# GEOMETRIC MORPHOMETRICS ANALYSIS OF WING VENATION FOR IDENTIFICATION OF SELECTED FORENSICALLY IMPORTANT FLIES IN MALAYSIA

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**KUALA LUMPUR**

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**DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE**

**INSTITUTE OF BIOLOGICAL SCIENCE**
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# GEOMETRIC MORPHOMETRICS ANALYSIS OF WING VENATION FOR IDENTIFICATION OF SELECTED FORENSICALLY IMPORTANT FLIES IN MALAYSIA

## ABSTRACT

Entomological evidence has become one of the elements in assisting the criminal investigation. The presence of various insects at the crime scene or on the dead body often giving clues on the manner of death particularly in suicide and homicide cases. Flies for instance, are the most common and apparent insect at the murder scene and frequently, a specific fly species would be present at the specific location and time. From here, the estimation of post mortem interval will be made upon various criteria; the time of death or the synchronisation between the locations of the murder and the location of the body was found. Thus, it is very crucial to acquire the speciation information of the fly as fast as we could to help the investigation processes. Hence, an effective, economical and rapid method is needed for the identification of these insects. This study was focused on using quantitative methods for species identification of the flies through geometric morphometrics analysis. The analysis on the wings of the flies was conducted by measuring the venation via landmark method. Fifteen forensically important fly species in Malaysia from the Calliphoridae and Sarcophagidae families were chosen from an archived collection as the subject of this analysis. The measurement obtained from the geometric morphometrics analysis in the form of thin-plate-spline data were subjected to the Generalised Procrustes Analysis (GPA), Principal Component Analysis (PCA) and Heat Map Analysis. From PCA, the differences between both families and the distribution of fly species within Calliphoridae family were quite obvious compared to Sarcophagidae family. Geometric morphometrics analysis were also carried out on 36 crime scene sample, and 14

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randomly chosen crime scene samples were sent for DNA sequence analysis for verification. From this study, geometric morphometrics method was shown to have the potential to become a useful identification and diagnostic tools for forensically important fly species in Malaysia.

**Keywords**: Geomertric morphometrics, Calliphoridae, Sarcophagidae, fly, identification.

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**ANALISIS *GEOMETRIC MORPHOMETRICS* KE ATAS SAYAP LALAT BAGI TUJUAN IDENTIFIKASI LALAT-LALAT YANG MEMPUNYAI NILAI FORENSIK DI MALAYSIA**

## ABSTRAK

Bahan bukti entomologi telah menjadi salah satu elemen dalam penyiasatan kes-kes jenayah. Kehadiran pelbagai jenis serangga di tempat kejadian jenayah atau di lokasi penemuan mayat membantu dalam penentuan cara kematian terutamanya dalam kes-kes pembunuhan dan bunuh diri. Lalat adalah antara serangga yang sering dilihat di tempat kejadian pembunuhan dan spesis lalat yang spesifik akan hadir pada tempat dan waktu yang spesifik. Melalui penemuan ini, anggaran *post mortem interval* akan dibuat atas beberapa kriteria; masa kematian atau keselarian tempat berlakunya pembunuhan dengan tempat mayat dijumpai. Oleh itu, maklumat mengenai spesis lalat yang ditemui perlulah dikenalpasti seberapa segera bagi membantu proses siasatan. Justeru, bagi mencapai matlamat tersebut, kaedah yang efektif, ekonomi dan pantas amat diperlukan bagi mengenalpasti spesis serangga ini. Dalam kajian ini, kami telah memfokuskan kaedah kuantitatif bagi mengenalpasti spesis lalat melalui analisis *geometric morphometrics*. Kami mengaplikasikan analisis ini ke atas sayap lalat dengan membuat pengukuran melalui kaedah *landmark*. Kami telah menggunakan koleksi arkib yang terdiri daripada 15 spesis lalat forensik di Malaysia merangkumi lalat daripada famili Calliphoridae dan Sarcophagidae. Data yang diperolehi dalam bentuk *thin-plate-spline* telah dianalisis menggunakan *Generalised Procrustes Analysis (GPA), Principal Component Analysis (PCA)* dan *Heat Map Analysis.* Melalui PCA, kami dapat melihat secara grafik perbezaan yang nyata di antara lalat daripada famili Calliphoridae dan Sarcophagidae; manakala di antara spesis pula, lalat daripada famili Calliphoridae

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menunjukkan perbezaan yang nyata berbanding Sarcophagidae. Kami turut menjalankan analisis sama ke atas 36 sampel lalat dari tempat kejadian dan 14 lalat daripada 36 sample tersebut telah dipilih secara rawak untuk analisis DNA bagi tujuan verifikasi. Secara keseluruhannya, analisis *geometric morphometrics* mempunyai potensi sebagai salah satu kaedah identifikasi dan diagnosis untuk lalat forensik di Malaysia.

**Kata kunci**: *Geomertric morphometrics*, Calliphoridae, Sarcophagidae, lalat, identifikasi.

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# LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| 2D | : | 2-dimensional |
| 3D | : | 3-dimensional |
| *ºC* | : | degree Celsius |
| $\mu l$ | : | microlitres |
| $\mu M$ | : | micromolar |
| *%* | : | percent |
| ANOVA | : | Analysis of Variance |
| ATL buffer | : | A Tissue Lysis buffer |
| AW buffer | : | A wash buffer for DNA extraction |
| bp | : | base pairs |
| C | : | Control |
| COI | : | Cytochrome c oxidase subunit I |
| COII | : | Cytochrome c oxidase subunit II |
| CVA | : | Canonical Variates Analysis |
| DNA | : | Deoxyribonucleic Acid |
| GPA | : | Generalised Procrustes Analysis |
| ITS | : | Internal Transcribed Spacer |
| JPEG | : | Joint Photographic Experts Group |

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| | | |
|---|---|---|
| KOH | : | Potassium Hydroxide |
| mg | : | milligram |
| mg/ml | : | milligram/microliters |
| MgCl2 | : | Magnesium Chloride |
| ml | : | millilitres |
| mM | : | millimolar |
| MRI | : | Magnetic Resonance Imaging |
| ng | : | nanogram |
| PC | : | Principal Component |
| PCA | : | Principal Component Analysis |
| PCR | : | Polymerase Chain Reaction |
| PE buffer | : | A wash buffer in DNA clean up procedures |
| PMI | : | Post Mortem Interval |
| QG buffer | : | A solubilisation and binding buffer |
| rDNA | : | ribosomal Deoxyribonucleic Acid |
| RMP | : | Royal Malaysia Police |
| RNase | : | Ribonuclease |
| rpm | : | revolutions per minute |
| s | : | seconds (time) |
| TPS | : | Thin-Plate-Spline |



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| | | |
|---|---|---|
| tRNA | : | transfer Ribonucleic Acid |
| UV | : | Ultraviolet |
| V | : | Volt |

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# CHAPTER 1: INTRODUCTION

Insects are the largest and the most diverse group of animals in the world. There are several estimates of the number of described insects, ranging from 720,200 (May, 2000) to more than 1 million (Myers, 2001a), with a total number of insects expected to be within the range of 5–6 million (Raven & Yeates, 2007) to around 8 million (Groombridge, Jenkins, & Jenkins, 2002). As part of the ecosystem, insects serve as tools in various biological processes in playing their role as predators, pests, pollinators, and food source (Triplehorn & Johnson, 2005). In addition, insects are also important scavengers, as they ensure the stabilisation of the ecosystem; by playing significant roles as decomposing agents for cadavers.

## 1.1 Forensic Entomology

Modern criminal investigation has included forensic science as part of the legal process, and the American Academy of Forensic Science recognises Forensic Entomology as one of the subdiscipline that could produce evidences in the court (also known as medico-criminal/medico-legal entomology) (Rivers & Dahlem, 2014).

Medico-criminal entomology is the field of study where insect evidence is used to assist legal investigations (Amendt, Richards, Campobasso, Zehner, & Hall, 2011). When dealing with criminal cases, medicolegal entomology is useful in determining – (i) the time; (ii) the location and (iii) the cause of death.

Here, insects are used to provide clues pertaining to the manner of death in homicide cases because some necrophagous insects are attracted to the carcass at different stage of decomposition. One of the ways insects help in deducing the manner of death is

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through the association of insect evidence found on/in crime scene material with the season, location of origin and peculiar insects' activities; for example in the temperature-dependant development of insects, or more frequently, forensically important fly species. Insects can also be useful in determining the Post Mortem Interval (PMI) when presence of a particular insect on bodies can be associated with the insects' specific succession pattern on the carcass. (See Literature review for elaboration).

## 1.2 Forensically Important Flies

Flies are classified under the order Diptera, which is commonly known as 'two-winged flies' and differs from other insects by only having a pair of wings. They have an adapted form of hind wings named as 'halteres' which aids in ensuring the stability of the insects itself. Diptera also serves as one of the largest orders in insects and contributes to the wide distribution of insects on earth. Insects which lie under this distinctive order are small and soft-bodied insects and they have their own role in their environment. Examples of dipterans include blowflies, houseflies, horseflies, craneflies, mosquito and midges (Smith, 1986). In their habitat, mosquitoes are pests to humans and animals, blow flies act as the scavengers and Hessian flies (a type of midge) function as pests of plants (Triplehorn & Johnson, 2005).

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**Figure 1.1: [Calliphoridae fly]** **(n.d.).**
https://www.pinterest.com/pin/243335186102466406/

**Figure 1.2: [Sarcophagidae fly]**
**(n.d.).** https://www.flickr.com/photos/tkclip/35312318766/

Medicocriminal entomology frequently utilise Diptera flies from the Calliphoridae and Sarcophagidae families because both can provide information that is vital in medicocriminal investigation. Calliphoridae flies act as scavengers and are the most commonly found species breeding on the carcass. These flies deposited the eggs on the carrion which then turn into larvae and feed on the carrion (Smith, 1986). Sarcophagidae, on the other hand, are commonly known as flesh flies and they differ from most flies because they usually deposit hatched or hatching maggots instead of



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eggs (ovoviviparous) on carrion, dung, decaying material, or open wounds of mammals. In addition, the adults mostly feed on fluids from animal bodies, carrion as well as from animal wastes.

## 1.3 Problem statement

### 1.3.1 Strategies for the identification of flies

Much of the success of using insect information in medicocriminal entomology depends on the ability to accurately identify the fly species, and more often than not, this requires the service of skilled and experienced entomological taxonomists. The conventional methods of fly species identification techniques are morphological observation and examination by a qualified and experienced entomologist.

There are several limitations in using morphology-based identification for forensic use. Firstly, the conditions of the specimens obtained from the crime scene are sometimes not intact or may be damaged, which will hinder accurate identification. Secondly, specimens collected from crime scenes and carcasses are usually in the immature stages, and there is an apparent lack of complete taxonomic guide for immature stages, particularly the family Sarcophagidae (Byrd & Castner, 2010; Smith, 1986). Finally, immature specimens often need to be reared until adulthood before they can be morphologically identified. While this step is time consuming, rearing the specimens to adulthood is not easy and often provides a different set of challenges.

Recent years have seen the introducton and inclusion of DNA based identification methods to facilitate fly species identification. While DNA based identification has been succesfully used to facilitate fly species from the family Calliphoridae, the utility of this approach for flies within the Sarcophidae family is still problematic. Many flesh flies

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appear to be generally similar in morpholoy especially to the untrained eye. Furthermore, there exists some dispute over the identification and classification (as well as nomenclature) system (Tan, 2012) and contributes further towards the complexity of Sarcophagidae species identification.

### 1.3.2 Geometric morphometrics

The basic principal in morphometrics is the measurement of length, width and depth which is first used ichtyological studies. There are limitations to using this method for identification; these include the measurements of 3-dimensional (3D) shape (which are sometimes not easy to measure) and the relative size of specimens which are not consistent. In terms of size correction method, different methods will produce different results. Secondly, there are no standard homologous points for measurement purposes. This would contribute to the difficulty in measuring the shape and lastly, the same measurement reading could be obtained from two or more different distances from the shapes (Adams, Rohlf, & Slice, 2004).

To circumvent these issues, a new method had been established which emphasised the utilisation of landmarks data for analysis. This new method is termed Geometric morphometrics, and it incorporates the measurements based on landmark coordinates on the surfaces of the shape and also along the outline of the curves. This strategy had been adopted in various biological fields, including the analysis of animal body parts and bones (Hingst-Zaher, Marcus, & Cerqueira, 2000; Gayzik, Mao, Danelson, Slice, & Stitzel, 2008), the wing venation analysis in Stenogastrinae wasps and the architecture of the nest (Baracchi, Dapporto, & Turillazzi, 2011) the taxonomy of plants from leaves (Viscosi & Cardini, 2011) and floral shape variation (Tsiftsis, 2016).

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## 1.4 Research question

The present study is undertaken to determine whether the geometric morphometric approach can be used on fly wing venation patterns as a method for the identification of forensically important Calliphoridae and Sarcophagidae species in Malaysia.

## 1.5 Research objective

This research is aimed to assess the use of geometric morphometrics in wing venation in distinguishing forensically important Calliphoridae and Sarcophagidae fly species in Malaysia. The outcomes of this assessment were compared with existing morphological and DNA data to observe the corroboration between the methods and to conclude the competency of wing venations as one of the identification method for forensic flies in Malaysia.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1 Entomology

Insects appear as the most dominant group of animals in every life processes since 350 million years ago (Triplehorn & Johnson, 2005). It covers the insects from fresh water and sea to the most presiding population which originated from the land. Entomology and entomologist are both associated in the study of insects which associated with the observation and collection, rearing and research to assess the evolution, behaviour, ecology, genetics, biochemistry, anatomy and physiology of the insects (Gullan & Cranston, 2014). More than 3 million species of insects are estimated to have been described by entomologists as the exploration of the tropics developed (Capinera, 2008).

As part of the **ecosystem,** insects serve as tools in various biological processes – **from** being predators, to becoming pests, pollinators, as well as becoming food source **to** the scavengers (Triplehorn & Johnson, 2005). As scavengers, they ensure the stabilisation of the ecosystem by playing important roles as the decomposing agents for cadavers. Insects, as a member of the Arthropoda phylum, have the characteristic exoskeleton, 3 pairs of legs, a body with 3 distinct parts and the presence of wings which allows them to move from one place to another for feeding and reproduction. The ability to locate transitory food resources from carcasses, including human remains, makes them pertinent as important forensic tools (Byrd & Castner, 2010).

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## 2.2 Forensic Entomology

Modern criminal investigation has included forensic science as part of the legal process and the American Academy of Forensic Science recognises forensic entomology as one of the many subdisciplines that can produce evidences in the court (Rivers & Dahlem, 2014).

Forensic entomology is the field of study where insect‘s evidence is used to assist in legal investigations (Amendt et al., 2011). Here, insects could provide clues pertaining to the manner of death in homicide cases because some necrophagous insects are attracted to the carcass at different stages of decomposition. One of the ways insects help in deducing the manner of death is through the observation of the season, location and insects‘ activities; for example in the temperature-dependent development of insects (frequently flies), and also the succession pattern on the carcass. Based on some of these clues, the insects that can be found on the body are pretty much predictable.

### 2.2.1 History and Progress of Forensic Entomology

The earliest use of forensic entomology was reported in ‗The Washing Away of Wrongs‘ written by Sung T‘su, a Chinese death investigator, who stated that insects had been used in a legal case as early as thirteenth century in China (Keh, 1985). (Smith, 1986) , however, insisted that the earliest instance of the use of forensic entomology was established in 1888 by Yovanovitch. The use of forensic entomology in criminal investigations was widely used in mid 1900s in countries such as Europe, United States and England. The field only became more developed in 1980s in United States (Byrd & Castner, 2010).

