APPLICATION OF FORENSIC ENTOMOLOGY IN CRIME SCENE INVESTIGATIONS IN MALAYSIA

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ABSTRACT

Forensic entomology is the application and study of insect biology to criminal matters. One of the most important aspects of forensic entomology is the usage of maggot found in dead human body to determine the post-mortem interval (PMI). Based on the number of maggot sent for PMI determination, the application of forensic entomology in crime scene investigation is still unsatisfactory in Malaysia. Hence the present study was the first to conduct a questionnaire survey to determine the degree of knowledge and awareness of forensic entomology in Malaysia.

A total of 402 participants comprising of the crime scene police officers, pathologists who did the post-mortem examination, scientific officers and university students who have taken forensic science as their main subjects were included. Results showed that pathologists, scientific officers and university students have better awareness and knowledge of forensic entomology than the crime scene police officers. Hence more professional training is needed particularly among the crime scene police officers. The survey identified two major obstacles that may hinder the growth of forensic entomology in Malaysia which are the lack of information on the forensically important fly as well as the lack of expertise in species identification. Nevertheless the survey revealed a bright prospect for forensic entomology as evidenced by increased awareness of its importance and interest in the younger generation.

The present study was the first to apply both morphological and molecular methods for fly species identification in samples collected from crime scene investigation in Malaysia. A total of 50 cases from December 2008 to March 2010 were included. The present study confirmed the usefulness of molecular method based on cytochrome oxidase genes sequencing as a complementary tool in assisting fly species identification. Phylogenetic analyses confirmed the presence of *Chrysomya megacephala, Chrysomya rufifacies, Chrysomya nigripes, Hemipyrellia ligurriens and Sarcophaga ruficornis*. In addition, one ‘unknown’ species of blow fly was discovered. The application of molecular method has proven to be more advantageous in the case of immature maggot and egg. Due to the lack of experienced entomologist in Malaysia, it is recommended that molecular method should be widely applied.

Since *Chrysomya megacephala* and *Chrysomya rufifacies* was found to be the two most prominent fly species recovered in crime scene investigation, DNA barcoding was done for each life cycle stage of both blow flies namely egg, 1st instar, 2nd instar, 3rd instar, pupae, empty puparium and adult fly. The present study confirms that all life cycle stage of a particular species of fly yield identical DNA barcode and hence all the stages can be used for accurate species identification. The present study represents an initial effort to establish a DNA barcoding for forensically important blow fly in Malaysia. However, the effective use of DNA barcoding would require an expert system of integrated information whereby species names and their respective DNA barcodes are coupled with data of life cycle and geographic distributions.

ABSTRAK

Forensic entomologi adalah aplikasi kajian biologi serangga ke atas hal-hal jenayah. Salah satu aspek yang paling penting dalam bidang forensik entomologi ialah penggunaan ulat yang dijumpai pada mayat manusia untuk menentukan selang masa kematian (PMI). Berdasarkan bilangan ulat yang dihantar untuk penentuan selang masa kematian didapati aplikasi forensik entomologi di tempat kejadian jenayah masih tidak memuaskan di Malaysia. Oleh itu kajian ini merupakan kaji selidik yang pertama dijalankan untuk menentukan tahap pengetahuan dan kesedaran tentang forensik entomologi di Malaysia.

Seramai 402 peserta yang terdiri daripada pegawai polis yang menyiasat di tempat kejadian jenayah, pakar patologi yang melakukan bedah siasat mayat, pegawai sains and pelajar universiti yang mengambil matapelajaran sains forensik sebagai subjek utama dimasukkan dalam kajian. Hasil kajian menunjukkan bahawa pakar patologi, pegawai sains dan pelajar universiti mempunyai kesedaran dan pengetahuan forensik entomologi yang lebih baik jika dibandingkan dengan dengan pegawai polis yang menyiasat di tempat kejadian jenayah. Oleh itu lebih banyak latihan profesional perlu diberi khususnya kepada pegawai polis yang menyiasat di tempat kejadian jenayah. Kaji selidik ini juga mengenal pasti dua halangan utama yang mungkin menghalang perkembangan forensik entomologi di Malaysia iaitu kekurangan sumber maklumat berkenaan lalat yang mempunyai kepentingan forensik serta kekurangan kepakaran dalam mengenalpasti spesies lalat. Walau bagaimanapun, kaji selidik ini mendedahkan prospek yang cerah untuk bidang forensik entomologi yang mana dapat dibuktikan dengan peningkatan kesedaran tentang kepentingan forensik entomologi dan minat di kalangan generasi muda.

Kajian ini adalah yang pertama menggunakan kedua-dua keadah morfologi dan molekul untuk mengenal pasti spesies lalat yang disampelkan di tempat kejadian jenayah di Malaysia. Sebanyak 50 kes dari Disember 2008 hingga Mac 2010 dimasukkan dalam kajian ini. Kajian ini mengesahkan kebergunaan kaedah molekul yang berdasarkan penjujukan gen *cytochrome oxidase* sebagai kaedah pelengkap dalam membantu pengenalpastian spesies lalat. Analisa filogenetik juga mengesahkan kehadiran lalat *Chrysomya megacephala*, *Chrysomya rufifacies, Chrysomya nigripes, Hemipyrellia ligurriens* dan *Sarcophaga ruficornis*. Di samping itu, satu spesies yang tidak dapat dikenalpasti telah ditemui. Kaedah molekul terbukti lebih berguna dalam

pengenalpastian spesies lalat yang belum matang dan telur. Disebabkan kekurangan pakar entomologi yang berpengalaman di Malaysia adalah disyorkan bahawa kaedah molekul perlu digunakan secara meluas dalam proses pengenalpastian spesies lalat.

Memandangkan *Chrysomya megacephala* dan *Chrysomya rufifacies* merupakan dua spesies lalat yang paling banyak ditemui di tempat kejadian jenayah maka *DNA barcoding* telah dilakukan bagi setiap peringkat kitaran hidup lalat tersebut iaitu telur, *instar* pertama, *instar* kedua, *instar* ketiga, kepompong, kepompong kosong dan lalat dewasa. Kajian ini mengesahkan bahawa semua peringkat kitaran hidup bagi satu spesies lalat tertentu mempunyai *DNA barcode* yang sama dan oleh itu semua peringkat hidup lalat boleh digunakan dalam proses pengenalpastian spesies lalat dengan tepat. Kajian ini merupakan satu usaha awal untuk menubuhkan *DNA barcoding* untuk spesies lalat yang berkepentingan secara forensik di Malaysia. Walau bagaimanapun, penggunaan *DNA barcoding* yang berkesan memerlukan satu sistem pakar maklumat yang bersepadu di mana nama spesies lalat dan *DNA barcode* masing-masing dipadankan bersama-sama dengan data kitaran hidup dan distribusi geografi.

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Kavitha Rajagopal

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%	percentage
≈	approximately
e.g.	for example
hrs	hour
kb	kilobase
ºC	degrees Celcius
cm	centimetre
sp.	species (singular)
spp.	species (plural)
vs.	versus
µl	microlitre
ml	mililiter
mg	milligram
ng	nanogram
µM	micrometer
cm	centimeter
min.	minute
s	second
bp	base pairs
kb	kilo base pairs
rpm	revolutions per minute
COI	cytochrome oxidase subunit I
COII	cytochrome oxidase subunit II
DNA	deoxyribonucleic acid
mtDNA	mitochondrial DNA
CPS	cephalopharyngeal skeleton
KOH	potassium hydroxide
ETOH	ethyl alcohol
PMI	post-mortem interval

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CHAPTER 1
INTRODUCTION

1.0 GENERAL INTRODUCTION

Forensic entomology is a branch of forensic science that applies the study of arthropods and insects in the investigation of criminal matters. In Canada and the United States, forensic entomology has become an indispensable part of forensics and criminology. Very often certified forensic entomologists will be invited to appear in court to contribute their expertise particularly in determining the time lapsed since death or post-mortem interval (PMI) of a human remain.

Unfortunately, the application of forensic entomology in crime scene investigation in Malaysia is still not satisfactory. This is evident by the much lesser number of maggots sent for identification by forensic entomologist as compared to the number of dead human bodies found. Moreover majority of the maggots were collected by pathologists during post-mortem examination. Ideally police officer involved in the crime scene investigation should be the one who collects the maggots as this may provide more exact estimation of the PMI.

In view of the above scenario, there is an urgent need to assess the knowledge and awareness of the personnel involved directly or indirectly in crime scene investigation regarding the importance of forensic entomology. With the advancement of technology, identification of maggot has been aided by molecular techniques. It is important to assess the reliability of the molecular technique as compared to the conventional morphological method and its possible applications in the crime scene investigation in Malaysia.

1.1 FORENSIC SCIENCE

There are various definitions of forensic science. Some prefer using broad definitions to avoid confusion, whereas others define forensic science in a more detailed way. From a broad perspective, forensic science is 'the application of scientific techniques and principles to provide evidence to legal or related investigations and determinations' (Tilstone *et al.,* 2006). Gaensslen (2003) prefers a more detailed definition. He defines forensic science as "a broad, interdisciplinary group of applications of physical and biological sciences and various technologies to issues in civil and criminal justice".

The importance of forensic science in solving crimes has been increasing noticeably. Certainty and the celerity of using forensic evidence have made fighting crimes much easier. Reliable evidence has led to the release of innocent people including death row inmates, who had been convicted wrongfully, and to the arrest of criminals. On the other hand, the improper handling of forensic evidence may result in unjust verdicts. Errors in obtaining forensic evidence could mean the release of criminals and/or the arrest of innocent people (Fradella *et al*., 2007).

All types of physical evidence have the potential to provide critical information about the incident depending on the success of the stages of physical evidence analysied, the recognition of evidence, analysis of evidence, interpretation of results, reporting of results and expert testimony. Actions taken at the earlier stages of an investigation will determine the quality of the final outcome (De Forest *et al*., 1983).

1.2 DEFINITION OF DEATH

Death is the termination of the biological functions that sustain a living organism. The word refers both to the particular processes of life's cessation as well as to the condition or state of a formerly living body, but there is no statutory or other legal definition of death. Although medical evidence of death is of considerable importance in a criminal case, the fact that death had occurred is for the court to determine. The signs of death fall into two groups that are somatic and molecular, of which the second is of particular medico-legal importance. The first group includes those signs by which death is normally recognized by laity and doctors alike (Polson, 1969).

1.2.1 Autopsy

An autopsy, also known as a post-mortem examination or abduction, is a medical procedure that consists of a thorough examination of a human corpse to determine the cause and manner of a person's death and to evaluate any disease or injury that may be present. According to Knight (1991), autopsies are of two main types:

i) The 'clinical' autopsy, where the cause of death is known and the examination is held to confirm the diagnosis and to discover the extent of the lesions, for academic interest, teaching and research purposes.

ii) The 'medico-legal' autopsy, whose functions is to discover some or all of the following facts:

- The identity of the body
- The cause of death
- The nature and number of injuries
- The time of death
- The presence of poisons

- The expectation of duration of life for insurance purposes
- The presence of natural disease and its contribution to death, especially where there is also trauma
- The interpretation of injuries, either criminal, suicidal or accidental
- The interpretation of any other unnatural conditions, including those associated with surgical or medical procedures

The performance of an autopsy should only be carried out by a pathologist who has been trained with the techniques. Furthermore, medico-legal autopsies should only be carried out by pathologists who have training and experience in forensic pathology, either as a career or as an addition to their pathology training (Knight, 1991).

1.2.2 Changes in the Human Body after Death

After death, human or animal bodies undergo many changes caused by autolysis of tissue, which is promoted by the internal chemical breakdown of cell and released enzymes as well as by the activity of bacteria and fungi from the intestine and the external environment (Amendt *et al.*, 2004). However, the precise rate of post-mortem decay is affected by a wide range of variables associated with the corpse itself and the surrounding environment. Moreover, after body temperature has equilibrated with the environment and following the initial putrefaction, no reliable estimation of the post-mortem interval is possible. Therefore, an insect found on the body provides an important source of information (Simpson & Knight, 1985; Saukko & Knight, 2004).

Insects are usually the first organisms to arrive on a body after death, and they colonize in a predictable sequence. A corpse, whether human or animal, is a large food resource for a great many creatures and supports a large and rapidly changing fauna as it decomposes. The body progresses through a recognized sequence of decomposition

stages, from fresh to skeletal, over time. During this decomposition, it goes through dramatic physical, biological and chemical changes (Coe & Curran, 1980; Henssge *et al*., 1995).

Each of these stages of decomposition is attractive to a different group of sarcosaprophagous arthropods, primarily insects. Some are attracted directly by the corpse, which is used as food or an oviposition medium, whereas other species are attracted by the large aggregation of other insects they use as a food source. According to Smith (1986), four ecological categories can be identified in a carrion community, as follow:

i) Necrophagous species, feeding and breeding on the carrion

ii) Predators and parasites of necrophagous species, feeding on other insects or arthropods. This group also comprises species which feed on carrion at first, but many become predaceous in later larval stages.

iii) Omnivorous species such as wasps, ants and some beetles feeding both on the carrion and its colonizers

iv) Other species, such as springtails and spiders, which use the carrion as an extension of their environment.

Smith (1986) adds that when the sequence of insects colonizing carrion is known for a given area and set of circumstances, an analysis of the arthropod fauna on a carcass can be used to determine the time of death. This procedure can provide accurate and precise methods for estimating elapsed time since death and is used in many homicide investigations worldwide. When remains are found weeks, months, or more after death, insect evidence is often the only method available to determine reliably the time of death (Merritt *et al*., 2000; Wolff *et al*., 2001; Oliveira-Costa & Mello-Patiu, 2004).

Insects colonize in a predictable sequence, with some species being attracted to the remains very shortly after death and others are attracted during the active decay stage and still others being attracted to the dry skin and bones. Insects continue to colonize a body until it is no longer attractive. When the insects migrate from the remains, they invariably leave evidence of their presence behind, such as cast larval skins, empty puparium cases and even peritrophic membrane (Smith, 1986).

Several succession studies were carried out in several different countries (Anderson & VanLaerhoven, 1996; Arnaldos *et al*., 2001; Archer, 2003; Archer & Elgar, 2003; Bharti & Singh, 2003; Grassberger & Frank, 2004; Watson & Carlton, 2005; Eberhardt & Elliot, 2008) to understand the order in which species response to the different stages of decomposition and to further correlate species and decomposition stage to estimate a post-mortem interval in real cases (Goff, 1993).

Meanwhile, the remains themselves have changed and entered a stage of decomposition that is attractive to other, later colonizers. Therefore, when remains are found, the forensic entomologist will study not only the insects that are present on the remains at the time of discovery, but the evidence left behind by earlier colonizers. They also will note the species that are absent, but normally expected to be present, in the colonization sequence. From this information an accurate time of death can be established. However, insect succession on a corpse is impacted by many factors, including geographical region, exposure, season and habitat (Bornemissza, 1957; Smith, 1986; Arnaldos *et al*., 2001; Carvalho & Linhares, 2001; Grassberger & Frank, 2004).

Several factors restrict the colonization of a corpse, such as its burial (Mann *et al*., 1990) and most Dipterans are not able to colonize bodies buried deeper than 30cm (Introna & Campobasso, 2000; Campobasso *et al*., 2001). Burial, therefore, will influence the time required for insects to reach the carcass as well as the species

composition of the necrophagous fauna (Payne *et al*., 1968; Campobasso *et al*., 2001). Such delay may not only occur in buried corpses, but in those that are covered or wrapped (Goff, 1991).

Studies on animal carcasses have demonstrated that species composition and insect succession on a cadaver vary with respect to the geographical region and season (Bornemissza, 1957; Arnaldos *et al*., 2001; Carvalho & Linhares, 2001; Grassberger & Frank, 2004). Even local characteristics of the death scene, like the ecology of the area or the degree of sun exposure, can alter the pattern of insect colonization (Smith, 1986; Erzinclioglu, 1996).

Dead bodies undergo a variety of changes which eventually return the tissue components to the food chain and rid the surface of the earth of corpses, be they animal, human or vegetable. The pathologist needs to know enough to recognize the various post-mortem changes, in order to avoid confusing them with signs of trauma or unnatural death. Most forensic pathologists have been called by police to examine a 'strangulation' only to find that the discoloured face, protruding tongue and blood issuing from the lips was merely putrefaction (Parikh, 2004; Henssge & Madea, 2007).

1.2.3 Stage of Decomposition

One of the most widely used methods to estimate PMI is analyzing decomposition stages in the dead body. Decomposition is the gradual breakdown of dead organic matter (Spitz, 1993), which begins moments after death and continues over a period of time. This process can be divided into five stages namely fresh, bloat, active decay, post decay and dry (Catts & Goff, 1992). Bornemissza (1957), working with dead guinea pigs in Australia recognizes 5 stages of carcass decomposition, namely

initial decay (0 to 2 days), putrefaction (2 to 12 days), black putrefaction, butyric fermentation (20 to 40 days) and dry decay (40 to 50 days).

Payne (1965) has conducted an impressive work on pig carrion in USA and he recognizes six stages of decomposition namely fresh, bloated, active, advanced, dry and remains. Early & Goff (1986) has recorded four stages of decomposition on domestic cat carrion in Hawaii namely fresh, bloated, decay and dry. There are also numerous other studies on decomposition conducted in tropical areas. Lee & Marzuki (1993) were the first to study decomposition on monkey carcasses in Malaysia.

Another such study conducted in Malaysia by Omar *et al*. (1994a), using monkey carrion at a rubber tree plantation where five stages of decomposition were recorded as fresh, bloated, decay, post decay and dry remains. Therefore, if the decomposition stage of the body can be determined, then a time of death can be roughly estimated. Various factors, such as rigor mortis, algor mortis and chemical and physiological changes, can also be used to estimate PMI, however, as the PMI increases; the estimated range becomes wider (Spitz, 1993; Wolff *et al*., 2001).

1.3 PMI DETERMINATION

After death, many changes begin to take place in the body due to physical, metabolic, autolysis, physiochemical and biochemical process. These changes progress in an orderly manner until the body disintegrates. The measurement of these changes along with time is used for estimating time since death (Parikh, 2004; Henssge & Madea, 2007).

The physical changes, such as algor mortis, rigor mortis, livor mortis and putrefaction, form the main basis of estimation of time since death, as follow:

1.3.1 Contact Flattening

This term is applied to those areas of skin which remains pale in the midst of hypostasis and are related to the parts of the body which are in contact with the surfaces on which it lies after death. Since the body normally lies on its back after death, the usual sites of contact flattening are the shoulder blades, buttocks and claves. If however, the body has been laid face down, the contact areas are the face, chest, breasts and abdomen. These areas are sharply defined and are normally prominent in the midst of the general purple-red discolouration of the adjacent skin (Polson, 1969).

1.3.2 Vitreous Humour

Various body fluids like blood, spinal fluid, aqueous humour and vitreous humour of eye show chemical changes immediately or shortly after death. These changes progress in a fairly orderly fashion until the body disintegrates. Each change has its own time factor or rate. Thus determination of these chemical changes could help the forensic pathologists to ascertain the time since death more precisely (Agrawal *et al*., 1983).

Vitreous humour became the most studied material for estimating time since death. This was mainly due to the fact that vitreous humour is topographically isolated and well protected and thus the autolytic changes proceed slower compared with blood and cerebrospinal fluid. The most studied parameter in vitreous humour is potassium. An increase in the concentration of potassium in vitreous humour occurs after death (Adelson *et al*., 1963; Adjuntantis & Coutselinis, 1972; Agrawal *et al*., 1983; Stephens & Richards, 1987; Madea *et al*., 1990).

The relationship between the rise of potassium concentration in the vitreous humour and the time since death has been studied by several workers and reviewed by

Madea *et al*. (1989). Agrawal *et al*. (1983) worked on the relationship between the potassium levels of vitreous humour collected separately from each eye and the increasing time since death was found using flame photometry. He observed that the vitreous humour potassium concentration increased in a linear fashion with increasing time since death, and this increase in the level was independent of factors such as age, sex, environmental temperature and humidity. It was also observed that there was no effect of other parameters, such as age, sex, temperature and humidity, on the levels of vitreous potassium.

Madea *et al*. (2007) developed an analytical method for the determination of potassium in vitreous humour by low-pressure ion chromatography. They developed a linear correlation equation for potassium concentration in the vitreous humour and post-mortem interval. The immediate eye change is as a result of the loss of the reflexes to light and touch, but this occurs in any unconscious state. There is often but not invariably, a fall of tension within the eye. The cornea soon becomes cloudy. As long as it is practicable to examine the interior of the eye with an opthalmoscope, the condition of the retinal vessels, at the back of the interior of the eye, should be noted. If the blood in these vessels no longer presents in solid columns and has become fragmented and 'lumpy' and if no movement in the clumps occurs, death can be presumed. This sign may be present within few minutes of death (Polson, 1969).

Colour changes in the retina (the inner lining of the eye) during the first 15 hours after death have been claimed to provide a 'post-mortem clock', but even with special technique, it is difficult to keep the cornea clear so as to permit prolonged examination of the interior of the eye. Chemical changes in the intra-occular fluids have also been studied with a view to estimate the time of death. None of these tests has, as yet, yielded a satisfactory guide (Polson, 1969).

1.3.3 Rigor Mortis (Rigidity)

Rigor mortis is a well known phenomenon, and is due to a complex chemical reaction in the body. In the living body muscles can function more aerobically. When muscle cells work aerobically, the end product is lactic acid. In the living body, lactic acid can be converted back, by means of excessive oxygen uptake after an anaerobic exercise. In the dead body this cannot happen, and the breakdown of glycogen in the muscles leads irreversibly to high levels of lactic acid in the muscles. This leads to a complex reaction where actin and myosin fuses to form a gel. This gel is responsible for the stiffness felt in the body. This stiffness will not be over before decomposition begins (Smith & Bendall, 1947; Bendall, 1973; Spitz, 1993).

As rigor mortis is due to a chemical reaction, the reaction time is due to temperature and the initial concentrations of lactic acid. High metabolic activity in the time just before death, for example when running, leads to higher levels of lactic acid, and shorter time for the rigor mortis to develop. Higher environmental temperature also leads to a shorter reaction time. Rigor mortis, often called stiffness of death, is caused by a decrease in the production of adenosine triphosphate (ATP) as well as an increasing acidity in the muscles after death making portions of the body stiff and unmovable. The process tends to follow a particular time line beginning between 2 to 4 hours post -mortem. After 12 to 24 hours, full rigor has set in and over the next 12 to 48 hours, it will subside (Smith & Bendall, 1947; Bendall, 1973; Spitz, 1993).

Stiffening of the body normally begins at about three hours after death and progresses so as to involve the whole of the body at the end of about 12 hours. It begins in the small muscles such as the eyelids and lower jaw, and progresses to involve the whole musculature. Progress is probably determined by the size, like mass of muscle involved. Stiffening normally persists for about 36 hours, like up to the time of the onset

of putrefaction. These are only approximate times since rigor mortis has been found at even 50 hours, and no reliance can be placed upon its degree and distribution as a precise indication of the time interval since death. Rigor mortis is a fairly certain sign of death, however it has to be distinguished from heat stiffening and cold stiffening (Polson, 1969).

Heat stiffening - Coagulation of the body tissues by heat, for example when the body tissue is exposed to fire in a burning building, causes contracture of the muscles, especially to the flexor muscles. In consequence, the limbs are flexed, and as a result the victim's body assumes a pugilistic attitude. Burns and the circumstances in which the body is found serve to distinguish this form of stiffening from rigor mortis (Polson, 1969).

Cold stiffening - When a body has been exposed to cold, for example when on mountain or moorland, freezing of the joint fluids can occur and the body thus becomes stiff. This condition is recognized by the circumstances in which the body is found and if the joints are moved, crackling (crepitation) may be heard due to breaking down of ice in the joint fluid. Warming removes the stiffening, but it may then recur due to delayed onset of rigor mortis (Polson, 1969).

The factors that interfere with the onset and duration of rigor mortis are temperature, existing ante-mortem pathologies, age, body muscular mass, presence of infections, temperature, climatic conditions and the degree of muscular activity immediately before death (Smith & Bendall, 1947, 1949; Polson *et al*., 1985; Gordon *et al*., 1988; Krompecher, 1994; Lawrie & Ledward, 2006).

1.3.4 Algor Mortis

Algor mortis refers to the gradual decrease in body temperature after death. It is one of the useful indicators for estimating time since death during the first 24 hours after death (Simpson & Knight, 1985; Morgan *et al*., 1988; Spitz, 1993; Jackson & Jackson, 2004; Saukko & Knight, 2004).

During life, a balance is maintained between heat loss and heat production. After death, however, the heat production ceases and body heat is lost to the environment. The body temperature falls steadily until it matches the environmental temperature. This cooling of body temperature is mainly a physical process and the influence of biological processes is relatively low. The rate of fall of body temperature with time is used for determining time of death. Different body sites have been used for measuring the temperature such as the abdominal skin surface, axilla, rectum, ear and nostril. However, rectum is the most commonly used site for measuring the temperature (Gordon *et al*., 1988; Morgan *et al*., 1988; Henssge & Madea, 2004).

The commonly used site for measuring temperature is the rectum but scientists have worked on other sites, such as skin, outer ear, brain, and eyeball (Bendall, 1973; Baccino *et al*., 1996). Temperature of the skin was also measured for determining time of death but it was never of use because the effect of external conditions was high, resulting in erroneous results (Al-Alousi, 2002). Body temperature is generally considered one of more reliable indicators of the time of death up to approximately 18 hours. The usual procedure for determining body temperature is to insert a thermometer into the liver. A comparison between that temperature and ambient temperature is used to determine the approximate time of death (Knight, 1991).

However, the rates of cooling established are valid for only a particular climatic region and are not applicable everywhere. The rates are valid only in cool or temperate climates because hot summer seasons or tropical temperatures slow down the loss of heat and, in some regions, can even raise post-mortem temperatures due to rapid putrefaction. Variables such as the size of the body, amount of subcutaneous adipose tissue, existence of clothing and coverings, air currents and humidity, and the medium where the body remained after death, which affect the post-mortem cooling, should be considered while estimating time since death using algor mortis (Mathur & Agrawal, 2011).

The use of decomposition stages, rigor mortis, algor mortis and chemical or physiological changes to estimate PMI are often unreliable because of a high rate of variability. External factors, such as higher or lower ambient temperature, age of the deceased, body mass of the deceased and the surroundings of the body is in, can influence the time frame (Spitz, 1993; Jackson & Jackson, 2004; Strachan & Read, 2004).

1.3.5 Livor Mortis (Lividity)

Livor mortis or lividity is the settling of blood in the lower portion of the body, resulting in a dark purple discoloration of the skin. As the heart is no longer agitating the blood, red blood cells sink by the action of gravity. The process begins immediately after the circulation stops. The discoloration does not occur in body areas that are in contact with the ground because the capillaries get compressed (Polson *et al*., 1985; Krompecher, 2002; Parikh, 2004).

Lividity develops in all bodies under the influence of gravity because the blood remains liquid rather than coagulating throughout the vascular system. After 30 to 60

minutes since death the blood becomes permanently incoagulable. This is due to the release of fibrinolysins, especially from small vessels and from serous surfaces such as pleura. This incoagulability of blood is independent of the cause of death. In some cases, due to infections, this fibrinolytic effect fails to develop, explaining the presence of abundant clots in the heart and large calibre vessels. In some cases of sudden death, the blood remains spontaneously coagulable only during a brief period immediately following death, but then it becomes completely free from fibrinogen and will never clot again (Mathur & Agrawal, 2011). Gordon *et al*. (1988) stated that the fluidity of the blood is not dependent on the cause of death and the mechanism of death, although it has been cited that the blood remains liquid longer in asphyxia deaths.

The colour and distribution of post-mortem lividity are important in medico-legal investigations and can be used for estimating cause of death such as carbon monoxide (CO) poisoning, cyanide intoxication and death from hypothermia. Livor mortis starts appearing as dull red patches after 20-30 minutes from the time of death. In the succeeding hours these red patches coalesce together to form larger areas of red-purple discoloration (Polson *et al*., 1985), although in some cases variation in colour is observed (Krompecher, 2002; Parikh, 2004; Saukko & Knight, 2004).

In some cases it has been observed that fading of the primary pattern of lividity occurs and there is subsequent development of a secondary pattern of lividity. This is due to the early movement of the body and is found to be more complete if the body is moved within the first six hours after death, than at a later period (Camps *et al*., 1976). Even after 24 hours, moving the body will result in a secondary pattern of lividity developing. Lividity attains its maximum intensity at around 12 hour's post-mortem, but there is some variation in descriptions of when it first appears, and when it is well developed. Lividity ordinarily becomes perceptible within 1/2 to 4 hours after death, is

well developed within the next 3 or 4 hours, and attains its maximum degree between 8 and 12 hours post-mortem (Adelson, 1974).

1.3.6 Putrefaction

Putrefaction is the post-mortem destruction of the soft tissues of the body by the action of bacteria and enzymes (both bacterial and endogenous). Tissue breakdown resulting from the action of endogenous enzymes alone is known as autolysis. Putrefaction results in the gradual dissolution of the tissues into gases, liquids and salts. The main changes which can be recognized in the tissues undergoing putrefaction are changes in colour, the evolution of gases, and liquefaction. The bacteria increase in hydrogen-ion concentration and the rapid loss of oxygen in the tissues after death favours the growth of anaerobic organisms (Gordon *et al*., 1988).

The rate of putrefaction is influenced by the body size of the deceased, obese individuals putrefy more rapidly than those who are lean. Conversely, putrefaction is more rapid in persons dying with widespread infection, congestive cardiac failure or anasarca. Putrefaction is accelerated when the tissues are oedematous, like in deaths from congestive cardiac failure, and delayed when the tissues are dehydrated. It tends to be more rapid in children than in adults, but the onset is relatively slow in unfed new-born infants because of the lack of commensal bacteria (Camps *et al*., 1976; Henssge & Madea, 2004).

Whereas warm temperature enhances putrefaction, intense heat produces ‘heat fixation’ of tissues and inactivates autolytic enzymes with a resultant delay in the onset and course of decomposition. Heavy clothing and other coverings, by retaining body heat, will speed up putrefaction. After normal burial, the rate at which the body decomposes will depend to a large extent on the depth of the grave, the warmth of the

soil, the efficiency of the drainage, and the permeability of the coffin. The restriction of air, in deep burials, particularly in clay soil, will retard decomposition, but never prevent it altogether (Camps *et al*., 1976; Henssge & Madea, 2004).

Typically, the first visible sign of putrefaction is a greenish discolouration of the skin of the anterior abdominal wall. The discolouration, due to sulph-haemoglobin formation, spreads to involve the entire anterior abdominal wall, and then the flanks, chest, limbs and face. As this colour change evolves, the superficial veins of the skin become visible as a purple-brown network of arborescent markings, which tend to be most prominent around the shoulders and upper chect, abdomen and groins. This change, owing to its characteristic appearance, is often described as 'marbling'. The skin has a glistening, dusky, reddish-green to purple-black appearance, displays slippage of large sheets of epidermis after any light contact with the body, like during its removal from the scene of death (Camps *et al*., 1976).

Putrefaction progresses internally beginning with the stomach and intestine. Progression of decomposition is associated with organ shrinkage. The more dense fibro-muscular organs such as the prostate and uterus remain recognizable until late in the process, thus aiding in the identification of sex (Spitz & Fisher, 1980).

1.3.7 Mummification

Mummification is a modification of putrefaction characterized by the dehydration or dessication of the tissues. The body shrivels and is converted into a leathery or parchment-like mass of skin and tendons surrounding the bone. The internal organs are often decomposed but may be preserved. Skin shrinkage may produce large arte-factual splits mimicking injuries. These are particularly seen in the groins, around the neck, and the armpits (Spitz & Fisher, 1980).

The forensic importance of mummification lies primarily in the preservation of tissues which aids in personal identification and the recognition of injuries. The time required for complete mummification of a body cannot be precisely stated, but in ideal conditions mummification may be well advanced by the end of a few weeks (Polson *et al*., 1985).

1.3.8 Adipocere (Saponification)

Adipocere or saponification formation is a modification of putrefaction characterized by the transformation of fatty tissues into a yellowish-white, greasy, (but friable when dry), wax-like substance, with a sweetish rancid odour. Mant (1960) stated that when its formation is complete it has a sweetish smell, but during the early stages of its production a penetrating ammoniacal odour is emitted and the smell is very persistent. It floats on water and dissolves in hot alcohol and ether. When heated it melts and then burns with a yellow flame. Ordinarily it will remain unchanged for years. Adipocere develops as the result of hydrolysis of fat with the release of fatty acids which being acidic and then inhibits putrefactive bacteria. A warm, moist, anaerobic environment favours adipocere formation. Adipocere develops first in the subcutaneous tissues, most commonly involving the cheeks, breasts and buttocks. Rarely, it may involve the viscera such as the liver (Camps *et al*., 1976).

Under ideal warm, damp conditions, adipocere may be apparent to the naked eye after 3 to 4 weeks (Mant, 1960; Adelson, 1974). Ordinarily, adipocere formation requires some months and extensive adipocere is usually not seen before 5 or 6 months after death (Spitz & Fisher, 1980). Other authors suggest that extensive changes require not less than a year after submersion or upwards of three years after burial (Polson *et al*., 1985). The medico-legal importance of adipocere lies not in establishing time of death but rather in its ability to preserve the body to an extent which can aid in personal

identification and the recognition of injuries. The presence of adipocere indicates that the post-mortem interval is at least weeks and probably several months (Polson *et al*., 1985).

1.3.9 Entomology

Insect used as an evidence to confirm the PMI established through body temperature, hypostasis, rigor mortis and others. Conversely the insect evidence recovered from a scene may contradict the standard temperature, hypostasis or rigor mortis indications bringing into question on the findings. This in turn can lead to the development of new and more accurate hypothesis regarding the circumstances of death and the PMI (Baden & Hennesse, 1989).

As well as the PMI, insect evidence can indicate the season of death. Insect evidence can indicate whether the death occurred in an urban or rural setting. Insect evidence can be used to determine whether a buried body was on the surface for some time after death and then buried. Insect evidence also indicates whether a body has been previously buried (Erzinclioglu, 2000; Goff, 2000; Anderson, 2001). Aquatic insects can indicate the season and conditions under which the body came to be in the water. An aquatic insect on a body found on land indicates death in wetter season or movement of the body (Thomas, 1995).

Insects begin to arrive at a corpse in less than ten minutes after death (Lane, 1992; Goff, 2000). In buried bodies, colonization can be found as much as ten years after death (Erzinclioglu, 2000). Insect colonization can be found on bodies sealed in plastic bags, rugs and cars (Anderson, 2001). Insect colonization occurs on bodies indoors and those which have been buried. This means that insect evidence can be used in wide variety of circumstances and over much longer periods of time as opposed to

other widely used methods for estimating the PMI (Erzinclioglu, 2000; Anderson, 2001).

1.4 FORENSIC ENTOMOLOGY

Lord & Stevenson (1986) divided forensic entomology into three major components:

i) Urban entomology

Legal proceedings involving insects and related animals that effect on manmade structures such as dwellings, house and other aspect of human environment. This category also includes the law of insecticidal abuse.

ii) Stored product entomology

Legal proceedings involving insects infesting stored commodities such as cereal and other kitchen products. This usually involves both criminal and civil proceeding involving food contamination of variety of commercial products.

iii) Medico-legal entomology

Medico-legal entomology or sometimes termed 'forensic medical entomology' and in reality 'medico-criminal entomology' (because it focuses more on violent crime), relates it to primary aspects (Leclercq, 1969; Haskell *et al*., 2008), as follow:

a) Determination of the time (PMI or post-mortem interval) or site of human death
b) Cases involving possible sudden death
c) Traffic accidents with no immediately obvious cause
d) Possible criminal misuse of insects

Forensic entomology is the use of insects and other arthropods to aid in legal investigation. Medico-legal, which is the focus of the present study, involves violent crimes against the person, where insect presence is directly related to human death, usually with the body present. The types of death may be classified as natural, accidental, suicide or criminal homicides (Catts & Goff, 1992; Benecke, 2001; Haskell *et al*., 2008).

Entomological evidence collected from a corpse can be used to make inferences about the location or cause of death, but is most frequently used to estimate the time of death. Upon death, the putrefaction of a body attracts a variety of large scavengers and smaller arthropods. Observations of the insect fauna taken at the time of the corpse's discovery can be used to estimate the amount of time that has passed since death, commonly referred to as the post-mortem interval (PMI). This estimation is accomplished by observing the types of species present on the corpse and estimating the immature insect specimens. Although a wide variety of insects may be found on or around a decomposing body, flies and beetles are the two most frequently encountered and forensically useful groups (Catts, 1990; Catts & Goff, 1992; Benecke 2001; Hall, 2008).

The forensically important insects (those associated with remains) can be placed into four categories namely Necrophages (feed on the tissues of the deceased) such as certain Diptera (flies) and Silphidae (carrion beetles), parasites/predators (feed/live on or within other insects attracted to the corpse) such as Hymenoptera (wasps or bees) and incidentals (use the corpse for reasons other than feeding) such as spiders and butterflies (Keh, 1985; Catts & Goff, 1992; Campobasso *et al*., 2001). Necrophages, more specifically blow flies (Calliphoridae) are usually the basis for determining PMI

because they are often the first to colonize human corpse, arriving minutes after death (Catts & Goff, 1992; Amendt *et al*., 2004).

Adult females prefer to lay eggs (oviposition) in the wounds and orifices of a body because newly hatched larvae cannot break skin barriers, these locations on the decomposing body allow access to a liquid protein food source which is essential for their development, as well as providing a moist and humid environment that enhances survival (Spitz, 1993; Ames & Turner, 2003). Blow flies are not the only insects to colonize decaying remains, others include flesh flies (Diptera: Sarcophagidae), carrion beetles (Coleoptera: Silphidae), rove beetles (Coleoptera: Staphylinidae) as well as others (Campobasso *et al*., 2001).

