et al*., 2009; Richard *et al*., 2009). The entire fauna of forensically important fly species in a particular area should be established for forensic entomology data to be relevant and effective (Barbosa *et al*., 2009).

Anderson (2001) stated that there are many factors influencing the diversity of species colonising carrion, which include the geographical and biogeoclimatic zones and aspects such as habitat, vegetation, soil type, and meteorological conditions of the area. These factors would have a major impact on the groups and species of insects inhabiting that particular area. In addition, these factors also influence the decomposition stage and its rate, which in turn may directly affect the species composition. Among the various meteorological conditions, temperature and humidity are known to be major factors influencing the fauna on a corpse. Other than these, the altitude of a crime scene should also be taken into consideration as different altitudes may be represented by varying species.

Calliphoridae have been well studied in contrast to Sarcophagidae. These Calliphoridae studies cover a wide range of aspects, which include the species checklist, diversity, abundance, geographical distribution and habitat preference (Senior-White *et al*., 1940; Kurahashi & Fauran, 1980; Pont, 1980; Hanski, 1981; Kurahashi, 1981;

Kurahashi, 1982; Kurahashi, 1986; Schumann, 1986; Rognes, 1991; Fan, 1992; De Jong & Chadwick, 1997; Kurahashi *et al*., 1997; Kurahashi & Chowanadisai, 2001; Kurahashi & Afzal, 2002; Mariluis & Mulieri, 2003; Verves, 2003; Schnack & Mariluis, 2004; Verves, 2005; Marinho *et al*., 2006; Baz *et al*., 2007; Mariluis *et al*., 2008).

An intensive series of studies concerning species checklist and distribution of Calliphoridae of the Oriental and Australasian regions have been carried out by Kurahashi and his colleagues. Checklists which cover the Oriental regions include those for Malaysia and Singapore (Singh *et al*., 1979; Kurahashi *et al*., 1997), Thailand (Tumrasvin *et al*., 1979; Kurahashi, 1995), Taiwan (Kurahashi, 1987a), Indochina (Kurahashi & Chowanadisai, 2001) and Pakistan (Kurahashi & Afzal, 2002) while checklists for the Australasian regions were for New Caledonia (Kurahashi & Fauran, 1980), Fiji (Kurahashi, 1981), Vanuatu (Kurahashi, 1982) and New Guinea, Bismarck Archipelago and Bougainville Island (Kurahashi, 1986).

The earliest groups of scientists who undertook species checklists and the distribution of Malaysian Calliphoridae were Senior-White *et al*. (1940), followed by Singh *et al*. (1979) and Kurahashi *et al*. (1997). Comprehensive data regarding the species checklists and ecological distributions as well as bionomics of Malaysian Calliphoridae became available from their studies; however, a revision with updated data is needed for current investigation particularly for forensic purposes, as the most recent checklist was made more than 10 years ago and is perhaps outdated (Kurahashi *et al*. 1997).

In contrast with Calliphoridae, faunistic studies on Sarcophagidae are relatively limited, particularly in Malaysia. Species checklists for most of the zoogeographical regions are still in the compilation stages, where intensive researches are still being carried out (Kano *et al*., 1967; Lopes, 1969, 1989; Lopes *et al*., 1977; Dear, 1980;

Verves, 1986; Sugiyama *et al*, 1990; Fan, 1992; Pape, 1996; Povolný & Verves, 1997; Pape, 1998; Kano *et al*., 1999; Nandi, 2002; Kurahashi & Leh, 2007). These Sarcophagidae species checklists were published according to the specific geographical regions, including the Palaearctic region (Kano *et al*., 1967; Verves, 1986; Fan, 1992; Povolný & Verves, 1997; Pape, 1998), the Oriental region (Lopes *et al*., 1977; Sugiyama *et al*, 1990; Kano *et al*., 1999; Nandi, 2002; Kurahashi & Leh, 2007), the Australasian region (Lopes, 1989), the Afrotropical region (Dear, 1980) and the Neotropical region (Lopes, 1969).

The Malaysian sarcophagid fauna was investigated by Senior-White (1940), Lopes *et al*. (1977), Kano & Lopes (1979), Shinonaga & Beaver (1979), Singh *et al*. (1979), Kurahashi & Kano (1984), Sugiyama *et al*. (1990) and Kurahashi & Leh (2007), starting from mid 20th century. Their works mainly involved the documentation of species, new records of known species in Malaysia and also describing newly discovered species. While a general species listing is available for certain states and localities, a comprehensive species checklist, including useful information on their ecological distribution and habitat preferences are currently lacking in Malaysia. Therefore, this study is carried out in the attempt to establish a comprehensive checklist, including details on species diversity and distribution for Malaysian Sarcophagidae.

3.2 Objective

The objectives of the study described in this chapter are:

1. To update and revise the species checklist of forensically important Calliphoridae and to study their ecological distributions in Malaysia.
2. To establish a comprehensive species checklist of Malaysian Sarcophagidae and to study their ecological distributions in Malaysia.

3.3 Materials and methods

3.3.1 Collection sites

In order to obtain a comprehensive species distribution data, different types of habitat from various geographical regions in Peninsular Malaysia and the state of Sarawak were selected as study areas. These habitats cover a large range of environmental condition, which included the mangrove swamp, coastal area, highland area and different types of forest, from tropical rain forest to montane forest as well as mossy forest. The varied nature of these habitats enables a selection of fauna which are highly representative of the entire environmental tolerance. The geographical coordinates and altitude of each collection site were recorded using a wireless Global Positioning System (GPS) (Holux, Taiwan). Habitat and environment of each collection site were also recorded.

3.3.2 Collection of fly specimens

Collection, preservation and storage methods of fly specimens were as described in section 2.3.1. In this study, collection of fly species belonging to the families Calliphoridae and Sarcophagidae was emphasised.

3.4 Results

3.4.1 Collection sites

A total of 53 collection sites, which covered a range of habitats including urbanised area, highland forest, lowland forest, mangrove swamp, costal area and island, were investigated in this study. For urbanised area, collections were done within the vicinity of the center of the town as well as in residential areas, while for the coastal areas, two types of habitats were selected in this study, which are the sandy shore and

the rocky shore. Details of each collection site, such as coordinate, altitude, and habitat are shown in Table 3.1. The altitudes of collection site ranged from 5m to 2036m.

The collection sites chosen for the distribution study lie across ten states within the Peninsular Malaysia and one state of East Malaysia (Figure 3.1). The inclusion of the state of Sarawak was regarded as a preliminary investigation for distribution studies of flies in the Borneo region.

Table 3.1: Collection sites with detail of state, locality, coordinates, altitude and habitat.

Location	Coordinates	Altitute	Habitat
Langkawi, Kedah	06° 19’ 43.06” N 99° 43’ 43.85” E	13m	Sandy shore
Bukit Mertajam, Penang	05° 26’ 42.23” N 100° 30’ 33.65” E	21m	Lowland forest
Kulim, Penang	05° 22’ 10.20” N 100° 34’ 20.83” E	29m	Urban area
Gopeng, Perak	04° 29’ 24.59” N 101° 06’ 18.84” E	50m	Urban area
Kuala Kangsar, Perak	04° 36’ 47.67” N 101° 54’ 09.33” E	117m	Stream area
Matang, Perak	04° 47’ 45.58” N 100° 37’ 34.50” E	10m	Mangrove swamp
Taiping, Perak	04° 29’ 04.85” N 100° 44’ 07.33” E	132m	Stream area
Mile 19 Tapah, Perak	04° 22’ 08.16” N 101° 19’ 58.70” E	608m	Lowland forest
Kuala Woh, Perak	04° 14’ 46.47” N 101° 19’ 23.14” E	142	Lowland forest
Kuala Selangor, Selangor	03° 20’ 39.40” N 101° 15’ 01.93” E	8m	Mangrove swamp
Sekinchan, Selangor	03° 25’ 40.61” N 101° 13’ 11.90” E	20m	Urban area
Shah Alam, Selangor	03° 04’ 40.61” N 101° 32’ 54.45” E	51m	Lowland forest
Pulau Ketam, Selangor	02° 59’ 54.12” N 101° 23’ 30.42” E	125m	Mangrove swamp
Morib, Selangor	02° 47’ 11.22” N 101° 24’ 54.13” E	18m	Mangrove swamp
Morib, Selangor	02° 45’ 23.07” N 101° 25’ 38.26” E	16m	Sandy shore
Klang, Selangor	03° 04’ 23.33” N 101° 26’ 48.35” E	34m	Urban area

Table 3.1 *(continued)*

Location	Coordinates	Altitute	Habitat
Petaling Jaya, Selangor	03° 07’ 27.18” N 101° 37’ 49.65” E	86m	Urban area
Subang Jaya, Selangor	03° 04’ 12.73” N 101° 35’ 32.46” E	67m	Urban area
Ulu Langat, Selangor	03° 03’ 32.52” N 101° 52’ 22.98” E	154m	Lowland forest
Gombak, Selangor	03° 19’ 30.28” N 101° 45’ 10.57” E	223m	Lowland forest
Taman Desa, Kuala Lumpur	03° 05’ 42.89” N 101° 41’ 12.59” E	65m	Urban area
University of Malaya, Kuala Lumpur	03° 07’ 15.11” N 101° 39’ 23.30” E	88m	Lowland forest
University of Malaya, Kuala Lumpur	03° 07” 22.14” N 101° 38” 37.23” E	47m	Stream area
Port Dickson, Negeri Sembilan	02° 29’ 49.35” N 101° 50’ 16.74” E	14m	Sandy shore
Port Dickson, Negeri Sembilan	02° 31’ 25.17” N 101° 48’ 10.18” E	17m	Rocky shore
Mount Ledang, Johor	02° 18’ 24.09” N 102° 37’ 25.30” E	153m	Lowland forest
Muar, Johor	02° 07’ 04.98” N 102° 43’ 01.83” E	67m	Urban area
Simpang Renggam, Johor	01° 49’ 18.90” N 103° 11’ 33.70” E	17m	Urban area
Sungai Peradin, Johor	01° 25’ 41.66” N 103° 31’ 35.16” E	13m	Mangrove swamp
Tanjung Piai, Johor	01° 15’ 12.48” N 103° 30’ 46.29” E	8m	Mangrove swamp
Johor Bahru, Johor	01° 29’ 49.93” N 103° 44’ 48.56” E	57m	Urban area
Kota Tinggi, Johor	01° 49’ 10.81” N 103° 49’ 20.72” E	133m	Stream area
Ulu Kali, Genting Highlands, Pahang	03° 26’ 23.05” N 101° 46’ 58.96” E	1745m	Highland forest
Awana, Genting Highlands, Pahang	03° 24’ 00.13” N 101° 47’ 02.90” E	1054m	Highland forest
Tanah Rata, Cameron Highlands, Pahang	04° 28’ 23.94” N 101° 22’ 33.44” E	1468m	Highland forest
Mount Brinchang, Cameron Highlands, Pahang	04° 30’ 59.59” N 101° 22’ 57.79” E	2036m	Highland forest
Kuala Terengganu, Terengganu	05° 14’ 19.21” N 103° 12’ 10.48” E	12m	Sandy shore
Marang, Terengganu	05° 20’ 33.21” N 103° 09’ 13.39” E	17m	Sandy shore
Kota Baharu, Kelantan	03° 02’ 24.56” N 101° 26’ 47.55” E	45m	Urban area

Table 3.1 *(continued)*

Location	Coordinates	Altitute	Habitat
Kampung Rambungan, Sarawak	01° 40’ 28.20” N 110° 07’ 25.76” E	5m	Mangrove swamp
Kampung Telega Air, Sarawak	01° 39’ 10.09” N 110° 12’ 12.72” E	14m	Mangrove swamp
Sampadi, Sarawak	01° 41’ 51.32” N 110° 03’ 35.66” E	10m	Sandy shore
Santubong, Sarawak	01° 42’ 37.40” N 110° 19’ 32.84” E	7m	Mangrove swamp
Regional Forestry Office, Kuching, Sarawak	01° 32’ 37.20” N 110° 19’ 21.38” E	9m	Mangrove swamp
Matang Wildlife Centre, Sarawak	01° 36’ 38.84” N 110° 09’ 43.36” E	12m	Lowland forest
Matang Wildlife Centre, Sarawak	01° 37’ 22.18” N 110° 09’ 11.14” E	10m	Rocky shore
Pueh, Sarawak	01° 49’ 41.90” N 109° 41’ 43.71” E	33m	Lowland forest
Pa Lungan, Bario Highlands, Sarawak	03° 44’ 21.34” N 115° 46’ 03.48” E	1072m	Highland forest
Arur Dalan, Bario Highlands, Sarawak	03° 44’ 21.34” N 115° 26’ 00.81” E	1235m	Highland forest
Tudal Hill, Bario Highlands, Sarawak	03° 49’ 10.87” N 115° 31’ 11.18” E	1271m	Highland forest
Mount Prayer, Bario Highlands, Sarawak	03° 44’ 51.42” N 115° 26’ 35.26” E	1428m	Highland forest
Ba Kelalan, Sarawak	03° 59’ 39.83” N 115° 37’ 15.61” E	1357m	Highland forest

Figure 3.1: Geographic location of collection sites in this study. Topography of collection sites is indicated in different colours and shapes; yellow dots indicate islands, red dots indicate lowland areas and red triangles indicate highland areas. The name of the location is included with each location symbol. Map from Wikipedia (http://en.wikipedia.org/wiki/File:Malaysia_location_map.svg).

3.4.2 Species checklist

3.4.2.1 Forensically important Malaysian Calliphoridae

A total of five genera, three subfamilies and 18 species of Calliphoridae, which are forensically important, were collected in this study (Table 3.2). In Malaysia, all the species within the genera *Calliphora*, *Chrysomya*, *Hemipyrellia*, *Hypopygiopsis* and *Lucilia* are forensically important except for *Chrysomya bezziana*. *Chrysomya bezziana* is a myiasis causing species and therefore it is not forensically important (Greenberg & Kunich, 2002). Of special note was the discovery of *Lucilia bismarckensis* which is also a forensically important species. The discovery of *L. bismarckensis* represents a new record for the Malaysian calliphorid species checklist.

Table 3.2: Checklist of forensically important Malaysian calliphorid species collected in this study.

Number	Calliphorid Species
1.	*Calliphora fulviceps* van der Wulp, 1881
2.	*Chrysomya chani* Kurahashi, 1979
3.	*Chrysomya defixa* (Walker, 1857)
4.	*Chrysomya megacephala* (Fabricius, 1794)
5.	*Chrysomya nigripes* Aubertin 1932
6.	*Chrysomya pinguis* (Walker, 1858)
7.	*Chrysomya rufifacies* (Macquart, 1843)
8.	*Chrysomya thanomthini* Kurahashi & Tumrasvin, 1977
9.	*Chrysomya villeneuvi* (Patton, 1922)
10.	*Hemipyrellia ligurriens* (Wiedemann, 1830)
11.	*Hemipyrellia tagaliana* (Bigot, 1877)
12.	*Hypopygiopsis fumipennis* (Walker, 1856)
13.	*Hypopygiopsis violacea* (Macquart, 1835)
14.	* *Lucilia bismarckensis* Kurahashi, 1987
15.	*Lucilia calviceps* Bezzi, 1927
16.	*Lucilia cuprina* (Wiedemann, 1830)
17.	*Lucilia papuensis* Macquart, 1824
18.	*Lucilia porphyrina* (Walker, 1857)

* New record (Sarawak, Borneo) discovered in this study

3.4.2.2 Malaysian Sarcophagidae

The species checklist of Malaysian Sarcophagidae was established with a total of 42 species as listed in Table 3.3. All these Sarcophagidae species belong to the subfamily Sarcophaginae, which are potentially forensically important, as some have been shown to be carrion eating flies (Povolný & Verves, 1997; Greenberg & Kunich, 2002), while others are parasites of other insects and invertebrates (Pape, 1996). These 42 sarcophagine species are represented by 17 genera, including one genus of *Sarcophaga* (s. lat.) with five species. During the course of this study, a new species (*Boettcherisca highlandica*) and a new record (*Iranihindia martellata*) were discovered and described from Peninsular Malaysia (Kurahashi & Tan, 2009; Tan *et al*., 2010a). Two new species (tentatively designated under genera *Myorhina* and *Sarcorohdendorfia*) and three new records discovered from this study are yet to be published.

Table 3.3: Checklist of Malaysian Sarcophagidae species collected in this study.

Number	Sarcophagid Species
1.	*Alisarcophaga gressitti* (Hall et Bohart, 1948)
2.	*Boettcherisca javanica* Lopes, 1961
3.	* *Boettcherisca highlandica* Kurahashi & Tan, 2009
4.	*Boettcherisca karnyi* (Hardy, 1927)
5.	*Boettcherisca krathonmai* Pape & Bänziger, 2000
6.	*Boettcherisca peregrina* (Robineau-Desvoidy, 1830)
7.	*Harpagophalla kempi* (Senior-White, 1924)
8.	*Hosarcophaga serrata* (Ho, 1938)
9.	** *Iranihindia martellata* (Senior-White, 1924)
10.	*Leucomyia alba* (Schiner, 1868)
11.	*Lioproctia pattoni* (Senior-White, 1924)
12.	** *Lioproctia saprianovae* (Pape & Bänziger, 2000)
13.	*Liopygia ruficornis* (Fabricius, 1794)
14.	*Liosarcophaga brevicornis* (Ho, 1934)
15.	*Liosarcophaga dux* (Thomson, 1869)
16.	*Liosarcophaga mimobrevicornis* Sugiyama, 1990
17.	*Myorhina borneensis* (Shinonaga & Lopes, 1975)
18.	*** *Myorhina globovesica* (Ye, 1980)

Table 3.3 (*continued)*

Number	Sarcophagid Species
19.	* *Myorhina* sp. nov.
20.	*Parasarcophaga albiceps* (Meigen, 1826)
21.	*Parasarcophaga javana* (Macquart, 1851)
22.	*Parasarcophaga lopesi* (Verves, 1980)
23.	*Parasarcophaga misera* (Walker, 1849)
24.	** *Parasarcophaga omari* Kurahashi & Leh 2007
25.	*Parasarcophaga scopariiformis* (Senior-White, 1927)
26.	*Parasarcophaga taenionota* (Wiedemann, 1819)
27.	*Rosellea notabilis* (Kano & Lopes, 1969)
28.	*Sarcorohdendorfia antilope* (Böttcher, 1913)
29.	*Sarcorohdendorfia inextricata* (Walker, 1859)
30.	**** *Sarcorohdendorfia seniorwhitei* (Walker, 1859)
31.	* *Sarcorohdendorfia* sp. nov.
32.	*Sarcosolomonia crinita* (Parker, 1971)
33.	*Sarcosolomonia rohdendorfi* Nandi, 1976
34.	*Seniorwhitea princeps* (Wiedemann, 1830)
35.	*Seniorwhitea orientalis* (Parker, 1917)
36.	** *Sinonipponia bengalensis* Nandi 1977
37.	*Sinonipponia hainanensis* (Ho, 1936)
38.	*Sarcophaga* (s. lat.) *aquila* Sugiyama, 1990
39.	*Sarcophaga* (s. lat.) *brachiata* Sugiyama, 1990
40.	*Sarcophaga* (s. lat.) *longifilia* (Salem, 1946)
41.	*Sarcophaga* (s. lat.) *quinqueramosa* Sugiyama, 1990
42.	*Sarcophaga* (s. lat.) *robustispinosa* Sugiyama, 1990

* New species
** New record to Peninsular Malaysia
*** New record to Sarawak, East Malaysia
**** New record to Peninsular Malaysia and Sarawak, East Malaysia

3.4.3 Ecological distribution

All of the species of Calliphoridae and Sarcophagidae collected in this study may be categorised into seven specific habitats – urbanised area, lowland forest, highland forest, mangrove swamp, sandy shore, rocky shore and stream area. Lowland forest is typically forest areas that are lower than 1000m whereas highland forest is defined as forest areas higher than 1000m.

3.4.3.1 Ecological distribution of forensically important Malaysian Calliphoridae

Eighteen species of forensically important Calliphoridae were categorised into seven specific habitat groups according to their abundances and ecological distribution (Table 3.4). In general, *Chrysomya megacephala* and *Chrysomya rufifacies* were observed in all types of habitats. However, these two species appear in high abundance in urbanised areas, and are greatly adapted to human settlement and environment, hence regarded as synanthropic species. *Hemipyrellia ligurriens* and *Lucilia cuprina* were also well known as synanthropic species, which can be largely collected from urbanised area. Although highland forest is defined as altitude more than 1000m, *Chrysomya pinguis* is a montane forest species which can be found at the altitude of 700m with low abundance. On the other hand, *Chrysomya villeneuvi* is a lowland forest species, but it also can be collected at sea level of 1500m with low frequency.

Table 3.4: List of Calliphoridae species found in seven habitats.

Habitat	Predominant species
Urbanised area *	*C. megacephala, C. rufifacies, H. ligurriens* and *L. cuprina*
Lowland forest	*C. megacephala, C. rufifacies, C. chani, C. defixa, C. nigripes, C. villeneuvi, H. tagaliana, H. fumipennis, H. violacea, L. papuensis* and *L. porphyrina*
Highland forest	*C. fulviceps* (>2000m), *C. megacephala* (1700m), *C. rufifacies* (1500m), *C. pinguis* (>700m), *C. villeneuvi* (from lowland up to 1700m), *C. thanomthini* (>1200m), *L. bismarckensis* and *L. porphyrina* (>1000m)
Mangrove swamp	*C. megacephala* and *C. rufifacies*
Sandy shore	*C. megacephala* and *C. rufifacies*
Rocky shore	*C. megacephala* and *C. rufifacies*
Stream area	*C. megacephala* and *C. rufifacies*

* synanthropic species: species which live in close association with humans

3.4.3.2 Ecological distribution of Malaysian Sarcophagidae

The 42 sarcophagine species collected in this study were grouped into the seven distinct habitats as previously described in section 3.4.3 (Table 3.4). Similar to Calliphoridae, some of the sarcophagine species live in several kinds of habitats. However, it was also found that one of the habitats would be the most preferable. Most of the sarcophagine species are observed at forested areas and mangrove swamp with 19 species and 14 species, respectively. Eight synanthropic sarcophagine species were collected, which are double the number compared to Calliphoridae. It is interesting to note that two swampy species, namely *Sarcophaga* (s. lat.) *brachiata* and *Sarcophaga* (s. lat.) *quinqueramosa* were found only in the states of Johor and Sarawak, respectively.

Table 3.5: List of Sarcophagidae species found in seven habitats.

Habitat	Predominant species
Urbanised area *	*B. peregrina, H. kempi, I. martellata, L. ruficornis, L. brevicornis, L. dux, P. misera* and *S. princeps*
Lowland forest	*B. javanica, B. krathonmai, L. saprianovae, L. pattoni, M. borneensis, P. albiceps, P. omari, P. taenionota, R. notabilis, S. crinita, S. rohdendorfi, S. inextricata* and *S. seniorwhitei*
Highland forest	*B. highlandica, M. globovesica, P. javana* (>1500m), *S. antilope, S. seniorwhitei* and *Sarcorohdendorfia* sp. nov.
Mangrove swamp	*B. karnyi, L. mimobrevicornis, Myorhina* sp. nov., *P. lopesi, P. scopariiformis, S. hainanensis, S. orientalis, S.* (s.lat.) *aquila,* ***S.* (s.lat.) *brachiata, S.* (s.lat.) *longifilia, S.* (s.lat.) *robustispinosa* and ***S.* (s.lat.) *quinqueramosa*
Sandy shore	*A. gressitti* and *L. alba*
Rocky shore	*S. bengalensis*
Stream area	*B. javanica* and *H. serrata*

* synanthropic species: species which live in close association with humans
** swampy species occurred at specific states of Malaysia: *S.* (s.lat.) *brachiata* in the state of Johor and *S.* (s.lat.) *quinqueramosa* in the state of Sarawak

3.5 Discussion

A checklist of forensically important flies is vital for the successful application of forensic entomology. In Malaysia, the family Calliphoridae are well studied (Senior-White *et al*., 1940; Singh *et al*., 1979; Kurahashi *et al*., 1997; Verves, 2005) compared to the family Sarcophagidae (Lopes *et al*., 1977; Singh *et al*., 1979; Sugiyama *et al*., 1990; Kurahashi & Leh, 2007). A comprehensive species checklist of Malaysian Calliphorids was been established in the year 1997 by Kurahashi and his colleagues. As such, the current study serves to review the existing calliphorid checklist and provide updates when and where necessary. Indeed, a new record of *Lucilia bismarckensis* was established in this study. This species is known to be distributed in Bangladesh and the Philippines (Kurahashi & Magpayo, 2000) apart from its type locality of Rabaul, Keravat, New Britain, Papua New Guinea (Kurahashi, 1987b).

Among the nine Malaysian *Chrysomya* species (subfamily Chrysomyinae), eight are forensically important, with the exception of *C. bezziana*, an obligate parasite of mammals which causes myiasis. In spite of previous entomology records noting the existence of three *Lucilia* species, namely *L. bazini*, *L. hananensis* and *L. sinensis* (Kurahashi *et al*., 1997; Kurahashi, 1998), these species were not encountered throughout this study. The importance of these three species in forensics remains unclear. *Lucilia sinensis*, however, had been previously reported as important in forensics (Omar *et al*., 1994b). Interestingly, during this study, *L. bismarckensisi* was recorded for the first time in Sarawak, although its involvement in forensics needs further confirmation.

In contrast to Calliphoridae, a comprehensive checklist of Malaysian Sarcophagidae is currently not available, which is largely due to the difficulty in correct species identification. Available information on works involving Sarcophagidae is yet to be compiled, as most researchers work in isolation, and over limited geographical areas.

The first ever documented effort to produce a checklist of Malaysian Sarcophagidae was carried out by Senior-White *et al*. (1940) who managed to record only eight species in their book. Later, Lopes and his colleagues (1977) commenced their own study of Sarcophagidae species in Malaysia and they added another 14 species to the list. Following on from that, Singh *et al*. (1979) recorded seven more species and Sugiyama *et al*. (1990) recorded another 12 species with the discovery of six new species from Malaya (Malaysia), North Borneo and Singapore. More recently, Kurahashi & Leh (2007) accomplished a Sarcophagidae species checklist of Sarawak, which contained 21 species. Despite the fact that there are at least 41 species recorded by these researchers, progress on Sarcophagidae research remained slow, hampered by the fact that identification of these morphologically similar species group is very difficult and requires the knowledge and experience of a trained entomologist. Another important factor for this problem is the fact that the taxonomic key of Malaysian Sarcophagidae has yet to be established and be made publically available. As such, this study attempts to establish a comprehensive species checklist at least for the peninsular region, which will undoubtedly be useful for downstream applications (e.g. forensics) and future reference for research on this fly family in Malaysia. In this study, a species checklist of sarcophagine of 42 species, with three new species and six new records, was successfully established.

Most species have a restricted altitude distribution or specific habitats due to their habitat preferences, which are related to the structure of landscape or topology of that particular environment (Baz *et al*., 2007). Consequently, this habitat selection may exhibit significant distribution variation for fly species (Nuorteva, 1963). For this reason, flies are suitable to serve as location indicators (Goff, 1993). In this study, the ecological distribution pattern of fly species were categorised into seven types of habitats namely, the urbanised area, the lowland forest, the highland forest, the

mangrove swamp, the sandy shore, the rocky shore and the stream area. According to Povolný & Verves (1997), Sarcophagidae have often adapted to certain special habitats, such as the sea shores and freshwater to avoid competition with other flies.

As shown in Tables 3.4 and 3.5, it was observed that most fly species showed a tendency to remain into one habitat type (e.g. *C. fulviceps* only found at highland forests while *L. alba* only found at sandy shores) although a few may have adapted to more than one habitat (e.g. *C. megacephala* and *C. rufifacies* can be found in almost all habitats while *L. dux* can be found at urbanised areas and mangrove swamps) (Povolný & Verves, 1997; Kurahashi, 2002; Bänziger & Pape, 2004).

Altitude greatly influences the calliphorid and sarcophagid populations' diversity. In this study, a series of sample collection was made on different altitudes along a montane forest of Cameron Highlands, Genting Highlands and Bario Highlands, from the foot range to the summit. Different calliphorid species were found to inhabit along the altitudinal gradient according to their altitudinal preferences. Hanski (1981) and Baz *et al*. (2007) also described similar observations, where significant differences in calliphorid species diversity were found at different elevations in Sarawak and Central Spain, respectively. For example, *C. fulviceps* is found only at high elevation areas of more than 2000m above sea level (Table 3.4). Similar observations were made by Hanski (1981) and Kurahashi *et al*. (1997), who had carried out the observation at Mount Mulu of Sarawak, Mount Brinchang of Pahang and Mount Kinabalu of Sabah. Davies & Lawrence (1992) also suggested that *Calliphora* species usually occur in cold and wet habitats in Malaysia. Such cold and wet habitats in Malaysia can only be found in highly elevated areas, which explain the occurrence of *C. fulviceps* in these areas.

At the intermediate elevation approximately 1000m above sea level, *L. porphyrina* is a common calliphorid species appearing in high densities. This observation corroborates with Hanski (1981) that the occurrence of this species is

expected at the range of 700m to 1500m, although it is not commonly found at 700m. For *Chrysomya* species, *C. thanomthini* is indigenous to undisturbed forests, found at elevations of more than 1200m above sea level while *C. pinguis* can usually be found at a wider range of elevation (700-2000m). Both species are commonly known as the mountainous forest species and are therefore considered good location indicators for mountainous forests (Kurahashi, 2002).

Chrysomya villeneuvi is notably most dominant in lowland forests (<1000m) although it can also be found albeit rarely, at higher elevations of up to 1700m above sea level. *C. villeneuvi* is commonly regarded as a tropical lowland forest species and this species particularly prefers to live at lower altitudes. It is believed that some populations of this species may have also adapted to higher altitude habitats, as observed in this study as well as others (e.g. Hanski, 1981).

The majority of calliphorid species are lowland forest species (Hanski, 1981; Kurahashi *et al*., 1997; Kurahashi, 2002). In this study, *C. chani, C. defixa, C. nigripes, C. villeneuvi, H. tagaliana, H. fumipennis, H. violacea, L. bismarckensis, L. calviceps,* and *L. papuensis* were collected at elevations lower than 1000m. However, these species showed differences in their habitat preferences, especially *Chrysomya* species, for which Hanski (1981) had the same observation. *C. defixa* is a sibling species of *C. pinguis* which inhabits lowland forests. *C. defixa* was found to be more abundant in lowland forests although both *C. defixa* and *C. pinguis* can co-exist at elevations of 700m. Two species of *Hypopygiopsis* are typical tropical rainforest species; *H. fumipennis*, in particular, is more indigenous than *H. violacea* and needs high humidity (>90%) to survive. Therefore, they can be served as the indicators of tropical rainforest (H. Kurahashi, pers. comm.). In the highland forest, the predominant sarcophagid species are *B. highlandica* and *B. krathonmai* for Peninsular Malaysia and Sarawak, respectively. Both of these species are sibling species. *B. highlandica* is

endemic to Peninsular Malaysia while *B. krathonmai* can be found in both Peninsular Malaysia and Sarawak. However, similar to *C. villeneuvi*, *B. krathonmai* appears to have adapted to a wider range of altitude (from lowland to 1500m).

Likewise to Calliphoridae, a great number of species of Sarcophagidae are present in lowland forest. Most of the species of genera *Lioproctia*, *Parasarcophaga* and *Sarcosolomonia* are the lowland forest species, which may coexist in the same forest.

It is curious to note that the mangrove swamp habitat attracts more species of Sarcophagidae than Calliphoridae. There are at least 14 species inhabiting this habitat and the predominant species are *B. karnyi*, *S.* (s. lat.) *aquila* and *S.* (s. lat.) *robustispinosa*. It is also interesting to note that sample collection throughout the mangrove swamp areas, which covered the states of Perak, Selangor, Johor and Sarawak, revealed that *S.* (s. lat.) *brachiata* and *S.* (s. lat.) *quinqueramosa* are found to be endemic to Johor and Sarawak, respectively. This finding suggests that these two species can serve as location indicators to those particular Malaysian states and types of habitat.

As previously mentioned, coastal areas can be divided into two types of habitat, namely the sandy shore and rocky shore. *A. gressitti* and *L. alba* are typical sandy shore species, while *S. bengalensis* is uniquely found within the rocky shore areas. *B. javanica* and *H. serrata* on the other hand are commonly found near stream areas. This habitat selection makes them suitable to be the location indicator for forensic investigations.

In urbanised habitats, synanthropic species are found. This is because those species found in urban areas are closely associated with the human environment and have adapted to living together with humans. However, the terms “eusyanthropic” and “hemisynanthropic” are not used in this study due to the fact that their level of synanthrophy was not investigated. This level of association is also known as the

"synanthropic index" (Nuorteva, 1963). Most species of Calliphoridae, especially species of *Chrysomya*, *Hemipyrellia* and *Lucilia*, have wide geographical distributions. They are habitually associated with the human activities. Some species are typically urbanised and they are therefore the greatest hygienic importance indicator (Rognes, 1991). So, in this ecological distribution study, anthropic habitats, especially the center of town and residential areas were included. *Chrysomya megacephala*, *C. rufifacies*, *H. ligurriens* and *L. cuprina* are very well reported in many studies as synanthropic species (Sulaiman *et al*., 2000; Gabre & Zied, 2003; Nurita *et al*., 2008). In the family of Sarcophagidae, synanthropic species include *B. peregrina*, *H. kempi*, *I. martellata*, *L. ruficornis*, *L. dux*, *L. brevicornis*, and *P. misera*. This observation agrees with that of Bänziger & Pape (2004), except for the species of *I. martellata* which was not included in their study.

3.6 Conclusion

Knowledge of the distribution and ecology of species of Calliphoridae and Sarcophagidae is important not only for forensic entomology, but also for other fields of science such as basic biology, physiology and toxicology. In this study, preliminary results of the species checklist and their ecological distribution of families Calliphoridae and Sarcophagidae are obtained and added to the knowledge of Malaysian forensic entomology. Some data (e.g. the location indicator and endemic species) obtained in this study will facilitate the ecological investigation of forensically important carrion flies and may provide some useful information for criminal investigations.

Chapter 4:

Diptera succession study on pig carcassess in Kuching, Sarawak

4.0 Abstract

Two domestic pig carcasses, *Sus scrofa* L., one scavenged and one clothed, were placed in close proximity to a grassland of secondary rainforest at Kuching, Sarawak, Malaysia, to study the decomposition rate and the dipteran succession pattern. Four stages of decomposition were recognised: fresh, bloated, decay, and dry remains. Decomposition was greatly influenced by meteorological conditions and dipteran activities. The scavenged carcass decomposed faster than the clothed carcass, and the bloated stage was skipped due to the presence of scavenged abdominal wound. The clothed carcass experienced a more complicated decomposition process compared to scavenged carcass. Despite the close proximity of the two carcasses in this study, significant differences in Diptera species were obtained. A total of 13 taxa representing four families and nine genera were recovered from the carcasses. *Chrysomya megacephala*, *Chrysomya rufifacies* and *Hemipyrellia ligurriens* were obtained from both scavenged and clothed carcasses. *Hypopygiopsis violacea* and *Boettcherisca karnyi* were only obtained from the scavenged carcass while *Chrysomya nigripes* and *Ophyra spinigera* were only obtained from clothed carcass. Significant findings for the decomposition were that the predominant species at the early stage was *C. megacephala*, towards late stage was *C. rufifacies* and the latest stage was *C. nigripes* and *O. spinigera*. *Boettcherisca karnyi* and *H. violacea* seemed to be important indicators for the environment, such as swamps and rainforest, respectively. As the time indicators, *H. ligurriens* seemed to be useful as the early decomposition indicator, while *C. nigripes* and *O. spinigera* were the indicators of late decomposition.

4.1 Introduction

A broad knowledge about the ecological and behavioural nature of arthropods is needed before forensic entomology can be applied in legal issues. One of the most important roles of arthropods in forensic entomology is to estimate the post mortem interval (PMI) (Hall, 2001). This is because arthropods are the first to arrive on abandoned corpses (Anderson, 2001) and their presences are useful to serve as time indicators.

However, the use of arthropods in PMI estimation can only be applied when the data of insect succession, decomposition (Campobasso *et al*., 2001), larval growth development (Sminth, 1986) and meteorology (Tabor *et al*., 2004) are accessible. Succession pattern is a term in forensic entomology that refers to the sequence of arthropods visiting and exploiting a carcass (Greenberg & Kunich, 2002). The succession pattern and the temperature-dependent development of larvae occur in a predictable manner (Smith, 1986; Hall, 2001). As a result, PMI estimations can be achieved quite accurately using these predictable data.

Arthropod succession patterns on a body are highly dependent and show a direct relationship with the stage of decomposition (Goff, 1993). To understand this relationship, various studies were carried out using animal models. To a certain extent, human cadavers have also been used to obtain data that would be directly relevant to human corpses (Rodriguez & Bass, 1983). Throughout the years, the most preferred animal carcass used as a corpse model for arthropod succession study is the domestic pig (Payne *et al*., 1965; Hewadikaram & Goff, 1991; Anderson and VanLaerhoven, 1996; Shalaby *et al*., 2000; Jenson & Miller, 2001; Wolff *et al*., 2001; Archer, 2003a; Tabor *et al*., 2004, 2005; Horenstein *et al*., 2007; Vitta *et al*., 2007; Heo *et al*., 2007, 2008a & b). Pig carcasses are chosen by most researchers because of their relatively hairless skin which is similar to humans compared to other mammals and it is

omnivorous, with a digestive tract fauna close to humans (Anderson and VanLaerhoven, 1996). Many studies have covered a wide range of investigations, including the difference in physical condition of carcasses (Avila & Goff, 1998, Heo *et al*., 2008b), seasonal distribution (Tabor *et al*., 2004, 2005; Archer & Elgar, 2003; Horenstein *et al*., 2007; Martinez *et al*., 2007;Wang *et al*., 2008), size of carcasses (Hewadikaram & Goff, 1991), effect of clothing (Grassberger & Frank, 2004) and placement of carcasses (Shalaby *et al*., 2000; Centeno *et al*., 2002; Anderson & Hobischak, 2004; Heo *et al*., 2008a). The parameters of these succession studies are summarised in Table 3.1.

In Malaysia, carcasses of the cynomolgus monkey, *Macaca fascicularis* (Lee & Marzuki 1993; Omar *et al*., 1994a & b) and domestic pig, *Sus scrofa* (Heo *et al*., 2007, 2008a & b) are the two main animal models that have been documented for arthropod succession studies. These studies have been carried out at a rubber tree plantation and a palm oil plantation in Selangor State, Peninsular Malaysia.

Heo *et al*., (2007) reported a short period of 14 days for complete decomposition of a pig carcass with a body weight (at death) of 8.5kg. From their study, it was found that the rate of decomposition occured in a rapid manner compared with carcass of similar weight used in other studies, although the authors did not elaborate on the reasons. Comparisons between the size of pig carcasses and decomposition duration in different geographic regions are shown in Table 3.2.

4.2 Objectives

The objectives of this study are to obtain a Dipteran succession pattern and to investigate differences between two domestic pig carcasses. One carcass is scavenged and naked, while the other is intact and clothed. Studies are focused on:

1. Recording the decomposition rates and observing different stages of carcass decomposition in tropical rainforest in Borneo.

2. To establish a preliminary Dipteran checklist (particularly the Calliphoridae and Sarcophagidae) and their succession patterns on these two decomposed carcasses.

Table 4.1: List of selected succession studies, including animal model, size, physical condition and placement of carcass, locality and experimental variable.

Author and reference	Animal model	Size of carcass (kg)	Physical condition of carcass	Placement of carcass	Locality of experiment	Variable *
Tullis & Goff, 1987	Pig	8.2 to 12.8	Intact	Tropical rainforest	O'ahu Island, Hawaii, USA	Habitat
Hewadikaram & Goff, 1991	Pig	8.4 and 15.1	Intact	Xerophytic habitat	O'ahu Island, Hawaii, USA	Habitat
Shean *et al*., 1992	Pig	22.7 and 20.3	Knife cut wound at the neck	Sunlit vs shaded	Washington, USA	Sunlight exposure
Omar *et al*., 1994(a)	Monkey and cats	4.5 & 3	Intact	Secondary forest & fruit orchard	Gombak, Selangor, Malaysia	Habitat
Omar *et al*., 1994(b)	Monkey	4.5	Intact	Rubber tree plantation	Gombak, Selangor, Malaysia	N/A
Anderson & VanLaerhoven, 1995	Pig	N/A[b]	Intact with gun shot at the thorax	Sunlit, open rural area	Fraser Valley, British Columbia, Canada	-
Moura *et al*., 1997	Rat	0.25	Intact	Open field urban environment vs heavily shaded forest	Curitiba, Paraná, Brazil	Habitat and sunlight exposure
Richards & Goff, 1997	Pig	9.07-11.79	Intact	Altitudinal gradient tropical habitats (646m-1877m)	Hawaii Volcanoes Park, Hawaii, USA	Habitat with altitudinal gradient
Avila & Goff, 1998	Pig	24.5[c] and 26.8, 15.5 and 15.6 [c]	Burnt & unburn	Xerophytic habitat and rainforest	O'ahu Island, Hawaii, USA	Physical condition of carcass
De Jong & Chadwick, 1999	Rabbit	1.15 to 2.02	Intact	High altitude (2713m-4191m)	Colorado, USA	Habitat with altitudinal gradient
Shalaby *et al*., 2000	Pig	N/A[b]	Hanging	Xerophytic habitat	Hawaii, USA	Placement of carcass
Jenson & Miller, 2001	Pig	23	Intact	Forest	Guam	No
Wolff *et al*., 2001	Pig	17.7	Intact	Metropolitan area (1450m)	Medellín, Colombia	No
Centeno *et al*., 2002	Pig	15-17	Intact with a stab in the heart	Field	Buenos Aires, Argentina	Season and sunlight exposure

Table 4.1 *(continued)*

Author and reference	Animal model	Size of carcass (kg)	Physical condition	Placement of carcass	Locality	Variable *
Archer, 2003a	Pig	0.88-2.02	Intact	Damp sclerophyll forest	Victoria, Australia	Season
Archer & Elgar, 2003	Pig	0.88 to 2.02	Intact	Swamp and bushlands	Victoria, Australia	Habitat
Anderson & Hobischak, 2004	Pig	20 to 25	Intact	Submerge in the water with different depth	British Columbia, Canada	Depth of water
Grassberger & Frank, 2004	Pig	37 and 44	Clothed with jeans and white T-shirt	On the ground	Vienna, Austria	Clothed vs naked
Tabor *et al*., 2004	Pig	41-45 vs 23-27	Intact	N/A [b]	Blacksburg, Virgina, USA	Season
Arnaldos *et al*., 2005	Chicken	N/A[b]	Partially flesh removed and viscera present	Natural area of agriculture field	Iberian Peninsula	Season
Tabor *et al*., 2005	Pig	41-45 vs 23-27	Intact	N/A [b]	Blacksburg, Virgina, USA	Season
Joy *et al*., 2006	Pig	23 vs 24	Intact	Sunlit vs shaded	Virginia, USA	Sunlight exposure
Heo *et al*., 2007	Pig	8.5	Intact	Oil palm plantation	Selangor, Malaysia	-
Horenstein *et al*., 2007	Pig	8	Intact	Sunlit vs shaded	Córdoba, Argentina	Sunlight exposure
Martinez *et al*., 2007	Pig	10	Intact	High altitude plain (3035m)	Paramo, Colombia	-
Vitta *et al*., 2007	Pig	3-4	Intact	Sunlit vs shaded	Phitsanulok, Thailand	Sunlight exposure
Heo *et al*., 2008a	Pig	5	Intact	Submerged in man-made freshwater pond	Selangor, Malaysia	-
Heo *et al*., 2008b	Pig	10	Partially burnt	Oil palm plantation	Selangor, Malaysia	Physical condition of carcass
Souza *et al*., 2008	Rabbit	2.67	Intact	N/A[b]	Rio Grande do Sul, Brazil	-
Velásquez, 2008	Rat	0.5	Intact	Sunlit vs shaded	Venezuela	Sunlight exposure

Table 4.1 *(continued)*

Author and reference	Animal model	Size of carcass (kg)	Physical condition	Placement of carcass	Locality	Variable *
Wang *et al*., 2008	Pig	32 to 67	Intact	Woods and grassland	Guangdong, China	Habitat
Sharanowshi *et al*., 2008	Pig	42-79	Intact	Moist mixed grassland ecoregion	Saskatchewan, Canada	Season and habitat
Gomes *et al*., 2009	Pig	13.4	Intact	Sugarcane crop area	Ipeúna, São Paulo, Brazil	-
Segura *et al*., 2009	Pig	12	Intact	Semi-rural area	Bogotá, Colombia	-
Voss *et al*., 2009	Guinea pig	0.423 to 0.553	Intact	Bushland vs agricultural field	Perth, Australia	Habitat
Matuszewski *et al*., 2010	Pig	14 to 39 (mean = 25.8)	Intact	Forest types of : alder vs pine-oak vs hornbeam-oak	Poland	Habitat

[b] N/A: information not available
[c] as control in the experiment
* an hyphen (-) indicates that no variable is found in the experiment

Table 4.2: Comparison of selected succession studies of size of pig carcass and duration of different decomposition stage occurring in geographic regions.