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### 2.2.2 Types of Application under Forensic Entomology

Forensic entomology can be divided into 3 categories; urban entomology/stored products entomology, structural entomology and medico-legal entomology (Goff, 2011). Stored product entomology is the study of insect pests in the environment which affect human habitat and health. An example of stored product entomology would be the invasion of insects into packed food. Structural entomology is the study of insects, e.g., termites, which, when infestation occurs, eventually lead to the destruction of a building structure (Goff, 2011). Additionally, insects have also been used to estimate the age some artifacts from west Mexican shaft tombs (Byrd & Castner, 2010). Lastly, we have medico-legal entomology, which is also known as forensic medical entomology or medico-criminal entomology. It is a field which utilises insect evidence at crime scenes that serves as leads or clues in police investigations (Byrd & Castner, 2010).

### 2.2.3 The Determination of Post Mortem Interval

The term post-mortem interval (PMI) refers to the period between time of death and the discovery of the remains (Amendt et al., 2011). Many researches were focusing on the rate of the decomposition of the body and these were deducted by factors like temperature and humidity. For example the comparison of both factors between the bodies located on the surface of the ground and below the ground did not produce a conclusive estimation when it comes to the rate of the decomposition (Cockle & Bell, 2015). There was also a condition in which the bodies share the same PMI but differ at the stage of the decomposition. It has been suggested that the state of the burial and other endogenous factors may also affect the estimation of the decomposition rate (Ferreira & Cunha, 2013).

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Various methods have been implemented to estimate the time of death of human remains. These include medical means such as tissue histology; chemically; bacteriologically and zoologically (Smith, 1986). The most obvious and common condition visibly when estimating time of death are the rigor mortis and livor mortis state. Rigor mortis refers to the state where the body muscle contracted after 2-6 hours following death and sustained up to 24-84 hours before the muscle eventually relaxed. However, there are factors such as temperature and muscle fibre types of the muscle that could affect the state of rigor mortis (Hayman & Oxenham, 2016). On the other hand, livor mortis refers to the pooling of blood in a gravitational state at the body. The condition is likely to occur as approximately 2 hours following death. The colour of the blood changes from red to purple depending on the surrounding environment such as temperature and other factor that might be the cause of death. Still, this condition is not conclusive since so many variables affecting the estimation of time of death (Hayman & Oxenham, 2016).

Biochemical markers have been established to estimate the PMI by comparing the pH and metabolites level of the blood. However, the pH level was not conclusive compared to the metabolites level (Donaldson & Lamont, 2013). Other than that, the content of dissolve organic and inorganic carbon together with other components in the soil taken from the area where the body is found could also be used to estimate the PMI. The content of nitrate-N, ammonium-N and dissolve inorganic carbon are higher at the early stage of death whereas the content of dissolve organic carbon, dissolved organic nitrogen (DON), orthophosphate-P (PO4-P), sodium (Na+), and potassium (K+) are peaked up to 1752 days following death. Nevertheless, body mass index is slightly affecting the content of the components in estimating the PMI (Fancher et al., 2017).

The presence of different community of microorganisms at the body could also be used as the ‗microbial clock' in estimating the PMI. These microorganisms known as



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human microbiome present at different part of the body and also produce different succession pattern (Hauther, Cobaugh, Jantz, Sparer, & DeBruyn, 2015). For instance, the presence of Proteobacteria was abundance at the buccal cavity and rectum whereas the colonisation of Firmicutes and Bacteroidetes were slightly low. However, further research need to be conducted since they become more similar as the decomposition progressed (Guo et al., 2016).

Other factors that could also contribute to the determination of PMI are the presence of different types of insects and also the development of body temperature (Byrd & Castner, 2010). Beetles and flies are the most common insects known to be present at the body soon after death and the succession pattern will be observed and recorded (Iancu, Carter, Junkins, & Purcarea, 2015). The studies of the drug content of the insects (known as entomotoxicology) have become yet another consideration for PMI (Amendt et al., 2011). This field explores the narcotics content of the insects but the setbacks are the environmental contamination which would affect the life cycle of the insects at the body (Dayananda & Kiran, 2013). The cuticular hydrocarbons composition of the insects could also be used for estimating the PMI (Drijfhout, 2009). For instance, the utilisation of cuticular hydrocarbons in the identification of Sarcophagidae‘s fly for taxonomic classification especially when only the fly‘s body parts are available (Braga, Pinto, de Carvalho Queiroz, Matsumoto, & Blomquist, 2013). The study of population in flies could also be achieved through this method by using the extraction of cuticular hydrocarbons from the flies‘ belt (Getahun, Cecchi, & Seyoum, 2014).

#### 2.2.3.1 Insects Involved in Post Mortem Interval

The contribution of insects towards determining PMI lies within four factors; the type of insects deposited on the carcass, the stages of decomposition and the duration taken at each stage as well as the temperature formed by the insects‘ colonies (Smith,



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1986). Another attribute that would also aid in the determination the PMI is the growth rate of immature insects found on the cadavers (Amendt et al., 2011). Arthropods involved in the succession of cadavers vary depending on the region (Bygarski & LeBlanc, 2013) and the season (for seasonal countries). For an example, Calliphoridae (flies) are usually present during the summer and autum whereas Coleoptera (beetles) is usually present during the winter (Benbow, Lewis, Tomberlin, & Pechal, 2013). Generally, flies, specifically the necrophagous species are known to be the most abundance arthropods followed by the beetles in the succession of cadavers (Azwandi, Nina Keterina, Owen, Nurizzati, & Omar, 2013).

In 1916, J.M Aldrich discovered that male genitals of *Boettcherisca* were useful for species identification. As for the Calliphoridae, a monograph was created by Hall in 1948 and the monograph continue to be used as an identification guide for the determination of Calliphoridae species to this day, despite various changes in the nomenclature proposed by other entomologists (Rognes, 1991; Whitworth & Rognes, 2014). In China, other than Calliphoridae and Sarcophagidae, members of the Muscidae family are also taken into consideration when deducing the PMI (Ying, Yaoqing, Yadong, Lagabaiyila, & Longjiang, 2013).

Other than flies, entomologists have also explored the possibility of using Coleoptera (beetles) (Midgley, Richards, & Villet, 2009) and Acari (mites) (Perotti, Braig, & Goff, 2009) in determining the PMI. Normally, beetles are present at the intermediate and later stages of decomposition of a carcass. Thus they are perfect for the study of the skeletal remains (Kulshrestha & Satpathy, 2001). The two families of beetles that are commonly found are the Dermestidae (skin beetles) and Cleridae (bone beetles). France forensic entomology laboratories had received 1,093 cases between 1994-2013 of which 81 cases pertain to the involvement of beetles (Dermestidae) (Charabidze, Colard, Vincent, Pasquerault, & Hedouin, 2014).



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Acari or mites on the other hand, are not so established in its use in determining the PMI as compared to the flies and beetles. The first cases of mites found concurrently with the Diptera was reported in 2016 in continental United States (Pimsler et al., 2016). Since then, mites have yet to become one of the potential factors in aiding the estimation of PMI.

To further understand how PMI is determined, one needs to understand the processes of decomposiition. According to Bornemisszа in 1957, there are 5 main stages involved in the decomposition of cadavers, each occuring within the estimated time taken during these stages. The first stage takes place from day 0 to day 2, and is known as the Initial Decay Stage. Cadavers at this stage are still looking fresh at the outer part but the internal part has already started to decay by the existing microorganisms inside the body. The second stage is estimated to occur at day 2 to day 12 which is known as the Putrefaction stage. The carcass starts to bloat due to the production of gas from the internal decaying activity together with the uncomfortable odour. The third stage is known as the Black Putrefaction stage which takes place from day 12 to day 20. At this point, the body starts to collapse and produces a very strong odor. Then at the fourth stage, which is called the Butyric Frementation stage, the body starts to dry out. Lastly, at the fifth stage (the Dry Decay stage), the rate of decay gets slower than before and the body is almost completely dried (Bornemissza, 1957).

## 2.3 Diptera

The Diptera is known as the ‗two-winged flies' and differs from other insects by only having a pair of wings. They have an adapted form of hind wings named as ‗halteres' which aids in ensuring the stability of the insects itself. Diptera also serves as one of the largest orders in insects and contributes to the wide distribution of insects on earth.



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Insects which lie under this distinctive order are small and soft-bodied insects and they have their own role in their environment. Examples of dipterans include blowflies, houseflies, horseflies, craneflies, mosquito and midges (Smith, 1986). In their habitat, mosquitoes are pests to humans and animals, blow flies act as the scavengers and Hessian flies function as pests of plants (Triplehorn & Johnson, 2005).

The three main families of flies that are involved in forensic investigations are the Calliphoridae family (blow flies), Sarcophagidae family (flesh flies) and Muscidae family (house flies) (Joseph, Mathew, Sathyan, & Vargheese, 2011). The estimation of post mortem interval can be deduced based on the temperature development from the succession of the carcass.

Medicocriminal entomology frequently utilise Diptera flies from the Calliphoridae and Sarcophagidae families because both can provide information that is vital in medicocriminal investigation. Calliphoridae flies are the most commonly found species breeding on the carcass. These flies deposited the eggs on the carrion which then turn into larvae and feed on the carrion (Smith, 1986).

### 2.3.1 Calliphoridae

Over 1000 species of these flies have been identified and they are widely distributed across the world (Byrd & Castner, 2010). The function of these flies encompasses the decomposition of cadavers and aiding in certain medical circumstances such as maggot debridement therapy (Robinson, 1935).

While there are thousands of species classified under the Calliphoridae family, in Malaysia, only a few genera are of forensic importance and these include *Chrysomya*, *Lucilia*, *Hemipyrellia*, *Calliphora* and *Hypopygiopsis* (Tan, 2012). These flies have metallic blue or green coloured body with size similar to the house fly. Many fly species



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from these families are already known and they are usually easier to identify based on morphological characteristics (Tan, 2012). A comparison study was also carried out to observe the type of flies that were present on the 34 bodies from the Universiti Kebangsaan Malaysia Medical Centre. From these bodies, they observed the type of flies that were present when the bodies were placed in two different situation and location (the indoor and outdoor) (Syamsa, Omar, Ahmad, Hidayatulfathi, & Shahrom, 2017). The dominant species that have been found from both situations were the Chrysomya species which consists of the *Chrysomya megacephala* (70.6%) followed by *Chrysomya rufifacies* 44.1%). Sarcophagidae flies on the other hand were also found on the bodies for about 38.2% with no exact species recorded.

### 2.3.2 Sarcophagidae

Flies belonging to the Sarcophagidae family (flesh flies) are morphologically similar to the untrained eye. To make matters worse, there exists some dispute over the identification and classification (as well as nomenclature) system for the Sarcophgidae (Tan, 2012). As such, further studies and research into the classification system is required to properly classify the genera and species of flies in this family. Many researches on the succession pattern of Sarcophagidae flies were conducted but the identification of the flesh fly remains scarce. One of those was the distribution study of the Calliphoridae and Sarcophagidae flies that has been carried out by Tan in 2012. From this study, 18 species of Calliphoridae and 42 species of Sarcophagidae were successfully collected within Penisular of Malaysia and Sarawak. A verification study was also conducted to observe the ability of COI and COII region of the mitochodrial gene in distinguishing 17 Sarchophagidae flies in Malaysia, 2 from Indonesia and 1 from Japan. All flies were successfully classified according to the group and generic except for the javanica. *S. javanica* was belief to be polyphelytic since *S. javanica*



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variant A was much more similar to the sequence of *S. peregrina* whereas *S. javanica* variant B was much closer to *S. krathonmai* (Tan, Rizman-Idid, Mohd-Aris, Kurahashi, & Mohamed, 2010).

## 2.4 Identification

Identification of a fly will provide information on the nature of the fly itself such as the life cycle, habitat and also the succession pattern on the carcass. The commonly used fly identification techniques are the morphological observation and examination by an entomologist and deoxyribonucleic acid (DNA) analysis by a researcher. Although morphometrics and geometric morphometrics existed since a few years ago, the applications of these methods for species identification are not as widespread as the morphological identification and DNA analysis.

### 2.4.1 Morphological Characters

Observing physical features is a qualitative way of describing the morphological characters of an organism. This method often involves comparing a shape with other similar and familiar object (Zelditch, 2012). There are 5 ways of identifying insects through morphological observation; by an expert, where the specimen is compared with an archived or labelled specimen; comparing with photograph, based on the descriptions; and lastly, using analytical keys (Triplehorn & Johnson, 2005).

However, an entomologist is not always available and the same goes for labelled specimens (Chan et al., 2014). At times, distinctive outcome cannot be acquired even with meticulous analysis if the samples are not properly preserved (Mazzanti, Alessandri, Tagliabracci, Wells & Campobasso, 2010). It is also not advisable to depend on illustrations and photographs for identification because no book could

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illustrate all insects and there are many insects which look similar to each other (Triplehorn & Johnson, 2005). While it is important to always refer to the reliable identification keys, (Smith, 1986) some of the flies are very similar and no appropriate keys are available to distinguish the species (Zajac et al., 2016). Due to these limitations, an innovative way to facilitate the identification of the species using DNA sequences has been established.

### 2.4.2 DNA-based Identification

The development of advance research tools, particularly in molecular biology, has contributed a lot to the rapid and systemic research in classic entomology (Liu & Kang, 2012). It has been established that one can also conduct molecular analysis of the DNA to determine a species based on the popular assumption that different species would be divergent in their genetic lineage, and thus have distinguishable DNA sequence. DNA analysis also aid in identifying most Sarcophagidae species due to the difficulties to be depending on the morphological characters of the flies (Meiklejohn, Wallman, & Dowton, 2011).

The standard barcode region for an animal, the *cytochrome c oxidase subunit I (COI)* from the mitochondrial gene has commonly been use in molecular identification. Other regions that have also been used for molecular analysis are the *cytochrome c oxidase subunit II (COII)* region of mitochondrial DNA (Aly, Wen, Wang, & Cai, 2012), tRNA–leucine genes (Hickey, Sperling, & Anderson, 1994), 28srDNA sequence (Friedrich & Tautz, 1997), the ITS2 region (Zajac et al., 2016) and the *period* gene Guo et al., 2014).

However, it has to be cautioned that DNA sequence analysis alone would not be sufficient to define species boundaries and often a combination of morphological



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characterization as well as supporting evidence from DNA sequence analysis would be required (Pai, Kurahashi, Deng, & Yang, 2014). It has also been reported that some 12 species of blow fly were infected by the endosymbiotic bacteria *Wolbachia* (Whitwort, Dawson, Magalon & Baudry, 2007), as such, the reliance on molecular sequence alone will not be accurate. Molecular identification also tended to damage the samples and often immature samples were used in the analysis. Furthermore in some closely related species, *COI* and *COII gene* could not assist in species identification. For example between *Chrysomya putoria* and *Chrysomya chloropyga* (Wells, Lunt, & Villet, 2004); between *Chrysomya megacephala* and *Chrysomya saffranea (Wallman, Leys, & Hogendoorn, 2005)*; and between *Lucilia coeruleiviridis*, *Lucilia cuprina*, and *Lucilia sericata* (Googe, 2014). Hwa, 2012, noted that a combination of more than one DNA region is needed to differentiate a few Sarcophagidae species.

### 2.4.3 Shape Analysis

Many identification methods had been proposed to distinguish species in animals and plants. The limitations each method has led to the discovery of new techniques to overcome the gaps and, as a consequence, assist the species identification of organism, which includes qualitative and quantitative methods.