The use of insects in forensic entomology has become one of the most helpful tools for estimating PMI. Within minutes of death, Calliphoridae flies colonize human corpse and are the most accurate to estimate PMI. The scientific study of insects, entomology, has given crime investigators new hope in obtaining the estimation of 'time of death'. The role of forensic entomology may enable the investigator to obtain 'real-time' information based upon stages of development in species associated with the death scene (Amendt *et al*., 2004). Larval development is dependent on temperature (Bowler & Terblanche, 2008) and every species has a slightly different growth rate (Erzinclioglu, 1990; Davies & Ratcliffe, 1994; Richards & Villet, 2009).

It is thus crucial to identify the larval species feeding from a corpse correctly to calculate the PMI properly. To ensure correct species identification, established molecular methods were transferred to the forensic field (Sperling *et al*., 1994; Stevens & Wall, 1996, 1997; Wallman & Adams, 1997; Benecke, 1998). Calliphoridae are one of the earliest visitors infesting a corpse with their larvae (Lane, 1975; Putmann, 1977; Benecke, 2004).

Forensic entomology is supported by a large collection of literature that has been peer reviewed. However, when trying to estimate the developmental stage of larvae, variation among data sets is evident. This variation plays a crucial role when trying to use entomological evidence to estimate PMI because there is a lack of known error rates for the methods currently utilized in forensic entomology. To improve the precision of PMI estimation, methods need to be developed where error rates can be mathematically determined. Variation in the growth rates of forensically important flies is obvious in many studies (Kaneshrajah & Turner, 2004; Clark *et al*., 2006).

1.4.1 History of Forensic Entomology

Historical events in forensic entomology reaching back to the 13th century have been described extensively in several studies, publications and reviews (Amendt *et al*., 2000, 2004; Benecke, 2001, 2008). Insects are known to have been used in the detection of crimes for a long time and a number of researchers have written about the history of forensic entomology (Benecke, 2001; Greenberg & Kunich, 2002). The Chinese used the presence of flies and other insects as part of their investigative armoury for crime scene investigation and instances of their use are recorded as early as the mid-tenth century (Greenberg & Kunich, 2002).

Forensic entomology was first reported to have been used in 13^{th} century in China and was used sporadically in the 19^{th} century and the early part of the 20^{th} century, playing a part in some very major cases. The oldest record is the documentation during the year 1235 A.D. The Chinese lawyer and investigator, Sung Tzu in his book 'His Yuan Chi Lu' (translated 'The Washing Away of Wrongs' by McKnight, 1981), recorded the first documented forensic entomology case.

A murder by slashing occurred in a Chinese village and the local death investigator was deputized to solve the crime. After some fruitless questioning, the investigator had all villagers bring their sickles to one spot and lay them out before the crowd. Flies were attracted to one of the sickles, probably because of invisible remnants of tissue still adhering to it and the owner subsequently broke down and confessed to the crime. In other portions of the text, Sung Tzu demonstrated knowledge of blow fly activity on bodies relative to those orifices infested, the time of such infestation and the effect of trauma on attractiveness of tissue to such insects (McKnight, 1981).

Until the mid-17th century, it was believed that under the right conditions maggots spontaneously arose from rotten meat. In 1668, Francesco Redi refuted the hypothesis of the spontaneous generation of life after the analysis of the results of his experiments in which rotting meat was either exposed to or protected from flies (Hall & Huntington, 2008). Redi proved by his experiments that maggots come from fly eggs deposited on rotten meat or putrefying carcasses (Redi, 1668; Goff, 2000; Cruz, 2006).

At the beginning of the 19th century, it was registered that flies are attracted by corpses at a very early stage of decomposition. In the year 1829, Mende compiled a list of necrophagous insects, including flies, beetles and other taxa and provided more precise account, but did not link flies to the time of death. Kamal (1958), described the opportunities and problems associated with using insects for the estimation of the post-mortem interval (PMI) and many of which are still relevant today (Gomes & Zuben, 2006).

The credit for the first modern forensic entomology case goes to French doctor Bergeret. He used forensic entomology to detect the post-mortem interval (PMI) in 1855. In that case the corpse of a child was found in a house. Bergeret was called to detect the PMI. In finding the PMI he assumed that metamorphosis involves one year

and also that females lay eggs in summer so that the larvae would transform to pupae the next spring and hatch in summer. He found the eggs of *Musca carnaria* L. on the corpse that lays eggs before the body dries out. Using these findings he calculated that the body must have been left there at least a couple of years back (Benecke, 2001; Gupta & Setia, 2004).

Pierre Megnin can be regarded as the first person who undertook a scientific research on forensic entomology. He worked on the subject for almost a couple of decades and compiled his findings in the form of a book titled '*La fauna des Cadavres*' in 1894. In this book he gave the theory of eight successional waves of insects on bodies left in the open. He also mentioned that on buried bodies insects came in two waves. He also described the morphological features of various classes of insects that helped in their identification. As the reports started pouring in that Megnin's work involved a lot of guesswork, people began modifying his findings to go with the flora and fauna prevelant at their places. This process started at the end of nineteenth century and has been continuing since (Benecke, 2001; Gupta & Setia, 2004).

Carrion (dead tissue) feeding blow flies (Calliphoridae) and flesh flies (Sarcophagidae) are those most useful in death investigations. Aldrich's (1916) monograph on the Sarcophagidae made use of distinctive male genitalia, thereby enabling entomologists to identify adult male specimens from this important family. Later, Knipling (1936) published descriptions and keys to many common early (first instar) maggots of flesh flies. Although considerable work had been done on the blow fly fauna of North America for instance Knipling (1939), Hall's (1948) monograph, 'The Blow flies of North America', made possible the accurate identification of adults and mature larvae of most species of this family as well. The situation is somewhat

better with respect to third instar or prepupal larvae (the largest maggot stage, and that most commonly observed), but only if such specimens are preserved properly.

When Watson and Crick discovered DNA in 1953, the use of DNA brought in a new era in the identification of the invertebrates. Soon DNA was being used to identify the insects at the scene of crime (Sperling *et al*., 1994; Wells & Sperling, 1999; Gupta & Setia, 2004). This is currently an area of active research, and this replaces the application of scanning electron microscopy for identifying the immature stages of fly (Liu & Greenberg, 1989; Byrd & Castner, 2001).

In the late 1970s, the emergence of entomotoxicology as a new branch of forensic entomology was seen. The presence of toxins in the invertebrate decomposers was detected and was used as a method of finding the cause of death. Now the use of forensic entomology was graduating from finding only PMI to finding the cause of death (Beyer *et al*., 1980; Goff, 1991, 1992, 1993; Goff & Lord, 1994, 2001; Bourel *et al*., 2001; Gagliano-Candela & Aventaggiato, 2001).

The use of forensic entomology in child abuse and sexual abuse cases can be seen from the case described by Mark Benecke. This case marked a landmark in the use of entomology in child abuse (Benecke & Lessig, 2001). The presence of certain fly species associated with the victim may indicate neglect or abuse. This can be corroborated by estimation of the age of recovered maggots to reveal the length of neglect (Gunn, 2006).

Studies have shown that post-mortem insect activity, particularly maggot masses, combined with natural decompositional changes can produce changes to clothing which mirror those seen in cases of sexual assault (Komar & Beattie, 1998). DiZinno *et al*. (2002) used mitochondrial DNA to match the human DNA found in the

blood in the insect's gut to that of the deceased's bone. In this case the deceased's DNA was available to match. This can be taken as a guide that in badly decomposed bodies, forensic entomology can be used for the identification of the deceased.

Today forensic entomology is not limited to finding PMI only. A forensic entomologist has acquired an important role in death investigation like finding time since death, season of death, geographical location of death, movement or storage of remains after death, time of decapitation or dismemberment, submersion interval, specific sites of injury on the body, post-mortem artifacts on the body and the crime scene, use of drugs, linking a suspect to scene of crime, in child neglect, sexual molestation, identification of suspects (Campobasso & Introna, 2001).

Forensic entomologists are always presented with the task of reconstructing the death scene conditions as closely as possible. A model for the calculation and handling of the data is crucial for the credibility of this discipline (Amendt *et al*., 2000). Now, at the beginning of the 21st century, forensic entomology is recognized in many countries as an important forensic tool (Goff, 1991; Greenberg, 1991; Anderson, 1995; Introna *et al*., 1998; Amendt *et al*., 2004; Gomes *et al*., 2006; Gennard, 2007; Haskell *et al*,. 2008; Byrd & Castner, 2009; Amendt *et al*., 2010).

1.4.2 History of Forensic Entomology in Malaysia

Forensic entomology in Malaysia is relatively a new area of study as compared to other parts of the world. This was started in 1950, when some young maggots were collected from a body of a woman shot by bandits in Penang, were received from the pathologist, Dr. Nevin, for examination. Senior entomologist, Dr. Reid identified the maggots as early second stage larvae of the blow fly *Chrysomya megacephala*, probably

between 16 and 24 hours old and this corresponded well with the evidence obtained by the police that the woman had been killed for less than 24 hours before (Reid, 1953).

In the years 1953 to 1990, it then became a routine service. In the year 1984, Lee reviewed all entomological evidence from the year 1973 which was received by Institute for Medical Research (IMR) in Kuala Lumpur. In 1989, he published a paper titled 'Recovery of forensically important entomological specimens from human cadavers in Malaysia - an update' (Lee, 1989). In the year 1993, Lee and Marzuki studied insect succession on monkey carcasses (Lee & Marzuki, 1993).

Then research in this area continued from 1994 to the present in relation to routine service. Omar *et al*. (1994c) reported the first record of *Synthesiomyia nudiseta* (Wulp) in Malaysia and its involvement in decomposing corpses inside buildings. Later in 2012, Syamsa and colleagues discovered the occurrence of *Synthesiomyia nudiseta* associated with a human corpse in a high-rise building in Malaysia (Syamsa *et al*., 2012), compared to other previous cases where the occurrence of *Synthesiomyia nudiseta* is at a ground level (Nor Afandy *et al*., 2003; Lee *et al*., 2004; Ahmad *et al*., 2007; Kumara *et al*., 2009). In Lee (1996) reported that the most dominant species found on human cadavers were those of Calliphoridae and Sarcophagidae. He also mentioned that these species were at times found as a single species in a mass and at other times they are found together with other fly species. Hamid *et al*. (2003) recovered 9 species of sarcosaprophagous flies in their forensic maggot specimens received from pathology departments.

In 2004, Lee and colleagues reviewed all case samples made available to him by the police personnel between the years from 1972 to 2002 to study the connection between the maggots and the time of death. In a review of 538 forensic specimens recovered from 1972 till 2002, Lee *et al*. (2004) identified *Chrysomya* sp., *Sarcophaga*

sp., *Lucilia* sp., *Hermetia* sp., *Hemipyrellia* sp., *Ophyra* sp., *Calliphora* sp., *Synthesiomyia nudiseta* and *Eristalis* sp. It has been reported that the pupae of *Desmometopa* sp. (Diptera: Milichiidae) were collected from a human corpse found indoor in active decay stage together with the larvae of Sarcophagidae, *Synthesiomyia nudiseta, Chrysomya megacephala* and *Chrysomya rufifacies* (Kumara *et al*., 2010).

Ahmad *et al*. (2007) also reviewed forensic entomological specimens received from Hospital Universiti Kebangsaan Malaysia (HUKM). Heo *et al*. (2007) used pig carcass placed in an oil palm plantation to study the faunal succession and stage of decomposition. Heo *et al*. (2008) used partially burned pig carcass in an oil palm plantation in Malaysia to study the insect succession and the rate of decomposition. Nazni *et al*. (2008) recovered *Piophila casei* of the Family Piophilidae for the first time from two cases of pathological specimens received from Forensic Department of Kuala Lumpur Hospital.

Tan *et al*. (2009) studied sequence variation in the *cytochrome oxidase subunit I* and *II* genes of two commonly found blow fly species, *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) in Malaysia. In the year 2010, Tan *et al*. (2010), suggested and demonstrated that mitochondrial DNA sequence can be successfully employed to distinguish some species of the sarcophagine flesh flies. The available DNA sequence of Sarcophagidae flies encompassing the complete COI, t-RNA-leucine and COII genes allows for the identification of these species, particularly useful for local forensic purposes.

In Malaysia, carrion-related arthropods have been known to include dipteran flies such as Calliphoridae, Sarcophagidae, Muscidae and Stratiomyidae (Lee & Marzuki, 1993; Omar *et al*., 1994a, 1994b). Recent reviews of Malaysian forensic studies showed that calliphorid flies, such as *Chrysomya rufifacies* and *Chrysomya*

megacephala were observed as the predominant species found in human cadavers (Hamid *et al*., 2003; Lee *et al*, 2004; Ahmad *et al*., 2007; Kumara *et al*., 2012b). Hence in the present study the molecular and morphological approach was applied to Calliphoridae mainly due to the fact that the samples were collected in real crime scene.

1.4.3 Insects of Forensic Importance

There are about 86,000 fly species described worldwide (Byrd & Castner, 2009). The study of the entomofauna associated with cadavers has been an extremely effective tool to clarify numerous cases of homicides, sexual abuses and traffic of organs (Wolff *et al*., 2001). Review of forensically important insect in Malaysia was conducted in the period of 1972-2002 (Lee *et al*., 2004). Eighteen species of cyclorrphagic flies were identified, consisting of:

i) *Chrysomya megacephala*

ii) *Chrysomya rufifacies*

iii) *Chrysomya villeneuvi*

iv) *Chrysomya nigripes*

v) *Chrysomya bezziana*

vi) *Chrysomya pinguis*

vii) *Chrysomya* sp.

viii) *Sarcophaga* sp.

ix) *Lucilia* sp.

x) *Hermetia* sp.

xi) *Hermetia illucens*

xii) *Hemipyrellia ligurriens*

xiii) *Hemipyrellia* sp.

xiv) *Ophyra spinigera*

xv) *Ophyra* sp.

xvi) *Calliphora* sp.

xvii) *Synthesiomyia nudiseta*

xviii) *Eristalis* sp.

The proper identification of the insect and arthropods species for forensic importance is the most crucial element in the field of forensic entomology. It is the species identification that allows the proper developmental data and distribution ranges to be applied to an investigation. If the species determination is incorrect, then the estimated post-mortem interval would be invalid (Amendt *et al*., 2000).

1.4.4 Flies and Beetles

Carrion flies are most frequently collected as forensic evidence due to their ability to quickly locate a corpse, sometimes within minutes after death. Although not much is known about the specific chemical compounds that attract carrion flies to a decomposing corpse, the carrion does provide an attractive environment for the flies to feed, mate and deposit eggs (Anderson & VanLaerhoven, 1996).

According to Byrd & Castner (2001), from the many different species of flies (Order: Diptera) that can be associated with a corpse, most of them belong to one of the following three families namely Calliphoridae (commonly called blow flies),

Sarcophagidae (flesh flies) or Muscidae (house flies and related species). These large flies produce a large amount of eggs, larvae and pupae that are easily seen and collected by investigators.

The insect order is composed of the flies and has over 86,000 known species. Flies can be found in almost any habitat and are characterized by having only one pair of wings. The second pair of wings is reduced to only knob-like organs called the halters, which are used to stabilize the insect in flight. Flies have large compound eyes with mouthparts of various types. However, most flies associated with a corpse have sponging mouthparts. The larvae of flies are called maggots and most are cream coloured, soft, legless and lack a visible head (Greenberg & Kunich, 2002).

All flies undergo a complete metamorphosis, a complex growth pattern that is commonly seen in a variety of insects. Complete metamorphosis is characterized by the insect's radical change from larva to pupa to adult. Females are able to deposit eggs (or in some cases larvae) soon after they discover the corpse. Eggs are laid onto moist areas of the body, providing the newly hatched fly larvae (maggots) direct access to soft tissue. Eggs are also frequently distributed in clumps, giving the emerging maggot mass the additional advantage of pooling their external digestive enzymes to help soften tissue before intake. As the larvae rapidly feed, they develop through three stages called instars, each separated by a molting of the cuticle. The ultimate size of a maggot will greatly depend on the species (Greenberg & Kunich, 2002).

During the 3rd larvae instar, the larvae will stop feeding and begin to migrate away from the corpse. The post feeding larvae will shorten slightly, darken in colour and pupate in the soil or another partially sheltered location. Inside the hardened puparium, the immature fly will undergo the final metamorphosis and emerge as an adult fly. The time it takes for a specific fly to develop through these stages depends on

a variety of factors, with its species and the ambient temperature having the greatest effect (Greenberg & Kunich, 2002).

Various species of beetles (Order: Coleoptera) are attracted to the corpse throughout the decomposition process and are therefore helpful in cases when the corpse remains undiscovered for a longer period of time (Goff & Catts, 1990; Kulshrestha & Satpathy, 2001). Similar to flies, adult beetles are attracted to the corpse as a source of food and as a medium to deposit eggs. Beetles will not only feed on the corpse itself, but many will also consume the eggs or maggots present on the corpse. Just as with flies, beetle eggs will hatch and grow through a complete metamorphosis pattern. Their larvae have six legs, are highly mobile and can vary greatly in both size and shape (Byrd & Castner, 2001).

Because different beetles are attracted to the body at different times throughout the decomposition process, the presence of a certain species on the corpse can be an indication of how long the body has been decomposing. This knowledge of insect succession, coupled with an estimation of the age of the immature insects developing on the body, forms the basis for post-mortem interval estimations (Carvalho *et al*., 2004; Turchetto & Vanin, 2004).

1.4.5 Blow Flies (Family Calliphoridae)

Figure 1.1: Adult Blow fly
(Source: Byrd & Castner, 2001)

Blow flies, as shown in Figure 1.1, often are of an attractive blue-green, metallic colour, with the common English names blue-bottles and green-bottles or bear the common Australian name blue-arsed flies. They also come in a non-metallic, brown form, but all blow flies are usually relatively large flies. Blow flies can pick up faint traces of the odour of decay and can fly up to 20 km from their birth-place in search of a suitable corpse in which to lay their eggs.

This is an extremely large family of medium-sized flies that contains more than 1000 species. Blow flies are found throughout the world. They, along with the sarcophagid and muscid flies, are the most important species that provide information relating to the accurate estimation of the post-mortem interval. Calliphorid flies are attracted to carrion and excrement, with some species exploiting open wounds. Blow flies are among the first insects discovered to colonize human remains. This is because:

i) They are the insects most commonly associated with corpses

ii) They are usually the first to colonize the body after death and are present in greater numbers than most other insect groups and

iii) They usually provide the most accurate information regarding time of death - a major objective of forensic entomology.

In an experimental study, calliphorid flies have been recorded arriving at carcasses within minutes of their exposure. The telescoping segments of the tip of the female's abdomen extend to form an ovipositor, which is used for egg laying. Large numbers of eggs are commonly placed in the nose and mouth, as well as other natural body openings that are exposed. Areas with open wounds also are selected for egg placement (Borror & White, 1970; Bland & Jacques, 1978; Peterson, 1979; Arnett & Jacques, 1981; Shewell, 1987; Borror *et al.*, 1989; Liu & Greenberg, 1989; Hall & Doisy, 1993; Hogue, 1993; Castner *et al.*, 1995).

1.4.5.1 *Chrysomya megacephala* (Fabricius)

Figure 1.2: Adult *Chrysomya megacephala*
(Source: Byrd & Castner, 2001)

Flies of the species *Chrysomya megacephala* are large with size over 9.5 mm long. The adults are bright metallic green with black margins on the second and third abdominal segments, and have large red eyes almost touching each other. The face below the eyes is usually yellow to orange (Siriwattanarungsee *et al*., 2005). *Chrysomya megacephala* has a life cycle of 4 growth stages, which are egg, larva, pupa and adult. The period from egg to adult usually takes 8 to 9 days. A female fly can lay from 150 to

300 eggs in each batch and the larva and pupa stages each usually last about 4 days (Sukontason *et al*., 2003).

This blow fly is widely distributed throughout the Asian regions, South Africa, and South America. It also is now well established in the southern United States. The adults have short stout bodies similar in appearance to *Chrysomya rufifacies* but with a noticeably larger head. The eyes are unusually large and a very prominent shade of red, making this flies easily recognizable in the field (Goff *et al.,* 1988; Goff, 1998).

The adult flies are attracted to carrion and sweet foods as well as urine and excrement. Although *Chrysomya megacephala* has a pronounced activity peak during the heat of the afternoon, this species is one of the first species to become active in the early morning hours and is one of the last species to depart carrion at nightfall. Once the adults have settled on carrion, they are not easily disturbed. The adults also have a habit of entering dwellings in search of suitable oviposition sites. The larvae are primarily carrion feeders, and the adult Oriental latrine fly shows a preference for fresh remains. Dry, decaying carrion has little attraction for this species. This calliphorid readily enters dwelling in search of food and egg-laying sites (Hall, 1948; Bohart & Gressitt, 1951; Greenberg, 1971, 1988; Hall & Townsend, 1977; Gagne, 1981; Prins, 1982; Smith, 1986; von Zuben *et al*., 1993).

1.4.5.2 *Chrysomya rufifacies* (Macquart)

Figure 1.3: Adult *Chrysomya rufifacies*
(Source: Byrd & Castner, 2001)

The hairy maggot blow fly is indigenous to the Australian and Asian regions of the Old World tropics (Greenberg & Povolny, 1971). These are large blue or green flies. *Chrysomya rufifacies* is most commonly found in the Orient, Australasia and the Neotropics. It is metallic bluish or green in colour. The adults are 6-12mm long, with at least the front part of the cheeks on the head being greenish in colour (Smith, 1986).

The adults of this species are usually the first to arrive on carrion (often within hours after death) in the southeastern United States. Unlike *Chrysomya megacephala*, this species rarely enters dwellings and the larvae only develop on carrion, not excrement. The larvae of this species are readily distinguished from other larvae in the family Calliphoridae that commonly occur in the United States by the presence of prominent fleshy protrusions along their body. The larvae are both predacious and cannibalistic and therefore should be separated from other species when live collections are made for shipment to a forensic entomologist. If the food supply becomes depleted, the other species of larvae will be consumed and often totally will be eliminated from the carcass. The larvae also are able to burrow several inches into the soil to colonize buried remains. This species is rapidly expanding its range throughout the United State,

and due to its predatory nature it is likely that forensic entomologist will encounter the hairy maggot blow fly with increasing frequency (James, 1947; Hall, 1948; Zumpt, 1965; Greenberg, 1971; Oldroyd & Smith, 1973; Hall & Townsend, 1977; Gagne, 1981; Richard & Ahrens, 1983; Baumgartner & Greenberg, 1984; Smith, 1986; Baumgartner, 1986, 1993; Goff & Odom, 1987; Wells & Greenberg, 1992; Singh & Bharti, 2000).

1.5 ATTRACTION TO THE REMAINS

Insects are attracted to the body immediately after death, frequently within minutes (Nuorteva, 1977; Erzinclioglu, 1983; Smith, 1986; Anderson & VanLaerhoven, 1996). Blow flies are the first colonizers and can be attracted over great distances by odour. Oviposition is elicited primarily by the presence of ammonia-rich compounds, as well as moisture, pheromones, and tactile stimuli (Ashworth & Wall, 1994).

The remains also appear to be more attractive once one female has begun to lay eggs, with many females immediately laying large numbers of eggs in one area (Browne *et al*., 1969). This may eventually result in large numbers of maggots on the body. Large maggot colonies (or maggot masses) can break down a body faster than individual maggots and also can generate heat that can protect the insects against adverse temperature drops. The odour emanating from a corpse changes as the body decomposes, becoming more attractive to some species and less attractive to others as time progresses. Although blow flies arrive very shortly after death, they are no longer attracted when the remains have passed certain stage decomposition or become mummified or dry (Nuorteva, 1977).

Blow flies are diurnal species and usually rest at night. Therefore, eggs are not usually laid at night and a body deposited at night may not attract flies until the following day. One study in an urban area found that some blow fly species oviposited

in low numbers on rat carcasses placed near sodium vapor lamps, but this is rare and nocturnal oviposition has not been observed in large scale studies in other area (Haskell *et al*., 1997). Although blow flies rarely lay eggs at night, they will often lay eggs in dark areas during daytime. These areas include under wrapping, inside closets, in dark basements, in containers and in chimneys (Erzinclioglu, 1996).

1.5.1 Geographical Differences in Succession

Insect colonization of carrion is dependent on many factors, but one of the most important is the geographical region or biogeoclimatic zone in which the remains are found. The biogeoclimatic zone defines the habitat, vegetation, soil type and meteorological conditions of the area. This obviously has a major impact on the types and species of insects present. It also affects the decomposition of the remains, which in turn impacts the insects that colonize them. Many families of carrion insects are relatively ubiquitous, but the individual species involved in decomposition vary from region to region. Decomposition itself also is quite different in various biogeoclimatic zones (Anderson, 2001).

1.5.2 Effects of Sun Exposure

The placement of the corpse has an effect on the decomposition and faunal colonization of the remains. The most obvious effect is that of sunlight and heat. Bodies found in direct sunlight will be warmer, heating up more rapidly and decomposing faster. They will lose biomass more rapidly than bodies in shade and progress through the decomposition stages faster (Shean *et al*., 1993; Anderson, 1995).

1.5.3 Urban versus Rural Scenarios

Some insect species are found in both urban and rural areas, yet others are very specific to one or the other, which indicates resource partitioning. The early colonizing blow flies include rural, urban and ubiquitous species. This can be useful in forensic analyses, as certain species of blow flies found on remains may be used to indicate that the remains have been moved from an urban to a rural environment or vice versa (Erzinclioglu, 1989; Catts & Haskell, 1990).

However, caution must be exercised since only some species are found exclusively in one or the other habitats, while many species can be collected in both. The decomposition rate of carcasses at urban habitats was significantly faster than that of those at other habitats. The speed of decomposition was determined by insect colonization and climatic conditions particularly temperature (Anderson, 2009).

Rural blow flies survive on natural animal carrion, whereas urban blow flies are primarily associated with human refuse in the form of discarded food. Rural areas close to human habitation with their associated human garbage, which encourages urban fly colonization and may increase the chances of accidental transport of urban species to rural environments by human (Byrd & Castner, 2001). Species such as *C.vicina* and *P. sericata* are commonly considered urban species, but have been collected in rural regions (Anderson, 1995; Haskell *et al*., 1997). Therefore, caution must be used in determining whether remains have been moved based on insect evidence.

1.5.4 Bodies Found Inside Buildings

The public and police alike often believe that insects will not colonize remains inside a building. However, insects will colonize remains indoors as easily as outdoors. The succession will be limited by the species that can and will enter dwelling and on

how well the dwelling is sealed. Blow flies are strong fliers that can follow an odour plume over a long distance and can easily enter buildings. Insects will be found even in high-rise apartments (Erzinclioglu, 1996).

1.5.5 Effects of Burial

Disposal of the remains is often of paramount concern to a killer, but a human body is a surprisingly difficult object to dispose of and a commonly chosen method is burial. Bodies buried feloniously, however rarely are deeply buried as burying a full-sized human body at the traditional 6 feet depth requires a great deal of work and time. The longer time a criminal spends with the victim, the greater the chance that evidence will be transferred, and also that the killer will be found with the remains. Therefore a hasty, shallow grave is usually all that is dug (Payne *et al*., 1968; Rodriguez & Bass, 1985; Smith, 1986; Rodriguez, 1997; VanLaerhoven & Anderson, 1999).

Buried remains are still colonized by insects, but burial influences the time required for insects to reach the remains, the sequence of colonization, the species involved and the rate of decomposition (Payne *et al*., 1968; Rodriguez & Bass, 1985; Smith, 1986; Rodriguez, 1997; VanLaerhoven & Anderson, 1999). The above also are affected by geographical region, soil type, whether the remains are disturbed after death and the elapsed time since death before burial (Rodrigues, 1997; VanLaerhoven & Anderson, 1999), as well as the depth of burial (Rodriguez & Bass, 1985; Mann *et al*., 1990).

Many studies have looked at insect colonization of buried bodies, although much of the early work centered on human exhumations, rather than empirical studies (Motter, 1898; Schmitz, 1928; Gilbert & Bass, 1967; Stafford, 1971). Research on buried baby pig carcasses was performed in South Carolina (Payne *et al*., 1968) and

results indicated that several insect species were confined to buried pigs, and that decomposition was greatly slowed by burial.

Most studies noted that decomposition was greatly slowed by even shallow burial (Payne *et al.*, 1968; Rodriguez & Bass, 1985; Rodriguez, 1997; VanLaerhoven & Anderson, 1999). Depth of burial also has an impact on decomposition and insect colonization (Rodriguez & Bass, 1985; Mann *et al*., 1990), but has not been extensively studied. Geographic region, habitat and season all play a major role in insect succession on exposed carrion (Payne *et al.*, 1968; Rodriguez & Bass, 1985; Rodriguez, 1997; VanLaerhoven & Anderson, 1999).

1.5.6 Bodies in Water

Since remains are often found in aquatic environment (Haglund *et al*., 1990; Hobischak & Anderson, 1999), it is important that forensic scientists and police visiting a crime scene have an increased knowledge of the aquatic organisms that could potentially colonize human and nonhuman models. When remains are found in water, faunal succession will be very different from that seen on land. This will be impacted by many factors including the body of water (lake, stream, ditch and ocean), temperature of water, season, presence/absence and type of clothing, scavenging and biogeoclimatic zone. In some cases, when the remains are only partially submerged, both terrestrial and aquatic fauna may colonize them (Anderson, 2001).

1.5.7 Bodies in Vehicles

Due to the nature of the crime, homicide victims are sometimes disposed of in somewhat unorthodox places. This can lead to a restricted or changed succession pattern. Cars and other vehicles are often used for suicide or for the disposal of a body. They provide an interesting environment for decomposition, as the vehicle itself may

act as a barrier to some species, but will act as a protectant from rain and predators, and also have an effect on temperature and humidity (Anderson, 2001). A recent study has shown that the vehicle temperature and the homicide victims mass may affect the development rate of the larval population. Besides that the adult fly numbers entering the vehicle is dependent on the population size and species of adult fly present in the general area and the access available to them to the interior of the cabin (Dadour *et al*., 2011). It was previously found that the carcass attendance by representatives of the Calliphoridae was delayed within the vehicle environment by 16 to 18 hours, while oviposition was not observed until 24 to 28 hours following death. In contrast, attendance by Calliphoridae at surface carcasses occurred within 1 hour of death and oviposition occurred within 6 to 8 hours of death (Voss *et al*., 2008).

1.5.8 Hanged Bodies

Hanging, either as a result of suicide or accident (or more rarely homicide), is not uncommon form of death. If the body is suspended above the ground, it could present a unique environment for insect colonization. Although extensive research has not been published, some researchers have noted that hanging affects the insect colonization of the remains. They noted that hanging altered the insect colonization by excluding soil-dwelling taxa, thus changing the drying pattern of the body and consequently, limiting the activities of flies species. This reduces the number of insects collected and influences which species colonizes the remains as well as their times of colonization (Goff & Lord, 1994).

1.5.9 Burnt Remains

Remains may often be burned either perimortem or post-mortem. The arthropod fauna which colonized the burned and unburned carcasses were basically the same, but appeared slightly earlier on the burned carcasses presumably due to the openings caused by the cracked skin. The burnt carcasses attracted much more fly oviposition than the unburnt carcasses are still extremely attractive to calliphorid flies (Avila & Goff, 1998). Killers often try to dispose of a victim by burning the body, but are unaware of the tremendously high temperatures and the time required to completely incinerate a human body.

Even in the extreme heat of a professional crematorium, recognizable pieces of human remains are still present. The level and amount of colonization of burned remains by insects will no doubt be strongly influenced by the amount of flesh remaining, with more complete incineration reducing insect colonization (Murray & Rose, 1993; Kennedy, 1996). Recently, the larval aggregation formed on a burned human remain by second instar *Chrysomya megacephala* were studied. It shows that the larval aggregation was 8°C above the ambient temperature. Since the metabolic heat generated by maggot mass can be sufficient to raise their micro-environmental temperature by several degrees above ambient, it is essential to take into consideration the maggot mass temperature in determining insect development (Kumara *et al*., 2012a)

1.5.10 Other Factors Which May Affect Succession Scavenging

Scavengers other than insects also are attracted to remains, and can remove large quantities of flesh and even clothing. This can have major effect on the decomposition rate and consequent insect colonization. Carrion in aquatic habitats also was scavenged more often in shade than in direct sun (Anderson, 2001). Scavenging, in addition to

affecting decomposition and insect colonization, may also produce post-mortem artifacts that may be initially mistaken for wounds or mutilation (Patel, 1994). Conversely, wound originally mistaken as rodent damage may actually have other causes (Patel, 1995).

Scavengers, acting as opportunistic predators of insects, are common on remains. Although they may remove substantial numbers of colonizers (particularly blow fly larvae), they usually have little impact overall. However, some insect scavengers (due to their voraciousness and numbers) can have a substantial impact on arthropod colonization of remains. One such example is the fire ants (Hymenoptera: Formicidae: *Solenopsis* spp.) which may remove significant numbers of blow fly eggs and larvae (Early & Goff, 1986; Greenberg, 1991; Wells & Greenberg, 1994; Stoker *et al*., 1995). Other species of ant also have been shown to have an impact (Cornaby, 1974; Lord & Burger, 1984), but most are present throughout decomposition as scavengers with little effect on overall decomposition rates (Payne *et al*., 1968; Payne & Mason, 1971; Anderson & VanLaerhoven, 1996).

1.5.11 Presence or Absence of Clothing

Human victims are frequently clothed. The clothing may be complete or partial. Clothing can be expected to have an effect on insect succession on a corpse, as it affects the temperature and humidity of the remains, the amount of shade and protection the body provides. Most early instar larvae require liquid protein for survival (Smith, 1986). As the clothing becomes saturated with decompositional fluids, it provides more sites for oviposition than a naked corpse, resulting in larger larval masses and hence faster decomposition (Anderson & Laerhoven, 1996; Anderson, 2001).

The clothing also can provide additional shelter for blow flies and their predators, increasing the number of Coleoptera on the remains and making the remains more attractive for species that prefer wetter environments, clothing provides shelter and extra attachment sites for aquatic fauna. However, the effect of clothing depends on whether the body is completely or partially submerged. When the body is exposed above the waterline, clothing protected maggots from predation, whereas below the waterline, organisms such as crayfish and caddis flies (Trichoptera) fed on unclothed regions preferentially (Anderson, 2001). Clothes permeated with lubricants, paint or combustibles may double the time for initial colonization and have been shown to retard decomposition (Marchenko, 1980; Greenberg, 1991).

Pig carcasses are frequently used to mimic human remains and are considered to be an excellent model for human decomposition (Catts & Goff, 1992). Most research has concentrated on naked pig carcasses (Payne, 1965; Payne & Crossley, 1966; Hewadikaram & Goff, 1991; Shean *et al*., 1993; Anderson & VanLaerhoven, 1996), although some work has included clothing (Komar & Beattie, 1998; VanLaerhoven & Anderson, 1999). Therefore, clothing can have a considerable impact on the decomposition, and it is important that future studies to include this aspect of colonization as a consideration. Insects also have been shown to move and tear clothing in a manner that may mislead investigators into assuming that that a sexual assault has taken place (Komar & Beattie, 1998). Maggot masses have been able to move clothing from underneath a body, despite the overlying carcass weight.

In carcasses clothed in skirts, the underwear and pantyhose were moved down to the distal hindlimbs while the skirt was pushed up. Natural decomposition changes such as bloating can tear clothing as well (Komar & Beattie, 1998). Heavy clothing may deter carnivore scavenging (Haglund, 1997), but carnivores also can cause clothing

disarray. Such post-mortem artifacts are usually easy to differentiate from that caused by insects (Komar & Beattie, 1998).

Pigs are chosen as good human models for many reasons, including the fact that they are relatively hairless. However, carcasses with a coat of fur also have been frequently used to generate carrion data (Bornemissza, 1957; Reed, 1958; Easton, 1966; Smith, 1975; Denno & Cothran, 1976; Putmann, 1978; Braack, 1981; Jiron & Cartin, 1981; Early & Goff, 1986). The predictable sequence of insect succession on a body has been recognized as an excellent method to estimate the time since death. However, there are many diverse parameters that can affect the timing and species composition of the carrion fauna. It is vitally important to be aware of all the factors that can impact insect colonization of remains and to take them into account when analyzing a death. In particular, research is needed to develop a geographical database of insect succession on carrion in a variety of habitats and scenarios in Malaysia.

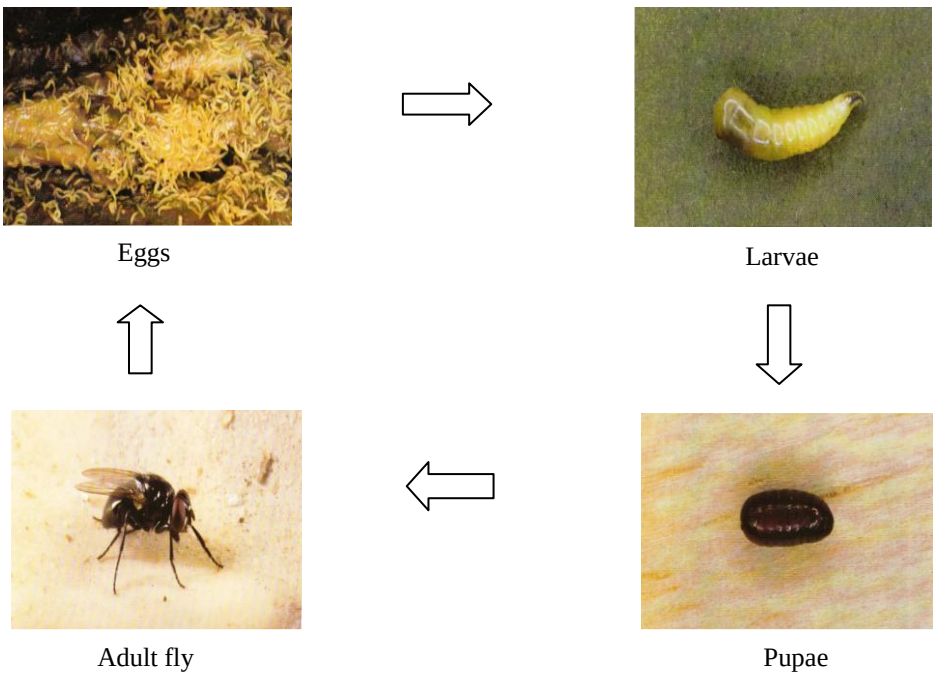
1.6 Insect Development

Insects pass through a series of stages when developing from egg to adult as shown in Figure 1.4. The development of blow flies includes four stages namely egg stage, larval stage, pupal stage and adult stage. During the larval stage three instars can be separated namely 1st, 2nd and 3rd instar, where the latter is divided due to behavioral changes in feeding and postfeeding larvae. Blow flies deposit egg clutches directly on the food substrate, such as a dead body (Smith & Wall, 1997), in a position where the eggs are protected and in a moist environment. This ensures a food supply for the hatching 1st instar larvae. Adelson (1972) and Fisher (1980) considered the egg hatching period to be 24 hours. Kulshrestha & Chandra (1987) found the eggs hatching period to be between 20-24 hours. They observed that hatching will not take place or will be

delayed by one or two days if weather is cold, and the warm weather, on the other hand may advance the process.