Author and reference	Weight of carcass (kg)	Additional parameters	Decomposition stage/Duration (Day)					Country
			Fresh	Bloated	Decay	Advance decay	Dry	
Tullis & Goff, 1987	8.2 to 12.8		1-2	2-7	5-13	10-23	18-90+	USA (Hawaii)
Hewadikaram & Goff, 1991	8.4		1-2	2-5	5-12	12-16	16-30+	USA (Hawaii)
	15.1		1-2	2-3	3-9	9-14	14-30+	
Anderson & VanLaerhoven, 1995	N/A		0-1	2-10	11-16	17-42	43+	Canada
Richards & Goff, 1997	9.07-11.79	1877m	1-3	3-13	13-19	19-42	-	USA (Hawaii)
		1169m	1-3	3-10	6-16	12-30	30-42	
		646m	1	1-4	4-8	8-42	-	
Avila & Goff, 1998 [a]	24.5[c] & 26.8	DHC [a]	1-2	3-4	5-9	10-30	-	USA (Hawaii)
	15.5 & 15.6 [c]	LA [a]	1	2	3-8	9-30		
Wolff *et al*., 2001	17.7		0-1	2-6	7-12	13-51	52-207	Colombia
Anderson & Hobischak, 2004	20 to 25		0-3	3-11	11-30	-	40+	Canada
Heo *et al*., 2007	8.5		1-2	3	4-5	6-8	9-14	Malaysia
Martinez *et al*., 2007	10		0-3	4-16	17-30	31-51	52-83	Colombia
Vitta *et al*., 2007	3-4		0-1	2	3	4-6	7-30	Thailand
Heo *et al*., 2008(a)	10		1-2	3	4-6	7-8	9-16	Malaysia
Gomes *et al*., 2009	13.4		0-5	6-10	11-14	15-21	22-28	Brazil
Segura *et al*., 2009	12		1-3	4-10	11-20	-	22-97	Colombia

[a] Two burnt carrion experimental sites, DHC: Diamond Head Crater; and LA: Lyon Arboretum.
[c] as controlled in the experiment

4.3 Materials and methods

4.3.1 Study site

This study was conducted in a secondary forested area in Kuching, Sarawak, with the consent of the land owner. The chosen site is within private land, and, as such, access to outsiders is restricted without prior consent of the land owner. The experimental site is located approximately seven miles away from the city of Kuching. The geographical coordinates of this site (01° 27” 59.47’ N 110° 20” 34.48’ E; elevation: 18m) were obtained using a wireless Global Positioning System (GPS) (Holux, Taiwan). The site is a secondary tropical rainforest with grassland and is close to a river. This experiment was conducted from 10th to 17th December 2008.

4.3.2 Animal study subjects

Two domestic piglet (*Sus scrofa* L.) carcasses were used in this experiment courtesy of Sing Loong Farming Sdn. Bhd. The piglets were slain with quick and hard blows to the head using a wooden rod and immediately placed at the experimental site. The carcasses weighed 6.82kg and 7.69kg, and were denoted as Carcass A and Carcass B, respectively. Carcass A was naked and partially scavenged by a monitor lizard while Carcass B was still intact and clothed. Both carcasses were placed separately in 1.5cm-welded-wire-mesh-close-bottom cages (H×L×W: 50×50×100cm). The edges of the cage were secured with cable ties. In order to prevent the disturbance of vertebrate scavengers, the four corners of each cage were staked to the ground.

Carcass A was left without protection immediately after killing on the experimental site to allow scavenging. A monitor lizard was found to scavenge on the pig carcass within a half an hour of placement. The abdominal part of the body which includes the rib and some of the internal organs were partially scavenged. The scavenged carcass was examined for oviposition or larviposition before being brought

to the experimental site and later caged. Carcass B on the other hand was clothed with a white T-shirt before being placed in the cage. The two carcasses were placed at a distance of 11.2m.

4.3.3 Data collection

During this 7-day experiment, the pig carcasses were examined once a day at 0900 and the meteorological conditions were recorded. The readings of relative humidity and the daily ambient temperature of the study site were obtained using a digital thermometer-hygrometer (Zeal, England) and a maximum-minimum thermometer that were placed near the carcass respectively. Rainfall was measured with a rain gauge that was placed in between the two carcasses.

A mercury thermometer was used to measure the body temperatures of the carcasses, larval mass temperatures and ground temperatures. Additionally, the pH of the body, larval mass and the soil were determined using pH 0-14 indicator strips (Merck, Malaysia).

Arthropod activities and physical changes associated with the decomposition (e.g. colour changes, swelling and discharged of liquids and gases) were observed, recorded and photographed with a digital camera (Canon IXUS 50) at each visit. To facilitate the collection of dipterans, the cages were opened upward at each sampling. Adult dipterans hovering above or landing on the carcasses were collected with an aerial insect net. The adult dipterans were killed with ethyl acetate vapour in a killing jar. The specimens were then brought back to the laboratory and mounted using entomological pins. For dipteran specimens of immature stages, such as egg and larval samples were collected from the top surface, underneath and from the insides of the carcasses using forceps. These egg and larval samples were then kept in rearing vials. Different parts of carcasses, such as the natural orifices (e.g. eyes, nose, mouth, and anus) and the wounds

(e.g. abdominal part of carcass A) were examined prior to obtaining eggs and larvae. Each colonising immature species of different life stages were sampled in a small number so as not to disturb the succession population and to minimize sampling effects. The collected eggs and larvae were reared on beef in the laboratory until adulthood prior to species identification. Each rearing vial contained not more than 20 individuals to allow sufficient food and space for growth. The quantity of beef was checked everyday to make sure there was an adequate food supply to the immatures. More beef was added if necessary until the larvae reached the post feeding stage. The post feeding larvae were transferred to clean rearing vials bedded with damped tissue papers to allow for pupation. The pupae were observed daily until their emergence as adults. All collected and reared adults were pinned, preserved, labelled and identified using the keys described in Kurahashi *et al*. (1997).

4.4 Results

4.4.1 Meteorological data

The daily meteorological condition of this study is shown in Figure 4.1. In general, the maximum ambient temperatures of the study site were high (with highest of 49.5°C in day 7) and the minimum ambient temperatures were consistent (with a daily record of 22.5°C). The maximum and minimum relative humidity was 100% and 91%, respectively, which is a typical condition of the tropical rainforest climate. Rainfall occurred everyday throughout the study, with the amount ranging from 0.6mm to 55.8mm per day. The detail readings of all meteorological data are given in appendix A.

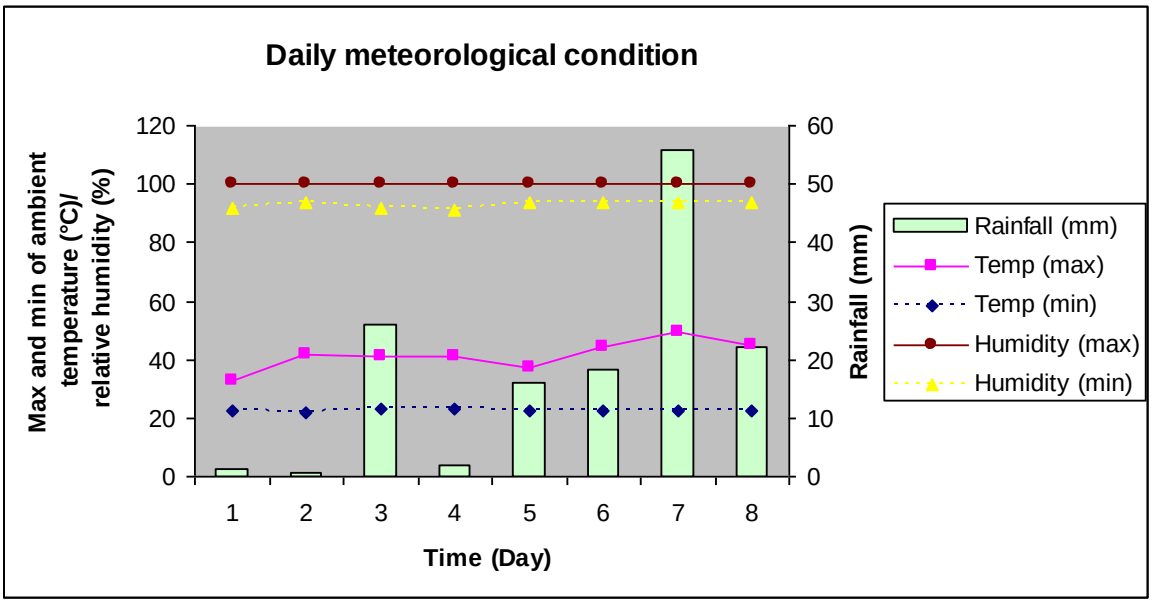


Figure 4.1: Daily meteorological condition of experiment, maximum/minimum ambient temperature and relative humidity as well as rainfall.

4.4.2 Temperature and pH

The temperature and pH of the carcass body, larval mass as well as soil for carcass A and carcass B are presented in Figures 4.2 and 4.3, respectively, while the detailed readings of all these parameters are shown in appendix A. The body temperatures of both carcasses were approximately 37°C on day 1, indicating the expected normal body temperature of an animal. As expected, the body temperatures of both carcasses showed a marked decreased on day 2. The body temperatures of both carcasses then increased as larval masses started to form. After this stage, the body temperature of carcass was not taken while the larval mass temperature was measured. Larval mass temperature reached its maximum (e.g. 41°C for carcass A; 41.5°C for carcass B) when a large number of larvae present at the carcass were in their second and third instars.

Soil pH at the study site was 5, which is a little acidic. However, during the bloated and decay decomposition stages liquids discharged from the body changed the soil pH and becoming alkaline. The pH of larval masses measured in this study was 10.

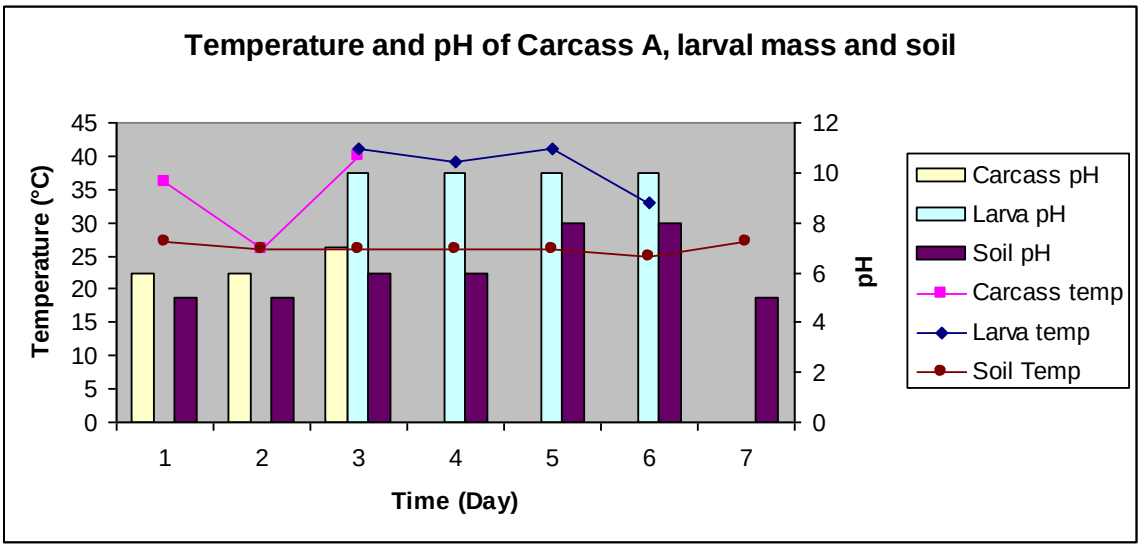


Figure 4.2: Temperature and pH of body, larval mass and soil from carcass A throughout decomposition stages.

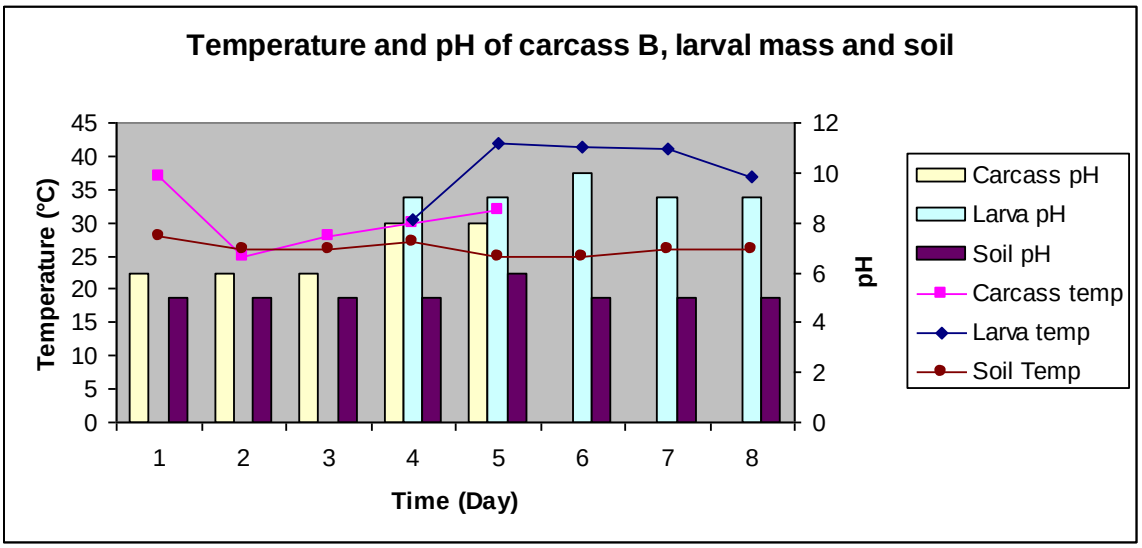


Figure 4.3: Temperature and pH of body, larval mass and soil from carcass B throughout decomposition stages.

4.4.3 Decomposition stages

The definition of stages used in this study was that of described by Reed (1958). Generally, decomposition is divided into four different stages namely fresh, bloated, decayed and dry remains. Fresh stage starts when the pig dies and the carcass shape has no visible physical changes. Bloated stage starts when the abdomen of the carcass begins to show a sign of inflation due to the gas produced by bacterial activity and is accompanied by odour of decaying flesh (Smith, 1986). Fluids also begin to be discharged from the natural orifices and any wound openings present on the carcass.

Decay is marked by great physical appearance changes to the carcass; particularly the colour of carcasses which tends to be black. The hair and skin begin to detach from the carcass and the cartilage tissues becomes exposed. The body starts to collapse and gases are released with the presence of a strong odour. The last stage of decomposition is the dry stage where the carcass starts to skeletonis and dry out. The internal organs, tissues, flesh, cartilages will be completely decomposed. The portions which cannot be digested by larvae, such as hair, nail and bone will dry up. The duration of each decomposition stage of carcass A and B in this study is presented in Table 4.3.

Table 4.3: Duration of decomposition stage.

Decomposition stage	Carcass A	Carcass B
Fresh	2 days	2 days
Bloated	-	2 days
Decay	2 days	3 days
Dry	onwards	onwards

4.4.3.1 Decomposition of carcass A

On the first day of the fresh stage, the smell of blood was apparent due to the scavenging activities of vertebrates. The carcass remained at the fresh stage for 2 days, with small visible physical changes. The bloated stage was not evident as the carcass was scavenged ventrally. Therefore, the carcass directly underwent the decay stage and remained at this stage for 2 days during which the wound of the carcass served as the main focus of larval colonisation as opposed to natural orifices (mouth, eye, ear and anus), as demonstrated on day 3. Fluids started to seep out through the mouth on this very day. On the following day, the back bones were revealed and natural orifices started to decompose at a higher rate. However, the four digits of the carcass remained intact at this stage. Eventually, all the skeletons of the carcass, including the teeth were exposed on day 5 leaving the hair as the only remains. Pictures were taken daily for carcass A to record the changes of decomposition (Figure 4.4).

Figure 4.4: Changes of decomposition of carcass A throughout the study. (A) Day 1, carcass shortly after scavenged by monitor lizard. (B) Day 2, fresh stage. (C) Day 3, beginning of decay stage. (D) Day 4, decay stage continued. (E) Day 5, dry stage, carcass start to skeletonised. (F) Day 6, dry remains.

4.3.3.2 Decomposition of carcass B

The fresh stage of the carcass began upon the death of the piglet and continued until the bloating of the abdomen was visible. No odour was present and the carcass settled for two days at the fresh stage before reaching the bloated stage. The eye balls were completely consumed by larvae by day 3. The bloated stage started at day 4 and remained at this stage for only a day, when the two hind digits of the carcass were rigidly propped up. However, this was not the case for the front digits as they were covered by the T-shirt. The presence of odour was apparent on this day. Colour change was observed on the skin of the carcass, especially at the inner part of the ruptured knees. It was noted that the knees were ruptured as a result of overpopulation of larvae in the carcass. The decay stage began on day 5. The facial structure of the carcass was highly decomposed, exposing the teeth. The internal organs also started to burst out through the ruptured skin on this day. At day 6, the body of the carcass started to collapse, with the head structure began to skeletonise. The skull was completely exposed and the hair started to detach from the body. At day 7, the bones were completely exposed from the head to the femur. The flesh was no longer present, leaving only the skin layer and the cartilages. This resulted in the body of the carcass deflating. In addition, the formation of adipocere (a wax-like organic substance formed by the anaerobic bacterial hydrolysis of fat in tissue) was observed under the T-shirt and near the hip of the carcass. The remains of the carcass dried at day 8, at which the dry stage of decomposition was significantly defined. Eventually, only the skeletons and the T-shirt remained at the experimental site. Pictures were taken daily for carcass B to record the changes of decomposition (Figure 3.5).

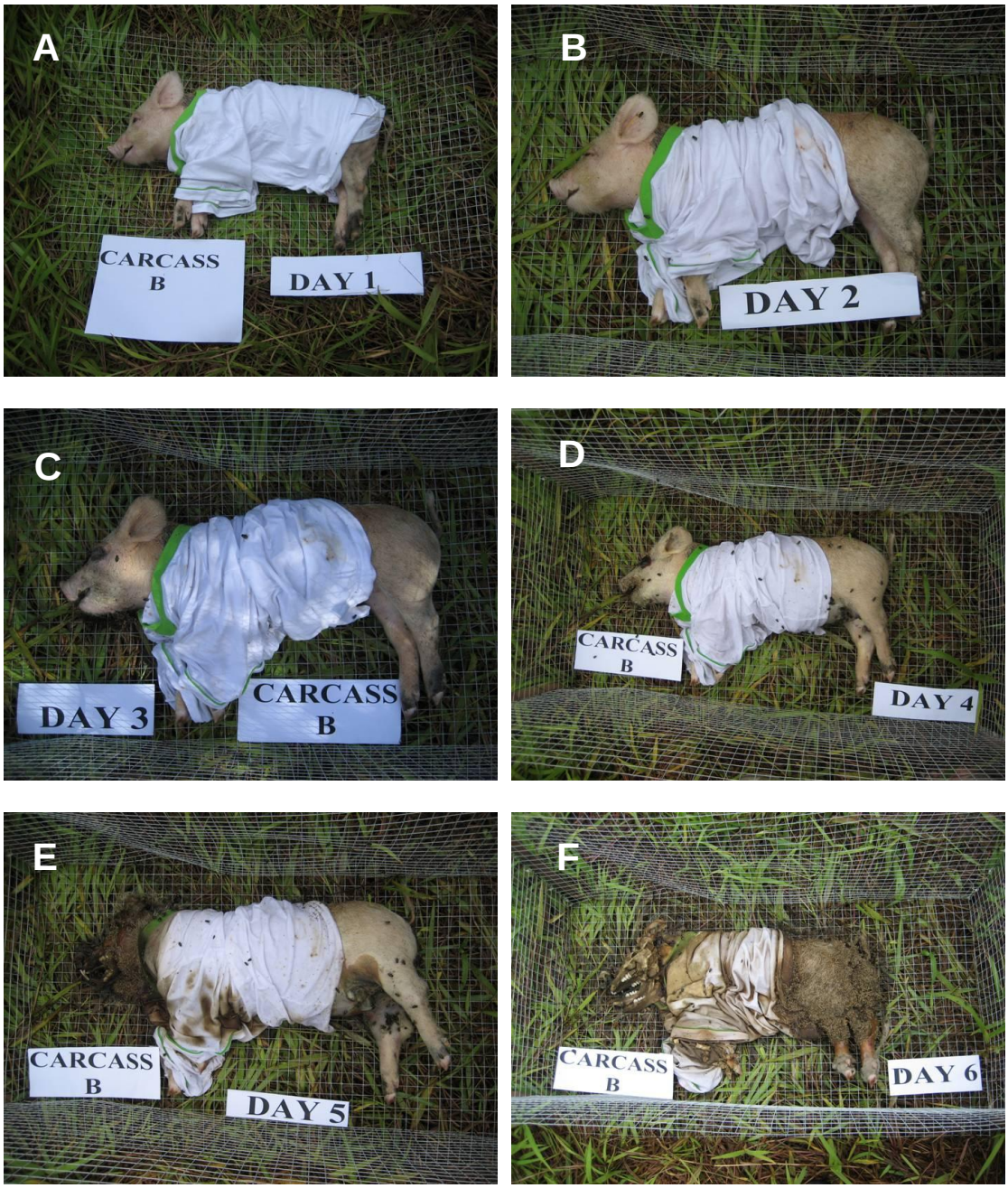


Figure 4.5: Changes of decomposition of carcass B throughout the study. (A) Day 1, clothed carcass shortly after placement. (B) Day 2, fresh stage. (C) Day 3, beginning of bloated stage. (D) Day 4, bloated stage continued. (E) Day 5, beginning of decay stage. (F) Day 6, decay stage continued. (G) Day 7, decay stage with skeletonised skull. (H) Day 8, dry remains.

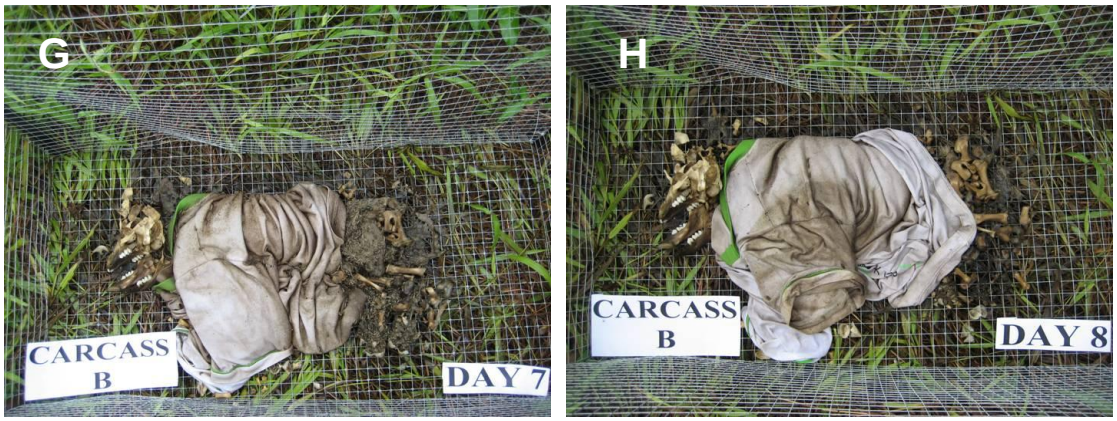


Figure 4.5 *(continued)*

4.4.4 Arthropod succession

A total of 13 dipteran species representing four families and eight genera were recovered during this study (Table 4.4). Non-dipteran arthropod species from other families were also collected, such as Formicidae, Vespidae and Acrididae. However, identification of the non-dipterans was only done up to the family level. Table 4.5 shows the occurrence of dipteran species, which were present as adult, egg and larval instars throughout different decomposition processes. A correlation between decomposition stages and Diptera larval succession pattern for carcass A and B is shown in Figure 4.6.

Table 4.4: Summary of insect species collected from 2 pig carcasses.

Family	Genus/Species	Carcass A	Carcass B
Calliphoridae	*Chrysomya megacephala*	A, I	A, I
	Chrysomya rufifacies	A, I	A, I
	Chrysomya nigripes	A	A, I
	Hemipyrellia ligurriens	A, I	A, I
	Hypopygiopsis violacea	A, I	A
Sarcophagidae	*Boettcherisca karnyi*	A, I	A
	Boettcherisca peregrina	A	
	Parasarcophaga albiceps		A
	Parasarcophaga misera	A	A
	Sarcosolomonia crinita		A
Muscidae	*Musca domestica*	A	A
	Ophyra spinigera	A	A, I
	Ophyra chalcogaster	A	A
Stratiomyidae	sp.	-	A
Formicidae	spp.	A	A
Acrididae	spp.	A	A
Vespidae	sp.	A	A

I: immature, A: adult

Table 4.5: Succession of insect species collected from 2 pig carcasses throughout the decomposition stages.

Decomposition stage	Family	Genus/Species	Carcass A	Carcass B
Fresh	Calliphoridae	*Chrysomya megacephala*	E, A	E, L_1, A
		Chrysomya rufifacies	E, A	E, L_1, A
		Chrysomya nigripes	A	-
		Hemipyrellia ligurriens	E, L_1, A	E, A
		Hypopygiopsis violacea	E, A	-
	Sarcophagidae	*Boettcherisca karnyi*	L_1, A	A
		Boettcherisca peregrina	A	-
		Parasarcophaga albiceps	A	-
		Parasarcophaga misera	A	-
		Sarcosolomonia crinita	-	-
	Muscidae	*Musca domestica*	A	A
		Ophyra spinigera	A	A
		Ophyra chalcogaster	A	A
	Stratiomyidae	sp.	-	-
	Formicidae	spp.	A	A
	Acrididae	spp.	A	A
	Vespidae	sp.	A	A
Bloated	Calliphoridae	*Chrysomya megacephala*	-	E, L_1, L_2, A
		Chrysomya rufifacies	-	E, L_1, L_2, A
		Chrysomya nigripes	-	A
		Hemipyrellia ligurriens	-	L_1, L_2
		Hypopygiopsis violacea	-	A
	Sarcophagidae	*Boettcherisca karnyi*	-	-
		Boettcherisca peregrina	-	-
		Parasarcophaga albiceps	-	-
		Parasarcophaga misera	-	-
		Sarcosolomonia crinita	-	A
	Muscidae	*Musca domestica*	-	A
		Ophyra spinigera	-	A
		Ophyra chalcogaster	-	A
	Stratiomyidae	sp.	-	A
	Formicidae	spp.	-	A
	Acrididae	spp.	-	A
	Vespidae	sp.	-	A
Decay	Calliphoridae	*Chrysomya megacephala*	L_2, L_3, A	L_2, L_3, A
		Chrysomya rufifacies	L_2, L_3, A	L_2, L_3, A
		Chrysomya nigripes	-	L_3, A
		Hemipyrellia ligurriens	-	L_2, L_3, A
		Hypopygiopsis violacea	-	-
	Sarcophagidae	*Boettcherisca karnyi*	A	A
		Boettcherisca peregrina	A	-
		Parasarcophaga albiceps	-	A
		Parasarcophaga misera	A	A
		Sarcosolomonia crinita	-	A
	Muscidae	*Musca domestica*	A	A
		Ophyra spinigera	A	A
		Ophyra chalcogaster	A	A

Table 4.5 *(continued)*

Decomposition stage	Family	Genus/Species	Carcass A	Carcass B
Decay	Stratiomyidae	sp.	-	A
	Formicidae	spp.	A	A
	Acrididae	spp.	A	-
	Vespidae	sp.	A	A
Dry	Calliphoridae	*Chrysomya megacephala*	A	A
		Chrysomya rufifacies	L_2, L_3, A	L_2, L_3, A
		Chrysomya nigripes	A	L_3
		Hemipyrellia ligurriens	A	-
		Hypopygiopsis violacea	-	-
	Sarcophagidae	*Boettcherisca karnyi*	-	A
		Boettcherisca peregrina	-	-
		Parasarcophaga albiceps	-	-
		Parasarcophaga misera	-	-
		Sarcosolomonia crinita	-	-
	Muscidae	*Musca domestica*	A	A
		Ophyra spinigera	A	L_3, A
		Ophyra chalcogaster	A	A
	Stratiomyidae	sp.	-	-
	Formicidae	spp.	A	A
	Acrididae	spp.	-	-
	Vespidae	sp.	A	-

E: egg, L_1: first instar larva, L_2: second instar larva, L_3: third instar larva, A: adult

4.4.4.1 Arthropod succession – carcass A

The first fly species that visited carcass A within a few minutes after being placed in the cage was of the family Sarcophagidae, followed by members of the family Calliphoridae, namely *H. violacea*, *C. megacephala*, and *C. rufifacies*. It was observed that these flies went directly to the wounded area of the carcass upon arrival. Ants were observed to scavenge the carcass on the same day and started to establish nests around the carcass. On Day 2 (fresh stage), the adult flies were seen on the carcass while larvae of the first and second instar stages were found to inhabit the internal organs and the scavenged wounds. Despite the decreasing number of adult flies inhabiting the vegetation or carcass on the following day (decay stage), an increased larval population density to a depth of up to 4 cm was observed. Non-hairy larvae, mostly of the species *C. megacephala*, were found on exposed internal organs, whereas hairy larvae of the

species *C. rufifacies* were found on the flesh and wound of carcass. Day 4 (decay stage) manifested decreased numbers of larvae on the carcass. The bigger sized larvae of *C. rufifacies* were observed to predate upon the smaller sized larvae of *C. megacephala*. Ant populations were observed to congregate at the periphery of the carcass and began to predate upon the post-feeding larvae. On day 5 (dry stage), no adult flies were observed on the vegetation and only a few of Muscidae adults (*M. domestica*, *O. spinigera* and *O. chalcogaster*) were found on the skeletons. The number of ants and feeding larvae was also markedly decreased. Post-feeding larvae of *C. rufifacies* were predominant, while the non-hairy larvae were absent and had migrated away from carcass.

4.4.4.2 Arthropod succession – carcass B

Within minutes after the placement of the carcass, female adults of *C. megacephala* and *C. rufifacies* were observed to lay eggs at the facial area. The ovipositors were clearly seen penetrating through the hairs of the carcass to deposit the eggs. On Day 2 (fresh stage), egg masses were found on the facial area and also on the clothing. Ants were seen to predate upon the eggs. The eggs started to hatch on the following day (fresh stage) while ants were found to establish their nests under the head of the carcass. Not many adult flies were found on the vegetation. Larvae started to colonise the carcass orifices, particularly the anus, in the ears, behind the ears, in the eye sockets and in the mouth on the very same day. Newly oviposited eggs were also found over the facial area. On day 4 (bloated stage), the population of the adult flies on the vegetation was found to have increased. Egg masses were found underneath the clothing near the abdomen of the carcass, while third-instar larvae were found in the anus. Ants were observed predating upon the non-hairy post-feeding larvae. On day 5 (decay stage), a big cavity was observed at the anus of the carcass as a result of larval colonisation.

Hairy larvae population on the carcass had increased while the number of the ant population decreased. On day 6 and 7 (decay stage), the population of adult flies inhabiting the vegetation had started to reduce. Larvae of *C. rufifacies* and *C. nigripes* were predominant on the skin and hair of the carcass. On day 8 (dry stage), *C. rufifacies* larval populations were seen migrating away or burrowing into the soil to pupate. *C. nigripes* and *O. spinigera* larvae were still inhabiting the bones and preparing to pupate in the soil.

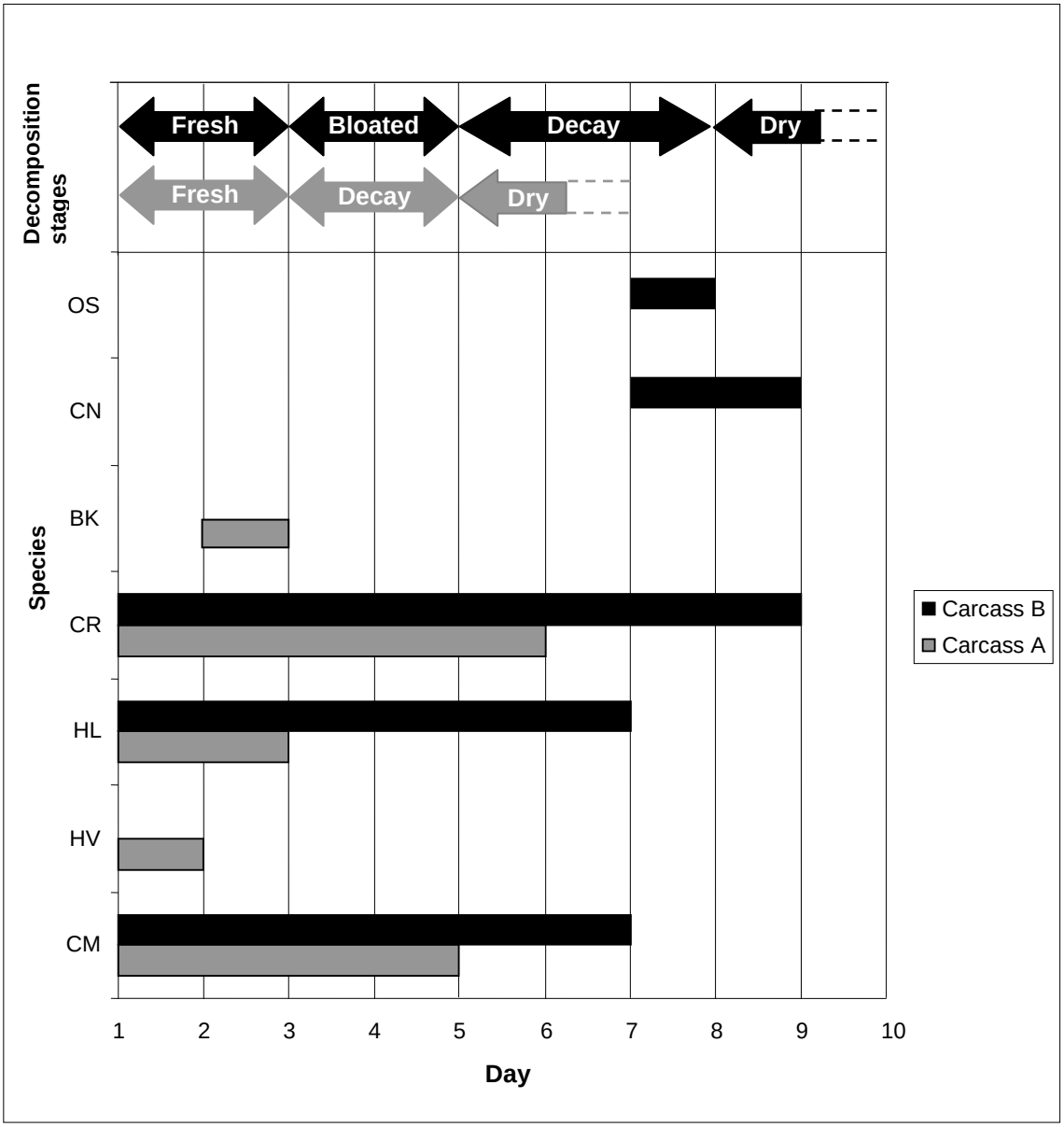


Figure 4.6: Observation of decomposition stages and Diptera larval succession patterns for carcass A and B. Species abbreviations, CM: *Chrysomya megacephala*, HV: *Hypopygiopsis violacea*, HL: *Hemipyrellia ligurriens*, CR: *Chrysomya rufifacies*, BK: *Boettcherisca karnyi*, CN: *Chrysomya nigripes* and OS: *Ophyra spinigera*.

4.4.5 Immature rearing

A total of seven species, five genera and three families (Calliphoridae, Sarcophagidae and Muscidae) of immature specimens were collected from the carcasses A and B. These immature specimens were then identified for their life stages (e.g. larval instar) and species (Table 4.5). Most of the immatures collected were eggs, first-instar larvae and second-instar larvae, except for *C. nigripes* and *O. spinigera*, which were third-instar larvae. The minimum duration of pupation for each immature species collected was presented in Table 4.6. Most of the larvae went through a 4-day larval development period before pupation. Among the seven immature species found on carcasses A and B, the minimum pupation period recorded was four days, which was established by *C. megacephala*.

Table 4.6: Minimum duration of pupation before emerge as adult.

Genus/Species	Duration of pupation (days)
Chrysomya megacephala [a]	4
Chrysomya rufifacies [a]	5
Chrysomya nigripesi [b]	5
Hemipyrellia ligurriens [a]	13
Hypopygiopsis violacea [a]	14
Boettcherisca karnyi [a]	11
Ophyra spinigera [b]	11

[a] Minimum feeding period is 4 days
[b] Third-instar larvae collected from carcass

4.4.5.1 Immature rearing from carcass A

Hypopygiopsis violacea was obtained only from day 1, while the flesh fly, *B. karnyi*, was obtained only from day 2. On the other hand, *H. ligurriens* was obtained from day 1 to day 3, *C. megacephala* was obtained from day 1 to day 4 and *C. rufifacies* was obtained from day 1 to day 5.

4.4.5.2 Immature rearing from carcass B

Eggs of *H. ligurriens* were obtained from day 1 and day 4, while *C. megacepahala* was obtained from day 1 to day 5. *Chrysomya rufifacies* was obtained from day 1 to day 8. Third-instar larvae of *C. nigripes* and *O. spinigera* were obtained from day 7 while only *O. spinigera* was obtained on day 8.

4.5 Discussion

In this study, the process of decomposition from fresh to skeletal or dry remains took place in a rapid manner in both carcasses (Table 4.3). Compared to previous reports, this study had accomplished a shorter decomposition period although similar weight and the same type of carrion were used (Tullis & Goff 1987, Vitta *et al*. 2007, Heo *et al*. 2007). The process of decomposition is relatively hastened by the microclimatological factors (Souza *et al*. 2008). For example, high amounts of rainfall would accelerate the process of decay (Archer 2003b, Ahmad & Ahmad 2009). Kuching, the experimental site, is the largest city in the Borneo Island, which possesses a tropical rainforest climate under the Köppen climate classification. This tropical rainforest climate is characterised by high temperature and humidity. Kuching is also a city that receives the highest amount of precipitation of rain fall in Malaysia each year. The experiment was conducted in December, which is a pronounced rainy season in Kuching. Therefore, large amounts of rainfall were expected during the succession

experiment. The ambient temperature and the relative humidity of the study site were very high and not affected by this high precipitation. All these meteorological factors, especially ambient temperature and humidity have been reported to play significant roles in accelerating the decomposition rate, (Rodriguez & Bass 1983, Shean *et al* 1993, Moura *et al*, 1997, Campobasso *et al*. 2001).

For both carcasses, each of the decomposition stages occurred rapidly, resulting in some details of each stage being not clearly detectable when compared with observations made in studies in temperate countries. Transition from one stage to another may overlap, and the transition from one stage to another was difficult to be determined as there was no clear difference between stages (Campobasso *et al*. 2001, Archer 2003a). Despite this ambiguity, the succession of the arthropod species at specific decomposition stage was clearly observed in the study (Figure 4.6).

The size of the experimental carcasses is a source of constant argument in the study of insect succession pattern. Hewadikaram & Goff (1991) reported that no size-related differences were evident in insect species composition or patterns of succession, as opposed to Anderson & VanLaerhoven (1996) who reported that carcass size would affect the insect succession. This incongruity should be validated in future experiments using carcasses of different size at the same time and location.

In our study, carcass A decomposed faster than carcass B and this is attributed to the fact that the former was scavenged. The abdominal part of carcass A, which includes the rib and some of the internal organs, were partially scavenged. As such, the bloated stage was not obvious since the gas produced by the bacterial activity escaped through the open wound. This scavenging effect was also thought to accelerate the decomposition process in carcass A (Komar 1998, De Jong & Chadwick 1999). The scavenged wound of carcass A had also altered the starting site of decomposition, from the centre of abdomen where the internal organs were exposed and moving outward to

the anterior and posterior parts of the carcass. The odour of blood from the wound had attracted the flies and accelerated their activities on the carcass, which in turn had sped up biomass removal. Consequently, larval colonisation was highly concentrated at the wound as opposed to the natural orifices (Anderson 2001). Larval colonisation is crucial in assisting forensic entomologists to recognise the fatal antemortem wound of a corpse (Campobasso & Introna 2001). However, caution needs to be taken as postmortem scavenging can be misleading (Anderson 2001).

When compared to carcass A, clothing material on carcass B did not delay oviposition of Diptera but rather served as a shelter to the eggs and the early instar larvae from the rain (Grassberg & Frank 2004). The decomposition of carcass B started at the natural orifices, both anterior (eyes, ears, mouth, nostrils) and posterior (anus, genitalia) towards the central part of the carcass (thorax). The first skeletonised part for carcass B was the head, where seven natural orifices are present (two eyes, ears, nostrils and a mouth) (Tullis & Goff 1987). Formation of adipocere was observed at day seven under the clothing and near the hip of the carcass. Greasy adipoceres are often formed at the areas covered by clothing material, accompanied by damp and high temperature (Mellen *et al*. 1993). As expected, this phenomenon was not observed in carcass A.

In studies by Heo *et al*. (2007, 2008a & b), different physical conditions of a carcass (e.g. fresh and burnt) and placement of a carcass (e.g. in the water) were tested for arthropod succession and decomposition studies in Malaysia. In their studies, dipterans were the major arthropod involved in decomposition. The presence of families of Muscidae, Calliphoridae, Sarcophagidae, Formicidae, Arachnidae, Lepidoptera, Staphylinidae and Stratiomyidae were reported in their study (2007). However, their reports did not include information on succession with the reference to time, location and environmental conditions. To date, Heo *et al*. (2007, 2008a & b) are the only studies on pig carcasses in Malaysia, although some have used monkey carcasses as the

animal model for succession studies (Omar *et al*., 1994a & b). More studies should be carried out to obtain an extensive baseline arthropod succession data from different biogeographical regions (e.g. seashore, forest, urban, rural, highland, lowland) and other circumstances of carcasses (e.g. scavenged, with clothing, in buildings or cars) because there are no dramatic changes in climate for a tropical country like Malaysia. Therefore, geographical distribution studies would be more important than seasonal distribution studies in Malaysia. Endemic species might be discovered from corpses which would be helpful in determining the location of crime scene.

Although most literature prefers to report the temperature differences (Δtemp) between internal temperature (IT) and ambient temperature (AT) (Anderson & VanLaerhoven, 1996; De Jong & Chadwick, 1999; Grassberg & Frank, 2004), emphasis should be given to the larval mass temperatures as these are disproportional to the ambient temperatures (Richards & Goff, 1997). In a recent finding by Thevan *et al*. (2010), larvae of *C. megacephala* were able to survive and grow in the mouth of a corpse, while the body was kept in a morgue cooler. They were able to show that heat generated by a larval mass is sufficient to allow larval development, even in a 4°C morgue cooler. This is also supported by the fact that the larval mass can thermoregulate their temperature in an extremely low temperature, and as such, the ambient temperature is relatively unimportant compared to the larval mass temperature.

Larval mass temperature is generated by exothermic aggregated feeding/growing larvae. Greenberg (1991) reported that this temperature would be at its maximum at the second-instar larval stage upon reaching the third-instar stage. Although adult activity is primarily governed by the ambient temperature, larvae can still be active and develop rapidly (Reed, 1958; Shean *et al*., 1993). Richards and Goff (1997) reported a little correlation but not a proportional link between maximum internal carcass temperature (IT) and ambient temperature (AT) for 3 pig carcasses at different elevations, IT=52°C,

AT=26°C at 1877m (Δtemp=26°C), IT=44°C, AT=18°C at 1169m (Δtemp=26°C) and IT=52°C, AT=29°C at 646m (Δtemp=23°C). In their study, the maximum internal carcass temperature was not affected by the ambient temperature. In this study, larval mass temperature was as high as 41°C in carcass A at day three (maxAT=41°C, minAT=23°C) and 42°C in carcass B at day five (maxAT=37°C, minAT=22.5°C) where both carcasses were in their decay decomposition stage. In other words, larval mass temperature is disproportional to the ambient temperature and is affected by the developmental stage of the larvae.

The first Diptera species to arrive on a corpse were those from the families Sarcophagidae and Calliphoridae for carcass A and B, respectively, and this result agrees with published reports (Tullis & Goff 1987, Anderson 2001, Watson & Carlton 2003, Tabor & Richard 2005, Heo *et al*. 2007, Ahmad and Ahmad 2009). Calliphorids were the quickest to respond to the presence of the carcasses (within minutes) and oviposited soon upon landing on the corpse (Anderson & VanLaerhoven 1996, Ahmad & Ahmad 2009). However, with exposed flesh and a bloody open wound present in carcass A, the Sarcophagidae were responding faster than the Calliphoridae.