Comparison of morphological features has been the basis of constructing the taxonomic hierarchy of most organisms for many years and at present (Adams et al., 2004). However, this technique, which is considered as a qualitative method, is arbitrary for the grouping of certain organism in the taxonomic classification. Hence, in early twentieth century, biology researches developed one quantitative method as one of the options that would assist the morphological identification (Bookstein, 1998). It is also believed that earlier works in quantitative shape analysis were initially conducted by Boas in 1905, Galton 1907 & Sneath in 1967 (Adams, Rohlf & Slice, 2013). In the mid



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twentieth century, researches have been incorporating morphological shape data with statistical data such as the analysis of variance (ANOVA), correlation coefficient and principal component analysis (PCA) to deduce the grouping of the organisms of interest—a method known as morphometrics during that era (Adams et al., 2004; Rohlf & Marcus, 1993).

The measurements of length, width and height were the elements or variables in morphometrics analysis (Adams et al., 2004). One of the obvious setbacks of morphometrics is it does not exhibit the geometry of the shape (Adams et al., 2013) and does not produce the changes of shape in the analysis (Adams et al., 2013; Rohlf & Marcus, 1993). Thus, in early 1980's, researches started to improvise a method that retain the geometry of the shape and the data of the shape—which is known as geometric morphometrics (Adams et al., 2004). Geometric morphometrics incorporates the measurements based on landmark coordinates on the surfaces of the shape and also along the outline of the curves (Adams et al., 2013).

#### 2.4.3.1 Morphometrics

Morphometrics started being implemented in shape analysis during 1960's and 1970's during which the measurements of length, width and height were commonly used. Subsequently, these were used as the form of various variables in describing shape changes among the groups of organisms (Adams et al., 2004; Rohlf & Marcus, 1993). The idea of measuring the length, depth and width was initially adapted from ichthyology text in 1962 and was later improvised into another method; the box truss (Strauss & Bookstein, 1982).

The morphometrics method, which is now known as traditional morphometrics (Marcus, 1990), applies variables in the multivariate statistical methods such as

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canonical variate analysis, discriminant functions, generalised distances, factor analysis and principal component analysis (Adams et al., 2004; Rohlf & Marcus, 1993; Zelditch, 2012). Apart from utilising the measurements of length and height, traditional morphometrics often uses ratios and angles to measure shapes (Rohlf & Marcus, 1993; Marcus, 1990).

The outcomes of these analyses produced shape changes that are also associated with size changes (Bookstein, 1985). Thus, a few size correction methods have been proposed but the issue with size correction methods are every size correction method produced inconsistent outcomes. Other than that, the usage of non-homologous point for measurement resulted in having non-homology linear distances among the samples and it is also possible to obtain the same set of measurements from different parts of the shape (Adams et al., 2004); and obtaining measurements from certain parts that would not contribute to any essential information on the shape itself (Zelditch, 2012). Lastly, linear distances measurement obviously could not produce the geometry of the shape both in traditional morphometrics and also in truss (Zelditch, 2012) since the information of the shape was not conserved during the gathering of the measurement information (Adams et al., 2004; Adams et al., 2013; Rohlf & Marcus, 1993).

#### 2.4.3.2 Geometric Morphometrics

The discovery of geometric morphometrics method arose in the attempt to answer questions about the alignment of the megalithic ‗standing stone' (Kendall & Kendall, 1980). The geometric morphometrics method improvised morphometrics methods produces accurate description quantitatively and allows the results to be visualised (Zelditch, 2012).

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Shape analysis in geometric morphometrics involves analysing outline-based, landmark-based and also surfaces data (Adams et al., 2004; Adams et al., 2013). Unlike traditional morphometrics, the data obtained in geometric morphometrics analysis are retained throughout the analysis and most intriguingly, it allows the interpretation and visualisation of the geometry of the shape (Mitteroecker & Gunz, 2009).

An outline approach is when the points are marked along the simple outlines and will be adequated into appropriate mathematical functions to compare the variations based on the coefficients of the function which act as the shape variables (Adams et al., 2004). For complicated outlines, several other methods were proposed to overcome the problem which is by using changes in the angle of tangents, analysing the Dx and Dy value and via the complex numbers obtained from the coordinates along the outlines (Rohlf & Slice, 1990). Unfortunately these proposed methods also come with their own problems since different methods ultimately produced different results (Rohlf, 1986).

Alternatively there is a landmark-based analysis, which is an approach that is focuses on the homologous point on either 2-dimensional (2D) or 3-dimensinal (3D) shape (Adams et al., 2004). It is essential to digitise appropriate and significant points or landmarks as it will affect the shape variation and visualisation during the comparison. The landmarks need to be a set of homologous landmarks among the samples; covers most of the samples‘ shape and easy to locate (Zelditch, 2012). It is quite straight forward for the 2D shape as it does not require complex equipment to capture the images. On the other hand, it is quite challenging for the 3D shape as it needs special equipment to gather the shape images before any digitisation could be made (Adams et al., 2004). For instance, the use of micro-computed tomography in capturing the orchid images, even though this data gathering method is not yet the standard method for attaining the 3D geometric morphometrics data (Niet, Zollikofer, León, Johnson, & Linder, 2010). Other methods that also have been used in acquiring



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the 3D data are the MicroScribe 3D Digitizers, Polhemus FastSCAN and the magnet resonance imaging (MRI) (Mitteroecker & Gunz, 2009).

The data collected from landmarks digitisation need to be in a standardised form to allow comparison latter. Thus, the removal of the non-shape variations is essential to allow more accurate comparisons (Adams et al., 2004). Initially, there were two methods that have been established to remove the non-shape variation for two objects; the Orthogonal Procrustes Analysis (Sneath, 2009) and the Orthogonal Resistant Fit (Siegel & Benson, 1982). For objects that have more than two, the proposed method was the Generalised Procrustes Analysis (GPA) (Gower, 1975). GPA removes the differences in rotation, translation, scale and superimposed the objects in a general coordinate system. The principle of GPA is to utilise the least-squares procedure to gather samples at the origin, scaling them via centroid unit and align the samples to reduce the total sum-of-squares of the standard formation (Adams et al., 2013). Another proposed method was the Generalised Orthogonal Resistant-Fit Analysis (Rohlf & Slice, 1990).

Visualising the results from statistical analysis through graphical presentation is the definite step in geometric morphometrics analysis. As previously mentioned, superimposition is one of the crucial steps before conducting comparison for shape variations. Thus, it is important to observe the outcome after performing the superimposition step and the scatter plot is the standard graphical presentation for viewing the superimposed outcome, which is also known as the GPA coordinates (Klingenberg, 2013). These coordinates will be used to compare the variation among the shapes and any shape changes could also be visualised through graphical presentations. Many graphical presentations have been developed; these are either based on the relative movement of the landmarks or the transformation grids. A combination both methods could also be used for visualisation of the outcomes Orthogonal



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Procrustes Analysis. Besides that, the lollipop graph, the warp outline drawing and the wireframe graph are the approaches that show the relative landmarks movement on the shape (Klingenberg, 2013).

As for the analysis of shape variables, various methods have been developed such as the ordination methods which consist of Principal Component Analysis (PCA) & Canonical Variates Analysis (CVA), Partial Least Squares, Statistics & General Linear Models (Adams et al., 2013; Querino, Moraes & Zucchi, 2002; Zelditch, 2012).

## 2.5 Application of Geometric Morphometrics

Geometric morphometrics method has been adopted in various biological field and is widely applied in zoology and anthropology studies compared to botanical studies (Niet et al., 2010). It has been assisting biologists in determining the ontogeny and phylogeny of organisms which acts as additional information to morphological data (Rohlf, 1998).

In zoology, it has been employed in the analysis of numerous body parts of animals ie., the skull in *Calomys expulsus,* which differs between male and female at different stages of age (Hingst-Zaher et al., 2000), the wing venation analysis in Stenogastrinae wasps which mostly synchronised with the existing data related to the nesting material and the architecture of the nest (Baracchi et al., 2011) and the utilisations of museum samples in flies (Hall, Macleod, & Wardhana, 2014).

Likewise, anthropology has also utilised this method in the study of bone structures in human, for instance the study of human rib cage to compare different shape changes in various ages in males (Gayzik et al., 2008), the utilisation of human mandibles for population study (Nicholson & Harvati, 2006) and also the comparison of craniofacial bones among modern human groups for regional study (Hennessy & Stringer, 2002).



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Researchers have also employed this method in the study of minute structures in human; the mapping of trabecular bone in the femur to determine the function of the trabecular bone and its locomotor activities (Sylvester & Terhune, 2016).

In botanical studies, geometric morphometrics is used to infer the taxonomy of plants from leaves (Viscosi & Cardini, 2011), floral shape variation (Tsiftsis, 2016) and also seed (Chemisquy, Prevosti, & Morrone, 2009). Three-dimensional geometric morphometrics are often used to study complex shape of the plant, for instance in the study of orchid flowers (Niet et al., 2010).

The wide application of geometric morphometrics method in various studies has been proven it as an effective tool in elucidating traits that allows the better study of organisms.

### 2.5.1 Geometric morphometrics analysis in insects

Wing morphology has been used as one of the attributes in geometric morphometrics analysis in species identification among insects (Zelditch, 2012). Insects from various orders have been classified according to geometric morphometrics analysis. For instance, in phylogenetic analysis of Stenogatrinae (Baracchi et al., 2011), in tsetse flies for distinguishing species between male and female flies (Kaba, Berté, Ta, Tellería, Solano & Dujardin, 2017) and also comparison of *Monochamus* genus from different geographical area (Rossa, Goczał, & Tofilski, 2016).

However, sometimes the findings from the wing venation analysis might not be consistent with the findings from morphological characters or DNA analysis; such as in the case of Hemerobiidae (brown lacewings) (Garzón-Orduña, Menchaca-Armenta, Contreras-Ramos, Liu, & Winterton, 2016). In the brown lacewing scenario, evolution might have occured and altered the classification of the species. Thus, it is essential to



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have supporting information rather than relying on one finding to conclude and verify the classification or speciation of organisms.

In addition to wings, other body parts from the adult and larvae stages can also be utilised for species classification in insects. For instance, surstylus and aculeus were used in distinguishing between *Rhagoletis pomonella* and *Rhagoletis zephyria.* As for maggots, researchers had utilised the cephalopharyngeal part in *Chrysomya albiceps, Chrysomya megacephala and Lucilia cuprina* for species identification (Nuñez & Liria, 2016).

### 2.5.2 Geometric morphometrics analysis in Diptera

It is evident that in dipterans, wings elicit various characteristics which are useful for species identification such as veins, wing colours and lobes character (Triplehorn & Johnson, 2005).

Wing shape in Calliphoridae, Sarcophagidae and Muscidae species exhibited variation within the closely related species and also reflects the variation in the geographical distribution (Brown, 2007). Moreover, the used of wing shape is more convincing for the study of evolution rather than wing size as it is not easily affected by environmental factors. It also proven that the wing shape and size variations are affected by several genes in *Drosophila melanogaster* (Robertson, 1962).

Within the Calliphoridae family in Venezuela; *Chrysomya albiceps* and *Chrysomya megacephala* can be distinguish via wing morphometrics (Vásquez & Liria, 2012). The same goes for other Calliphoridae species, namely *Cochliomyia hominivorax* and *Cochliomyia macellaria*. From this study, both *Cochliomyia* species could also be distinguished via wing morphometrics (Lyra, Hatadani, Azeredo-Espin, & Klaczko, 2010).



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As for Sarcophagidae, despite the usual method in analysing the morphology and also the male genital of the fly which can only be carried out by the expert entomologists, the researchers have implemented geometrics morphometrics to observe the ability of this approach in differentiating the species. For instance, this method was able to differentiate five species of Sarcophagidae flies; *Microcerella halli, Oxysarcodexia culmiforceps, Oxysarcodexia paulistanensis, Oxysarcodexia riograndensis* and *Oxysarcodexia thornax* (Silveira, 2011). Other than wings, researchers have also employing this analysis onto the larvae of the Sarcophagidae fly (Samerjai et al., 2016).

## 2.6 Molecular Analysis

Agreement between various methods of species identification increases the robustness of the outcomes. In insects, several conserved regions of DNA have been exploited for species identifications and very importantly, these regions are short but have enough variation for species identification, unique and easy to sequence (Aly et al., 2012).

The DNA regions that have been utilised for species identification include the nuclear internal transcribed spacer 2 (Merget et al., 2012), nicotinamide adenine dinucleotide dehydrogenase (Rach, DeSalle, Sarkar, Schierwater, & Hadrys, 2008), *cytochrome c oxidase subunit I (COI), cytochrome c oxidase subunit II (COII)* region of mitochondrial DNA (Aly et al., 2012), tRNA–leucine genes (Sperling, Anderson & Hickey, 1994), 28srDNA sequence (Friedrich & Tautz, 1997), ITS2 region (Zajac et al., 2016) and *period* gene (Guo et al., 2014).

Nevertheless, sometimes it is likely that the morphological traits do not agree with the findings from DNA analysis due to the integrity of the data in GenBank and also the



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occurrence of hybridization among the species (Googe, 2014). It is then important to obtain sufficient DNA sequence to distinguish the species because we may not need the whole sequence for the identification. For *COI*, instead of using the complete sequence of *COI* (1,535-base pairs); 658-bp (Meiklejohn et al., 2011) or 700-bp are sufficient for species identification (Guo et al., 2014). The same goes for the *COII* region; it is sufficient to utilise a 189-bp segment (Aly et al., 2012).

Due to the limitations of DNA analysis to distinguish several closely related species, combinations of sequences might be needed to aid the determination of species (Guo et al., 2014). For instance, the 12S and 16S region are not able to properly distinguish *Boettcherisca peregrina* from the other species of that genus (Tan, 2012). Similar problem was also seen in the efforts to distinguish *Boettcherisca peregrina and Boettcherisca nathani* via the *COI* region (Zajac et al., 2016). Thus, further research needs to be conducted in distinguishing closely related species especially in Sarcophagidae flies.

There are also cases in which DNA analysis is not applicable to distinguish fly species in the Calliphoridae family. Closely related *Lucilia cuprina* and *Lucilia sericata* are undistinguishable through DNA analysis (Wells, Wall & Stevens, 2007) and researches tried comparing the size of the genome, which are different between both flies, to distinguish both of the Lucilinii flies (Picard, Johnston, & Tarone, 2012).

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# CHAPTER 3: METHODOLOGY

## 3.1 Sampling

Samples for this research are divided into two types; archived specimens and crime scene (fresh) samples. Archived specimens were obtained from a collection made in previous studies, where flies were collected using rotten meat as bait at various types of habitat throughout Malaysia (Tan, 2012). Crime scene samples were obtained through a student attachment program with the Forensic Laboratory of the Royal Malaysian Police.

### 3.1.1 Archived Specimen

The first part of this research was conducted using archived specimens of forensically important flies obtained from the Genetics and Molecular Biology Division, Institute of Biological Science, University of Malaya, Kuala Lumpur. Archived samples were collected from the dead bodies and also collected from the succession studies. Samples were sorted and classified according to family and species. Specimens of known identity were used to establish the ―base line" information for geometric morphometrics, and would be used later as reference samples for comparison with the crime scene samples. The morphological and molecular identifications of these samples were previously described by Tan in 2012.

Table 3.1 shows selected fly species from Caliphoridae family (7 species from 2 genera) and Sarcophagidae family (8 species from 3 genera). For analysis, 95 male flies comprising 15 species were chosen. The small number of samples is due to the limited availability of the flies from archived specimens (Figure 3.1).