The first three instars each undergo a moult in order to reach the next developmental stage and the stages can be distinguished by the number of respiratory slits at the posterior end of the larvae. The third instar stage lasts for longer than the first two. The larvae feed on the substrate as 3rd instars, then leave the food source to find a suitable place for pupation, entering the post-feeding stage (Arnott & Turner, 2008).

About one third of the pre-adult development time is spent in the post-feeding larval stage. Then pupation sets in and the imago develops within the pupal case till eclosion (Greenberg, 1991). This last stage persists for about half of the time of the total development. The larval's growth rate depends on its body temperature, which is directly influenced by environmental conditions as the ambient temperatures and the heat generated by maggot aggregations (Slone & Gruner, 2007). Also, each species has its own temperature dependent growth rate.



*Note: Photos from Byrd & Castner, 2001

Figure 1.4: Life cycle of a fly

1.7 Estimating the Post-Mortem Interval (PMI)

Estimation of time since death is an integral part of medico-legal investigations. Post-mortem Interval is defined as 'amount of time that has elapsed since the death of the decedent'. The key goal of estimating time since death at the scene of crime is to have a preliminary idea of the time of assault and for narrowing the field of suspects. A precise estimation of PMI is important for criminal law as it validates the witness's statement, limits the number of suspects and assesses their alibis (Mathur & Agrawal, 2011).

The estimation time for PMI was based on entomological evidence and in agreement with the PMI obtained by standard means, provided that all evidence from the death scene is taken into consideration, such as the delayed arrival of flies to a corpse when in enclosed environments. The estimate of PMI can be different from the

real interval due to physical circumstances in the surroundings of the remains (Oliveira-Costa & Mello-Patiu, 2004). Entomologist then determined the age of the specimens to provide evidence as when the female's flies first found the dead body and laid their eggs, the minimum estimate of the PMI. This can be taken as the latest time by which death must have occurred. The estimation of maggot age relies on detailed knowledge of the fly life cycle and the factors that influence it (Greenberg, 1990; Singh & Bharti, 2001; Wooldridge *et al*., 2007; Amendt *et al*., 2008).

Within a few hours of death, corpses start to have strong odour. Large numbers of adult blow flies, with their finely tuned olfactory senses, are attracted by the odours of decomposition. These odours are mainly created by the action of bacteria on dead tissues. Blow flies often swarm to wounds, open sores and ulcers on living vertebrates as well as dead ones and their targets can include humans. On arriving at a corpse, some flies will feed, others will mate and some females will deposit their heavy loads of eggs. A maggot will develop inside each egg and break through the protective shell, the chorion, and a few days later, to start feeding on the decomposition body (Browne *et al*., 1969; Erzinclioglu, 1983; Smith, 1986; Wall & Warnes, 1994; Anderson, 2001).

On an uninjured body, blow fly eggs are usually laid at the openings of body orifices and it is in those areas that the emerging maggots start to feed. Blow flies are also attracted to injuries, so if maggots are found at sites other than the body orifices, they might indicate that some traumatic wounding took place before death (Lee, 1989; Hall, 2000). There are two insect-based methods for making PMI estimation. As the body passes through stages of decomposition, the biological and chemical properties of the body change, making it attractive to different types of insects at different times throughout the process. A simple observation on the types of species present on a corpse

(immature and adult) can be used to estimate how long the body has been deceased (Marchenko, 2001; Carvalho *et al*., 2004).

The second method involves estimating the age of immature specimens located on the corpse. Their age, sometimes coupled with an estimation of the time that elapsed between deaths and when the eggs were deposited on the corpse, also serves as a basis for PMI estimation (Catts & Goff, 1992; Carvalho *et al*., 2004). Regardless of the method used, the process of PMI estimations begins at the crime scene. Often, it is not possible for an entomologist to be called to the scene. Therefore, the crime scene investigator is frequently responsible for the proper collection and preservation of entomological evidence. Insect samples are collected from the corpse and surrounding area and should represent the different species present, as well as the different sizes and stages for each species. The samples are labeled and stored in preservation fluids, such as ethanol to preserve the external features that are required for their identification (Amendt *et al*., 2007).

Accurate PMI estimations not only rely on proper evidence collection, but also require a precise recording of the crime scene details. Because temperature is a key factor affecting the rate of insect development, specific climatological data must be recorded along with relevant body or maggot mass temperatures. The temperature of the crime scene for the days or weeks before the discovery of the body can be estimated using data collected from nearby weather stations. Any additional details that could affect the decomposition of the corpse and concurrent insect colonization, such as partial burial or wrapping of the body, are also recorded (Amendt *et al*., 2007).

Once the entomologist receives the insect samples and crime scene details, each specimen is identified to the level of species. Proper identification is important since two species that are similar in appearance may have markedly different growth rates.

Improper identification would make the specimen's age estimation and subsequent PMI estimation invalid. An entomologist's personal experience and the use of published keys aid in the identification process (Greenberg & Kunich, 2002).

However, immature larvae and pupae of some species are so similar in appearance that they cannot be differentiated by microscopic examination. One method for preventing a misidentification is to keep a portion of the immature insects collected at the crime scene alive and return them to the lab where they can be reared to adults, at which point they can easily be identified (Haskell, 1990). If rearing fails or is not attempted, it may be possible to identify immature specimens using scanning electron microscopy (Liu & Greenberg, 1989) or the insect's own DNA (Sperling *et al*., 1994; Wells & Sperling, 2001; Wells *et al*., 2001, Tan *et al*., 2009, 2010).

The insects associated with a corpse greatly depend on the biogeoclimatic zone. Ideally, a comparison should be made to succession data collected in the general geographic area of the crime (Nuorteva, 1977; Erzinclioglu, 1983; Marchenko, 2001). In addition to geography, other factor such as burial (Rodriguez & Bass, 1985) or wrapping of the corpse (Goff, 1992) must be taken into account. Although the crime details may not exactly match the parameters of the published data, the data provides an indication of how these factors may affect the decomposition process.

Although most corpses immediately attract flies following death, there are various factors that could delay this process. A corpse located indoors is sheltered from fly activity and could extend the time it takes for a carrion fly to discover the corpse (Goff, 1991). Although it was commonly believed that carrion flies do not lay eggs at night (Nuorteva, 1977), recent publications have shown that it is possible, although insect activity is drastically reduced from that in the daytime (Greenberg, 1990; Singh & Bharti, 2001).

1.8 CRIME AND FORENSIC SCIENCE

It is true that science and technology today have made incredible leaps toward the ability to identify unknown perpetrators. It is not necessarily an over abundance of physical evidence that has given the science this ability (Durnal, 2010).With scientific evaluation of physical evidence, police has become more effective in fighting crime and criminals. The use of scientific evidence has led to the release of thousands of innocent people, including death row inmates, who had been convicted. Law enforcement personnel, especially patrol officers, have been criticized for ignoring or overlooking physical evidence at crime scenes. The improper handling of forensic evidence may results in unjust verdicts. Errors in the process of obtaining forensic evidence could result in the release of criminals and/or the arrest of innocent people (Fradella *et al*., 2007).

Physical evidence found at crime scenes needs to be evaluated to become more valuable for criminal investigations. The evaluation of physical evidence starts with crime scene investigations continues at crime laboratories and finishes in the courts. In this respect, to be able to evaluate physical evidence effectively, each counterpart must operate flawlessly. However, mistakes have been made in the evaluation of physical evidence (Saferstein, 1995).

According to Saferstein (1995), police officers make mistakes when they overlook evidences, fail to use appropriate technologies for certain types of evidence, and fail to document collected evidence properly. Consequently, Saferstein argues that "the major problem is in making sure there are properly trained officers at the scene". Like Saferstein, many scholars address the need for training to prevent mistakes at the crime scene. Different training and education options are available for the people who handle forensic evidence.

Hess & Wrobleski (2006) stated the importance of line officers in criminal investigations through physical evidence because the quality of evidence plays a vital role in criminal investigation and prosecution, an understanding of the types of evidence to look forward maintaining the integrity and subsequent admissibility of such evidence is as vital a role for the line officer as it is for the investigator.

1.8.1 Physical Evidence

Physical evidence refers to objects that can indicate that a crime has been committed or suggest links to a crime, its perpetrator and/or its victim (Saferstein, 1977). Because physical evidence cannot be wrong, cannot commit perjury and/or cannot be completely absent, such evidence is of inestimable value for modern criminal investigations. Physical evidence is more reliable than most other sources of information, such as eyewitnesses (Svensson *et al*., 1982). However, humans can make errors in the interpretation of physical evidence. Failures in finding, studying and understanding physical evidence can weaken its value (Kirk, 1974).

Criminal investigations can gather information on crimes, the perpetrators, and victims through identification and individualization of physical evidence. Identification means associating an item to a common source. Individualization means that an item is unique (Svensson *et al*., 1982). Physical evidence can provide the following types of information:

i) The body of the crime: Physical information may provide insights into whether a crime has taken place.

ii) The modus operandi (method of operation): It can be possible to link a crime or criminal to previous crimes by examining the method of operation.

iii) Links between a suspect and a victim: Physical evidence, such as trace evidence, can link a suspect with a victim.

iv) Links between a person and the crime scene: Physical evidence may indicate whether or not a suspect was at the crime scene.

v) Negation or support of a witness's testimony: Physical evidence usually reveals whether a witness is telling the truth.

vi) Identification of a suspect: Physical evidence can reveal the identity of a suspect.

vii) Investigative leads: By following physical evidence, investigators can narrow down the focus of the investigation (Lee & Harris, 2006).

viii) Identification of unknown substances: A small amount of physical evidence may reveal the identity of unknown substances.

ix) Reconstruction of a crime: Physical evidence can help investigators understand how the crime was committed or progressed (Lee *et al*., 2001; Miller, 2005).

There are many ways that physical evidence can help with a criminal investigation. For instance, broken windows or doors of a house can indicate burglary (*corpus delicti*). The type of explosive or style of a burglar may indicate how the crime was committed (modus operandi). A suspect's bloody hands can indicate contact with a victim (linking a suspect to a victim). Shoe prints may prove that the suspect was at the crime scene may lead investigators to a female suspect (narrowing the focus). Such kinds of information also help investigators reconstruct a crime (Lee *et al*., 2001; Miller, 2005).

Each type of physical evidence has the potential to provide critical information for the success of criminal investigations. However, having critical information about the incident depends on the success of the stages of physical evidence analysis. These stages are as follows:

i) Recognition of evidence

ii) Documentation of the crime scene and evidence

iii) Collection and preservation of evidence

iv) Analysis of the evidence and interpretation of results

v) Reporting the results

vi) Expert testimony

Physical evidence gains its value as it successfully passes through stages of analysis. Each stage is important. The actions that are taken at the earlier stages determine the quality of the final outcome (De Forest *et al*., 1983).

1.8.2 The Responsibilities of Crime Scene Police Officers

Crime prevention, criminal apprehension, law enforcement, order maintenance, public services and traffic enforcement are considered to be the duty of the police (Hale, 1994). There are many types of crime scenes. Crime scenes can be classified based on the location, size and boundaries of the crime scene, the type of crime committed and criminal activity or other on-scene conditions. Basically, the scene in which the initial crime has taken place is considered to be a primary scene and any other relevant scenes are referred to as secondary crime scenes. Regardless of the type of crime scene, any forensic investigation of a criminal act starts at the crime scene (Miller, 2005).

Recognizing, preserving, collecting, interpreting and reconstructing relevant physical evidence are the objectives of crime scene investigations. First responders and the crime scene investigators may perform the crime scene survey together (Miller, 2005). However, large police departments usually employ specially trained technical employees to gather and preserve physical evidence at a crime scene (Fisher, 2008).

According to Garrison (2003), police officers should be the first to arrive at the crime scene. Without delay, crime scene police officers should begin to implement their tasks as preliminary investigator and a first responder. "The longer the police take to arrive the greater the possibility that evidence will be destroyed or contaminated" (Palmiotto, 1998). Forensic techniques cannot be successfully resolved without the intuition, knowledge and experience of law enforcement personnel (Platt, 2003). Proper handling of the crime scene by patrol officers increases the successful resolution of the criminal cases (Fisher, 2003; Dantzker, 2005).

The crime scene usually does not remain stable (Fisher, 2003; Garrison, 2003). It is a dynamic, rapidly changing situation (Svensson *et al*., 1982; Fox & Cunningham, 1998) and should be considered fragile (Fox & Cunningham, 1998). The first thing a crime scene officer should do when responding to a crime is to record the time of arrival at the scene (Brown, 2006). The time can be critical for every stage of the investigation. For instance, recording the time can be important to support or discredit the testimony of suspects, eyewitness or victims. Crime scene officers should be observant when approaching and entering a crime scene (Jamieson, 2004). The crime scene officers responding to a crime scene also can establish whether a crime has been committed, determine the type of crime committed, the method of the perpetrator, the extent of personal injuries and the nature and value of any stolen property (Weston & Wells, 1986).

Crime scene officers should avoid unnecessary actions at a crime scene. For instance, they should never try to satisfy their curiosity by walking around the crime scene or touching, picking up and/or moving items. They should not use toilets, glasses, dishes, paper towels, pens, pencils or any other items that belong at the crime scene. It has been documented many times that first responding officers toured the crime scene, touching a variety of objects and leaving their fingerprint behind (Svensson *et al.*, 1982).

The crime scene should be protected from unauthorized people. Unauthorized intrusions can render physical evidence useless if such evidence has been moved or contaminated (Fox & Cunningham, 1998). However, authorized people doing their job also may contaminate physical evidence by altering the crime scene inadvertently. Crime scene officers should try to inform and guide authorized people not to harm the criminal investigation while doing their job (Lee *et al*., 2001).

Crime scene officer's proper handling of the crime scene increases the success of resolving criminal cases (Fisher, 2003; Dantzker, 2005). Palmiotto (1998) argues that the most important people in a criminal investigation usually are the first responding officers. The officers who are first to arrive at a crime scenes may have a huge effect on the eventual success of the investigation. They can affect the success of the whole investigation in either a negative or a positive way. There are many examples of how the actions or omissions of first responders can cause crimes to remain unsolved (Fisher, 2003). In order to respond effectively to cases that include physical evidence, crime scene officers need to have awareness of and knowledge about forensic science and physical evidence.

1.8.3 Forensic Laboratory of the Royal Malaysia Police (RMP) Force

Figure 1.5: Logo of the Forensic Laboratory of RMP

The idea of forming a forensic laboratory was mooted by a former Inspector General of Police Tun Muhammed Haniff bin Omar in 1974. As the first step in the introduction and usage of forensic science, the Royal Malaysia Police established a mini forensic laboratory at the Crime Investigation College (CIC) then situated at the Police Training Centre, Jalan Semarak (PULAPOL). This laboratory was used for demonstrations and training in plaster casting, finger printing, ballistic test, studying evidence from broken glasses, paint work, blood splatter pattern and comparing of samples from microscope and others.

In 1994, Tan Sri Rahim Noor (the Inspector General of Police then) agreed with the proposal to build the Royal Malaysia Police (RMP) forensic laboratory. In 1996, the Ministry of Home Affairs officially approved this project. The Deputy Director 1 of Criminal Investigation Department (CID) was appointed the Chairman of the Establishment and Building of a new Forensic Laboratory Committee in Cheras which

started in 1999. In the month of July 2000, 28 senior police officers started their courses (Basic Forensic Science Course and Management of Laboratory) at Victoria Forensic Science Centre Melbourne, Australia.

The installation and training in the usage of the analysis equipment at the RMP Forensic laboratory, Cheras started on March 2001. Thirty four Senior Police Officers were involved in the Advance Forensic course for 3½ months. The equipment of the RMP Forensic laboratory was commissioned and received by the former Deputy Director 1 of Criminal Investigation Department, Dato' Ramli Yusuff. The laboratory started operations in June, 2001.

The RMP Forensic Laboratory is divided into 5 divisions namely Physics, Chemistry, Biology, Narcotic and Management. Figure 1.5 shows the logo of the Forensic Laboratory of Royal Malaysia Police. The mission of the RMP Forensic Laboratory is to establish the laboratory as an outstanding scientific service centre in this region with dedicated and highly motivated personnel committed towards integrity, excellence and quality. The concept behind the setting-up of the Royal Malaysia Police Forensic Laboratory is to achieve efficiency and provide effective service in the field of investigations and research. The objectives of the Forensic Laboratory are as follow:

i) To give assistance scientifically in the investigation of criminal cases from the beginning in order for investigation to be conducted effectively.

ii) To organize and update data of analysis which can assist in criminal investigations such as DNA profiling and drug and ballistic profiling. This facility can be used as a databank for criminal intelligence.

iii) To expand the usage of forensic science knowledge following the current trend.

iv) To increase the knowledge of forensic science amongst police officers through training and in depth and systematic experiments.

v) To carry out research and development of Criminal Investigation Department specifically in the field of forensic science.

vi) To assist in fulfilling the government's and society's aspiration that crimes are solved and criminals charged expeditiously in court as a deterrent to prevent them from committing future crime.

vii) To follow the development of science and technology by applying scientific techniques recognized worldwide.

The main service rendered by the Physics Division is managing and conducting investigations at crime scenes, collecting of physical evidence and conducting analysis for the purpose of linking the evidence to the crime committed. This division is also involved in research and development (R&D), periodically training and sharing investigation technology with other investigation departments of the Royal Malaysia Police. As this division plays a major role in initial investigations they are in a state of preparedness at all times. At the initial stage, this service is confined to areas of the Klang Valley and later will be expanded to the whole country when the laboratory is fully operational. This division is again divided into various sections such as Crime Scene Investigation, Document, Fingerprint, Photo/Video and Computer Crime.

The Chemistry Division is made up of the Criminalistics, Vehicle, Casting, Fire Investigation & Mischief and Ballistic Sections. The main mission of the Narcotic division is to assist the intelligence unit of the Narcotic Department in identifying drug distributions of drugs produced in the country and to identify syndicates processing drugs using drug profiling. Other duties include conducting research on the trends of

current drug usage. This division can be divided into 3 sections; they are the Drugs in the Body Section, the Drug Analysis Section and the Examination of Illegal Drug Laboratory Section (Halal, 2004).

Forensic Unit of RMP will be the first to be dispatched on call. At any crime scene in Malaysia, the general procedure for the RMP Forensic Unit is to collect all the appropriate evidence and release to the Investigating Officer (I.O). Any biological, narcotic, ballistic evidence are sent to the Chemistry Department (JKM) for analysis purposes. Evidence such as fingerprints is sent to Bukit Aman Headquarters for processing. Any insect evidence such as maggots collected from the dead human bodies is sent to Institute for Medical Research (IMR) for PMI estimations.

1.8.4 Legal Aspects of the Forensic Entomology in Malaysia

This section presents the admissibility of a forensic entomologist's testimony in the courts of law in Malaysia for the purpose of determining the post-mortem interval (PMI). Legal mechanism controlling on what, when and whose evidence may be allowed to be given during a proceeding in the court of law. General rule says that any evidence, which proves the facts that are relevant in a proceeding, is *prima facie* and admissible in that proceeding. Exception to the law is hearsay evidence, illegally obtained evidence and unreliable evidence.

According to the expert evidence under section 45, Evidence Act 1950, when the court has to form an opinion of science, the opinions upon that point of a person specially skilled in that science are relevant. Three requirements under section 45:

i) The opinion is upon a point of science

ii) The court has to form an opinion upon that point and

iii) The person who gives evidence in court is an expert in that science

If an opinion can meet all the three requirements, then such opinion is relevant and evidence to prove the opinion may be admissible. The admissibility of expert evidence rests upon the relevancy of the scientific opinion.

Sir James Stephen (drafter of the Evidence Act 1950 wrote that 'Science includes all subjects on which a course of special study or experience is necessary to the formation of an opinion'). Court's requirement in scientific evidence is an "expert evidence is only admissible to furnish the court with scientific information which is likely to be outside the experience and knowledge of a judge. If judge can form his own conclusions without help, then expert evidence is not required". The more scientific and complex the subject matter, the more extensive and deeper will the court be required to enquire into the ascertainment of the expert's qualification or experience in the particular field.

Furthermore, according to section 329 of the Criminal Procedure Code (CPC), the officer in charge of a police station (OCS) or any police officer not below the rank of a sergeant, shall make any enquiry under the directions of the officer in charge of the police district (OCPD) in the event death has occurred in the following situations, namely,

i) That a person has committed suicide

ii) That a person has been killed by another or by an animal or by machinery or by an accident

iii) That a person has died under circumstances raising a reasonable suspicion that some other person have committed an offence

iv) That the body of a dead person has been found and it is not known how the death has occurred

v) That a person has died due to a sudden death

Section 330 of the CPC further provides that every officer making an enquiry under section 329 above shall, if there appears to him any reason to suspect that the deceased came by his death in a sudden or unnatural or by violence or that his death resulted in any way from or was accelerated by any unlawful act or omission on the part of any other person, at once inform nearest Government Medical Officer (GMO) and shall take or send the body to the nearest Government Hospital or other convenient place for the holding of a post-mortem examination of the body by the GMO. Finally, section 331(1) of the CPC provides that upon receiving the information referred to in section 330 above the GMO shall, as soon as practicable, make a post-mortem examination of the body of the deceased.

The purpose for the arrangement of post-mortem examination is to determine the 'cause of death', which according to section 328 of the CPC includes not only the apparent cause of death as ascertainable by inspection of the body of the deceased but also all matters necessary to enable an opinion to be formed as to the manner in which the deceased came by his death and so to whether his death was resulted in any way from or was accelerated by an unlawful act or omission on the part of any other person.

One of the important facts that the investigating police officer would want to know from the GMO is the time when the death has occurred. As commonly understood, mostly all victim-based crimes occur only when there is union in space and in time between a criminal and a victim. Thus, the person with whom the deceased person was at that time of death may either be the killer or eye-witness, which in either case, is a person who can provide material information during the murder investigation.

Furthermore, the time of death is also important when it comes to the trial stage, since according to section 153(1) of the CPC, the charge sheet shall contain such particulars as to the time and place of the alleged offence. Consequently, section 9 of the Evidence Act 1950 (EA) provides, *inter alia*, that facts in which fixed time at which any fact in issue or relevant fact happened are themselves relevant so far as they are necessary for that purpose (Criminal Procedure Code, 1976).

1.9 OBJECTIVES OF THE PRESENT STUDY

The present study was designed with the following objectives:

i) To assess the current status of awareness and knowledge towards the application of forensic entomology in Malaysia.

ii) To compare morphological and molecular techniques for the identification of flies collected from various crime scenes.

iii) DNA barcoding for *Chrysomya rufifacies* and *Chrysomya megacephala*- two predominant flies species found in dead human bodies from crime scenes in Malaysia.

CHAPTER 2
LITERATURE REVIEW

2.0 LITERATURE REVIEW

Entomology is derived from the Greek word *entomon* (insect) and *logos* (word, reason) meaning the study of insect (Gupta & Setia, 2004). Forensic entomology is the science and study of insects and other arthropods with legal related applications. It can be divided into three subfields like urban, stored-product and medico-legal or medico-criminal. Urban forensic entomology typically concerns pest infestations in buildings or gardens that may be the basis of litigation between private parties and service providers such as landlords or exterminators. Such questions may include the appropriateness of certain pesticide treatments. Civil law actions and litigations involving arthropods in dwelling or as house and garden pests are included in urban forensic entomology (Catts & Goff, 1992).

Stored-product forensic entomology is often used in litigation cases over infestation or contamination of commercially distributed foods by insects. It is also civil in nature, and depending on the case it may have a criminal aspect. This area concerns itself with insect contamination in food and beverage items. In a stored product case the forensic entomologist must determine the species of insect involved and then make a determination as to the presence of the insect being accidental contamination, intentional contamination or if the insect particulate matter is below allowable or permissible levels. Some insect particulate matter is allowed in food simply because they are naturally occurring and it is not economically feasible to grow food items completely free of insects. Therefore, there are cases in which complaints about insect

particularly in food are found to be allowable within governmental guidelines (Byrd & Castner, 2001).

Medico-legal forensic entomology includes arthropod involvement in events such as murder, suicide, rape, physical abuse and contraband trafficking. In murder investigations it deals with what insects lay eggs when, where and in what order they appear in dead bodies. Other than that, it cannot be denied that forensic entomology has been an important investigative tool for many years, particularly through its use in court trials in providing an estimation of post-mortem interval (PMI) in homicide cases (Catts & Goff, 1992; Hall, 2008; Haskell *et al*., 2008).

Forensic entomology is inexorably related with the fields of medical entomology, taxonomy and forensic pathology (Catts & Haskell, 1990), and is used mainly to estimate the time of death based on the developmental rates and the successional ecology of specific insects that feed on carcasses. The period of 72 hours after death is usually the most important time and often the only period to accurately estimate the time of death (Wolff *et al*., 2001).This application of entomology in investigations demands great accuracy in PMI estimations resulting in significant research addressing this issue. The association of flies with dead human bodies and animal carrion has been noted throughout history (Amendt *et al*., 2000, 2004; Benecke, 2001, 2008). Early reports of using insects as evidence in criminal situations have been retold in the several landmark texts on the subject of forensic entomology (Smith, 1986; Hall, 1990; 2001; Greenberg & Kunich, 2002).

Dr. Greenberg has summarized the following findings of his research at the University of Illinois, which was published in the Journal of Medical Entomology (Greenberg, 1991):

(i) Insects are associated with each stage of the body decomposition stages

(ii) Most blowflies lay eggs in a batch

(iii) Blow flies usually lay eggs in the following areas of the body, mouth, nose, open wounds and anus. In addition, blow flies have predators and the actual decomposition may be prolonged by predators consuming the blow flies eggs

Recently, the use of arthropods in criminal forensic studies has become more recognized amongst forensic scientists, forensic pathologists, criminalists, jurists and the public. Subsets of insect and mite species were shown to be valuable tools in the investigation of post-mortem intervals, even of badly decomposed corpses, child neglect, relocation of a body, identification of suspects, hygienical questions and the explanation of two sets of postmortem lividities on one corpse (Anderson, 1997; Benecke, 1998).

Hundreds of arthropod species are attracted by corpses, primarily flies (Diptera), beetles (Coleoptera) and their larvae. The animals feed on the body and live or breed in and on the corpse, thus depending on their biological preferences and on the state of body decomposition (Benecke, 2001). The flies lay their eggs, usually on a moist body part or open wound (Haskell & Williams, 1990). The fly larvae (maggots) feed on decomposing flesh and then begin their growth pattern over time.

Two ways are used for estimation of PMI of human remains based on information on the development and succession of carrion insect species (Tabor *et al.*, 2004). In very early stage of death (1-3 days), the PMI can be estimated by the pathologist based on the biological and physical changes of a corpse. The entomological findings can corroborate the results of the pathologist. In the early stages of decomposition blow fly eggs, and sometimes small larvae can be seen in the natural orifices or wounds of the body and it can be assumed that it has only been on the site for a very short time (1-2 days) (Smith, 1986). After 72 hours or more after death, the

medical procedures are no longer of value (Campobasso & Introna, 2001) and then the value of entomological tools become increasingly important in the PMI estimate.

According the Benecke (2001), there are several other types of information that can be derived from arthropods found at a crime scene. For example, besides the estimation of the colonization time or PMI, the following information can be determined:

i) Suspects have been linked to a scene of crime as a result of the fact that they had been bitten by arthropods specific to the vicinity (Webb *et al*., 1983; Prichard *et al*., 1986)

ii) Insects that live in restricted areas but are found on a corpse in a different area can prove that the body had been moved after death (Catts & Goff, 1992)

iii) Blow fly larvae can give information on how long children or elderly people were neglected by their relatives or nursing personnel (Benecke *et al.*, 2004)

iv) Aspects of hygiene (appearance of larval and flies in clean, empty rooms or of maggots in food) can be explained by linking the entomological findings to known death cases or other environmental factors from the surroundings of the death scene

v) A report describes that in ancient times a murder weapon was identified (McKnight, 1981)

vi) Drugs that cannot be detected in severely decomposed tissue of corpse may still be found in the insects that did feed on the corpse, termed entomotoxicology (Goff & Lord, 1994)

vii) The location of a stab wound can be determined by unusual feeding sites of beetles and maggots

viii) The question of whether a person was killed and brought outside during day or night time while it was raining or not may be scrutinized (Schroeder *et al*., 2002)

A major focus of medico-criminal forensic entomology is to calculate an accurate and precise estimate of the time between death and discovery of a corpse, otherwise known as the post-mortem interval (PMI) (Catts, 1992), and sometimes may even provide clues to the cause of death (Anderson, 2004). Long term PMI estimations are best performed by calculating the age of blow fly larvae developing on the corpse (Smith, 1986; Catts & Goff, 1992; Gennard, 2007).

It has to be accounted for however, that different blow fly species develop at different rates under the same conditions (Niederegger *et al*., 2010). Larval development is dependent on temperature (Bowler & Terblanche, 2008) and every species has a slightly different growth rate (Erzinclioglu, 1990; Davies & Ratcliffe, 1994; Richards & Villet, 2009). Accurate species identification is therefore crucial especially when legal matters are involved (Wells & LaMotte, 2001), but this may be difficult using traditional morphology-based approach (Prins, 1982; Wallman, 2001).

Conventionally, adult insect species are identified based on specific morphological features, such as presence and number of bristles, wing venation, and body colouration (Smith, 1986; Wallman & Donnellan, 2001). The immature stages are, however, almost impossible to identify and require trained eyes as identification is based on specific characters such as the pattern differences of the spine, posterior spiracle and cephalopharyngeal skeleton (Wells & Sperling, 1999, 2001; Zehner *et al*., 2004; Nelson *et al.*, 2008). For most cases, species identification for the larval stages only becomes feasible when they reach the third instar (Wells *et al*., 1999; Turchetto *et al*., 2001).

In larvae however, species specific morphological features are considerably smaller and often internal organs or structures need to be consulted. The analysis of external features such as spiracles and spine bands can be performed by the use of a stereomicroscopy (Prins, 1982; Erzinclioglu, 1985; Szpila, 2010) or scanning electron microscopy (Liu & Greenberg, 1989; Sukontason *et al.,* 2002; Boonchu *et al*., 2003; Thyssen & Linhares, 2007).

Analyses of internal organs however require for challenging dissections and elaborate histological treatment (Queiroz *et al.,* 1997). An important internal structure used for species determination in blow fly larvae is the cephalopharyngeal skeleton (CPS) (O'Flynn & Moorhouse, 1980; Cantrell, 1981; Queiroz *et al.,* 1997; Wallman, 2001). It is embedded in the anterior end of the larvae and comprises the mouth hooks which are used for feeding and locomotion (Schoofs *et al.,* 2009).

The CPS furthermore provides attachment site for the pharyngeal muscles (Ludwig, 1949; Hanslik *et al*., 2010). The integument which covers the body of the larvae consisting of a two-layered epicuticle (Wolfe, 1954) as well as the above mentioned pharyngeal muscles needs to be removed in order to have a clear view of the CPS. This preparation is elaborate and the risk of damaging the structure considerable. Szpila & Pape (2007) used Hoyer's medium (gum Arabic, glycerol, chloral hydrate, distilled water) to clear these structures.

The techniques eliminated removing the structure or tissues from the larval body. Another clearing technique is the use of potassium hydroxide (KOH) (Sukontason *et al*., 2004). Both techniques however demand the mounting of the specimen onto slides. In order to reduce time and operating expenses as well as the usage of above mentioned hazardous chemicals a far simpler procedure is required.

Furthermore larvae stored in 70% ethanol for a long time can be analyzed without difficulty as long as no browning of the larvae occurred (Niederegger *et al*., 2011).

However, morphological identification can be complicated due to similarity among the species, especially in the early larval stages. Although electron microscopy-based identification of some *Chrysomya* larval stages has been proposed (Sukontason *et al*., 2005), it is not practical and requires many special skills for sample preparation. Alternatively, rearing larvae to the adult stage followed by traditional identification based on the adult morphological characteristics can be performed, but rearing is a time-consuming procedure. Moreover, specimens may be killed or damaged before its arrival to the laboratory (Wallman & Adams, 2001).

To simplify and make the species identification more practical and reliable, DNA-based identification is preferentially considered (Tan *et al.,* 2009, 2010; Preativatanyou *et al*., 2010). Several studies using DNA-based identification of some forensically important blow fly specimens have been reported (Sperling *et al*., 1994; Benecke & Wells, 2001; Wells & Sperling, 2001, Schroeder *et al*., 2003, Ames *et al*., 2006; Tan *et al*., 2009, 2010). The molecular tools can overcome many difficulties associated with morphological problems.

Sperling *et al*. (1994) were the first to demonstrate how mtDNA sequence data from (easy to identify) adult specimens of forensically important flies could be used to identify immature forms of the same species. DNA sequencing is basically done in three steps, polymerase chain reaction (PCR) followed by a sequencing reaction, then gel electrophoresis. PCR is a step that cleaves the long chain of chromosomes into much shorter and workable pieces. These pieces are used as patterns to create a set of fragments (Klug & Michael, 2007).

These fragments are different in length from each other by one base which is helpful in identification. Those sets of fragments are then separated by gel electrophoresis. This process uses electricity to separate DNA fragments by size as they move through a gel matrix. With the presence of an electric current, the negative DNA stand marches towards the positive pole of the current. The smaller DNA fragments moves through the gel pores much more easily or faster than larger molecules. At the bottom of the gel, the fragments go through a laser beam that emits a distinct colour according to the base that passes through (Klug & Michael, 2007).

Forensic entomology not only uses arthropod biology, but it pulls from other sciences introducing fields like chemistry and genetics, exploiting their inherent synergy through the use of DNA in forensic entomology. The use of DNA examination has been recently applied to the typing of necrophageous insects by Random Amplified Polymorphic DNA (Benecke, 1998). This testing is used on the actual larvae found on the deceased. In 2001, a method was devised by Jeffery Wells and Felix Sperling to use mitochondrial DNA to differentiate between different species of the subfamily Chrysomyinae. This is particularly useful when working on determining the identity of specimens that do not have distinctive morphological characteristics at certain life stages (Sperling, 1993; Sperling *et al*., 1994; Wells & Sperling, 2001; Tan *et al*., 2009, 2010).

Mitochondrial DNA (mtDNA) represents only a tiny fraction of organismal genome size, yet it has been by far the most popular marker of molecular diversity in animals over the last three decades. Following Avise *et al*. (1987) and Moritz *et al*. (1987), among others, population geneticists and molecular systematicists have adopted this tool with little reserve. A mitochondrial fragment, COX1, was recently elected as the standardized tool for molecular taxonomy and identification (Ratnasingham &

Hebert, 2007). Mitochondrial gene content is strongly conserved across animals, with very few duplications, no intron, and very short intergenic regions (Gissi *et al*., 2008).

Several studies have addressed this issue by using DNA sequences to identify insects, most choosing to use mitochondrial DNA (mtDNA) as the basic for sequencing (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Wallman & Donnellan, 2001; Wells & Sperling, 2001; Harvey *et al*., 2003a; Tan *et al*., 2009, 2010). These studies have revealed the potential for the use of mtDNA in providing more accurate identifications for the estimation of PMI. Mitochondrial DNA (mtDNA) is useful for insect species identification as it is, for the most part, resistant to degradation and its use can enable forensic scientists to provide identification of fly species within a day. The mtDNA mutation rate, according to Avise (1991) and Sperling *et al.* (1994), is such as to be able to distinguish between closely related species of insects.

In comparison to nuclear DNA, mitochondrial DNA (mtDNA) has some significant advantages in forensic investigations. Firstly, it is present in high copy number and can provide better results when nuclear DNA is scanty, like analysis of hair shafts, teeth and skin (Wilson *et al.,* 1995). Mitochondrial DNA (mtDNA) genes have long dominated the field of molecular systematic because of their maternal inheritance, limited recombination, rapid evolution and the robustness of mtDNA against degradation, making them ideal markers for many species-level questions (Avise *et al*., 1987). Forensic scientists typically turn to mtDNA for:

i) Identification of an individual when the recovered specimens contains too little useful DNA for nuclear DNA analysis

ii) Identification of remains using a maternal relative as a reference

iii) Identification of species

The greater abundance of mtDNA in tissues means that mtDNA can often be extracted and analysed from very small, degraded or otherwise poor sources of DNA that are not suitable for nuclear DNA analysis (Holland & Parsons, 1999).

The area of forensic entomology is also being considered with the application of DNA, in that, insects found in or around a corpse can be examined and compared for molecular structure. Such comparisons require live or deep frozen specimens and may be analyzed at any life stage within one day (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Wallman & Donnellan, 2001; Wells & Sperling, 2001; Harvey *et al*., 2003b).

In Malaysia, forensic entomology is gradually gaining importance. Calliphoridae is the most important family involved in forensic related cases in Malaysia (Hamid *et al*., 2003; Lee *et al*., 2004) and these include *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart). Lee *et al.* (2004) reported the identification of several forensically important specimens collected from cases involving humans, which included families of Calliphoridae, Sarcophagidae, Muscidae, Stratiomyidae, Pthiridae and order of Coleoptera.