In this study, it was found that larvae of two species from Calliphoridae were predominant on both carcasses A and B, in which *C. megacephala* was only predominant at the early stage of decomposition, whereas *C. rufifacies* was present throughout the whole decomposition stage. The population of *C. rufifacies* also increased markedly, becoming the predominant species at the later stages of succession. This has been reflected in previous works in various habitats (Hewadikaram & Goff 1991, Omar *et al*. 1994a & b, Richard & Goff 1997, Heo, *et al*. 2007, Vitta *et al*. 2007, Wang *et al*. 2008). These two species are synanthropic (Sulaiman *et al*. 2000) and are widely distributed around the world. As such, they are not particularly suitable to serve as biogeographic indicators, although they play a major role in biomass removal of

carcasses. The predominance of *C. rufifacies* is thought to be due to the natural cannibalistic and predacious behaviour of this species towards conspecifics, triggered by insufficient food supply at the later stage of decomposition (Campobasso *et al*. 2001, Jenson 2001). This was also seen to lead to the eventual suppression of the population density of *C. megacepahala*. This observation corroborates the findings of Hewadikaram & Goff (1991), as well as Jenson (2001), who conducted a similar experiment in the tropical countries and the climates similar to Malaysia.

Hemipyrellia ligurriens was found at the early stage of decomposition, which includes fresh and bloated stages on both carcasses. O'Flynn (1983) reported similar findings, where the first-instar larvae of this species were recovered on day 2 during summer in Brisbane, Australia. Therefore, this result suggests that perhaps *H. ligurriens* could serve as the species indicator for early decomposition.

The ant activities on both carcasses observed in this study did not appear to be interested in the carcass, but more towards predating upon the eggs and larvae. Similar findings were observed in the study of Grassberg & Frank (2004). Ants were found present throughout the decomposition, which is in agreement with the observation of Heo *et al*. (2009), and therefore could not serve as time or regional indicators during the decomposition stage (Arnaldos *et al*., 2005). No carrion beetle species were collected during this study due to rapid decomposition. This observation is also similar to what was noted by Jenson & Miller (2001).

Despite close proximity for both carcasses, significant differences of dipteran species recovered from the carrions were observed. First-instar larvae of *Hypopygiopsis violacea* (Calliphoridae) and second-instar larval stage of *B. karnyi* (Sarcophagidae) were only recovered from carcass A. Although *P. misera* was the predominant flesh fly found at the experimental site, it should be noted that no immature generations of this species were recovered from both carcasses, and this is consistent with the observation

reported by Tullis & Goff (1987). The presence of *H. violacea* and *B. karnyi* on the carcass A is possibly due to the attraction to the blood odour of flesh and exposed internal organs resulted from vertebrate scavenging. It was observed that larvae of Sarcophagidae and Calliphoridae coexisted without competition, and this finding corroborates that reported by Souza *et al*. (2008), who described the similar observations in their insect succession study in Guam. However, more investigation concerning the behaviour and ecology of sarcophagids and calliphorids should be carried out to obtain a better understanding of the population dynamics of these flies on carcass. In addition, from the distribution study conducted on Calliphoridae and Sarcophagidae throughout Malaysia, *B. karnyi* was categorised as a swamp area species (H. Kurahashi, pers. comm.) while *H. violacea* was categorised as a forest species (Kurahashi *et al*., 1997). Hence, these two species could serve as environmental indicators for these regions.

Larvae of *C. nigripes* and *O. spinigera* were only found in carcass B due to their preference to the adipocere formation which developed at the later stage of decomposition (Grassberg & Frank 2004). De Jong & Chadwick (1999) reported that scavenged carcass would accelerate desiccation in decomposition which could explain the absence of *C. nigripes* and *O. spinigera* from carcass A in this study. *Chrysomya rufifacies* does not predate on *C. nigripes* and *O. spinigera* (Grassberg & Frank 2004). In fact, they appeared to co-exist on carcass B during the late decomposition stage. This might be due to the fact that *C. nigripes* shows a distinctive pattern, with a black stripe along the dorsal structure which is an almost similar in appearance to *C. rufifacies*. Hence, the presence of *C. nigripes* and *O. spinigera* at the late stage of decomposition could serve as time indicators as previously suggested (Tullis & Goff 1987, Omar *et al*. 1992, Byrd & Castner 2001, Wang *et al*. 2008).

A short duration time for adult emergence is expected in a tropical rainforest climate. The minimum life cycle duration of each species can be estimated from Table 4. This information would serve as baseline data which will be useful for PMI estimation in forensic investigation due to the fact that different species have different pupation period. However, an in-depth study on other factors governing succession, such as microenvironmental relationships and the interval time of fly oviposition period is indeed crucial (Reed 1958, O'Flynn, 1983). An extensive further study on developmental and growth rate of different Malaysian carrion fly species, especially sarcophagid flies, are also required in view of their frequent occurrence on carrion in tropical regions (Goff *et al*., 1986).

4.6 Conclusion

Arthropod succession patterns vary from location to location (Wang *et al*., 2008). Bioclimatic and biogeographic factors are the main determinants of species distribution. Borneo and Peninsular Malaysia are two different regions with different species distribution. Therefore, comprehensive regional arthropod succession studies which are suitable for each location should be carried out before it can be applied in forensic entomology locally. In almost all cases, corpses are frequently clothed (Anderson, 2001). For this reason, clothed carcasses are essential for mimicking human corpse for arthropod succession study. In this study, some important indicators were reported for time and geography. *H. violacea* and *B. karnyi* are important in specific biogeography while *Hemi. ligurriens* could serve as the early decomposition stage indicator. On the other hand, *C. nigripes* and *O. spinigera* could serve as late decomposition stage indicators. Despite the close proximity of the two carcasses in this study, significant differences of Diptera species obtained were observed. These differences are associated to differences in physical condition of the carcasses. Therefore, different physical

condition, bioclimate and biogeography studies should be carried out in the future in order to obtain a more comprehensive decomposition and arthropod succession data.

Chapter 5:

DNA-based identification of forensically important Calliphoridae species in Malaysia

5.0 Abstract

In Malaysia, forensic entomology is gradually gaining importance. A quick and accurate identification system is desirable in any forensic entomological analysis. Calliphoridae are the most important fly family involved in forensic related cases in Malaysia. Fly species of the genera *Chrysomya*, *Calliphora*, *Lucilia*, *Hemipyrellia*, and *Hypopygiopsis* are the important indicators in the determination of PMI.

In this study, PCR amplification from different life stages of fly (e.g. egg, larva, pupa and adult) and different preservation ages (e.g. fresh, 2 year-old and 10 year-old archive specimens) were tested to establish their reliability for species identification. DNA-based identification of 19 Malaysian Calliphoridae species using approximately 2.3kb of mitochondrial DNA regions of *cytochrome c oxidase subunit* I and II (COI+II), encompassing the tRNA leusine gene, was analysed to evaluate the efficiency of this marker. All species successfully clustered into distinct clades except for some species of genera *Chrysomya* (*C. defixa* and *C. pinguis*), *Hemipyrellia* (*H. ligurriens* and *H. tagaliana*) and *Lucilia* (*L. papuensis*, *L. bismarckensis* and *L. calviceps*). Further study was carried out to discriminate *C. defixa* and *C. pinguis* using 28S ribosomal DNA (D1-D7, 2.2kb) since they are the most important species in forensic cases in Malaysia. The results showed that the 28S rDNA sequences are sufficient to differentiate all the *Chrysomya* species in Malaysia.

The PCR-RFLP technique also been analysed to facilitate species identification of eight *Chrysomya* species in Malaysia. Three unique restriction profiles (*C. rufifacies*, *C. thanomthini* and *C. villeneuvi*) were obtained by *Ssp*I, which would be useful for species identification of these species. Further differentiations of *C. chani*, *C. megacephala* and *C. nigripes* were achieved using *Taq*$^{\alpha}$I and *Msp*I.

5.1 Introduction

Analysis of insect succession patterns at various stages of corpse decomposition may facilitate criminal investigations through the estimation of the minimum post-mortem interval (PMI), and sometimes may even provide clues to the cause and place of death (Anderson, 2004). Accurate species identification is therefore crucial especially for estimating the PMI when legal matters are involved (Wells & LaMotte, 2001). Conventionally, adult insect species, especially flies, are identified based on specific morphological features, such as presence and number of bristles, wing venation, and body colouration (Smith, 1986; Wallman & Donnellan, 2001). The immature stages are, however, almost impossible to identify and often require trained eyes as identification is based on specific characters, such as the pattern differences of the spine, posterior spiracle and cephalopharyngeal skeleton (Wells & Sperling, 1999 & 2001; Nelson *et al*., 2008). For most cases, identification of larval species is only feasible on third-instar larva (Wells *et al*., 1999; Turchetto *et al*., 2001). Younger larvae require rearing to the third instar or to maturity (adulthood) to enable the species identification and this often involves additional work and time (Wallman & Adams, 2001).

To overcome all the difficulties faced in morphology-based identification, the use of comparative DNA sequence analysis to facilitate species identification has become increasingly popular in recent years due to their ease of use, rapidity and reliability (Vincent *et al*., 2000; Wallman & Donnellan, 2001; Harvey *et al*., 2003a, 2003b; Ames *et al*., 2006; Nelson *et al*., 2007, 2008; Singh *et al*., 2010). It is an attractive alternative to conventional morphology-based identification methods as it can be applied to any life stage and any preservation method of an insect (Sperling *et al*., 1994). The usefulness of DNA-based identification methods have been proved in many insects studies, including flies (Noël *et al*., 2004), butterfly (Lukhtanov *et al*., 2009), dragonfly (Rach *et al*., 2008), wasp (Arévalo *et al*., 2004; Erasmus *et al*., 2007) and

beetles (Monaghan *et al*., 2005). To date, many studies of DNA-based identification of Calliphoridae have been carried out across different DNA regions, species and countries and the results are promising (Table 5.1).

In line with DNA barcoding efforts (Hebert *et al*., 2003; Hebert & Gregory 2005), mitochondrial DNA has been one of the more common targets for analysis, and has shown promising results in several forensic identification studies (Wells *et al*., 2001; Harvey *et al*., 2003b). Due to its higher sequence variability as compared to nuclear DNA (Moriyama & Powell, 1997), mitochondrial DNA is also widely used for taxonomic, phylogenetic, population, evolutionary studies, and species differentiation (Simon *et al*., 1994; Hillis *et al*., 1996; Malgorn & Coquoz, 1999; Wells & Sperling, 2001; Zehner *et al*., 2004), as well as intra- and interspecific comparison.

The complete or partial of *cytochrome c oxidase* subunit I gene has been widely used and is recognised as a good marker for species identification, particularly in forensically important blow fly species. This was indicated by many studies (Malgorn & Coquoz, 1999; Vincent *et al*., 2000; Litjens *et al*., 2001; Harvey *et al*., 2003a & b, 2008; Chen *et al*., 2004). However, some other studies have used a combination of *cytochrome c oxidase* subunit I and II for DNA-based identification of blow fly species, as more genetic information are provided and the sequence data are easily available from GenBank for comparison and reference (Sperling *et al*, 1994; Wells & Sperling, 2001; Wells *et al*., 2002). Nevertheless, precaution has to be taken into account where some closely related species may not be distinguished using these DNA sequences (Wallman & Donnellan, 2001; Stevens *et al*., 2002; Wells & Williams, 2007; Wells *et al*., 2007).

The use of nuclear DNA has also been suggested by other researchers for species identification, such as the 28S ribosomal DNA (rDNA). The studies of rDNA sequences have been used to infer phylogenetic history among closely related species

(Stevens & Wall, 2001; Stevens *et al*., 2002; Steven, 2003). These nuclear rDNA are present in multiple copies in the genome, making them easy to amplify by PCR. The 28S large subunit rDNA (lsu) shows a peculiar organization for the presence of conserved and highly variable regions, known as the divergence region, D (Sonnenberg *et al*., 2007). The divergence region of 28S rDNA consists of 12 divergent domains (D1 to D12), which are widely used in molecular genetic analyses (Gillespie *et al*., 2005). These divergent domains have demonstrated significant divergence between closely related species (Otranto *et al*., 2005).

In Malaysia, forensic entomology is gradually gaining in importance. Calliphoridae are the most important fly family involved in forensic related cases (Lee, 1989 & 1996; Hamid *et al*., 2003; Lee *et al*., 2004; Mohd Salleh *et al*., 2007) and these include the species of *Chrysomya*; *Chrysomya megacephala* (Fabricius), *Chrysomya rufifacies* (Macquart), *Chrysomya villeneuvi* Patton, *Chrysomya nigripes* Aubertin, and *Chrysomya pinguis* (Walker). Other calliphorid genera, which have also been documented in forensic cases, are *Lucilia*, *Hemipyrellia* and *Calliphora* (Lee, 1989; Lee *et al*., 2004). Some of these species, particularly the sister species and closely related species, are very similar in their morphological appearance. Therefore, the DNA-based identification method is necessary to overcome the setback of morphology-based identification.

5.2 Objectives

The main objective of this study is to determine the importance and the effectiveness of mitochondrial DNA regions (COI+II) in DNA-based identification of forensically important calliphorid species in Malaysia and to compare the DNA sequences obtained from published data available in The National Center for Biotechnology Information (NCBI).

Specific objectives include:

1. To evaluate the efficiency of DNA extraction and PCR amplification from different life stages of fly (egg, larva, pupa and adult) and different preservation ages (fresh, 2 year-old and 10-year old archive specimens) and to establish their reliability for species identification.
2. To evaluate the utility of (i) *cytochrome c oxidase* subunit I and II and (ii) 28S ribosomal DNA in species identification, and subsequently in establishing a rapid DNA-based identification method suitable for forensically important calliphorid species in Malaysia.
3. To establish a PCR-RFLP species identification assay for eight forensically important *Chrysomya* species in Malaysia.

Table 5.1: DNA region used in DNA-based identification analyses of blow flies.

Studies	DNA region [a]	Length (bp)	Calliphoridae species
Sperling *et al*., 1994	COI+II	2.3kb	2 *Lucilia* spp. and *Phormia regina*
Stevens & Wall, 1996	12S rDNA	329	*Calliphora vicina* and 2 *Lucilia* spp.
Stevens & Wall, 1997	12S rDNA	322	*Calliphora vicina, Hemipyrellia fernandica* and 10 *Lucilia* spp.
Malgorn & Coquoz, 1999	COI	297 & 304	*Calliphora vicina* and 3 *Lucilia* spp.
Wells & Sperling, 1999	COI	2.3kb	2 *Chrysomya* spp., *Lucilia sericata* and *Phormia regina*
Lessinger & Azeredo-Espin, 2000	CR	1000-1600	*Chrysomya megacephala*, 2 *Cochliomyia* spp. and *Lucilia eximia*
Vincent *et al*., 2000	COI	140	2 *Calliphora* spp., *Cynomya mortuorum*, 3 *Lucilia* spp. and *Protophormia terraenovae*
Litjens *et al*., 2001	COI	870	2 *Cochliomyia* spp.
Stevens & Wall, 2001	28S rDNA	2.2kb	2 *Calliphora* spp., *Cynomya mortuorum*, 4 *Lucilia* spp. and *Protophormia terraenovae*
Wallman & Donnellan, 2001	COI COII	639 635	7 *Calliphora* spp., 2 *Chrysomya* spp. and *Onesia tibialis*
Wells & Sperling, 2001	COI+II	2.3kb	*Chrysomya* spp., *Cochliomyia macellaria, Compsomyiops callipes, Eucalliphora latifrons, Phormia regina, Protocalliphora sialia* and *Protophormia* spp.
Stevens *et al*., 2002	COI+II 28S rDNA	2.3kb 2.1kb	*Calliphora vicina* and 3 *Lucilia* spp.
Wells *et al*., 2002	COI+II	2.3kb	*Calliphora vicina*, *Chrysomya rufifacies*, 3 *Dyscritomyia* spp., *Eucalliphora latifrons,* 6 *Lucilia* spp. and *Phormia regina,*
Harvey *et al*., 2003a	COI	278	2 *Calliphora* spp., 2 *Chrysomya* spp. and *Lucilia sericata*
Harvey *et al*., 2003b	COI	1167	7 *Chrysomya* spp. and 2 *Lucilia* spp.
Stevens, 2003	COI+II 28S rDNA	2.3kb 2.2kb	*Auchmeromyia luteola*, *Cordylobia anthropophaga*, 5 *Calliphora* spp., 5 *Chrysomya* spp., 2 *Cochliomyia* spp., *Cynomya mortuorum*, *Cynomyopsis cadaverina*, *Hemipyrellia fernandica*, 10 *Lucilia* spp., *Onesia tibialis*, *Pollenia rudis* and 3 *Protocalliphora* spp.
Chen *et al*., 2004	COI	1588	3 *Chrysomya* spp., *Hemipyrellia ligurriens* and 4 *Lucilia* spp.
Wells *et al*., 2004	COI	593	5 *Chrysomya* spp.
Cai *et al*., 2005	COI	278	*Chrysomya megacephala* and 2 *Lucilia* spp.
Saigusa *et al*., 2005	COI	304	2 *Calliphora* spp., *Chrysomya pinguis* and 3 *Lucilia* spp.

Table 5.1 *(continued)*

Studies	DNA region [a]	Length (bp)	Calliphoridae species
Wallman *et al*., 2005	COI COII ND4 ND4L	822 638 884 968	18 *Calliphora* spp., 9 *Chrysomya* spp., 2 *Hemipyrellia* spp. and 3 *Lucilia* spp.
Ames *et al*., 2006	COI	523	2 *Calliphora* spp.
Nelson *et al*., 2007	COI	658	*Calliphora ochracea*, 9 *Chrysomya* spp., *Hemipyrellia fergusoni* and *Lucilia porphyrina*
Wells *et al*., 2007	COI	1545	*Cynomya cadaverina*, *Chrysomya rufifacies*, *Dyscritomyia* spp., *Hemipyrellia ligurriens*, 14 *Lucilia* spp. and *Phormia regina*
Wells & Williams, 2007	COI	304	*Aldrichina grahami, Calliphora vicina,* 4 *Chrysomya* spp., *Cochliomyia macellaria, Compsomyiops callipes, Cynomya cadaverina, Phormia regina* and *Protophormia terraenovae*
Ying *et al*., 2007	COII	635	7 *Calliphora* spp., 3 *Chrysomya* spp., 2 *Lucilia* spp. and *Onesia tibialis*
Desmyter & Gosselin, 2008	COI	304	*Lucilia sericata, Chrysomya albiceps, Phormia regina* and *Protophormia terraenovae*
Duarte *et al*., 2008	CR	854-1905	3 Calliphora spp., *Chloroprocta idioidea,* 5 *Chrysomya* spp., 2 *Cochliomyia* spp., 3 *Hemilucilia* spp., 2 *Lucilia* spp. and *Phormia regina*
Harvey *et al*., 2008	COI	1167	9 *Callihpora* spp., 12 *Chrysomya* spp., 2 *Cochliomyia* spp. and 4 *Lucilia* spp.
Nelson *et al*., 2008	ITS2	1010-1354	9 *Chrysomya* spp.
Song *et al*., 2008b	ITS2	301-337	*Aldrichina grahami, Achoetandrus rufifacies,* 2 *Calliphora* spp., 2 *Chrysomya* spp., *Hemipyrellia ligurriens*, 7 *Lucilia* spp. and *Phormia regina*
Song *et al*., 2008c	ITS2	297-337	*Aldrichina grahami, Achoetandrus rufifacies,* 2 *Calliphora* spp., 2 *Chrysomya* spp., *Hemipyrellia ligurriens*, 4 *Lucilia* spp. and *Phormia regina*
Reive *et al*., 2009	COI	229	2 *Calliphora* spp., 3 *Lucilia* spp. and *Protophormia terraenovae*
Park *et al*., 2009a	COI	1539	*Calliphora vicina*, *Hemipyrellia ligurriens* and 4 *Lucilia* spp.
Park *et al*., 2009b	COI	1314	*Aldrichina grahami,* 6 *Calliphora* spp., *Cynomya cadaverina, Eucalliphora latifrons, Lucilia illustris* and *Triceratopyga calliphoroides*

Table 5.1 *(continued)*

Studies	DNA region [a]	Length (bp)	Calliphoridae species
DeBry *et al*., 2010	COI	1.2kb	*Calliphora vicina*, *Chrysomya putoria*, *Cochliomyia hominivorax*,
	28S rDNA	2.1kb	2 *Dyscritomyia* spp., 3 *Hemipyrellia* spp. and 11 *Lucilia* spp.
Singh et al., 2010	COI	1536	*Calliphora vomitoria*, 22 *Chrysomya* spp., 2 *Cochliomyia* spp., *Lucilia sericata* and
	CPS	850	*Phormia regina*.

[a] Abbreviation of DNA region of the studies.

COI: *cytochrome c oxidase* subunit I; COII: *cytochrome c oxidase* subunit II; COI+II: *cytochrome c oxidase* subunit I and II encompassing tRNA leucine; CR: control region; 12S rDNA: 12S ribosomal DNA; 28S rDNA: 28S ribosomal DNA; ITS2: internal transcribed spacer 2; ND4: *NADH dehydrogenase* subunits 4 and ND4L: *NADH dehydrogenase* subunits 4Land CPS: carbamoylphosphate synthetase.

5.3 Materials and methods

5.3.1 Fly and larval specimens

Live blow flies and immature stages (larvae and eggs) were collected from several locations to represent different geographical conditions (Table 5.2). Live flies were collected from the field using meat baits. Identification of adult flies was carried out according to the identification keys of Kurahashi *et al*. (1997). Ambiguous identifications were further confirmed by Kurahashi.

In an attempt to evaluate the efficiency of DNA extraction and PCR amplification from different life stages of fly, the immature stages of identified flies (egg, larva, pupa and puparium) were obtained from existing *C. megacephala* laboratory colonies. Fly specimens from archived material were also included in this study to compare the efficiency of DNA extraction and amplification. The adult flies were killed with ethyl acetate while the larvae were placed in a microcentrifuge tube, prior to kill in 60°C water for 10 minutes.

To compare the efficiency of DNA extraction and PCR amplification from different preservation ages (fresh, 2 year-old and 10-year-old archive specimens), fresh specimens were collected from the field and the archival specimens were obtained from the personal collection of Professor Dr. Baharudin Omar, Department of Biomedical Science, Faculty of Allied Health Science, National University of Malaysia.

Table 5.2: Collection locality and reference data for Calliphoridae specimens used in this study.

Species	Voucher	Location/State	Country
Calliphora fulviceps	Calli-13 [a]	Cameron Highlands, Pahang	Malaysia
	Calli-CH	Cameron Highlands, Pahang	Malaysia
	CF-UKali [a]	Genting Highlands, Pahang	Malaysia
Calliphora vicina	Cvicina-JP [a]	Japan	Japan
	Cvicina-UK [a]	London	United Kingdom
	AJ417702 [a, c]	Laboratory colony	United Kingdom
	AJ300131 [b, c]	University of Bristol colony	United Kingdom
Chrysomya bezziana	CB-3/9	Bario, Sarawak	Malaysia
	AF295548 [a, c]	Bogor	Indonesia
	AJ551434 [b, c]	Bogor	Indonesia
Chrysomya chani	CC-UM-26/9 [a]	University of Malaya, Kuala Lumpur	Malaysia
	Z85 [a]	Genting Highlands, Pahang	Malaysia
	W-Chry [a]	Raub, Pahang	Malaysia
	CJ3-CC [a]	Kuala Woh, Perak	Malaysia
	CC-Penang [a]	Penang	Malaysia
	CC-F11-UM [b]	University of Malaya, Kuala Lumpur	Malaysia
	CC-F12-UM [b]	University of Malaya, Kuala Lumpur	Malaysia
	CC-AD2 [b]	Bario, Kuching	Malaysia
Chrysomya defixa	CD-AS2	Gombak, Selangor	Malaysia
	CD-AS3 [b]	Gombak, Selangor	Malaysia
	KH37	Gombak, Selangor	Malaysia
	KH38	Gombak, Selangor	Malaysia
	E24-CD	Templer Park, Selangor	Malaysia
	E26-CD	Templer Park, Selangor	Malaysia
	E39-CD [b]	Gombak, Selangor	Malaysia
	CD-F17-AS [b]	Gombak, Selangor	Malaysia
	CD-F18-AS [b]	Gombak, Selangor	Malaysia
	CD-forest	Bario, Kuching	Malaysia
	CD1 [a]	Matang Wildlife Centre, Sarawak	Malaysia
	CD-SWK [a]	Matang Wildlife Centre, Sarawak	Malaysia
	LP-4-CD [a]	Kuching, Sarawak	Malaysia
Chrysomya megacephala	Fly [a]	Taman Melawati, Selangor	Malaysia
	CM41 [a]	Gombak, Selangor	Malaysia
	CM-Muar [a]	Muar, Johor	Malaysia
	CM-forest-3/9 [a]	Bario, Sarawak	Malaysia
	AF295551 [a]	Papua New Guinea	Papua New Guinea
	CM-F1-UM [b]	University of Malaya, Kuala Lumpur	Malaysia
	CM-F2-UM [b]	University of Malaya, Kuala Lumpur	Malaysia
	CM-F15-AS [b]	Gombak, Selangor	Malaysia
	CM-UK1 [b]	Genting, Pahang	Malaysia
	CM-UK2 [b]	Genting, Pahang	Malaysia
	AJ551435 [b, c]	Fuertaventura	Spain

Table 5.2 *(continued)*

Species	Voucher	Location/State	Country
Chrysomya nigripes	CN-UM-20/8 [a]	University of Malaya, Kuala Lumpur	Malaysia
	CN-Gom [a]	Gombak, Selangor	Malaysia
	C-SWK-5 [a]	Kuching, Sarawak	Malaysia
	CN1-B [a]	Kuching, Sarawak	Malaysia
	CN2-B [a]	Kuching, Sarawak	Malaysia
	CN-F8 [b]	University of Malaya, Kuala Lumpur	Malaysia
	CN-F35 [b]	Gombak, Selangor	Malaysia
Chrysomya pinguis	C-CP1	Genting Highlands, Pahang	Malaysia
	C-CP3	Gombak, Selangor	Malaysia
	CP1-UKali	Genting Highlands, Pahang	Malaysia
	CP2-UKali	Genting Highlands, Pahang	Malaysia
	CP3-UKali	Genting Highlands, Pahang	Malaysia
	CP-Hwa	Genting Highlands, Pahang	Malaysia
	Z86	Genting Highlands, Pahang	Malaysia
	Z87	Genting Highlands, Pahang	Malaysia
	CP-GB	Cameron Highlands, Pahang	Malaysia
	CP-1/9	Pa Lungan, Sarawak	Malaysia
	CP-7/9	Arur Dalan, Sarawak	Malaysia
	CP-10/9	Arur Dalan, Sarawak	Malaysia
Chrysomya rufifacies	CR3	Mortuary, University Malaya Medical Center, Kuala Lumpur	Malaysia
	LSP2	University of Malaya, Kuala Lumpur	Malaysia
	U5 *	Petaling Jaya, Selangor	Malaysia
	010/09 *	Kuching, Sarawak	Malaysia
	CR-SWK-A	Kuching, Sarawak	Malaysia
	AF083658 [c]	Florida	USA
Chrysomya thanomthini	Chrys thanom	Bario, Sarawak	Malaysia
	CT2-AD2	Arur Dalan, Bario, Sarawak	Malaysia
	CT4-9/9	Mount Prayer, Bario, Sarawak	Malaysia
Chrysomya villeneuvi	CV-Gom	Gombak, Selangor	Malaysia
	M19-CV2	Tapah, Perak	Malaysia
	GH3	Genting Highlands, Pahang	Malaysia
	Z79	Genting Highlands, Pahang	Malaysia
	C-SWK-2	Kuching, Sarawak	Malaysia
Hemipyrellia ligurriens	LS42	Gombak, Selangor	Malaysia
	Gmbk-LC	Gombak, Selangor	Malaysia
	TmnDesa	Kuala Lumpur	Malaysia
Hemipyrellia tagaliana	HL-female	University of Malaya, Kuala Lumpur	Malaysia
	CR-CH1	Gombak, Selangor	Malaysia
	HT	University of Malaya, Kuala Lumpur	Malaysia
	HT-UM1	University of Malaya, Kuala Lumpur	Malaysia
	Hemi-SWK2	Kuching, Sarawak	Malaysia
	HT-SgLang2	Templer's Park, Selangor	Malaysia
Hypopygiopsis fumipennis	HF-Gom	Gombak, Selangor	Malaysia
	Hfumi-UL	Hulu Langat, Selangor	Malaysia
	HF-WOH	Kuala Woh, Perak	Malaysia

Table 5.2 *(continued)*

Species	Voucher	Location/State	Country
Hypopygiopsis violacea	HT-UM3	University of Malaya, Kuala Lumpur	Malaysia
	HV2-SA	Shah Alam, Selangor	Malaysia
	HV-UL	Hulu Langat, Selangor	Malaysia
	H.violaciea	Kuching, Sarawak	Malaysia
Lucilia bismarckensis	L.bismarckensis	Matang Wildlife Centre, Sarawak	Malaysia
	L.bismarc-Bario	Bario, Sarawak	Malaysia
	L.bismarc-Kchg	Kuching, Sarawak	Malaysia
Lucilia calviceps	Lcalviceps	Kampung Telega Air, Sarawak	Malaysia
Lucilia cuprina	Par2-LC *	University of Malaya, Kuala Lumpur	Malaysia
	LCUM-A	University of Malaya, Kuala Lumpur	Malaysia
	LC-Muar1	Muar, Johor	Malaysia
	LC-Muar2	Muar, Johor	Malaysia
	LC-1/9	Pa Lungan, Sarawak	Malaysia
	LC-Bario	Bario, Sarawak	Malaysia
	AJ417704 [c]	Honolulu, Hawaii	USA
	AJ417707 [c]	Perth	Australia
Lucilia papuensis	Lpapuensis-GL	Mount Ledang, Johor	Malaysia
	Lpapuensis-CH	Cameron Highlands, Pahang	Malaysia
	LP-G2	Tapah, Perak	Malaysia
Lucilia porphyrina	Lporphy-Gom	Gombak, Selangor	Malaysia
	Lporphy-WOH1	Kuala Woh, Perak	Malaysia
	Lpor-M19-Blue	Tapah, Perak	Malaysia
	Lpor-Green	Tapah, Perak	Malaysia
	Lporphy-CH	Cameron Highlands, Pahang	Malaysia
	Lpor-UKali	Genting Highlands, Pahang	Malaysia
	Lpor-low3	Kuching, Sarawak	Malaysia
	Lpor-low4	Kuching, Sarawak	Malaysia
	AY074900 [c]	Japan	Japan

In Voucher columns, [a] indicates specimen used only for COI+II DNA analysis; [b] indicates specimen used only for 28S ribosomal DNA analysis; [c] indicates DNA sequence retrieved from GenBank and * indicates immature specimens.

5.3.2 DNA extraction

For DNA extraction of adults, only legs from one side of the flies were used while the remaining parts of the flies were maintained as voucher specimens. For the immature stages (egg, first-instar larva, second-instar larva, puparium and pupa), the whole specimens were used to extract DNA.

Total genomic DNA was extracted using QIAamp® Mini Kit purchased from Qiagen GmbH, Germany. Legs of the adult fly, or the immature specimen were put

inside 1.5ml microcentrifuge tube before immersed in liquid nitrogen and then ground into powder using sterile plastic pestles. Then, the sample was homogenized with 180µl of buffer ATL and 20µl of Proteinase K. The mixture was homogenized by vortexing, and incubated at 56°C shaking water bath until the tissue was completely lysed. After the incubation, the microcentrifuge tube was centrifuged briefly to collect drops from the walls and the lid.

Then, 4µl of 100mg/ml ribonuclease A was added. The sample was mixed by pulse-vortex for 15 seconds and incubated at room temperature for 2 minutes. Short centrifugation was done before 200µl of buffer AL was added. The sample was mixed again by pulse-vortex for 15 seconds and incubated at 70°C for 10 minutes in a heating block. Short centrifugation was done before 200µl of absolute ethanol was added. The sample was mixed by pulse-vortex for 15 seconds and followed by short centrifugation.

The mixture was then carefully transferred into the QIAamp Spin Column placed in 2ml collection tube without wetting the rim, and centrifuged at 6000×*g* for 1 minute. The QIAamp Spin Column was then placed in to a clean 2ml collection tube and the tube containing the filtrate was discarded. The cap of QIAamp Spin Column was opened and 500µl of buffer AW1 was added, without wetting the rim. The column was then centrifuged at 6000×*g* for 1 minute. The QIAamp Spin Column was then placed in a clean 2ml collection tube and the tube containing the filtrate was discarded. The cap of QIAamp Spin Column was opened and 500µl of buffer AW2 was added without wetting the rim. The column was centrifuged at maximum speed (20 000×*g*) for 3 minutes. QIAamp Spin Column was then placed in a clean 2ml collection tube and the tube contained the filtrate was discarded. The column was centrifuged at maximum speed (20 000×*g*) for 1 minute. QIAamp Spin Column was then placed in a clean 1.5ml collection tube and the tube containing the filtrate was discarded. The cap of QIAamp Spin Column was opened; 200µl of buffer AE was added and incubated for

1 minute. The column was centrifuged at 6000×*g* for 1 minute. The elution was collected and stored at -20°C. The elution step was repeated to collect more DNA but the incubation time was increased to 5 minutes.

5.3.3 PCR amplification

PCR amplification mixtures were prepared to contain the following: 100ng template DNA, 1 unit of *Taq* polymerase (Promega), 1× PCR reaction buffer (Promega), 1.5mM $MgCl_2$ (Promega, USA), 200µM dNTPs (Promega) and 0.4µM of each forward and reverse primers (Research Biolabs Technology, Singapore). All PCR amplifications were carried out with a negative control (without any template DNA). The PCR products were then separated electrophoretically on 1% agarose gel (Promega) and visualised under ultraviolet illumination after ethidium bromide staining.

Table 5.3: Primer sequences used to amplify overlapping segments of the mitochondrial COI and COII genes (Simon, 1994; Sperling *et al*., 1994) and 28S rDNA (Stevens & Wall, 2001).

Primer	Region	Sequence (5' – 3')
TY-J-1460	COI+II	TACAATTTATCGCCTAAACTTCAGCC
C1-N-2800	COI+II	CATTTCAAGCTGTGTAAGCATC
C1-J-2495	COI+II	CAGCTACTTTATGAGCTTTAGG
TK-N-3775	COI+II	GAGACCATTACTTGCTTTCAGTCATCT
D1.F	28S rDNA	CCCCCTGAATTTAAGCATAT
D2.R	28S rDNA	GTTAGACTCCTTGGTCCGTG
D3-5.F	28S rDNA	GACCCGTCTTGAAACACGG
D3-5.R	28S rDNA	TTACACACTCCTTAGCGGA
D3-5.742F	28S rDNA	TCTCAAACTTTAAATGG
D7.R	28S rDNA	CGACTTCCCTTACCTACAT

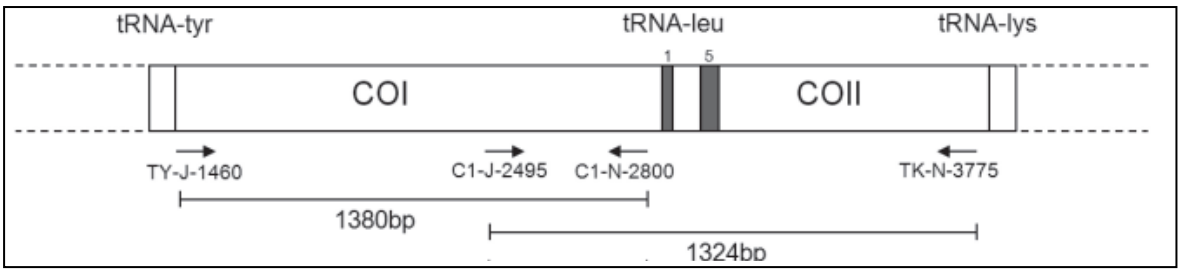


Figure 5.1: Schematic representation of the mitochondrial COI, COII, tRNA leucine genes and intergenic regions modified from Schroeder *et al*., 2003a. Shaded boxes (and corresponding numbers) represent non-coding nucleotides that are present between the genes. Locations of the primers and sizes of the amplification fragments using different primer combinations are shown.

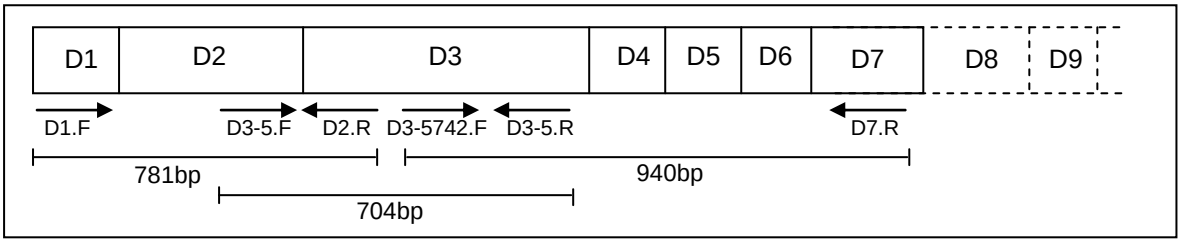


Figure 5.2: Schematic representation of the 28S rDNA with its divergence domain. Locations of the primers and sizes of the amplification fragments using different primer combinations are shown.

5.3.3.1 PCR optimisation – gradient temperature PCR

Two sets of primer (TY-J-1460 & C1-N-2800 and C1-J-2495 & TK-N-3775) were used for COI+II, and were designed based on the description of Sperling *et al*. (1994) (Table 5.3). Relative positions and orientation of primers are shown in Figure 5.1. Three sets of primer were used for 28S rDNA (D1.F & D2.R, D3-5.F & D3-5.R and D3-5.742F & D7.R) and were designed based on the description of Stevens and Wall (2001).

PCR parameters included an initial denaturation step of 94ºC of 5 minutes, followed by 35 cycles of 94ºC for 1 minute, gradient temperatures ranged from 45.0ºC to 65.0ºC for 1 minute 30 seconds and 72ºC for 2 minutes. The last cycle was a final elongation step of 72ºC for 5 minutes.

5.3.3.2 PCR amplification of 5 sets of primer

After the optimal annealing temperatures had been determined in the PCR optimisation for each primer sets, a similar PCR condition described in the section 5.2.3.1 was used to amplify different species of Calliphoridae. The only different conditions, as described in section 5.3.3.1, was that the annealing temperatures used in each PCR were specific to each set of primer used according to the result from PCR optimisation.

5.3.3.3 PCR amplification using DNA from fresh and archival specimens

PCR was carried out using DNA obtained from specimens of different preservation ages. This DNA was extracted from fresh, 2 year-old and 10 year-old specimens. Primers used in this study were from the primer set of TY-J-1460 & C1-N-2800, following the PCR programme from section 5.3.3.1, with the annealing temperature 46.0ºC.

5.3.3.4 PCR amplification using DNA from different life stages of the fly

The quality and quantity of DNA extracted from flies of different life stages were used to evaluate the efficiency of DNA extraction and PCR amplification. The primers used in the PCR were C1-J-2495 and C1-N-2800. PCR programme used in this study was the same as that described in section 5.3.3.1, although the annealing temperature was 45.0ºC.

5.3.4 Purification of PCR products

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen GmbH, Germany) or QIAquick® Gel Extraction Kit (Qiagen GmbH, Germany). The QIAquick® Gel Extraction Kit was used when the PCR product yields unspecific

amplification fragment. The quality of purified PCR products was confirmed on ethidium bromide stained agarose gel electrophoresis before the samples were sent for sequencing.

5.3.4.1 QIAquick® PCR purification

The PCR product sample was mixed with 5 volumes of buffer PB. QIAquick Spin Column was then placed in a clean 2ml collection tube. The mixture was applied to the QIAquick Spin Column to bind DNA and centrifuged at ~18 000×*g* for 1 minute, and the flow-through was discarded. The QIAquick Spin Column was placed back in to the same collection tube and 0.75ml of 35% guanidine hydrochloride aqueous solution was added to remove the primer-dimers. The column was centrifuged at ~18000×*g* for 1 minute and the flow-through was discarded. The QIAquick Spin Column was placed back to the same collection tube and 0.75ml of buffer PE was added. The column was centrifuged at ~18 000×*g* for 1 minute and the flow-through was discarded. The QIAquick Spin Column was placed back to the same collection tube and centrifuged at ~18 000×*g* for 1 minute. Then, the QIAquick Spin Column was placed into a clean 1.5ml microcentrifuge tube. For DNA elution, 50µl of buffer EB was added to the center of the QIAquick membrane and incubated for 1 minute at room temperatue. The column was centrifuged at ~18 000×*g* for 1 minute and the elution was collected and stored in -20°C.

5.3.4.2 QIAquick® gel extraction

The DNA fragment was excised from a 0.8% agarose gel with a clean and sharp scalpel. The gel slice was then put into a 1.5ml microcentrifuge tube and weighed. According to the weight, 3 volumes of buffer QG to 1 volume of gel were added. The gel was incubated at 50°C for 10 minutes. The microcentrifuge tube was mixed by

vortex every two to three minutes to help the gel dissolve. After the gel was completely dissolved, one volume of isopropanol was mixed into the sample.

QIAquick Spin Column was then placed in a clean 2ml collection tube. The sample was applied to the QIAquick Spin Column to bind the DNA, centrifuged at ~18 000×*g* for one minute and the flow-through was discarded. The QIAquick Spin Column was placed back into the same collection tube and 0.5ml of buffer QG was added. The column was centrifuged at ~18 000×*g* for 1 minute and the flow-through was discarded. The QIAquick Spin Column was placed back in to the same collection tube and 0.75ml of buffer PE was added. The column was incubated for five minutes at room temperature and centrifuged at ~18 000×*g* for 1 minute and the flow-through was discarded. The QIAquick Spin Column was placed back to the same collection tube and centrifuged at ~18 000×*g* for a minute. Then, the QIAquick Spin Column was placed into a clean 1.5ml microcentrifuge tube. For DNA elution, 50µl of buffer EB was added to the center of the QIAquick membrane and incubated for 1 minute at room temperatue. The column was centrifuged at ~18000×*g* for a minute and the elution was collected and stored in -20°C.

5.3.5 Cloning and sequencing

Purified PCR products were then used for direct sequencing. Only faint PCR products were cloned into the pGEM®-T Easy vector system (Promega, USA) to facilitate DNA sequencing procedures. The cloning procedures were carried out according to the manufacture's protocol.

Sequencing was performed using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. All mitochondrial DNA samples were sequenced for both forward and reverse DNA strands using the universal M13

forward and reverse primers while for 28S rDNA samples, PCR amplification primers (Table 5.3) and additional internal sequencing primers were used (Table 5.4). Electrophoresis and detection of the sequencing reaction products were carried out in the capillary electrophoresis system of the Applied Biosystems 3730xl DNA Analyzer with 80-cm capillary array.

Table 5.4: Internal sequencing primers used for mitochondrial *cytochrome c oxidase* I and II subunits, 28S rRNA regions D1–D7 and clones.

Primers	Region	Sequence (5' – 3')
M13F	Clone	GTTTTCCCAGTCACGAC
M13R	Clone	GGAAACAGCTATGACCATG
C1-N-1687[a]	COI+II	CAATTTCCAAATCCTCCAATTAT
C1-J-2183[a]	COI+II	CAACATTTATTTTGATTTTTTGG
C2-N-3389[a]	COI+II	TCATAAGTTCARTATCATTG
C2-J-3408[a]	COI+II	CAATGATARTGAAGWTATGA
D1.R[b]	28S rDNA	CTCTCTATTCAGAGTTCTTTTC
D2.F[b]	28S rDNA	GAGGGAAAGTTGAAAAGAAC
D3-5.486.R[b]	28S rDNA	TCGGAAGGAACCAGCTACTA
D7.F[b]	28S rDNA	GACTGAAGTGGAGAAGGGT

[a] from Wells & Sperling, 2001
[b] from Stevens & Wall, 2001

5.3.6 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

A portion of the COI region was amplified using primer set TY-J-1460 & C1-N-2800, using PCR conditions as described in section 5.3.3 and 5.3.3.1, with the annealing temperature 46.0ºC. Eight Malaysian forensically important *Chrysomya* species were included in this study.

Prior to RFLP digestion, the PCR products obtained were checked on a 1% agarose gel for successful PCR amplification. Concentrations of each PCR product were also determined spectrophotometrically. Three restriction endonucleases, *Ssp*I, *Taq*$^{\alpha}$I and *Msp*I (New England Biolabs, UK) were used in RFLP analysis. The restriction digestion conditions of these restriction endonucleases were according to the

manufacturer's recommendation in a final volume of 20µl. Then, the digested samples were fractionated on 2% agarose gel with TBE buffer, stained with ethidium bromide and viewed under UV-illumination. The restriction patterns were photographed and analysed. The PCR-RFLP assays developed were evaluated and validated repeatedly with different field samples collected from different geographical locations in Malaysia (Table 5.2).

5.3.7 Data and phylogenetic analysis

DNA sequence reads of COI+II and 28SrDNA were edited manually using the Chromas 2.32 to remove the primer regions and to resolve discrepancies between contig sequences. Sequences were aligned using ClustalW (Thompson *et al*., 1994). Analysis of DNA sequence variation and nucleotide composition was performed using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura *et al*., 2007). Variation across sites was analysed using DnaSP 4.0 (Rozas *et al*., 2003). Variance of the Pi value was calculated and presented in a graph with window length of 100 and step size of 25. In this analysis, gaps were excluded.

Prior to phylogenetic analysis, sequence alignment was tested for the best-fit evolutionary model using Modeltest 3.8 (Posada & Crandall, 1998). Pairwise genetic distances of COI+II and 28S rDNA were measured in PAUP* 4.0b10 (Swofford, 1998) by using an appropriate model of nucleotide substitution as determined in Modeltest 3.8 (Posada & Crandall, 1998). Minimum pairwise sequence divergence between species and maximum pairwise sequence divergence within species was calculated and determined.