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Table 3.1: Species used from archived samples

| No | Sarcophagidae (sample size) | Calliphoridae (sample size) |
|---|---|---|
| 1 | *Boettcherisca javanica (5)* | *Chrysomya megacephala (10)* |
| 2 | *Boettcherisca karnyi (5)* | *Chrysomya nigripes (10)* |
| 3 | *Boettcherisca peregrine (5)* | *Chrysomya pinguis (10)* |
| 4 | *Sarcophaga ruficornis (5)* | *Chrysomya rufifacies (6)* |
| 5 | *Sarcophaga dux (5)* | *Chrysomya villeneuvi (5)* |
| 6 | *Parasarcophaga albiceps (5)* | *Lucilia cuprina (4)* |
| 7 | *Sarcophaga lopesi (5)* | *Lucilia porphyrina (10)* |
| 8 | *Parasarcophaga misera (5)* | |

**Figure 3.1: Specimen box**

### 3.1.2 Crime Scene Samples

The second part of this research involved obtaining fresh crime scene samples and conducting geometric morphometrics analysis as previously conducted on the archived specimens. We obtained permission from the Training Unit of the Royal Malaysia



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Police (RMP), Bukit Aman, to collect fly samples at the crime scenes encompassing the area around Klang Valley. In January 2016, during an attachment at the RMP Forensic Laboratory, a total of 447 insects were successfully captured at a scene near the Kg. Sungai Tekali, Hulu Langat, Selangor. A dead body was found by the villagers and police suspected that the body had been dumped at the area and probably had been dead for more than 24 hours.

**Figure 3.2: Sampling of flies at the crime scene**

All samples were captured using entomological nets and were anaesthetised using ethyl acetate at the scene (Tan, 2012). They were then pinned and killed by freezing upon arrival at the lab (Baracchi et al., 2011). Morphological identification was conducted by making comparison with the identified archived specimens, and via morphological keys (Szpila, 2010). Due to the fact that the majority of archived specimens with confirmed identity were males, only male flies were chosen for subsequent studies. The remaining samples were female flies and also other insects, were not used further.

## 3.2 Slide Preparation

Permanent slides with mounted wings were made for the archived and crime scene samples. The key objective of preparing permanent slides was because the images from the flattened wings yielded better visuals compared to the intact wings on the fly specimen (Hall, Macleod, & Wardhana, 2014).

Fly wings from the archived specimens were slightly brittle due to the age of the specimens that had been in storage since 2007 in the specimen box. Hence, the specimens were relaxed before detaching the wing by placing it inside a Perspex box with damp tissues (using water) overnight (Perrard, Baylac, Carpenter, & Villemant, 2014). The left wing of the fly was dissected using a pin, a blade and a forcep and was placed in a thin layer of Euparal and covered with a coverslip. The slide was then put inside the oven at 52°C to remove the bubbles (Hall et al., 2014).

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**Figure 3.3: Perspex box**

Each slide label contained the date of the slide preparation; the wings of a specific sex and species (same orientation for each wing) and the type of samples (archived or crime scene samples). Slides were then kept inside a slide box for future reference.

## 3.3 Image Capturing

The analysis conducted in this study only focused on 2 dimensional images of the wings. We used Moticam 2300 (Motic, China) to obtain the 2 dimensional images. These images were saved in Joint Photographic Experts Group (JPEG) format for further analysis.

## 3.4 Geometric Morphometrics Analysis

For geometric morphometrics, the landmark method was adopted and a series of softwares were used in analysing the data. These involved digitising the landmarks and subsequent analysis to observe the variation of shapes.

### 3.4.1 Thin-Plate-Spline (TPS)

The first step in geometric morphometrics via landmark method is digitising the homologous landmarks on the shape of interest. The thin-plate-spline (TPS) software was used on the wings for this purpose to obtain TPS data (Baracchi et al., 2011). Initially, the JPEG images that were previously captured had to be converted into TPS data format using the tpsUtil software. The purpose of converting these images into TPS data format was to allow the process of digitising the landmarks in tpsDig2.

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### 3.4.2 Landmarks Selection

For the present work we have chosen to use 19 landmarks as previously described in a previous study (Hall et al., 2014). All digitised images were saved in TPS data format individually for further analysis that was conducted in the R environment, via ellipse, geomorph, and monogeneaGM packages (Khang, Soo, Tan, & Lim, 2016). For GPA and PCA analysis, comparison of results was made with analysis using only 11 landmarks.

**Figure 3.4: Wing venation with 19 landmarks**

### 3.4.3 Generalised Procrustes Analysis, Principal Component Analysis & Heat Map

As previously mentioned, the TPS data was analysed using in R software package (R Foundation, Austria) and as the images were not uniform, a Generalised Procrustes Analysis (GPA) were applied, which eliminated the differences in the size, location and scale (Rohlf & Slice, 1990). Congregations of points at each landmark provided the relative movement of those landmarks within all specimens. GPA produced standard coordinates and these coordinates were used to perform a Principal Component

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Analysis (PCA) which allowed us to observe any variances that occur within the subject of study.

Principal Component Analysis (PCA) generates various shape variables in the form of PC1, PC2…PC*n*. Shape variables with more than 5% variance will contribute to the total variance percentage. Apart from PCA, we could also observe the clustering of the flies through a heat map. In this study, we utilised the data from wing venation to form a heat map to observe the species clustering.

## 3.5 Species Verification

The specific identities of both the archived and fresh specimend were further confirmed using DNA sequence analysis. Sequences obtained from this analysis were compared and corroborated with those available on public databases.

### 3.5.1 DNA Extraction

We used QIAamp® DNA Mini and Blood Mini (Qiagen, The Netherlands) extraction kit for DNA extraction with minor modifications. Two legs from each fly were placed inside a 1.5 ml microcentrifuge tubes for lysing. Twenty microliters of ATL buffer was added inside the tube containing the legs and the cap of the tube was closed and the whole tube was immersed in liquid nitrogen using a forceps for several seconds. The homogeniser stick was then used to grind and homogenise the legs in the frozen buffer and this step was repeated several times until the legs broke into tiny fragments. Subsequently, 160 microliters of ATL buffer and 20 microliters of proteinase K were added into the tube. The mixture was vortexed and incubated in a shaking water bath at 56°C overnight.

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After overnight incubation, the tube was taken out and briefly centrifuged to remove the drops from the inside of the lid. The supernatant was transferred into a new 1.5 ml microcentrifuge tube and 4 microliters of RNase A (100mg/ml) was added, mixed by pulse-vortexing for 15 s and incubated at room temperature for 2 minutes. After the incubation, the tube was centrifuged before adding 200 microliters of AL buffer (this will form white precipitate). The mixture was then mixed by pulse-vortexing for 15 s and incubated at 70°C using dry bath for 10 minutes and then was centrifuged to remove the drops from inside the lid. Then, 200 microliters of iced-cold ethanol (100%) was added into the tube, mixed via flicking and incubated for 20 minutes in -20°C to increase DNA precipitation. The tube was then centrifuge to remove the drops from inside the lid. The mixture was then transferred into a QIAamp Mini spin column with a 2 ml collection tube (provided) without wetting the rim. This column was centrifuged at 6,000 xg for 1 minute. Then the column was placed inside a new 2 ml collection tube (provided) and the collection tube containing filtrate was discarded.

Five hundred microliters (500) of AW1 buffer was added into the column without wetting the rim and centrifuged at 6,000 xg for 1 minute. Then the column was placed inside a new 2 ml collection tube (provided) and the collection tube containing filtrate was discarded. The same step as conducted with AW1 was repeated using 500 microliters of AW2 buffer but the tube was centrifuged at 16,000 xg for 3 minutes. The filtrate was discarded and the centrifuged step was repeated at the same speed for 1 minute. Then, the column was transferred into a new 1.5 ml microcentrifuge tube for elution with distilled ultra-pure water.

First elution was done using 30 microliters of ultra-pure water at 3 minutes incubation time. The tube and column were centrifuged at 6,000 xg for 1 minute and the second elution using the same column and collection tube was conducted by adding 20

microliters of ultra-pure water at 1 minute incubation time. The tube was centrifuged at 6,000 xg for 1 minute.

### 3.5.2 Polymerase Chain Reaction (PCR)

Upon obtaining the extracted DNA, the concentration of DNA and its purity was determined using Implen NanoPhotometer® P-Class (P300). Approximately 50 ng of template DNA was used for Polymerase Chain Reaction (PCR) using a pair of primers which amplified the conserved region of *COII* of mitochondrial DNA with the expected product size of 1324-bp. As shown in Figure 3.5, apart from the *COII* region, the primers were also amplifying partial region of *COI*.

PCR reagents that were included in the reaction are 1 unit of *Taq* polymerase (EURx, Poland), 1×PCR reaction buffer (EURx, Poland), 1.0 mM MgCl2 (EURx, Poland) (we did MgCl2 titration for optimisation ranging from 0.5 mM to 1.75 mM.), 200 µM dNTPs (EURx, Poland) and 10 µM of each forward (C1-J-2495) and reverse (TK-N-3775) primers (Integrated DNA Technologies, United States of America) and ultra-pure water. A negative control (without any template DNA) was also included in the PCR amplifications. For optimisation, we conducted gradient PCR with the annealing temperature ranging from 54ºC to 65 ºC, starting with initial denaturation at 94ºC for 5 minutes, 35 cycles at 94ºC for 1 minute, annealing with gradient temperature for 1 minute and 30 seconds, elongation at 72ºC for 2 minutes and final elongation at 72ºC for 5 minutes.

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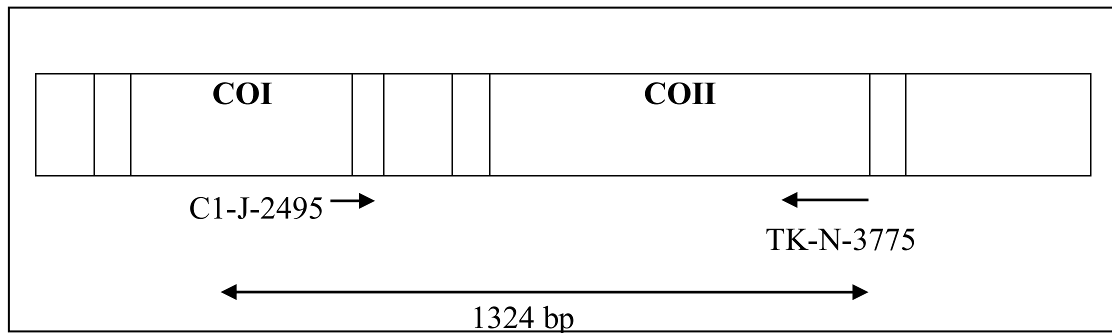


**Figure 3.5: Schematic diagram of the COI and COII mitochondrial DNA region. The size of the amplified region is 1324 bp (Tan, 2012; Steven &Wall, 2001).**

**Table 3.2: Primer sequences for amplification of partial *COI* and *COII* mitochondrial DNA region (Tan, 2012).**

| Primer | Sequence (5'-3') |
|---|---|
| C1-J-2495 | CAGCTACTTTATGAGCTTTAGG |
| TK-N-3775 | GAGACCATTACTTGCTTTCAGTCATCT |

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### 3.5.3 Gel Electrophoresis of PCR products

The PCR products were separated via gel electrophoresis using 1% agarose gel together with 100 base pairs ladder and viewed under ultraviolet illumination via the Perkin Elmer Geliance 200 Imaging System after staining with ethidium bromide. We were expecting to observe the size of 1324 base pairs from the gel.

### 3.5.4 Purification of PCR products via Gel Extraction

A sufficient amount of PCR products were used (60-80) in 1% agarose gel in gel electrophoresis with approximately 100V of current for 1 hour. The gel was viewed and

a specific band was excised with the aid of UV light or blue light. The excised gel was then transferred into an empty 1.5 ml microcentrifuge tube (weigh before putting the gel in) and the tube was then weighed again to infer the weight of the gel.

We used the QIAquick® Gel Extraction Kit for the gel extraction. Firstly, three (3) volumes of Buffer QG to 1 volume of gel (100 mg gel ~ 100) were added into the tube with maximum amount of 400 mg gel. The gel with the Buffer QG was incubated at 50ºC in a heat block for 10 minutes and then vortexed at every 2-3 minutes until the gel had completely dissolved. Then, 1 volume of isopropanol was added into the tube and the mix. The mixture was transferred in a column at the maximum volume of 750 microliters and centrifuged at 15,000 xg for 1 minute. The filtrate in the collection tube was discarded. This step is to be repeated if the mixture volume exceeds 750 microliters. Five hundred microliters (500) of Buffer QG was added into the column and centrifuged at 15,000 xg for 1 minute and the filtrate in the collection tube was discarded. To wash, 750 microliters of Buffer PE was added into the column and incubate at room temperature for 5 minutes before centrifuging at 15,000 xg for 1 minute and the filtrate in the collection tube was discarded. The centrifuging step was repeated at the same speed for 1 minute. For elution, the column was transferred into a new 1.5 ml micro centrifuge tube and 30 microliters distilled ultra-pure water was added with 5 minute incubation time. The tube was centrifuged at 15,000 xg for 1 minute.

Gel extraction product underwent gel electrophoresis using 1% agarose gel together with 100 base pairs ladder and viewed under the Perkin Elmer Geliance 200 Imaging System. We were expecting to observe a single band with the size of 1324 base pairs from the gel. For any presence of other unspecific band, we incubated the gel extraction product at 99ºC for 5 minutes and conducting gel electrophoresis to confirm the unspecific band was not present in the purified PCR product.

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### 3.5.5 Sequencing

The gel extraction product was then used in direct sequencing. 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 manufacture‘s recommendations. All DNA samples were sequenced for both forward and reverse DNA strands using the forward and reverse primers as previously used in the PCR amplification. 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. The total volume of reaction (20 microliters), concentration of DNA (50 ng) used and primer (3.2 pmol). Also, the method used to purify the cycle sequencing product is ethanol/EDTA precipitation method.

### 3.5.6 Data and Phylogenetic Analysis

Sequenced DNA was edited manually using the Chromas 2.6 to remove the primer regions and were aligned using BioEdit Sequence Alignment Editor Version 7.2.5. A phylogenetic tree via Neighbor-Joining and General Time Reversible+Gamma (TN93+G) model was constructed using Mega 6 software with 1000 bootstrap replications. The outcomes from this analysis were compared with the wing data to observe the corroboration between geometric morphometrics and molecular analysis in species determination.

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# CHAPTER 4: RESULTS

This research was carried out to evaluate the use of geomorphometrics morphometrics for the identification of selected forensically important fly species within the families Calliphoridae and Sarcophagidae. As previously mentioned two sets of samples were used for analysis: i) archived samples, and ii) samples collected freshly from a crime scene (done with the help of PDRM). Analysis results obtained from archived samples (identified specimens) will serve as reference to determine if this approach can facilitate the identification of freshly collected samples.

## 4.1 Samples

As outlined in the methods section, for the archived samples, a total of 95 specimens (representing 15 species, 5 genera and 2 families) were included in this study. One wing was selected and mounted permenantly on slides as shown in Figure 4.1. The anatomical description of the venation pattern is given in Figure 4.2.

Crime scene samples acted as the test samples to the archived samples; in which the comparisons of these samples were aimed to observe the consistency of species grouping via wing venation with the species grouping via morphological characters.