As interest in biodiversity has increased in the fields of entomology, it has become increasingly important to precisely identify species. Alternative and accurate identification methods that non-experts can use are required. One of the most promising approaches is the use of molecular instead of morphological data for identifying taxa, which has long been a fundamental idea of many biologists (Busse *et al*. 1996; Blaxter 2003).

Advances in DNA-sequencing technologies have enabled researchers studying biodiversity to conduct simple, cost-effective and rapid DNA analyses. This progress in biotechnology, and the taxonomy crisis itself, played a large role in the creation of DNA

barcoding (Jinbo *et al*., 2011). Hebert *et al*. (2003a, 2003b) proposed a technique using a primer set to amplify a 648-base pair (bp) region of the mitochondrial cytochrome-*c* oxidase subunit 1 (*COI*) gene to ensure rapid and accurate identification of a broad range of biological specimens. They named this technique 'DNA barcoding'.

DNA barcoding is becoming a common practice for routine use in crime detection in many countries due to sensitivity and discrimination of the molecular techniques (Evett & SWeir, 1998). Numerous studies have since addressed the DNA-based identification of calliphorids (Malgorn & Coquoz, 1999; Stevens & Wall, 2001; Wallman & Donnellan, 2001; Harvey *et al*., 2003a, 2003b). A variety of regions of DNA have been suggested for study including the nuclear internal transcribed spacers (ITS) (Ratcliffe *et al*., 2003), mitochondrial rRNA genes and the mitochondrial control region (Stevens & Wall, 1997).

The insect mitochondrial DNA is a small circular genome containing around 16,000 base pairs of double-strand DNA, which comes predominantly from maternal sources. The molecule comprises approximately 37 genes (22 for transfer RNA, 2 for ribosomal RNA and 13 for peptides). These genes include those for two subunits of cytochrome c oxidase, subunits I and II (COI and COII) (Lessinger *et al*., 2000).

COI was originally chosen by molecular biologists to investigate genetic profiles, because it is the biggest of the three mitochondrially encoded cytochrome oxidase subunits and the protein sequence combines both variable and highly conserved regions (Clary & Wolstenholme, 1985; Saraste, 1990; Gennis, 1992; Beard *et al*., 1993; Morlais & Severson, 2002).

The majority of molecular studies, however, have used the cytochrome oxidase I (COI) encoding region of mitochondrial DNA (mtDNA) calliphorids (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Vincent *et al*., 2000; Wallman & Donnellan, 2001; Harvey *et al*., 2003a, 2003b; Tan *et al*., 2009, 2010). Analysis of mitochondrial DNA (mtDNA) and particularly of the cytochrome oxidase I gene (COI) appears to be a useful tool in species identification among the subfamilies of Calliphoridae (Harvey *et al*., 2003a, 2008; Wallman *et al*., 2005; Wells & Williams, 2007; Wells *et al*., 2007).

In line with DNA barcoding efforts (Hebert *et al*., 2003b; Hebert & Gregory, 2005), mitochondrial DNA has been one of the more common targets for analysis, and has shown promising results in several forensic identification studies (Wells *et al.,* 2001; Harvey *et al.,* 2003b). Wells *et al.* (2001) showed that DNA from the corpse could be recovered from maggots using mtDNA.

In DNA barcoding, a short stretch of DNA (barcode) is commonly used to allocate an unidentified individual to a species (Galtier *et al*., 2009). Identifications are usually made by comparing unknown sequences against known species DNA barcodes via distance-based tree construction (Hebert *et al*., 2003b; Hebert *et al*., 2004a, 2004b), and alignment searching namely BLAST (Altschul *et al*., 1990, 1997).

The use of comparative DNA sequence analysis to facilitate species identification has become increasingly popular in recent years due to their ease of use, rapidity and reliability (Vincent *et al*., 2000; Wallman & Donnellan, 2001; Harvey *et al*., 2003a, 2003b; Ames *et al*., 2006; Nelson *et al*., 2007, 2008). It is an attractive alternative to conventional morphology-based identification methods as it can be applied to any life stage and any preservation method of an insect (Sperling *et al*., 1994; Tan *et al*., 2009, 2010). An ideal DNA barcode should allow fast, reliable, automatable,

and cost-effective species identification by users with little or no taxonomic experience (Hebert *et al*., 2003b; Hajibabaei *et al*., 2005; Hebert & Gregory, 2005).

The DNA barcoding used today for criminal investigations and other purposes is based on the idea of PCR. In 1986, DNA was first used to solve crimes in England. The first use of DNA in the United States was performed by the Lifecodes Corporation in 1987 (Inman & Rudin, 2000). 'DNA testing has had a tremendous impact on the solution of crimes of violence especially those involving sexual assault' (Deadman, 2004). DNA barcoding has become increasingly common since it was proposed in 2003. Currently, more than one million records are available in the BOLD system, which is the official depository of DNA barcode data. The new large-scale project, iBOL, will accelerate the creation of reference barcode libraries and will facilitate the application of this simple identification method. In the near future, DNA barcoding will become a standard identification protocol for various organisms (Jinbo *et al*., 2011).

Identifications using molecular data can help elucidate the relationships of morphologically variable individuals of the same species, such as individuals in different developmental stages, castes in social animals and sexually dimorphic individuals (Miller, 2005; Edwards *et al*., 2008; Zhang *et al*., 2008; Emery *et al*., 2009; Johnson *et al*., 2009; Malumphy *et al*., 2009; Pieterse *et al*., 2010). Insects, especially those of holometabolous orders, are extremely variable, and numerous attempts have been made to associate their life stages using molecular markers (Miller, 2005; Ahrens *et al*. 2007; Sutou *et al*. 2007; Johnson *et al*. 2009; Gattolliat & Monaghan, 2010; Hayashi & Sota, 2010; Kathirithamby *et al*., 2010; Murría *et al*., 2010; Pauls *et al*., 2010).

DNA barcoding can be a simple but powerful method for non-experts, especially those who routinely identify a large number of samples. Many molecular-based methods for identifying various organisms using various tools and target molecules have been introduced. However, methods that can be applied to a range of targets are necessary because of the drastic increase in and globalization of potential targets for identification (Bonants *et al*. 2010).

Some taxonomists are concerned that DNA barcoding will compete with traditional taxonomic studies (Ebach & Holdrege, 2005a, 2005b). However, DNA barcoding is inseparably linked to taxonomy, a powerful tool that complements taxonomic studies (Schindel & Miller, 2005; Hajibabaei *et al*., 2007). The integration of various types of data, such as morphological, ecological, physiological and molecular data, including DNA barcodes, will improve species discovery and description processes (Waugh, 2007; Padial *et al*., 2010).

The field of forensic science has experienced technological advances beginning in the 1800's, primarily in the natural sciences such as biochemistry, physics and areas dealing with medical interpretation (Eckert, 1997). The use of scientific technology continues to provide physical evidence examinations involving paint analysis, hair and fiber comparisons, tool mark and firearms identification, drug analysis, as well as questioned document examination (Kelly & Wearne, 1998). The following areas of scientific evidence evaluation has been accepted by most courts, fingerprint identification, photography, tests for alcohol intoxication, firearms identification, trace evidence analysis, serology, odontology, drug analysis and DNA analysis. The area of forensic entomology has been accepted on an individual case basis (Catts & Haskell, 1990).

The scientific study of insects, entomology, is fairly new. The study of insects, coupled with the study of medicine, eventually became known as 'medico-criminal entomology'. The combination of entomology and criminal investigation is commonly used especially in the area of specialization, forensic entomology (Catt & Haskell, 1990).

There are several subfields of forensic science (Gaensslen, 2003; Lambert *et al*., 2003). According to Gaensslen, the subfields of forensic science are pathology, dentistry, psychiatry, toxicology, entomology and physical anthropology. These subfields focus on tasks such as analyzing human biological evidence and other types of biological materials, pollen, fingerprints, documents, firearms, tool marks. The subfields also involve engineering and the investigation and analysis of computer crimes and crime scene patterns (Gaensslen, 2003).

The criminal justice system aims to identify the guilty and exonerate the innocent. In this respect, forensic science is accepted as the most effective tool in criminal justice system. The importance of forensic science has increased 'in the detection, investigation and reduction of crime and recent initiatives' (Mennell & Shaw, 2006). In today's world, it is impossible to ignore scientific evidence in criminal investigations (Fradella *et al*., 2007).

The Cetus Corporation in California put the idea of polymerase chain reaction (PCR) testing into practice, Kerry Mullen conceived the idea in 1983. Since that time, forensic entomology has remained a popular area of interest, with numerous articles being published on the subjects of carrion-insect identification, insect faunal succession and temperature-dependent development rates. As forensic entomology maintains its popularity, investigators are beginning to show interest in using entomological evidence for purposes other than PMI estimations. Maggots have been investigated as a way to

detect illicit drugs in a corpse if there is a suspicion that the death was caused by drug overdose. There has also been interest in using maggot gut contents as a source of carrion DNA to identify missing victims or aid in the PMI estimation process (Wells *et al*., 2001).

Forensic entomology as a science and profession faces many challenges as it attracts the public interest and also offers an opportunity for expanding research related to forensically important arthropods (Byrd & Castner, 2009). Forensic entomology is still a young discipline and there is still much room for progress (Amendt *et al*., 2004, 2010). The scientific literature available on this topic, although constantly growing, remains small when compared to many other biological and legal subjects. Likewise, the number of qualified participating forensic entomologists capable of fully utilizing insect evidence is currently very small (Hall & Huntington, 2008, 2010).

CHAPTER 3
MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 METHODOLOGY FOR THE ASSESSMENT OF FORENSIC ENTOMOLOGY AWARENESS IN MALAYSIA

3.1.1 Sampling Population

Since this study focused on knowledge and awareness of individuals who are involved directly and indirectly in the field of forensic entomology therefore the public was not included. The study sample was comprised of four groups namely crime scene police officers from Forensic Laboratory of Royal Malaysia Police and from the district throughout Malaysia, pathologists from government hospitals (throughout Malaysia), scientific officers from government hospitals and research institutions, students (undergraduate and postgraduate) from various public universities who are undertaking courses or researches related to forensic science. A total of 402 respondents have shown willingness to participate in this study. Of whom 219 respondents were the crime scene police officers, 126 respondents were students, 42 respondents were scientific officers and 15 respondents were pathologists.

The sampling population was divided into four groups:

i) Crime scene police officers

The crime scene police officers who are chosen to answer this questionnaire are police officers from rank of Inspectors to Assistant Superintendent of Police (ASP). This is because they are the police officers who are involved with crime scene investigations. Actually, the police officers are combination of two groups, first group is specifically crime scene investigation officers

from forensic department (Inspector and Assistant Superintendent of Police) and the second group is police officers from the ground (Assistant Superintendent of Police) those are gazette officers to investigate the murder cases. A minimum of one year working experience are required to answer this questionnaire so that the respondents are aware about forensic entomology and its importance.

ii) Forensic pathologist

Most of the forensic pathologists come from various hospitals like Kuala Lumpur General Hospital, Hospital Tengku Ampuan Rahimah, Klang, and Hospital Sultanah Aminah Johor Bharu. Forensic pathologists are normally involved in postmortem examinations as their core duty. Normally, the forensic pathologists will collect the maggots from the dead human body during postmortem examination. The forensic pathologists are experienced and experts in their daily duty and they are aware about forensic entomology and its importance.

iii) Scientific officers

Most of the scientific officers were working with government hospitals, private hospitals, research institutions, Ministry of Health and other related agencies. They have a vast knowledge about forensic entomology during their years of study. Almost all of them were involved in researching maggots as their main focus of the research.

iv) Undergraduate and Postgraduate Students

Students from public universities are chosen to answer this questionnaire. The students are from University of Malaya, Universiti Kebangsaan Malaysia and Universiti Sains Malaysia because one of the factors was to select the students with forensic entomology background to answer the questionnaire. They are undergraduate students who have taken at least one paper which is related to forensic entomology field and for postgraduate students is whether they are involved in forensic entomology field directly or indirectly. This young generation will determine the future of forensic entomology field in this country.

3.1.2 Inclusion Criteria

i) The pathologists must have at least 2 years of working experience as a forensic pathologist to be eligible to answer this questionnaire. Fixed period of 2 years is to ensure that forensic pathologist who answered this questionnaire have some ground experience. This is because in the initial forensic pathologist will undergo an intensive course and observation period.

ii) The crime scene police officers must have at least 1 year of working experience in a related field to be eligible to answer this questionnaire. Stipulated period of one year is to ensure that all the crime scene police officers who answered the questionnaire was exposed to the crime scene involving a human corpse with maggots.

iii) Students who are answering this questionnaire must have a knowledge or awareness about forensic entomology field. Basically the students must

have taken forensic entomology as their major subject or minor subject during the course of their study.

iv) Scientific officers who are working in the hospitals and the research institution must have at least 1 year working experience to be eligible to answer this questionnaire.

3.1.3 Exclusion Criteria

i) All respondents must be Malaysians. A non Malaysian is not eligible to answer this questionnaire.

ii) Those with less than 1 year working experience are also not eligible to answer this questionnaire.

iii) Students without forensic entomology background are not eligible to answer this questionnaire.

3.1.4 Questionnaire Survey

The questionnaire used for this study comprised of two parts (Appendix A). Each part contained 10 questions. The first part contained general idea regarding forensic entomology while the second part demands more specific knowledge on the field. Crime scene police officers and pathologists were required to answer only the first part. Meanwhile the scientific officers and students were required to answer all the 20 questions. To ensure that the questions presented can be understood by individuals from the four groups then the questionnaire was formatted in two different languages, which are the English and Bahasa Malaysia versions. The questionnaire was constructed based on opinions from renowned forensic entomologists in Malaysia.

For crime scene police officers, students and scientific officers, an appointment was made to conduct the questionnaire survey in person. A brief description regarding

the purpose of the survey was given to respondents before they begin to answer the questionnaire. The questionnaire was collected right after they have completed the survey. Meanwhile for the pathologist, they were contacted by telephone and the purpose of the survey was explained. Subsequently the questionnaire was sent to them via e-mail. All completed questionnaire were received by e-mail.

3.1.5 Reliability and Validity of the Study Tool

A self-developed questionnaire was subjected to the process of content validation. During the content selection, the questions were limited to the knowledge about the entomology, and its importance of the crime scene investigation. The content validation of the study tool was performed by the research team at the Department. Furthermore, factor analysis was carried out using Bartlett's test of sphericity and the Kaiser–Meyer–Olkin measure. Bartlett's test of sphericity was significant at <0.001, while the Kaiser–Meyer–Olkin measure was 0.710. Measure of sampling adequacy is used to compare the magnitudes of the observed correlation coefficients interrelation to the magnitudes of the partial correlation coefficients. Large Kaiser–Meyer–Olkin values are good because correlations between pairs of variables (i.e., potential factors) can be explained by the other variables.

According to Scheridan &Lyndall (2001), the contents of a tool are considered adequate if the Kaiser–Meyer–Olkin measure value is more than 0.6.

Table 3.1: Kaiser-Meyer-Olkin measure value

Kaiser-Meyer-Olkin Measure of Sampling Adequacy		0.710
Bartlett's Test of Sphericity	Approx. Chi-Square	2131.251
	df	120
	Sig.	0.000*

In addition, a reliability scale evaluation was applied to estimate the internal consistency of the items; overall, it is seen that reliability for the three major themes was varying from 0.65 to 0.70, and over all reliability for the whole tool was Cronbach's Alpha (α= 0.67).

Table 3.2: Cronbach's Alpha

Section	Cronbach's Alpha
The understanding of forensic entomology	0.68
The acceptance and application of forensic entomology in crime scene investigations	0.70
The future directions of forensic entomology in Malaysia	0.65
Overall Cronbach's Alpha	0.67

3.1.6 Qualitative and Quantitative Analysis

Qualitative analysis of the questionnaire identified three major themes namely the understanding of forensic entomology, the acceptance and application of forensic entomology in crime scene investigations and the future directions of forensic entomology in Malaysia. Meanwhile for quantitative analysis a bivariate analysis (chi-squared test) in SPSS (Statistical Package for the Social Sciences) version 17 was used to assess the statistical significance of the association between subject matter of the question with the socio-demographic profiles of the respondents using 95% Confidence Intervals (CI).

3.2 METHODOLOGY FOR THE FLY IDENTIFICATION BASED ON MORPHOLOGICAL AND MOLECULAR APPROACHES

3.2.1 Maggot Collections

Samples collected from fifty (50) real crime scene cases during the year 2008 to 2010 were used in this study. Specimens were collected and preserved by the crime scene police officers during crime scene investigations or by the pathologists during the post-mortem examination in various hospitals such as Hospital Besar Kuala Lumpur, Hospital Tengku Ampuan Rahimah, Klang, Hospital Sultanah Aminah, Johor Bharu and Hospital Besar Melaka, from Unit of Medical Entomology (IMR) and Entomology Unit of University Kebangsaan Malaysia (UKM).

Maggot samples that have been collected from dead human bodies were kept in a universal bottle which has been filled with 70% ethanol. This method of preservation is preferred because it is easier for body length measurement, morphological study and for avoiding discolouration (Hall, 2000).

The maggot samples that preserved in the universal bottle were examined under the microscope to determine the maggot's species as the preliminary observation. Once ensured how many species of maggots present in the samples, to determine whether there is a single fly infestation or double fly infestation then individual whole maggot was used for morphological analysis and another individual whole maggot was used for molecular analysis. The remaining maggots will be stored in a universal bottle containing 70% ethanol for future usage.

3.2.2 Morphological Identification

3.2.2.1 Slide preparation

Specimens as shown in Figure 3.1 were generally preserved in 70% alcohol in a container (universal bottle). Each bottle, together with prescribed forms, was labeled with hospital identification number, police report number and short notes of the case, name of deceased (if known), date and time of collection and name of collector. Sample received were immediately registered in a log book for record purposes. Various stages of flies (eggs, larvae and pupae) from the decomposing bodies were generally collected by the attending pathologist or crime scene police officers. Then the maggot samples were prepared for morphological process by using the established standard procedures, as described by Lee *et al*. (1984).

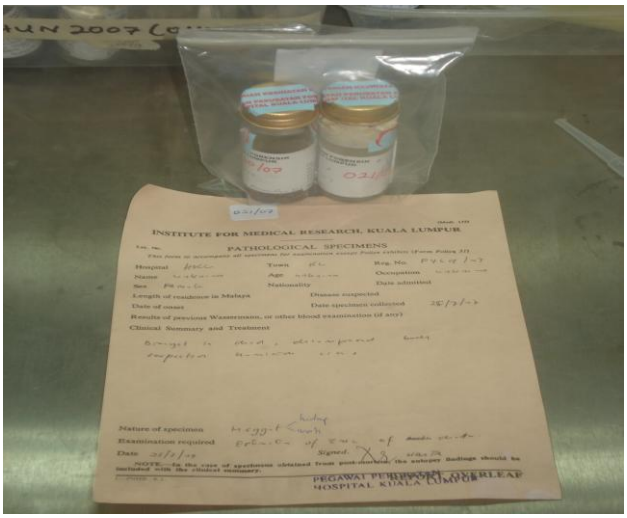


Figure 3.1: Specimens received with the prescribed form

Samples were then incubated in a biological cabinet until they were processed using established standard procedures as shown in Figure 3.2.

Figure 3.2: Samples received were immediately kept inside the cabinet

The maggots were then transferred to a Petri dish using forceps. Posterior segment of the maggots was cut vertically with a sharp blade or surgical blade as shown in Figure 3.3.

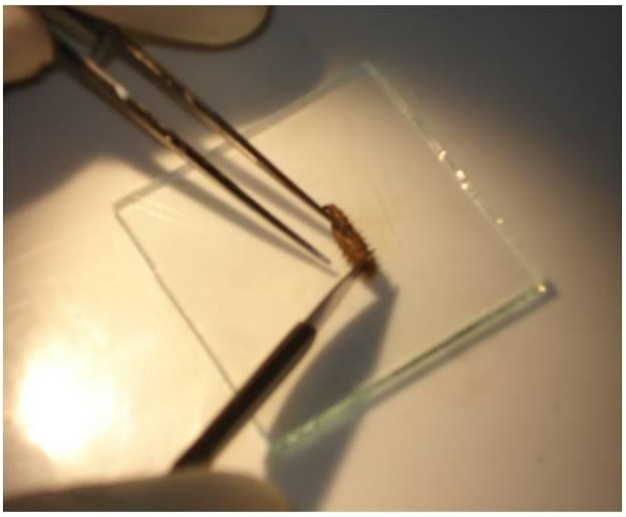


Figure 3.3: Posterior segment of the maggots was cut vertically

The maggots were then soaked in 10% potassium hydroxide (KOH) overnight for cuticle softening purposes. On the next day, the internal taxonomic and residues of maggots were removed carefully to avoid damages to the important parts, such as posterior spiracle, spines, anterior spiracle and the cephalopharyngeal sclerites as shown in Figure 3.4.

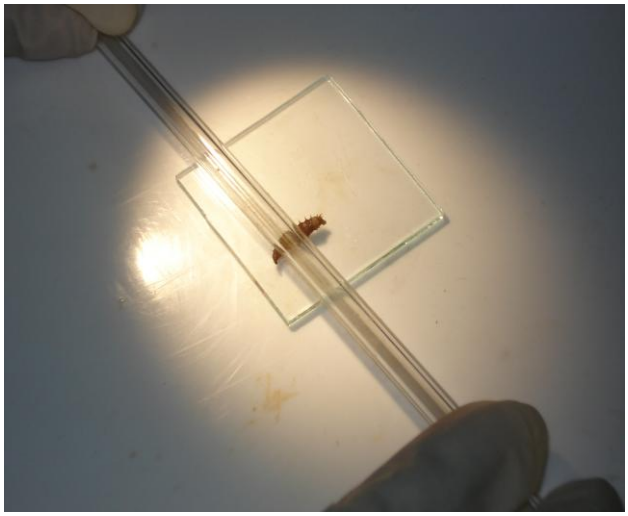


Figure 3.4: The internal organs and residues of maggot were removed

The maggots were then soaked in acetic acid for 10 minutes to neutralize the KOH. The process was continued with a series of ethyl alcohol (ETOH) at different concentrations, ranging from 30%, 50%, 70% and 90%. Thirty minutes in each concentration was required to dehydrate the maggot's body. The maggots were then soaked in absolute alcohol for 30 minutes. The maggots were then transferred into clove oil for 30 minutes, which served as a clearing agent. The maggot was soaked in xylene for 30 minutes before being mounted on slides as shown in Figure 3.5.

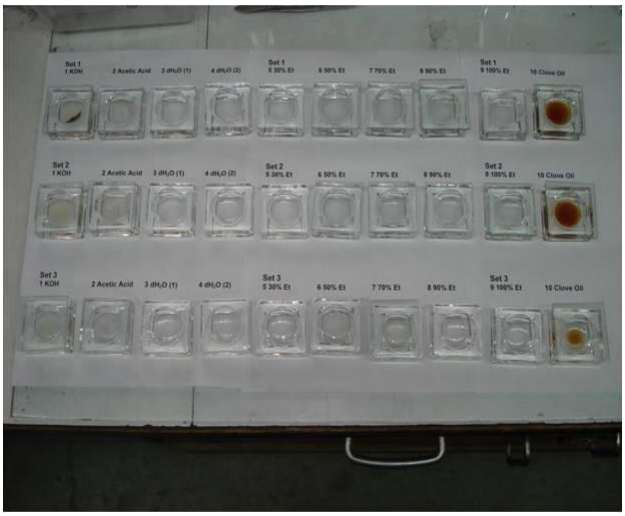


Figure 3.5: Series of ethyl alcohol (ETOH) at different concentrations

The chemically treated maggots were placed on glass slides, followed by few drops of Canada Balsam, as a mounting media and finally covered with a cover slide as shown in Figure 3.6.

Figure 3.6: Mounted slide

The slide was labeled and left in an incubator for 1 to 2 days for drying purposes. The slide was examined under a light microscope for taxonomy studies and identification as shown in Figure 3.7.

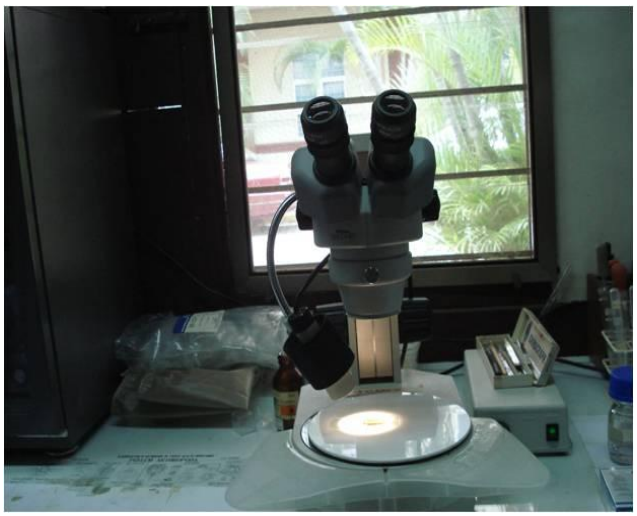


Figure 3.7: Examination of the slide under a light microscope

3.2.2.2 Species Identification

Specimens were identified using keys and characters in Zumpt (1965) and Omar (2002). The method used in this study concentrates more on the observation of larval features such as cephalopharyngeal skeleton (mouthhooks), body spines, posterior spiracle and anterior spiracle as shown in Figures 3.8a, 3.8b, 3.8c, 3.8d and 3.9a, 3.9b, 3.9c, adapted from Ong (2007).

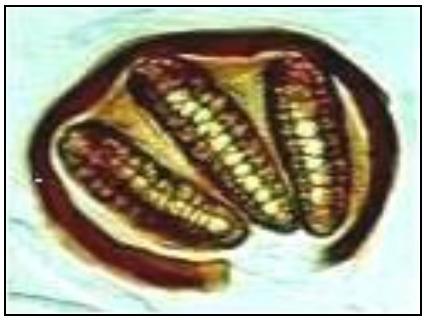


Figure 3.8a: Posterior spiracle of *Chrysomya megacephala*

Figure 3.8b: Body spines of *Chrysomya megacephala*

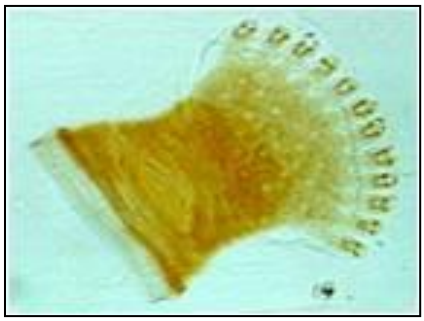


Figure 3.8c: Anterior spiracle of *Chrysomya megacephala*

Figure 3.8d: Mouthhooks of *Chrysomya megacephala*

Figure 3.9a: Anterior spiracle of *Chrysomya rufifacies*

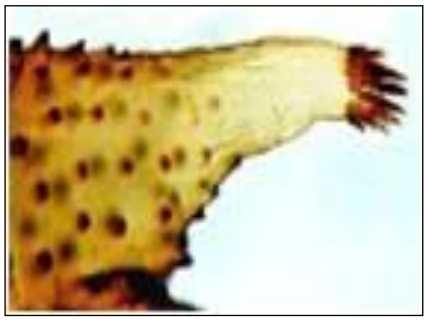


Figure 3.9b: Body spine of *Chrysomya rufifacies*

Figure 3.9c: Mouthhooks of *Chrysomya rufifacies*

3.2.3 Molecular Analysis

3.2.3.1 DNA Extraction

Samples were prepared for DNA extraction by using the method described by Sperling *et al*. (1994) with slight modification. Larvae were soaked in distilled water for 10 minutes prior to DNA extraction. Larvae tissues were then placed in 1.5 ml microfuge tubes immersed briefly in liquid nitrogen and then grounded into powder using sterile plastic pestles. Total genomic DNA was then extracted using QIAamp ® DNA Mini Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. After overnight incubation in ATL buffer (Qiagen, Germany), the samples were treated with RNASE A. At the end of the extraction process the DNA was eluted in 200µl of elution buffer and kept at -20°C. The fraction of extracted DNA was spectrophotometrically quantitated and diluted to 50ng/µl prior to PCR amplification.

3.2.3.2 PCR Amplification

PCR amplification mixtures were prepared to contain the following: 100ng template DNA, 1 unit of Taq DNA polymerase (Promega, USA), 1 x PCR reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 200 µM of each dNTPs (Promega) and 0.4 µM of each forward and reverse primer and ddH2O to a final volume of 50 µl. Amplification reactions were performed in T1 Thermocycler (Biometra) thermal cycler.

Three sets of primers were used in this study and were designed based on the description of Sperling *et al.* (1994) as shown in Table 3.1. Relative positions and orientation of primers are shown in Figure 3.10. The PCR cycling conditions were as follow: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min.

The annealing temperature was 47.4°C for complete COI gene (TY-J-1460 & C1-N-2800) and partial COI gene (C1-J-2495 & C1-N-2800) and 50.2°C for COII gene (C1-J-2495 & TK-N-3775) and was used to amplify fragments of 2.3 kilobase length (2303-2306 base pairs plus primers). The PCR products were separated electrophoretically on 1% agarose gel (Promega) and visualized after ethidium bromide staining. Then 1% agarose gel photo for all the samples of maggots that have been analyzed using molecular method is exhibited under the Appendix D.

Table 3.3: Primer sequences used to amplify overlapping segments of the mitochondrial COI, COII and t-RNA genes (Sperling *et al*., 1994).

Primer ID	Sequence (5' – 3')
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC
C1-N-2800	CATTTCAAGCTGTGTAAGCATC
C1-J-2495	CAGCTACTTTATGAGCTTTAGG
TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT

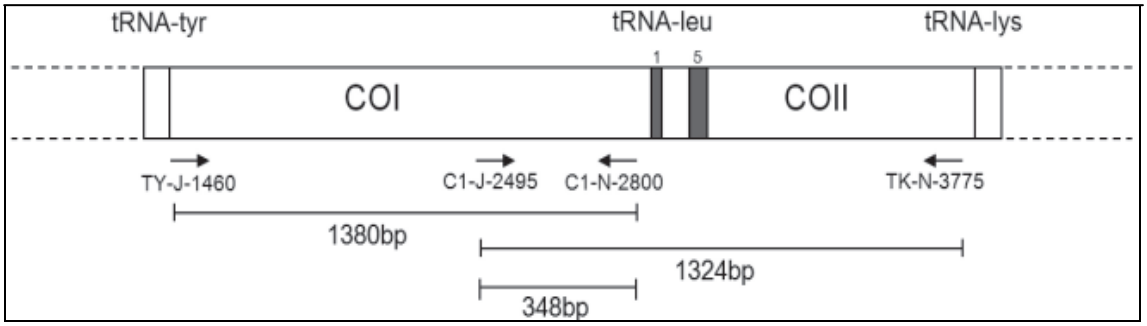


Figure 3.10: Schematic representation of the mitochondrial COI, COII, t-RNA genes and intergenic regions modified from Schroeder *et al*., 2003. Shaded boxes (and corresponding numbers) represent non-coding nucleotides that are present between the genes. Locations of the primers and sizes of the amplification fragments using different primer combinations are shown.

3.2.3.3 Purification of PCR Products

PCR products were purified prior to cloning and direct sequencing. PCR products were purified using the QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit (Qiagen), according to the manufacturer's protocols. The successes of PCR product purification were confirmed by agarose gel electrophoresis.

3.2.3.4 Cloning and Sequencing

Purified PCR products were then cloned into the pGEM®-T Easy Vector System (Promega®) to facilitate DNA sequencing procedures. Sequencing was performed using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1, Applied Biosystems, Forster City, CA, USA, according to the manufacturer's recommendations. All samples were sequenced for both forward and reverse DNA strands using forward and reverse primers according to Sperling *et al*. (1994). Electrophoresis and detection of the sequencing reaction products was carried out in the capillary electrophoresis system ABI PRISM 3730xl capillary DNA Sequencer with a capillary length of 80 cm.

3.2.3.5 DNA Sequence Alignment and Phylogenetic Analysis

DNA sequence chromatograms were read and discrepancies between forward and reverse sequences were resolved using the Chromas software version 2.33 (http://www.technelysium.com.au/chromas.html). The DNA sequences obtained were aligned using ClustalW alignment analysis from BioEdit Version 7.0.9. and a Neighbour- Joining tree (Saitou & Nei, 1987) were made using MEGA 4 (Tamura *et al*., 2007), bootstrap support derived from 1000 replicates and values >50% are shown in the phylogenetic trees.

3.3 METHODOLOGY FOR DNA BARCODING

3.3.1 Blow Fly Samples

Adult *Chrysomya megacephala* and *Chrysomya rufifacies* blow flies were used in this study, as adult morphological characters allowed more accurate identification to species level than larval characters. Specimens were identified using taxonomic keys and characters described by Zumpt (1965) and Omar (2002).

3.3.2 Laboratory Establishment of Blow Flies Colonies

Two blow flies species of *Chrysomya megacephala* and *Chrysomya rufifacies* were successfully colonized in the insectariums at the Medical Entomology Unit, Institute for Medical Research (IMR) in Kuala Lumpur. Laboratory colonies are maintained at natural temperature. The Institute for Medical Research (IMR) also provided the fresh cow liver for this study. The procedure of fly larval development used in this study followed Greenberg & Wells (1998).

Precautions must be taken to start colonies with an adequate number of clean specimens and maintain them with very limited levels of mortality. The aluminium fly cage size 36x36x36 cm was covered with fine material for ventilation and prevention of other small insects from entering the cage to oviposit. The lid was sealed tightly with adhesive tape to prevent the larvae from crawling out. Fresh cow liver was replaced daily into the cage until third instars developed into prepupae the non-feeding period.

3.3.3 Fly Rearing

As for adult blow fly male and female species that have been identified and the gender were chosen from their own colony and early investigation using morphological identification was conducted to determine the validity of the species. Then both blow fly were inserted into a cage that has been prepared and left to be mated. Next, one has to wait till the female blow fly lay eggs.

After the hatching of female blow fly lay eggs, the eggs were taken for molecular identification whereas the balances were left to grow. As the larvae grow, from 1^{st} instar to 2^{nd} instar and to 3^{rd} instar, parts of those larvae were removed from the same batch of eggs in order to obtain similar hatching for the generation (refer to Table 3.2 and Table 3.3). When blow fly are at the level of pupa, 5 pupae were taken and inserted into various jars. Jars containing pupae were covered and tightly sealed until the emergence of adults. Pupae were separated in various jars to ensure the empty puparium which was obtained belongs to the specific adult blow flies. After which, the jars were placed into a rearing cage and the adults and the empty puparium were collected. The complete development from egg to adult takes approximately 1 week.

3.3.4 Sample Collection

Samples of maggot from each life stages like eggs, maggots, pupa, empty puparium and adult were collected and killed by placing them in hot water (≈90°C) for 3 min to fix their protein and prevent darkening of the specimens in ethanol. Then the maggot samples were placed into 70% ethanol.

After that these maggots were processed for morphological identification. Maggot samples set aside for molecular analysis were inserted into two different universal bottles whereby one bottle is preserved in 70% ethanol and another bottle is

without any preservative solution. Both sets of specimen were refrigerated at -4°C till it is used for DNA extraction. The purpose of 1 set of samples being preserved in70% ethanol and another set of samples without preservative is to examine the effect of ethanol preservation on the DNA of the blow flies.

Eventhough it is known that egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium and adult originate from same blow fly species but species identification using morphological method is still been conducted for a more accurate confirmation.

3.3.5 Morphological Identification

The 3rd instar of maggot and adult blow fly which were collected earlier had to be preserved and processed for observation and identification purposes. Then the maggot samples were prepared for morphological process by using the established standard procedures, as described by Lee *et al*. (1984) as in the section 3.2.2.

3.3.6 Species Identification

The 3rd instar maggot specimens and the adult fly were identified as in the section 3.2.2.2.

3.3.7 Molecular Analysis

Samples were prepared for DNA analysis according to method in the section 3.2.3. DNA was prepared from different immature stages of *Chrysomya megacephala* and *Chrysomya rufifacies,* including eggs, first, second and third instars, pupae, empty puparium and adults. Voucher deposit for all the specimens were stored at the Medical Entomology Unit, Institute for Medical Research, which is the WHO Collaborative Centre for Vectors since 1985 and also a provider of training for the post-graduate

Diploma in Applied Parasitology and Entomology under the auspices of SEAMEO-TROPMED. The details about the specimen are shown in Table 3.2 and Table 3.3.

Table 3.4: *Chrysomya megacephala* preserved in 70% ethanol and without any preservative solution

Blow fly life stage's	Quantity of sample preserved in 70% ethanol used for the analysis	Voucher Deposit for sample preserved in 70% ethanol	Quantity of sample without any preservative solutions used for the analysis	Voucher Deposit for sample without any preservative solutions
Eggs	5	CM-eggs	8	CM-eggs
1st instar	7	CM-1st instar	9	CM-1st instar
2nd instar	8	CM-2nd instar	3	CM-2nd instar
3rd instar	3	CM-3rd instar	3	CM-3rd instar
Pupae	2	CM-pupae	2	CM-pupae
Empty puparium	2	CM-puparium	2	CM-puparium
Adult	2	CM-adult	2	CM-adult

*Note: CM = *Chrysomya megacephala*

Table 3.5: *Chrysomya rufifacies* preserved in 70% ethanol and without any preservative solution

Blow fly life stage's	Quantity of sample preserved in 70% ethanol used for the analysis	Voucher Deposit for sample preserved in 70% ethanol	Quantity of sample without any preservative solutions used for the analysis	Voucher Deposit for sample without any preservative solutions
Eggs	7	CR-eggs	7	CR-eggs
1st instar	7	CR-1st instar	6	CR-1st instar
2nd instar	7	CR-2nd instar	6	CR-2nd instar
3rd instar	3	CR-3rd instar	3	CR-3rd instar
Pupae	2	CR-pupae	2	CR-pupae
Empty puparium	2	CR-puparium	2	CR-puparium
Adult	2	CR-adult	2	CR-adult

*Note: CR = *Chrysomya rufifacies*

CHAPTER 4
RESULTS

4.0 RESULTS

4.1 ASSESSMENT OF FORENSIC ENTOMOLOGY AWARENESS IN MALAYSIA

This research was conducted to study the awareness and knowledge on forensic entomology among Malaysian. Furthermore, the aim of this study was to enhance the development of forensic entomology in Malaysia in the future. It would be of great value for the crime scene police officers to be exposed to the forensic entomology field so that the entomological evidence on crime scene can be utilized for fruitful police investigations.