Neighbour-joining (NJ) and maximum parsimony (MP) phylogenetic trees were constructed using PAUP* 4.0b10 (Swofford, 1998). For both analyses, all characters were included and 10000 replicates were bootstrapped. In NJ analysis, under the

DNA/RNA distances option, maximum likelihood was selected and in weighting least squares, inverse-squared weighting (power=2) was performed. A full heuristic search was performed in the MP analysis with 10 random additions per replicate and tree bisection-reconnection (TBR) branch swapping. Accelerated transformation (ACCTRAN) was used and gaps were treated as new states or fifth base. A Bayesian inference analysis was performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Four Markov chains (three heated chains and one cold chain) were run for a million generations and the trees were sampled every 100^{th} generation. The consensus tree was constructed after burn-in of 25% as recommended. For 28S rDNA sequences, the partition was performed for consideration of gaps. Different variable rates and unlinked model parameters across partitions were applied. All three trees, the NJ, MP and Baysian analysis, were rooted with a Sarcophagidae species, and only branches with over 70% bootstraps were considered for phylogenetic inference (Hillis & Bull, 1993).

5.4 Results

5.4.1 Samples

In the present study, 98 DNA sequences of COI+II and 49 DNA sequences of 28S rDNA were successfully generated and some of them were deposited into GenBank (Tables 5.5 and 5.6). All the species are carrion feeders except for *Chrysomya bezziana*, which is the obligate parasite involved in myiasis.

Table 5.5: Fly species with mitochondrial DNA sequence data deposited in GenBank, which covers the genes of *cytochrome c oxidase* subunits one and two (COI+II) and the intervening transfer RNA leucine (tRNA-leu). Asterisks (*) indicates the species that are not likely to be forensically important and double asterisk (**) indicates foreign forensically important species. These species were included as they are the sister species.

Species	Voucher	GenBank Accession Number
Calliphora fulviceps	Calli-CH	JN014901
	Calli-13-CF-CH	JN014902
Calliphora vicina **	Cvicina-London	JN014899
	Cvicina-Japan	JN014900
Chrysomya bezziana *	CB-3/9	JN014862
Chrysomya chani	CC-Penang	JN014865
	CC-UM	JN014866
	WChry	JN014867
Chrysomya defixa	CD-AS2	JN014847
	LP20-CD	JN014848
	CD-SWK	JN014849
	KH38-CD	JN014850
	E24-CD	JN014851
	E26-CD	JN014852
Chrysomya megacephala	Egg-CM	JN014844
	CM-Muar	JN014845
	CM-forest-3/9	JN014846
Chrysomya nigripes	CN-Gom	JN014863
	CN2-B	JN014864
Chrysomya pinguis	CP1-UK	JN014853
	CP2-UK	JN014854
	CP3-UK	JN014855
	CP-GB	JN014856
	CP-forest	JN014857
	CP-1/9	JN014858
Chrysomya rufifacies	LSP2-CR	JN014868
	010/09	JN014869
	CR-SWK-A	JN014870
Chrysomya thanomthini	CT2-AD2	JN014859
	CT4-9/9	JN014860
	Chrys-thanom	JN014861
Chrysomya villeneuvi	CV-Gom2	JN014871
	C-SWK-2-CV	JN014872
	GH3-CV	JN014873
Hemipyrellia ligurriens	TmnDesa-HL	JN014894
	HL-female-UM	JN014894
	Hemi-SWK-2	JN014895
Hemipyrellia tagaliana	HT	JN014896
	HT-SgLang2	JN014897
	HT-UM1	JN014898

Table 5.5 *(continued)*

Species	Voucher	GenBank Accession Number
Hypopygiopsis fumipennis	Hfumi-UL	JN014884
	HF-WOH	JN014885
	HF-Gom	JN014886
Hypopygiopsis violacea	Hviolacea	JN014890
	HV-UL	JN014891
	HV2-SA	JN014892
Lucilia bismarckensis	Lbismarckensis	JN014874
Lucilia calviceps	Lcalviceps	JN014875
Lucilia cuprina	LC-Bario	JN014887
	LC-Muar2	JN014888
	LC-UM-A	JN014889
Lucilia papuensis	Lpapuensis-GL	JN014876
	Lpapuensis-CH	JN014877
	Lpapuensis-Bario	JN014878
	Lpapuensis-Kuching	JN014879
Lucilia porphyrina	Lporphy-CH	JN014880
	Lporphy-UKali	JN014881
	Lporphy-Gom1	JN014882
	Lpor-low-4	JN014883

Table 5.6: Fly species with nuclear DNA sequence data deposited in GenBank, which is the partial of 28S ribosomal DNA (28S rDNA). Asterisks (*) indicates species that are not likely to be forensically important.

Species	Voucher	GenBank Accession Number
Chrysomya bezziana *	CB-3/9	JN014910
Chrysomya chani	CC-AD2	JN014939
	CC-F11-UM	JN014940
	CC-F12-AS	JN014941
Chrysomya defixa	CD-KH-37	JN014911
	CD-KH-38	JN014912
	CD-forest	JN014913
	CD-AD2	JN014914
	CD-AD3	JN014915
	CD-E24	JN014916
	CD-E26	JN014917
	CD-E39	JN014918
	CD-F17-AS	JN014919
	CD-F18	JN014920
Chrysomya megacephala	CM-UKali-1	JN014905
	CM-UKali-2	JN014906
	CM-F1-UM	JN014907
	CM-F2-UM	JN014908
	CM-F15-AS	JN014909
Chrysomya nigripes	CN-F8	JN014942
	CN-F35	JN014943

Table 5.6 *(continued)*

Species	Voucher	GenBank Accession Number
Chrysomya pinguis	CP1-UKali	JN014924
	CP2-UKali	JN014925
	CP3-UKali	JN014926
	UKali-KH1	JN014927
	CP-1/9	JN014928
	CP-7/9	JN014929
	CP-KH-M19-4	JN014930
	CP-forest	JN014931
	CP-GB	JN014932
Chrysomya rufifacies	CR-F5	JN014933
	CR-F6-UM	JN014934
	CR-F13-AS	JN014935
	CR-F32-PPLUM	JN014936
Chrysomya thanomthini	CT2-AD2	JN014921
	CT3-AD2	JN014922
	CT4-9/9	JN014923
Chrysomya villeneuvi	CV-F20-AS	JN014937
	CV-F33	JN014938
Hemipyrellia tagaliana	HT-UM1	JN014944
	HT-UM2	JN014945
Lucilia cuprina	LC	JN014946
Calliphora fulviceps	Calli-CH	JN014947
Parasarcophaga albiceps	PA-PU	JN014904

5.4.2 PCR amplification

5.4.2.1 PCR optimisation – gradient temperature PCR

In the PCR optimisation study, two sets of primer were used for COI+II (TY-J1460 & C1-N-2800 and C1-J2495 & TK-N-3775) and three sets of primer were used for 28S rDNA (D1.F & D2.R, D3-5.F & D3-5.R and D3-5.742F & D7.R).

Lengths of the PCR amplified fragments were 1380bp and 1324bp for primer sets TY-J1460 & C1-N-2800 (partial of COI) and C1-J2495 & TK-N-3775 (partial region of COI, complete regions of tRNA leucine and COII – COI+tRNA-leu+COII), respectively. Optimisation of PCR amplification for these two primer sets was necessary as the recommended PCR parameters did not yield satisfactory results. After optimisation, the optimal annealing temperatures were determined to be 46ºC for

primer set TY-J1460 & C1-N-2800 and 58ºC for primer set C1-J2495 & TK-N-3775 (Figures 5.3 and 5.4), as opposed Schroeder *et al*. (2002), the recommended annealing temperature were 45ºC and 47ºC, respectively.

For three primer sets of 28S rDNA, a wide range of annealing temperatures (45ºC -65ºC) were sufficient to produce good yield and specific amplicon (Figures 5.5, 5.6 and 5.7). The optimal annealing temperatures: 58 ºC for D1.F & D2.R, 53ºC for D3-5.F & D3-5.R and 50ºC for D3-5.742F & D7.R, were determined based on the yield of amplicons.

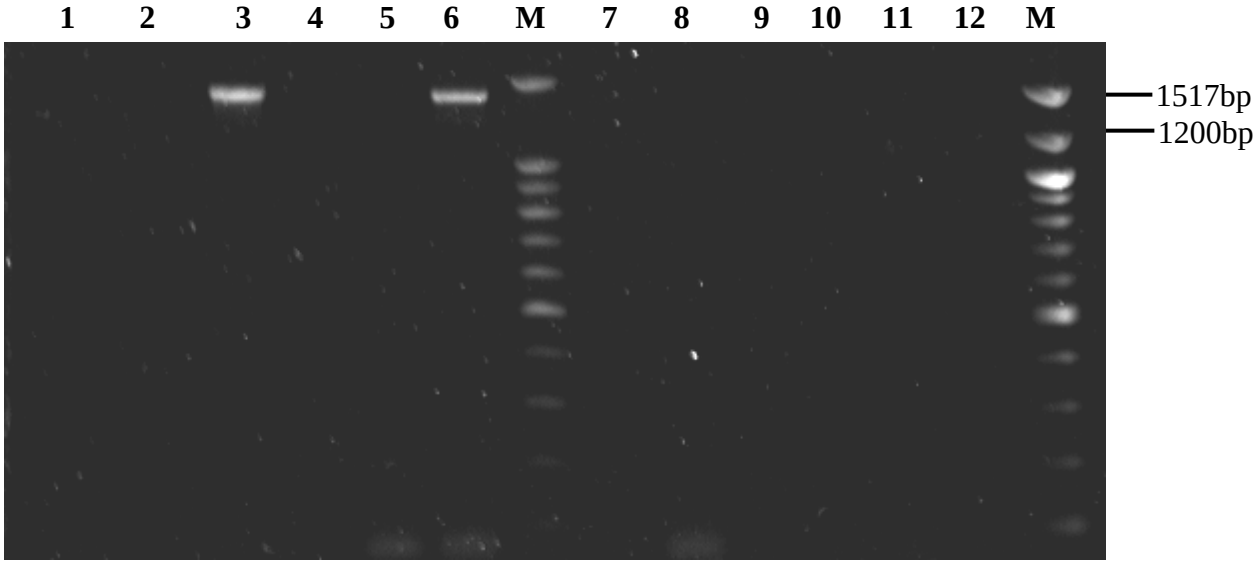


Figure 5.3: Gradient temperature PCR with temperatures ranging from 45ºC to 60ºC for primers TY-J-1460 & C1-N-2800 in PCR optimisation. Lanes: 1, 45.0ºC; 2, 45.4ºC; 3, 46.2ºC; 4, 47.5ºC; 5, 49.2ºC; 6, 51.4ºC; M, 100bp molecular weight marker (Promega, USA); 7, 53.9ºC; 8, 56.0ºC; 9, 57.7ºC; 10, 58.8ºC; 11, 59.7ºC; 12, 60.0ºC and and M, molecular weight marker (New England Biolabs, UK). Optimal annealing temperature of 46.0ºC was determined (lane 3), with the expected size of 1380bp.

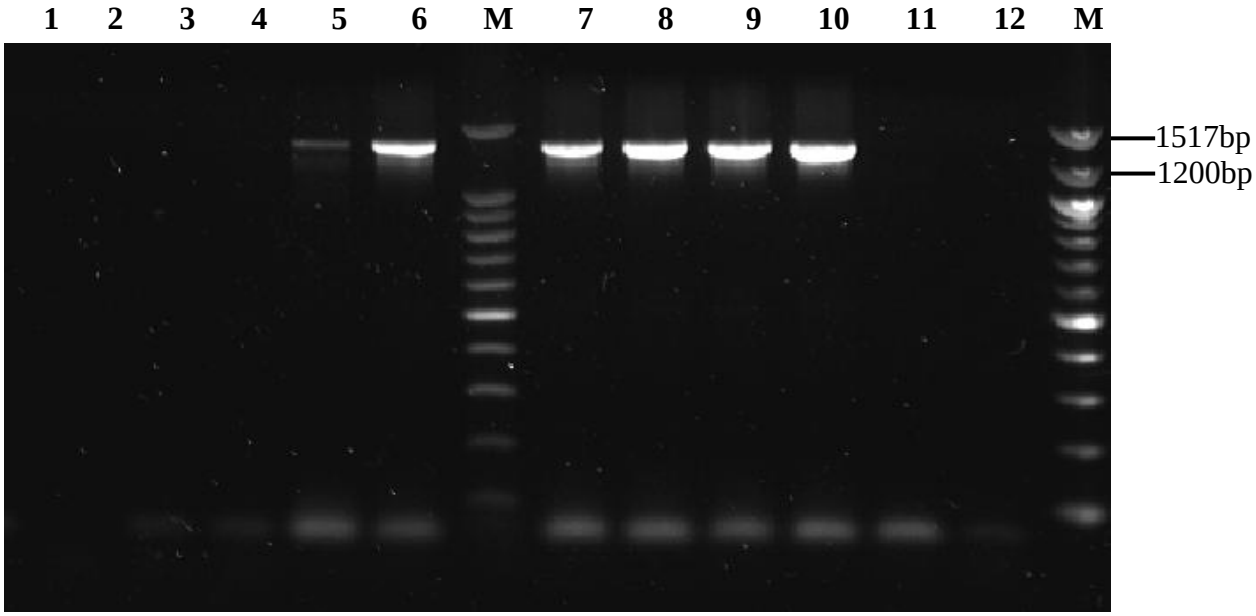


Figure 5.4: Gradient temperature PCR with temperatures ranging from 45ºC to 60ºC for primers C1-J-2495 & TK-N-3775 in PCR optimisation. Lanes: 1, 45.0ºC; 2, 45.4ºC; 3, 46.2ºC; 4, 47.5ºC; 5, 49.2ºC; 6, 51.4ºC; M, 100bp molecular weight marker (Promega, USA); 7, 53.9ºC; 8, 56.0ºC; 9, 57.7ºC; 10, 58.8ºC; 11, 59.7ºC; 12, 60.0ºC and and M, 100bp molecular weight marker (New England Biolabs, UK). Optimal annealing temperature of 58ºC was determined (lane 10), with the expected size of 1324bp.

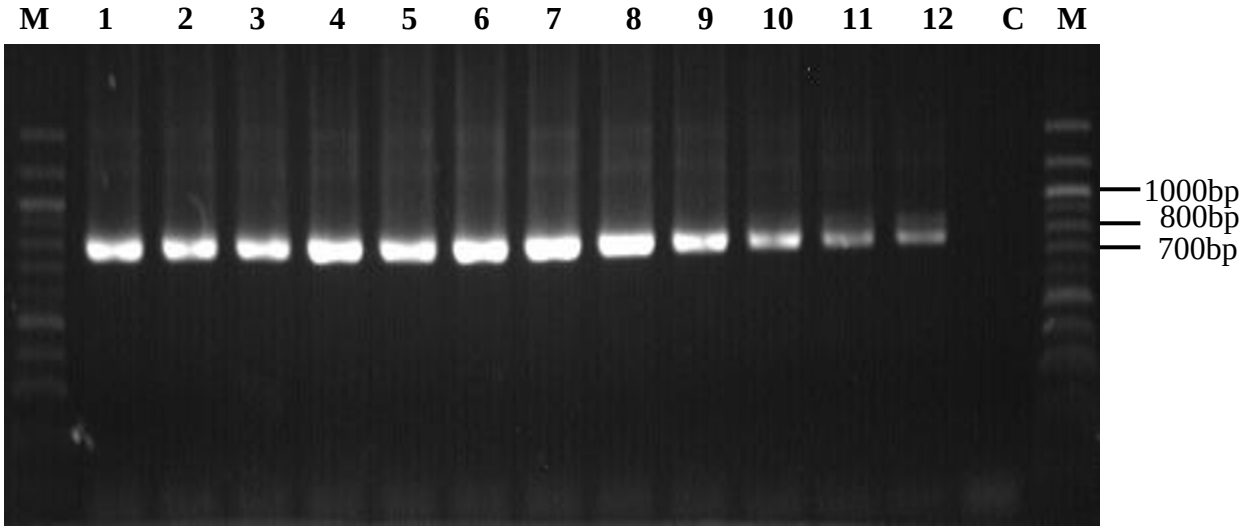


Figure 5.5: Gradient temperature PCR with temperatures ranging from 45ºC to 65ºC for primers D1F & D2R in PCR optimisation. Lanes: 1, 45.0ºC; 2, 45.3ºC; 3, 46.4ºC; 4, 48.2ºC; 5, 50.4ºC; 6, 53.0ºC; 7, 55.8ºC; 8, 58.4ºC; 9, 61.0ºC; 10, 63.0ºC; 11, 64.7ºC; 12, 65.6ºC; C, negative control and M, 100bp molecular weight marker (Seegene, Korea). Optimal annealing temperature of 58ºC was determined (lane 8), with the expected size of 781bp.

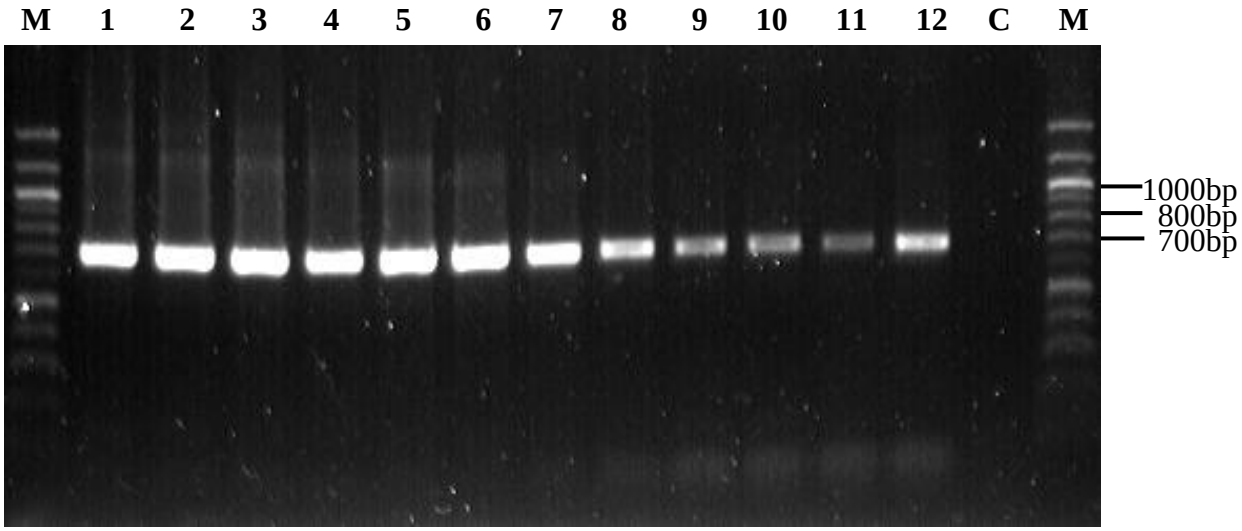


Figure 5.6: Gradient temperature PCR with temperatures ranging from 45ºC to 65ºC for primers D3F & D3R in PCR optimisation. Lanes: 1, 45.0ºC; 2, 45.3ºC; 3, 46.4ºC; 4, 48.2ºC; 5, 50.4ºC; 6, 53.0ºC; 7, 55.8ºC; 8, 58.4ºC; 9, 61.0ºC; 10, 63.0ºC; 11, 64.7ºC; 12, 65.6ºC; C, negative control and M, 100bp molecular weight marker (Seegene, Korea). Optimal annealing temperature of 53ºC was determined (lane 6), with the expected size of 704bp.

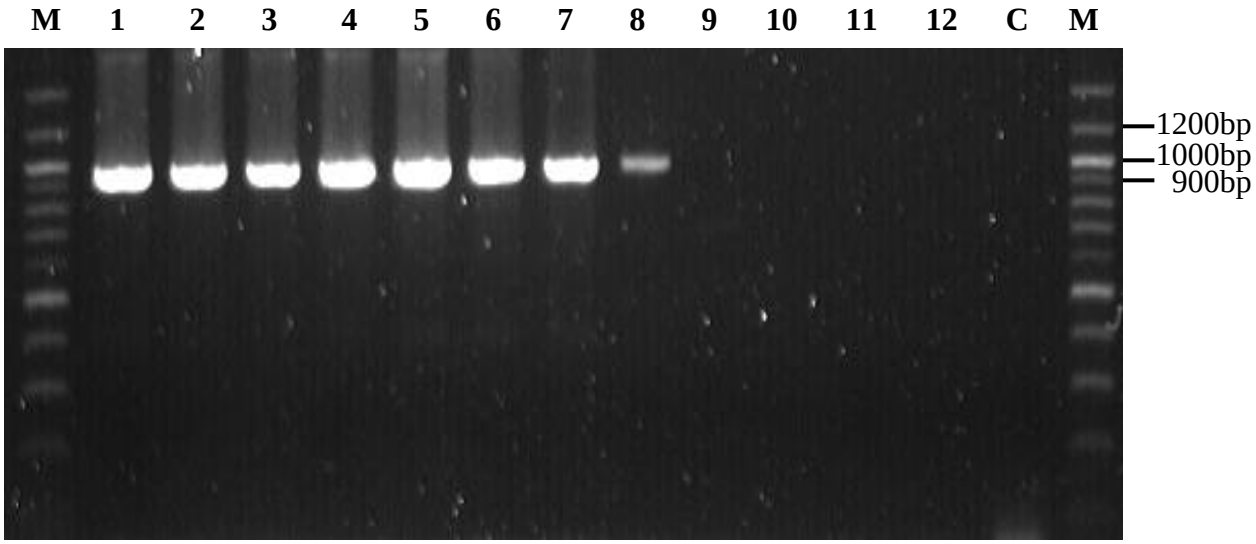


Figure 5.7: Gradient temperature PCR with temperatures ranging from 45ºC to 65ºC for primers D3.5742F & D7R in PCR optimisation. Lanes: 1, 45.0ºC; 2, 45.3ºC; 3, 46.4ºC; 4, 48.2ºC; 5, 50.4ºC; 6, 53.0ºC; 7, 55.8ºC; 8, 58.4ºC; 9, 61.0ºC; 10, 63.0ºC; 11, 64.7ºC; 12, 65.6ºC; C, negative control and M, 100bp molecular weight marker (Seegene, Korea). Optimal annealing temperature of 50ºC was determined (lane 5), with the expected size of 970bp.

5.4.2.2 PCR amplification with 5 sets of primers

Different species of Calliphoridae were treated for PCR using two sets of primers for COI+II region and three sets of primers for 28S rDNA region. Primer sets of TY-J-1460 & C1-N-2800 and C1-J2495 & TK-N-3775, which amplified the region of COI+II were used for PCR and the PCR products obtained were 1380bp and 1354bp, respectively (Figure 5.8).

Primer sets of D1.F & D2.R, D3-5.F & D3-5.R and D3-5.742F & D7.R were used to amplify the region of 28S rDNA (Figures 5.9 and 5.10). Fragment length obtained from the PCR amplification of these primers were 781bp (D1.F & D2.R), 704bp (D3-5.F & D3-5.R) and 980bp (D3-5.742F & D7.R).

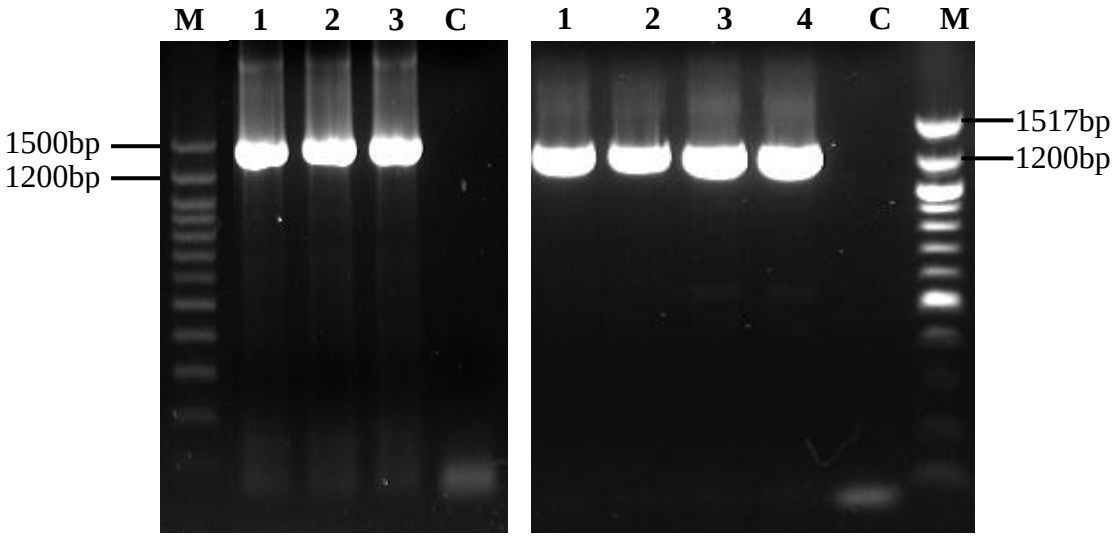


Figure 5.8: PCR amplification carried out by primer sets of COI+II. (a) TY-J-1460 and C1-N-2800 with the expected PCR products of 1380bp. Lanes: M, molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea); 1-3, different species of Calliphoridae and C, negative control. (b) C1-J-2495 and TK-N-3775 with the expected size of 1324bp. Lanes: 1-6, different species of Calliphoridae; C, negative control and M, molecular weight marker of 100bp (New England Biolabs, UK).

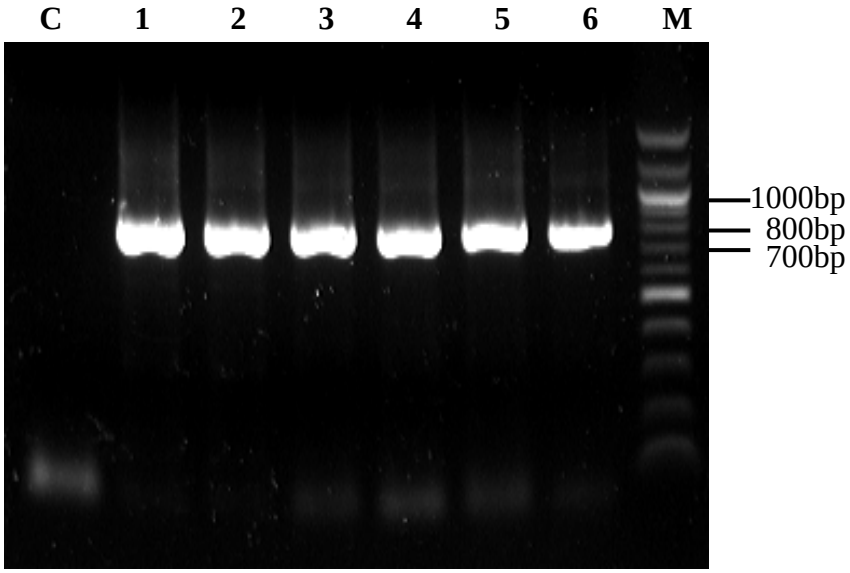


Figure 5.9: PCR amplification carried out using primers D1.F and D2.R with the expected size of 781bp. Lanes: M, molecular weight marker of 100bp (New England Biolabs, UK), 1-2, different species of Calliphoridae and C, negative control.

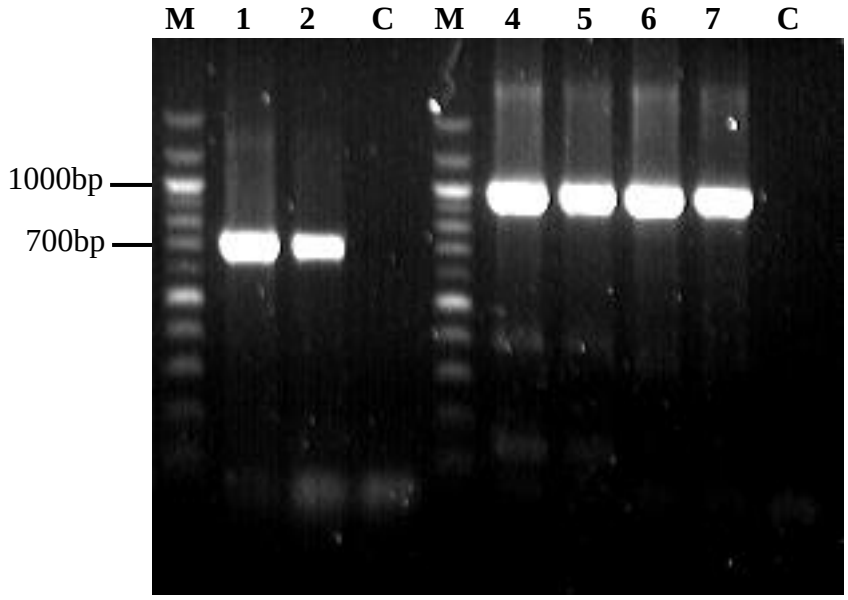


Figure 5.10: PCR amplification carried out by primers D3-5.F & D3-5.R and D3-5.742.F & D7.R with the expected size of 704bp and 940bp, respectively. Lanes: M, molecular weight marker of 100bp DNA ladder (Seegene, Korea); 1-2, different species of Calliphoridae (PCR by D3-5.F & D3-5.R); 4-7, different species of Calliphoridae (PCR by D3-5.742.F & D7.R) and C, negative control.

5.4.2.3 PCR amplification using DNA from fresh and archival specimens

As the samples obtained in this study were archival material as well as freshly collected material, a comparison was made to determine if the archival samples could yield DNA of sufficient quality for this study. In this experiment, DNA was extracted from fresh specimens, 2 year-old specimens and 10 year-old specimens and amplified under conditions previously described for PCR using primers TY-J-1460 and C1-N-2800. The results are shown in Figure 5.11. Only six out of nine 10 year-old samples produced PCR bands, albeit very faintly (lanes 2, 4, 8, 10, 11 and 12). PCR results from 2 year-old samples were sufficient for further analysis but significantly lower when the intensity of the bands were compare to DNA extracted from fresh specimens.

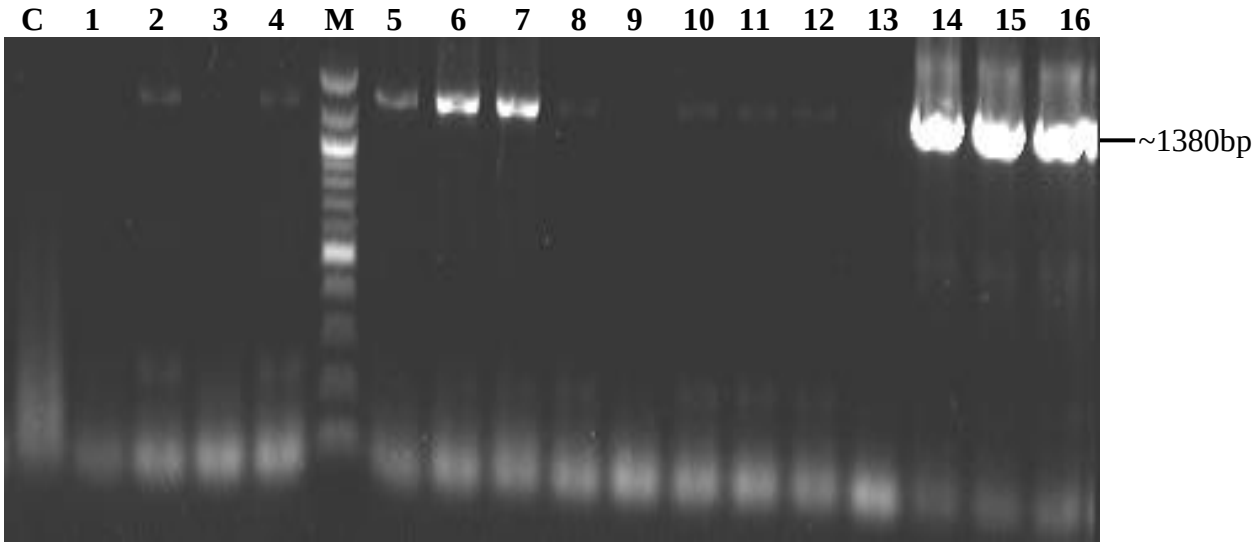


Figure 5.11: PCR amplification of fresh, 2 year-old, 10 year-old specimens and negative control using TY-J-1460 and C1-N-2800 primers, with an expected product of 1380bp. Lanes: 1-4 and 8-13, 10 year-old specimens; 5-7, 2 year-old specimens; 14-16, fresh specimens; C, negative control and M, 100bp molecular weight marker (New England Biolabs, UK).

5.4.2.4 PCR amplification using DNA from different life stages of the fly

The success of PCR amplification was also compared with DNA material obtained from the different life stages of fly. For this purpose, PCR was performed using DNA extracted from egg, 1 day-old larva, 2 day-old larva, puparium (empty casing of the pupa), pupa and the amplification results were compared. Results in Figure 5.12 shows that the amplification was successful for all stages, although some stages showed higher success than others, judged based on the intensity of the PCR amplification bands.

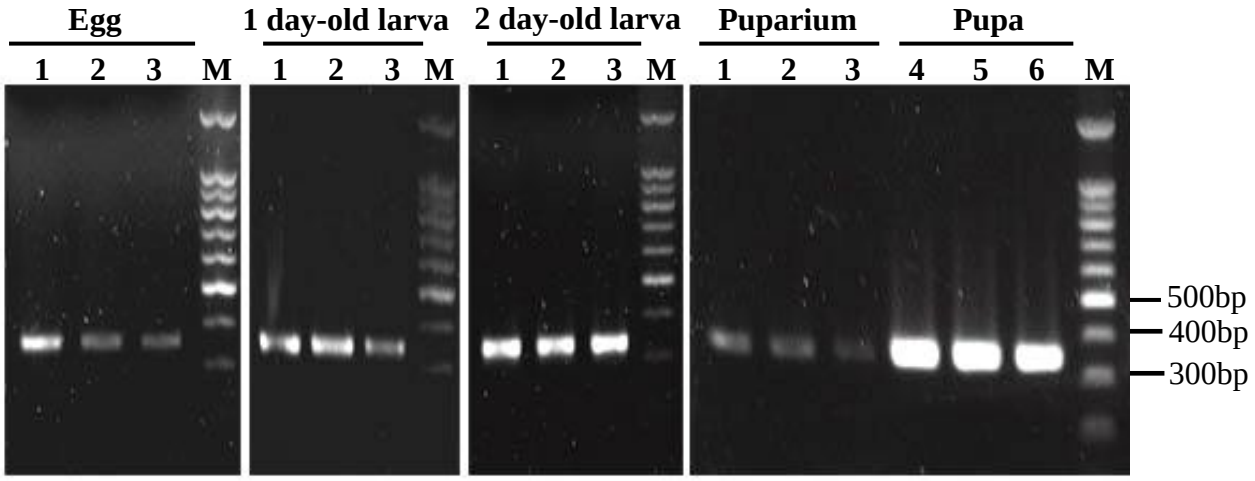


Figure 5.12: PCR products of different life stages of the fly on 1% agarose gel electrophoresis. PCR amplification was carried out using C1-J-2495 and C1-N-2800 primers, with an expected product of 348bp. M, 100bp molecular weight marker (New England Biolabs, UK). Results indicate that the immature stages of flies are also amenable for DNA extraction and PCR.

5.4.3 Purification of PCR products

All the PCR products were purified before direct sequencing or cloning. This is to ensure that there is only one PCR product obtained and good quality DNA templates for downstream application such as sequencing and cloning. Therefore, it was necessary to purify PCR products from impurities such as excessive or non-incorporated primers and dNTP, primer-dimers nucleotides, as well as proteins such as *Taq* DNA polymerase from the PCR amplification. If the PCR product was specific, PCR purification was carried out using QIAquick® PCR purification (Figures 5.13 and 5.14). Nevertheless, if the PCR produced unspecific amplification fragments or traces of smear were observed in the agarose gel (e.g. Figure 5.10, Lanes 4-7), the desired band had to be excised from the gel and purified using QIAquick® Gel Extraction Kit (Figures 5.15 to 5.17).

5.4.3.1 QIAquick® PCR purification

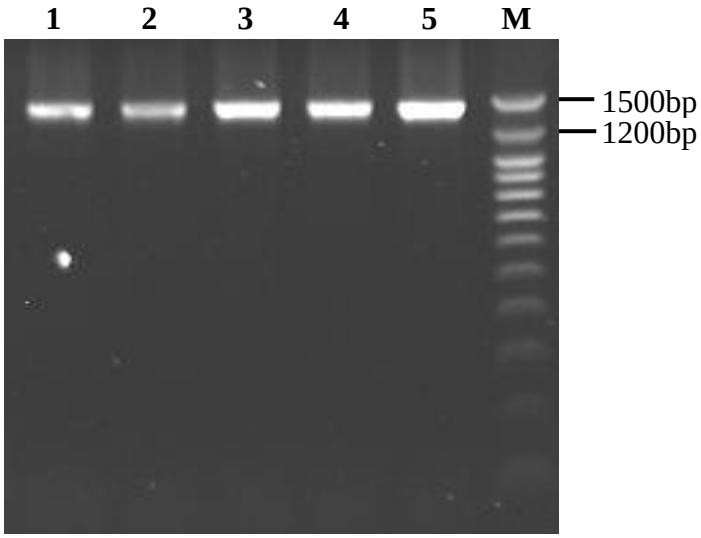


Figure 5.13: Purified PCR products of primer set TY-J-1460 and C1-N-2800 (~1380bp) after PCR purification. Lanes: 1-5, purified PCR products of species of Calliphoridae and M, molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea).

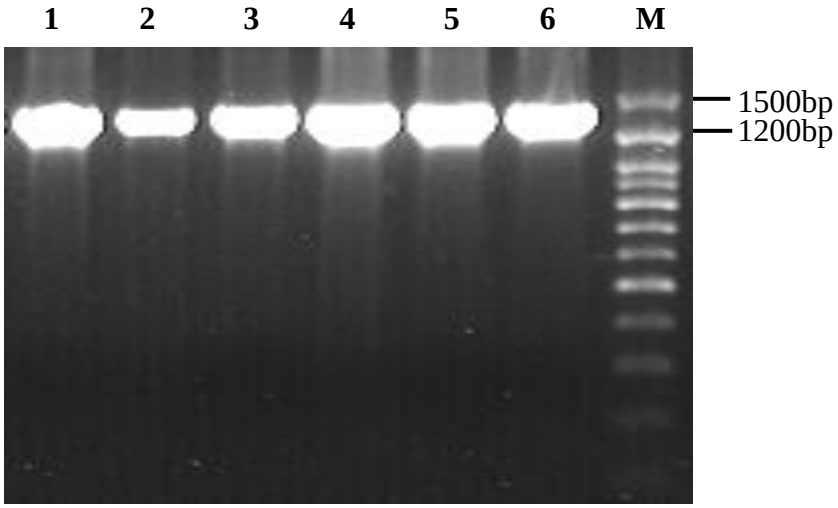


Figure 5.14: Purified PCR product of primer set C1-J-2495 and TK-N-3775 (~1324bp) after PCR purification. Lanes: 1-6, purified PCR products of species of Calliphoridae and M, molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea).

5.4.3.2 QIAquick® gel extraction

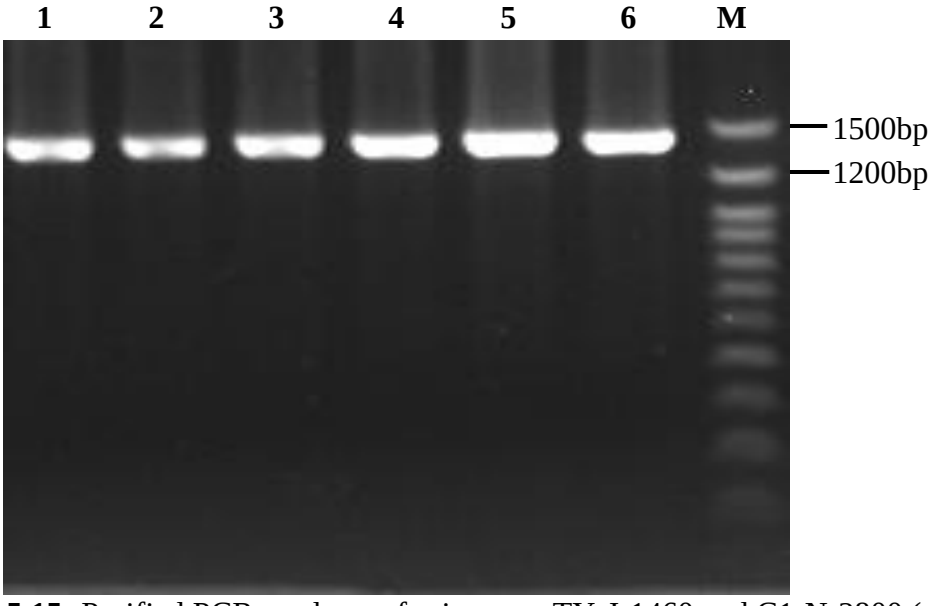


Figure 5.15: Purified PCR products of primer set TY-J-1460 and C1-N-2800 (~1380bp) after gel extraction. Lanes: 1-6, purified PCR products of species of Calliphoridae and M, molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea).

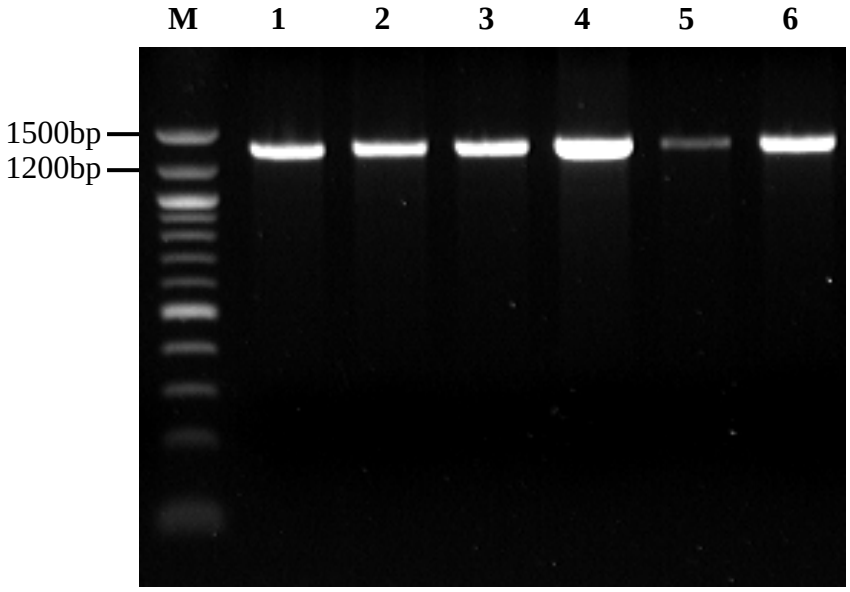


Figure 5.16: Purified PCR product of primer set C1-J-2495 and TK-N-3775 (~1324bp) after gel extraction. Lanes: M, molecular weight marker of 100bp DNA Ladder (New England Biolabs, UK) and 1-6, purified PCR products of species of Calliphoridae.

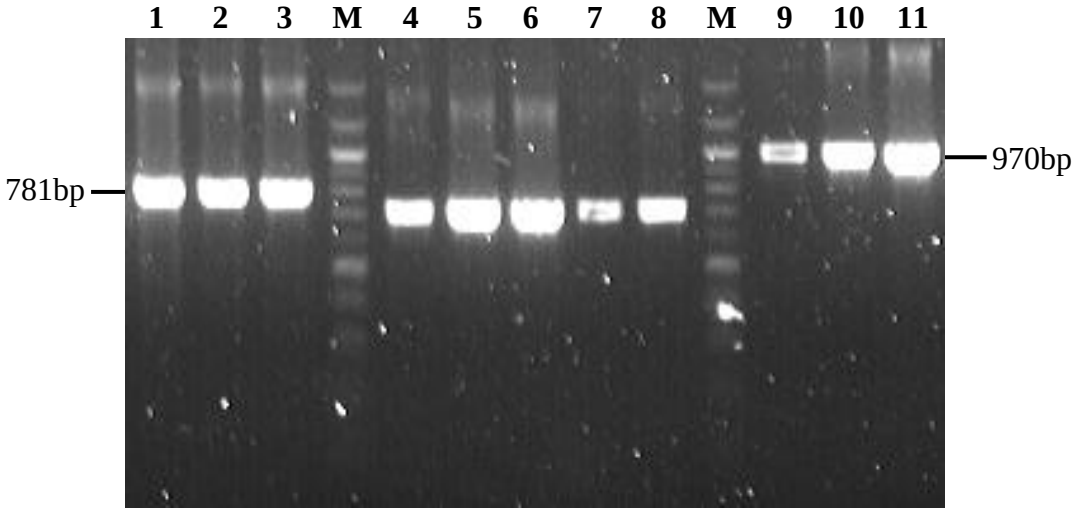


Figure 5.17: Purified PCR product of primer sets of D1.F & D2.R (781bp), D3-5.F & D3-5.R (704bp) and D3-5.742F & D7.R (970bp) after gel extraction. Lanes: 1-3, purified PCR products of Calliphoridae species (D1.F & D2.R); M, molecular weight marker of 100bp DNA Ladder (New England Biolabs, UK); 4-8, purified PCR products of species of Calliphoridae (D3-5.F & D3-5.R) and 9-11, purified PCR products of species of Calliphoridae (D3-5.742F & D7.R).