**Figure 4.1: Slides for archived and crime scene samples**

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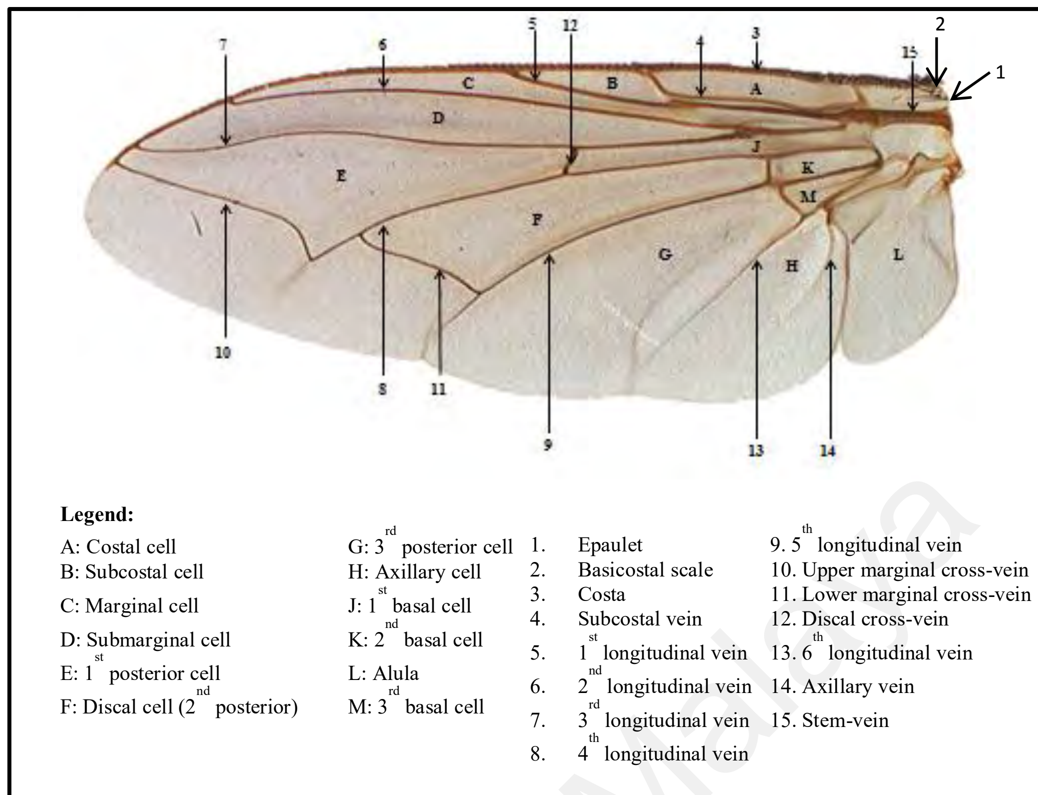


**Figure 4.2: Wing veins and partitions (White, Aubertin, & Smart, 1940)**

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The wing slides were then used to capture images of the wings, upon which 19 landmarks (see Figure 3.4) are identified for each of the wings, digitised, and analysed using the workflow described in sections 3.4 until 3.5.

## 4.2 Archived Samples

As previously mentioned, archived samples functioned as the reference samples for comparison with the crime scene samples later.

### 4.2.1 Generalised Procrustes Analysis

Upon digitising the landmarks on the wing images and obtaining the TPS data, all landmarks coordinates of the wings were subjected into Generalised Procrustes Analysis (GPA) to remove the variance in scale, position and orientation (Rohlf & Slice, 1990). Landmarks coordinate graphics after GPA is as shown in Figure 4.3. It is evident from the scatter plot that all 19 landmarks were consistently identifiable in all specimen wings, and no anomalous coordinates were detected after GPA.

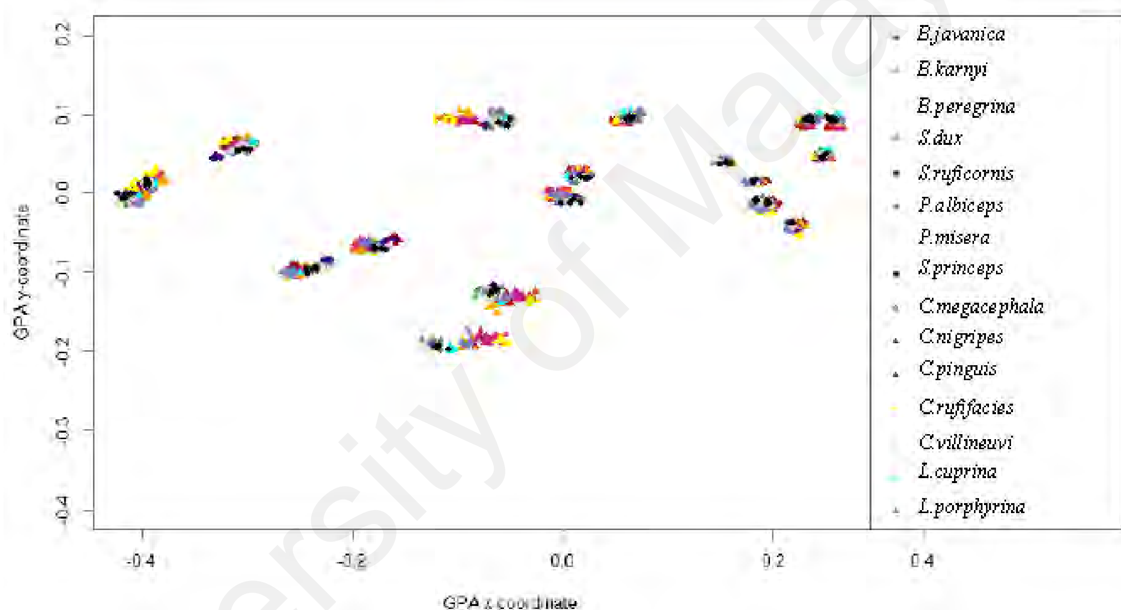


**Figure 4.3: Scatter plot of 19 landmarks after performing GPA. Different colors represent different fly species.**

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### 4.2.2 Principal Component Analysis and Heatmap

Sets of GPA coordinates were then used in Principal Component Analysis (PCA) that generates various shape variables. PCA revealed three (3) shape variables having more than 5% variance; PC1 (62%), PC2 (13%) and PC3 (5%), and taken together these meet a total of 80% of the variance percentage. Plotting the PCs (PC1 against PC2, and PC1 against PC3) on a 2D plot showed an obvious and expected result; that flies from

Calliphoridae formed a distinct group from Sarcophagidae at family level (Figure 4.4). Zooming in at the generic and species level however (PC1 and PC2, above, left panel, Figure 4.4), although fly species within a particular genus of the Calliphoridae family tend to show groupings around their centroids, there are however some overlaps with other species, that would make accurate delineation of a species boundary rather difficult. In contrast, Sarcophagidae fly genera and species did not show any obvious groupings, and even the centroids overlaps. This indicates its little usability of this approach to distinguish different species, or genera.

One interesting observation that could be made is that flies in the genus Lucilia (*Lucilia cuprina* and *Lucilia porphyrina*) appears to be in a distinct group, separate from flies of Calliphoridae and Sarcophagidae. Lucilia is currently identified as part of the Calliphoridae, and this is supported by both morphology and DNA studies (Tan, 2012).

Using all PCs together in 3 dimentional plot only serves to support previous findings, that

i) Calliphoridae and Sarcophagidae flies are distinguishable in separate groups,
ii) although centroids are clearly separated, individual flies may overlap suggesting that species identification using this approach for Calliphoridae flies may not be easy for some species
iii) This approach is not suitable for Sarcophagidae flies as the centroids of some species overlap
iv) Flies from genus Lucilia forms a separate group from Calliphoridae and Sarcophagidae, eventhough Lucilia has been assigned to the family Calliphoridae through morphological and DNA analysis.



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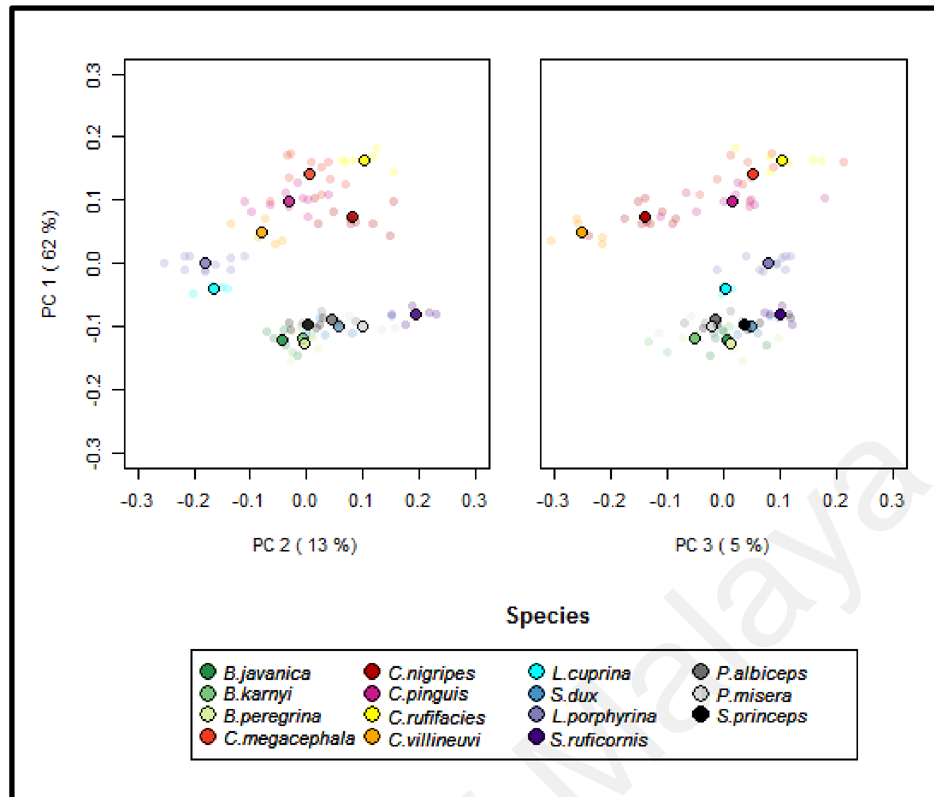


**Figure 4.4: A representation of 2D (above) and 3D (below) PCA graph of Calliphoridae and Sarcophagidae flies. Solid colored circles are the centroids of the species.**

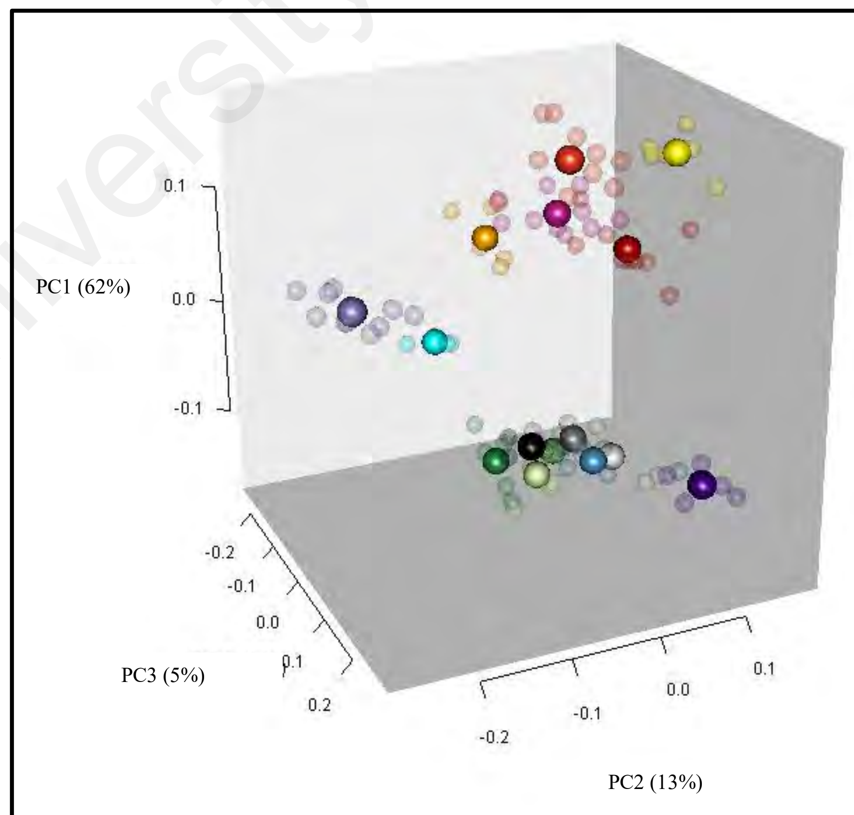


**Figure 4.4, continued**

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A heat map was generated as an alternative way to view clustering of wing venation data, and this is shown in Figure 4.5. With the exception of *Lucilia cuprina* (turqiose), all Calliphoridae flies are groups in the same clade, and all flies have been grouped correctly according to their species. As expected, flies within the Sarcophagidae family were only correctly grouped to their family clade, while the grouping of the fly species within the subclades are very inconsistent. These results corroborate previous observation that geometric morphometrics of wing veins are not able to correctly separate flies of the Sarcophagidae family according to their species.

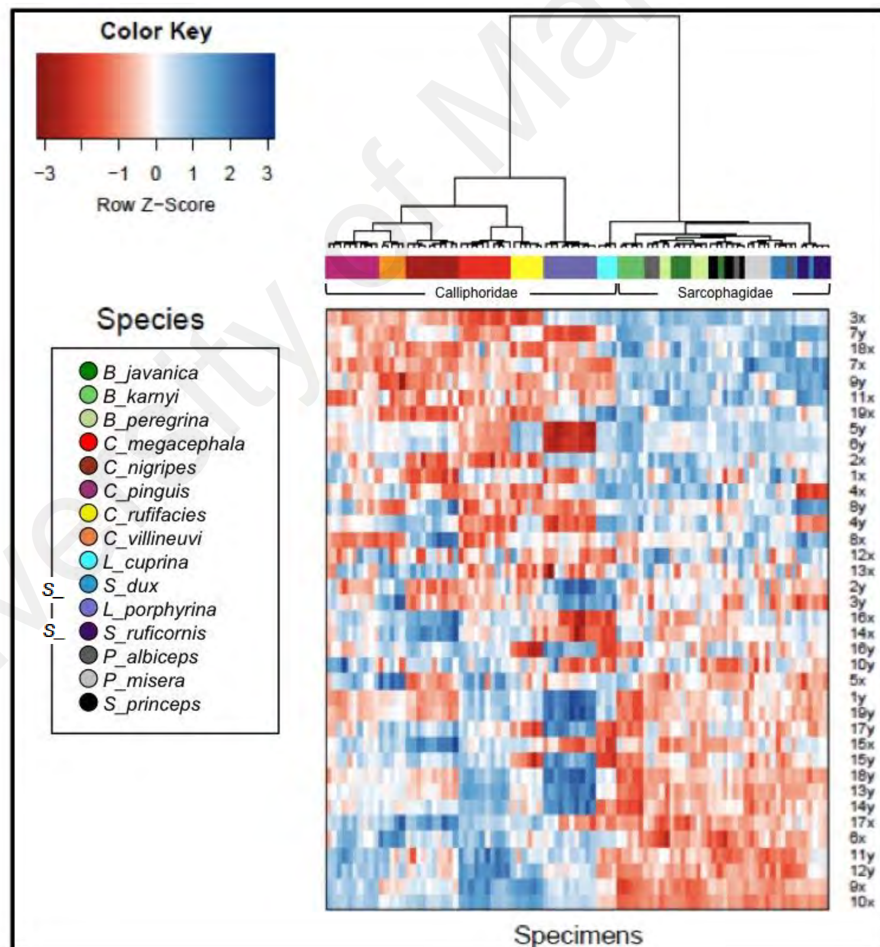


**Figure 4.5: A heatmap of the wing venation of Calliphoridae and Sarcophagidae flies.**

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### 4.3 Crime Scene Samples

A similar workflow used for archived samples are then used for test samples obtained from a crime scene. The purpose of this is to assess whether the geometric morphometrics approach can be used to identify flies to the species level. A total of 26 Calliphoridae and 10 Sarcophagidae flies from crime scene samples were used as test samples in this analysis.