4.1.1 Characteristics of the Participants

A total of 402 questionnaires were distributed to the crime scene police officers from all the 14 districts in Malaysia, pathologists from 3 government hospitals, scientific officers, undergraduate and postgraduate students from 3 public universities. The questionnaire used for this chapter is shown in Appendix A.

Based on Table 4.1, out of 402 respondents, 80.3% of respondents aged below 40 and 19.7% of the respondents aged 40 and above. The minimum age of a respondent is 20 years old and maximum age is 57 years old, with a median value of 28.5. Majority of the respondents were crime scene police officers (54.5%) involved in crime scene investigations. Second largest group of respondents was university students (31.3%) from local universities, who were doing undergraduate and postgraduate studies. This shows an increased interest in pursuing studies in forensic entomology in Malaysia.

The third group of respondents was scientific officers working in hospitals in forensic department with various job descriptions and in research institutions. About 3.7% of the respondents were pathologists from local government hospitals. Most of the time pathologists collect the maggot samples from the dead human body during post-mortem examinations. Initial participants showed only 33% of the respondents who held SPM qualification, compared to nearly 11% who held STPM qualification. About 22% of the respondents held diploma qualification and almost 34% had tertiary education.

There are about 81.8% of the respondents with working experience of 15 years or less and the remaining 18.2% had more than 15 years of working experience. As for the frequency in investigating murder cases, about 80.1% of the respondents who investigated less than 5 cases and about 19.9% had investigated 5 cases and above since they started their job service. Congruents with age distribution of the respondents, most investigations of murder cases were conducted by younger investigators.

Table 4.1: Socio-demographic distribution of the respondents

Criteria	Number (N)	Percentage (%)
Age		
Below 40	323	80.3
40 and above	79	19.7
Education Level		
Diploma and below	264	65.7
Degree and above	138	34.3
Occupation		
Uniformed	219	54.5
Non Uniformed	183	45.5
Working Experience		
15 years or less	329	81.8
More than 15 years	73	18.2
Frequency in investigating murder case		
Less than 5 cases	322	80.1
5 cases and above	80	19.9

4.1.2 Qualitative Analysis

Thematic content analysis of the questionnaire identified three major themes namely the understanding of forensic entomology, the acceptance and application of forensic entomology in crime scene investigations and the future directions of forensic entomology in Malaysia. Descriptions of each theme with data are shown in Appendix B and Appendix C.

Theme 1: Understanding of forensic entomology

Question 1: Forensic entomology is a study about insects found on dead bodies. What is your degree of understanding about forensic entomology?

Question 2: Do you know that the study of insect in forensic entomology includes study of the eggs, the larvae, the pupae, the adult, the empty puparium (skin of pupa) and other insects like beetle.

Question 3: Do you know that maggots (larvae) found on a dead human body originated from the flies and can be used to determine the postmortem interval (time of death)?

The main aim of these 3 questions was to explore the familiarity level of respondents about the understanding about forensic entomology and for question 1 the overall level of understanding about forensic entomology was good among the respondents. Only 56 respondents have heard about forensic entomology first time in their life.

Most of the respondents shared that they are aware of the fly life cycle and maggots found on a dead human body originated from the flies and can be used to determine the postmortem interval.

Theme 2: Acceptance and application of forensic entomology in crime scene investigation.

In this part of theme the respondents ws evaluated on the ground of acceptance and application of forensic entomology in crime scene investigations. Respondents were questioned using about 7 questions which emphasis more on crime scene police officers, pathologists and scientific officers. Most of the questions were related to each and other.

Question 4: Have you ever collected the maggots (larvae) or any other insects found on a dead human body to assist in investigations?

Basically for question 4, maggots found on a dead human body are collected by the crime scene police officers during the forensic investigation or by the pathologists or scientific officers during the post-mortem examinations. The results of the question showed that 70% of the respondents have never collected the maggots found on dead human bodies.

This is because most of the respondents are not aware of the usefulness of maggot found on human dead body in assisting the investigations and seldom applied it in crime scene investigations.

Question 5: Do you know that the maggots (larvae) found on a dead human body can be used to determine the cause of death of a person and/or surrounding area of the crime and/or the position of the wounds in the body?

Question 6: Do you know that flies can locate the dead human body within 24 hours, depending on the surrounding area where the dead body is found?

When asked to the respondents either the respondents knew that the maggots found on a dead human body can be used to determine the cause of death of a victim, the surrounding area of the crime and the position of the wounds in the body, almost 72% of the respondents said “yes”, for question 5. Around 75% of the respondents also knew that flies can locate the dead human body within 24 hours, based on question 6.

Question 7: Do you always encounter a dead human body infected with maggots (larvae)?

Question 8: Have you ever found an empty puparium (skin of pupa) in a crime scene during investigation or during post mortem?

Based on question 7 and 8, only few respondents did encounter a dead human body infested with maggots and empty puparium during crime scene investigation or during post-mortem examination.

Question 9: Do you know that the maggots or larvae found on a dead human body can assist in crime scene investigations on other countries which apply forensic entomology to assist in murder cases?

Most of the respondents were found familiar with the concept of forensic entomology. This can been seen from question 9, whereby 80% of the respondents answered that they knew the maggots found on a dead human body can assist in crime scene investigation on other countries which applied forensic entomology to assist in murder cases.

Question 10: Does this questionnaire introduce to you forensic entomology – the study of insects in assisting crime scene investigations?

Responses for question 10, revealed that majority of the respondents agreed that the questionnaire introduce to them that forensic entomology is a study of insects in assisting crime scene investigations.

Theme 3: Future direction of forensic entomology in Malaysia

The main aim of this theme was to explore the future prospect of forensic entomology in Malaysia. To achieve this aim the graduate students and the scientific officers were questioned about their involvement in forensic entomology study by answering the second part of the questionnaire.

Question 1: How many types of flies do you know in the field of forensic entomology?

Question 2: Since you are involved in forensic entomology field, can you identify the types of the fly species?

Overall most of the respondents knew more than one species of flies in the field of forensic entomology and the respondents can identify the fly themselves.

Question 3: In your studies or research, what types of process do you use to identify the fly species and what are the advantages of the related identification process?

58% of the respondents use morphological identification to identify the fly species because this method is easy, cheap and faster in getting the identification result.

Question 4: What is the main motive in your studies or research related to the forensic entomology field?

According to question 4, majority of the respondents said that their main motive of studies or research is to determine the post-mortem interval (PMI).

Question 5: What are the problems that you always encounter in your studies and research related to the forensic entomology field?

There are 2 main problems based on question 5, which is identifying the fly species and lack of information on forensic entomology.

Question 6: What is the main reason for you to choose forensic entomology as your research field or as an important subject for your studies?

Responses from respondents for question 6 are motivation to get to know more about forensic entomology, compulsory subject for some students and enthusiasm among the students.

Question 7: In your opinion, what is the position and status of forensic entomology in our country?

Overall most of the respondents strongly believed that Department of Education in our country has to play an important role to encourage more students to do research about forensic entomology. In addition, some other respondents working towards to make sure that forensic entomology will continue to move forward and develop fully into a discipline by itself.

Question 8: What is your contribution to improve the forensic entomology field in our country?

Basically the respondents disclose that for question 8, they will do more research related to forensic entomology field and at the same time they will promote forensic entomology to other students.

Question 9: In your opinion, what are the contributions from forensic entomology field in our country?

The main contribution that majority of the respondents indicated in question 9 is the involvement of forensic entomology in murder case investigations and to determine the post-mortem interval (PMI), besides creating more career opportunity for younger generations.

Question 10: In your opinion, which government departments need the services of the forensic entomology researchers more in our country?

The main government department that most of the respondents identify is police department. Besides that, respondents are also considering higher learning institutions and research centres too.

4.1.3 Quantitative Analysis

This part presents the analysis of the data collected based on statistical methods. Descriptive statistic was used to explore respondents' understanding of forensic entomology, the acceptance and application of forensic entomology in crime scene investigations and the future directions of forensic entomology in Malaysia. The chi-square test of association is useful for investigating association among categorical variables. In this study, chi-square test is used to investigate the association between

age, education level, occupation, working experience and frequency in investigating murder case.

Question1: The respondents were questioned on their degree of understanding about forensic entomology. Those who responded with 'high' and 'medium' were grouped as 'high'. Those who responded with 'low' and 'no idea' were grouped as 'low'. The counts, percentages, chi-square values and the p-values are shown in Table 4.2.

Table 4.2: Degree of understanding about forensic entomology classified by the respondents' socio-demographic profiles

		High	(%)	Low	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	167	52%	156	48%	323	0.329	0.327	No significant difference
	40 and above	38	48%	41	52%	79		(>0.05)	
2. Education Level	Diploma and below	131	50%	133	50%	264	0.581	0.256	No significant difference
	Degree and above	74	54%	64	46%	138		(>0.05)	
3.Occupation	Uniformed	79	36%	140	64%	219	42.865	0.001	Significant difference
	Non Uniformed	126	69%	57	31%	183		(<0.05)	
4. Working Experience	15 years or less	168	51%	161	49%	329	0.003	0.528	No significant difference
	More than 15 years	37	51%	36	49%	73		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	157	49%	165	51%	322	3.241	0.047	Significant difference
	5 cases and above	48	60%	32	40%	80		(<0.05)	

Based on Table 4.2, there is a significant difference in only occupation and frequency in investigating murder cases. Among the uniformed, 36% of the respondents had high knowledge on forensic entomology whereas 64% had low knowledge. Meanwhile, among the non-uniformed, 69% of the respondents had high knowledge on forensic entomology and 31% had low knowledge. For frequency in investigating murder cases, 49% of the respondents with less than 5 cases and 60% of the respondents for 5 cases and above, were knowledgeable. In conclusion, respondents who had

investigated more than 5 murder cases and non-uniformed personnel had more knowledge on forensic entomology.

Question 2: The respondent's knowledge of forensic entomology, as the study of insects, was investigated. Those who responded that 'they had heard about the forensic entomology through friends', 'mass media', 'professional knowledge' and 'others' were grouped as 'yes', while those who responded with 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.3.

Table 4.3: Knowledge on forensic entomology as a study of insects classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	269	83%	54	17%	323	0.108	0.446	No significant difference
	40 and above	67	85%	12	15%	79		(>0.05)	
2. Education Level	Diploma and below	225	85%	39	15%	264	1.517	0.138	No significant difference
	Degree and above	111	80%	27	20%	138		(>0.05)	
3.Occupation	Uniformed	163	74%	56	26%	219	29.37	0.001	Significant difference
	Non Uniformed	173	95%	10	5%	183		(<0.05)	
4. Working Experience	15 years or less	275	84%	54	16%	329	0.001	0.557	No significant difference
	More than 15 years	61	84%	12	16%	73		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	264	82%	58	18%	322	2.998	0.054	No significant difference
	5 cases and above	72	90%	8	10%	80		(>0.05)	

Based on Table 4.3, there is a significant difference under the category of occupation. Among the uniformed, 74% of the respondents had knowledge on study of insects in forensic entomology and 26% of the respondents did not have any knowledge. Meanwhile, among the non-uniformed, 95% of the respondents had knowledge on study of insects in forensic entomology and 5% of the respondents were ignorant.

Question 3: The respondents' knowledge on whether or not they knew that maggots found on a dead human body originated from the flies and could be used to determine the post-mortem interval (time of death) was explored. Those who responded that 'they had heard through friends', 'mass media', 'professional knowledge' and 'others' were grouped as 'yes'. Those who responded with 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.4.

Table 4.4: Knowledge on the use of maggots found on a dead human body to determine the post-mortem interval classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	295	91%	28	9%	323	0.003	0.552	No significant difference
	40 and above	72	91%	7	9%	79		(>0.05)	
2. Education Level	Diploma and below	241	91%	23	9%	264	0.001	0.578	No significant difference
	Degree and above	126	91%	12	9%	138		(>0.05)	
3.Occupation	Uniformed	189	86%	30	14%	219	15.084	0.001	Significant difference
	Non Uniformed	178	97%	5	3%	183		(<0.05)	
4. Working Experience	15 years or less	301	91%	28	9%	329	0.087	0.457	No significant difference
	More than 15 years	66	90%	7	10%	73		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	293	91%	29	9%	322	0.183	0.433	No significant difference
	5 cases and above	74	93%	6	8%	80		(>0.05)	

Based on Table 4.4, there is a significant difference in only occupation. Among the uniformed, 86% of the respondents were aware that the maggots found on dead human body originated from the flies and can be used to determine the postmortem interval and 14% of the respondents do not have any knowledge on this area. Meanwhile among the non uniformed, 97% of the respondents were aware that the maggots found on dead human body originated from the flies and can be used to determine the postmortem interval and 3% of the respondents were ignorant.

Objective 4: The respondents were asked whether they had ever collected the maggots (larvae) or any other insects found on a dead human body to assist them in their investigations. Those who responded with 'yes', 'sometimes' and 'depend on senior officers' were grouped as 'yes'. Those who responded with 'no' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.5.

Table 4.5: Experience on colleting maggots found on a dead human body classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	58	18%	265	82%	323	16.681	0.001	Significant difference
	40 and above	31	39%	48	61%	79		(<0.05)	
2. Education Level	Diploma and below	44	17%	220	83%	264	13.362	0.001	Significant difference
	Degree and above	45	33%	93	67%	138		(<0.05)	
3.Occupation	Uniformed	45	21%	174	79%	219	0.707	0.236	No significant difference
	Non Uniformed	44	24%	139	76%	183		(>0.05)	
4. Working Experience	15 years or less	62	19%	267	81%	329	11.406	0.001	Significant difference
	More than 15 years	27	37%	46	63%	73		(<0.05)	
5.Frequency in investigating murder case	Less than 5 cases	53	16%	269	84%	322	30.28	0.001	Significant difference
	5 cases and above	36	45%	44	55%	80		(<0.05)	

Based on Table 4.5, there is a significant difference in age, education level, working experience and frequency in investigating murder cases. Among respondents who are below 40 years old, 18% of them had collected maggots from dead human bodies and for the respondents who are 40 years old and above, 39% of them had done that. Those with diploma and lower academic qualification, 17% of the respondents had collected maggots from dead human bodies. For those with degree and above, 33% of the respondents had done that.

Around 19% of the respondents with 15 years or less working experience and 37% respondents with more than 15 years of working experience had collected maggots from dead human bodies. For frequency in investigating the murder cases, among less than 5 cases, 16% of the respondents had collected maggots from dead human bodies and 45% of the respondents for 5 cases and above had collected maggots from dead human bodies. 55% of them had not.

Question 5: The respondents were asked whether they knew that the maggots found on a dead human body can be used to determine the cause of death of a person or the surrounding area of the crime or the position of the wounds in the body. Those who responded 'through friends', 'mass media', 'professional knowledge' and 'others' were grouped as 'yes'. Those who responded with 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.6.

Table 4.6: Knowledge on the use of maggots found on a dead human body classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	235	73%	88	27%	323	1.003	0.193	No significant difference
	40 and above	53	67%	26	33%	79		(>0.05)	
2. Education Level	Diploma and below	188	71%	76	29%	264	0.07	0.443	No significant difference
	Degree and above	100	72%	38	28%	138		(>0.05)	
3.Occupation	U Uniformed		55%	98	45%	219	63.616	0.001	Significant difference
	Non U Uniformed		91%	16	9%	183		(<0.05)	
4. V Ex 4. Working Experience	15 years or less	240	73%	89	27%	329	1.522	0.138	No significant difference
	More than 15 years	48	66%	25	34%	73		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	232	72%	90	28%	322	0.133	0.406	No significant difference
	5 cases and above	56	70%	24	30%	80		(>0.05)	

Based on Table 4.6, there is a significant difference in the category of occupation. Among the uniformed, 55% of the respondents did knew that the maggots (larvae) found on a dead human body can be used to determine the cause of a death person and/or the surrounding area of the crime and/or the position of the wounds in the body and 45% of the respondents did not knew about it.

Among the non- uniformed, 91% of the respondents had the knowledge and 9% of the respondents are not aware about it.

Question 6: The respondents were asked whether they knew that flies can locate dead human body within 24 hours, depending on the surrounding area where the dead body is found. Those who responded that 'they heard about it through friends', 'mass media', 'professional knowledge' and 'others' were grouped as 'yes'. Those who responded with 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.7.

Table 4.7: Knowledge on the ability of flies to locate dead human body within 24 hours classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	241	75%	82	25%	323	0.512	0.288	No significant difference
	40 and above	62	78%	17	22%	79		(>0.05)	
2. Education Level	Diploma and below	197	75%	67	25%	264	0.234	0.361	No significant difference
	Degree and above	106	77%	32	23%	138		(>0.05)	
3.Occupation	Uniformed	146	67%	73	33%	219	19.646	0.001	Significant difference
	Non Uniformed	157	86%	26	14%	183		(<0.05)	
4. Working Experience	15 years or less	247	75%	82	25%	329	0.086	0.449	No significant difference
	More than 15 years	56	77%	17	23%	73		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	236	73%	86	27%	322	3.776	0.033	Significant difference
	5 cases and above	67	84%	13	16%	80		(<0.05)	

Based on Table 4.7 there is a significant difference in the category of occupation and frequency in investigating murder cases. Among the uniformed, there were 67% and 86% of the non-uniformed respondents did know that flies can locate dead human body within 24 hours.

For frequency in investigating murder cases, 73% of the respondents with less than 5 cases and 84% respondents with 5 cases and above, knew about the said knowledge.

Question 7: The respondents were asked whether they always encounter a dead human body infested with maggots. Those who responded with 'always', 'one dead human body infested with maggots every week', 'one dead human body infested with maggots every month' were grouped as 'yes'. Those who responded with 'seldom' and 'no' were grouped as 'seldom'. The counts, percentages, chi-square values and the p-values are shown in Table 4.8

Table 4.8: Experience on encountering dead human body infested with maggots classified by the respondents' socio-demographic profiles

		Yes	(%)	Seldom	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	45	14%	278	86%	323	17.695	0.001	Significant difference
	40 and above	27	34%	52	66%	79		(<0.05)	
2. Education Level	Diploma and below	34	13%	230	87%	264	13.243	0.001	Significant difference
	Degree and above	38	28%	100	72%	138		(<0.05)	
3.Occupation	Uniformed	53	24%	166	76%	219	12.948	0.001	Significant difference
	Non Uniformed	19	10%	164	90%	183		(<0.05)	
4. Working Experience	15 years or less	48	15%	281	85%	329	13.589	0.001	Significant difference
	More than 15 years	24	33%	49	67%	73		(<0.05)	
5.Frequency in investigating murder case	Less than 5 cases	36	11%	286	89%	322	49.85	0.001	Significant difference
	5 cases and above	36	45%	44	55%	80		(<0.05)	

Based on Table 4.8 there is a significant difference in all the variables. 14% of the respondents aged below 40 years old and 34% of the respondents aged 40 and above answered that they always encounter a dead human body infested with maggots.

Education level between ‘diploma and below’ and ‘degree and above’ also showed a significant difference. 13% of the respondents with diploma and below academic qualification did always encounter a dead human body infested with maggots (larvae). 28% of the respondents with degree and above academic qualification have responded that they do always encounter a dead human body infested with maggots (larvae).

24% of the uniformed respondents and 10% of the non-uniformed respondents responded that they always encounter a dead human body infested with maggots. For respondents with 15 years or less working experience, about 15% of them always encountered a dead human body infested with maggots (larvae) while 85% of the respondents responded as seldom. The questionnaire also showed that 33% of the respondents with more than 15 years of working experience always encountered a dead human body infested with maggots.

Personnel involved with investigating murder cases showed that 11% of the respondents with a frequency of investigating less than 5 cases always encountered a dead human body infested with maggots (larvae). For those with frequency of investigating more than 5 cases, 45% of the respondents always encountered with a dead human body infested with maggots and 55% of the respondents responded as seldom.

Question 8: The respondents were asked whether they have ever found an empty puparium in a crime scene during investigation or during post-mortem. Those who responded with 'always', 'indicating with one dead human body along with an empty puparium every week', 'one dead human body along with an empty puparium every month' were grouped as 'yes'. Those who responded with 'seldom' and 'no' were grouped as 'seldom'. The counts, percentages, chi-square values and the p-values are shown in Table 4.9.

Table 4.9: Experience on finding an empty puparium classified by the respondents' socio-demographic profiles

		Yes	(%)	Seldom	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	17	5%	306	95%	323	7.348	0.01	Significant difference
	40 and above	11	14%	68	86%	79		(<0.05)	
2. Education Level	Diploma and below	15	6%	249	94%	264	1.955	0.118	No significant difference
	Degree and above	13	9%	125	91%	138		(>0.05)	
3.Occupation	Uniformed	13	6%	206	94%	219	0.786	0.245	No significant difference
	Non Uniformed	15	8%	168	92%	183		(>0.05)	
4. Working Experience	15 years or less	19	6%	310	94%	329	3.96	0.048	Significant difference
	More than 15 years	9	12%	64	88%	73		(<0.05)	
5.Frequency in investigating murder case	Less than 5 cases	15	5%	307	95%	322	13.287	0.001	Significant difference
	5 cases and above	13	16%	67	84%	80		(<0.05)	

Based on Table 4.9, there are significant differences in the category of age, working experience and frequency in investigating murder case. Among the respondents below the age of 40 years old, 5% of the respondents had found an empty puparium (skin of pupa) in a crime scene during investigation or during postmortem examination and 95% of the respondents responded as seldom. 14% of the respondents above 40 years old had found an empty puparium and 86% of the respondents responded as seldom.

6% of the respondents with 15 years or less working experience had found an empty puparium and 94% of the respondents reported as seldom. There is a slight increase for the respondents with more than 15 years of working experience wherein 12% of them had found an empty puparium and 88% of the respondents responded as seldom.

Among the respondents who investigated less than 5 murder cases, showed 5% of them had found an empty puparium. Meanwhile 16% of the respondents who investigated more than 5 cases had found an empty puparium and 84% of the respondents responded as seldom.

Question 9: The respondents were asked if they knew that in other countries, the maggots found on a dead human body can assist in crime scene investigations. Those who responded that 'they knew it through friends', 'mass media', 'professional knowledge' and 'others' were grouped as 'yes'. Those who responded with 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.10.

Table 4.10: Knowledge on the application of forensic entomology in other countries classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	262	81%	61	19%	323	2.316	0.088	No significant difference
	40 and above	58	73%	21	27%	79		(>0.05)	
2. Education Level	Diploma and below	214	81%	50	19%	264	1.008	0.191	No significant difference
	Degree and above	106	77%	32	23%	138		(>0.05)	
3.Occupation	Uniformed	148	68%	71	32%	219	42.822	0.001	Significant difference
	Non Uniformed	172	94%	11	6%	183		(<0.05)	
4. Working Experience	15 years or less	269	82%	60	18%	329	5.21	0.019	Significant difference
	More than 15 years	51	70%	22	30%	73		(<0.05)	
5.Frequency in investigating murder case	Less than 5 cases	257	80%	65	20%	322	0.045	0.47	No significant difference
	5 cases and above	63	79%	17	21%	80		(>0.05)	

Based on Table 4.10, there are significant differences in occupation and working experience. 68% of the uniformed respondents and 94% of non-uniformed respondents are aware that the maggots or larvae found on a dead human body can assist in crime scene investigations in other countries which applied forensic entomology to assist in murder cases.

As for personnel with 15 years or less working experience, 82% of the respondents have knowledge that the maggot or larvae found on a dead human body can assist in crime scene investigation in other countries which have applied forensic entomology to assist in murder cases and as for personnel with more than 15 years of working experience, there are 70% of respondents had the knowledge.

Question10: The respondents were asked whether this questionnaire introduces to them about forensic entomology, the study of insects in assisting crime scene investigations. For those who responded under the category of 'first time exposure to forensic entomology', some respondents have responded that their 'knowledge about forensic entomology field have increased by 25%', while another group of respondents have responded that their 'knowledge have increased by 50%' in the forensic entomology field, whereas a majority have stated that their 'knowledge about forensic entomology field has increased to 75% 'and for those who have responded they that have 'gained it through professional knowledge' were grouped as 'yes'. Those who responded with 'no' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.11.

Table 4.11: Effectiveness of the questionnaire in introducing forensic entomology classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	271	84%	52	16%	323	1.092	0.194	No significant difference
	40 and above	70	89%	9	11%	79		(>0.05)	
2. Education Level	Diploma and below	222	84%	42	16%	264	0.323	0.340	No significant difference
	Degree and above	119	86%	19	14%	138		(>0.05)	
3.Occupation	Uniformed	196	89%	23	11%	219	8.158	0.003	Significant difference
	Non Uniformed	145	79%	38	21%	183		(<0.05)	
4. Working Experience	15 years or less	276	84%	53	16%	329	1.231	0.177	No significant difference
	More than 15 years	65	89%	8	11%	73		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	272	84%	50	16%	322	0.157	0.421	No significant difference
	5 cases and above	69	86%	11	14%	80		(>0.05)	

Based on Table 4.11, there is only one significant difference for occupation. 89% of the uniformed and 79% of the non-uniformed respondents agreed that the questionnaire introduces forensic entomology to them.

Second questionnaire set were answered by the scientific officers and undergraduate and postgraduate students.

Question 1: The respondents were asked how many types of flies they know in the field of forensic entomology. Those who responded with 'one species', 'two species' and 'more than three species' were grouped as 'at least one species or more'. Those who responded with 'no idea' and 'others' were grouped as 'no idea'.The counts, percentages, chi-square values and the p-values are shown in Table 4.12.

Table 4.12: Knowledge on types of flies in forensic entomology classified by the respondents' socio-demographic profiles

		At least one species or more	(%)	No Idea	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	106	68%	49	32%	155	0.004	0.683	No significant difference
	40 and above	2	67%	1	33%	3		(>0.05)	
2. Education Level	Diploma and below	71	60%	48	40%	119	16.833	0.001	Significant difference
	Degree and above	37	95%	2	5%	39		(<0.05)	
3.Occupation	Uniformed	3	50%	3	50%	6	0.971	0.284	No significant difference
	Non Uniformed	105	69%	47	31%	152		(>0.05)	
4. Working Experience	15 years or less	105	68%	49	32%	154	0.084	0.623	No significant difference
	More than 15 years	3	75%	1	25%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	100	67%	50	33%	150	3.901	0.044	Significant difference
	5 cases and above	8	5%	0	0%	8		(<0.05)	

Based on Table 4.12, there are significant differences in education level and frequency in investigating murder cases. 60% of the respondents with diploma and below academic qualification knew more than one fly in the forensic entomology field and 40% of the respondents do not know of the said knowledge. 95% of the respondents with degree and higher academic qualification knew more than one fly and 5% of the respondents do not know about it.

Personnel with frequency in investigating less than 5 cases showed that 67% of the respondents knew more than one fly in the forensic entomology field and 33% of the respondents do not know. Besides that, personnel with frequency in investigating more than 5 cases showed that 5% of the respondents knew more than one flies in the forensic entomology field.

Question 2: The respondents were asked whether they can identify the types of fly species. Those who responded with 'yes', 'seeking the help from the lecturers to identify them' and 'can a bit' were grouped as 'yes'. Those who responded with 'no idea' and 'others' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.13.

Table 4.13: Ability to identify the fly species classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	124	80%	31	20%	155	0.324	0.495	No significant difference
	40 and above	2	67%	1	33%	3		(>0.05)	
2. Education Level	Diploma and below	91	76%	28	24%	119	3.204	0.054	No significant difference
	Degree and above	35	90%	4	10%	39		(>0.05)	
3.Occupation	Uniformed	3	50%	3	50%	6	3.417	0.098	No significant difference
	Non Uniformed	123	81%	29	19%	152		(>0.05)	
4. Working Experience	15 years or less	123	80%	31	20%	154	0.057	0.6	No significant difference
	More than 15 years	3	75%	1	25%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	119	79%	31	21%	150	0.314	0.492	No significant difference
	5 cases and above	7	88%	1	13%	8		(>0.05)	

Based on Table 4.13, there is no significant difference between the variables with Question 2.

Question 3: The respondents were asked the types of processes do they used to identify the fly species and the advantages of the related identification process. Those who responded with 'morphological identification', 'molecular DNA identification' and 'help from the lecturers' were grouped as 'yes'. Those who responded with 'others' and 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.14.

Table 4.14: Knowledge on the techniques used to identify the fly species classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	125	81%	30	19%	155	0.717	0.529	No significant difference
	40 and above	3	100%	0	0%	3		(>0.05)	
2. Education Level	Diploma and below	94	79%	25	21%	119	1.28	0.187	No significant difference
	Degree and above	34	87%	5	13%	39		(>0.05)	
3.Occupation	Uniformed	3	50%	3	50%	6	3.9	0.083	No significant difference
	Non Uniformed	125	82%	27	18%	152		(>0.05)	
4. Working Experience	15 years or less	124	81%	30	19%	154	0.962	0.427	No significant difference
	More than 15 years	4	100%	0	0%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	122	81%	28	19%	150	0.198	0.47	No significant difference
	5 cases and above	6	75%	2	25%	8		(>0.05)	

Based on Table 4.14, there is no significant difference between the variables with Question 3.

Question 4: The respondents were asked about the main motive of their studies or research related to the forensic entomology. Those who responded with 'to identifying the types of fly species', 'to determine the postmortem interval (PMI)' and 'to determine the insect successions' were grouped as 'yes'. Those who responded with 'others' and 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.15.

Table 4.15: Assessment of the motive of studies or research in forensic entomology classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	129	83%	26	17%	155	5.024	0.081	No significant difference
	40 and above	1	33%	2	67%	3		(>0.05)	
2. Education Level	Diploma and below	96	81%	23	19%	119	0.853	0.253	No significant difference
	Degree and above	34	87%	5	13%	39		(>0.05)	
3.Occupation	Uniformed	4	67%	2	33%	6	1.043	0.288	No significant difference
	Non Uniformed	126	83%	26	17%	152		(>0.05)	
4. Working Experience	15 years or less	128	83%	26	17%	154	2.932	0.145	No significant difference
	More than 15 years	2	50%	2	50%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	122	81%	28	19%	150	1.815	0.202	No significant difference
	5 cases and above	8	100%	0	0%	8		(>0.05)	

Based on Table 4.15, there is no significant difference between the variables with Question 4.

Question 5: The respondents were asked about the problems that they always encounter in their studies and research related to the forensic entomology field. Those who responded with 'problems in identifying the fly species', 'problems in analyzing the fly DNA' and 'lack of information on forensic entomology' were grouped as 'yes'. Those who responded with 'others' and 'no idea' were grouped as 'no'.The counts, percentages, chi-square values and the p-values are shown in Table 4.16.

Table 4.16: Problem encountered in studies and researches related to forensic entomology classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	116	75%	39	25%	155	2.638	0.165	No significant difference
	40 and above	1	33%	2	67%	3		(>0.05)	
2. Education Level	Diploma and below	85	71%	34	29%	119	1.725	0.134	No significant difference
	Degree and above	32	82%	7	18%	39		(>0.05)	
3.Occupation	Uniformed	4	67%	2	33%	6	0.177	0.491	No significant difference
	Non Uniformed	113	74%	39	26%	152		(>0.05)	
4. Working Experience	15 years or less	115	75%	39	25%	154	1.235	0.277	No significant difference
	More than 15 years	2	50%	2	50%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	111	74%	39	26%	150	0.004	0.656	No significant difference
	5 cases and above	6	75%	2	25%	8		(>0.05)	

Based on Table 4.16, there is no significant difference between the variables with Question 5.

Question 6: The respondents were asked the main reason for them to choose the area of forensic entomology either as their research field or as an important subject for their studies. Those who responded with 'enthusiasm', 'motivation to get to know more about forensic entomology', 'influences from friends and lecturers' and 'compulsory subject' were grouped as 'yes'. Those who responded with 'others' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.17.

Table 4.17: Reason for the involvement in forensic entomology classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	131	85%	24	15%	155	0.704	0.406	No significant difference
	40 and above	2	67%	1	33%	3		(>0.05)	
2. Education Level	Diploma and below	98	82%	21	18%	119	1.205	0.202	No significant difference
	Degree and above	35	90%	4	10%	39		(>0.05)	
3.Occupation	Uniformed	5	83%	1	17%	6	0.003	0.651	No significant difference
	Non Uniformed	128	84%	24	16%	152		(>0.05)	
4. Working Experience	15 years or less	130	84%	24	16%	154	0.26	0.502	No significant difference
	More than 15 years	3	75%	1	25%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	126	84%	24	16%	150	0.07	0.63	No significant difference
	5 cases and above	7	88%	1	13%	8		(>0.05)	

Based on Table 4.17, there is no significant difference between the variables with Question 6.

Question 7: The respondents were asked about the status of forensic entomology in our country. Those who responded that 'it is still in the early stage without hope for improvement', 'the department of education in our country have to play an important role to encourage more students to do research in the forensic entomology field' and 'will continue to move forward and develop fully into a discipline by itself' were grouped as 'moving forward'. Those who responded with 'others' and 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.18.

Table 4.18: Knowledge on the status of forensic entomology in Malaysia classified by the respondents' socio-demographic profiles

		Moving Forward	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	143	92%	12	8%	155	2.553	0.228	No significant difference
	40 and above	2	67%	1	33%	3		(>0.05)	
2. Education Level	Diploma and below	107	90%	12	10%	119	2.2	0.121	No significant difference
	Degree and above	38	97%	1	3%	39		(>0.05)	
3.Occupation	Uniformed	5	83%	1	17%	6	0.588	0.408	No significant difference
	Non Uniformed	140	92%	12	8%	152		(>0.05)	
4. Working Experience	15 years or less	142	92%	12	8%	154	1.529	0.293	No significant difference
	More than 15 years	3	75%	1	25%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	137	91%	13	9%	150	0.755	0.495	No significant difference
	5 cases and above	8	100%	0	0%	8		(>0.05)	

Based on Table 3.18, there is no significant difference between the variables with Question 7.

Question 8: The respondents were asked about the contribution to improve the forensic entomology in our country. Those who responded with 'more publicity to introduce forensic entomology to other students', 'do more research related to forensic entomology' and 'take part in the seminars which are held in our country and overseas to introduce their research findings and to discuss important issues related to forensic entomology' were grouped as 'yes'. Those who responded with 'others' and 'no idea' were grouped as 'no'. The counts, percentages, chi-square values and the p-values are shown in Table 4.19.

Table 4.19: Contributions on improving forensic entomology in Malaysia classified by the respondents' socio-demographic profiles

		Yes	(%)	No	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	115	74%	40	26%	155	2.518	0.173	No significant difference
	40 and above	1	33%	2	67%	3		(>0.05)	
2. Education Level	Diploma and below	81	68%	38	32%	119	7.072	0.005	Significant difference
	Degree and above	35	90%	4	10%	39		(<0.05)	
3.Occupation	Uniformed	4	67%	2	33%	6	0.146	0.506	No significant difference
	Non Uniformed	112	74%	40	26%	152		(>0.05)	
4. Working Experience	15 years or less	114	74%	40	26%	154	1.153	0.288	No significant difference
	More than 15 years	2	50%	2	50%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	108	93%	8	7%	116	3.051	0.079	No significant difference
	5 cases and above	42	100%	0	0%	42		(>0.05)	

Based on Table 4.19, 68% of the respondents with diploma and below in academic qualification did some contribution to improve the forensic entomology field in our country and 32% of the respondents did not. Respondents with degree and above

in academic qualification showed 90% of them did provide some contribution to improve the forensic entomology and 10% of the respondents did not.

Question 9: The respondents were asked about the contribution from forensic entomology in our country. Those who responded 'to be involved in murder case investigations and to determine the postmortem interval (PMI)', 'to make our country proud by representing Malaysia when working together with other countries which are well known in the practice of forensic entomology' and 'more career opportunity' were grouped as 'yes'. Those who responded with 'no idea' were grouped as 'none'.

The counts, percentages, chi-square values and the p-values are shown in Table 4.20.

Table 4.20: Contributions of forensic entomology in Malaysia classified by the respondents' socio-demographic profiles

		At least one contribution	(%)	None	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	149	96%	6	4%	155	6.034	0.128	No significant difference
	40 and above	2	67%	1	33%	3		(>0.05)	
2. Education Level	Diploma and below	114	96%	5	4%	119	0.06	0.551	No significant difference
	Degree and above	37	95%	2	5%	39		(>0.05)	
3.Occupation	Uniformed	5	83%	1	17%	6	2.205	0.241	No significant difference
	Non Uniformed	146	96%	6	4%	152		(>0.05)	
4. Working Experience	15 years or less	148	96%	6	4%	154	4.101	0.167	No significant difference
	More than 15 years	3	75%	1	25%	4		(>0.05)	
5.Frequency in investigating murder case	Less than 5 cases	143	95%	8	5%	151	0.391	0.69	No significant difference
	5 cases and above	7	100%	0	0%	7		(>0.05)	

Based on Table 4.20, there was no significant difference between the variables with Question 9.

Question 10: The respondents were asked about which government departments need the services of the forensic entomology in our country. Those who responded with 'police department', 'hospital department' and 'higher learning institutions/research centres' were grouped as 'at least one department'. Those who responded with 'others' and 'no idea' were grouped as 'none. The counts, percentages, chi-square values and the p-values are shown in Table 4.21.