5.4.4 PCR-RFLP

5.4.4.1 PCR-RFLP assay

Restriction profiles of *Ssp*I, *Msp*I and *Taq*$^{\alpha}$I were summarised in Table 5.7. A dichotomous key of these PCR-RFLP assays for species identification of eight Malaysian *Chrysomya* species was described in section 5.4.4.2.

Unique restriction profiles were generated for *C. rufifacies*, *C. thanomthini* and *C. villeneuvi* by *Ssp*I (Figure 5.18). Identical restriction patterns were produced by *Ssp*I in *C. chani* and *C. megacephala* (948bp, 432bp); *C. nigripes*, *C. pinguis* and *C. defixa* (948bp, 432bp, 148bp) as well as *C. pinguis* and *C. defixa* (1380bp). Therefore, it was impossible to discriminate between these species with *Ssp*I alone.

In such circumstances, further differentiation could be done using second restriction endonuclease. For the case of *C. chani* and *C. megacephala*, *Taq*$^{\alpha}$I was used. Different restriction patterns were produced and enable differentiation between these species (Figure 5.19a). For the case of *C. nigripes*, *C.pinguis* and *C. defixa*, *Msp*I was used (Figure 5.19b). Restriction pattern of *C. nigripes* (1224bp, 104bp, 52bp) was able to distinguish from *C. pinguis* and *C. defixa* although *C. pinguis* and *C. defixa* produced identical banding patterns (745bp, 531bp, 104bp).

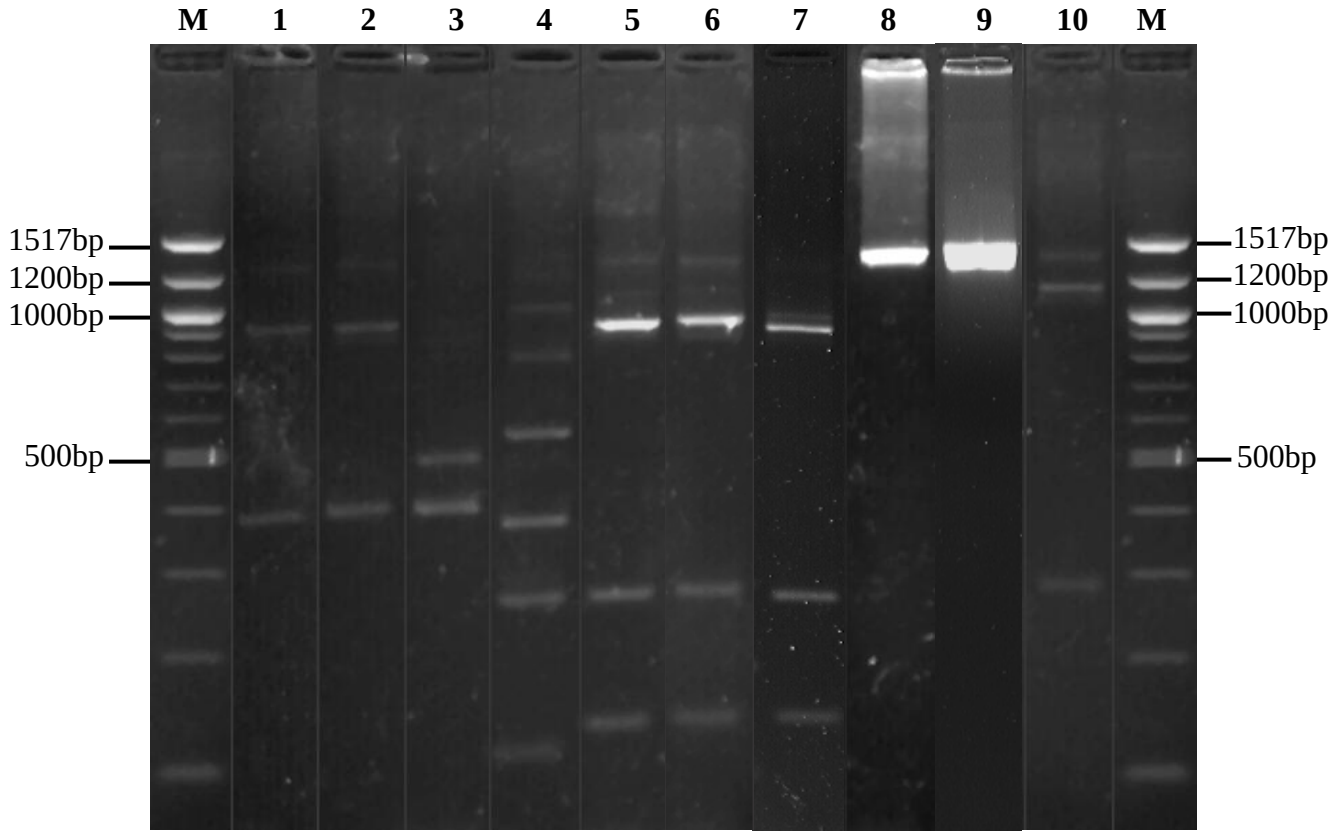


Figure 5.18: PCR-RFLP assay of *Ssp*I restriction endonuclease digestion of PCR fragment amplified by primer set TY-J-1460 and C1-N-2800 (1380bp) on 2% agarose gel. Lanes: M, molecular weight marker of 100bp DNA Ladder (New England Biolabs, UK) and 1-10, different species of genus *Chrysomya*; 1, *C. megacephala*; 2, *C. chani*; 3, *C. rufifacies*; 4, *C. villeneuvi*; 5, *C. nigripes*; 6, *C. defixa*; 7, *C. pinguis*; 8, *C. defixa*; 9, *C. pinguis*; and 10, *C. thanomthini*.

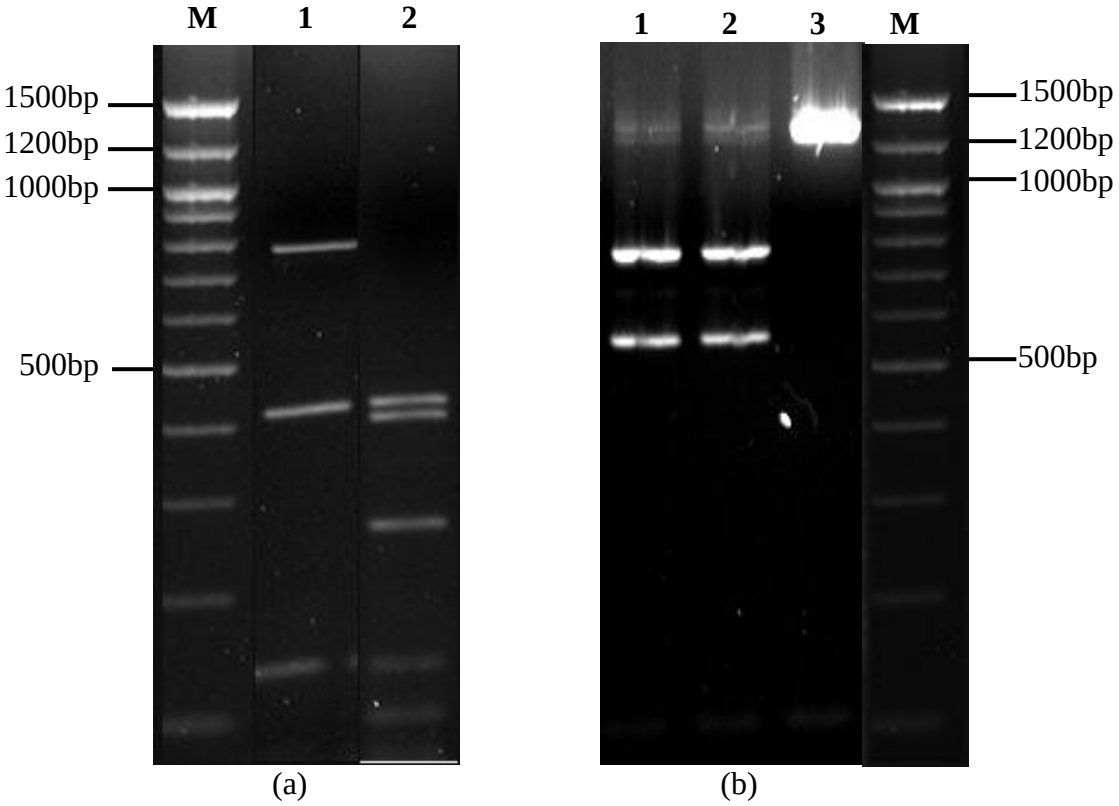


Figure 5.19: Further differentiation of PCR-RFLP assay of (a) *Taq*$^{\alpha}$I and (b) *Msp*I restriction endonucleases digestion of PCR fragment amplified by primer set TY-J-1460 and C1-N-2800 (1380bp) on 2% agarose gel. Lanes: M, molecular weight marker of Forever 100bp personalised DNA Ladder (Seegene, Korea) and different species of genus *Chrysomya*; (a) 1, *C. megacephala*; 2, *C. chani*; (b) 1, *C. defixa*; 2, *C. pinguis*; 3, *C. nigripes*.

Table 5.7: Characterisation of the restriction sites in eight Malaysian *Chrysomya* species.

SPECIES	RESTRICTION ENDONUCLEASES					
	*Ssp*I (AAT▼ATT)		*Taq*$^{\alpha}$I (T▼CGA)		*Msp*I (C▼CGG)	
	Cleavage site	Length (bp)	Cleavage site	Length (bp)	Cleavage site	Length (bp)
Chrysomya chani	432	948, 432	138, 564, 663, 930, 1338	426, 408, 267, 138, 99, 42	104	1276, 104
Chrysomya defixa	-	1380	138, 564, 686, 932, 1338	426, 406, 246, 138, 122, 42	635, 104	745, 531, 104
	284, 432	948, 284, 148				
Chrysomya megacephala	432	948, 432	138, 564, 1338	774, 426, 138, 42	104	1276, 104
Chrysomya nigripes	284, 432	948, 284, 148	138, 663, 686, 930, 1338	525, 408, 244, 138, 42, 23	104,,1328	1224, 104, 52
Chrysomya pinguis	-	1380	138, 564, 686, 932, 1338	426, 406, 246, 138, 122, 42	104, 635	745, 531, 104
	284, 432	948, 284, 148				
Chrysomya rufifacies	432, 857	523, 432, 425	138, 564, 663, 930, 1338	426, 408, 267, 138, 99, 42	-	1380
Chrysomya thanomthini	284	1096, 284	138, 564, 686, 930, 1338	426, 408, 244, 138, 122, 42	104, 635, 812	568, 531, 177, 104
Chrysomya villeneuvi	284, 857, 1253	573, 396, 284, 127	138, 663, 686, 930, 1338	525, 408, 244, 138, 42, 23	-	1380

5.4.4.2 Dichotomous key of PCR-RFLP assay for species identification of eight Malaysian *Chrysomya* species

1. Digestion with *Ssp*I:
   - Cut ……..... 2
   - Uncut …… *C. defixa* or *C. pinguis*

2. Produced 4 bands ……. *C. villeneuvi*
   Produced less than 4 bands ……..... 3

3. Produced 2 bands ……... 4
   Produced 3 bands ……... 5

4. Produced a band at 1096bp ……...……... *C. thanomthini*
   Produced a band at 948bp …….. 6

5. Produced a band at 523bp ……...……. *C. rufifacies*
   Produced a band at 948bp ……...……... 7

6. Digestion with *Taq*$^{\alpha}$I:
   - Produced 5 bands ……...……..... *C. megacephala*
   - Produced 6 bands ……...…… *C. chani*

7. Digestion with *Msp*I:
   - Produced a band at 1224bp ….……..... *C. nigripes*
   - Produced a band at 745bp …… *C. defixa* or *C. pinguis*

5.4.5 DNA sequence analyses

5.4.5.1 *Cytochrome c oxidase* subunit I and II

(a) Sequence diversity

Complete sequences of *cytochrome c oxidase* subunit I and II genes and the intervening tRNA-leucine for 19 Malaysian Calliphoridae and one Japanese species were obtained in this study. A total of 97 Calliphoridae DNA sequences were obtained, which comprised 20 species from five genera and three subfamilies. Among these 97 DNA sequences, five were retrieved from GenBank (AF083658, AY074900, AJ417704, AJ417707 and AJ417702) as references with local DNA sequences. The phylogenetic tree was rooted with *Parasarcophaga albiceps* as a Sarcophagidae outgroup.

The total length of the amplified DNA sequences obtained ranged from 2300bp-2309bp, which corresponds to positions 1461 to 3763 of *Drosophila yakuba* (Accession number NC_001322: Clary & Wolstenholme, 1985). The average base compositions were T=38.8%, C=15.1%, A=31.9% and G=14.2% with a strong AT-bias (70.7%), that is typical of insect mitochondrial DNA (Clary & Wolstenholme, 1985; Crozier & Crozier, 1993; Sperling & Hickey, 1994; Wallman & Donnellan, 2001; Stevens *et al*., 2008) (Table 5.8). There was no base composition heterogeneity between sequences among species (p=1.00).

The final sequence alignment obtained was 2309bp, which encompassed the complete sequences of COI, tRNA-leucine and COII genes (COI+II). This alignment revealed the inclusion of a maximum 6-bp gap within the spacer region between the tRNA-leucine and *COII* gene and a 3-bp gap at the end of *COII* gene. In the alignment, 783 of the nucleotides were variable, whereby 588 sites were considered parsimony informative (Table 5.9).

Table 5.8: DNA sequence length polymorphism of mitochondrial DNA of COI+II and its nucleotide composition in different species of Calliphoridae.

Species	Sequence length (bp)	Nucleotide composition (%)			
		T	C	A	G
Calliphora fulviceps	2300	39.7	14.5	31.8	14.0
Chrysomya bezziana	2303	38.3	15.5	32.1	14.1
Chrysomya chani	2303	39.4	14.6	31.8	14.2
Chrysomya defixa	2303	38.2	15.7	31.4	14.7
Chrysomya megacephala	2303	38.5	15.3	31.7	14.5
Chrysomya nigripes	2303	39.4	14.9	31.3	14.4
Chrysomya pinguis	2303	38.1	15.8	31.4	14.7
Chrysomya rufifacies	2303	39.0	14.6	32.3	14.1
Chrysomya thanomthini	2303	38.0	15.8	31.7	14.5
Chrysomya villeneuvi	2303	39.0	14.5	32.5	14.0
Hemipyrellia ligurriens	2303	39.1	15.0	31.8	14.1
Hemipyrellia tagaliana	2303	39.0	15.0	31.8	14.2
Hypopygiopsis fumipennis	2304	39.4	14.4	32.0	14.2
Hypopygiopsis violacea	2304	38.6	15.2	32.0	14.2
Lucilia bismarckensis	2309	38.4	14.9	32.7	14.0
Lucilia calviceps	2303	38.5	14.9	32.2	14.4
Lucilia cuprina	2304	39.2	14.8	31.7	14.3
Lucilia papuensis	2309	38.6	15.0	32.3	14.1
Lucilia porphyrina	2307	38.5	15.6	31.6	14.3
Average		38.8	15.1	31.9	14.2

Table 5.9: DNA variation of DNA region of mitochondrial COI+II.

DNA variation	Nucleotide (bp)	Percentage (%)
Conserved	1526	66.1
Variable	783	33.9
Parsimony informative	588	25.5
Singleton	195	8.4
Total alignment	2309	100

(b) Distribution of variation

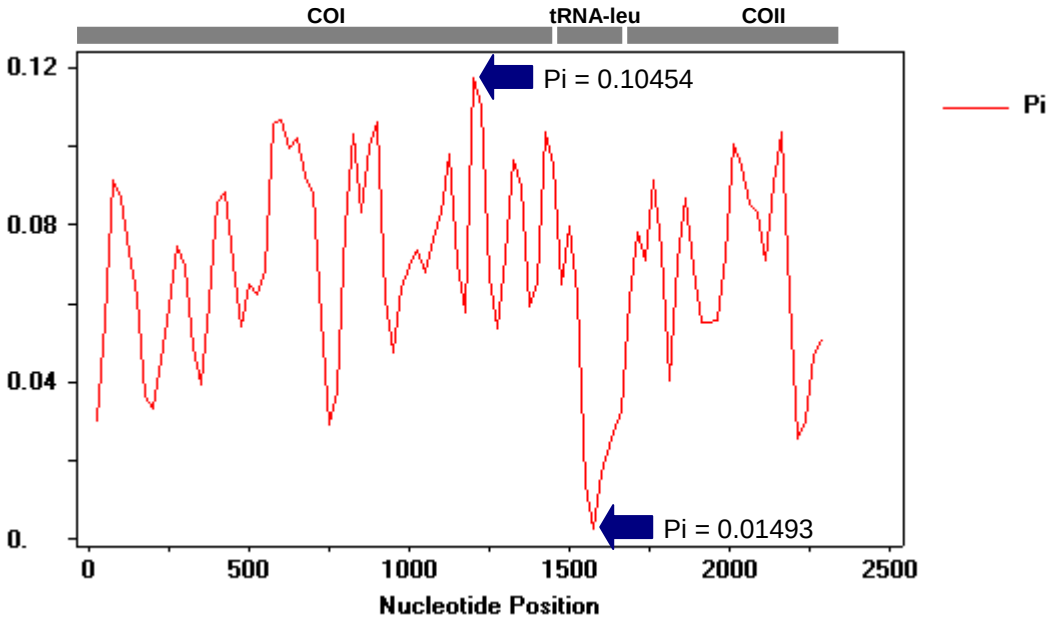


Figure 5.20: Distribution of nucleotide variation of mitochondrial DNA COI+II of 2309bp sequences based on a 100bp sliding window plot with 25bp steps. Arrows indicate the highest and lowest nucleotide diversity (Pi) values.

The distributed variation was calculated using the nucleotide diversity (Pi), which was the average number of nucleotide differences per site (Nei, 1987; Nei & Miller 1990). The variation was distributed evenly across the *COI* and *COII* genes, with almost no variation at the tRNA-leucine gene (Figure 5.20). The highest Pi value is found in COI region with 0.10454 while the lowest Pi value is found in tRNA-leucine region with 0.01493.

(c) Estimation of best fit model

In choosing the best fit model among 56 models for COI+II DNA sequences, the Akaike Information Criterion (AIC) was chosen. The dataset resulted in the best likelihood score for the general time reversible (GTR) model with invariable sites and rate heterogeneity. Base frequencies were unequal; A = 0.3198, C = 0.1379, G = 0.1367, T = 0.4065; and the estimated proportion of invariable sites (I) were 0.5069. The substitution model incorporated the following rate matrix: [A–C] = 1.7696, [A–G] = 35.6687, [A–T] = 10.0344, [C–G] = 1.8785, [C–T] = 75.2073, [G–T] = 1.0000. The shape parameter of the gamma distribution was a = 0.8838. This model was used to determine the number of substitution types and the inclusion of the gamma rate distribution and proportion of invariable sites in the NJ and Bayesian analyses.

(d) Accumulation of nucleotide substitutions

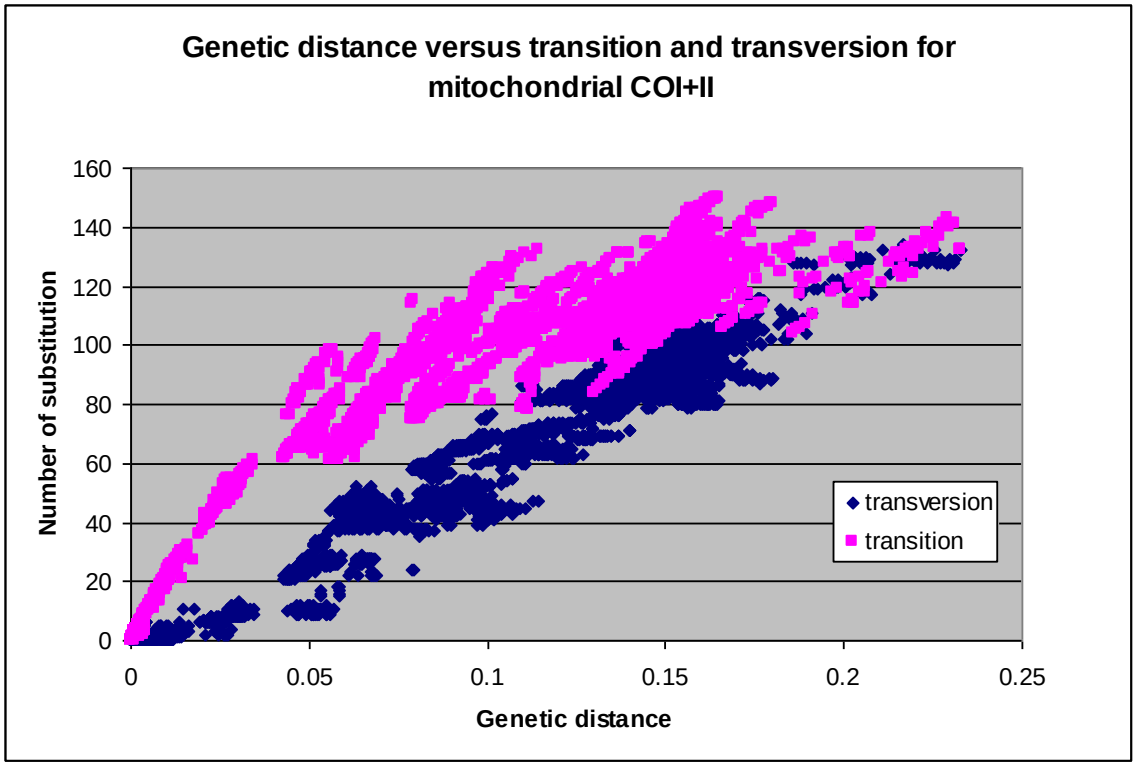


Figure 5.21: Genetic distance versus transition and transversion of mitochondrial COI+II sequences of 98 taxa.

Plots of nucleotide substitutions – transition and tranversion at each site versus the genetic distance of COI+II were calculated and analysed (Figure 5.21). From the graph, the transition rate is higher than the transversion rate with the ratio of transition over transversion of 1.5. Transitions accumulate more rapidly than transversions and begin to reach saturation at a genetic distance of 0.1.

(e) Pairwise sequence divergence

In general, the maximum pairwise sequence divergence within species of Calliphoridae for COI+II were smaller than 1% except *C. defixa*, *C. pinguis*, *H. fumipennis*, *L. cuprina*, *L. papuensis* and *L. porphyrina* (Table 5.10 in bold). The value of maximum pairwise sequence divergence within species of *C. defixa* (1.41%) and *C. pinguis* (2.74%) was perhaps due to the congeneric shared haplotype by these two species. Although the maximum pairwise sequence divergences within species of *H. fumipennis*, *L. cuprina*, *L. papuensis*, *L. porphyrina* were more than 1%, the affinities of the discrimination of species were still able to achieve because these values did not overlap with the minimum pairwise sequence divergence between species.

In general, minimum pairwise sequence divergence between species among the three subfamilies is more than 10% while within subfamilies is below 10%. The largest minimum divergence value between the species was 17.93% between species of *C. nigripes* and *L. calviceps* while the smallest is between the *H. ligurriens* and *H. tagaliana* (0.17%).

Table 5.10: Minimum pairwise sequence divergence between species and maximum pairwise sequence divergence within species (bold) of COI+II. The sequence divergence was calculated based on GTR+I+G model in PAUP* 4.0b10 (Swofford, 1998).

* Species	CB	CC	CD	CM	CN	CP	CR	CT	CV	HF	HL	HT	HV	Lbi	Lcu	Lca	Lpa	Lpo	Cfu	Cvi
CB	**0.75**																			
CC	5.80	**0.39**																		
CD	4.67	4.92	**1.41**																	
CM	4.56	4.64	0.61	**0.39**																
CN	6.53	6.14	5.97	5.70	**0.66**															
CP	4.72	4.41	0.26	1.11	6.19	**2.74**														
CR	9.15	8.01	8.50	8.15	9.55	9.07	**0.85**													
CT	5.30	5.13	2.79	2.69	6.80	2.70	9.03	**0.43**												
CV	8.92	8.14	8.38	8.11	8.86	9.02	4.27	9.24	**0.44**											
HF	13.52	9.83	12.71	14.12	13.54	11.76	13.15	12.54	13.00	**1.79**										
HL	14.16	12.60	14.67	14.17	14.56	14.21	15.35	13.98	15.00	8.25	**0.09**									
HT	14.12	12.56	14.70	14.11	14.69	14.25	15.65	14.03	15.30	8.34	0.17	**0.39**								
HV	13.55	12.67	14.64	14.76	15.13	14.19	16.00	14.32	14.53	8.39	6.24	6.32	**0.26**							
Lbi	15.16	12.10	14.73	14.87	16.48	15.01	15.72	15.43	14.91	8.04	13.01	13.02	12.45	**0.44**						
Lcu	12.60	10.94	12.54	12.13	13.63	12.47	14.16	12.74	14.57	5.54	7.86	8.00	8.52	8.40	**1.04** [a] **3.04** [b]					
Lca	15.69	13.90	17.09	16.87	17.93	16.79	16.79	16.78	16.30	9.42	13.45	13.57	13.13	4.39	9.44	**N/A**				
Lpa	14.44	12.76	15.27	15.34	15.65	15.77	15.20	14.77	14.27	7.21	10.50	10.57	10.41	6.11	8.01	6.69	**5.87**			
Lpo	14.85	12.73	13.97	14.12	16.23	13.88	15.13	13.76	14.27	7.07	10.42	10.52	10.40	9.69	6.49	10.44	8.08	**2.83**		
Cfu	15.15	13.94	14.40	15.80	16.39	16.10	16.22	16.32	16.58	11.87	13.35	13.47	13.55	12.45	11.59	12.96	11.83	12.85	**0.09**	
Cvi	12.49	12.94	15.75	13.97	13.57	13.94	15.69	14.23	14.62	11.27	12.89	12.84	12.08	11.93	9.92	12.37	10.37	10.93	10.44	**0.49**

* Species abbreviation.
CB: *C. bezziana*, CC: *C. chani*, CD: *C. defixa*, CM: *C. megacephala*, CN: *C. nigripes*, CP: *C. pinguis*, CR: *C. rufifacies*, CT: *C. thanomthini* and CV: *C. villeneuvi*.

N/A: not calculated because it is only one specimen.
[a] pairwise comparison within local sequences
[b] pairwise comparison between local and foreign sequences

5.4.5.2 Sequences of 28S rDNA

(a) Sequence diversity

Partial DNA sequences of 28S rDNA for nine Malaysian *Chrysomya* species were obtained in this study. A total of 43 Calliphoridae DNA sequences were successfully generated in the present study and five reference sequences were retrieved from the GenBank (AJ551434, AJ551435, AJ551436, AJ300131 and AJ300139). *Parasarcophaga albiceps* sequence was added as a Sarcophagidae outgroup. Total length of the DNA sequences varies from 2149bp to 2161bp among species.

The average nucleotide compositions of this region were T (30.1%), C (15.7%), A (32.2%) and G (22.0%) (Table 5.11). The sequence alignment consists of 262 conserved sites and 110 variable sites, whereby 67 sites were considered parsimony informative (Table 5.12).

The 28S rDNA sequences produced a final alignment of 2172bp, which revealed a total of 30-bp indel across the gene fragment. These indels appeared in 1-bp, 2-bp, 4-bp, 5-bp and 9-bp (Table 5.13).

Table 5.11: DNA sequence length polymorphism of nuclear DNA of 28S rDNA and its nucleotide composition in different species of Calliphoridae.

Species	Sequence length (bp)	Nucleotide composition (%)			
		T	C	A	G
Chrysomya bezziana	2151	30.1	15.7	32.2	22.0
Chrysomya chani	2149	30.1	15.7	32.2	22.0
Chrysomya defixa	2149	30.1	15.7	32.1	22.1
Chrysomya megacephala	2149	30.1	15.7	32.1	22.1
Chrysomya nigripes	2149	30.1	15.7	32.2	22.0
Chrysomya pinguis	2149	30.1	15.7	32.2	22.0
Chrysomya rufifacies	2151	30.0	15.7	32.4	21.9
Chrysomya thanomthini	2150	30.1	15.7	32.2	22.0
Chrysomya villeneuvi	2150	30.0	15.7	32.4	21.9
Calliphora fulviceps	2150	30.4	15.5	32.0	22.0
Calliphora vicina	2146	30.5	15.6	31.9	22.0
Hemipyrellia tagaliana	2152	30.5	15.6	32.2	21.8
Lucilia cuprina	2161	30.3	15.5	32.5	21.7
Lucilia sericata	2155	30.3	15.6	32.3	21.8
Average		30.1	15.7	32.2	22.0

Table 5.12: DNA variation and nucleotide compositions of the region 28S rDNA.

DNA variation (bp)	Nucleotide (bp)	Percentage (%)
Conserved	2062	94.94
Variable	110	5.06
Parsimony infomative	67	3.08
Singleton	43	1.98
Total alignment	2172	100.0

Table 5.13: Distribution of indels in the 28S rDNA with the nucleotide positions.

Specimens	Nucleotide position
	11111111111111111 55588888888887777777888888888 13311222455587888889900000011 33489013901248456787834567890 1
CD_forest	----TT-A---------TATA---------
CD_KH37	----TT-A---------TATA---------
CD_KH38	----TT-A---------TATA---------
CD_E24	----TT-A---------TATA---------
CD_AS2	----TT-A---------TATA---------
CD_AS3	----TT-A---------TATA---------
CD_E26	----TT-A---------TATA---------
CD_E39	----TT-A---------TATA---------
CD_F17_AS	----TT-A---------TATA---------
CD_F18	----TT-A---------TATA---------
CM_F2_UM	----TT-A---------TATA---------
CM_F1_UM	----TT-A---------TATA---------
CM_F15_AS	----TT-A---------TATA---------
CM_UK1	----TT-A---------TATA---------
CM_UK2	----TT-A---------TATA---------
CM_AJ551435	----TT-A---------TATA---------
CT3_AD2	-T--TT-A---------TATA---------
CT4_9_9	-T--TT-A---------TATA---------
CT2_AD2	-T--TT-A---------TATA---------
CP_GB	----TT-A---------TATA---------
CP_forest	----TT-A---------TATA---------
CP_KH_M19_4	----TT-A---------TATA---------
CP_1_9	----TT-A---------TATA---------
CP_7_9	----TT-A---------TATA---------
UK-KH1	----TT-A---------TATA---------
CP1_UK	----TT-A---------TATA---------
CP2_UK	----TT-A---------TATA---------
CP3_UK	----TT-A---------TATA---------
CC_F11_UM	-TA-TT-A----------TA---------
CC_F12_AS	-TA-TT-A----------TA---------
CC_AD2	-TA-TT-A----------TA---------
CN_F8	---ATT-A----------TA---------
CN_F35	---ATT-A----------TA---------
CR_F5	-TA-TT-A------AA---TG---------
CR_F6_UM	-TA-TT-A------AA---TG---------
CR_F13_AS	-TA-TT-A------AA---TG---------
CR_F32_PPLUM	-TA-TT-A------AA---TG---------
CR_AJ551436	-TA-TT-A------AA---TG---------
CV_F20_AS	-TA-T--A------AA---TG---------
CV_F33	-TA-T--A------AA---TG---------
CB_3_9	---AT--ATTAT-------TA---------
CB_AJ551434	---AT--ATTAT-------TA---------
Calli_CH	A--ATT-A----A------TG---------
Cvicina_AJ300131	---ATTTA----A------TG---------
LC	-------A----ATAAATATATAAGAAAAA
LS-AJ300139	-------A----ATAAATATATAAGAAAAA
HT_UM1	---ATTTA----AT-T-TA----------
HT_UM2	---ATTTA----AT-T-TA----------
PA_PU	-GA--TT-----A-AT-TATA---------

(b) Distribution of variation

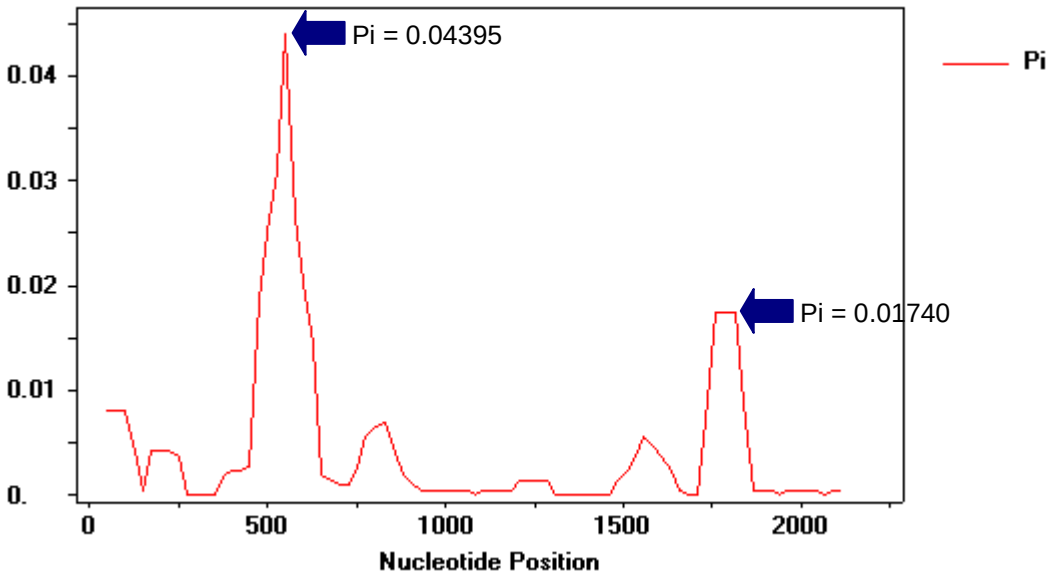


Figure 5.22: DNA variation along the nucleotide position of nuclear DNA 28S rDNA of 2172bp sequences. Arrows indicate the distinct sites with high polymorphism.

Nucleotide diversity (Pi) is a measurement of the average number of nucleotide difference per site (Nei, 1987; Nei and Miller 1990). Nuclear 28S rDNA showed low variation across the region or even at some points, there were no variations (Figure 5.22). However, two very distinct peaks were observed from the graph, which were the sites at approximately nucleotide positions of 500 and 1800 with the Pi values of 0.04395 and 0.01740, respectively (blue arrows). These distinct regions were caused by the high variations that occurred at that site.

(c) Estimation of best fit model

For choosing the best fit model for 28S rDNA among 56 models, the Akaike Information Criterion (AIC) was chosen. The best-fit model selected was transversional model (TVM) for which the base frequencies were unequal; A = 0.3206, C = 0.1556, G = 0.2192, T = 0.3046; and the estimated proportion of invariable sites (I) were 0.9413. The substitution model incorporated the following rate matrix: [A–C] = 0.6765, [A–G] = 2.9850, [A–T] = 3.3020, [C–G] = 0.5657, [C–T] = 2.9850, [G–T] = 1.0000. The rate variation among sites was equal. This model was used to determine the number of substitution types and the inclusion of equal rates for variation among sites and the proportion of invariable sites in the NJ and Bayesian analyses. Partitioned gaps were treated as the Standard Discrete Model (gaps=0, T=1, A=2, C=3, G=4) with nst=1 and rate=equal.

(d) Accumulation of nucleotide substitutions

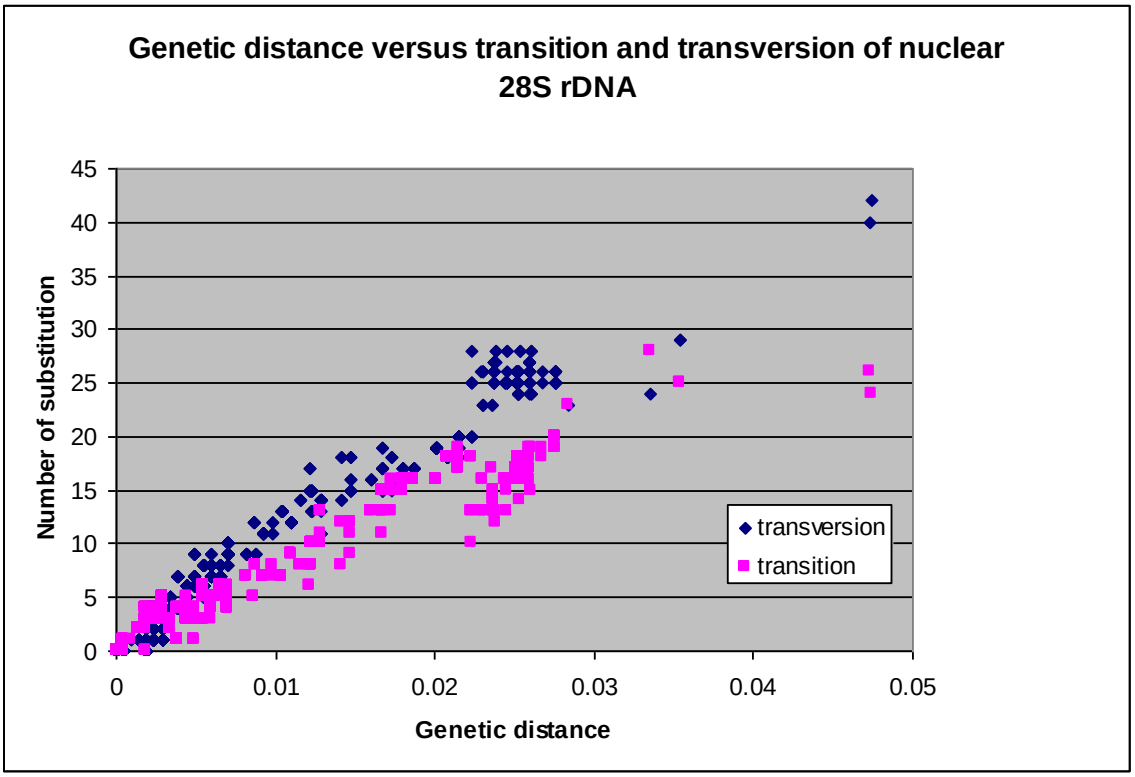


Figure 5.23: Genetic distance versus transition and transversion of nuclear 28S rDNA sequences of 49 taxa. Genetic distance was calculated based on TVM+I model in PAUP* 4.0b10 (Swofford, 1998).

From the graph, the transversion rate was higher than the transition rate with the average of transition over transversion rate ratio of 0.8, which was approximately to one (Figure 5.23). This result was similar to the previous studies in gall wasps and Brachyceran flies (Rokas *et al*., 2003; Wiegmann *et al*., 2003). Both transition and tranversion show no evidence of saturation. Genetic distances within species and between congeneric species were below 0.01. Genetic distances of species between subfamilies and with the outgroup are greater than 0.02.

(e) Pairwise sequence divergence

Due to the extremely low genetic distances (<0.01), the pairwise divergence was calculated based on frequency of substitutions. As shown in Table 5.14, there are no variations within species of *Chrysomya*, except *C. pinguis* with one nucleotide difference at the position 1014 (G/A) for voucher CP2-UK and 2149 (G/T) for voucher CP3-UK.

Table 5.14: Pairwise divergence of 28S rDNA of *Chrysomya* species based on the frequency of substitutions between and within species (bold). Intraspecific and interspecific comparisons were reported in maximum and minimum values, respectively.

Species *	CB	CC	CD	CM	CN	CP	CR	CT	CV
CB	**0**								
CC	17	**0**							
CD	15	14	**0**						
CM	13	12	6	**0**					
CN	28	19	21	20	**0**				
CP	18	12	6	6	18	**1**			
CR	22	11	14	11	14	8	**0**		
CT	14	9	5	4	17	4	7	**0**	
CV	18	12	15	12	13	9	3	8	**0**

* Species abbreviation.
CB: *C. bezziana*, CC: *C. chani*, CD: *C. defixa*, CM: *C. megacephala*, CN: *C. nigripes*, CP: *C. pinguis*, CR: *C. rufifacies*, CT: *C. thanomthini* and CV: *C. villeneuvi*.

5.4.6 Phylogenetic trees

Three phylogenetic trees (NJ, MP and Bayesian analysis) were constructed separately for DNA sequences of COI+II and 28S rDNA (see Appendices B1-B6). In the results, only the Bayesian consensus tree was shown. However, the supported values of posterior probabilities (from Bayesian analysis) and the bootstrap values (from NJ and MP analyses) were incorporated at the branch nodes (Figures 5.24 and 5.25).

Bayesian analyses of COI+II and 28S rDNA resulted in highly resolved and well supported phylogenies with slightly different patterns in both genes. Three subfamilies of Calliphoridae: Chrysomyinae, Calliphorinae and Luciliinae; were fully resolved as monophyletic in both genes with high posterior probabilities (>98%). Calliphorinae was a sister group to Luciliinae.

Almost similar tree topologies were recovered from the NJ, MP and Bayesian analyses for COI+II DNA sequences (Figure 5.24). The phylogeny of Calliphoridae flies recognised 19 genetic clades (A–S) that were highly supported. These 21 clades consisted of three subfamilies: Chrysomyinae (ten clades: A-J), Luciliinae (nine clades: K-Q) and Calliphorinae (two clades: R-S), which only Bayesian analysis successfully resolved (lines with pink, blue and red, respectively).

The subfamily Chrysomyinae clades were defined as A: *C. megacephala*, B: *C. defixa*, C: *C. defixa* + *C. pinguis*, D: *C. pinguis*, E: *C. thanomthini*, F: *C. bezziana*, G: *C. nigripes*, H: *C. chani*, I: *C. rufifacies* and J: *C. villeneuvi*. All species were monophyletic, except for *C. defixa* and *C. pinguis* which were paraphyletic due to the sharing of haplotype in clade C. Two distinct groups were identified; the species groups of hairy maggot (*C. ruffifacies* and *C. villeneuvi*) and non-hairy maggot (clades A-H).

The subfamily Luciliinae comprised clades K: *L. papuensis* + *L. bismarckensis* + *L. calviceps*, L: *L. porphyrina*, M: *H. fimupennis*, N: *L. cuprina*, O: *H. violacea*, P: *H. ligurriens* and Q: *H. ligurriens* + *H. tagaliana*.

Among the three species in clade K, only *L. bismarckensis* was monophyletic and closely related to *L. calviceps*. Paraphyly of genus *Lucilia* was demonstrated in the Bayesian analysis by the unexpected affinity of *L. cuprina* to genera *Hypopypiogiopsis* and *Hemipyrellia*. However, the NJ and MP analyses showed an unresolved relationship of *L. cuprina* due to the polytomy of most of the Lucilinae species. Nevertheless, all analyses showed *H. violacea* was more related to *H. ligurriens* and *H. tagaliana* rather than *H. fumipennisi*. *H. fumipennisi* appeared to be more basal among the species of *Hypopygiopsis* and *Hemipyrellia*.

The subfamily Calliphorinae consisted of clades R: *C. vicina* and S: *C. fulviceps*. The separation between Luciliinae and Calliphorinae was only supported by the Baysian anaylysis.