#### 4.3.1 Principal Component Analysis

GPA was conducted on both samples (archived and crime scene samples) before performing the PCA (Figure 4.6). Two factor PCA plot showed that the crime scene samples were distributed into 2 major families; Calliphoridae and Sarcophagidae. Within Calliphoridae, most crime scene samples clustered among the *Chrysomya* genera; whereas for Sarcophagidae; among the *Sarcophaga* genera. None were distributed among the *Lucilia* genera in Calliphoridae and *Boettcherisca* genera in Sarcophagidae family. However, determinations of the species were not straightforward, even for Calliphoridae flies. The species identity was determined by looking at the position of the closest centroid. While *r (C. rufifacies)* and *m (C. megacephala)* identities are quite unambiguous, the same cannot be said for some *C. nigripes* and *C. pinguis* because their overlapping position in the plot. Their identities were later confirmed after analysis by morphological studies, because *C. nigripes* and *C. pinguis* are quite easy to distinguish based on morphological characteristics.

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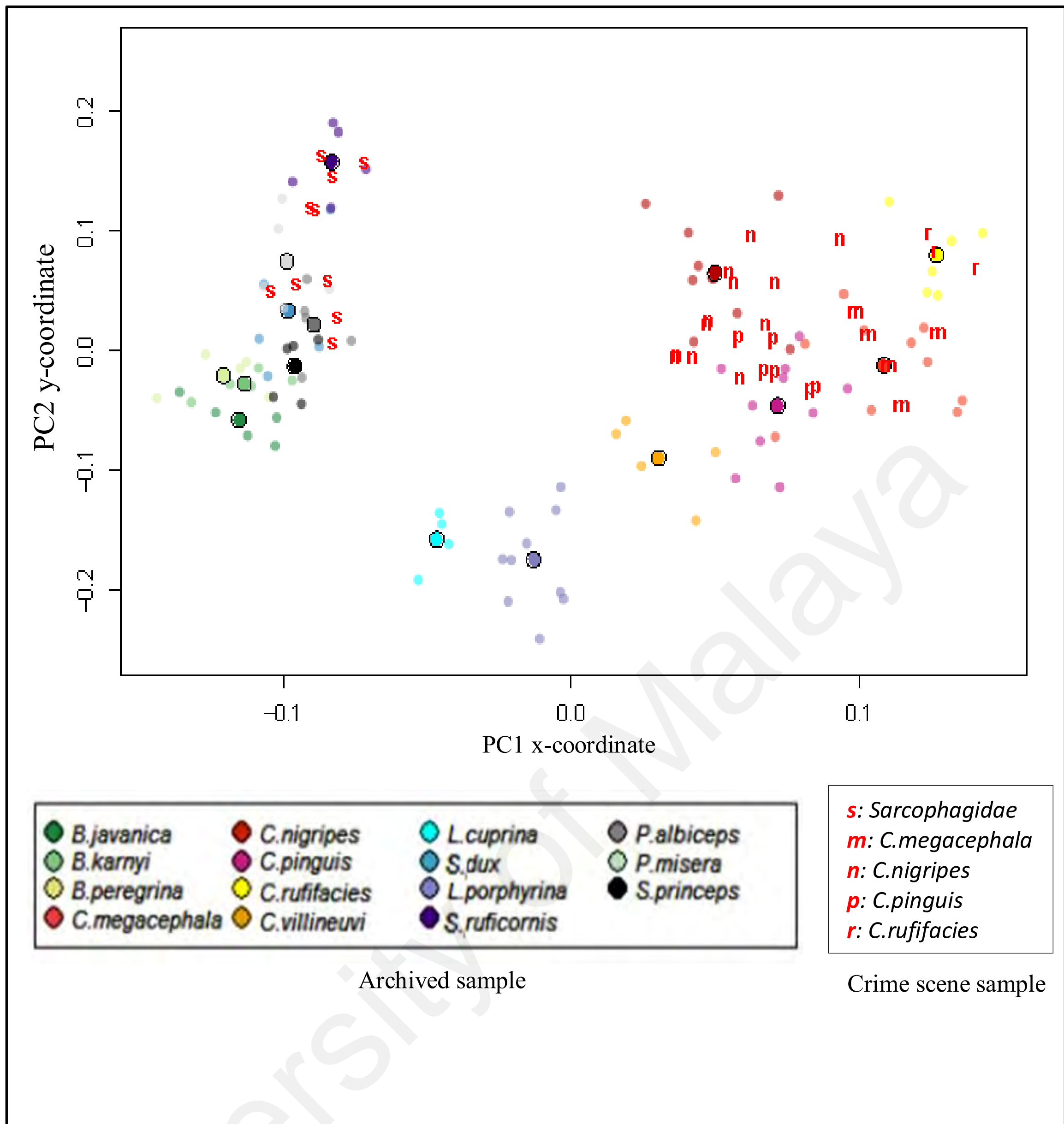


**Figure 4.6: The PCA of the archived samples with crime scene samples (n=36).**

### 4.3.2 Molecular Analysis

Molecular analysis was conducted for verification of the test samples. Only few samples were randomly chosen for the analysis, because for some species the morphological features are quite obvious for species determination.

#### 4.3.2.1 DNA Extraction

Below are the samples used for DNA extraction for the crime scene samples of Calliphoridae and Sarcophagidae. We only conducted some representatives of the male samples from the scene with the total amount of 14 samples.

**Table 4.1: Samples of Calliphoridae and Sarcophagidae**

| Family | Sample No |
|---|---|
| Calliphoridae | 420 |
| | 421 |
| | 427 |
| | 428 |
| | 429 |
| | 465 |
| | 466 |
| | 467 |
| | 497 |
| | 501 |
| | F3(2) |

| Family | Sample No |
|---|---|
| Sarcophagidae | 9 |
| | 13 |
| | 21 |
| | 34 |
| | 41 |
| | 53 |
| | 111 |
| | 116 |
| | 120 |
| | 123 |

#### 4.3.2.2 Polymerase Chain Reaction

Extracted DNA from the test samples in Table 4.2 were used to conduct Polymerase Chain Reaction (PCR) followed by sequencing. Optimisation of PCR conditions showed that the best annealing temperature from the gradient PCR was at 57ºC and for MgCl2 titration, the concentration of 1.0 mM was the ideal concentration for the PCR amplification. Primers uses for the PCR were COII primers.

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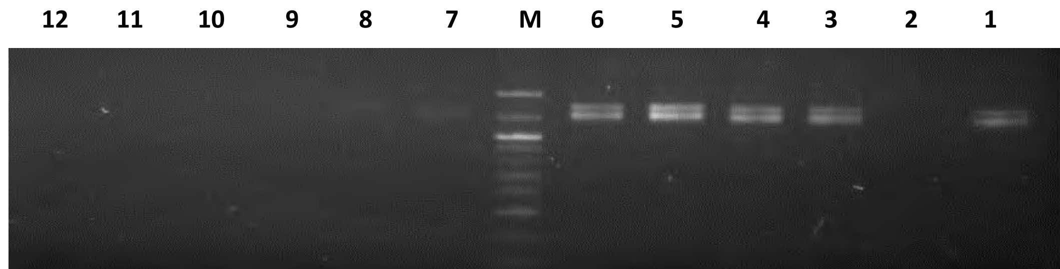


**Figure 4.7: Gradient PCR amplification (ranging from 54°C-65°C). Lane 1: 54°C, lane 2: 54.2°C (control), lane 3: 54.8°C, lane 4: 55.7°C, lane 5: 56.9°C, lane 6: 58.4°C, lane 7: 60.3°C, lane 8: 61.9°C, lane 9: 63.1°C, lane 10: 64.2°C, lane 11: 64.7°C and lane 12: 65°C. Lane M: 100bp DNA Ladder (Seegene, Korea).**

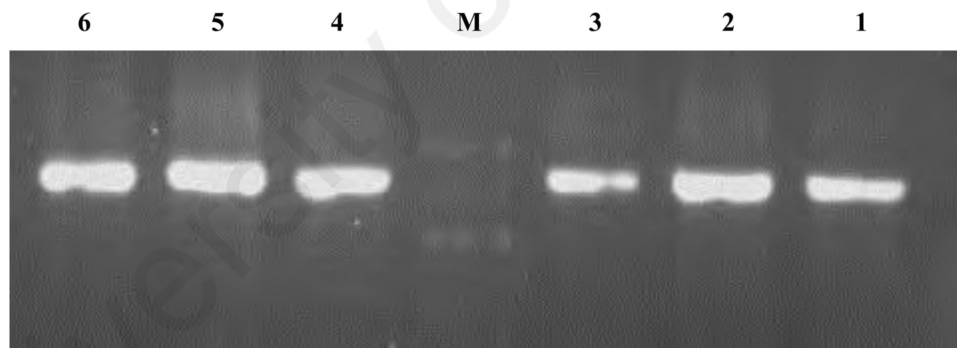


**Figure 4.8: $MgCl_2$ titration for PCR amplification. Lane 1: 0.5mM, lane 2: 0.75mM, lane 3: 1.0mM, lane 4: 1.25mM, lane 5: 1.5mM and lane 6: 1.75mM. Lane M: 100bp DNA Ladder (Seegene, Korea).**

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#### 4.3.2.2.1 PCR amplification of the COII sequence for Calliphoridae flies

Below are the gel electrophoresis images from the PCR amplifications of Calliphoridae samples (Figure 4.9 to Figure 4.12).

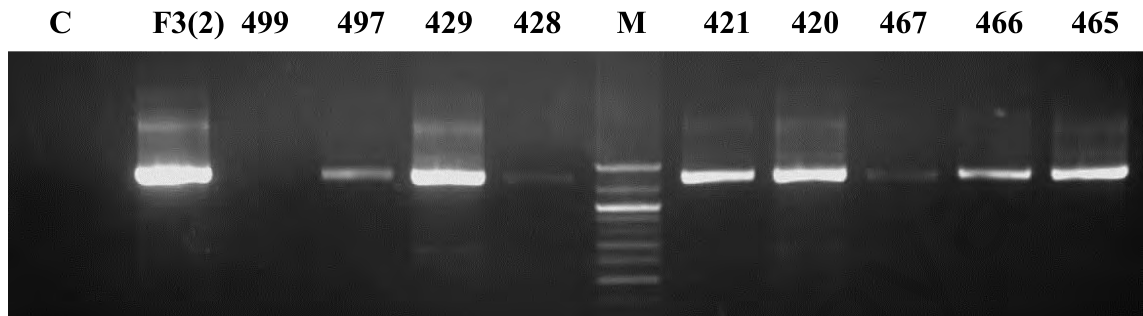


**Figure 4.9: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification produc is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

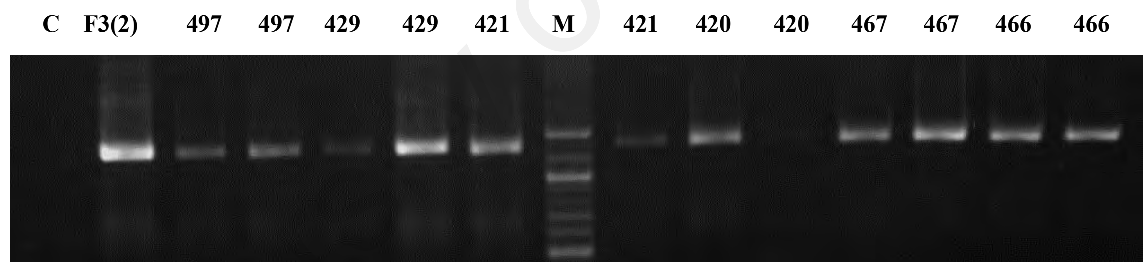


**Figure 4.10: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification produc is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

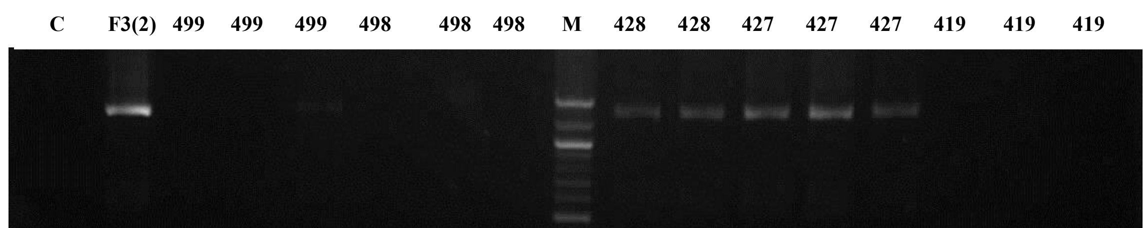


**Figure 4.11: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification produc is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

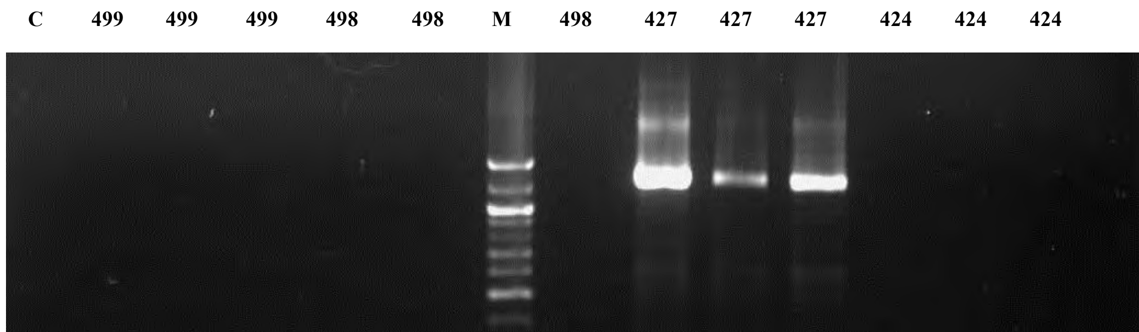


**Figure 4.12: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification produc is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

#### 4.3.2.2.2 PCR amplification of the COII sequence for Sarcophagidae flies

Since we were not able to determine the species identity of flies from Sarcophagidae via morphological characters, DNA analysis was used as result from sequencing analysis have been shown to be more definitive. Figure 4.13 to 4.15 showed the images of PCR amplifications using forward (C1-J-2495) and reverse (TK-N-3775) primers for Sarcophagidae flies, prior to sequencing analysis.