Table 4.21: Opinion on the need for forensic entomology services in government department classified by the respondents' socio-demographic profiles

		At least one department	(%)	None	(%)	Total	Chi-square (x^2)	p-value	Conclusion
1. Age	Below 40	148	95%	7	5%	155	5.084	0.145	No significant difference
	40 and above	2	67%	1	33%	3		(>0.05)	
2. Education Level	Diploma and below	114	96%	5	4%	119	0.745	0.311	No significant difference
	Degree and above	36	92%	3	8%	39		(>0.05)	
3.Occupation	Uniformed	5	83%	1	17%	6	1.747	0.272	No significant difference
	Non Uniformed	145	95%	7	5%	152		(>0.05)	
4. Working Experience	15 years or less	148	96%	6	4%	154	17.24	0.013	Significant difference
	More than 15 years	2	50%	2	50%	4		(<0.05)	
5.Frequency in investigating murder case	Less than 5 cases	143	95%	7	5%	150	0.97	0.347	No significant difference
	5 cases and above	7	88%	1	13%	8		(>0.05)	

Based on Table 4.21, 96% of the respondents with 15 years or less working experience need the services of the forensic entomology researchers more in our country and 4% did not need the forensic entomology service. 50% of the respondents with more than 25 years of working experience need the services of the forensic entomology researchers more in our country and 50% do not need the service of forensic entomology.

4.2 COMPARISON BETWEEN MORPHOLOGICAL AND MOLECULAR METHODS IN BLOW FLY SPECIES IDENTIFICATION

PMI determination can be made based on maggots' presence on the dead human body. For accurate PMI determination, the species of flies present on the dead human body at different life stages of the larvae need to be determined. Maggots can usually be identified through morphological and molecular methods. The aim of this study was to compare the maggot identification procedure between traditional morphological and molecular methods using samples collected from real crime scene.

The mtDNA region sequenced in this study includes the cytochrome oxidase b subunit I and II genes (COI and COII). One individual per species was sequenced over this region.The voucher deposit of all the specimens were stored at the Medical Entomology Unit, Institute for Medical Research (IMR), which is the WHO Collaboratory Centre for Vectors since 1985 and also a provider of training for the post-graduate Diploma in Applied Parasitology and Entomology under the auspices of SEAMEO-TROPMED. The details of the origin, collection date of the data samples and voucher deposit are presented in Table 4.22.

Table 4.22: List and origin of specimens

Case No.	Collection Date	Specimen Obtained From	Voucher Deposit
1	25/12/2008	Hospital Besar Kuala Lumpur	FE 040/2008
2	26/12/2008	Hospital Besar Kuala Lumpur	FE 039/2008
3	13/01/2009	Hospital Besar Kuala Lumpur	FE 02/2009
4	30/01/2009	Hospital Universiti Kebangsaan Malaysia	FE HKLG 15/2009
5	01/02/2009	Hospital Besar Kuala Lumpur	FE 04/2009
6	03/03/2009	Hospital Besar Kuala Lumpur	FE 05/2009
7	16/03/2009	Hospital Besar Kuala Lumpur	FE 06/2009
8	20/03/2009	Hospital Besar Kuala Lumpur	FE 10/2009
9	27/03/2009	Hospital Serdang	FE 029/2009
10	28/03/2009	Hospital Besar Kuala Lumpur	FE 07/2009
11	29/03/2009	Hospital Besar Kuala Lumpur	FE 08/2009
12	31/03/2009	Hospital Besar Kuala Lumpur	FE 09/2009

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13	31/03/2009	Hospital Besar Kuala Lumpur	FE 012/2009
14	12/04/2009	Hospital Serdang	FE 011/2009
15	13/04/2009	Hospital Johor Bahru	FE JB 073/2009
16	13/04/2009	Hospital Besar Kuala Lumpur	FE 013/2009
17	21/04/2009	Hospital Besar Kuala Lumpur	FE 014/2009
18	01/05/2009	Hospital Melaka	FE 015/2009
19	04/05/2009	Hospital Besar Kuala Lumpur	FE 016/2009
20	20/05/2009	Hospital Besar Kuala Lumpur	FE 017/2009
21	23/05/2009	Hospital Serdang	FE 20/2009
22	23/05/2009	Hospital Serdang	FE 022/2009
23	23/05/2009	Hospital Serdang	FE 021/2009
24	23/05/2009	Hospital Melaka	FE 018/2009
25	25/05/2009	Hospital Johor Bahru	FE 03/2009
26	26/05/2009	Hospital Besar Seremban	FE 019/2009
27	28/05/2009	Hospital Besar Kuala Lumpur	FE 023/2009
28	09/06/2009	Hospital Besar Kuala Lumpur	FE 026/2009
29	09/06/2009	Hospital Johor Bahru	FE 02/2009
30	10/06/2009	Hospital Melaka	FE 027/2009
31	30/06/2009	Hospital Besar Kuala Lumpur	FE 028/2009
32	17/07/2009	Hospital Johor Bahru	FE JB 074/2009
33	27/07/2009	Hospital Melaka	FE 30/2009
34	11/08/2009	Hospital Universiti Kebangsaan Malaysia	FE F904/2009
35	14/08/2009	Hospital Pahang	FE 031/2009
36	01/09/2009	Hospital Melaka	FE 032/2009
37	03/09/2009	Hospital Besar Kuala Lumpur	FE 033/2009
38	23/09/2009	Hospital Melaka	FE 035/2009
39	27/09/2009	Hospital Pahang	FE 034/2009
40	05/10/2009	Hospital Besar Kuala Lumpur	FE 036/2009
41	01/12/2009	Hospital Serdang	FE 038/2009
42	03/12/2009	Hospital Besar Kuala Lumpur	FE 039/2009
43	09/12/2009	Hospital Melaka	FE 040/2009
44	09/12/2009	Hospital Melaka	FE 041/2009
45	13/12/2009	Hospital Banting	FE 45 BANTING
46	15/02/2010	Hospital Besar Kuala Lumpur	FE 03/2010
47	16/02/2010	Hospital Besar Kuala Lumpur	FE 04/2010
48	18/02/2010	Hospital Besar Kuala Lumpur	FE 05/2010
49	25/02/2010	Hospital Universiti Kebangsaan Malaysia	FE F228/2010
50	11/03/2010	Hospital Universiti Kebangsaan Malaysia	FE F302/2010

Maggots were collected form all the 50 different crime scene and subjected to both morphological and molecular methods for species identification. The gender and ethnicity of the deceased as well as the condition and location of the deceased when it was found are listed in Table 4.23. All the sequences for COI and COII genes have been deposited in GenBank under accession numbers as shown in Table 4.23.

Table 4.23: Summary of forensic cases (Case 1 - Case 50)

Case	Sex	Ethnic	Remarks (Conditions of deceased when it was found)	Location of deceased	Species of maggot		Instar	Accession Number from GenBank
					Morphological Identification	Molecular Identification		
1i	Male	Myanmar	Post-mortem showed decomposed body. Heavily ingested by maggots over the wound on the head.	Hill (Rural)	*Hemipyrellia ligurriens*	Unknown species	III	JN228993
1ii					*Hemipyrellia ligurriens*	Unknown species	III	JN228993
2i	Male	Unknown	Post-mortem showed early stage of decomposed body. Most of the maggots were seen around the face and the body.	Inside house (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	II	JN 229004 and JN 571553
2ii					*Chrysomya megacephala*	*Chrysomya megacephala*	II	JN 229004 and JN 571554
3i	Male	Unknown	Post-mortem showed early stage of decomposed body. Most of the maggots were seen around the face and body.	Drain (Rural)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229005 and JN 571555
3ii					*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229005 and JN 571556
4i	Male	Unknown	Post-mortem showed advanced decomposed body and no external injuries. Maggots were found all over the body.	Inside house (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 228994
4ii					*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 229007 and JN 571557
5i	Female	Unknown	Post-mortem showed advanced stage of decomposed body. Most of the maggots were seen around the stomach.	River (Aquatic)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229008
5ii					*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229008
6i	Female	Unknown	Post-mortem showed partially decomposed body. Most of the maggots were seen around the head and clothing.	Bushes (Rural)	*Chrysomya villeneuvi*	*Chrysomya megacephala*	III	JN 229018
6ii					*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 229017
7i	Male	Indian	Post-mortem showed decomposed body. Maggots were found all over the body.	Drain (Rural)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 228996
7ii					*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 228997
8	Female	Unknown	Post-mortem showed advanced decomposition and partially skeletonised. Maggots were taken from internal area of remaining trunk.	Bushes (Rural)	*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 228998
9	Male	Unknown	Post-mortem showed advanced decomposed body. Most of the maggots were seen around the head. Cause of death was due to severe head injury and blunt trauma.	Side of road (Rural)	*Chrysomya rufifacies*	Unable to be amplified by PCR	III	None
10i	Male	Chinese	Post-mortem showed advanced decomposed body. Maggots were found all over the body. Cause of death was due to sustained multiple injuries.	Foothill (Rural)	*Chrysomya villeneuvi*	*Chrysomya megacephala*	III	JN 229013
10ii					Cannot be identified	Unknown species	I	JN 228993

Continued from page 140

Case	Sex	Ethnic	Remarks (Conditions of deceased when it was found)	Location of deceased	Species of maggot		Instar	Accession Number from GenBank
					Morphological Identification	Molecular Identification		
11	Female	Malay	Post-mortem showed advanced decomposed body. Maggots concentrated around head. Cause of death was due to sustained multiple injuries.	Foothill (Rural)	*Chrysomya megacephala*	*Chrysomya rufifacies*	III	JN 229014 and JN 571558
12	Female	Unknown	Post-mortem showed decomposed body. Cause of death was unascertained.	Rubbish dumping area (Rural)	*Sarcophaga* sp.	Unable to be amplified by PCR	III	None
13	Male	Chinese	Post-mortem showed moderate to advanced stage of decomposition. Covered with a blanket and plastic bag.	Car boot (Rural)	*Chrysomya megacephala*	Unable to be amplified by PCR	III	None
14	Male	Chinese	Post-mortem showed early stage of decomposition with a mummification at both feet. Cause of death was due to because of compression on the neck.	Car boot (Rural)	*Megaselia scalaris*	Unable to be amplified by PCR	III	None
15i	Male	Indian	Post-mortem showed advanced decomposed body covered with maggots. Cause of death was due to blunt trauma to the head and multiple slashed wounds.	Bushes (Rural)	*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 571551
15ii					*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229009 and JN 571559
16	Male	Bangladeshi	Post-mortem showed early stage of decomposed body. Cause of death was due to multiple stab wounds.	Inside house (Residential)	Flies eggs	*Chrysomya megacephala*	Eggs	JN 229019
17i	Female	Indian	Post-mortem showed moderate to advanced stage of decomposition. Found hanging in a house. Ligature was found around neck with ligature mark encircling neck.	Inside house (Residential)	*Megaselia scalaris*	Unable to be amplified by PCR	III	None
17ii					*Megaselia scalaris*	Unable to be amplified by PCR	III	None
18	Female	Malay	Post-mortem showed moderate to advanced stage of decomposition.	Inside house (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	II	JN 229021
19	Male	Malay	Post-mortem showed advanced stage of decomposition. Maggots were found all over the body.	Building (Residential)	*Hemipyrellia* sp.	*Hemipyrellia liguriens*	II	JN 229034
20	Male	Malay	Post-mortem showed advanced bloated stage of decomposition. Cause of death was due to heart attack.	Inside house (Residential)	*Hemipyrellia* sp.	Unable to be amplified by PCR	III	None

Continued from page 141

Case	Sex	Ethnic	Remarks (Conditions of deceased when it was found)	Location of deceased	Species of maggot		Instar	Accession Number from GenBank
					Morphological Identification	Molecular Identification		
21	Female	Indian	Post-mortem showed advanced stage of decomposition. No external injuries were found because all organs were decomposed.	Inside house (Residential)	*Chrysomya pinguis*	*Chrysomya megacephala*	III	JN 228999
22	Female	Indian	Post-mortem showed advanced stage of decomposition. No external injuries were found because all organs were decomposed.	Inside house (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229000
23	Female	Indian	Post-mortem showed advanced stage of decomposition. No external injuries were found because all organs were decomposed.	Inside house (Residential)	*Chrysomya pinguis*	Unable to be amplified by PCR	III	None
24	Female	Unknown	Post-mortem showed advanced state of decomposed with skeletonization. The lower limb was relatively intact with mummification changes.	Sugarcane plantation (Rural)	*Hermetia illucen*	Unable to be amplified by PCR	III	None
25i	Male	Unknown	Post-mortem showed moderate to advanced decomposed body. Thorax and abdomen was fully exposed with internal autolysis. Cause of death was due to blunt force trauma to the head.	Oil palm plantation (Rural)	*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 229027 and JN 571560
25ii					*Chrysomya nigripes*	*Chrysomya nigripes*	III	JN 229002
26	Male	Unknown	Post-mortem showed moderate to advance decomposed body. Cause of death was due to blunt force trauma to the head.	Rubbish dumping area (Rural)	*Calliphoridae* sp.	Unknown sp.	I	JN 228993
27	Male	Unknown	Post-mortem showed decomposed stage.	River (Aquatic)	Cannot be identified	*Chrysomya megacephala*	I	JN 229029 and JN 571561
28	Female	Indian	Post-mortem showed moderate to advance decomposed body. Cause of death was due to multiple sharp lymen to the head.	Inside house (Residential)	Cannot be identified	*Chrysomya megacephala*	I	JN 229030
29	Male	Unknown	Post-mortem showed decomposed stage. Found at river in a partly burnt on the upper portion of the body.	River (Aquatic)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229003
30	Male	Unknown	Post-mortem showed decomposed body	Inside house (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229026

Continued from page 142

Case	Sex	Ethnic	Remarks (Conditions of deceased when it was found)	Location of deceased	Species of maggot		Instar	Accession Number from GenBank
					Morphological Identification	Molecular Identification		
31i	Male	Unknown	Post-mortem showed moderate decomposed body. The deceased was found with fully clothed with hands and legs tied with cotton tile.	Rubbish dumping area (Rural)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229032 and JN 571562
31ii					*Chrysomya megacephala*	Unable to be amplified by PCR	III	None
32	Male	Chinese	Post-mortem showed early stage of decomposition. Cause of death was due to coronary atheroma with anomaly.	Inside house (Residential)	*Sarcophaga* sp.	*Sarcophaga ruficornis*	III	JN 229033
33	Male	Unknown	Post-mortem showed decomposed body. The room in where the deceased was found was locked from within and the windows were closed.	Inside room (Residential)	*Sarcophaga* sp.	*Sarcophaga ruficornis*	III	JN 229022 and JN 571552
34	Male	Unknown	Post-mortem showed advanced decomposed body covered with eggs and maggots. Found hanging in the room.	Inside room (Residential)	*Sarcophaga* sp.	*Sarcophaga ruficornis*	III	JN 229035
35	Female	Unknown	Post-mortem showed advanced stage of decomposition. Cause of death was due to ligature strangulation to the neck.	Ravine (Rural)	*Chrysomya villeneuvi*	Unable to be amplified by PCR	III	None
36	Male	Unknown	Post-mortem showed early stage of decomposition and also had predator activity (e.g. Varunus).	Bushes (Rural)	*Chrysomya pinguis*	Unable to be amplified by PCR	III	None
37	Female	Unknown	Post-mortem showed advanced stage of decomposition. The deceased covered by cement expect the part of lower limbs.	Shop house (Residential)	*Synthesiomyia nudiseta*	Unable to be amplified by PCR	III	None
38	Male	Unknown	Post-mortem showed advanced stage of decomposition. The room in where the deceased was found was locked from within and the windows were closed.	Inside room (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	II	JN 229036
39	Male	Indonesian	Post-mortem showed advanced decomposed body covered with maggots. The deceased head covered by plastic bag, wearing only leans.	Ravine (Rural)	*Chrysomya villeneuvi*	Unable to be amplified by PCR	III	None
40	Male	Indian	Post-mortem showed decomposed stage. The deceased was known as drug addict. Cause of death was due to tuberculosis.	Inside room (Residential)	*Sarcophaga* sp.	Unable to be amplified by PCR	II	None

Continued from page 143

Case	Sex	Ethnic	Remarks (Conditions of deceased when it was found)	Location of deceased	Species of maggot		Instar	Accession Number from GenBank
					Morphological Identification	Molecular Identification		
41	Female	Indonesian	Post-mortem showed advanced stage of decomposition.	Bushes (Rural)	*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 229037 and JN 571563
42	Male	Unknown	Post-mortem showed advanced stage of decomposition, partly skeletonised and partly mummified.	Ravine (Rural)	*Chrysomya nigripes*	*Chrysomya nigripes*	III	JN 229038
43	Male	Unknown	Post-mortem showed advanced stage of decomposition. The septic tank was covered with a zinc slab.	Septic tank (Aquatic)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229015
44	Male	Unknown	Post-mortem showed advanced stage of decomposition. The septic tank was covered with a zinc slab.	Septic tank (Aquatic)	*Chrysomya megacephala*	*Chrysomya megacephala*	II	JN 229039
45	Male	Bangladeshi	Post-mortem showed the body started to shrink and bloated. Most of the maggots were found at oral cavities and all over the body.	Inside factory (Residential)	*Chrysomya rufifacies*	Unable to be amplified by PCR	III	None
46	Male	Unknown	Post-mortem showed partly skeletalized body	Under bridge (Rural)	*Chrysomya rufifacies*	Unable to be amplified by PCR	III	None
47	Male	Indonesian	Post-mortem showed decomposed stage. Cause of death was slash wound of the neck.	Apartment (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229016
48	Female	Unknown	Post-mortem showed advanced decomposed stage.	Inside house (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229020
49	Female	Unknown	Post-mortem showed advanced decomposed body covered with eggs and maggots.	Inside room (Residential)	*Chrysomya megacephala*	*Chrysomya megacephala*	III	JN 229040 and JN 571564
50	Female	Unknown	Post-mortem showed advanced decomposed body covered with eggs and maggots.	Inside room (Residential)	*Chrysomya rufifacies*	*Chrysomya rufifacies*	III	JN 229041 and JN 571565

The following section explains briefly the facts about the 50 crime cases. The data collected in respect of the 50 cases showed that 28 cases involved unknown persons and in the remaining 22 cases the identities of the deceased were known. Of these, 18 were females and 32 were males and are shown in Figure 4.1.

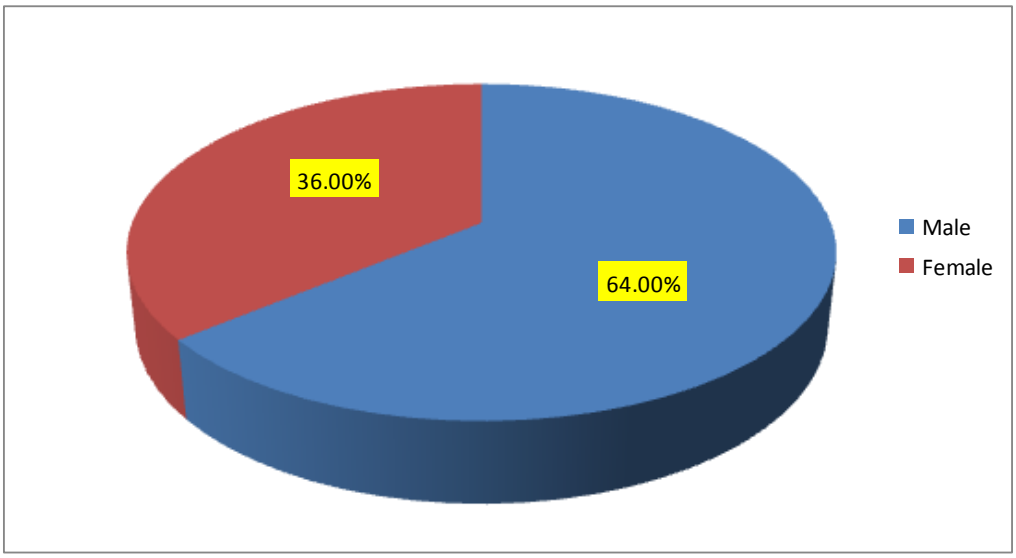

Figure 4.1: Percentage (%) of deceased by gender

According to the stage of decomposition, 1 case showed dry stage (skeletonization), 9 were actively decaying, 25 cases showed moderate stage and 6 cases were in fresh stage. Injuries were present in 14 cases and more infestations of maggots were found in these areas of the bodies and also in dark moist areas and orifices of the bodies like nostrils, eyes, genitals and hairy areas. The areas where the dead human bodies were found were divided into residential area are 23 cases, rural area 22 cases and aquatic area are 5 cases. The data are shown in Figure 4.2.

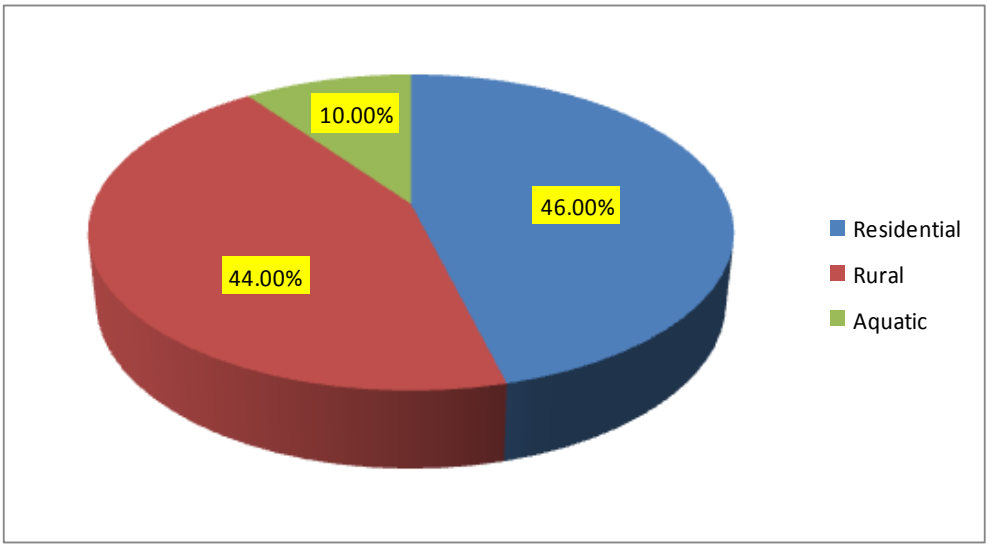

Figure 4.2: Percentage (%) of location where the bodies were found

The majority of the fly species was obtained from unidentified deceased was accounted for 28 cases (56.00%) followed by Indian deceased for 8 cases (16.00%), Chinese deceased for 4 cases (8.00%), Malay deceased for 4 cases (8.00%) and other ethnic deceased for 6 cases (12.00%) as shown in Figure 4.3.

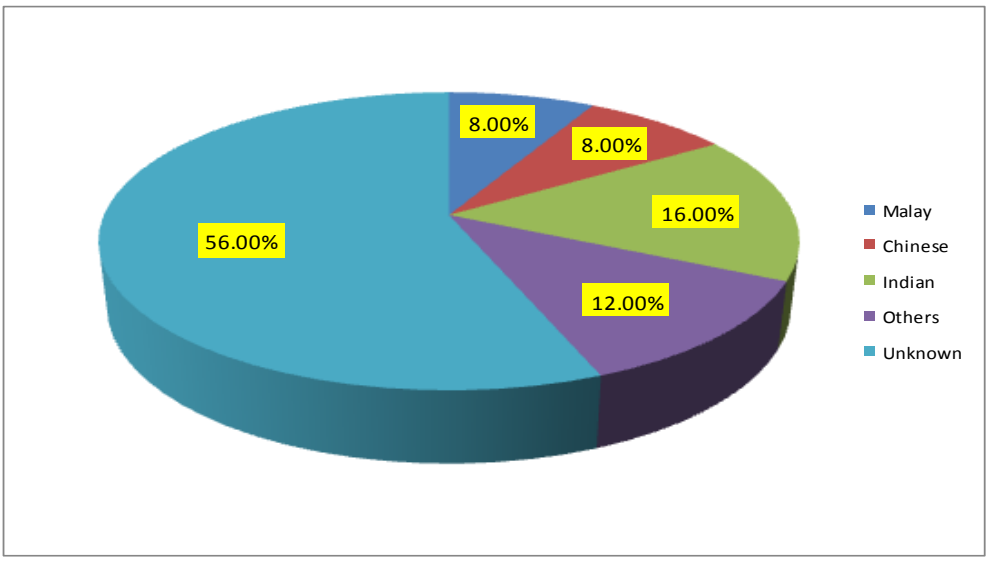

Figure 4.3: Percentage (%) of deceased by the ethnic group

The total of crime scene cases where the entomological evidence have been collected and compared in accordance to months are 2 cases were reported in January, 6 cases were reported in February, 8 in March, 4 in April, 10 in May, 3 in June, 3 in July, 2 in August, 4 in September, 1 in October, no case in November and 7 cases were reported in December (Figure 4.4).

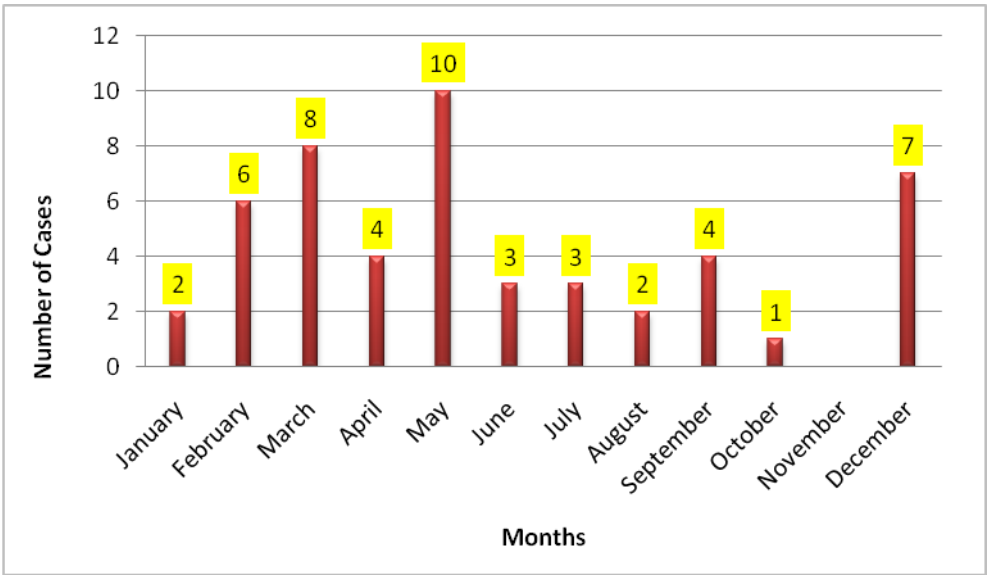

Figure 4.4: Number of cases versus months

In total 50 cases of crime scene investigation were included. Two maggot samples were analyzed in 12 cases (cases 1, 2, 3, 4, 5, 6, 7, 10, 15, 17, 25 and 31) (Table 4.23), due to either double fly infestation or the sample obtained was not in optimum condition for morphological identification. Only one maggot sample was analyzed for the remaining cases. Hence in total 62 maggot samples were analyzed by both morpohological and molecular methods.

In total 58 samples were successfully identified by morphological method. Only four samples were unable to be identified due to immature stages such as egg and first instar (10ii, 16, 27 and 28) (Table 4.23). Figure 4.5 showed the percentage of fly larvae species identified that are *Chrysomya megacephala* accounted for 23 samples (39.7%), *Chrysomya rufifacies* for 11 samples (19%), *Sarcophaga* sp. for 5 samples (9%), *Chrysomya villeneuvi* for 4 samples (6.9%), *Megaselia scalaris* for 3 samples (5.2%), *Chrysomya pinguis* for 3 samples (5.2%), *Chrysomya nigripes* for 2 samples (3.4%), *Hemipyrellia ligurriens* for 2 sample (3.4%), *Hemipyrellia* sp. for 2 sample (3.4%), *Hermetia illucens* for 1 sample (1.7%), *Synthesiomyia nudiseta* for 1 sample (1.7%) and Calliphoridae sp. for 1 sample (1.7%).

For the comparison of both methods, only those samples that were identified both morphological and molecular methods were included. Four samples (10ii, 16, 27 and 28) were unable to be identified by morphological method due to the immature stages and distorted morphological features. Four samples (1i, 1ii, 10ii and 26) gave rise to an identical nucleotide sequence and were identified as 'unknown species' based on phylogenetic analysis (Figure 4.7 and Figure 4.8). Since sample 10ii was an immature stage, it was unable to be identified by morphological method. However the sample was later successfully identified as an 'unknown species' by phylogenetic analysis.

Meanwhile 17 samples (9, 12, 13, 14, 17i, 17ii, 20, 23, 24, 31ii, 35, 36, 37, 39, 40, 45 and 46) were unable to be identified by molecular method due to failure in PCR amplification. Hence in total 38 samples were considered for comparison. Results showed that species identification by molecular method was in agreement with morphological method in 89% (34/38) of the samples. Discrepancy in species identification was observed in four samples (6i, 10i, 11 and 21) (Table 4.23). Based on morphological method, the majority of infestation was single species accounting for 45

cases (90%) as shown in Figure 4.6 and double infestation species accounting for 5 cases (10%).

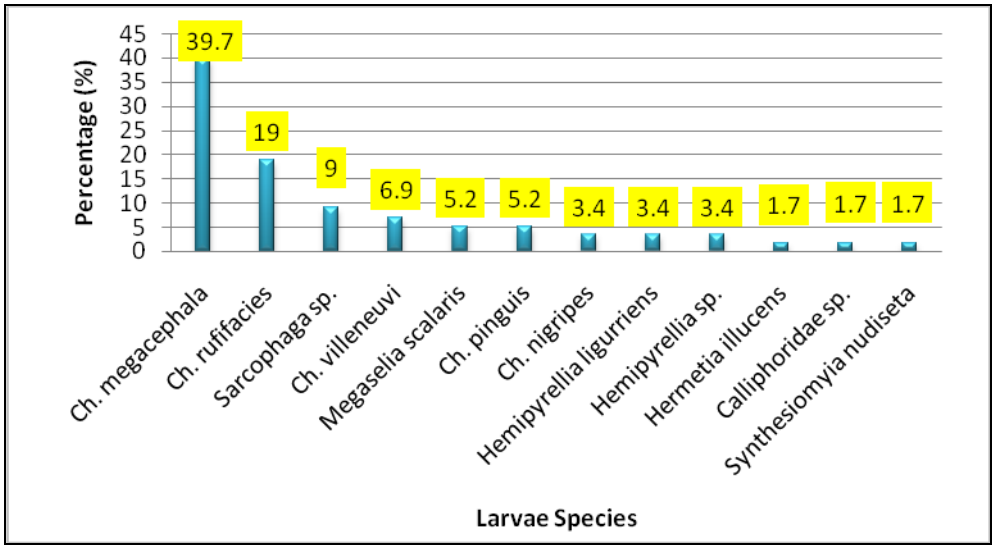

Figure 4.5: Percentage (%) of larvae species found on the dead human bodies during crime scene investigation

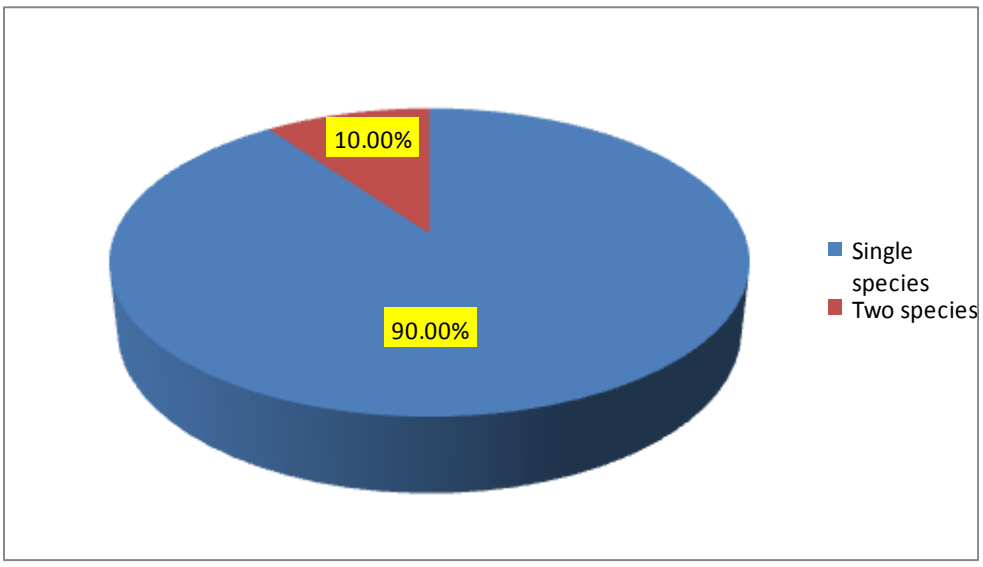

Figure 4.6: Percentage (%) of fly infestation on the dead human bodies during crime scene investigations

4.3 DESCRIPTION OF CASE STUDIES

During the period of December 2008 to February 2010 studies on insect fauna on human dead body yielded significant information concerning the decomposition process. Though a continuous process, it is divided into various stages by different workers for better understanding. In the present, studies on the decomposition process were evident in the form of 4 successive stages. These were fresh stage (early stage), moderate stage, advanced stage and dry stage (skeletonization).

4.3.1 Comparison according to site of death

4.3.1.1 Residential Area

Case 21, 22 and 23 were related and the deceased were found in their house that was locked from inside. The post-mortem showed advanced stage of decomposition. No external injuries were found because all organs were decomposed. Cause of death was unascertained but appeared to be suicidal from ingestion of unknown poison. DNA molecular identification indicated the presence of 3rd instar *Chrysomya megacephala* for case 21 and 22, and 3rd instar of *Chrysomya pinguis* for case 23. As the deceased was found inside the house, the point of access for the fly is important because if access is not possible, for instance if the body is securely enclosed in a plastic sack or inside locked house, then clearly eggs cannot be laid even if flies were attracted to the general vicinity of the body.

Case 28 was that of a 43 years old Indian female who was found dead in her house with the air conditioner on. The post-mortem examination showed moderate to advanced stage of decomposed body and the cause of death was due to blunt force trauma to the head. Most of the maggots were seen around the head. Based on DNA

molecular identification, the 1st instar of larvae was diagnosed as *Chrysomya megacephala*.

Case 30 was that of a 74 years old man who was found dead in his room locked from within with a partially open window. By morphological and DNA molecular identification methods the 3rd instar of larvae was identified as *Chrysomya megacephala*. The body of a 56 years old Chinese man was found dead in a house for case 32. Post-mortem examination showed the deceased in an advanced stage of decomposition and the cause of death was determined as 'coronary atheroma with anomaly'. Most of the maggots were found in the head. Molecular identification of the 3rd instar maggots revealed them as *Sarcophaga ruficornis*.

Case 37 was that of an unidentified adult female who was found dead in a trade-shop house. Post-mortem examination showed the deceased was covered by cement except on the lower limbs. Most of the maggots were found at the lower limbs. The 3rd instar larvae were identified as *Synthesiomyia nudiseta* based on morphological identification. Post-mortem examination showed advanced decomposed stage for the 63 years old lady who was found dead in her own house, for case 48. Morphological and DNA molecular identification methods showed the presence of 3rd instar of *Chrysomya megacephala*.

Case 49, was that of an adult female found lying on the bed in her room. Post-mortem examination showed an advanced decomposed body covered with eggs and maggots. Both morphological and molecular identification methods indicated the presence of 3rd instar of *Chrysomya megacephala*.

4.3.1.2 Rural Area

Case 1 was that of a Myanmar male of about 30 years of age who climbed the hill top to hide when ambushed by police, and believed to have slipped and fallen down and died. Post-mortem examination showed a decomposed body heavily ingested by maggots over the wound on the head. Cause of death was injury due to fall from height. Morphological identification showed the presence of 3rd instar of *Hemipyrellia ligurriens*.

A 52 years old Indian man was found dead near a drain for case 7. Post-mortem examination showed decomposed body and the cause of death was undetermined. Maggots were found all over the body. For this case double fly infestation were encountered. Based on morphological and DNA molecular methods the 3rd instar maggots were identified as *Chrysomya megacephala* and *Chrysomya rufifacies*. Case 10 was that of a Chinese man of about 57 years of age, who was found dead at a foothill. Post-mortem examination showed the deceased in an advanced stage of decomposition and the cause of death was sustained multiple injuries. Maggots were found all over the body. DNA molecular identification indicated the presence of 3rd instar *Chrysomya megacephala*.

Case 12 was that of an unidentified female baby who was found dead in a plastic bag in the rubbish. Post-mortem examination showed decomposed body and the cause of death was unascertained. Morphological identification indicated the presence of 3rd instar *Sarcophaga* sp.. The body of an unidentified male was found dead at an oil palm plantation for case 25. Post-mortem examination showed a moderate to advanced stage decomposed body and the cause of death was due to blunt force trauma to the head. The deceased was exposed to direct sunlight and the thorax and abdomen was fully exposed

with internal autolysis. Most of the maggots were seen around the head. This case showed double fly infestation and the DNA molecular identification together with morphological identification showed the presence of 3rd instar *Chrysomya rufifacies* and 3rd instar *Chrysomya nigripes*.

Case 39 was that of an unidentified Indonesian adult male who was found dead in a jungle ravine. The post-mortem examination showed advanced stage decomposition and the body was covered with maggots. Cause of death was due to blunt trauma to the head. Morphological identification indicated the presence of 3rd instar of *Chrysomya villeneuvi*.

4.3.1.3 Aquatic Area

There were 4 cases whereby all the deceased were found inside water either in the lake, water tank or river. Case 5 was that of an unidentified adult female who was found dead in river. Post-mortem examination showed an advanced stage decomposed body and most of the maggots were seen around the stomach. The cause of death was undetermined. Both the morphological and DNA molecular identification showed the presence of 3rd instar of *Chrysomya megacephala*. Case 27 was that of an unidentified body of a deceased male who was found in a river. Post-mortem examination showed a decomposed body. DNA molecular identification analyses indicated the presence of 1st instar of *Chrysomya megacephala*.