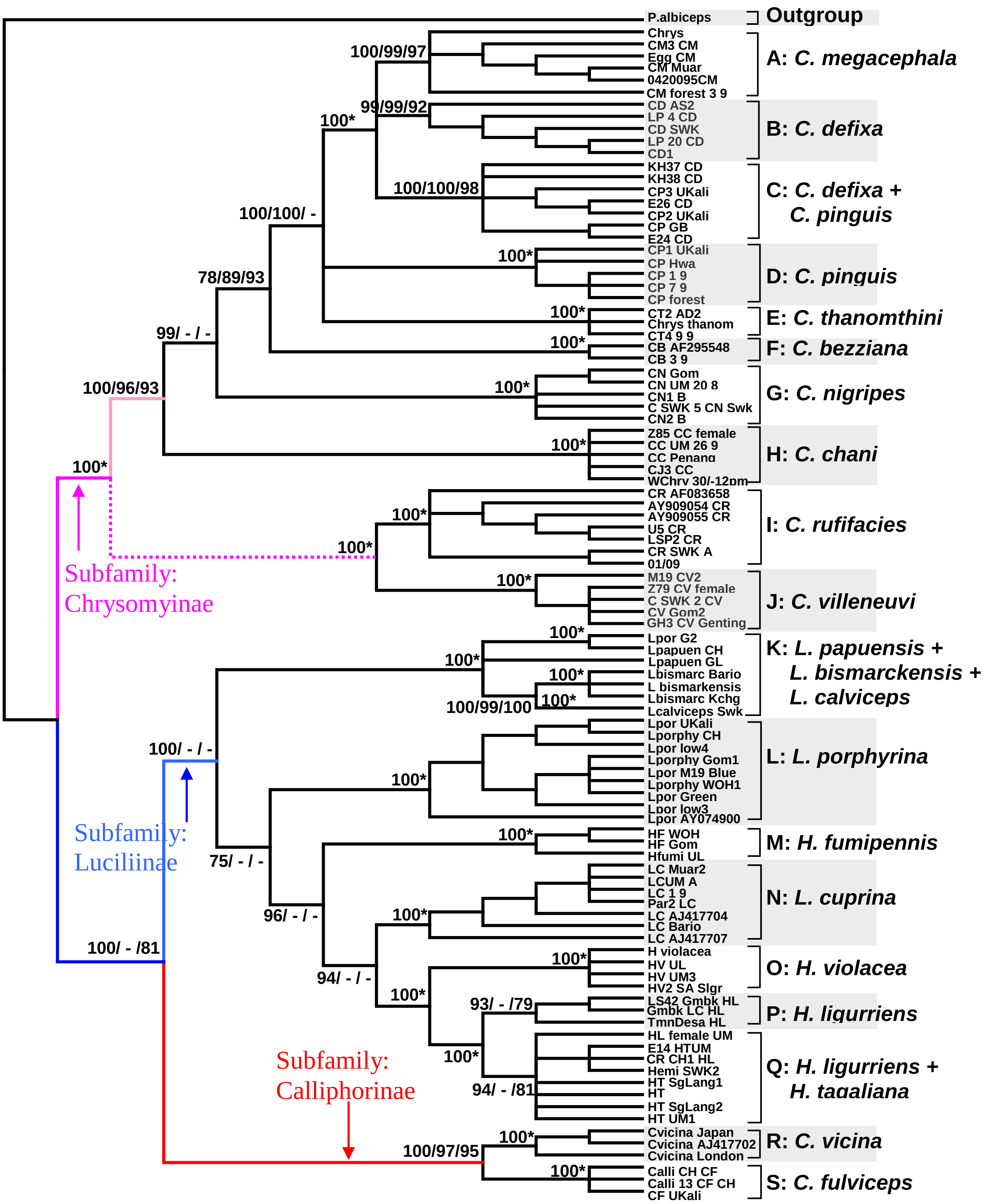
Tree topologies for 28S rDNA sequences recovered from the NJ, MP and Bayesian analyses were slightly different (Figure 5.25). Similar to the COI+II phylogenetic tree (Figure 5.24), only the Bayesian analysis was able to resolve the subfamily level of Chrysomyinae, Luciliinae and Calliphorinae. Nevertheless, all three methods seem to support the lineages of Luciliinae + Calliphorinae and Chrysomyinae in 28S rDNA. Twelve clades were defined (A-L) excluding the Sarcophagidae outgroup (Figure 5.25). The subfamily Chrysomyinae was divided into nine species clades, A: *C. megacephala*, B: *C. bezziana*, C: *C. defixa*, D: *C. thanomthini*, E: *C. pinguis*, F: *C. rufifacies*, G: *C. villeneuvi*, H: *C. chani* and I: *C. nigripes*. Three other clades belonged to subfamilies Luciliinae and Calliphorinae, J: *H.tagaliana*, K: *L. cuprina* + *L. sericata* and L: *C. fulvicpes* + *C. vicina*.

Although all *Chrysomya* species were monophyletic, most of the relationships between species were unresolved. The MP tree showed only the close relatedness between the hairy maggot species, *C. rufifacies* and *C. villeneuvi*, whereas the NJ phylogeny showed a closer relationship between *C. defixa*, *C. pinguis*, *C. megacephala*,

C. bezziana and *C. thanomthini* than the rest (see appendix). It is noteworthy that *C. defixa* and *C. pinguis* were clearly separated here, unlike in the COI+II phylogeny.

In the subfamily Chrysomyinae, *C. nigripes* was more basal than *C. chani*, *C. villeneuvi*, and *C. rufifacies*. This was incongruent with the COI+II phylogeny (Figure 5.23), which *C. villeneuvi*, and *C. rufifacies* (hairy maggot species) appeared as the most basal. Nevertheless, the consistent basal topology of these four predacious species may indicate that predatory behaviour might be one of the ancestral traits of Chrysomyinae. Furthermore, it is plausible to consider that the hairy trait was derived from the smooth trait. In 28S rDNA phylogeny *C. bezziana* was closer to *C. megacephla* and recently diverged, whereas an earlier divergence was suggested in the COI+II phylogeny (Figure 5.24).

Figure 5.24: Bayesian consensus phylogeny of COI+II genes. Node support values are Bayesian posterior probabilities followed by NJ and MP bootstrap, respectively. Clade values represented by hyphens are below 70% cut off value while asterisks denoted that three of the values are the same. Colour-coded branches represent subfamilies, following the classificatory system of Kurahashi & Leh, 2010 (pink: Chrysomyinae; blue: Luciliinae and red: Calliphorinae).



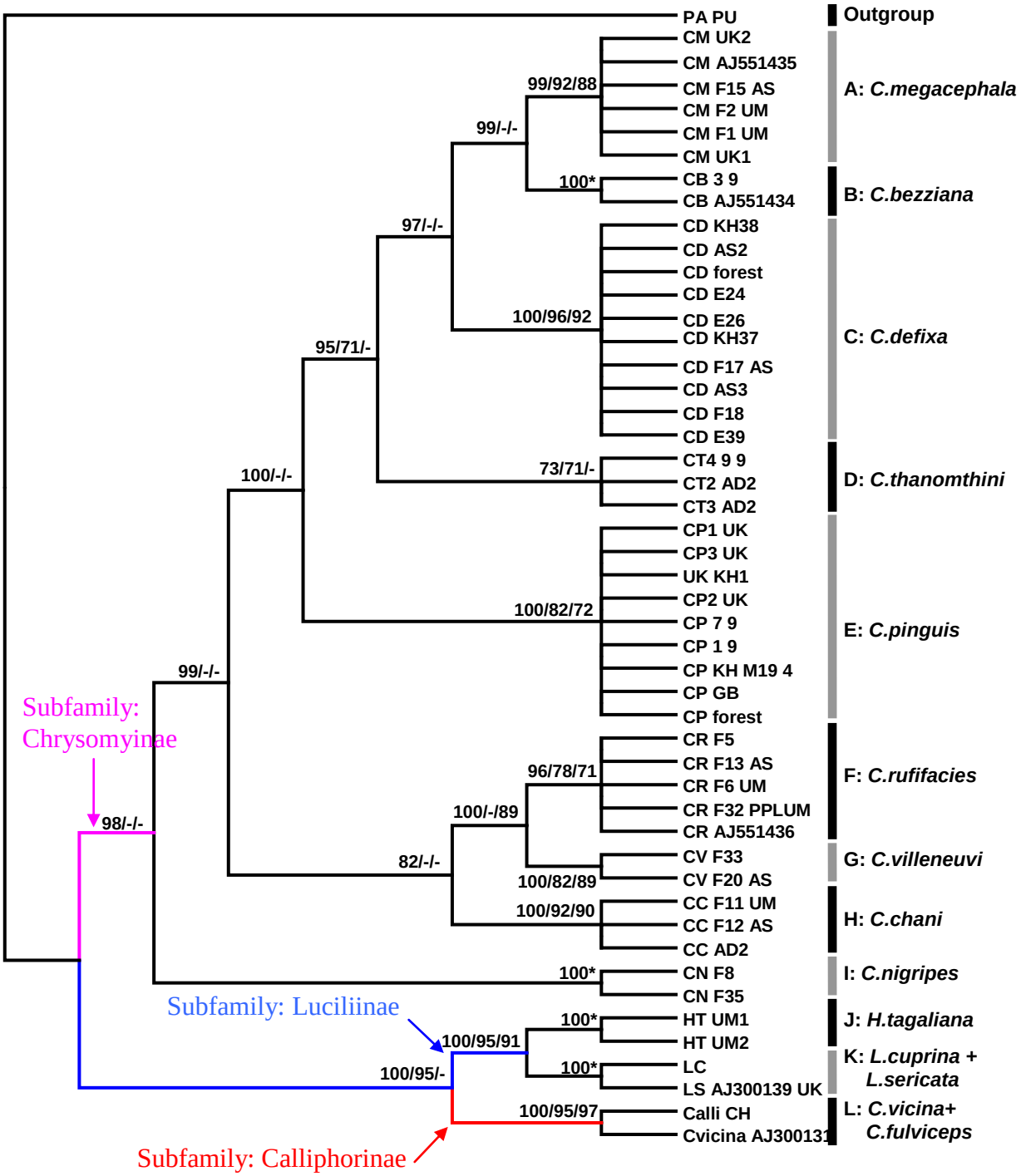


Figure 5.25: Bayesian consensus phylogeny of 28S rDNA gene. Node support values are Bayesian posterior probabilities followed by NJ and MP bootstrap, respectively. Clade values represented by hyphens are below 70% cut off value while asterisks denoted that three of the values are the same. Twelve major clades were identified as (A) *C. megacephala*; (B) *C. bezziana*; (C) *C. defixa*; (D) *C. thanomthini*; (E) *C. pinguis*; (F) *C. rufifacies*; (G) *C. villeneuvi*; (H) *C. chani*; (I) *C. nigripes*; (J) *H. tagaliana*; (K) *L. cuprina* + *L. sericata*; and (L) *C. fulviceps* + *C. vicina*.

5.5 Discussion

With the advent of PCR, only a little amount of DNA is required for amplification. In forensic entomology, PCR can amplify DNA from damaged, incomplete or degraded specimens and even from different life stages of insect (Sperling *et al*., 1994; Schroeder *et al*., 2003a; Thyssen *et al*., 2005; Tan *et al*., 2009). Thus, DNA-based identification offers a superior alternative when the conventional morphology-based identification is hindered. DNA-based identification is notably useful when handling the mummified corpses and archived samples from forensic cases (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Vincent *et al*., 2000). Although the bulk of the DNA from these specimens would have been degraded, amplification of DNA fragments can be resolved by choosing smaller fragments, optimising the DNA extraction and PCR protocols (Post *et al*., 1993).

PCR success rates can be improved, if it was targeted to mitochondrial DNA regions such as the 348bp *COI* gene (amplified by primers C1-J-2495 & C1-N-2800) as the chances of amplification is increased by (i) their relatively high copy numbers; (ii) the tendency to remain intact due to their circular structure; and (iii) the relatively small fragment (Sperling *et al*., 1994). Despite their rapid and easy application in species identification, a shorter region would limit the amount of phylogenetic signals.

PCR-RFLP is one of the commonly used molecular genetic techniques for species identification because it offers a rapid and economical diagnostic method, particularly in routine screening of large quantity of samples (Hoy, 1994). This technique is widely used in distinguishing insect species such as forensically important flies – Calliphoridae, Sarcophagidae and Muscidae (Sperling *et al*., 1999; Malgorn & Coquoz, 1999; Schroeder *et al*., 2003b & b; Noël *et al*., 2004; Thyssen *et al*., 2005; Ames *et al*., 2006), species that causing myiasis – Oestridae (Otranto *et al*., 2000), mosquitoes – Culicidae (Goswami *et al*., 2005; Alam *et al*., 2007; Shaikevich 2007),

beetles – Coleoptera: Chrysomelidae (Clark *et al*., 2001) and so on. However, this method requires the comparison of the banding profile of the unknown specimen to a known reference assay (Wells & Stevens, 2010).

The successfulness of PCR-RFLP assay depends on two factors: (i) no intra-specific variations occur at the restriction site – as such polymorphism will produce different banding patterns for the same species and (ii) restriction patterns produced are monomorphic for each species – if not, validation of specimens is required from different localities, populations and colonies.

In the present study, the genus *Chrysomya* was chosen as the model for establishing the PCR-RFLP assay, because of its prominence in Malaysian forensic cases (Lee, 1989; Lee *et al*., 2004). This technique has successfully distinguished six of the Malaysian *Chrysomya* species – overcoming morphological identification problems, such as lack of diagnostic features in immature stages (e.g. egg and first instar larva).

The usage of single restriction endonuclease is not sufficient to distinguish among many species especially for the congeneric species, as they tend to share same bands. Therefore, resolution should be increased by using more than one restriction endonuclease. In the case of Malaysian *Chrysomya*, two restriction endonucleases were required to distinguish the six species. In the study, restriction endonuclease, *Ssp*I is a good candidate, which can differentiate three of the Malaysian *Chrysomya* species in *COI* (*C. villeneuvi*, *C. thanomthini* and *C. rufifacies*) at the first digestion. This is because *Ssp*I possess restriction sites of AAT↓ATT which is commonly found in AT-rich region of *COI*. Further digestion with second restriction endonuclease (e.g. *Taq*$^{\alpha}$I and *Msp*I) was required for discriminating another three *Chrysomya* species.

Apart from the PCR-RFLP method almost all published DNA-based identification methods of forensically important fly species use DNA sequencing (Wells & Sperling, 1999 & 2001; Wallman & Donnellan, 2001; Wallman *et al*., 2005; Wells &

Stevens, 2007). The advantages of using DNA sequences as species identification tool are these data providing more information, in which the variations can be classified in the different categories: (i) substitution (e.g. transition and transversion) and (ii) insertion or deletion (indels) (Page & Holmes, 1998). In addition, the nucleotide substitution pattern and the variation rate among sites across the DNA sequences, which always applied in the phylogenetics analyses, are the factors to determine the efficacy of markers in DNA-based identification (Blouin *et al*., 1998).

With the aid of the DNA barcoding system, the use of DNA sequences as a species identification tool should meet several criteria: (i) the gene is orthologous, (ii) the magnitude of intraspecific variation should not be higher than interspecific variation, as it will confound the delineation of species and (iii) the species is reciprocally monophyletic (Hebert *et al*. 2003; Wells *et al*., 2007). In the present study, similar observation of no overlapping values between the intraspecific and interspecific variation was found in the of COI+II sequences, except for *C. defixa, C. pinguis* and *H. tagaliana* which showed larger intraspecific variation than the interspecific variation.

Although the initial barcoding system has been based on partial sequences of the COI gene (Herbert *et al*., 2003), some studies have incorporated the COII gene (Wells & Sperling, 1999; Wells wt al., 2002), or other suitable markers (i.e. 18S rDNA and 28S rDNA for beetles; ITS1 and 2 for Tetranychid mites) to enhance phylogenetic signals of certain organisms (Raupach *et al*., 2010; Li *et al*., 2010). Throughout the years, most of the diagnostic studies on forensically important insect species have used either complete or portions of COI and II genes (Sperling *et al*., 1994; Wells & Sperling, 1999, 2001; Wells *et al*., 2002; Wallman *et al*., 2005; Harvey *et al*., 2008; Park *et al*., 2009a & b; Tan *et al*., 2009 & 2010a). In the case of investigating the evolutionary history of species, mitochondrial phylogenies need to be corroborated with nuclear DNA analysis because phylogenetic signatures of recombination and hybridisation are untraceable

from the maternally inherited mtDNA. For example, large subunit ribosomal rRNA genes (LSU) are frequently employed in the studies of forensically important species to corroborate with mitochondrial DNA results (Stevens & Wall, 2001; Stevens *et al*., 2002; Stevens, 2003). This is because sharing of mitochondrial haplotypes has been known to occur between species, thus causing paraphyletic or polyphyletic groupings.

Such sharing of haplotypes may be due to (i) incomplete lineage sorting (Pollard *et al*., 2006), (ii) ancestral polymorphism (Avise & Wollenberger, 1997), (iii) introgressive hybridisation, (iv) bacterial infection biasing mtDNA variation, e.g. *Wolbachia* (Hurst & Jiggins, 2005), (v) integration of mitochondrial fragments in nuclear DNA (numts) (Sunnucks & Hales, 1996; Zhang & Hewitt, 1996; Bensasson *et al*., 2001; Richly & Leister, 2004; Song *et al*., 2008a), (vi) duplication of the gene within the mitochondrial genome (Campbell & Barker, 1999), (vii) heteroplasmy (Frey & Frey, 2004) [viii] convergence or parallelism (Schluter *et al*., 2004; Alexander *et al*., 2006). These events could notably confound the interpretation of phylogenetic trees.

In the present study, a nuclear gene marker (28S rDNA) was subsequently analysed since the COI+II results showed haplotype sharing between *C. defixa* and *C. pinguis*. It is plausible that introgressive hybridisation, incomplete lineage sorting and ancestral polymorphism may explain the anomaly between the mtDNA and nuDNA phylogenies. It is common to have such incongruence in phylogenetic studies using two different genes (Sato & Vogler, 2001; Sonnenberg *et al*., 2007; Wahlber *et al*., 2009), in which the gene tree may not reveal the species tree (Figure 5.25) (Page & Holmes, 1998). This is because a gene tree is constructed from DNA sequences of selected marker, which may evolved in different rates, and it does not necessarily reveal the actual evolutionary pathway of the species (the species tree). The male flies of *C. defixa* and *C. pinguis* are morphologically distinct whereas the females are more alike. Although *C. defixa* occurs in lowland forests while *C. pinguis* is predominantly found in

highland forests, both of these species had been found to coexist in an altitude range of 700-1000m, presumably a hybrid zone. Hybridisation of *Chrysomya* species has not been observed in the field, but fertile hybrids between *C. megacephala* and *C. pacifica* have been produced successfully in laboratory (personal comm. with Kurahashi). Incomplete lineage sorting and ancestral polymorphism are the alternative explanations of haplotype sharing, which have been reported in other flies, such as *Sapromyza* (Pestano *et al*., 2003) and *Drosophila* (Pollard *et al*., 2006).

In the phylogenetic analyses, a foreign species of *C. vicina* was included to facilitate the phylogenetic relationship. *C. vicina* is a very common and predominant species found on human cadavers in the temperate countries, such as United States and Europe (Amendt *et al*., 2000; Schroeder *et al*., 2003b; Byrd & Castner, 2010; Pohjoismäki *et al*., 2010) but it has never been found in Malaysia. The only *Calliphora* species found in Malaysia is *C. fulviceps* and it was documented to be of forensic importance in Malaysia by Lee (1989 & 1996) and Lee *et al*. (2004). Although the phylogenetic tree designated *C. fulviceps* and *C. vicina* under the genus of *Calliphora*, more species from this genus are required to determine the actual sister taxon of *C. fulviceps* in *Calliphora*.

In Chrysomyinae, the COI+II and 28S rDNA phylogenies suggest that the initial ancestral trait was associated with predacious behaviour, whereas the emergence of hairy (e.g. *C. ruffifacies* and *C. villeneuvi*) and non-hairy (e.g. *C. chani* and *C. nigripes*) maggots is a recently derived trait. Apart from that, the consistent and strong clustering of *C. rufifacies* and *C. villeneuvi* deems them as sister species, both sharing the hairy maggot feature. This result favours the revision of these species from genus *Chrysomya* to *Achoetandrus* as proposed by Fan (1992).

5.6 Conclusion

A current approach of DNA-based identification method for forensically important Malaysian calliphorid flies was designed as a novel system to provide rapid and accurate species identifications by (i) recovering the target DNA from different life stages of the flies; (ii) recovering the target DNA from archive or degraded specimens; (iii) PCR-RFLP assay and (iv) using DNA databases of COI+II and 28S rDNA. If an unknown specimen was included and compared with the COI+II sequence database, it is most likely to identify unequivocally and maybe with the exception of *C. defixa*, *C. pinguis*, *H. ligurriens*, *H. tagaliana*, *L. papuensis*, *L. bismarckensis* and *L. calviceps*. However, the unambiguous identification of *Chrysomya* species can be achieved by using the 28S rDNA sequences. Therefore, the DNA-based identification of calliphorid species using these reference sequence data can be accomplished for specimens collected from a human corpse with widely separated geographic locations in Malaysia.

It is anticipated that DNA-based analysis will accelerate the pace of species identification and discovery, as well as contribute to the development of forensic entomology in this country.

Chapter 6:

DNA-based identification of forensically important Sarcophagidae species in Malaysia

6.0 Abstract

Insect larvae and adults found on human corpses provide important clues for the estimation of the postmortem interval (PMI). Among all necrophagous insects, flesh flies (Diptera: Sarcophagidae) are considered forensically important. DNA variations of 39 Malaysian, four Indonesian, two Japanese and one Taiwanese flesh fly species are analysed using the 2.3kb mitochondrial COI and COII. These two DNA regions were useful for identifying most of the species but could not genetically distinguish *Boettcherisca javanica*. Thirty nine Malaysian species of forensic importance were successfully clustered into 19 distinct clades, which contain single species, a single genus and mixture of genera. The molecular phylogeny is generally congruent with most morphologically-based classification systems and therefore several taxonomic revisions of genera were also suggested. Part of these results, which investigated 17 Malaysian, two Indonesian and one Japanese flesh fly species, had been published in *Forensic Science International* (see Appendix C).

Twelve forensically important sarcophagine species were analysed via PCR-RFLP of COI using *Ssp*I restriction endonuclease. Eight species produced unique patterns; they are *Boettcherisca peregrina, Harpagophalla kempi, Iranihindia martellata, Liopygia ruficornis, Liosarcophaga brevicornis, Parasarcophaga misera, Parasarcophaga taenionota* and *Seniorwhitea princeps*.

6.1 Introduction

Sarcophagidae is one of the families of greatest importance among the dipterans involved in forensic cases (Smith, 1996; Benecke, 1998b; Carvalho & Linhares 2001; Lee *et al*., 2004; Mohd Salleh *et al*., 2007; Bryd & Castner, 2010). They can be attracted to carrion in most conditions, such as exposure to sun and shade, dry and wet surrounding, indoor and outdoor environments (Bryd & Castner, 2010). They can be found on carrion at the early and late stages of decomposition (Carvalho & Linhares 2001; Bryd & Castner, 2010). A number of forensically important sarcophagid species have been studied and reported in forensic cases from many different countries around the world, including tropical and temperate regions (Table 6.1).

In Malaysia, the occurrence of carrion-related arthopods has hitherto been known to include dipteran flies such as Sarcophagidae (Lee & Marzuki, 1993; Omar *et al*., 1994a & b). Reviews of Malaysian forensic studies have shown that calliphorid flies, such as *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart), were observed as the predominant species in human cadavers, especially in the early stages of decomposition (Hamid *et al*., 2003; Lee *et al*., 2004; Mohd Salleh *et al*., 2007). Although sarcophagid flies are also important as forensic indicators, their identification has mostly been carried out only to the genus level (Lee, 1989 & 1996; Lee *et al*., 2004; Mohd Salleh *et al*., 2007). This is because studies and knowledge on Malaysian Sarcophagidae are very limited. To date, only a few studies on general taxonomy and biology of these interesting flesh flies have been carried out. Taxonomic keys available are only applicable for adult male species and they require specialised taxonomic knowledge (Sugiyama & Kano, 1984; Sugiyama *et al*., 1990; Kurahashi, 2002).

To overcome the shortcomings in morphology-based methods, a quick and accurate identification system is desirable in any forensic studies and investigations as

well as ecology. In recent years, there has been an increase in the use of DNA sequence data in the studies of carrion flies as an aid to accurately identify insect species, even in the cases of immature stages (Wallman & Donnellan, 2001; Wells & Sperling, 2001; Wells & Williams, 2007).

Sarcophagid flies are notoriously difficult to identify due to their highly similar morphological appearance, especially the immature stages (Byrd & Castner, 2001; Wells *et al*., 2001; Saigusa *et al*., 2005). These authors suggested and demonstrated that mitochondrial DNA sequences could be successfully employed to distinguish some species of the sarcophagid flies.

Molecular techniques have the advantage of being applicable to any life stages and have the potential to distinguish morphologically similar species or even genera, such as in the case of sarcophagine flesh flies (Zehner *et al*., 2004). DNA-based identification techniques are also easily transferable between laboratories and are not limited to the requirements of specific taxonomical expertise.

A variety of DNA-based techniques had been studied to determine forensically important species, such as PCR restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA fingerpriting (RAPD), inter simple sequence repeat (ISSR), sequence-characterized amplified region (SCAR) and sequence analysis (Benecke, 1998a; Wells *et al*., 2001; Chen & Shih, 2003; Ratcliffe *et al*., 2003; Schroeder *et al*., 2003a; Zehner *et al*., 2004; Thyssen *et al*., 2005; He *et al*., 2007; Meiklejohn *et al*., 2009; Bajpai & Tewari, 2010). These studies demonstrated that the DNA techniques used in different DNA region, including *cytochrome c oxidase* gene subunit I, NADH dehydrogenase 5 and internal transcribed spacer 2, are useful for sarcophagid identification (Table 6.2).

Despite the availability of mitochondrial DNA sequencing techniques, a robust taxonomic classification of these flies is still required. Such methods of species

identification would be truly useful if more comprehensive baseline data could be established. Therefore, this study analysed the mitochondrial *cytochrome c oxidase* gene subunits I and II (COI and COII) sequences of many oriental flesh fly species from Malaysia, Indonesia, Taiwan and Japan that have not been well represented in the previous studies (Wells *et al*., 2001; Zehner *et al*., 2004; Saigusa *et al*., 2005; Meiklejohn *et al*., 2010). The sequence data obtained would be useful as reference standards for future analyses and the practical identification of species in forensics. Phylogenies estimated from these DNA sequences also provide useful information towards the understanding of the taxonomy and systematics of the sarcophagid flies.

6.2 Objectives

In this study, the main objective is to determine the usefulness of mitochondrial DNA regions (COI+II) in DNA-based identification of forensically important species in this country.

Specific objectives include:

1. To establish a PCR-RFLP species identification assay for 12 potential forensically important sarcophagine species in Malaysia.
2. To evaluate the utility of *cytochrome c oxidase* subunit I and II for identifying forensically important Malaysian sarcophagine species, which could be useful as reference standards for future analyses and the practical identification of species in forensics.
3. To re-evaluate the existing taxonomic classification status and systematics of Malaysian sarcophagine flesh flies by phylogenetic analyses.

Table 6.1: Forensically important Sarcophagidae species found on human cadavers reported in forensic cases in the previous work.

Species	Country	Reference
Sarcophaga africa	Hawaii	Goff & Odom 1987
(= *S. haemorrhoidalis*)		Goff, 1991
(= *Bercaea cruentata*)	USA	Introna *et al*., 1998
		Byrd & Castner, 2001
Sarcophaga argyrostoma	Belgium	Leclercq, 1976
(=*Parasarcophaga argyrostoma*)	Moravia	Povolný & Verves, 1997
(=*Liopygia argyrostoma*)	Germany	Benecke, 1998b
	Germany	Amendt *et al*. 2000
	Vienna	Grassberger & Reiter, 2002
Sarcophaga caerulescens	Finland	Pohjoismäki *et al*., 2010
Sarcophaga carnaria	France	Yovanovitch, 1888
	Germany	Schumann, 1990
	USA	Introna *et al*., 1998
Sarcophaga (*Liosarcophaga*) *dux*	Guam	Bohart & Gressitt, 1951
	Japan	Chigusa *et al*. 2006
	Malaysia	IMR, 2008 *
Sarcophaga gressitti	Guam	Bohart & Gressitt, 1951
Sarcophaga (*Boettcherisca*) *peregrina*	Hawaii	Goff & Odom 1987
	Hawaii	Goff, 1991
	Japan	Chigusa *et al*. 2006
Sarcophaga (*Pandelleana*) *protuberans*	Italy	Vanin *et al*., 2008
Sarcophaga (*Parasarcophaga*) *ruficornis*	Thailand	Sukontason *et al*., 2007
	Malaysia	IMR, 2009 *
Sarcophaga (*Pandelleisca*) *similis*	Japan	Chigusa *et al*. 2006
Sarcophaga sp. or spp.	Malaysia	Lee, 1989 & 1996
		Lee *et al*., 2004
		Mohd Salleh, 2007
	Thailand	Sukontason *et al*., 2001
	Germany	Schroeder *et al*., 2003b
	Portugal	Cainé *et al*., 2009

* Forensic cases from Medical Entomology Unit, Infectious Diseases Research Centre, Institute for Medical Research, Malaysia

Table 6.2: DNA region used in DNA-based identification analyses of flesh flies.

Studies	DNA region [a]	Length (bp)	Sarcophagidae species
Wells *et al*., 2001	COI	783	*Sarcophaga africa, S. argyrostoma, S. bullata, S. cooleyi, S. crassipalpis, S. peregrine, S. ruficornis, Blaesoxipha plinthopyga, Brachicoma devia, Peclia chrysostoma, Ravinia iherminieri* and *Wolfahrtia viril*
Ratcliffe *et al*., 2003	ITS1+5.8SrDNA+ITS2	1200-1500	*S. bullata* and *S. crassipalpis*
Zehner *et al*., 2004	COI ND 5	296 386	*Sarcophaga carnaria, S. subvicina, S. variegata, Parasarcophaga albiceps, Bercaea africa, Liopygia argyrostoma, L. crassipalpis, Liosarcophaga teretirostris, L. tibialis, Pandelleana protuberans, Thyrsocnema incisilobata* and *Helicophagella melanura*
Cai *et al*., 2005	COI	278	*Boettcherisca peregrina*
Saigusa *et al*., 2005	COI	304	*Parasarcophaga crassipalpis* and *P. similis*
He *et al*., 2007	Inter simple sequence repeat*		*Parasarcophaga crassipalpis*
Song *et al*., 2008b	ITS2	317-343	*Sarcophaga* (*Liopygia*) *crassipalpis, S.* (*L.*) *ruficornis, S.* (*Parasarcophaga*) *albicpes, S.* (*P.*) *misera, S.taenionota* (*=S. sericea*), *S.* (*Liosarcophaga*) *brevicornis, S.* (*L.*) *dux, S.* (*Bercaea*) *cruentata, S.* (*Boettcherisca*) *formosensis* and *S.* (*B.*) *peregrina*
Meiklejohn *et al*., 2009	COI	658	*Sarcophaga australis, S. bidentata, S. littoralis, S.beta, S. hardyi, S. praedatrix, S. megafilosia, S. meiofilosia, S. impatiens, S. iota, S. kohla, S. dux, S. aurifrons, S. crassipalpis, S. misera* and *Oxysarcidexia varia*
Bajpai & Tewari, 2010	COI ND5	296 386	*S. argyrostoma, S. dux, S. albiceps, S. ruficornis,* and *S.taenionota* (*=S. knabi*)

[a] Abbreviation of DNA region of the studies. COI: *cytochrome c oxidase* subunit I and ITS2: internal transcribed spacer 2.

* Inter simple sequence repeat (ISSR) is a method instead of DNA region. However, stretches of DNA between adjacent microsatellite elements are amplified using this method.

6.3 Materials and methods

6.3.1. Specimens

Sarcophagine flesh flies were caught using chicken liver, chicken or beef as bait. The specimens were killed using ethyl acetate, pinned and kept under dry conditions with silica gel until DNA extraction was performed. The remaining portions of the vouchers were then stored in the Unit of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya for future reference. Adult males were identified based on the morphological characteristics of the male genitalia using the identification keys of Kurahashi (pers. comm.). The taxonomical names and systematics of the flies were assigned according to the traditional classifications of Lopes (1969 & 1989), Lopes *et al*. (1977), Verves (1986), Shewell (1987) and Rohdendorf (1937 & 1965). The morpho-species of female specimens were more difficult to determine due to fewer distinguishing features of the genitalia compared to the male. Nevertheless, some of the female flies were successfully identified, by unambiguous identification of their male offspring reared in the laboratory and included in the present study. A total of 129 fly specimens were analysed, representing 52 species which included 49 species of Sarcophagidae and three species of Calliphoridae as outgroups (Table 6.3). Of the sarcophagid species, nine were non-Malaysia species: *Boettcherisca dumoga* (Indonesia), *Boettcherisca koimani* (Indonesia), *Boettcherisca timorensis* (Indonesia), *Boettcherisca formosensis* (Taiwan), *Lioproctia aureolata* (Indonesia) *Myorhina uniseta* (Japan), *Liopygia crassipalpis* (Japan), *Angiometopa hikosana* (Japan) and *Angiometopa shinonagai* (Japan) were collected by Dr. Hiromu Kurahashi. In this study, subfamilies of Paramacronychiinae and Miltogramminae as well as some other foreign sarcophagine species (e.g. *B. dumoga, B. formosensis, B. timorensis, L. ausreolata, L. crassipalpis*

and *M. uniseta*) were included in order to produce a better resolution and representation of taxa as well as a more reliable topology in phylogenetic analyses.

Table 6.3: List of species, voucher number and locality for specimens used in this study.

Species	Voucher	Collection site (State)
Alisarcophaga gressitti	SWK-288	Sarawak
	S462	Negeri Sembilan
Boettcherisca dumoga [a]	BD	Indonesia
Boettcherisca formosensis [a]	BF	Taiwan
Boettcherisca highlandica	BKGOM	Selangor
	GJASAR1	Pahang
	RL1	Pahang
	UB1	Pahang
Boettcherisca javanica variant A	S26	Kuala Lumpur
	S40	Kuala Lumpur
	S162	Kuala Lumpur
	S171	Kuala Lumpur
Boettcherisca javanica variant B	KWOH3	Perak
	S32	Kuala Lumpur
	S114	Kuala Lumpur
	S125	Pahang
Boettcherisca javanica Borneo	SWK72	Sarawak
	SWK82	Sarawak
	BJ2BKLL	Sarawak
	BJ1BKLL	Sarawak
Boettcherisca karnyi	BKARA	Sarawak
	BKARNYI	Selangor
	S268	Kuala Lumpur
Boettcherisca koimani [a]	BKOIM	Indonesia
	J119	Indonesia
Boettcherisca krathonmai	BN	Sarawak
	SARCOA2	Sarawak
	SARCOD2	Sarawak
	SWK288	Sarawak
Boettcherisca peregrina	LAB2	Kuala Lumpur
	SCH2	Pahang
	SCH9*	Pahang
Boettcherisca timorensis [a]	BTIMOR	Indonesia
Harpagophalla kempi	S102*	Pahang
	S134*	Kuala Lumpur
	S274	Kuala Lumpur
Hosarcophaga serrata	SWK302	Sarawak
Iranihindia martella	S153	Kuala Lumpur
	S155	Kuala Lumpur
	IRAJB	Johor

Table 6.3 *(continued)*

Species	Voucher	Collection site (State)
Leucomya alba	LALBA1	Kedah
	LALBA2	Kedah
	ALBA1	Negeri Sembilan
Lioproctia aureolata [a]	LA	Indonesia
Lioproctia pattoni	LPGOM	Selangor
	S494	Kuala Lumpur
	SARCOB	Sarawak
Lioproctia saprianovae	LS1	Sarawak
	LS2	Sarawak
	S415	Selangor
Liopygia crassipalpis [a]	J69	Japan
Liopygia ruficornis	SY5	Kelantan
	S21*	Kuala Lumpur
	POLLIPR	Selangor
Liosarcophaga brevicornis	S23*	Kuala Lumpur
	SCH14	Pahang
	PBMWC	Sarawak
Liosarcophaga dux	S69	Selangor
	S132	Terengganu
	SY9	Kelantan
	SM8	Johor
Liosarcophaga mimobrevicornis	MIMO1	Perak
	MIMO2	Johor
Myorhina borneensis	MSP5	Sarawak
	MSP7	Sarawak
	MB2	Perak
	MB3	Perak
Myorhina globovesica	MYO	Sarawak
Myorhina uniseta [a]	J49	Japan
Parasarcophaga albiceps	S152	Kuala Lumpur
	S158	Pahang
	PA1	Sarawak
	PAAD	Sarawak
Parasarcophaga javana	S157	Pahang
	PJAVANA	Pahang
Parasarcophaga lopesi	PL	Negeri Sembilan
	PL1	Selangor
	PL2	Selangor
Parasarcophaga misera	S9*	Kuala Lumpur
	S107	Pahang
	S206	Negeri Sembilan
Parasarcophaga omari	PS1	Sarawak
	PS2	Sarawak
Parasarcophaga scopariiformis	S122	Negeri Sembilan

Table 6.3 *(continued)*

Species	Voucher	Collection site (State)
Parasarcophaga taenionota	S103	Pahang
	S127	Kuala Lumpur
	SM4	Johor
Rosellea notabilis	SR61	Selangor
	S676	Selangor
Sarcorohdendorfia antilope	Sarco-J2	Sarawak
	Sarco-J3	Sarawak
	Sarco-J4	Sarawak
Sarcorohdendorfia inextricata	S37	Kuala Lumpur
	S106	Pahang
	S131*	Terengganu
Sarcorohdendorfia seniorwhitei	SS2	Sarawak
	SS4	Sarawak
	S660	Selangor
	SSUM	Kuala Lumpur
Sarcosolomonia crinita	SC1	Sarawak
	SC2	Sarawak
	S105	Pahang
	SCGOM	Selangor
Sarcosolomonia rohdendorfi	SRUM	Kuala Lumpur
Seniorwhitea princeps	S25*	Kuala Lumpur
	S71	Kuala Lumpur
	S130	Terengganu
Sininipponia hainanensis	S-SWK-177	Sarawak
Sininipponia bengalensis	Sino-PD	Negeri Sembilan
	Sino-NP	Selangor
Sarcophaga (s. lat.) *aquila*	SWK125	Sarawak
	PAQUILA2	Johor
	P.aquila-NP	Selangor
Sarcophaga (s. lat.) *brachiata*	S.bra1	Johor
	S.bra2	Johor
Sarcophaga (s. lat.) *longifilia*	SL1-NP	Selangor
	S-MB4	Selangor
	SL-TPiai	Johor
Sarcophaga (s. lat.) *quinqueramosa*	SWK260	Sarawak
Sarcophaga (s. lat.) *robustispinosa*	SROBUS1	Johor
	SROBUS2	Johor
Angiometopa hikosana [a]	J105	Japan
Angiometopa shinonagai [a]	J111	Japan
Miltogramminae	Sarco-Gom	Selangor
Chrysomya megacephala	CM3	Kuala Lumpur
Chrysomya rufifacies	CR5	Kuala Lumpur
Chrysomya nigripes	CN-UM-20-8	Kuala Lumpur

* Female specimen

[a] Foreign species

6.3.2 DNA extraction

For DNA extraction of adults, only legs from one side of the flies were used while the remaining parts of the flies were maintained as voucher specimens. Total genomic DNA was extracted followed the protocols in chapter 5, section 5.2.2.

6.3.3 PCR amplification

Two sets of primers (TY-J-1460 & C1-N-2800 and C1-J-2495 & TK-N-3775) were used in this study (Sperling *et al*., 1994). Primer sequences were shown in Table 5.3. PCR amplification mixtures were prepared same as section 5.2.3.

6.3.3.1 PCR optimisation – gradient temperature PCR

PCR cycle programme for both primer sets was as described in section 5.2.3.1 with gradient temperatures ranged from 45.0ºC to 65.0ºC. Optimal annealing temperature was determined for each set of primer.

6.3.3.2 PCR amplification with 2 sets of primer

After the optimal annealing temperatures have been determined in the PCR optimisation for each primer sets as described in section 6.3.3.1, a similar PCR condition described in the section 5.2.3.1 was used to amplify different species of Sarcophagidae. The only different condition, as described in the section 5.2.3.1, was the annealing temperatures used in each PCR, which are specific to each set of primer used according to the result from PCR optimisation.

6.3.4 Purification of PCR products

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen GmbH, Germany) or QIAquick® Gel Extraction Kit (Qiagen GmbH, Germany) depending on the specificity of amplicons. Similar procedures were carried out, as described in sections 5.2.4.1 and 5.2.4.2.

6.3.5 Cloning and sequencing

Good quality purified PCR products was performed for direct sequencing. Cloning was carried out if necessary. Protocols of sequencing and cloning were done following the method in section 5.2.5.

6.3.6 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

PCR-RFLP analysis was carried out for the fragments amplified with primer set TY-J-1460 & C1-N-2800. The use of restriction endonuclease, *Ssp*I (New England Biolabs, UK; Promega, USA; Vivantis, Malaysia) was analysed in this study. Out of 44 sarcophagine species, 12 species that were potentially important in forensics were examined. These species comprised *B. peregrina*, *H. kempi*, *I. martellata*, *L. saprianovae*, *L. ruficornis*, *L. brevicornis*, *L. dux*, *P. misera*, *P. taenionota*, *S. inextricata*, *S. crinita* and *S. princeps*. Restriction digestion condition of this restriction endonuclease was done according to the manufacturer's recommendation in a final volume of 20µl. Then, the digested samples were fractionated on 2% agarose gel with TBE buffer, stained with ethidium bromide and viewed under UV-illumination. The restriction patterns were photographed and analysed.

6.3.7 Data and phylogenetic analysis

DNA sequence reads were edited manually using the Chromas 2.32 to remove the sequences of primers and to resolve discrepancies between contig sequences. Sequences were aligned using ClustalW (Thompson *et al*., 1994). Sequences of COI+II were analysed to increase the phylogenetic signal. Analysis of DNA sequence variation and nucleotide composition was performed using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura *et al*., 2007). Variation across site was analysed using DnaSP 4.0 (Rozas *et al*., 2003). Variance of Pi value was calculated and presented in a graph with window length of 100 and step size of 25. In this analysis, gaps were excluded.

Prior to phylogenetic analysis, sequence alignment was tested for the best-fit evolutionary model using Modeltest 3.8 (Posada & Crandall, 1998). Pairwise distance of COI+II was measured in PAUP* 4.0b10 (Swofford, 1998) by using the model of nucleotide substitution determined in the Modeltest 3.8 (Posada & Crandall, 1998). Minimum pairwise sequence divergence between species and maximum pairwise sequence divergence within species were determined and presented in the tables.

Neighbour-joining (NJ) and maximum parsimony (MP) phylogenetic trees were constructed using PAUP* 4.0b10 (Swofford, 1998). For both analyses, 10,000 replicates were bootstrapped. Full heuristic search was performed with 10 random sequence additions and tree bisection–reconnection (TBR) branch swapping. A Bayesian inference analysis was performed using MrBayes 3.1.2 (Huelsenbeck, & Ronquist, 2001). Four Markov chains (three heated chains and one cold chain) were run for three million generations and the trees were sampled every 100^{th} generation. All trees were rooted with the Calliphoridae species, whereas only branches with over 70% bootstraps were considered for phylogenetic inference (Hillis & Bull, 1993).

6.4 Results

6.4.1 Samples

In the present study, 126 DNA sequences of COI+II genes of Sarcophagidae were generated successfully and selected ones were deposited into GenBank (Tables 6.4). Among the 46 species of sarcophagines, seven were species from Indonesia, Taiwan and Japan.

Table 6.4: DNA sequences of COI+II genes deposited in Genbank for specimens of flies used in the present study.

Species	Voucher	GenBank Accession Number
Alisarcophaga gressitti	S462	FJ479727
	S-SWK-286	FJ479728
Boettcherisca dumoga [a]	BD	EF405950
Boettcherisca formosensis [a]	BF-Taiwan	JF500461
Boettcherisca highlandica	UB1	JF500467
	GJasar1	JF500468
Boettcherisca javanica variant A	S26	EF405925
	S40	EF405926
Boettcherisca javanica variant B	S32	EF405922
	S125	EF405923
Boettcherisca karnyi	Bkar-A-SWK	JF500464
	Bkarnyi-PKetam	JF500465
Boettcherisca koimani [a]	Bkoim-Indo	JF500462
	J119-Bkoimani-Indo	JF500463
Boettcherisca krathonmai	BN	EF405924
	S-SWK-288	GU174023
Boettcherisca peregrina	S-CH2	EF405927
	S-CH9	EF405928
Boettcherisca timorensis [a]	Btimor-Indo	JF500466
Harpagophalla kempi	S102	EF405946
	S134	EF405947
	S274	GU174025
Hosarcophaga serrata	S-SWK-302	FJ479734
Iranihindia martella	S153	FJ440843
	S155	FJ440844
Leucomya alba	Lalba2	JF500471
	Alba2-LGW	JF500472
Lioproctia aureolata [a]	LA	EF405951
Lioproctia pattoni	LP-Gom	FJ479723
	S494	FJ479724
Lioproctia saprianovae	LS	EF405944
	LS2	EF405945

Table 6.4 *(continued)*

Species	Voucher	GenBank Accession Number
Liopygia crassipalpis [a]	J69	GU174024
Liopygia ruficornis	S21	EF405940
	SY5	EF405941
Liosarcophaga brevicornis	S-CH14	EF405935
	S23	EF405936
Liosarcophaga dux	S69	EF405937
	S132	EF405938
	SY9	EF405939
Liosarcophaga mimobrevicornis	Mimo1-Perak	JF500469
	Mimo2-Johor	JF500470
Myorhina borneensis	MB3M19-Prk	JF500473
	Msp7-SWK	JF500474
Myorhina globovesica	Myo-Mglobovesica-SWK	JF500475
Myorhina uniseta [a]	J49-Muniseta-Jpn	JF500476
Parasarcophaga albiceps	PA1	EF405931
	S152	EF405932
Parasarcophaga javana	P.javana-CH	FJ479732
	S157	FJ479733
Parasarcophaga lopesi	PL2-KS	JF500477
	PL-PD	JF500478
Parasarcophaga misera	S107	EF405929
	S9	EF405930
Parasarcophaga omari	PS1	FJ479742
Parasarcophaga scopariiformis	S122	FJ479745
Parasarcophaga taenionota	S103	EF405933
	S127	EF405934
Rosellea notabilis	S767	FJ479725
	C1-BK-M14	FJ479726
Sarcorohdendorfia inextricata	S37	EF405942
	S131	EF405943
Sarcorohdendorfia seniorwhitei	SS2Quarry2-SWK	JF500478
	SS4-SWK	JF500480
Sarcosolomonia crinita	SC2-SWK	JF500481
	SCGom	JF500482
Sarcosolomonia rohdendorfi	SRUM26/6	JF500483
Seniorwhitea princeps	S25	EF405948
	S71	EF405949
Sininipponia hainanensis	S-SWK-177	JF500484
Sininipponia bengalensis	Sino-PD	JF500485
	Sinno-NP	JF500486

Table 6.4 *(continued)*

Species	Voucher	GenBank Accession Number
Sarcophaga (s. lat.) *aquila*	S-SWK-125	FJ479735
	P.aquila-PT	FJ479736
	P.aquila-NP	FJ479737
Sarcophaga (s. lat.) *brachiata*	S.bra1-PT	FJ479740
	S.bra2-PT	FJ479741
Sarcophaga (s. lat.) *longifilia*	SL1-NP	FJ479729
	S-MB4	FJ479730
	SL-TPiai	FJ479731
Sarcophaga (s. lat.) *quinqueramosa*	S-SWK-260	FJ479743
Sarcophaga (s. lat.) *robustispinosa*	S.robus1-PT	FJ479738
	S.robus2-PT	FJ479739

[a] foreign species

6.4.2 PCR amplification

6.4.2.1 PCR optimisation

PCR amplification of COI+II genes was optimised using two sets of primers, TY-J1460 & C1-N-2800, which amplified a 1380bp fragment of partial region of COI and C1-J2495 & TK-N-3775, which amplified a 1324bp fragment of partial region of COI, complete regions of tRNA leucine, as well as COII – COI+tRNA-leu+COII (Figures 6.1 & 6.2). Both primer sets produced PCR bands at several temperatures, an optimal annealing temperature was selected based on the bands' intensity and specificity for each sets of primer. Based on the gel electrophoresis, the optimal annealing temperatures were determined as 46ºC and 58ºC for primer sets TY-J1460 & C1-N-2800 C1-J2495 & TK-N-3775, respectively.