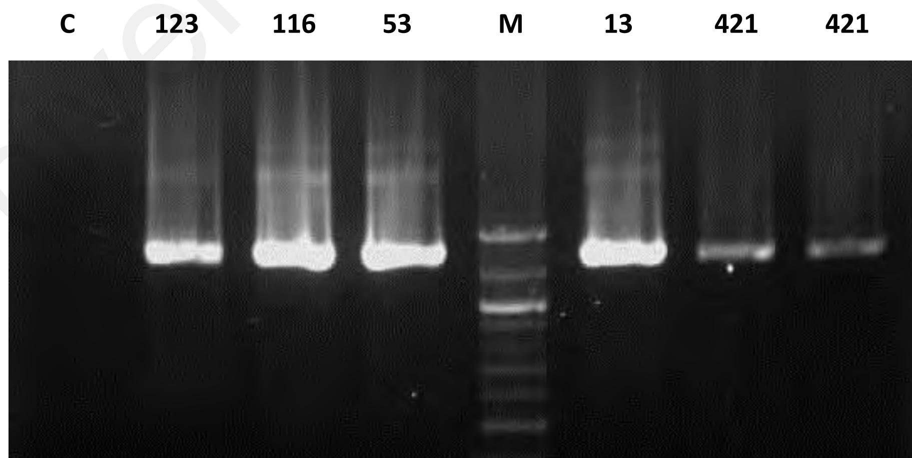


**Figure 4.13: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification produc is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

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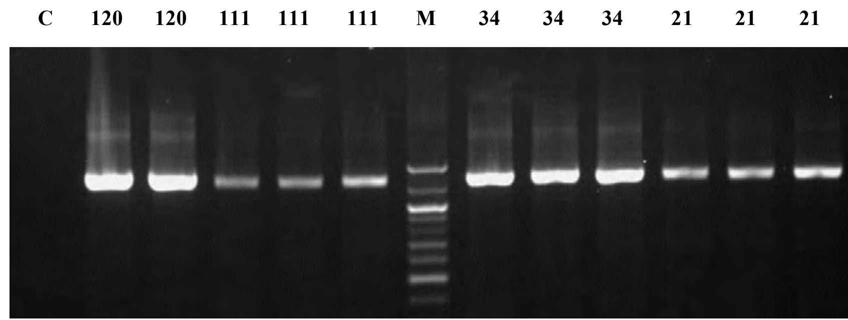


**Figure 4.14: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification product is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

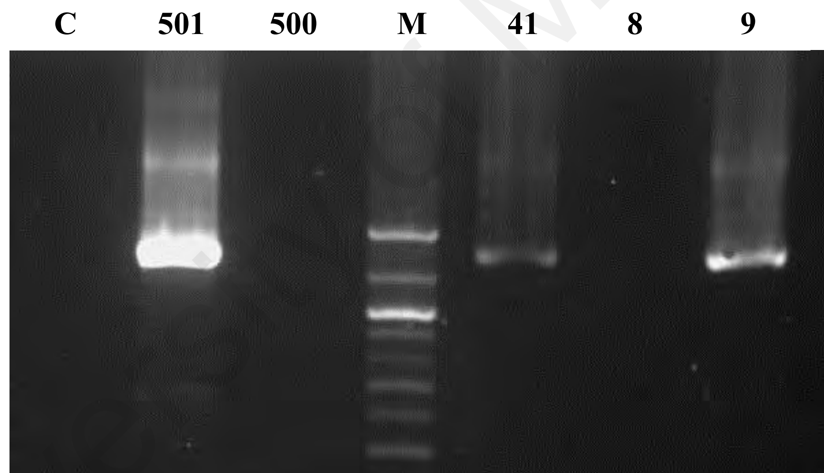


**Figure 4.15: PCR amplification using C1-J-2495 and TK-N-3775 primers. The expected size of amplification produc is 1324bp. Lane C is the negative control. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

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#### 4.3.2.3 Gel Extraction

Purified PCR products were obtained through the gel extraction processes and the PCR products were subsequently used for sequencing.

##### 4.3.2.3.1 Calliphoridae

Figure 4.16 to 4.19 showed the successful purification of PCR product of Calliphoridae samples from gel electrophoresis. Although for some samples there are traces of high molecular weight bands, these are negligible as they did not compromise the clarity of sequencing data obtained in subsequent steps (Figure 4.18).

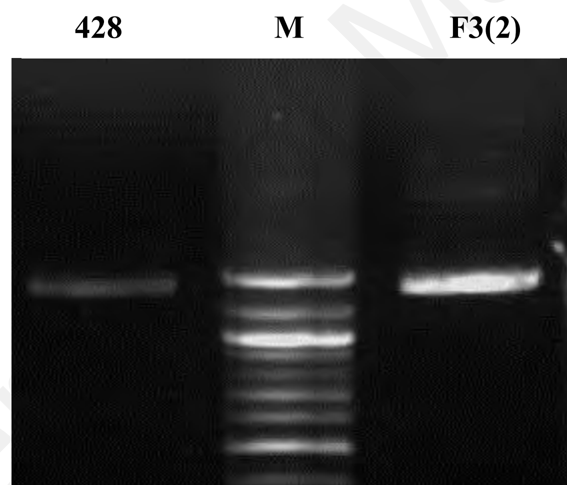


**Figure 4.16: Purified PCR products via C1-J-2495 and TK-N-3775 primers (1324-bp) from gel extraction. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

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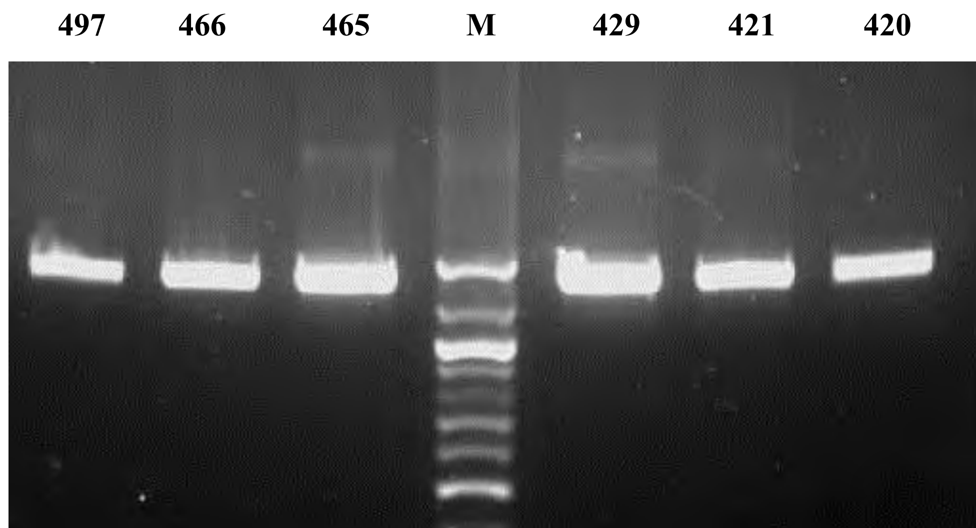


**Figure 4.17: Purified PCR products via C1-J-2495 and TK-N-3775 primers (1324-bp) from gel extraction. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

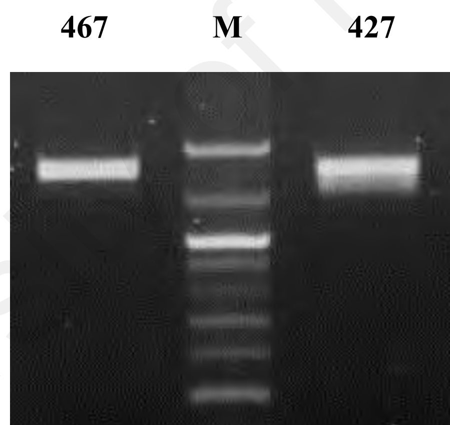


**Figure 4.18: Purified PCR Products via C1-J-2495 and TK-N-3775 primers (1324-bp) from gel extraction. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

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#### 4.3.2.3.2 Sarcophagidae

Figure 4.20 to 4.24 showed the purified PCR product of Sarcophagidae samples from gel electrophoresis. Similarly, for some samples there are traces of high molecular weight bands, but these are negligible as they did not compromise the clarity of sequencing data obtained in subsequent steps (Figure 4.22 and 4.24).

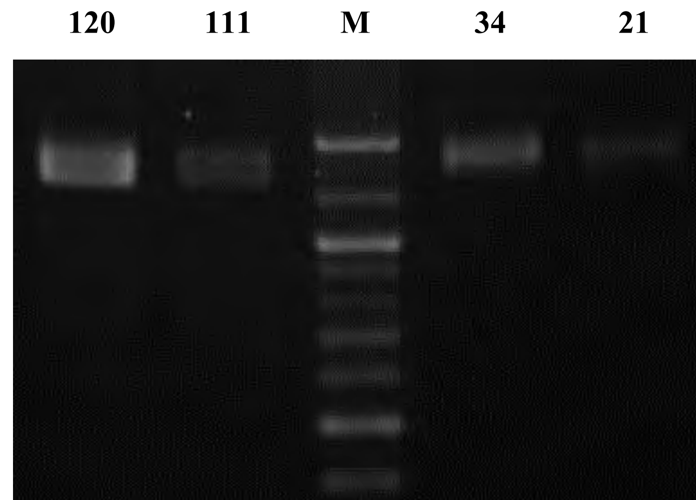


**Figure 4.19: Purified PCR products via C1-J-2495 and TK-N-3775 primers (1324-bp) from gel extraction. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

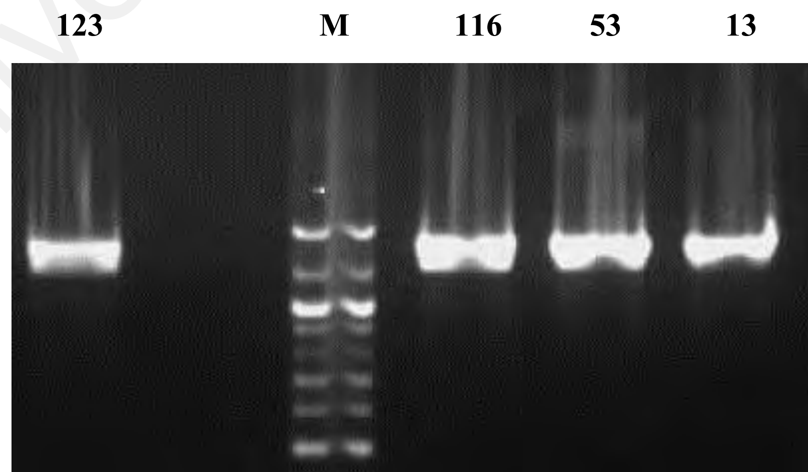


**Figure 4.20: Purified PCR products via C1-J-2495 and TK-N-3775 primers (1324-bp) from gel extraction. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

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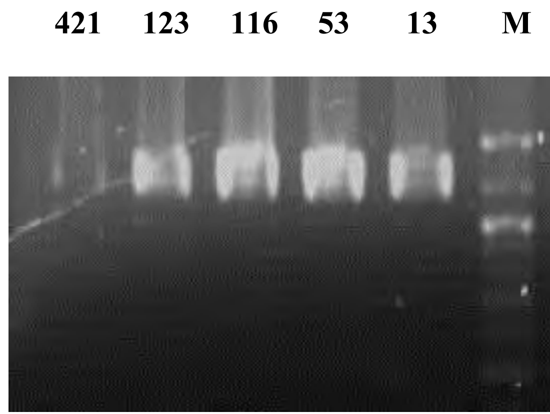


**Figure 4.21: Purified PCR products after heating at 99ºC. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

**Figure 4.22: Purified PCR products via C1-J-2495 and TK-N-3775 primers (1324-bp) from gel extraction. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

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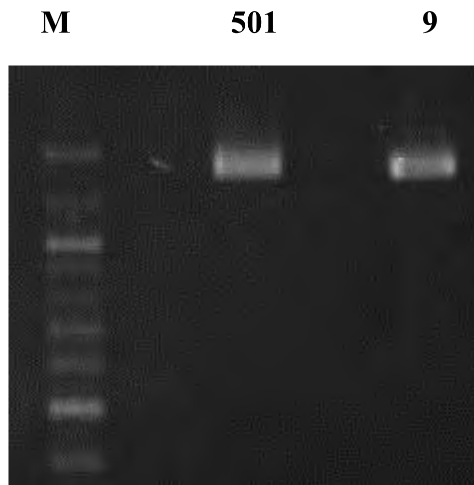


**Figure 4.23: Purified PCR Products after heating at 99ºC. Lane M: 100bp DNA Ladder (Seegene, Korea). Other lane names refer to specimen ID.**

#### 4.3.2.4 Sequencing

We had successfully obtained partial sequences of *cytochrome c oxidase* subunit II genes ranging from 521bp to 692bp from 14 crime scene samples. These samples comprises of 2 families; 6 samples were from Calliphoridae and 8 samples were from Sarcophagidae. Six (6) Calliphoridae flies were determined to be from the *Chrysomya* genus which comprises 3 species (*Chrysomya nigripes, Chrysomya megacephala* and *Chrysomya rufifacies*) and 8 Sarcophagidae flies were comprises of 4 species (*Sarcophaga dux, Sarcophaga princeps, Sarcophaga ruficornis* and *Sarcophaga taenionota*). A total of 21 sequences were used for phylogenetic tree construction, which included 7 sequences from GenBank with accession numbers shown in Table 4.3 as the reference sequences.

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**Table 4.2: Species name and accession**

| Species | GenBank Accession Number |
|---|---|
| *Chrysomya nigripes* | KT444441.1 |
| *Chrysomya megacephala* | KR820765.1 |
| *Chrysomya rufifacies* | KF997672.1 |
| *Sarcophaga dux* | EF405939.1 |
| *Sarcophaga princeps* | EF405948.1 |
| *Sarcophaga ruficornis* | JN571552.1 |
| *Sarcophaga taenionota* | KM279656.1 |

#### 4.3.2.5 Data and Phylogenetic Analysis

An estimation of best fit model for *cytochrome c oxidase* subunit II genes was conducted and Tamura-Nei+General Time Reversible (TN93+G) were determined to be the best model. The base frequencies for the model were; A = 0.3362, T = 0.3969, G = 0.1253 and C = 0.1417. A phylogenetic tree via Neighbor-Joining tree was constructed and the bootstrap values were shown at each branch (Figure 4.25). The consistent grouping (in the clades) of the test samples with reference sequence was taken to show that correct specied identification of the test samples has been achieved.

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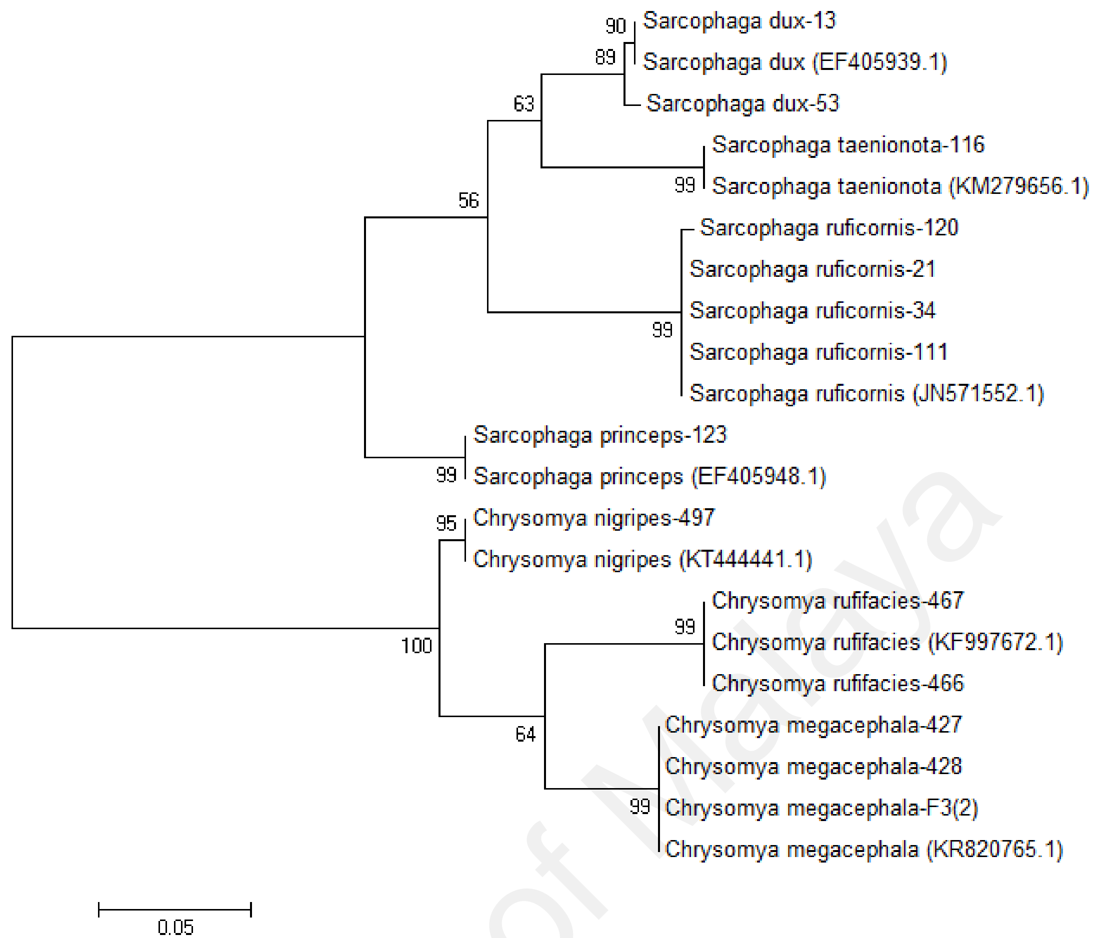


**Figure 4.24: A neighbour-joining tree constructed by MEGA using a TN93+G model based on 521 bp *COII* sequences with 1000 boostrap replicates (percentage is as shown at the tree branch). The numbers at the end of the species names indicate the sample numbers and the accession number as well.**

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# CHAPTER 5: DISCUSSION

In recent times forensic entomology has become more important and had contributed considerably in assisting criminal investigation processes. Insects, especially flies (Amendt et al., 2011) act as the main character in forensic entomology and can be used as a tool for estimating minimum time since death in homicide cases. Crucial to determining the success of using forensic entomology to facilitate investigation is the ability to correctly and quickly identify the specimens, prefereably up to the species level.