Case 43 and case 44 were related, whereby 2 bodies of an adult male fully clothed was found in a septic tank covered with a zinc slab in a farm house. Post-mortem examination showed a body in an advanced stage of decomposition, swarming with maggots and adults flies. Morphological and molecular identification indicated the presence of 3rd stage *Chrysomya megacephala* for case 43. Both morphological and DNA molecular identifications for case 44 indicated the presence of 2nd instar *Chrysomya megacephala*.

4.3.2 Comparison according to cause of death

4.3.2.1 Slash Wounds

An unidentified adult man was found dead at side of a road for case 9. Post-mortem examination showed an advanced stage of decomposed body and most of the maggots were seen around the head. Cause of death was severe head injury and blunt object trauma. Morphological identification indicated the presence of 3rd instar of *Chrysomya rufifacies*.

Case 6 was that of an unidentified female body found dead in bushes. Post-mortem examination showed a partially decomposed body with slash wounds to the neck. Most of the maggots were seen around the head and clothing. This case was another double fly infestation. DNA molecular identification indicated the presence of 3rd instar of *Chryomsya megacephala* and 3rd instar of *Chrysomya ruficacies*.

A 53 years old lady was found dead at a foothill for case 11. Post-mortem examination showed an advanced stage decomposed body and maggots were found all over the body but concentrated around the head. Cause of death was attributed to multiple injuries. The deceased was allegedly missing 3 to 7 days before the body was found. DNA molecular identification indicated the presence of 3rd instar of *Chrysomya rufifacies*.

Case 15 was that of an unidentified Indian man who was found dead because of blunt object trauma to the head and multiple slash wounds. Post-mortem examination showed an advanced decomposed body covered with maggots. Morphological and DNA molecular species identification processes indicated the presence of two blow flies infestations that is 3rd instars *Chrysomya rufifacies* and *Chrysomya megacephala*.

An adult man was found dead at a rubbish dumping area with multiple blunt injuries to the body, for case 26. The post-mortem examination showed a moderate to advanced stage decomposed body and most of the maggots were seen around the wounds using morphological identification process the 1st instar larvae were identified as a species of *Calliphoridae* and the molecular processes identified it as an unknown species.

Case 47 was that of an Indonesian man of about 33 years old, who was found dead due to slash wound in the neck in an apartment of an almost completed

construction building. Post-mortem examinations showed a decomposed body with most maggots found around the neck. Morphological and molecular identification processes showed the presence of 3rd instar maggot of *Chrysomya megacephala*.

4.3.2.2 Strangulation

Case 35 was that of an unidentified adult female who was found dead in a ravine. The deceased was fully clothed. The post-mortem examination showed an advanced decomposed body with maggots all over. Cause of death was due to ligature strangulation to the neck. Morphological identification of the maggots indicated the presence of 3rd instar *Chrysomya villeneuvi*.

4.3.2.3 Drug

Case 19 was that of a 38 years old Malay man who was found in the basement of an abandoned building. The post-mortem examination showed an advanced stage decomposed body. Maggots were found all over the body. The deceased was a drug addict. Morphological and DNA molecular identification showed the presence of 2nd instar maggots of *Hemipyrellia ligurriens*.

Case 40 was that of Indian man of about 30 years of age from India. He was a known drug addict and was found dead in an empty house. Post-mortem examination showed a decomposed body with most maggots found in the facial region and upper body. Cause of death was attributed to tuberculosis. Morphological identification indicated the presence of 2nd instar maggots of *Sarcophaga* sp..

4.3.2.4 Burnt

Case 29 was that of about an unidentified adult man who was found partly burnt on the upper portion of the body. Post-mortem examination showed a decomposed body and medium to large sized maggots were seen all over the body. Both morphological and DNA molecular identification processes indicated the presence of 2nd instar of *Chrysomya megacephala*.

4.3.2.5 Natural Cause

Case 20 was that of about a 55 years old Malay man who was found dead in his house due to heart attack. The deceased was staying alone in his house and the fan was still switched on. The post-mortem examination showed a body in an advanced bloated stage of decomposition and maggots were found all over the body. Morphological identification showed the presence of 3rd instar *Hemipyrellia* species.

4.3.3 Comparison according to stage of decomposition

4.3.3.1 Early Decomposition Stage

Case 2 was that of an adult man found dead in a house. Post-mortem examination showed a body in early stages of decomposition. Most of the maggots were seen around the face and the body. Cause of death was due to stab wound to the heart. Morphological and DNA molecular identification indicated the presence of 2nd instar of *Chrysomya megacephala*.

Case 3 was that of an adult man found dead in the drain. Post-mortem examination showed an early stage decomposed body and most of the maggots were seen around the face and the body. Cause of death was due to slash wound to the neck.

Both morphological and DNA molecular identification indicated the presence of early 3rd instar of *Chrysomya megacephala*.

The body of a Chinese man who was found dead in a car boot showed early stage decomposition with mummification at both feet, based on postmortem examination report for case 14. The cause of death was because of compression on the neck. DNA molecular identification showed 3rd instar of *Megaselia scalaris.* Case 16 was that of a 32 year old Bangladesh man who was found dead in his house due to multiple stab wounds. The post-mortem showed early stage decomposed body and fly eggs were found on the left thigh of the deceased. DNA molecular identification identified the fly egg as those of *Chrysomya megacephala*.

Case 34 was about of an unidentified adult man who was found hanging in a room in his house. There were no other suspicious injuries seen on the body. Post-mortem examination showed a severely decomposed body covered with eggs and maggots. DNA molecular identification identified the maggots as 3rd instar of *Sarcophaga ruficornis*. Case 36 was that of an adult man who was found dead in an open air space in the middle of a bush area. Post-mortem examination showed a body in an early stage of decomposition. The body showed predation by monitor-lizard (*Varanus* sp.). There were also hundreds of maggots in various sizes and no puparium-casings were seen. Morphological identification indicated the presence of early 3rd instar of *Chrysomya pinguis*.

4.3.3.2 Moderate Decomposition Stage

Case 17 was that of a 39 years old Indian female found hanging in her house. The deceased was covered with a blanket and plastic bag. The post-mortem examination showed moderate to advanced stage decomposition. Besides that, ligature was found around the neck with ligature mark encircling the neck. Maggots were found on body mainly nostrils, mouth, chest and abdomen. Morphological identification process showed the presence of 3rd instar of *Megaselia scalaris*.

Case 18 was that of a 77 year old Malay female who was found dead on the floor of her house. Besides that, the room where the deceased was found was locked from within and there were ventilation ducts above the closed windows. Post-mortem examination showed moderate to advanced stage decomposition and the maggots were found all over the body. Both morphological and DNA molecular identification processes indicated the presence of 2nd instar maggots of *Chrysomya megacephala*.

Next was case 31, that of an unidentified adult man who was found dead in pruned position within the rubbish. The deceased was fully clothed with hands and legs tied with cotton tile. Post-mortem examination showed a moderately decomposed body with maggots all over the body. Morphological and DNA molecular identifications showed the presence of 3rd instar maggots of *Chrysomya megacephala*.

Followed by case 33, was that of a 35 year old unidentified adult man found dead in a house. The room in where the deceased was found was locked from within and the windows were closed. The post-mortem examination showed a moderately decomposed body and maggots were found all over the body. DNA molecular identification of the 3rd instar of larvae indentified it as *Sarcophaga ruficornis*.

Finally case 45 that of an adult Bangladeshi man found dead in a factory and post-mortem examination showed the body had started to shrink and was bloated. Most of the maggots were found in the oral cavity and all over the body. Based on morphological identification analysis of 3rd instar the maggots were those of *Chrysomya rufifacies*.

4.3.3.3 Advanced Stage of Decomposition

Case 4 was that of an 80 years old man, who was found dead in a house. The post-mortem examination showed an advanced stage decomposed body with no external injuries and the maggots were found all over the body. Based on the morphological and DNA molecular identifications, two fly-species were determined. The presence of two blow flies was diagnosed that are 3rd instar of *Chrysomya megacephala* and *Chrysomya rufifacies*. Next case was 13, that of a Chinese man who was found dead in a car boot. The deceased was covered with a blanket and plastic bag. The post-mortem examination result showed a moderate to advanced stage decomposed body. Morphological analysis showed the presence of 3rd instar *Chrysomya megacephala*.

Post-mortem examination of case 38 showed an advanced stage of decomposition of a 67 years old man who was found dead in a room in his house which was locked from within and the windows were closed. Most of the maggots were found in the open orifices. Morphological and DNA molecular analysis showed the presence of 2nd instar of *Chrysomya megacephala*. Case 41 was that of a 31 years old Indonesian woman, who was found dead in bushes nearby to her house. The post-mortem showed an advanced stage of decomposition and most of the maggots were found all over the body. The same species identification was made by the morphological and DNA molecular identifications for this case that is 3rd instar of *Chrysomya rufifacies*.

Case 50 was about a 54 years old woman who was found dead in her room. Post-mortem examination showed an advanced stage decomposed body covered with eggs and maggots. Both the morphological and DNA molecular identification processes showed the presence of 3rd instar of *Chrysomya rufifacies*.

4.3.3.4 Mummified Stage of Decomposition

Case 24 was that of an 81 years old female who was found dead in an open air space in the middle of a sugar cane plantation with dense shrubs that showed an advanced stage decomposed body with skeletonization. The body itself had undergone severe decomposition but the lower limb was relatively intact with mummification. There were also hundreds of maggots of various sizes. Morphological identification process result showed 3rd instar of *Hermetia illucens*. Second case for this category was case 8. This case was that of an unidentified female, who was found dead in the bushes. The post-mortem examination showed an advanced stage of decomposition with partial skeletonisation and the cause of death was given as 'unascertained'. Morphological and DNA molecular identification processes showed the maggots were 3rd instars of *Chrysomya rufifacies*.

Case 42 was that of an unknown male who was found dead inside a small ravine by the roadside. The post-mortem examination showed a body in an advanced stage of decomposition and the body was partly skeletonised and partly mummified. Most of the maggots were found all over the body and infestation of maggots were seen over upper part of the chest with other insects. Both morphological and DNA molecular species identification processes diagnosed the presence of 3rd instar of *Chrysomya nigripes*. The last case for this category was case 46. The unknown adult man was found dead under a bridge. The post-mortem examination showed a partly skeletonised boy and the body

was swarmed with maggots and adult flies. Morphological identification showed the presence of 3rd instar *Chrysomya rufifacies*.

4.3.4 Phylogenetic Analysis

Phylogenetic trees were reconstructed based on COI (complete and partial sequences), COII and the entire cytochrome oxidase genes including the t-RNA gene. The reference sequences were retrieved from Genbank for comparative purposes for the previous reported blow fly in Malaysia according to Lee *et al.* (2004) namely *Calliphora vicina* AJ417702, *Chrysomya bezziana* AF295548, *Chrysomya megacephala* AF295551, *Chrysomya nigripes* GU174026, *Chrysomya pinguis* AY092759 and DQ345101, *Chrysomya rufifacies* AF083658, *Chrysomya villeneuvi* FJ195382, *Hemipyrellia liguriens* AY097334 and DQ345116, *Hermatia illucens* GQ465783, *Lucilia cuprina* AJ417707 and FJ153277, *Megaselia scalaris* AF217464, *Ophyra spinigera* EU627714, *Sarcophaga ruficornis* EF405941, *Synthesiomyia nudiseta* EU627713 and DQ345099 and *Calliphora* sp. AY842649.

Phylogenetic tree was constructed by neighbour-joining (NJ) method implemented in the MEGA 4 and the tree were tested by 1000 bootstrap replicates as shown in Figure 4.7, Figure 4.8, Figure 4.9 and Figure 4.10. According to tree in the Figure 4.7, it involved the sequencing of a total length around 2.3 kilo base pairs encompassing the mitochondrial cytochrome oxidase I (COI), cytochrome oxidase II (COII) and t-RNA genes. Phylogenetic analyses confirmed the presence of *Chrysomya megacephala*, *Chrysomya rufifacies* and *Chrysomya nigripes*. In addition, one new species or 'an unknown species' of blow fly was discovered. Maggot samples for Case 26 are 1st instar and due to the immature larval stage, the process of morphological identification cannot be conducted. Each of the blow fly species was clearly separated

from *Chrysomya megacephala*, *Chrysomya rufifacies* and *Chrysomya nigripes* and 3 isolates were clustered together. *Chrysomya pinguis*, *Chrysomya bezziana*, *Chrysomya villeneuvi, Calliphora vicina, Lucilia cuprina, Ophyra spinigera, Synthesiomyia nudiseta, Hermatia illucens, Hemipyrellia liguriens* and *Megaselia scalaris* were used as outgroup of the phylogenetic tree.

Chrysomya megacephala, *Chrysomya rufifacies* and *Chrysomya nigripes* could be easily distinguished although they belong to the same genus, implying that the COI and COII genes sequences were useful for identification of these congeneric species. All of the *Chrysomya megacephala*, *Chrysomya rufifacies* and *Chrysomya nigripes* isolates formed a single cluster with branches indicating minor nucleotide variations between the congeneric species.

Figure 4.8 shows a phylogenetic tree involving the sequencing of approximately 348-base pairs 'barcode' fragment of the mitochondrial cytochrome oxidase subunit I (parital COI) gene for 44 specimens, representing 50 crime scene investigations in Malaysia. Phylogenetic analyses confirmed the presence of 5 different blow fly species namely *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya nigripes*, *Hemipyrellia ligurriens* and *Sarcophaga ruficornis*. In addition, two 'unknown species' of blow fly were discovered based on phylogenetic tree for Case 1 and Case 10. Maggot samples for Case 1, which is labelled as 1i and 1ii are 3rd instar of *Hemipyrellia ligurriens* based on morphological analysis. Maggot samples for Case 10, which is labelled as 10ii, are 1st instar and morphological identification cannot be done.

When sequence alignment was done using ClustalW from BioEdit Version 7.0.9, the arrangement of DNA nucleotide sequences for maggot samples 1i, 1ii, 10ii and 26 was identical and this shows that these maggots are in fact the same species that is

currently ‘unknown species’. Each of the blow fly species were clearly separated from *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya nigripes*, *Hemipyrellia ligurriens* and *Sarcophaga ruficornis* and 5 isolates were clustered together. *Chrysomya pinguis*, *Chrysomya villeneuvi*, *Lucilia cuprina*, *Synthesiomyia nudiseta*, *Hermatia illucens* and *Megaselia scalaris* were used as outgroup of the phylogenetic tree. All of the *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya nigripes*, *Hemipyrellia ligurriens* and *Sarcophaga ruficornis* isolates formed 5 clusters with branches indicating minor nucleotide variations between the congeneric species.

Figure 4.9 shows a phylogenetic tree involving the sequencing of approximately 1324-base pairs of the mitochondrial cytochrome oxidase subunit II (COII) gene for 11 specimens, representing 50 crime scene investigations in Malaysia. Phylogenetic analyses confirmed the presence of 4 different blow fly species namely *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya nigripes* and *Sarcophaga ruficornis*. Each of the blow fly species was clearly separated from *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya nigripes* and *Sarcophaga ruficornis* and 4 isolates were clustered together.

Chrysomya pinguis, *Chrysomya bezziana*, *Hemipyrellia ligurriens*, *Synthesiomyia nudiseta*, *Lucilia cuprina*, *Musca domestica* and *Calliphoran* sp. were used as outgroup of the phylogenetic tree. All of the *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya nigripes* and *Sarcophaga ruficornis* isolates formed 4 clusters with branches indicating minor nucleotide variations between the congeneric species.

Figure 4.10 shows a phylogenetic tree involving the sequencing of entire region (approximately 1380-base pairs) of complete cytochrome oxidase subunit I (COI) gene

for 22 specimens, representing 50 crime scene cases in Malaysia. Phylogenetic analyses confirmed the presence of 3 different blow fly species namely *Chrysomya megacephala*, *Chrysomya rufifacies* and *Chrysomya nigripes*.

Each of the blow fly species was clearly separated from *Chrysomya megacephala*, *Chrysomya rufifacies* and *Chrysomya nigripes* and 3 isolates were clustered together. *Chrysomya pinguis*, *Chrysomya bezziana*, *Chrysomya villeneuvi*, *Synthesiomyia nudiseta*, *Lucilia cuprina, Ophyra spinigera, Sarcophaga ruficornis, Hermatia illucens, Hemipyrellia ligurriens and Calliphora vicina* were used as outgroup of the phylogenetic tree. All of the *Chrysomya megacephala*, *Chrysomya rufifacies* and *Chrysomya nigripes* isolates formed 3 clusters with branches indicating minor nucleotide variations between the congeneric species.

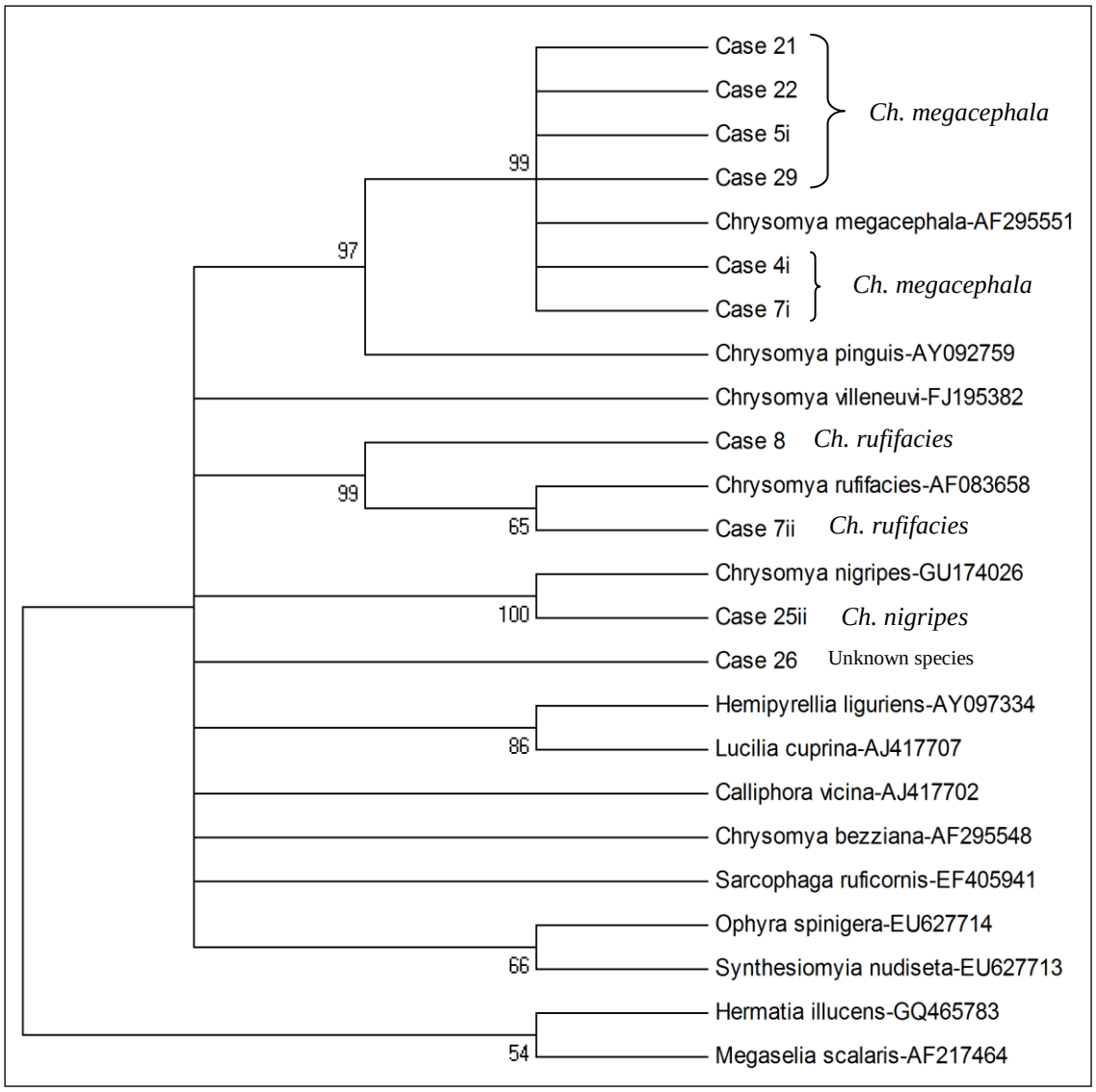

Figure 4.7: Neighbour-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships among blow flies recovered from crime scene investigation, based on 2.3 kilo base pairs of COI, COII and t-RNA nucleotide sequences data with the outgroups. Numbers on branches indicate percentage of bootstrap support.

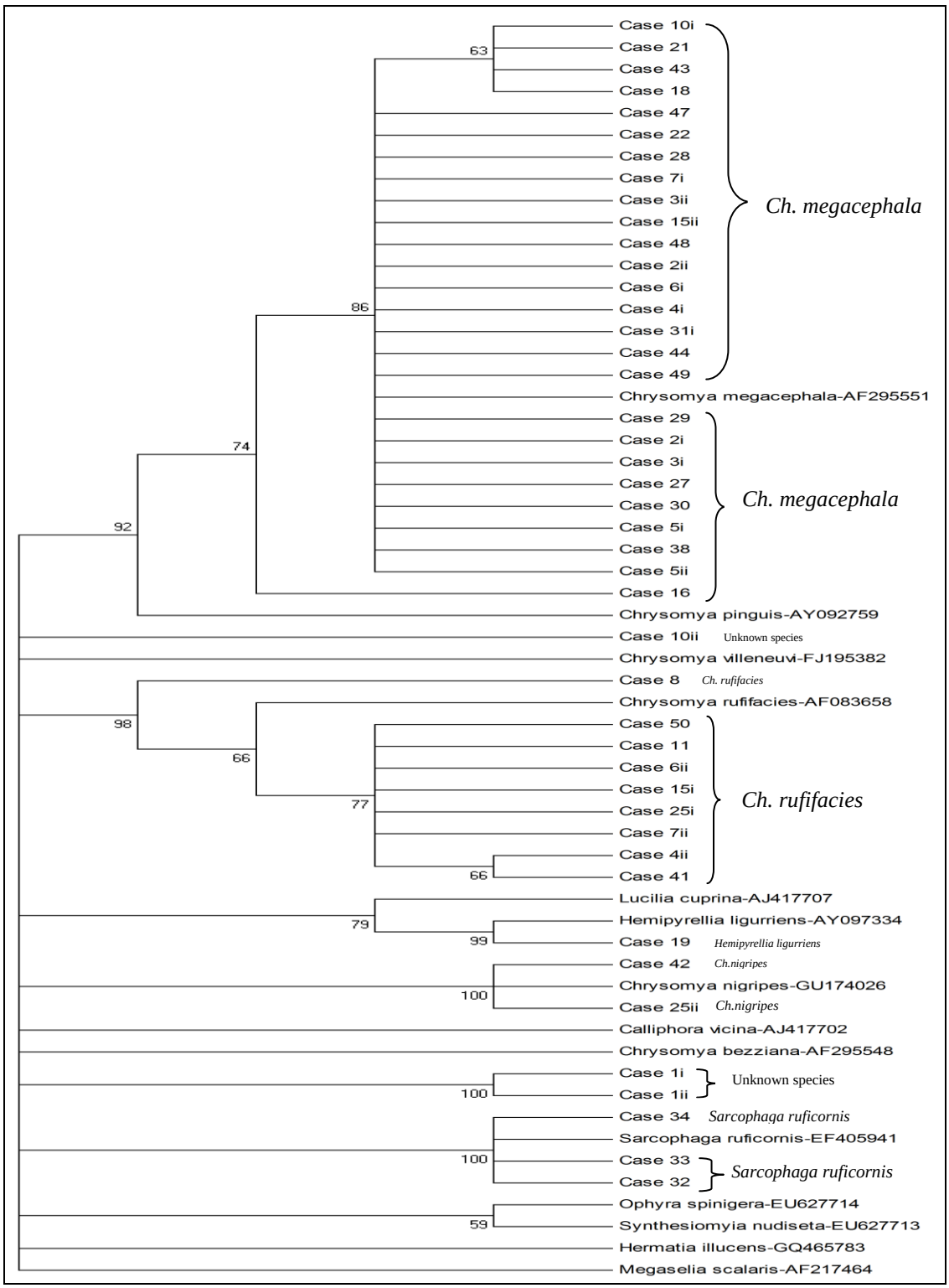

Figure 4.8: Neighbour-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships among blow flies recovered from crime scene investigation, based on 348-base pairs of partial COI nucleotide sequences data with the outgroups. Numbers on branches indicate percentage of bootstrap support.

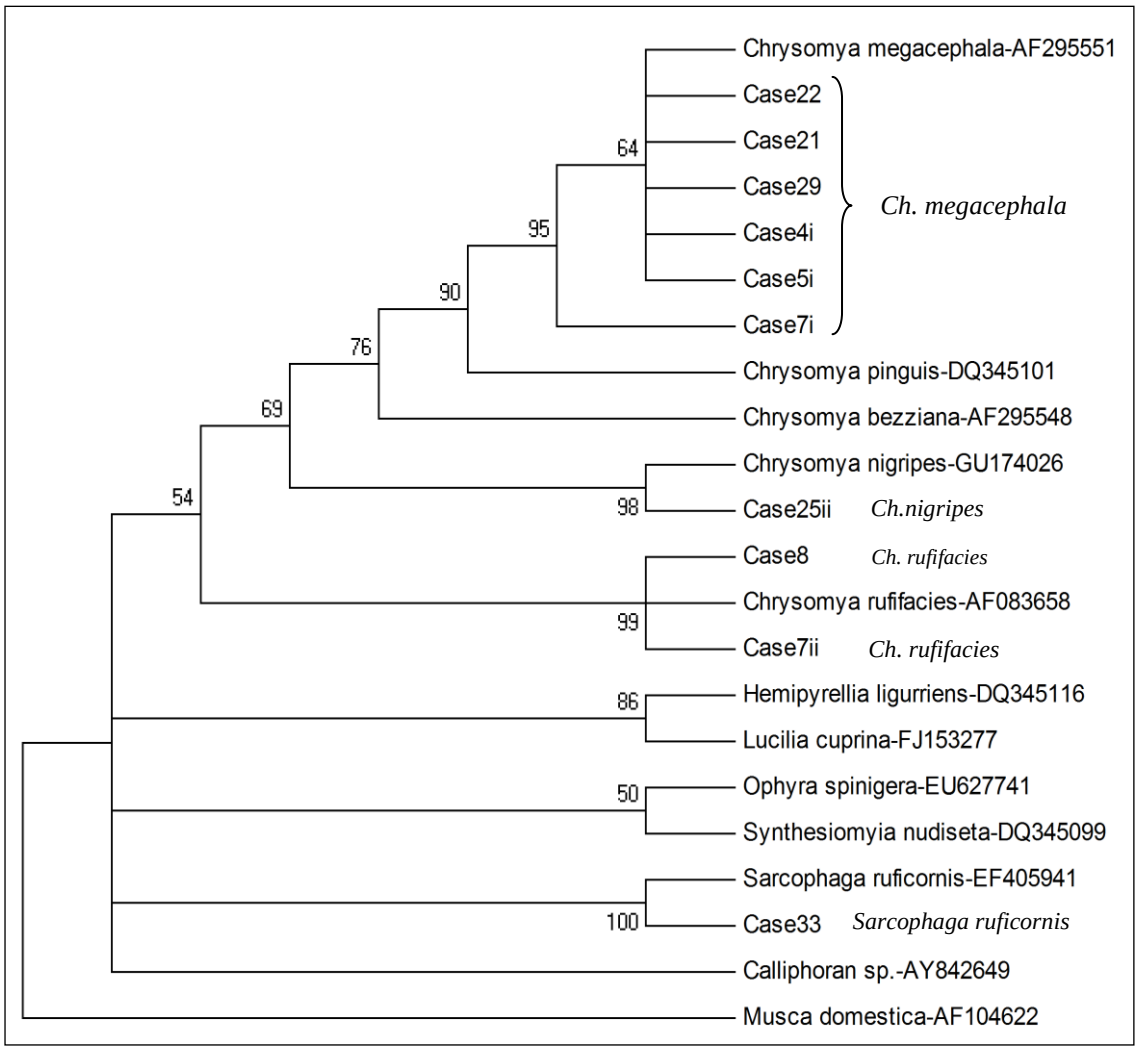

Figure 4.9: Neighbour-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships among blow flies recovered from crime scene investigation, based on 1324-base pairs of COII nucleotide sequences data with the outgroups. Numbers on branches indicate percentage of bootstrap support.

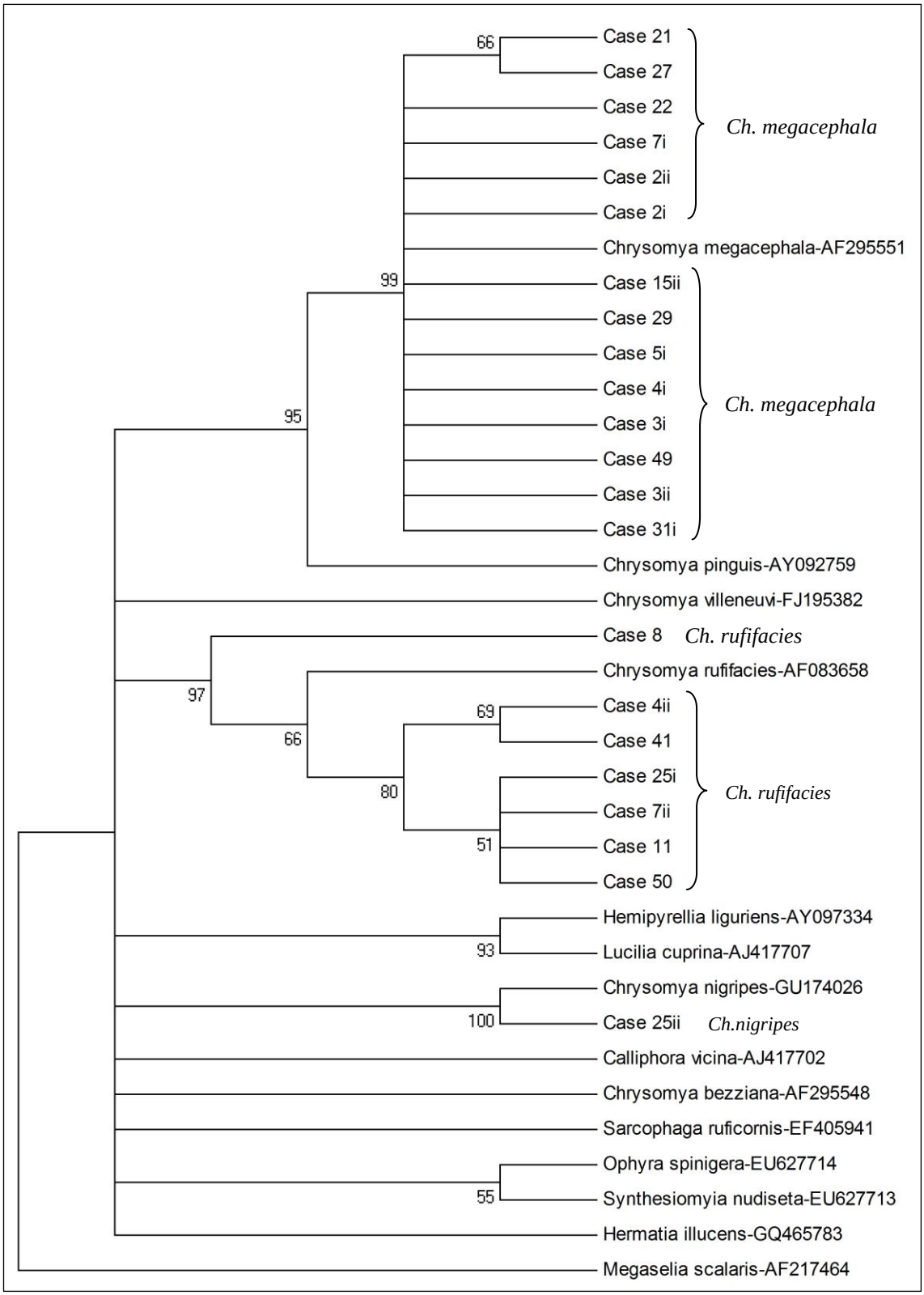

Figure 4.10: Neighbour-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships among blow flies recovered from crime scene investigation, based on 1380-base pairs of complete COI nucleotide sequences data with the outgroups. Numbers on branches indicate percentage of bootstrap support.

4.3.5 Comparison between Morphology and Molecular Analysis

Based on the maggot samples that were obtained from 50 different crime cases, identification using morphological and molecular methods was done. As for the purposes of species identification through morphology and molecular methods, 62 samples of maggots were processed. The maggot samples were processed using molecular method by PCR amplification of cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes of mitochondrial DNA. Amplification results for analysis on mitochondrial DNA for COI (partial and complete) and COII genes are exhibited under the Appendix E.

The results of the comparison between morphological and molecular species identifications are shown in Table 4.23. Based on Table 4.23, the results of species identification using morphological and DNA molecular identifications processes showed the same results for 34 samples (2i, 2ii, 3i, 3ii, 4i, 4ii, 5i, 5ii, 6ii, 7i, 7ii, 8, 15i, 15ii, 18, 19, 22, 25i, 25ii, 29, 30, 31i, 32, 33, 34, 38, 41, 42, 43, 44, 47, 48, 49 and 50). Entomological evidence for 3 samples (16, 27, 28) were in the form of immature larvae stages. Hence the method of species identification using morphological method will be difficult. In this case, species identification using molecular species identification method was more helpful.

However, negative results can also occur with DNA-based method due to PCR failure like for 17 samples namely 9, 12, 13, 14, 17i, 17ii, 20, 23, 24, 31ii, 35, 36, 37, 39, 40, 45 and 46. The reason why the process using the molecular method failed may be due to DNA degradation or failure of the polymerase chain reaction (PCR) process (Mazzanti *et al*., 2010). Dehydration and exposure to air and light can also potentially affect DNA during long-term storage leading to diverse types of damage (Zimmermann *et al*., 2008).

Therefore, it can be stated that the blow flies species, can be identified using either morphological or molecular methods, each having its own merits and demerits. The species identification proses using morphological and molecular methods have to be done simultaneously for crime scene samples to determine the species of blow flies more accurately. As has been discovered, the determination of the blow flies species found in a crime scene is important to estimate the time of death more accurately. However, for the other 4 samples (6i, 10i, 11 and 21), the results of species identification using moprhological and molecular processes were different.

The differences in results of species identification using morphological and molecular method are as follows:

i) Case 6 is a double blow fly infestation. Early examination under the microscope showed the presence of 2 species of blow flies that are different. When it was processed using morphological method, the result of examination showed one species only, that was 3rd instar of *Chrysomya villeneuvi*. However the result of molecular process showed the presence of 2 types of blow fly species that are *Chrysomya megacephala* and *Chrysomya rufifacies.*

ii) Case 10 is a double blow fly infestation. Early examination under the microscope showed the presence of 2 different species of blow flies. However, after being processed for morphological identification, the result showed the presence of 3rd instar of *Chysomya villeneuvi* and another sample which is 1st instar larvae cannot be identified. Meanwhile, the molecular analysis showed the presence of *Chrysomya megacephala* and the 1st instar larvae showed unknown species based on phylogenetic tree.

iii) Case 11 is a single fly infestation. Examination by morphological features showed the presence of 3rd instar *Chrysomya megacephala*. The result of molecular analysis showed *Chrysomya rufifacies*.

iv) Case 21 is a single fly infestation. Examination using morphological features showed the presence of *Chrysomya pinguis* besides that result of examination using molecular analysis showed the presence of *Chrysomya megacephala*.

Based on the phylogenetic trees, maggots samples 1i, 1ii, 10ii and 26 were clustered together in one separate branch in the phylogenetic trees. Maggot samples that were obtained from the crime scenes showed the existence of new species of blow fly or the nucleotide sequences that have been obtained has not appeared in the GenBank system. When DNA sequences alignment was done using ClustalW from BioEdit Version 7.0.9. it had been found out that the nucleotide sequences from all the four samples of maggots were the same. This showed that the same species of blow fly present for all the crime scene cases stated above.

4.3 DNA BARCODING

Since both *Chrysomya megacephala* and *Chrysomya rufifacies* are the most predominant blow fly associated with dead human bodies, the DNA barcoding for these two blow flies were done. Mainly this was done to determine whether the sample of different life cycle stage of blow fly like eggs, 1st instar, 2nd instar, 3rd instar, pupae, empty puparium and adult can be used for molecular species identification.

Samples that have been obtained from each life cycle stage of blow fly specimen were preserved using two different methods, where by one set of specimen were preserved in 70% ethanol and another set of specimen were preserved without any preservative solution. This was done to compare the quality of DNA extracted from specimen preserved in 70% ethanol and without any preservative solution prior the PCR amplification process.

Basically each life cycle stage of these two blow flies species like the egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium and adult blow fly from one life cycle stage were collected and processed and the DNA sequences were deposited in the GenBank as a source of reference. Phylogenetic analyses were also done to find out the validity of the species identification using the DNA molecular method.

The mtDNA region sequenced in this study includes the cytochrome oxidase subunit I and II genes (COI and COII) and the t-RNA gene. The results of species identification based on morphological and molecular methods are shown in Table 4.24 to Table 4.25.