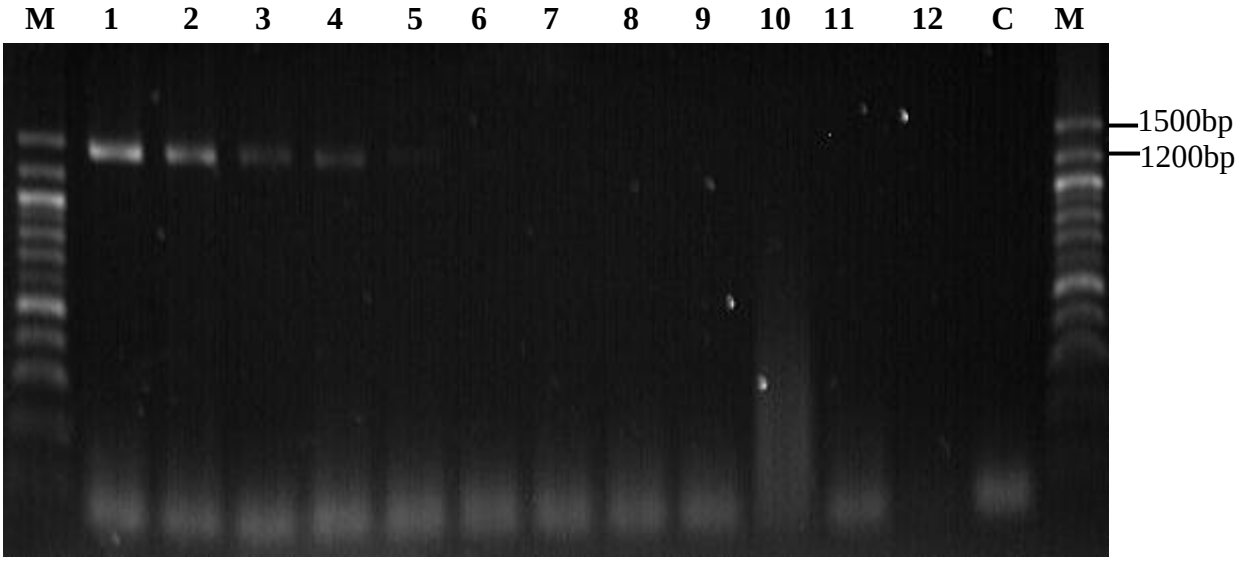


Figure 6.1: PCR optimisation using gradient temperatures from 45ºC to 65ºC (Lanes: 1, 45.0ºC; 2, 45.3ºC; 3, 46.4ºC; 4, 48.2ºC; 5, 50.4ºC; 6, 53.0ºC; 7, 55.8ºC; 8, 58.4ºC; 9, 61.0ºC; 10, 63.0ºC; 11, 64.7ºC; 12, 65.6ºC) for primers TY-J-1460 & C1-N-2800 for COI. Lane M is loaded with 100bp molecular weight marker (Seegene, Korea) and lane C is loaded with a negative control. Optimal annealing temperature was 45.0ºC, which was lane 1.

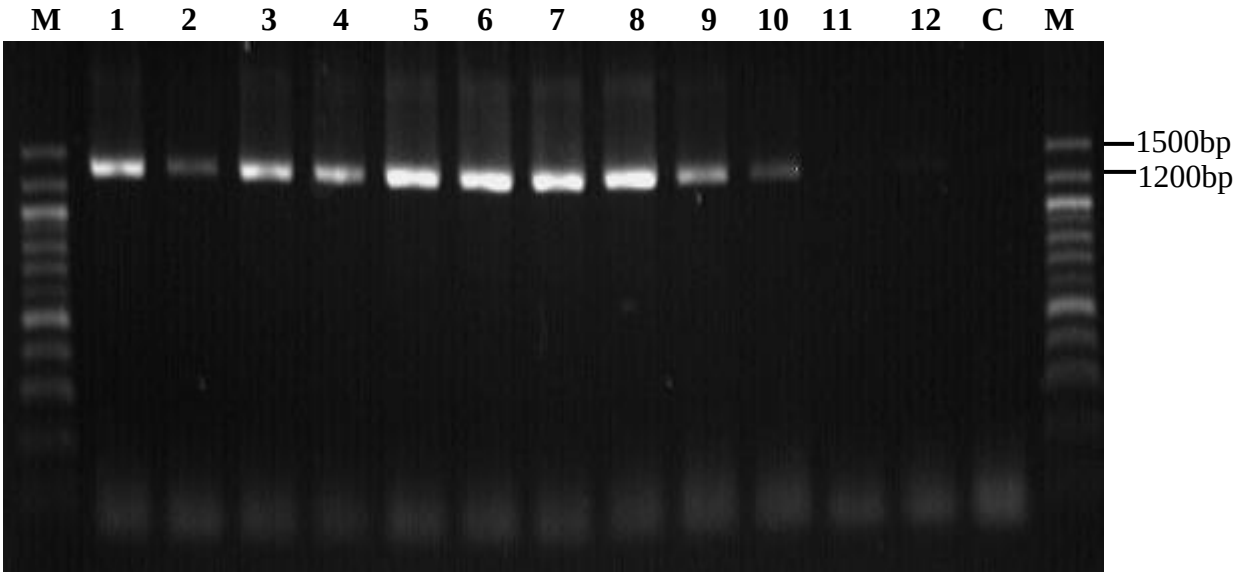


Figure 6.2: PCR optimisation using gradient temperatures from 45ºC to 65ºC (Lanes: 1, 45.0ºC; 2, 45.3ºC; 3, 46.4ºC; 4, 48.2ºC; 5, 50.4ºC; 6, 53.0ºC; 7, 55.8ºC; 8, 58.4ºC; 9, 61.0ºC; 10, 63.0ºC; 11, 64.7ºC; 12, 65.6ºC) for primers C1-J-2495 & TK-N-3775 for COI+tRNA-leu+COII. Lanes C is negative control and M is 100bp molecular weight marker (Seegene, Korea). Optimal annealing temperature was 58ºC, which was lane 8.

6.4.2.2 PCR amplification with 2 sets of primer

Different species of Sarcophagidae were amplified using primers TY-J-1460 & C1-N-2800 at 45ºC and C1-J2495 & TK-N-3775 at 58ºC, as the optimal annealing temperatures for COI and COI+tRNA-leu+COII, respectively. The sizes of the PCR products obtained from these two sets of primers were 1380bp (TY-J-1460 & C1-N-2800) and 1354bp (C1-J2495 & TK-N-3775). The PCR products yielded satisfactory results, which are shown in Figures 6.3 and 6.4. However, PCR amplification yield depends on DNA template quality and quantity. In Figure 6.4, PCR products gave goods yield in lanes 1 to 4 while the PCR amplifications were not so successful in lanes 5 and 6.

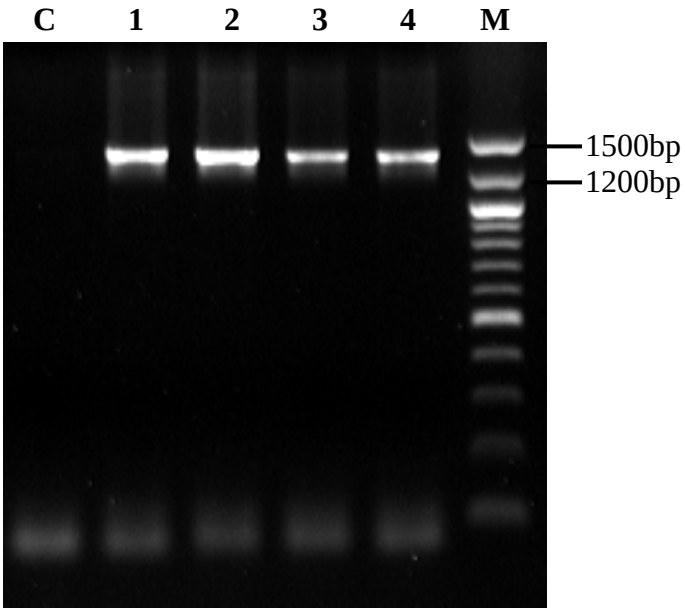


Figure 6.3: PCR amplification of COI using primers TY-J-1460 and C1-N-2800 at 45°C with the expected 1380bp products from different species of Sarcophagidae (lanes 1-4). Lane M contains the molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea) and lane C is the negative control.

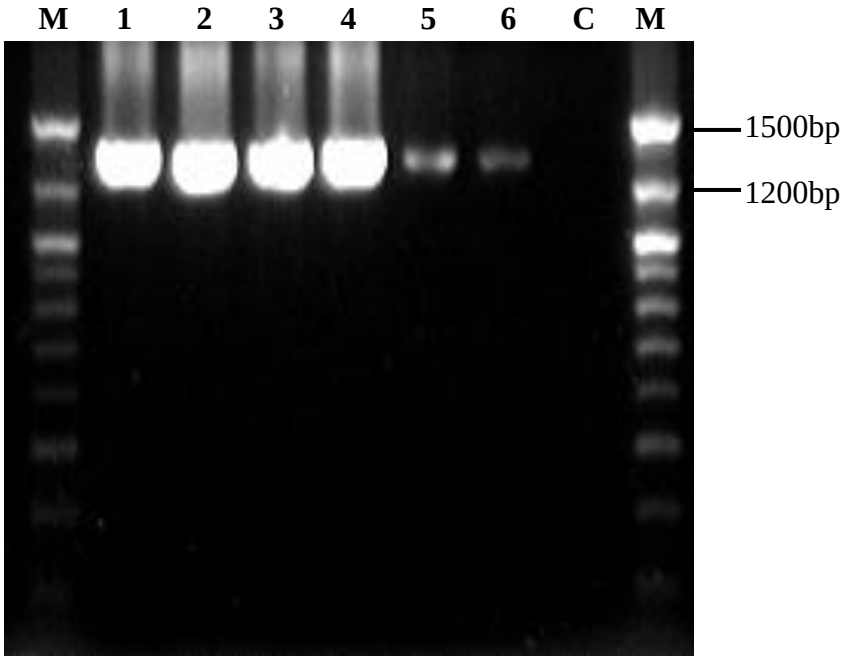


Figure 6.4: PCR amplification of COI-tRNA-COII using primers C1-J-2495 and TK-N-3775 at 58°C with the expected 1324bp products from different species of Sarcophagidae (lanes 1-6). Lanes M contain the molecular weight marker of Forever 100bp personalised DNA Ladder (Seegene, Korea) and lane C is the negative control.

6.4.3 Purification of PCR products

Before sequencing or cloning, PCR products were purified to remove any traces of contaminant and finally eluted the product in higher concentration. This allows for good sequencing result and higher chances for ligation of inserts in the cloning process. Purification method of PCR products is determined depending on the specificity of the amplicon. The QIAquick® PCR Purification Kit was used when the amplicon yielded a specific band (Figure 6.5). On the other hand, if the PCR generated unspecific amplification fragments or a trace of a smear were observed in the agarose gel, the QIAquick® Gel Extraction Kit was used to ensure that only the desired amplicon was purified (Figure 6.6).

6.4.3.1 QIAquick® PCR purification

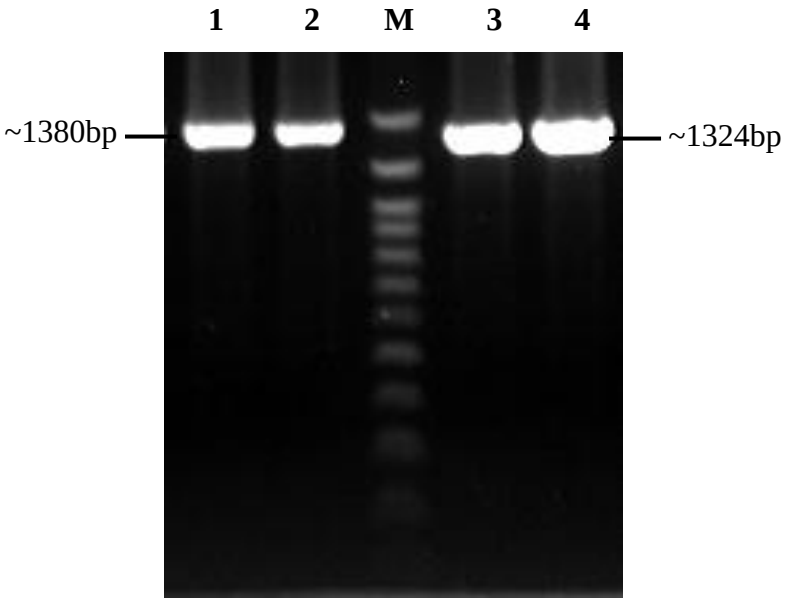


Figure 6.5: Purified PCR products using primer sets TY-J-1460 & C1-N-2800 (lanes 1-2, ~1380bp) and C1-J-2495 & TK-N-3775 (lanes 3-4, ~1324bp) of different Sarcophagidae species. Lane M contains molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea).

6.4.3.2 QIAquick® gel extraction

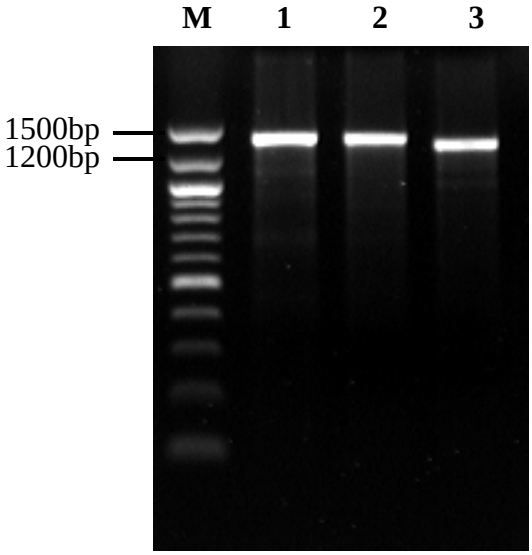


Figure 6.6: Purified PCR products from excised gel using primer sets TY-J-1460 & C1-N-2800 (lanes 1-2, ~1380bp) and C1-J-2495 & TK-N-3775 (lane 3, ~1324bp) of different Sarcophagidae species. Lane M contains molecular weight marker of Forever 100bp Personalized DNA Ladder (Seegene, Korea).

6.4.4 PCR-RFLP

6.4.4.1 PCR-RFLP assay

Restriction profiles of 12 sarcophagine species that are potentially important in forensics were obtained from the digestion of COI fragment with *Ssp*I restriction endonuclease (Figures 6.7 - 6.9). A summary of the *Ssp*I restriction profile is presented in Table 6.5.

Among 12 species RFLP profiles, eight were with unique patterns with a variable number of cleave sites, from a minimum of one cut (*S. princeps*) to a maximum of five cuts (*B. peregrina*). These unique restriction profiles are efficient in offering an unambiguous differentiation of these species. *Harpagophalla kempi*, *I. martellata* and *L. ruficornis* possess two restriction cleave sites while *L. brevicornis* and *P. misera* possess three restriction cleave sites. The only species with four restriction cleave sites is *P. taenionota*, which produced a PCR-RFLP assay of 522bp, 433bp, 218bp, 188bp and 19bp.

The other four species, of which RFLP profiles produced same restriction pattern, were *L. dux*, *L. saprianovae*, *S. crinita* and *S. inextricata* (Figure 6.9). The PCR-RFLP profile obtained four restriction fragments with the sizes of 584bp, 432bp, 237bp and 127bp.

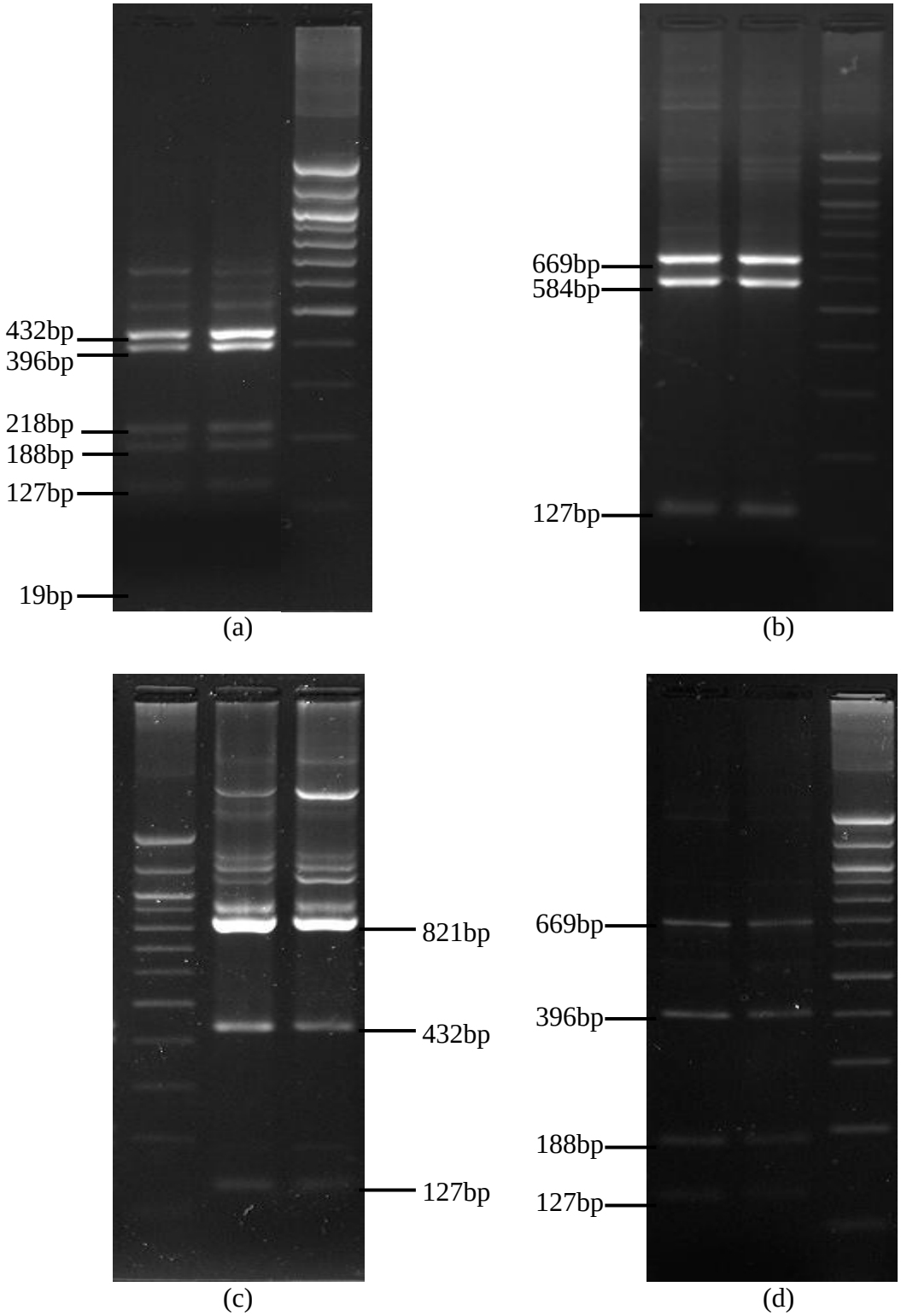


Figure 6.7: Different RFLP profiles of COI digested with restriction endonuclease, *Ssp*I for (a) *B. peregrina*, (b) *H. kempi*, (c) *I. martellata* and (d) *L. brevicornis*.

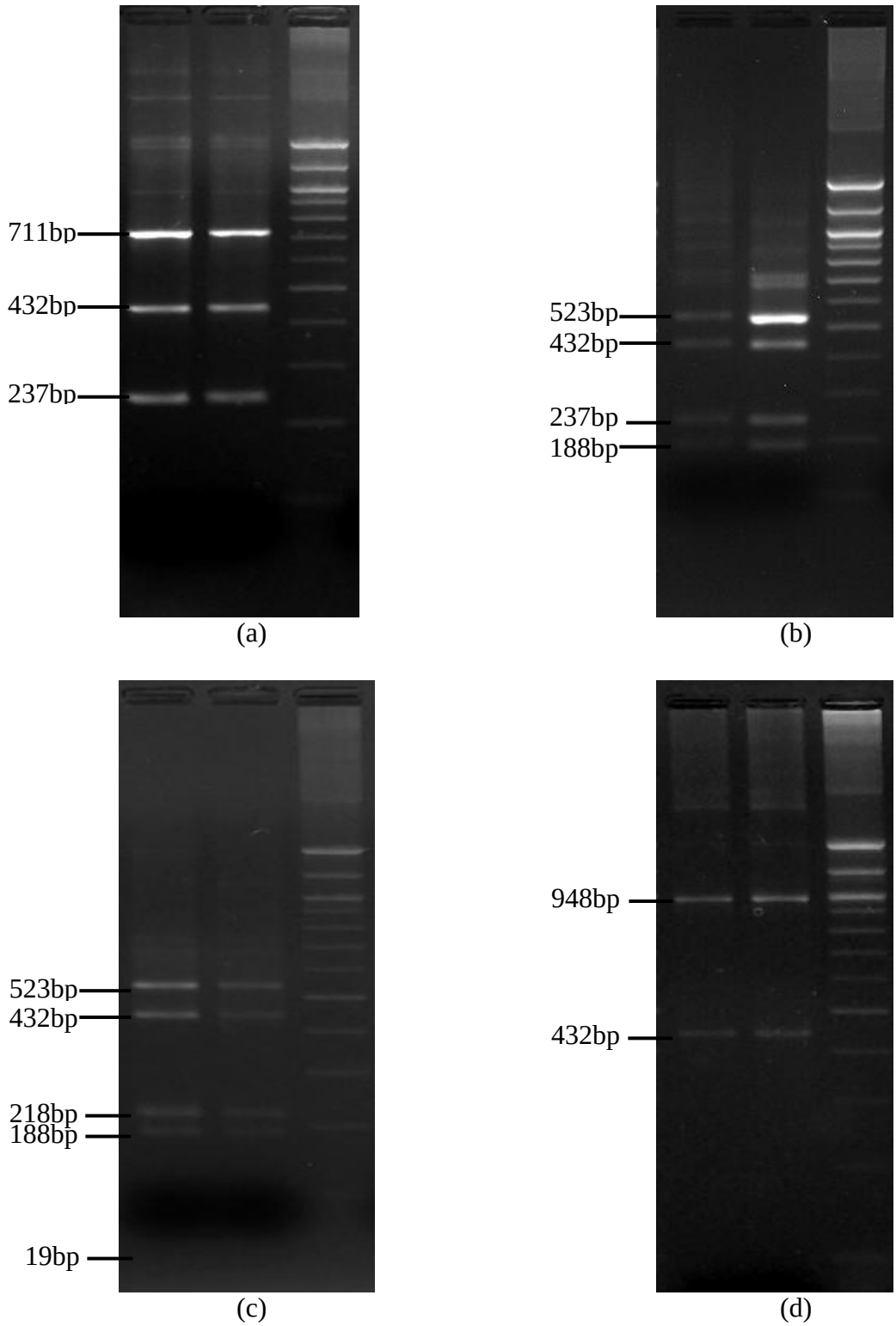


Figure 6.8: Different RFLP profiles of COI digested with restriction endonuclease, *Ssp*I for (a) *L. ruficornis*, (b) *P. misera*, (c) *P. taenionota* and (d) *S. princeps*.

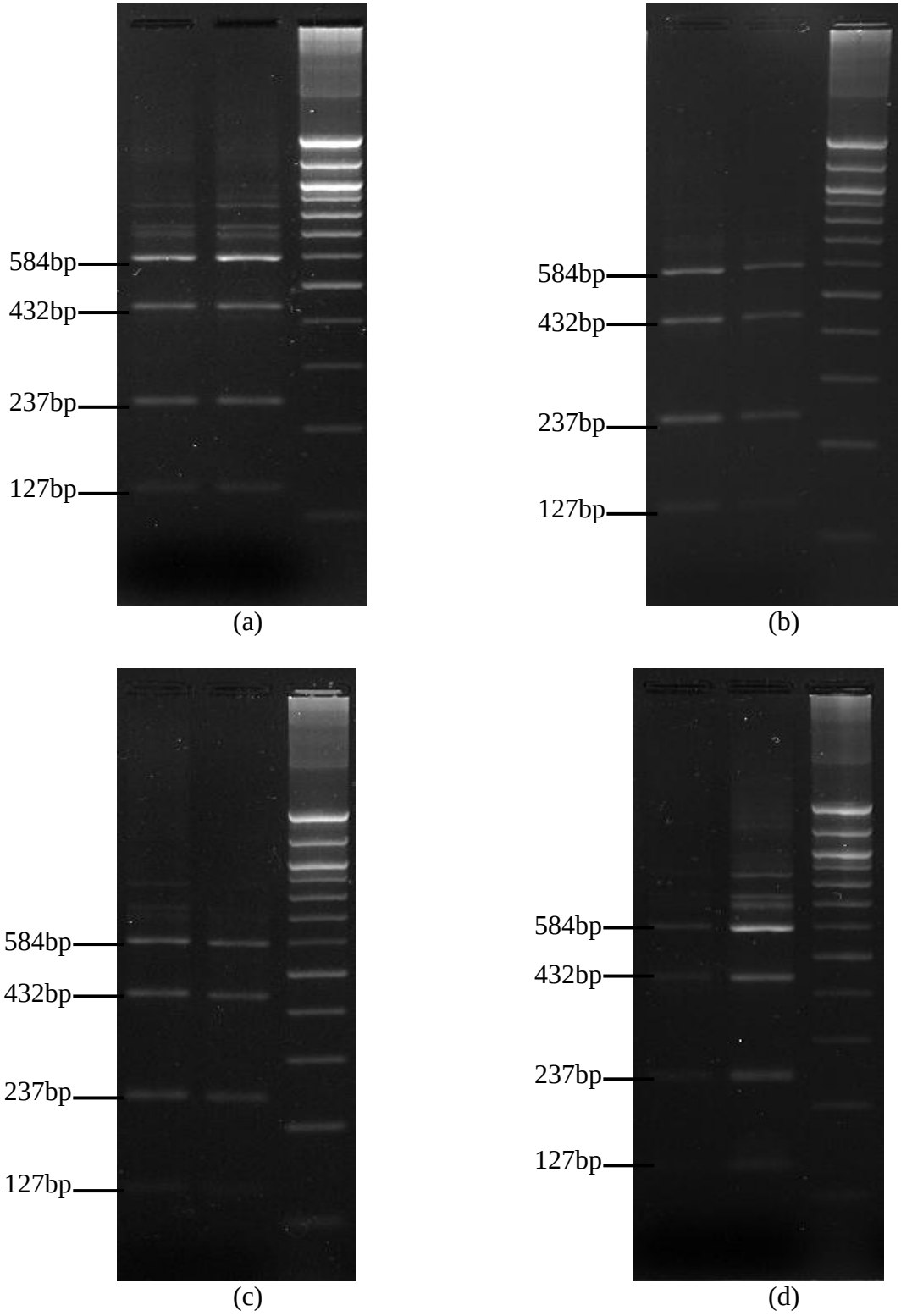


Figure 6.9: Identical RFLP profiles of COI digested with restriction endonuclease, *Ssp*I for (a) *L. dux*, (b) *L. saprianovae*, (c) *S. crinita* and (d) *S. inextricata*.

Table 6.5: Characterisation of the restriction sites of potential forensically important sarcophagine species.

Species *	**BP**	**HK**	**IM**	**LB**	**LR**	**PM**	**PT**	**SP**	**LD, LS, SC, SI**
Position of cleavage site	432 650 669 857 1253	669 1253	432 1253	669 857 1253	432 669	432 669 857	432 650 669 857	432	432 669 1253
Number of cleavage	5	2	2	3	2	3	4	1	3
Number of fragment produced	6	3	3	4	3	4	5	2	4
Length of fragment produced (bp)	432 396 218 188 127 19	669 584 127	821 432 127	669 396 188 127	711 432 237	523 432 237 188	523 432 218 188 19	948 432	584 432 237 127

* Species abbreviation.
BP: *B. peregrina*, HK: *H. kempi*, IM: *I. martellata*, LB: *L. brevicornis*, LR: *L. ruficornis*, PM: *P. misera*, PT: *P. taenionota*, SP: *S. princeps*, LD: *L. dux*, LS: *L. saprianovae*, SC: *S. crinita* and SI: *S. inextricata*.

6.4.5 DNA sequence analyses

6.4.5.1 Sequence diversity

In this study, a total of 129 DNA sequences were obtained either by direct sequencing or sequencing of clones. Among these sequences, three calliphorid sequences were designated as an outgroup. The 126 sarcophagid sequences represent three subfamilies, 17 genera and 48 species.

The total length of the amplified DNA sequences obtained ranged from 2302bp-2308bp (Table 6.6). The average base compositions of sarcophagid species were T=37.9%, C=16.1%, A=31.9% and G=14.1% with a strong AT-bias (69.8%), which is typical of insect mitochondrial DNA (Clary & Wolstenholme, 1985; Crozier & Crozier, 1993; Sperling & Hickey, 1994; Wallman & Donnellan, 2001; Stevens *et al*., 2008) (Table 6.6). There was no base composition heterogeneity between sequences among species (p=1.00).

The final sequence alignment obtained was 2308bp, which encompassed the complete sequences of COI, tRNA-leucine and COII genes (COI+II). This alignment revealed the inclusion of a maximum 6-bp gap within the spacer region between the tRNA-leucine and *COII* gene. In the alignment, 1029 of the nucleotides were variable, whereby 795 sites were considered parsimony informative (Table 6.7).

Table 6.6: DNA sequence length polymorphism of mitochondrial DNA of COI+II and its nucleotide composition in different species of Sarcophagidae.

Species	Sequence length (bp)	Nucleotide composition (%)			
		T	C	A	G
Alisarcophaga gressitti	2304	37.8	15.4	32.7	14.1
Boettcherisca dumoga	2304	37.2	16.9	31.5	14.4
Boettcherisca formosensis	2303	37.7	16.4	31.4	14.5
Boettcherisca highlandica	2303	37.8	16.3	31.7	14.2
Boettcherisca javanica variant A	2303	37.0	17.1	31.4	14.5
Boettcherisca javanica variant B	2303	37.5	16.7	31.4	14.4
Boettcherisca javanica variant C	2303	37.7	16.7	31.4	14.2
Boettcherisca karnyi	2303	37.8	16.4	31.5	14.3
Boettcherisca koimani	2303	37.2	16.9	31.4	14.5
Boettcherisca krathonmai	2303	37.7	16.6	31.5	14.2
Boettcherisca peregrina	2303	37.7	16.5	31.5	14.3
Boettcherisca timorensis	2303	37.3	17.0	31.6	14.1
Harpagophalla kempi	2305	37.5	16.0	32.2	14.3
Hosarcophaga serrata	2304	38.8	15.3	31.9	14.0
Iranihindia martella	2305	37.0	17.0	32.1	13.9
Leucomya alba	2308	37.2	16.7	32.3	13.8
Lioproctia aureolata	2303	37.5	16.4	32.0	14.1
Lioproctia pattoni	2303	37.3	16.5	32.0	14.2
Lioproctia saprianovae	2303	37.3	16.6	32.0	14.1
Liopygia crassipalpis	2305	37.4	16.1	32.3	14.2
Liopygia ruficornis	2305	37.3	16.2	32.5	14.0
Liosarcophaga brevicornis	2304	38.8	15.0	32.4	13.8
Liosarcophaga dux	2305	38.8	14.9	32.4	13.9
Liosarcophaga mimobrevicornis	2304	39.1	14.7	32.4	13.8
Myorhina borneensis	2305	36.8	16.6	32.7	13.9
Myorhina globovesica	2304	37.8	15.7	32.1	14.4
Myorhina uniseta	2304	37.8	15.6	32.8	13.8
Parasarcophaga albiceps	2304	38.3	15.2	32.2	14.3
Parasarcophaga javana	2304	37.9	15.7	32.4	14.0
Parasarcophaga lopesi	2304	38.1	16.1	31.9	13.9
Parasarcophaga misera	2304	38.4	15.8	31.9	13.9
Parasarcophaga omari	2304	38.0	16.3	31.5	14.2
Parasarcophaga scopariiformis	2304	38.2	16.1	31.8	13.9
Parasarcophaga taenionota	2304	38.8	15.3	31.9	14.0
Rosellea notabilis	2304	38.1	16.1	31.7	14.1
Sarcorohdendorfia inextricata	2304	38.3	15.8	32.0	13.9
Sarcorohdendorfia seniorwhitei	2304	39.1	14.9	32.1	13.9
Sarcosolomonia crinita	2303	38.3	15.7	31.6	14.4
Sarcosolomonia rohdendorfi	2305	37.1	16.8	31.8	14.3
Seniorwhitea princeps	2305	38.0	15.7	32.2	14.1

Table 6.6 (*continued*)

Species	Sequence length (bp)	Nucleotide composition (%)			
		T	C	A	G
Sininipponia spp.	2304	38.0	16.1	31.7	14.2
Sarcophaga (s. lat.) *aquila*	2304	38.2	16.1	31.5	14.2
Sarcophaga (s. lat.) *brachiata*	2304	37.6	16.7	31.7	14.0
Sarcophaga (s. lat.) *longifilia*	2304	36.8	16.7	32.3	14.2
Sarcophaga (s. lat.) *quinqueramosa*	2304	37.8	16.5	31.7	14.0
Sarcophaga (s. lat.) *robustispinosa*	2304	38.5	15.3	32.4	13.8
Protomiltogramma sp. [a]	2303	41.1	12.8	33.2	12.9
Angiometopa hikosana [b]	2302	37.1	18.4	30.3	14.3
Angiometopa shinonagai [b]	2302	37.1	18.2	30.2	14.5
Average		37.9	16.1	31.9	14.1

[a] Miltogrammatinae
[b] Paramacronychiinae

Table 6.7: DNA variation of DNA region of mitochondrial COI+II.

DNA variation	Nucleotide (bp)	Percentage (%)
Conserved	1279	55.4
Variable	1029	44.6
Parsimony infomative	795	34.5
Singleton	234	10.1
Total alignment	2308	100

6.4.5.2 Distribution of variation

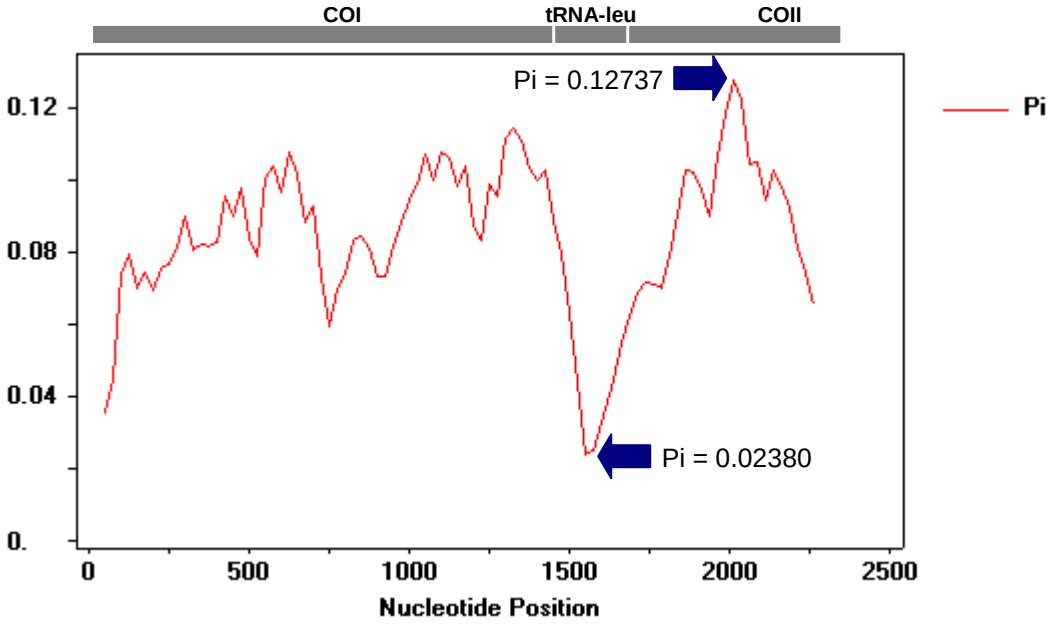


Figure 6.10: Distribution of nucleotide variation of mitochondrial DNA COI+II of 2308bp sequences based on a 100bp sliding window plot with 25bp steps. Arrows indicate the highest and lowest Pi values.

Nucleotide diversity (Pi) – the average number of nucleotide differences per site was calculated using DnaSP 4.0 to study the distribution of variation across site (Nei, 1987; Nei and Miller 1990). The highly conserved tRNA-leucine region showed very low levels of variation. In contrast, the highest level of variation was found at COII with the Pi value of 0.12737 (Figure 6.10). Most of the variations found in COI+II were at third codon positions.

6.4.5.3 Estimation of best fit model

Fifty six models were compared for the best fit model of COI+II DNA sequences using the Akaike Information Criterion (AIC). The best likelihood score chose the general time reversible (GTR) model with invariable sites and rate heterogeneity. Base frequencies were unequal; A = 0.3295, C = 0.1344, G = 0.1141, T = 0.4220; and the estimated proportion of invariable sites (I) was 0.4682. The substitution model incorporated the following rate matrix: [A–C] = 3.0424, [A–G] = 27.3508, [A–T] = 7.1908, [C–G] = 0.8689, [C–T] = 66.0503, [G–T] = 1.0000. The shape parameter of the gamma distribution was 0.7704. This model was used in subsequent NJ and Bayesian analyses.

6.4.5.4 Accumulation of nucleotide substitutions

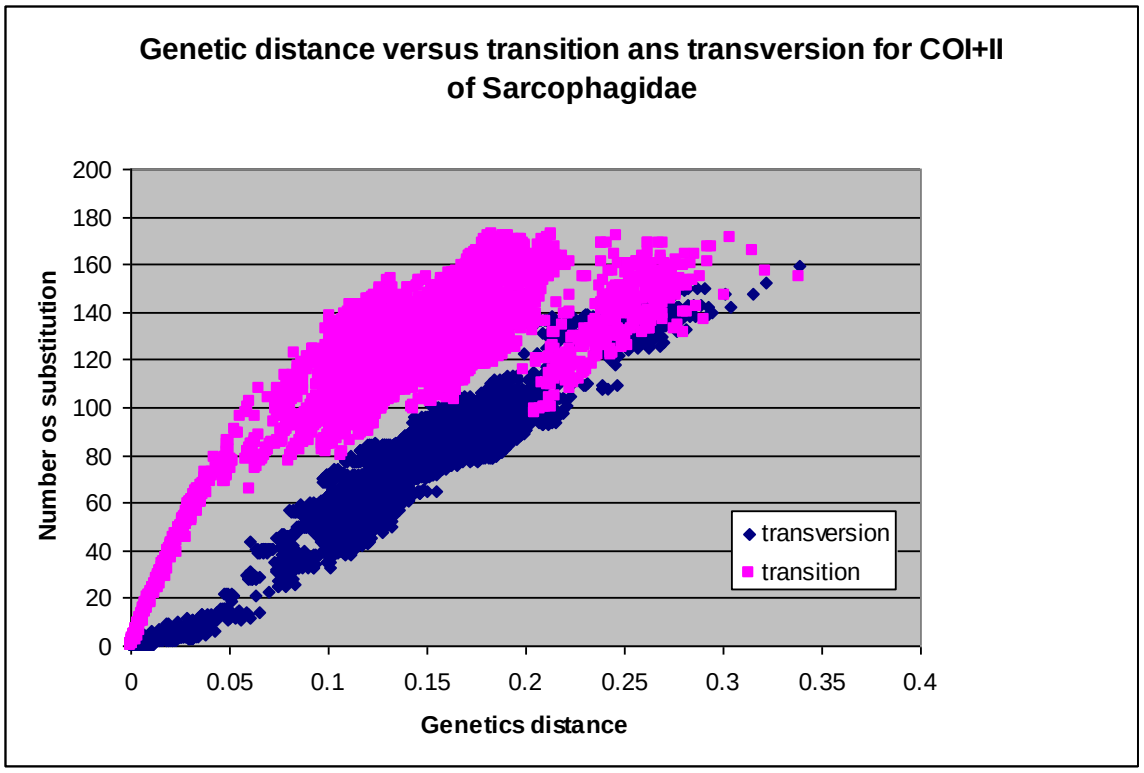


Figure 6.11: Genetic distance versus transition and transversion of mitochondrial COI+II sequences of 129 taxa. Genetic distance was calculated based on GTR+I+G model in PAUP* 4.0b10 (Swofford, 1998).

Substitution rates of transition were different from transversion depending on the magnitude of genetic distances (Figure 6.11). From the graph, the different genetic distances represent pairwise comparisons of different taxonomic levels: distances below 0.1 are due to comparisons within the same genera. Distances ranging from 0.1 to 0.2 were the distances between the genera and the distance values above 0.2 were comparisons with the outgroup species. At lower divergences, most of substitutions were transitions rather than transversions. The transition rate was higher than the transversion rate with the ratio of transition over transversion of 1.6 which is similar to the Calliphoridae studied in chapter 5.

6.4.5.5 Pairwise sequence divergence

In order to successfully delineate and identify a species using the genetic barcoding system, the intraspecific variation should be less than the interspecific variation, which is known as the barcoding gap concept (Herbert *et al*., 2004; Meyer & Pauley, 2005). When the intraspecific variation is bigger than or overlaps with the interspecific variation, it would be difficult to assign whether such genetic differences were accounted by the same or different species.

In this study, the maximum intraspecific variation and the minimum interspecific variation of COI+II were calculated to determine the barcoding gap (Table 6.8). Among 46 sarcophagine species, 45 species conformed to the barcoding gap criterion, whereby their genetic distances within species (intraspecific variation) are lower than those between species (interspecific variation). In general, the intraspecific variations are less than 1%, with the maximum observed in *Alisarcophaga gressitti* (2.84%) and the minimum found in *Parasarcophaga javana* (0.04%). Interspecific variation ranged mostly from10 to 20%, with the highest and lowest divergence values observed in comparisons of *M. uniseta-M. borneensis* (23.84%) and *B. peregrina-B. karnyi* (0.51%), respectively.

Boettcherisca javanica variant B showed overlapping value of the intraspecific and interspecific variations. The intraspecific variation for this species was typically small (1.39%) but showed exceptionally small interspecific variation when compared with *B. highlandica* (0.84%). Therefore, it is believed that these two species have recently diverged. In fact, most pairwise sequence divergences among *Boettcherisca* species showed small magnitudes of barcoding gaps, which suggest their recent divergence.

Table 6.8: Matrix of minimum pairwise sequence divergence between species and maximum pairwise sequence divergence within species (bold) of COI+II. The sequence divergence was calculated based on GTR+I+G model in PAUP* 4.0b10 (Swofford, 1998).