Morphology and DNA analysis have been known as the common identification method for insects. However, morphological methods are usually suitable for adult flies and not for maggots, simply because adults offer more discernable characters. Identification of larval stage specimens usually require rearing to adulthood, and rearing maggots to adult for the purpose of identification is time consuming (Mazzanti et al., 2010). The other apparent limitation is the dwindling number entomologists, specifically with expertise in larval identification. For instance, there are parts of the apod larvae of the flies which are hardly distinguishable even at the order level by the expert entomologist (Gullan & Cranston, 2014).

Thus, an alternative solution to this situation is to do the identification by conducting DNA analysis and comparing the DNA sequence. While DNA based identification has received much praise, there are also inherent constraints. From previous research, the COI DNA sequences obtained for *P. dux* was found to be divided into 3 subgroups, and the sequences were also observed to be highly variable within these three subgroups although were highly similar among the individual groups (Pai et al., 2014). This situation indicates that the DNA might not be as useful for some closely related fly species as reported in some Sarcophagidae fly (Jordaens et al., 2013). There had been

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also cases for which the morphological identification of the flies did not match the identification results obtained from DNA sequences using the COI gene. For example, the morphological identification of 2 out of the 31 Lucilia flies were not aligned with the DNA sequence identification in which the morphology indicated that they are *L. coeruleiviridis* but the genetic sequence shows that they belong to *L. mexicana* and *L. sericata* (Googe, 2014). In another unexpected situation, a fly with the morphology of *L. cuprina* was carrying the COI sequence of *L. sericata* but possess the 28 S rRNA sequence of *L. cuprina* (Amendt et al., 2011).

Hence, researchers continue to search for the optimal identification method and of late, considerable attention has been given to geometric morphometrics. As previously explained, this method in insect‘s identification was derived from traditional morphometrics and wings were the most common part of the insects that have been used for this purpose. Wings have been used as one of the tool for identification of insects since 1899 to differentiate between order and family (Comstock & Needham, 1898), and at present, it has been widely used to determine the genus and species of the insects. Futhermore, it requires less time to prepare and analyse the images of the wing as the equipments required for the analysis are handy and this type of analysis can be conducted in the field (Perrard et al., 2014).

In a previous published study, it has been proven that wing shapes were sufficiently variable to allow discrimination of wasps at the genus and species level. It also allows the construction of a phylogenetic classification based on the wings shape which is also parallel with the architecture and the material of the wasps‘ nest (Baracchi et al., 2011). Apart from that, it can also be used in the study of evolutionary of the hornets but this still requires further studies of the insect itself and also the contribution of the environment towards the evolutionary aspect of the shape (Perrard et al., 2014). Other advantage of using geometric morphometric is that 3D graphic images could also be



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obtained using a special scanner, for example the micro-computed tomography scanner, before analysing the images. Such application has been used on the orchids to observe the variation of the flower shape (Lee, Kuo, Hsu, & Wang, 2014) which is essential for the study of evolution of the plant (Niet et al., 2010).

The application of geometric morphometrics on flies had been widely used among the Calliphoridae flies. For instance, due to the morphological similarity of *Cochliomyia hominivorax* and *Cochliomyia macellaria*, a research study using the wings of both species was conducted to observe the ability of this tool to distinguish the species (Lyra et al., 2010). This method also allows the researches to observe the variation of wing at the subcosta rupture, join of R2+3 with wing border, join of dm-cu, and join of Cu with bm-cu, which are the main points that allow the tool to distinguish *Chrysomya albiceps* and *Chrysomya megacephala* in the Venezuela (Vásquez & Liria, 2012b). Apparently, geometric morphometrics not only helps in the identification of Calliphoridae; it also facilitates the study of the fly population (Hall et al., 2014). As for Sarcophagidae flies, such method was implemented on 5 species of fly *(Microcerella halli, Oxysarcodexia culmiforceps, O. paulistanensis, O. riograndensis, O.thornax)* in Curitiba, Brazil and the results indicated that they were able to distinguish the genera, species and sex of the flies (Silveira, 2011).

Geometric morphometrics offers landmark and/or outline method but in the current research project, landmark method was used to measure the wing using 19 landmarks. Nineteen landmarks (19) were chosen to maximise and retain the geometry of the shape as much as possible (Hall et al., 2014). Choosing the landmarks and numbers of landmark are essential when applying geometric morphometrics method. There are 3 major criteria in selecting the landmarks (Zelditch, 2012). Firstly, the landmarks have to be homologous. Homologous means that the point on every shape is similar; which will allow the comparison of shapes among the individuals. It is also important to



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consistently digitize at the same points to avoid any error that could contribute to incorrect data coordinates. The second criteria concerns choosing the appropriate number of landmarks. It is best to choose landmarks that retain the geometry of the shape since one could actually visualize the pattern of the shape before analyzing it through geometric morphometrics analysis. Landmarks also act as information on the shape and sufficient coverage on the shape would evetually contribute to the detection of shape changes (Zelditch, 2012). For the comparison of shape variation within Calliphoridae flies, previous researchers had utilized 8 landmarks (Vásquez & Liria, 2012), 16 landmarks (Lyra et al., 2010) and also 19 landmarks (Hall et al., 2014). Wing variation was observed between *Chrysomya albiceps* and *Chrysomya megacephala* using 8 landmarks. On the other hand, population study of *Cochliomyia hominivorax* and *Cochliomyia macellaria* showed both species were successfully distinguished through 16 landmarks despite the closely similar morphological appearance between both species. The geographical origin and also the identification of the *Chrysomya bezziana* was successfully obtained through the used of 19 landmarks.

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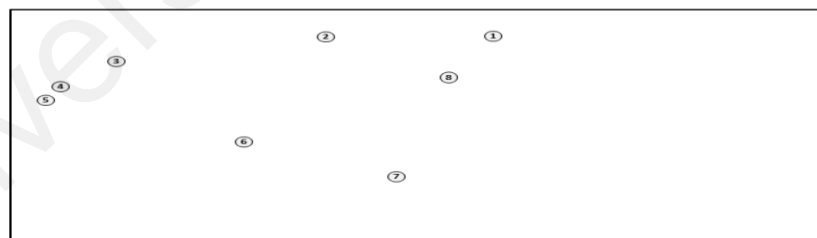

**Figure 5.1: Wing geometry with 8 landmarks**

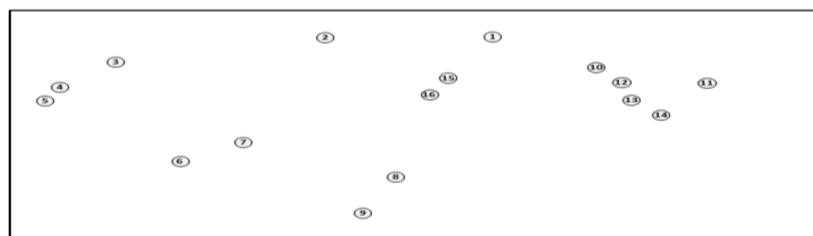

**Figure 5.2: Wing geometry with 16 landmarks**

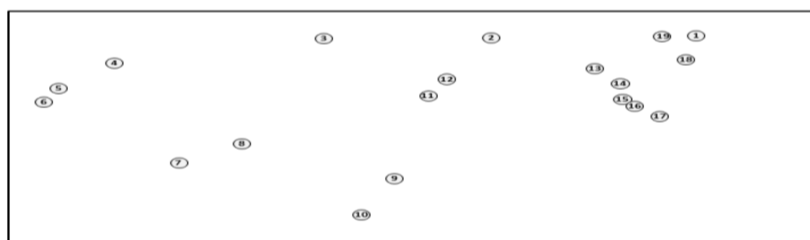

**Figure 5.3: Wing geometry with 19 landmarks**

Lastly, the landmarks selected for study should be reliable and replicable across all samples. This is important during the digitizing phase where, by choosing the landmarks that are easy to recognize, it will actually decrease the error of measurement in the later stages of analyses.

Another issue that could limit the usefulness of this approach is the accuracy of measurements using attached wing (not removed from the specimen) or flattened wing (wings removed from specimen body and mounted onto glass slides). Johnson et al. (2013) noted that the measurement of wing from pinned specimens and digitized images from dragon fly produced similar reading. However, this only applies to the linear measurement and not applicable to landmark based measurement. This finding was supported by a population research of *Chrysomya bezziana* using flattened wing and ‗on fly‘ wing by Hall et al. (2014), which suggested that flattened wing produced more accurate results compared to the ‗on fly‘ wing for landmark based measurement. They also emphasised that for populations‘ research using the landmark based measurement, only one (1) type of specimen should be used; it‘s either the flattened specimen or ‗on fly‘ specimen and not by mixing both measurement techniques. In this study, flattened wing were prepared before the images of the wings were captured and analyzed.

In the current study, the sample size for this research is quite small due to the limited collection of archived specimens. As such, only 95 male flies within the 15 species of

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the Calliphoridae and Sarcophagidae families from the archived sample were available for study. Furthermore, the very limited availability of female specimens for certain Sarcophagidae species also contributed to the decision to only inlcude male flies in this study. Although designing a study that included both male and female flies would be ideal, an advantage if using only one sex in the analyses was the ability to avoid sexual dimorphism (Hall et al., 2014).

In order to allow visualization of the relevant variables responsible for highest variation in resulting dataset, the Principle Component Analysis (PCA) procedures were applied. PCA was chosen as the tool for analysing the data which focuses on the variance between individual rather than group (Zelditch, 2012). Other than the small sample size, another factor is the nature of the flies from Sarcophagidae which are closely related and by conducting such analysis; the best aggregation among the individuals could and would be observed.

PCA on the archived sample shows 80% cumulative variance achieved and this allowed Calliphoridae flies to be distinguished up to the species level. However, the aggregation in Sarcophagidae was not as distinct as the ones observed for Calliphoridae flies. Most of the centroids among Sarcophagidae flies were overlapped and clustered closely to each other. From the literature, similar conditions were also observed in the clustering of *B. niveatus* and *B. vorticosus* bumblebees from the PCA analysis on the front and hind wings. The overlap between both species is more obvious on the front wing compared to the hind wing. Other geometric morphometrics methods such as relative warps analysis, Bookstein‘s shape‘s coordinates and Kruskal-Wallis test were also conducted on both species and no significant difference were observed from the front and hind wings (Aytekin, Terzo, Rasmont, & Çağatay, 2007). The variation between 2 populations *of Calliphora vicina* from Germany and England were observed at 62.45% following PCA analysis.  Hence, some overlap was observed from the scatter



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plot from both populations (Limsopatham et al., 2018). On the other hand, wing shape analysis of *Ptychoptera albimana* through PCA also allowed the divergence of the flies into 2 main groups and thus proving that the use of molecular marker in species identification must also be supported by other data (Kolcsár, Dénes, Keresztes, & Török, 2015). Finally, wing analysis of the Thailand Sarcophagidae flies resulted in the ability to distinguish various species mostly at genus level through Canonical Variate Analysis. There are also 4 out of 12 species that were successfully clustered distinctly (Sontigun et al., 2019).

An alternative data visualization was also possible through the use of a ‗heat map'. Data from the archived samples were used and then tabulated so that it can be used to generate a heat map. As expected, the heat map formed 2 distinct branches representing 2 fly families; the Calliphoridae and Sarcophagidae. However, in this analysis, *L.cuprina* (belonging to the Calliphoridae) was to be clustered together with other Sarcophagidae species. This would suggest that there are probably characteristics in wings of *L.cuprina* that are similar to Sarcophagidae flies. Interestingly, literature search revealed a previous study that showed that *L. cuprina* has similar content of polytene chromosome as Sarcophagidae flies (Foster et al., 1980) and probably this homology also contributed to the similarity of wing venation between *L.cuprina* and Sarcophagidae flies. As for other species within the Sarcophagidae family, there are some species that shared the homology in wing shape; between *S.dux* and *S.ruficornis* and between *B.javanica* and *S.albiceps* with *S.princeps*.

To provide support on the utility of this approach for fly species identification, a number of new specimens obtained from an actual crime scene was used, using what current knowledge (archived samples) available as a reference. These analyses act as the ‗test' to simulate a situation where identification is required without the presence of an entomologist. In this research, archived samples had been identified morphologically by

the expert (Tan, 2012) and were validated by DNA analysis. Crime scene samples on the other hand, were the unknown samples. Thus, in order to identify the fly species, test analysis was conducted by comparing the wings through geometric morphometrics analysis and also DNA analysis of the crime scene samples to the archived samples. From the test analysis, a PCA analysis was conducted on 36 flies from the crime scene which consist of only male flies. The PCA again shows the distribution of the flies into 2 families. Sarcophagidae flies seemed to be distributed within all Sarcophagidae species except the *Boettcherisca* genus. On the other hand, flies from Calliphoridae were distributed within the *Chrysomya* genus, with the exception of *Chrysomya villeneuvi*. From the graph we could observe that the distributions of Calliphoridae flies are quite distinct compared to Sarcophagidae flies. The centroids of Sarcophagidae species are closer to each other compared with Caliphoridae flies.

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# CHAPTER 6: CONCLUSION

The present study reports the potential use of geometric morphometrics of wing venation for the identification of selected flies from the Calliphoridae and Sarcophagidae families. While this approach showed some success with Calliphoridae flies to be identified to the species level, the results for Sarcophagidae are not that clear. Perhaps, for future considerations of geometric morphometrics analysis, one could probably explore other body parts of the Sarcophagidae fly that might exhibit unique characters for species identification such as the antennae, legs and chaetotaxy (bristles' arrangement) (Triplehorn & Johnson, 2005) or genitalia since it differs morphologically between species (Giroux, Pape, & Wheeler, 2010). In fact, a research applying geometric morphometrics method has been used on the Cephalopharyngeal of 3 species of blowflies. This is to aid the identification of the immature stage of flies by providing findings that could support the DNA analysis (Nuvez & Liria, 2016).

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# LIST OF PUBLICATIONS AND PAPERS PRESENTED

## List of Publications

1. Khang, T. F., **Puaad, N. A. D. M.**, Teh, S. H., & Mohamed, Z. (2020). Random forests for predicting species identity of forensically important blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) using geometric morphometric data: Proof of Concept. *Journal of Forensics Sciences*, *66(3),* 960-970.

PAPER

Random forests for predicting species identity of forensically important blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) using geometric morphometric data: Proof of concept

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