*	BJb	BH	BK	BF	Bko	BJa	BP	Bka	BJc	BT	BD	RN
BJb	**1.39**											
BH	0.84	**1.01**										
BK	1.84	1.63	**1.25**									
BF	2.04	1.79	1.78	**-**								
Bko	3.16	2.91	2.87	3.10	**0.21**							
BJa	2.77	2.48	2.71	2.71	3.02	**0.34**						
BP	2.73	2.44	2.77	2.47	3.02	1.72	**0.30**					
Bka	2.78	2.39	2.61	2.41	2.97	1.76	0.51	**0.51**				
BJc	2.92	2.67	2.64	2.91	3.52	3.23	3.32	3.14	**0.97**			
BT	4.31	4.07	4.32	4.64	5.36	4.63	4.58	4.76	4.56	**-**		
BD	7.55	7.41	7.58	8.17	9.06	8.24	8.26	8.29	7.61	8.31	**-**	
RN	8.01	7.38	7.25	7.80	9.20	8.76	8.71	8.74	8.11	8.45	9.89	**0.52**
PM	9.35	9.34	9.36	9.79	8.99	10.93	10.36	10.52	9.89	11.11	11.65	11.52
PT	9.80	9.93	9.98	10.36	11.17	10.86	10.03	9.91	10.01	11.10	10.70	10.52
PA	10.28	10.62	10.17	10.50	11.39	11.24	10.28	10.11	10.67	11.52	11.81	11.76
PL	11.12	10.04	10.72	11.08	12.36	11.62	10.78	10.92	10.79	11.35	11.70	11.53
HS	12.77	12.09	11.67	12.29	12.80	13.22	12.37	12.28	12.11	12.50	13.24	11.02
SiB	10.64	9.80	10.33	10.32	10.86	10.43	10.49	10.35	9.74	10.52	11.76	11.06
SiH	11.57	10.60	10.86	11.34	11.92	11.34	11.24	11.23	10.45	11.09	12.69	11.52
Saq	10.22	9.65	9.61	10.13	10.90	10.74	10.24	10.32	9.54	10.11	12.29	10.17
Sro	11.65	10.94	11.17	11.95	11.67	12.29	11.50	11.42	11.47	11.86	12.30	11.34
SB	11.20	10.76	10.30	11.30	11.65	11.67	11.05	11.10	10.87	11.64	12.06	12.50
PO	10.73	10.48	10.57	11.14	11.42	11.63	10.93	11.08	10.66	11.01	12.15	11.76
SQ	12.80	12.33	12.10	12.98	13.08	13.36	12.75	12.72	12.28	12.18	13.08	14.03
PS	10.43	9.86	9.73	10.61	11.52	11.25	10.87	10.70	10.33	10.87	11.49	11.29
PJ	10.30	10.17	9.88	10.44	10.82	11.02	10.37	10.22	10.40	10.50	11.53	11.45
LB	10.58	10.15	9.82	10.62	11.24	11.47	10.64	10.72	10.31	11.01	10.99	10.61
LM	10.23	9.84	9.88	10.05	11.26	11.22	10.40	10.33	10.18	10.16	11.10	10.23
LD	10.54	10.06	9.97	10.59	11.15	10.81	10.61	10.59	9.87	10.68	9.62	10.72
LR	12.62	12.49	12.37	12.85	13.77	13.24	12.21	12.25	13.25	12.88	13.59	12.31
LC	14.21	13.73	13.48	13.96	14.65	14.32	14.14	14.13	13.74	13.65	14.39	12.70
SC	14.61	14.38	14.45	14.97	16.03	15.81	15.39	15.36	15.04	15.30	16.47	15.20
SR	17.62	17.05	17.64	17.83	18.88	18.04	18.02	18.01	18.19	17.97	17.54	19.76
LP	16.70	15.06	15.83	18.63	17.47	17.25	17.12	16.65	16.24	15.71	16.59	16.73
LA	17.54	16.25	16.98	17.78	18.25	17.72	17.04	16.96	16.81	16.35	17.27	17.20
LS	15.76	14.31	15.07	16.58	16.40	16.44	16.00	15.86	15.59	16.30	16.31	16.63
SI	14.48	14.50	14.10	14.88	15.79	15.63	15.53	15.53	15.09	15.05	15.12	15.16
Lal	15.58	15.39	15.41	15.91	16.82	15.71	15.04	15.08	16.08	15.76	17.82	15.27
SS	12.80	12.05	12.55	12.75	13.22	13.73	12.93	12.66	12.38	12.97	12.90	13.49
SA	13.19	12.57	12.91	13.41	13.62	13.71	13.40	13.52	13.10	13.22	12.88	12.85
SP	15.43	14.87	15.43	15.87	16.04	15.86	14.93	15.09	15.75	14.58	14.14	15.24
HK	15.97	15.61	15.97	16.94	16.65	16.94	15.81	15.80	16.04	15.69	17.34	17.41
MU	21.07	20.51	20.09	21.12	20.86	21.12	21.26	21.17	20.26	21.13	21.53	20.41
MG	13.03	12.69	13.51	14.13	14.47	14.13	13.60	13.85	13.10	13.97	14.89	14.68
SL	17.23	16.31	17.08	17.77	18.16	17.77	17.20	16.96	16.47	16.84	17.16	17.55
IM	15.84	15.25	15.53	16.48	17.03	18.48	15.35	15.68	15.88	14.96	15.73	15.71
MB	16.87	16.56	16.03	17.81	17.43	17.81	16.95	17.12	17.16	17.05	18.37	18.85
AG	17.99	17.31	17.47	18.43	18.69	18.43	18.23	18.60	17.90	19.01	20.25	16.76

Table 6.8 *(continued)*

*	PM	PT	PA	PL	HS	SiB	SiH	Saq	Sro	SB	PO	SQ	PS
PM	**0.30**												
PT	5.88	**0.39**											
PA	5.95	4.76	**0.75**										
PL	10.66	10.39	10.85	**0.43**									
HS	13.14	12.79	12.38	12.01	**-**								
SiB	12.11	11.47	10.45	10.54	10.40	**1.11**							
SiH	13.18	11.98	11.36	11.70	11.56	2.17	**-**						
Saq	10.91	10.76	11.44	10.64	10.80	9.16	10.36	**1.55**					
Sro	11.93	11.51	12.03	13.21	12.57	11.75	12.73	10.44	**1.42**				
SB	12.83	11.95	12.54	10.23	12.78	11.52	12.23	11.30	10.38	**1.06**			
PO	12.36	11.24	11.35	9.69	11.81	10.13	11.23	10.10	12.61	5.54	**0.93**		
SQ	12.54	12.46	12.05	10.40	13.21	11.10	11.67	12.29	13.36	6.29	4.18	**0.47**	
PS	11.39	10.97	11.38	9.04	11.56	10.73	11.20	10.58	11.57	10.39	3.79	4.09	**-**
PJ	11.00	9.36	8.42	11.27	11.33	10.37	11.35	10.44	11.23	11.67	10.55	11.56	9.89
LB	9.66	9.03	8.62	10.97	11.70	10.28	10.79	10.38	10.47	11.47	11.66	12.20	11.26
LM	9.61	8.34	8.53	10.77	11.23	10.22	10.88	10.62	10.42	10.75	11.21	11.97	11.10
LD	8.74	8.00	8.66	10.24	11.32	10.24	11.29	9.99	9.63	12.65	10.72	11.88	10.03
LR	11.23	10.31	10.47	12.51	13.78	11.85	12.46	12.02	12.83	15.37	13.14	13.15	11.88
LC	12.08	11.39	10.40	13.31	14.27	12.11	12.42	12.19	12.78	13.65	12.69	12.57	11.69
SC	16.87	16.29	15.82	15.80	16.60	16.60	17.54	15.81	15.39	17.03	15.87	16.13	14.26
SR	18.66	18.54	18.69	17.14	18.61	17.64	18.50	17.83	19.72	14.79	16.74	17.61	16.10
LP	16.62	14.70	15.77	14.31	17.21	15.70	16.43	15.77	16.33	15.16	15.47	16.10	14.51
LA	16.67	14.92	16.30	14.13	17.83	15.86	16.84	16.23	16.80	15.06	15.76	16.79	15.27
LS	15.54	15.30	14.39	14.03	16.21	15.97	16.48	16.03	15.55	14.70	16.34	16.33	15.14
SI	15.07	14.62	13.55	14.32	15.10	15.41	16.39	14.65	15.10	16.47	15.27	15.84	14.05
LaI	14.53	14.56	14.00	15.34	16.06	15.47	16.31	15.65	16.15	12.57	15.56	16.55	14.43
SS	12.03	11.58	12.37	11.73	12.62	12.97	13.48	12.12	12.06	14.62	13.03	13.37	12.48
SA	13.77	13.55	12.14	12.03	13.65	13.23	14.68	13.18	14.26	14.26	13.78	13.99	12.86
SP	14.19	12.34	13.28	14.02	15.02	13.84	14.73	15.39	15.18	15.37	14.67	16.12	13.83
HK	15.54	14.77	15.17	14.59	17.13	15.34	16.31	16.80	15.28	20.76	16.44	18.00	15.65
MU	19.82	20.05	19.30	19.86	18.71	17.96	18.90	17.99	19.00	15.32	19.89	20.09	18.76
MG	14.11	14.63	13.21	14.28	15.54	14.81	15.28	14.13	14.76	16.29	13.73	15.71	14.36
SL	15.18	15.37	15.71	17.35	17.39	17.05	18.84	15.88	17.19	15.58	17.00	17.28	15.71
IM	16.06	14.92	13.90	15.25	15.48	14.81	15.92	16.19	15.72	18.07	15.60	15.93	15.16
MB	15.82	16.51	15.06	18.16	19.73	17.32	18.62	19.58	19.73	17.85	17.34	17.84	17.04
AG	18.70	16.68	16.32	17.36	18.37	17.82	17.79	18.68	18.43	18.91	18.61	20.95	17.72

Table 6.8 *(continued)*

*	PJ	LB	LM	LD	LR	LC	SC	SR	LP	LA	LS	SI
PJ	**0.04**											
LB	7.93	**0.44**										
LM	7.80	1.33	**0.85**									
LD	7.35	6.44	6.36	**0.90**								
LR	9.99	10.07	9.31	10.05	**0.61**							
LC	10.38	9.91	9.01	10.43	4.50	**-**						
SC	14.35	15.77	15.52	15.32	14.91	15.87	**1.67**					
SR	15.91	17.15	15.93	16.25	17.74	17.99	16.10	**-**				
LP	13.42	12.36	12.01	13.47	12.92	13.41	14.93	19.90	**1.27**			
LA	14.26	13.04	12.58	14.05	13.21	14.13	15.79	20.42	1.76	**-**		
LS	13.86	13.92	13.42	13.32	13.75	14.37	14.60	18.54	7.63	7.70	**0.88**	
SI	12.15	11.75	11.43	11.67	14.14	14.07	15.53	16.77	13.47	14.65	13.26	**0.13**
LaI	13.96	13.70	12.79	14.29	12.46	13.90	18.98	20.73	17.94	17.92	17.49	16.44
SS	11.43	9.74	9.71	9.72	11.99	11.87	12.10	14.52	11.08	11.28	10.04	7.60
SA	11.21	10.14	10.17	10.41	11.98	12.73	14.76	15.43	11.88	12.38	11.07	6.89
SP	11.22	11.64	10.67	10.87	10.94	12.04	18.12	16.90	14.58	15.53	14.60	15.76
HK	13.53	14.06	13.35	13.07	13.35	14.31	18.82	19.48	15.74	16.47	16.29	15.12
MU	17.24	17.61	17.12	17.81	17.97	18.09	22.92	21.42	20.25	21.41	20.01	18.54
MG	11.48	11.69	11.48	12.49	13.62	14.09	16.22	17.95	16.41	16.79	16.02	14.75
SL	14.30	14.54	14.39	14.48	14.95	15.76	16.14	18.25	15.98	16.71	15.63	14.32
IM	12.51	13.62	13.10	13.46	14.00	14.75	17.33	18.85	16.16	16.84	15.91	16.02
MB	14.54	14.42	14.35	15.60	16.26	17.20	18.67	19.43	17.28	18.08	18.15	16.19
AG	15.43	14.60	14.22	15.22	16.16	16.25	17.43	20.55	17.82	18.75	18.06	18.08

Table 6.8 *(continued)*

*	Lal	SS	SA	SP	HK	MU	MG	SL	IM	MB	AG
Lal	**0.26**										
SS	16.49	**0.86**									
SA	16.43	7.18	**0.34**								
SP	14.47	11.98	13.52	**0.39**							
HK	16.03	13.02	13.52	7.43	**0.76**						
MU	21.27	16.84	14.98	19.03	20.57	**-**					
MG	17.43	12.39	18.56	14.13	15.75	15.50	**-**				
SL	18.31	12.07	13.41	16.48	18.10	20.81	16.39	**0.39**			
IM	16.58	12.47	12.64	13.51	15.79	20.30	13.22	16.49	**0.17**		
MB	19.30	14.76	14.70	16.88	20.02	23.84	18.71	17.16	17.79	**1.27**	
AG	21.38	16.02	15.07	16.28	19.00	21.61	18.50	20.64	19.49	19.58	**2.84**

* Species name abbreviations.
BJb: *B. javanica* variant B; BH: *B. highlandica*; BK: *B. krathonmai*; BF: *B. formosensis*; Bko: *B. koimani*; BJa: *B. javanica* variant A; BP: *B. peregrina*; Bka: *B. karnyi*; BJc: *B. javanica* variant Borneo; BT: *B. timorensis*; BD: *B. dumoga*; RN: *R. notabilis*; PM: *P. misera*; PT: *P. taenionota*; PA: *P. albiceps*; PL: *P. lopesi*; HS: *H. serrata*; SiB: *S. bengalensis*; SiH: *S. hainanensis*; Saq: *S.* (s. lat.) *aquila*; Sro: *S.* (s. lat.) *robustispinosa*; Sbr: *S.* (s. lat.) b*rachiata*; PO: *P. omari*; Squ: *S.* (s. lat.) *quinqueramosa*; PS: *P. scopariiformis*; PJ: *P. javana*; LB: *L. brevicornis*; LM: *L. mimobrevicornis*; LD: *L. dux*; LR: *L. ruficornis*; LC: *L. crassipalpis*; SC: *S. crinita*; SR: *S. rohdendorfi*; LP: *L. pattoni*; LA: *L. aureolata*; LS: *L. saprianovae*; SI: *S. inextricata*; Lal: *L. alba*; SS: *S. seniorwhitei*; SA: *S. antilope*; SP: *S. princeps*; HK: *H. kempi*; MU: *M. uniseta*; MG: *M. globovesica*; SL: *S.* (s. lat.) *longifilia*; IM: *I. martellata*; MB: *M. borneensis* and AG: *A. gressitti*.

Hyphen indicates uncalculated intraspecific variation (bold) due to single specimen.

6.4.6 Phylogenetic trees

The Bayesian analysis showed better resolution than the NJ and MP analyses for COI+II sequences of Sarcophagidae (Figure 6.12). All three analyses support the monophyly (100% bootstrap value) of Sarcophaginae (A-S), which is different from the subfamilies Paramacronychiinae and Miltogramminae. In general, older divergences were not resolved in NJ and MP analyses.

Nineteen clades (A-S) were defined from the Bayesian analysis phylogenetic tree (Figure 6.12) and eight of them (clades A, B, H, J, N, O, R and S) were supported by all three analyses. Among the 19 clades, three clades comprised mixed genera, eight clades represented a single species and the remaining eight clades represented a single genus.

Clade A is a monophyletic group of species from genus *Boettcherisca* with bootstrapped values of higher than 73% from all three analyses. It is a recently diverged genus with nine *Boettcherisca* species and interestingly, *B. javanica* was found to have three genetic variants, namely variant A, B and C. Clade B belongs to *R. notabilis*, which is the sister species of *Boettcherisca*. *Rosellea notabilis* possesses ancestral characteristics of *Boettcherisca* and therefore is more basal than *Boettcherisca* in the phylogeny.

Clades C, F, G, J Q, R and S were represented by a single species of *H. serrata*, *S.* (s. lat.) a*quila*, *S.* (s. lat.) *robustispinosa*, *P. javana*, *S.* (s. lat.) *longifilia*, *M. borneensis* and *A. gressitti*, respectively. Clades E, H, I, L, N, O and P were represented by the genera *Sinonipponia*, *Parasarcophaga*, *Liosarcophaga*, *Liopygia*, *Lioproctia*, *Sarcorohdendorfia* and *Sarcosolomonia*, respectively. Mixed groups of genera are defined in clades D, K and M. Clade D comprised three *Parasarcophaga* species and two *Sarcophaga* (*sensu lato*) species, clade K contains two *Myorhina* species and *Iranihindia martellata*, and clade M was consisted of *S. princeps*, *H. kempi* and *L. alba*.

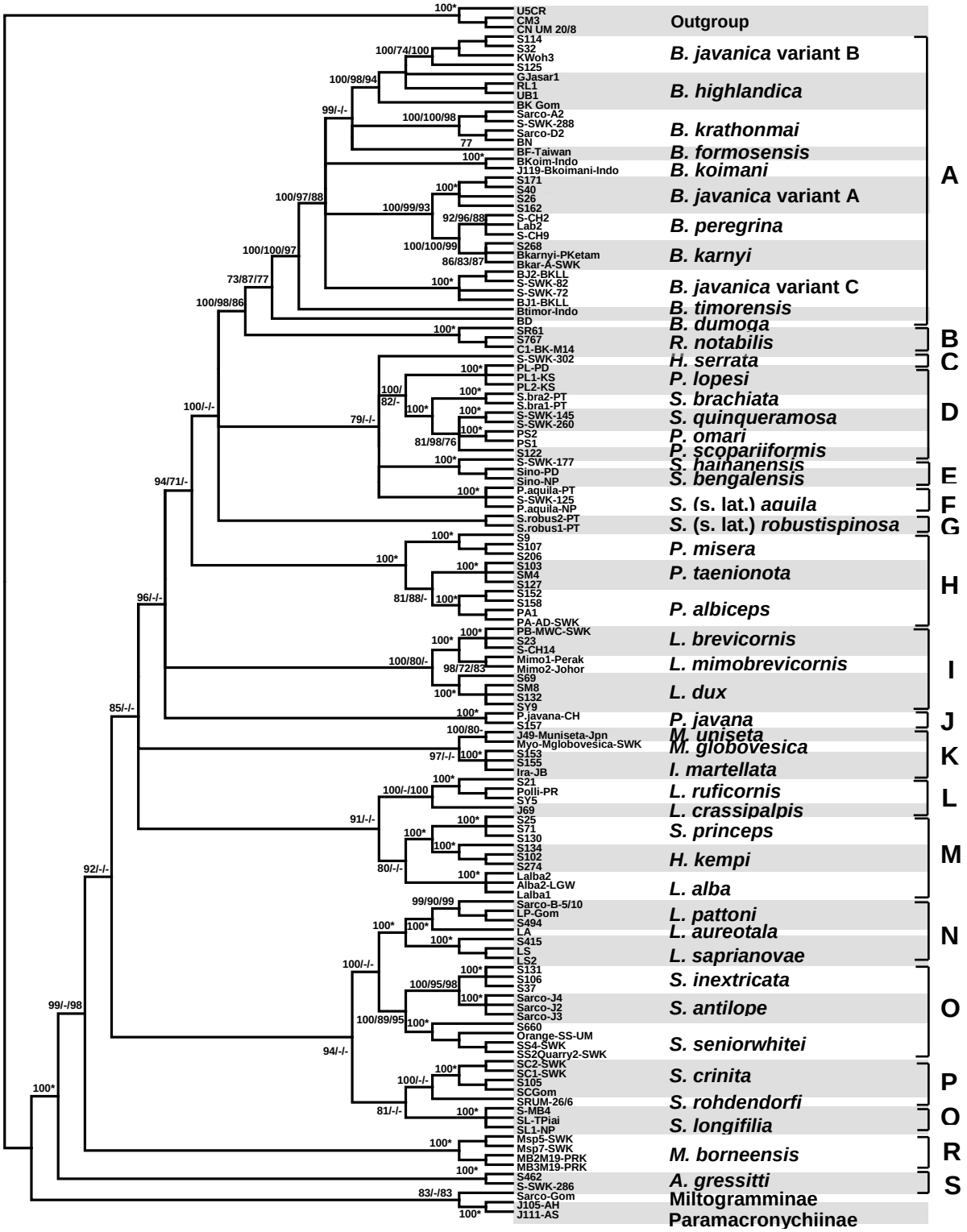


Figure 6.12: Bayesian consensus phylogeny of COI+II genes. Node support values are Bayesian posterior probabilities followed by NJ and MP bootstrap, respectively. Clade values represented by hyphens are below 70% cut off value while asterisks denoted that three of the values are the same.

6.5 Discussion

After Sperling *et al*. (1994) who first proposed the DNA-based identification method using PCR-RFLP and DNA sequencing for three forensically important blow fly species, there has been a global increase of similar studies for Calliphoridae carried out in temperate and tropic regions (Vincent *et al*., 2000; Wallman & Donnellan, 2001; Harvey *et al*., 2003a & b; Chen *et al*., 2004; Wallman *et al*., 2005; Ames *et al*., 2006; Nelson *et al*., 2007 & 2008; Harvey *et al*., 2008, Park *et al*., 2009a & b; Tan *et al*., 2009). However, such applications are not fully explored for Sarcophagidae since almost all of the species are difficult to identify morphologically.

In forensic entomological studies, PCR-RFLP can be used to differentiate closely related species sampled from different life stages (Sperling *et al*. 1994; Malgorn & Coquoz, 1999; Thyssen *et al*., 2005; Tan *et al*., 2010a). Mitochondrial DNA (mtDNA) regions are favoured for such studies because it is easy to retrieve sufficient DNA from damaged or poorly preserved samples, which may facilitate forensic investigations (Otranto & Stevens 2002). Previous PCR-RFLP studies usually tested two or three species and rarely included more than four species (Sperling *et al*. 1994; Malgorn & Coquoz, 1999; Otranto *et al*., 2000; Chen & Shih, 2003; Ratcliffe *et al*., 2003; Schroeder *et al*., 2003a; Thyssen *et al*., 2005; Preativatanyou *et al*., 2010; Tan *et al*., 2010a). Among these studies, Ratcliffe *et al*. (2003) claimed to have differentiated ten species from families Calliphoridae, Sarcophagidae and Muscidae but failed to provide supporting data (e.g. gel electrophoresis images and restriction profiles). Additionally, Wells & Williams (2007) commented on the exclusion of closely related species that were common and important in forensics.

Hence, the present PCR-RFLP study included 12 potential forensically important sarcophagine species encompassing several sister species and morphologically similar species (e.g. *L. dux* and *L. brevicornis*; *I. martellata* and

L. ruficornis). However, it is not easy to produce a "one step digestion" PCR-RFLP species identification assay (using single restriction endonuclease) when the number of species has increased. Second or third digestions with a different restriction endonuclease are always needed for further discrimination (Otranto *et al*., 2000; Ratcliffe *et al*., 2003). It is important to note that the PCR-RFLP profiles obtained must be species specific for reliable species discrimination. The effective discrimination of species by PCR-RFLP relies on obtaining species specific restriction profiles but this could be compromised by using many restriction endonucleases that are laborious and impractical.

In forensic investigation, a rapid and relatively straight forward screening technique is preferred such as DNA sequencing, which not only is easily conducted and replicated but also provides genetic variation of the highest level necessary for a more precise and unambiguous species identification. In the present study, sequence data of complete COI+II have the potential to identify species of Sarcophagidae and place them into the respective clades with high confidence. The availability of such DNA reference data will facilitate forensic cases by allowing immature stages to be identified (Tan *et al*., 2009). The DNA reference data also assists in the species assignment of female flies that are morphologically more difficult to identify (Kurahashi & Kano, 1984; Smith, 1986).

The COI+II phylogeny from the present study is robust for forensic applications in Malaysia because (i) it covers 46 Oriental sarcophagine species, which is well represented of species range, particularly the Malaysian species; (ii) it includes both sexes of flies, thus allowing the potential usage of female fly specimens in species identification; (iii) it provides more genetic information – longer DNA sequences (2.3kb), which may offer improved resolution of species clustering, and (iv) it provides a phylogeny that conforms to the barcoding rationale.

Results from the phylogenetic study showed polyphyly of *B. javanica*; variant A was related to *B. peregrina*, variant B was closer to *B. highlandica* and variant C represents a lineage on its own; emphasising the need for further study and perhaps revision of this species. The distinction of variant A and B is not due to geographical isolation because both were collected from the same site in Kuala Lumpur, whereas variant C represents a specific haplotype from Sarawak. This polyphyly may be accounted for by convergent evolution, ancestral polymorphism, incomplete lineage sorting or even introgressive hybridisation (Stevens *et al*., 2002; Wells & Williams, 2007). These variants could be an example of cryptic species or sibling species because they exhibit genetic differences but similar morphology.

Boettcherisca species show high resemblance and are difficult to distinguish without examining the male genitalia (Kurahashi & Kano, 1984). The historical events of their speciation, rather than inaccurate morphological identification, are the primary reasons for the difficulty in delineating the cryptic species and variants in *Boettcherisca*. Hence, it is important to explore alternative markers and analytical methods, such as those of nuclear regions (e.g. 28S rDNA and ITS region) and protein analysis (allozyme electrophoresis) to confirm the relationships among these difficult species (Wallman & Adams, 1997 & 2001; Stevens & Wall, 2001; Song *et al*., 2008b). Besides, in order to investigate the polyphyletic status and the extent of variability of *B. javanica*, more specimens are needed, which include field colonies from different localities across wider geographical and ecological ranges.

Many attempts had been carried out to understand and resolve the classification and systematics of Sarcophagidae, particularly the study of morphology of male genitalia (Table 6.9). This is because the females possess highly similar external anatomy characters, which are insufficient to differentiate the species. Rohdendorf (1937) was one of the earliest scientists to classify Sarcophagidae into different genera

based on the external and male genitalia characters. Roback (1954) also tried to understand the Nearctic sarcophagine phylogeny via the study of male genitalia, in order to achieve a better classification. Within the Oriental region, particularly in Malaysia, Sugiyama and Kano (1984) were the authorities to study the phylogeny of Sarcophagidae based on comparative morphology of the same character. Although Pape (1996) underlined the usage of *Sarcophaga* in a broad sense by placing all the species under one genus as *Sarcophaga* sensu lato, such designation does not reflect the natural and biologically meaningful groupings of these species. Therefore, further phylogenetic studies are required in order to unravel evolutionary relationships among these species.

Despite the challenges in resolving the phylogenetic relationships among Sarcophagidae species, the present study attempted to provide information to solve taxonomic ambiguities and classification problems of Malaysian Sarcophagidae by using molecular genetics data. The 19-clade phylogeny (A-S) suggests that the taxonomic application of a single genus *Sarcophaga* sensu lato by Pape (1996) may require revision although some of the genera appeared to be para- and polyphyletic. Revisions or assignments of new genus level were postulated based on the present phylogenetic study as well as previous morphological inputs (Table 6.9).

First, the revision of *Sarcophaga* (s. lat.) *longifilia* to a new genus is essential due to its distinctiveness of male genitalia characters, which is different from other sarcophagine genera in Malaysia although Lopes & Kano (1979) and Pape (1996) has put this species under the genus or subgenus of *Sarcorohdendorfia*. Genus *Sarcorohdendorfia* is monophyletic and *Sarcophaga* (s. lat.) *longifilia* is not even phylogenetically close to this genus.

Another four species of *Sarcophaga* (s. lat.) – *S.* (s. lat.) a*quila*, *S.* (s. lat.) *brachiata*, *S.* (s. lat.) *quiqueramosa* and *S.* (s. lat.) *robustispinosa* included in this study

need to be assigned to a new genus. To date, the genus level of these species is still questionable and the designation of these species to any of the subgenera remains inconclusive (Sugiyama *et al*., 1990; Pape, 1996). Four of these *Sarcophaga* (s. lat.) species may be revised to genus *Parasarcophaga* because their females possess similar morphological characteristics of genus *Parasarcophaga* (H. Kurahashi, pers. comm.) and it is congruent with the present phylogeny. Since *Parasarcophaga* is a big polyphyletic genus, containing more than 30 species in China and India (Fan, 1992; Nandi, 2002), the genus is in fact quite unstable and potentially subjected to revisions. *Liopygia ruficornis*, formerly named as *Parasarcophaga ruficornis* in Lopes *et al*. (1977) and Verves (1986), was transferred to the genus *Liopygia* after a number of comprehensive morphological studies (Lopes, 1989; Verves, 1989). It is noteworthy that only clade H is the true *Parasarcophaga* or *Parasarcophaga* (sensu stricto). The rest of the *Parasarcophaga* species require further investigation.

In the case of *Rosellea notabilis*, revision of the genus to *Rosellea* is proposed despite being named *Burmanomyia notabilis* by Kano and Lopes (1969) and *Lioproctia notabilis* by Pape (1996). This is because *Rosellea* possesses typical ancestral traits of *Boettcherisca*, which is corroborated with phylogenetic analysis. *Rosellea notabilis* is genetically distinct from other *Lioproctia* species, thus the suggestion of the former species belonging to genus *Lioproctia* by Pape (1996) is unfounded. Nevertheless, DNA sequences of other species from genera *Rosellea* and *Burmanomyia* are needed to confirm its taxonomic affinity.

Finally, the revision of genus *Myorhina* seems to be necessary due to the grouping patterns in the phylogenetic analysis. *Myorhina borneensis* may be revised as *Pseudothyrsocnema borneensis* while *M. globovesica* and *M. uniseta* may be revised as *Bellieriomima globovesica* and *Bellieriomima uniseta*. However, further morphological studies are needed to support this hypothesis.

Table 6.9: Proposed classification of Sarcophagidae species based on comparisons of taxonomical classifications and present COI+II phylogeny

Original description	Synonym * (Genus/subgenus)	Pape (1996)	Proposed classification
Sarcophaga gressitti Hall & Bohart, 1948	*Alisarcophaga gressitti* [1, 6, 11]	*S.* (s. lat.) *gressitti*	*Alisarcophaga gressitti*
Sarcophaga dumoga Sugiyama & Kurahashi, 1988	*Boettcherisca dumoga* [1, 16]	*S.* (s. lat.) *dumoga*	*Boettcherisca dumoga*
Boettcherisca formosensis Kirner & Lopes, 1961	*Boettcherisca formosensis* [1, 2, 5]	*S.* (s. lat.) *formosensis*	*Boettcherisca formosensis*
Boettcherisca highlandica Kurahashi & Tan, 2009	*Boettcherisca highlandica* [6]	*S.* (s. lat.) *highlandica*	*Boettcherisca highlandica*
Boettcherisca javanica Lopes, 1961	*Boettcherisca javanica* [1, 6, 7, 11, 16]	*S.* (s. lat.) *javanica*	*Boettcherisca javanica*
Sarcophaga karnyi Hardy, 1927	*Boettcherisca karnyi* [1, 3, 6, 11]	*S.* (s. lat.) *karnyi*	*Boettcherisca karnyi*
Boettcherisca koimani Kano & Shinonaga, 1977	*Boettcherisca koimani* [1, 8]	*S.* (s. lat.) *koimani*	*Boettcherisca koimani*
Sarcophaga (Boettcherisca) krathonmai Pape & Bänziger 2000	*Boettcherisca krathonmai* [6, 9, 11, 16]	*S.* (s. lat.) *krathonmai*	*Boettcherisca krathonmai*
Boettcherisca timorensis Kano & Shinonaga, 1977	*Boettcherisca timorensis* [1, 8]	*S.* (s. lat.) *timorensis*	*Boettcherisca timorensis*
Myophora peregrine Robineau-Desvoidy, 1830	*Boettcherisca peregrina* [1, 2, 3, 4, 6, 11, 16]	*S.* (s. lat.) *peregrina*	*Boettcherisca peregrina*
Sarcophaga kempi Senior-White, 1924	*Harpagophalla kempi* [1, 2, 3, 6, 16]	*S.* (s. lat.) *kempi*	*Harpagophalla kempi*
Sarcophaga serrata Ho, 1938	*Hosarcophaga serrata* [1]	*S.* (s. lat.) *serrata*	*Hosarcophaga serrata*
Sarcophaga martellata Senior-White, 1924	*Iranihindia martellata* [1, 3]	*S.* (s. lat.) *martellata*	*Iranihindia martellata*
Sarcophila alba Schiner, 1868	*Leucomyia alba* [1, 2, 3, 4, 6]	*S.* (s. lat.) *alba*	*Leucomyia alba*
Sarcophaga (Lioproctia) aureolata Pape & Kurahashi 2000	N/A	*S.* (s. lat.) *aureolata*	*Lioproctia aureolata*

Table 6.9 *(continued)*

Original description	* Synonym (Genus/subgenus)	Pape (1996)	Proposed classification
Sarcophaga pattoni Senior-White, 1924	*Lioproctia pattoni* [1, 3, 6, 16] *Burmanomyia pattoni* [2, 15] *Coonooria pattoni* [3, 15]	*S.* (s. lat.) *pattoni*	*Lioproctia pattoni*
Sarcophaga (Lioproctia) saprianovae Pape & Baenziger, 2000	*Lioproctia saprianovae* [6, 9, 11, 16]	*S.* (s. lat.) *saprianovae*	*Lioproctia saprianovae*
Sarcophaga crassipalpis Macquart, 1839	*Liopygia crassipalpis* [1] *Jantia crassipalpis* [2]	*S.* (s. lat.) *crassipalpis*	*Liopygia crassipalpis*
Musca ruficornis Fabrius, 1794	*Liopygia ruficornis* [1, 2, 3, 4, 6, 11, 16]	*S.* (s. lat.) *ruficornis*	*Liopygia ruficornis*
Sarcophaga brevicornis Ho, 1934	*Liosarcophaga brevicornis* [1, 2, 3, 4, 16] *Parasarcophaga brevicornis* [6, 18]	*S.* (s. lat.) *brevicornis*	*Liosarcophaga brevicornis*
Sarcophaga dux Thomson, 1869	*Liosarcophaga dux* [1, 2, 3, 4] *Parasarcophaga dux* [6]	*S.* (s. lat.) *dux*	*Liosarcophaga dux*
Sarcophaga mimobrevicornis Sugiyama, 1990	*Liosarcophaga mimobrevicornis* [1, 16] *Parasarcophaga mimobrevicornis* [6, 11]	*S.* (s. lat.) *mimobrevicornis*	*Liosarcophaga mimobrevicornis*
Thyrsocnema (Pseudothyrsocnema) borneensis Shinonaga & Lopes, 1975	*Pseudothyrsocnema borneensis* [1] *Myorhina borneensis* [6, 11]	*S.* (s. lat.) *borneensis*	*Pseudothyrsocnema borneensis*
Pierretia globovesica Ye, 1980	*Bellieriomima globovesica* [1, 2, 3, 4] *Myorhina globovesica* [6] *Pierretia globovesica* [2]	*S.* (s. lat.) *globovesica*	*Bellieriomima globovesica*
Sarcophaga uniseta Baranov, 1939	*Bellieriomima uniseta* [1, 4]	*S.* (s. lat.) *uniseta*	*Bellieriomima uniseta*
Sarcophaga albiceps Meigen, 1826	*Parasarcophaga albiceps* [1, 2, 3, 4, 6, 11, 16, 18]	*S.* (s. lat.) *albiceps*	*Parasarcophaga albiceps*
Sarcophaga javana Macquart, 1851	*Robineauella javana* [1] *Parasarcophaga javana* [6]	*S.* (s. lat.) *javana*	*Parasarcophaga javana*
Parasarcophaga (Baliisca) lopesi Verves, 1980	*Parasarcophaga lopesi* [6, 11] *Baliisca lopesi* [1]	*S.* (s. lat.) *lopesi*	*Parasarcophaga lopesi*
Sarcophaga misera Walker, 1849	*Parasarcophaga misera* [1, 2, 3, 4, 6, 11, 16, 18]	*S.* (s. lat.) *misera*	*Parasarcophaga misera*

Table 6.9 *(continued)*

Original description	Synonym * (Genus/subgenus)	Pape (1996)	Proposed classification
Parasarcophaga omari Kurahashi & Leh, 2007	*Parasarcophaga omari* [6, 11]	*S.* (s. lat.) *omari*	*Parasarcophaga omari*
Sarcophaga dux spp. *scopariiformis* Senior-White, 1927	*Liosarcophaga scopariiformis* [1] *Curranea scopariiformis* [2, 3] *Parasarcophaga scopariiformis* [6]	*S.* (s. lat.) *scopariiformis*	*Parasarcophaga scopariiformis*
Musca taenionota Wiedemann, 1819	*Parasarcophaga taenionota* [1, 2, 3, 4, 18]	*S.* (s. lat.) *taenionota*	*Parasarcophaga taenionota*
Burmanomyia notabilis Kano & Lopes, 1969	*Lioproctia notabilis* [1] *Rosellea notabilis* [6] *Burmanomyia notabilis* [12]	*S.* (s. lat.) *notabilis*	*Rosellea notabilis*
Sarcophaga antilope Böttcher, 1913	*Sarcorohdendorfia antilope* [1, 2]	*S.* (s. lat.) *antilope*	*Sarcorohdendorfia antilope*
Sarcophaga inextricata Walker, 1859	*Sarcorohdendorfia inextricata* [1, 2, 3, 6, 11, 16]	*S.* (s. lat.) *inextricata*	*Sarcorohdendorfia inextricata*
Sarcophaga basalis Walker, 1859	*Sarcorohdendorfia seniorwhitei* [1, 2, 3] *Parkerimyia seniorwhitei* [3]	*S.* (s. lat.) *seniorwhitei*	*Sarcorohdendorfia seniorwhitei*
Sarcophaga crinita Parker, 1917	*Sarcosolomonia crinita* [1, 2, 3, 6, 11], *Parkerimyia crinita* [3]	*S.* (s. lat.) *crinita*	*Sarcosolomonia crinita*
Sarcosolomonia (Parkerimyia) rohdendorfi Nandi, 1976	*Sarcosolomonia rohdendorfi* [1, 3, 13]	*S.* (s. lat.) *rohdendorfi*	*Sarcosolomonia rohdendorfi*
Sarcophaga princeps Wiedemann, 1830	*Seniorwhitea princeps* [1, 2, 3, 4, 6, 16]	*S.* (s. lat.) *princeps*	*Seniorwhitea princeps*
Sinonipponia bengalensis Nandi, 1977	*Sinonipponia bengalensis* [1, 3, 14]	*S.* (s. lat.) *bengalensis*	*Sinonipponia bengalensis*
Sarcophaga hainanensis Ho, 1936	*Sinonipponia hananensis* [1, 2]	*S.* (s. lat.) *hainanensis*	*Sinonipponia hananensis*
Sarcophaga aquila Sugiyama, 1990	*Sarcophaga* (s. lat.) *aquila* [1, 6, 11, 17]	*S.* (s. lat.) *aquila*	*Parasarcophaga aquila*
Sarcophaga brachiata Sugiyama, 1990	*Sarcophaga* (s. lat.) *brachiata* [1, 17] *Parasarcophaga brachiata* [6]	*S.* (s. lat.) *brachiata*	*Parasarcophaga brachiata*

Table 6.9 *(continued)*

Original description	Synonym * (Genus/subgenus)	Pape (1996)	Proposed classification
Sarcophaga longifilia Salem, 1945	*Sarcorohdendorfia longifilia* [1, 10] *Sarcophaga* (s. lat.) *longifilia* [6, 11]	*S.* (s. lat.) *longifilia*	New genus to be proposed
Sarcophaga quinqueramosa Sugiyama, 1990	*Sarcophaga* (s. lat.) *quinqueramosa* [1, 17]	*S.* (s. lat.) *quinqueramosa*	*Parasarcophaga quinqueramosa*
Sarcophaga robustispinosa Sugiyama, 1990	*Sarcophaga* (s. lat.) *robustispinosa* [1, 17], *Parasarcophaga robustispinosa* [6]	*S.* (s. lat.) *robustispinosa*	*Parasarcophaga robustispinosa*

* reference: [1] Pape, 1996; [2] Fan, 1992; [3] Nandi, 2002; [4] Verves, 1986; [5] Kirner & Lopes, 1961; [6] Kurahashi & Tan, 2009; [7] Lopes, 1961a; [8] Kano & Shinonaga, 1977; [9] Pape & Bänziger, 2000; [10] Lopes & Kano, 1979; [11] Kurahashi & Leh, 2007; [12] Kano & Lopes, 1969; [13] Nandi, 1976; [14] Nandi, 1977; [15] Fan, 1965; [16] Tan *et al*., 2010[b]; [17] Sugiyama *et al*., 1990; and [18] Kano *et al*., 1967.
N/A: not applicable.

6.6 Conclusion

PCR-RFLP offers a quick preliminary species identification method for forensic entomologists in estimating PMI. It is beneficial, especially in dealing with large numbers of samples and some ambiguous species. In addition, it can be applied to any life stages of the insects and even to the damaged samples. PCR-RFLP may serve as the alternative or further testing for identification when the classical morphology-based identification has been hindered.

Nonetheless, DNA sequencing is still the relatively best method for precise species identification, accounted for its robust genetics information. The available DNA sequences of Sarcophagidae flies encompassing the complete *COI*, tRNA-leucine and *COII* genes allow for the identification of these species, particularly useful for local forensic purposes. The phylogeny provides insight into the evolutionary relationships of these species, which could be assigned into any of the 19 clades. The molecular phylogeny is generally congruent with most morphologically-based classification systems. Suggestions of a taxonomic revision may be premature, as the current phylogeny can benefit from the sampling of more taxa.

Chapter 7:

General remarks

7.1 General remarks

Forensic entomology is scientifically mature for practical use in many countries, such as France, Germany, Italy, the United Kingdom, Australia, Brazil and the United States of America which began to apply this field since the 1990s (Anderson, 1997; Benecke, 2001; Dadour *et al*., 2001; Campobasso *et al*., 2004; Ames *et al*., 2006; Adair & Kondratieff, 2006; Adair *et al*., 2008; Pujol-Luz *et al*., 2008). One particularly comprehensive case report describing the systematic collection of entomological specimens followed by succession analysis to infer post-mortem interval was from Texas, USA in the year 2007 (Bucheli *et al*., 2009). In this case, the collected insects (e.g. *Calliphora vicina*, *Hydrotaea aenescens* and *Piophila casei*) formed a unique assemblage representing a particular time of year, which led to the conclusion that the estimated post-mortem interval of a remains (reported missing for close to a year) was between 7 and 10 months.

In Europe, the greatest proponent for forensic entomology is perhaps Mark Benecke, a German forensic biologist who has been providing forensic entomology training to professionals as well as increasing public awareness on this topic since 1996. Active researches to simulate fly succession pattern on non-human corpses have been seen in subsequent years (as listed in Table 4.1). Studies on specific conditions of experimented carcass (e.g. buried, hanged, in the water, burnt) to mimic actual crime scenes have also been carried out to develop an understanding of the interaction between necrophagous insects and the decomposing body. Ultimately, a "body farm" – research facility where human decomposition was studied in a variety of settings, including entomological manifestations, was established at the University of Tennessee Anthropological Research Facility.

In Malaysia, several forensic entomology researches have been made and published in recent years, although most of the efforts have remained isolated and

progress is slow. Most documented research deals with forensically important flies as they are more relevant in the estimations of time of death. Slow progress in this field can be attributed to several factors, including lack of awareness on this subject, but a more important problem is the lack of relevant expertise in entomology. Practical use of forensic entomology very much depends of having baseline reference data (e.g. species identification, succession, geographical distribution, etc.) especially of families Calliphoridae and Sarcophagidae for analysis involving forensically important flies. Species identification alone represents a big challenge. Although current data on calliphorid identification are quite comprehensive, data on sarcophagid identification are very limited. Problems with identification ultimately mean that there are also very little relevant reference data available for forensic applications.

Realising this, the current project was carried out to overcome several limiting factors in the progress and successful application of forensic entomology. For identification purposes, this study utilised both morphological characteristics, supported with DNA data, and this approach has proven effective and efficient for most species within the calliphorids and the sarcophagids (as described in chapters 2, 5 and 6). Significant outcomes of this study include an updated checklist with the report of seven new records. Additionally, the identification of a new species of Sarcophagidae has also been reported (see chapter 3). Correlating this checklist with ecologically distinct habitat types in various localities has also provided some interesting findings on habitat specific species. From this study, strong associations of certain species to particular types of habitats have been observed (for a detailed explanation, see chapter 3). Although these findings are perhaps preliminary and require further research, the prospect of using presence of habitat specific insects as indicators of a first crime scene is exciting, and has been proposed by many (Smith, 1986; Lord, 1990; Gennard, 2007; Castner, 2010).

Barcoding is gaining popularity in recent years and it has been successfully used in many studies to assist species identification. This study embarked upon the utility of DNA variations for species identification, and for most of the fly species DNA data were found to be discriminative and useful for identifing species. In general, the capability of the barcoding system is promising. However, in certain cases, ambiguity of species identification is still present, such as the case with *Boettcherisca javanica* in this study. Sensitivity and specificity of DNA-based identification systems can be increased by using several markers (e.g. mitochondrial and nuclear). However, this is not always true, as shown in a preliminary analysis of *Boettcherisca javanica* using combined mitochondrial and nuclear markers (data from a related study). Nevertheless, molecular techniques have helped to speed up species identification and increase accuracy, especially for females and immatures. There is indeed good consistency between sequences and morphological findings. Molecular data have also led to additional questions regarding the classification system of these flies. Results of this study lends stronger support for the traditional generic classifications proposed by Lopes (1969 & 1989), Lopes *et al*. (1977), Rohdendorf (1937 & 1965), Shewell (1987) and Verves (1986) in the taxonomy of the sarcophagine flesh flies, compared to the system suggested by the Catalogue of the Sarcohagidae of the World (Pape, 1996).

There is definitely great potential for forensic entomology to be used in this country. The data obtained from this study, which cover most of the forensically important species from this country, are readily applicable to forensic cases. The techniques derived from this study need actual application in real cases to further gauge their practicability and limitations. It is evident from this study, as well as other similar studies, that the potency and usefulness of DNA-based identification application in forensics are strongly supported. The availability of such tools enhances the investigation of forensics in terms of reliability, efficiency, rapidity and cost efficiency.

Nevertheless, proper operational guidelines should be established regardless of entomological or molecular methods to prevent misinterpretation.