Table 4.24: Identification of various life cycle stages of *Chrysomya megacephala*

Blow fly life stage's	Specimen preserved in 70% ethanol		Specimen preserved without 70% ethanol	
	Morphological Identification	**Molecular Identification**	**Morphological Identification**	**Molecular Identification**
Egg	Cannot be done	*Chrysomya megacephala*	Cannot be done	*Chrysomya megacephala*
1st instar	Cannot be done	*Chrysomya megacephala*	Cannot be done	*Chrysomya megacephala*
2nd instar	Cannot be done	*Chrysomya megacephala*	Cannot be done	*Chrysomya megacephala*
3rd instar	*Chrysomya megacephala*	*Chrysomya megacephala*	*Chrysomya megacephala*	*Chrysomya megacephala*
Pupa	Cannot be done	*Chrysomya megacephala*	Cannot be done	*Chrysomya megacephala*
Empty pupa	Cannot be done	*Chrysomya megacephala*	Cannot be done	*Chrysomya megacephala*
Adult	*Chrysomya megacephala*	*Chrysomya megacephala*	*Chrysomya megacephala*	*Chrysomya megacephala*

(Note: Failed= Amplification process failed)

Table 4.25: Identification of various life cycle stages of *Chrysomya rufifacies*

Blow fly life stage's	Specimen preserved in 70% ethanol		Specimen preserved without 70% ethanol	
	Morphological Identification	**Molecular Identification**	**Morphological Identification**	**Molecular Identification**
Egg	Cannot be done	*Chrysomya rufifacies*	Cannot be done	*Chrysomya rufifacies*
1st instar	Cannot be done	*Chrysomya rufifacies*	Cannot be done	*Chrysomya rufifacies*
2nd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	*Chrysomya rufifacies*	*Chrysomya rufifacies*
3rd instar	*Chrysomya rufifacies*	*Chrysomya rufifacies*	*Chrysomya rufifacies*	*Chrysomya rufifacies*
Pupa	Cannot be done	*Chrysomya rufifacies*	Cannot be done	*Chrysomya rufifacies*
Empty puparium	Cannot be done	*Chrysomya rufifacies*	Cannot be done	Failed
Adult	*Chrysomya rufifacies*	*Chrysomya rufifacies*	*Chrysomya rufifacies*	*Chrysomya rufifacies*

(Note: Failed= Amplification process failed)

The results obtained for *Chrysomya megacephala* sample's which was preserved in 70% ethanol is shown in Table 4.26. *Chrysomya megacephala* which was preserved in 70% ethanol gives better PCR amplification in comparison to *Chrysomya megacephala* which was kept without any preservative solutions. Based on Table 4.26, only partial COI gene has been successfully amplified for the all samples of each life cycle stage of blow fly that is egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium and adult blowflies. As for COII gene, only samples 1st instar, pupa, empty puparium and adult of *Chrysomya megacephala* have been successfully amplified. Furthermore only the eggs sample of *Chrysomya megacephala* has not been successful in being amplified for complete COI gene.

The PCR amplification results that have been obtained for *Chrysomya megacephala* sample's which was not preserved in 70% ethanol are not satisfactory. Only partial COI gene of *Chrysomya megacephala* samples which had been preserved in 70% ethanol and without 70% ethanol have been successfully amplified for all the samples from each life cycle stage. But for COII and complete COI genes only samples of pupa, empty puparium and adult of *Chrysomya megacephala* had been succefully amplified. In conclusion, samples of *Chrysomya megacephala* which had been preserved in 70% ethanol resuting in better PCR amplification in comparison to samples of *Chrysomya megacephala* which had not been preserved in 70% ethanol.

Table 4.26: Amplification of various life cycle stages of *Chrysomya megacephala*

Blow fly life stage's	Preserved in 70% ethanol			Preserved without 70% ethanol		
	mtDNA gene			mtDNA gene		
	Partial COI	COII	Complete COI	Partial COI	COII	Complete COI
Egg	√	-	-	√	-	-
1st instar	√	√	√	√	-	-
2nd instar	√	-	√	√	-	-
3rd instar	√	-	√	√	-	-
Pupa	√	√	√	√	√	√
Empty pupa	√	√	√	√	√	√
Adult	√	√	√	√	√	√

Note: √ = done; - = unable to be amplified by PCR

Also, similar results had been obtained for samples of *Chrysomya rufifacies* which was perserved in 70% ethanol. The result obtained is shown in Table 4.27. Partial COI gene had been successfully amplified for samples of each life cycle stage of *Chrysomya rufifacies*. Besides that for COII gene, only the samples of 2nd instar and adult stages of *Chrysomya rufifacies* had been succesfully amplified. Complete COI gene also had been succesfully amplified for samples of each life cycle stage except for 1st instar stage of *Chrysomya rufifacies*.

In comparison, none of the samples of *Chrysomya rufifacies* which had been kept without any preservative give rise to successfully PCR amplification for COII gene. As for partial COI gene, samples of each life cycle stage that had been successfully amplified except for samples of empty puparium of *Chrysomya rufifacies*. Furthermore for complete COI gene, only egg, 2nd instar and adult samples of *Chrysomya rufifacies* had been successfully amplified. In conclusion, samples of

Chrysomya rufifacies which had been preserved showed better DNA results compared to samples of *Chrysomya rufifacies* which had not been preserved.

Table 4.27: Amplification of various life cycle stages of *Chrysomya rufifacies*

Blow fly life stage's	**Preserved in 70% ethanol**			**Preserved without 70% ethanol**		
	mtDNA gene			**mtDNA gene**		
	Partial COI	**COII**	**Complete COI**	**Partial COI**	**COII**	**Complete COI**
Egg	√	-	√	√	-	√
1st instar	√	-	-	√	-	-
2nd instar	√	√	√	√	-	√
3rd instar	√	-	√	√	-	-
Pupa	√	-	√	√	-	-
Empty pupa	√	-	√	-	-	-
Adult	√	√	√	√	-	√

Note: √ = done; - = unable to be amplified by PCR

After the DNA sequences had been obtained, the phylogenetic trees were constructed to determine the species of the maggots samples based on the DNA sequences. The phylogenetic trees represent the mitochondrial genetic structure of *Chrysomya megacephala* and *Chrysomya rufifacies* based upon COI (partial and complete) and COII genes sequences as shown in Figure 4.11 and Figure 4.12. Therefore two phylogenetic trees were constructed for *Chrysomya megacephala* which had been preserved in and without 70% ethanol and for *Chrysomya rufifacies* which had been preserved in and without 70% ethanol as seen in Figure 4.11 and Figure 4.12. Neighbour-joining results showed that the maggot samples from same blow fly species formed one clade. In general, members of the same species were closer to each other than to members of other subfamilies.

Furthermore based on the phylogenetic trees, species of the maggot samples were identified. The complete sequences of COI and COII genes for *Chrysomya megacephala* and *Chrysomya rufifacies* have been deposited in GenBank under accession numbers of JN 571566 and JN 571567.

4.4.1 Phylogenetic Analysis

Upon successful PCR amplification, the obtained PCR products were sequenced and sequence alignment was performed using ClustalW from BioEdit Version 7.0.9. Results showed that the sequence of DNA obtained for each sample for every life cycle stage was identical for a particular species.

Then phylogenetic trees were constructed based on COI (partial and complete) and COII nucleotide sequences. The reference sequence were retrieved from the previous reported blow fly in Malaysia according to Lee *et al.* (2004) namely *Calliphora vicina* AJ417702, *Chrysomya bezziana* AF295548, *Chrysomya megacephala* AF295551, *Chrysomya nigripes* GU174026, *Chrysomya pinguis* AY092759, *Chrysomya rufifacies* AF083658, *Chrysomya villeneuvi* FJ195382, *Hemipyrellia liguriens* AY097334, *Hermatia illucens* GQ465783, *Lucilia cuprina* AJ417707, *Megaselia scalaris* AF217464, *Ophyra spinigera* EU627714, *Sarcophaga ruficornis* EF405941, *Synthesiomyia nudiseta* EU627713 were added as an outgroups.

Meanwhile the phylogenetic trees were constructed by neighbour-joining (NJ) method implemented in the MEGA 4 and the trees were tested by 1000 bootstrap replicates as shown in Figure 4.11 and Figure 4.12. Figure 4.11 shows the phylogenetic tree for *Chrysomya megacephala* samples preserved in 70% ethanol and without 70% ethanol. It involves the sequencing of a total length around 2300-base pairs encompassing the 'barcode' fragments of the mitochondrial cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes only for 1st instar, pupae, empty puparium and adult blow fly of *Chrysomya megacephala*. Besides that, the phylogenetic tree also included the sequencing of a 348-base pairs 'barcode' fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene for eggs, 2nd instar and 3rd instar of

Chrysomya megacephala together with the sequences of approximately 1380-base pairs for complete COI gene for 2nd instar and 3rd instar of *Chrysomya megacephala*.

Figure 4.12 shows a phylogenetic tree for *Chrysomya rufifacies* preserved in 70% ethanol and without 70% ethanol. The tree involved the sequencing of a total length around 2300-base pairs encompassing the 'barcode' fragments of the mitochondrial cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes only for 2nd instar and adult blow fly of *Chrysomya rufifacies* and sequencing of a 348-base pairs 'barcode' fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene for eggs, 1st instar, 3rd instar, pupae and empty puparium of blow fly *Chrysomya rufifacies.*

The phylogenetic tree also includes the sequencing of approximately 1380-base pairs of the complete COI gene for 1st instar, 3rd instar, pupae and empty puparium of blow fly *Chrysomya rufifacies*. All the samples collected from each life cycle stage of blow fly either *Chrysomya megacephala* or *Chrysomya rufifacies* form a single cluster with branches indicating minor nucleotide variations between the congeneric species.

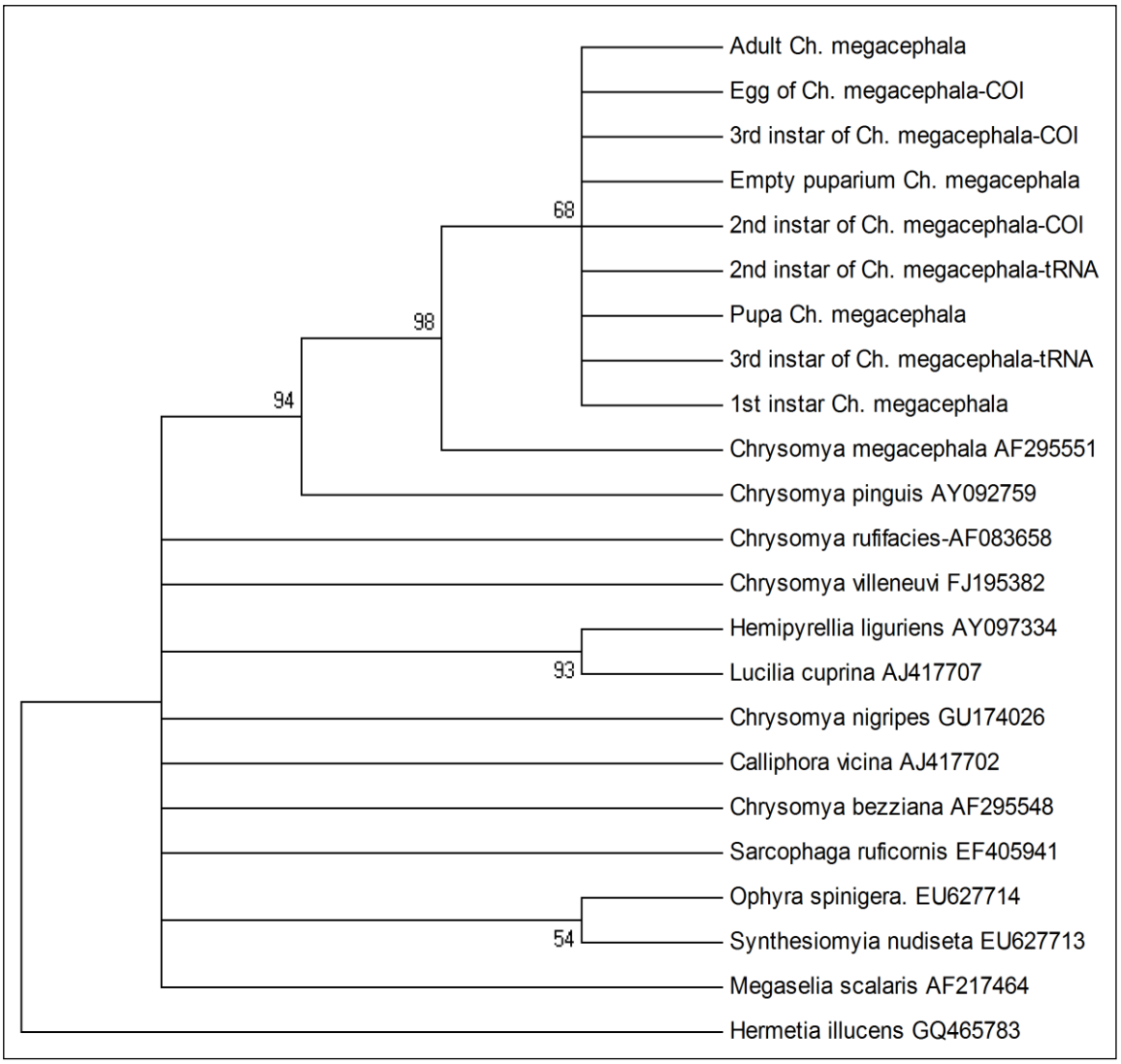

Figure 4.11: Neighbour-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships for *Chrysomya megacephala* preserved in 70% ethanol and without 70% ethanol, based on complete cytochrome oxidase nucleotide sequences (2300 base pairs) with the outgroups. Numbers on branches indicate percentage of bootstrap support.

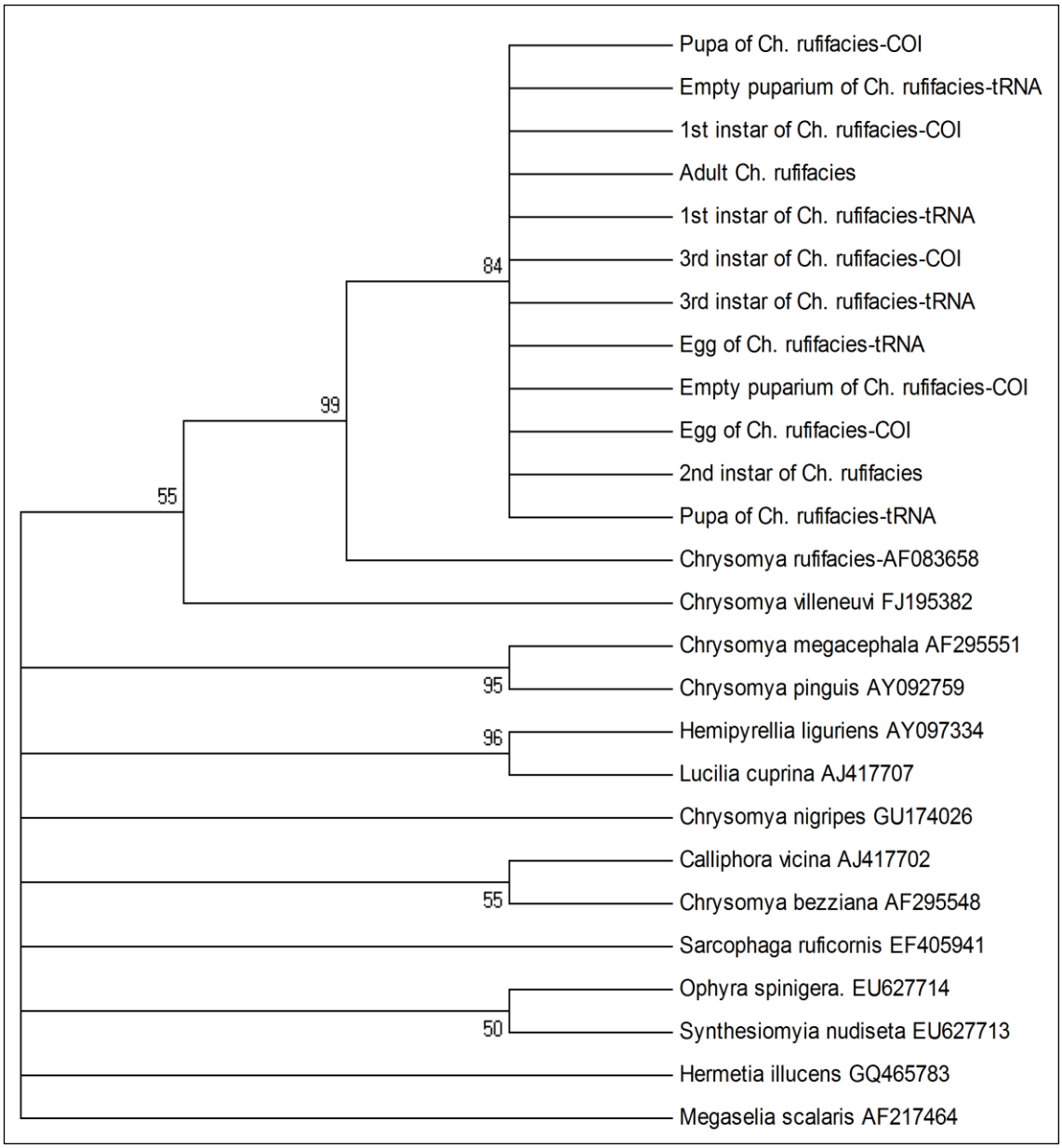

Figure 4.12: Neighbour-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships for *Chrysomya rufifacies* preserved in 70% ethanol and without 70% ethanol, based on complete cytochrome oxidase nucleotide sequences (2300 base pairs) with the outgroups. Numbers on branches indicate percentage of bootstrap support.

CHAPTER 5
DISCUSSION

5.0 DISCUSSION

5.1 ASSESSMENT OF FORENSIC ENTOMOLOGY AWARENESS IN MALAYSIA

The conducted study was the first attempt which was done in Malaysia to study the awareness and knowledge of Malaysian on forensic entomology. Half of the respondents had a good degree of understanding that forensic entomology is a study about insects found on dead human bodies. Apart from that, the respondents also acquired better understanding of the field of forensic entomology because they had gained professional knowledge from answering the questionnaire. Most of the respondents understood about forensic entomology because of the nature of their profession like pathologists, scientific officers and students.

Another impotant focus of the survey was to emphasize on the application of forensic entomology in crime scene investigations. Results showed that the respondents were aware that maggots found on a dead human body originated from flies and can be used to determine the PMI. This awareness was largely contributed by professional knowledge and mass media. It was further found that only a few experienced crime scene police officers with knowledge of forensic entomology were involved in maggot collection during crime scene investigation. A majority of maggots were collected by pathologist during post-mortem examination. The present study identified an important pitfall in the application of forensic entomology in crime scene investigation in Malaysia. In fact, the crime scene police officers should be the first person who should collect the maggots. According to Amendt *et al*. (2007), the first and most important

stage of the procedure in forensic entomology involves careful and accurate collection of insect evidence at the scene. Moreover, some insect evidence may be left out at the scene during transportation of the dead human body. Only the crime scene police officer will be able to provide data on general habitat, ambient weather conditions, location of the dead body as well as the microhabitat that is surrounding the dead body.

Furthermore, a high percentage of respondents were aware that the maggots found on a dead human body can be used to determine the cause of death of a person, the surrounding area of the crime or the positions of the wounds in the body. This indicates a bright future for the application of forensic entomology particularly in crime scene investigation in Malaysia. More professional training should be provided particularly to the crime scene police officers so that more entomological insect evidence data could be collected from the crime scene. This is mainly because the practical value of the entomological evidence is very much dependent on the accurate collection by the crime scene police officers (Amendt *et al.*, 2007).

Based on the survey, it was generally found that dead bodies infested with maggots were encountered, but there was a general lack of collection of maggot taking place at the crime scene. This could be due to more emphasis given to other physical evidence, hence entolomogical evidence was largely ignored during crime scene investigation. In fact, insect specimens, such as blowfly larvae or adults, must be considered as physical evidence just as blood stains, fingerprints, hairs, fibers or any other biological materials (Lord & Burger, 1983). Therefore, insects should be processed as evidence at the crime scene examination as well as at the autopsy (Haskell *et al*., 2001).

Due to the exposure of popular television drama series of crime scene investigation (CSI), the respondents knew that the maggots found on a dead human body can assist in crime scene investigations in other countries which apply forensic entomology to assist in murder cases. However, the application of forensic entomology in Malaysia has been hindered by general lack of information on the biology of the forensically important fly which can be found in Malaysian fauna. Moreover forensic entomology has not been established as a profession. Rarely will they be invited for appearance in the court.

Based on the responses, the questionnaire survey has succeeded in deepening the understanding of forensic entomology particularly among the crime scene police officers. It was further noted that a large number of respondents particularly crime scene police officers were being introduced to forensic entomology through the participation in this survey. This shows that there was a serious lack of emphasis on forensic entomology among the crime scene police officers. Hence forensic entomology should be included in the curriculum of training to become a crime scene police officer followed by professional training on collection of maggots.

In order to determine the future prospect of forensic entomology in Malaysia, the knowledge of scientific officers and university students who were actively involved in the field were evaluated. Although a majority of the respondents knew there were more than one species of forensically important fly, however they generally lacked expertise in identifying them. Very often the few experienced entomologists will be consulted for the speciation of the fly. It was also found that conventional morphological method was the choice of method for species identification. Molecular method of speciation is rather new and seldom employed due to the general lack of expertise.

The present study revealed that forensic entomology research in Malaysia was mainly to determine the PMI, identify the types of blow fly species and to study the insect succession. Active and continued research is essential to provide current data particularly on the biology of the forensically important blow flies as well as generating more experts in forensic entomology.

The present study also proved that the motivation to get to know more about forensic entomology was the main reason for the respondents to choose forensic entomology as their research field. Generally the younger generation has the enthusiasm and interest to pursue forensic entomology as their field of research. The survey showed that respondents also indicated that the Ministry of Education in our country has to play an important role to encourage more students to do research on forensic entomology. It was generally believed by the respondents that forensic entomology will continue to move forward and develop fully into a discipline by itself.

In order to improve the status of forensic entomology in Malaysia, more research related to forensic entomology and its application in the crime scene investigation has to be put forward. Publicity to introduce forensic entomology to other students and participation in seminars hold both in Malaysia and overseas needs to be encouraged. On the other hand, forensic entomology in our country can create more career opportunities. Police department will be the one that needs the services of the forensic entomology researchers more followed by institutions of higher learning or research centres and hospital departments.

5.1.1 Actions

According to the model of study of forensic entomology, students need to be taught so that they will have basic knowledge and awareness of why they should know

about forensic entomology. This will create a new generation of scientists with forensic entomology knowledge. Besides that the police department should emphasize more on forensic entomology in their crime investigations and the Forensic Laboratory of Royal Malaysia Police should expose the crime scene police officers to forensic entomology and its importance besides training them on how to collect entomological evidence during crime scene investigations.

5.1.2 Limitation of Methodology

While gathering data to analyze the results, some limitations of the methods used in this part were found. The weaknesses of the thesis method are described below:

- Questionnaire is the only way to collect data for this thesis. There could be some bias from incomplete responses.

- A few responses were incomplete in the questionnaire. Some of those who participated in the survey did not answer all the questions required and did not give any examples of what they knew.

- Validity of the answers from respondents has to be considered and analyzed carefully, because it is out of the control of the researcher.

- Sample size of respondents from each study group was not averaged.

- Due to limited number of trained pathologists in Malaysia, an equal number of respondents in each group was not obtained.

5.1.3 Further Study

- For the research, use of questionnaire is not the only way to investigate. Interviews and observations could be considered to help the research.

- Use of social media namely Face Book to cover more people for interviews and increase of sample size.

5.2 COMPARISON BETWEEN MORPHOLOGICAL AND MOLECULAR METHODS IN BLOW FLY SPECIES IDENTIFICATION

The correct determination of blow fly species that are found in dead human body is important to determine accurate post-mortem interval (PMI). The determination of wrong blow fly species will cause an inaccurate determination of the post-mortem interval (PMI). To ensure the optimum usage of entomological evidence in crime scene investigations, the determination of the PMI should be done as accurate as possible.

The present study showed that the predominant fly species were those of *Chrysomya* species. *Chrysomya megacephala* was the commonest fly seen followed by *Chrysomya rufifacies*. The Calliphoridae family was represented by *Chrysomya megacephala*, *Chrysomya rufifacies*, *Chrysomya pinguis*, *Chrysomya villeneuvi* and *Chrysomya nigripes*. The predominance of both *Chrysomya megacephala* and *Chrysomya rufifacies* in dead human bodies was in aggreement with previous reports in Malaysia by Lee *et al*. (1984; 2004), Lee (1989) and Hamid *et al*. (2003).

It is generally believed that Chrysomyinae flies can act as primary species in the absence of the Calliphorinae (Coe, 1978; Braack, 1981). However, O'Flynn & Moorhouse (1979), Bharti & Singh (2003) reported that members of the subfamily Chrysomyinae can act as primary flies even when Calliphorinae is also available. In accordance with previous reports (Tumrasvin *et al*., 1976; Sukontason *et al*., 2001, 2003), *Chrysomya megacephala* was the most common blow fly species and more easily captured than other species, throughout Thailand. *Chrysomya megacephala* is a primary invader of human corpses in Malaysia (Cheong *et al*., 1973).

The adults are large, thick and of a dark green or blue coloration, Goff *et al*. (1988) and Goff (1998) used these species as forensic indicators in four cases. *Chrysomya megacephala*, the Oriental latrine fly is widely distributed in the Oriental and Australian regions. Adults are common around human habitations (Zumpt, 1965) and prefer warmer conditions (Das *et al*., 1978).

Based on the present study, *Chrysomya megacephala* and *Chrysomya rufifacies* was present a year round in Malaysia. *Chrysomya rufifacies* is also widely distributed throughout the Australian and Oriental regions. It is more adapted to tropical conditions and is found throughout the year (Greenberg & Polvolny, 1971). These species has been used as a forensic indicator by many scientists (Goff & Odum, 1987; Singh & Bharti, 2000). Large numbers of female Calliphoridae were attracted to the remains within minutes after cadaver placement, with oviposition beginning shortly thereafter. This observation is in line with other carrion studies (Payne, 1965; Anderson & VanLaerhoven, 1996).

Their ability to survive and compete successfully in the carrion environment accounted for the predominance of *Chrysomya* species. Other fly maggots were also found, albeit in much lower frequency, probably showing that they may not be as successful as *Chrysomya* larvae in the carrion habitat. The presence of a particular fly species always revealed information on the ecology of the crime scene.

Insect species found on a decomposing body that do not correspond with species normally found in the area can be a good indicator that the body in question was moved from one area to another. Clothes on a cadaver do not delay oviposition, but clothes permeated with lubricants, paint or combustibles may double the initial colonization time and retard decomposition by as much as 50% according to Marchenko (2001).

Conversely, Anderson (2001) stated that clothing that gets soiled with blood, urine or fluids leaking in the course of decomposition, provides more sites for oviposition than a naked corpse, resulting in larger larval masses and hence faster decomposition. Besides, egg patches or first instar larvae are not easily washed away by rain, as might be the case with unclothed cadavers (Anderson & VanLaerhoven, 1996).

The present study confirmed a high percentage (89%) of agreement between morphological and molecular methods in fly species identification. Hence molecular method should be widely used for fly species identification for maggots found in crime scene investigation. The discrepancy in the results obtained for species identification between the morphology and molecular methods may be due to the suboptimal condition of the maggot samples that was collected from the crime scenes which may lead to false identification by morphological method. Besides that the utilization of maggot samples that were used for PCR amplification was not from the slides prepared for morphological identification. So it may not be from the same colony of maggots collected from corpses.

The molecular method was proven more useful particularly in the cases of immature stages such as cases 10ii, 16, 27 and 28. Nevertheless there were 17 samples that were unable to be amplified by PCR. This was mainly due to the poor preservation of the maggot samples which may lead to DNA degradation as previously noticed by (Mazzanti *et al.,* 2010). Moreover since the maggot samples were obtained from the various crime scenes, it could have been exposed to air, light and dehydration which can potentially affect DNA integrity and quality (Zimmermann *et al*., 2008). In addition, phylogenetic analysis was proven useful in discovering unknown species. The finding of unknown species for case number 1i, 1ii, 10ii and 26 from phylogenetic analysis that have been done maybe due to sequences contained in GenBank database that were not

representative of all known insect species or there were no extensive records available for insects spread worldwide and some haplotypes could be confused between closely related genera (Wells & Stevens, 2008). According to Wells *et al*. (2007) close similarity between different genera have been observed in the haplotypes of some groups.

Species identification through morphological and molecular methods, have its own advantages and disadvantages. As for the cost of analysis, morphological process does cost much lesser in comparison with molecular analysis. In the present study, mitochondrial DNA analysis for each case was only done once due to financial constraint. Other than that, the small numbers of maggot samples that were collected from crime scenes made the process of molecular analysis to be conducted only once.

Most important in forensic case work involving entomological evidence is the identification of the insect species collected in association with the corpse or the surroundings. Identification is the foundation of all further insect based estimations. Larval development is dependent on temperature (Bowler & Terblanche, 2008) and every species has a slightly different growth rate (Erzincliogl u, 1990; Davies & Ratcliffe, 1994; Richards & Villet, 2009).

In forensic investigations, all immature stages of flies (egg, larvae and puparium) can serve as entomological evidence at death scene. These insects are primarily used to estimate the PMI, but can also be involved in the analysis of toxic substances, determining the manner of death and in indicating relocation of a corpse in homicide cases (Turchetto & Vanin, 2004; Sukontason *et al*., 2007).

Correct identification of the species to which larvae belongs to is critical, because even closely related species can have different developmental characteristics

(Ash & Greenberg, 1975). One quick and easy way is the morphological identification using appropriate identification keys. However, it may be impossible to identify an insect by means of its morphology, due to damage, and in this case it is necessary to use molecular identification tools.

Although electron microscopy-based identification of some chrysomyine larval stages has been proposed (Sukontason *et al*., 2005), it is not practical and requires many special skills for sample preparation. To ensure correct species identification, established molecular methods were transferred to the forensic field (Sperling *et al*., 1994; Stevens & Wall, 1996, 1997; Wallman & Adams, 1997; Benecke, 1998). So an increasing amount of DNA sequence data has been obtained with the goal of using molecular phylogenetic methods for species identification of larvae (Wells & Stevens, 2008).

A variety of regions of DNA have been suggested for study including the nuclear internal transcribed spacers (ITS) (Ratcliffe *et al*., 2003), mitochondrial rRNA genes and the mitochondrial control region (Stevens & Wall, 1997). The majority of molecular studies, however, have used the cytochrome oxidase I (COI) encoding region of mitochondrial DNA (mtDNA) (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Vincent *et al*., 2000; Wallman & Donnellan, 2001; Harvey *et al*., 2003a; 2003b).

Species identification for this study was obtained by the amplification of COI (partial and complete) and COII genes from mitochondrial DNA independently. The results of the analysis on mitochondrial DNA showed cytochrome oxidase I (COI) gene as the easiest gene to analyze whereby out of 62 samples, cytochrome oxidase I (COI) gene for 45 maggot samples were successfully amplified. As for cytochrome oxidase II (COII) gene, only 11 maggot samples were successfully amplified whereas the

remaining 50 maggots sample were not successfully amplified or analyzed. Furthermore for complete COI gene, 23 maggot samples were successfully analyzed and amplified.

The present study showed that analysis of mitochondrial DNA (mtDNA) particularly cytochrome oxidase I gene (COI) appeared to be a useful tool in species identification among the subfamilies of Calliphoridae as reported in previous studies (Harvey *et al*., 2003a, 2008; Wallman & Donnellan, 2001; Wells & Williams, 2007; Wells *et al*., 2007). The potential of cytochrome oxidase gene (COI) has been shown to be helpful for species determination in many previous studies also (Zehner *et al*., 2004; Saigusa *et al*., 2005). Even though it is known that mitochondrial DNA (mtDNA) can often be extracted and analyzed from very small, degraded or poor sources of DNA that are not suitable for nuclear DNA analysis (Holland & Parsons, 1999), DNA in very old samples degrades more or less strongly over time and often becomes inaccessible for genetic studies (Zimmermann *et al*., 2008).

The reason for several fly maggot specimens that could not be identified was because of poor killing and preservation techniques. For example, maggots were killed by immersion for 10 to 15 seconds in warm water (about 70°C) and storage in 70% ethanol are much preferred and are better for measurement, morphological study and for avoiding discolouration (Hall, 2000). Placing them alive directly into a solution of alcohol or ethanol will kill and preserve them but as they die they will shrink and so appear younger that they actually are (Hall, 2000). Insects are important in carcass decomposition and calliphorids, which are among the most abundant and the best studied carrion insects, have been extensively used as indicators of the postmortem interval (PMI) and of corpse's translocation. These flies are therefore a valuable tool for forensic medicine (Smith, 1986; Catts & Haskell, 1990).

Wells & Williams (2007), Wells *et al*. (2007) and Wells & Stevens (2008), have emphasized the importance of using a broad enough genetic database of all relevant species as an essential key for accurate species identification by phylogenetic analysis of COI sequence. To distinguish sister species more effectively, phylogenetic analysis has been widely employed (Stevens *et al*., 2002; Wells & Williams, 2007; Wells & Stevens, 2008). However, the current study successfully demonstrated not only the application of mitochondrial cytochrome oxidase genes for species identification, but also provided phylogenetic information for the common forensically important blow flies from several geographical areas of Malaysia.

5.3 DNA BARCODING

Chrysomya megacephala and *Chrysomya rufifacies* are forensically important blow fly species in many parts of the world. Larvae of both species have been reported in association with human corpses in several case situations (Smith, 1986; Gunatilake & Goff, 1989; Goff & Flynn, 1991; Lord, 1991; Barreto *et al*., 2002; Lee *et al*., 2004; Sukontason *et al*., 2007).

Other than that, literature review also shows that these two blow fly species are the most predominant blow fly associated with dead human bodies not only in Malaysia but also in other countries such as in in Africa, Asia, Australia and South America (Prins, 1982; Pont, 1985; Wells, 1991; Wells & Kurahashi, 1994; von Zuben *et al*., 2001). Not only have their specimens been used to estimate the post-mortem interval (PMI) in cases (Goff & Odom, 1987; Goff *et al*., 1988; Goff, 2000) but also to detect organophosphate poisoning in a putrefying bodies through larval analysis (Gunatilake & Goff, 1989; Goff & Flynn, 1991; Byrd & Castner, 2001).

DNA barcoding is an identification approach that uses short DNA sequences from a standardized region of the genome as a molecular diagnostic tool in species identification (Luo *et al*., 2011). An ideal DNA barcode should allow fast, reliable, automatable, and cost-effective species identification by users with little or no taxonomic experience (Hebert *et al*., 2003b; Hajibabaei *et al*., 2005; Hebert & Gregory, 2005). DNA barcode-based identification is quite effective at discriminating a limited set of species, such as species occurring in a small area, agricultural pest species and invasive species (Meier, 2008; Kress *et al*., 2009). For a forensic entomologist, identifying an insect specimen is typically an important early step in an investigation (Smith, 1986; Amendt *et al*., 2007). Traditional morphological keys are unavailable or difficult to use for many immature stages of these insects or even adult specimens such as some female Calliphoridae (Smith, 1986). Therefore, many authors have proposed a DNA-based method for forensic insect identification (Sperling *et al*., 1994; Malgorn & Coquoz, 1999; Vincent *et al*., 2000; Stevens & Wall, 2001; Wallman & Donnellan, 2001; Harvey *et al*., 2003a; Zehner *et al*., 2004). mtDNA sequences are typically easier to achieve due to the high copy number of mtDNA and protection by the organelle (Holland & Parsons, 1999).

Besides that mtDNA offers several advantages over nuclear DNA, the latter undergoes relatively slow mutation rates compared with mtDNA, so identification would require a much longer nucleotide sequence than is necessary with mtDNA. This enables mtDNA to provide differences in sequences between closely related species (Waugh, 2007) and therefore be useful for molecular identification purposes.

Identification of blow fly species collected in a corpse is an initial and mandatory step when using blow flies as entomological evidence in forensic investigations. The reason why *Chrysomya megacephala* and *Chrysomya rufifacies*

were chosen for purposes of DNA barcoding is because *Chrysomya megacephala* and *Chrysomya rufifacies* were the most common blow fly species found in cadavers from different ecological habitats. Since the *Chrysomya megacephala* and *Chrysomya rufifacies* are predominant blow fly species which are associated with dead human bodies and always found in crime scene investigations, a preliminary species identification were done using DNA sequences in the term of DNA barcoding for these two blow fly species without counting the larvae stages whether it was in the form of egg, 1st instar, 2nd instar, 3rd instar, pupa, empty puparium or adult stage.

There are a number of identification keys for mature larvae of forensically important blow flies (Smith, 1986; Greenberg & Kunich, 2002). However, morphological identification of blow fly larvae is not easy because only about 2 percent of the species known to science have been described in their immature stages (Smith, 1989).

Based on the present study it was found that the DNA extracted from maggots preserved in 70% ethanol resulted in better yield of DNA compared to maggots stored without preservative particularly if longer fragment of DNA is desired. Furthermore, the duration of time the maggot samples were stored in ethanol did not seem to greatly affect DNA recovery from the corpse as seen in the genotyping results. Ethanol is also recommended as a preservation solution because of its ability to denature nucleases and dehydrate specimens. The same view was given by Dessauer *et al*. (1996) and Marrelli *et al*. (2006). Smith *et al*. (1987) and Dillon *et al*. (1996) also confirmed the fact that ethanol is a useful preservative for DNA.

Ethanol appears to be the preservative of choice (Lord & Rodriguez, 1989; Anderson, 1995; Benecke, 1998; Benecke & Lessig, 2001; Sukontason *et al*., 2001) but the concentrations adopted by workers vary and it appears that hot water, despite being

recommended, is not always used as a killing agent (Hall *et al*., 1986; Anderson, 1995; Tessmer *et al*., 1995; Wells & LaMotte, 1995).

In controlled experiments, workers have killed larvae in hot water and preserved them in ethanol (Byrd & Butler, 1996, 1997, 1998). Apparently the crime scene environment is very different to that of a laboratory and, when an entomologist is unable to attend a crime scene, crime scene personnel must instead collect the entomological evidence. However, such personnel may not preserve larvae as recommended in the literature, either because the required chemicals or dilutions are not available to them, or because suggested techniques, such as killing larvae in hot water, may be impractical or impossible in the field (Day & Wallman, 2008). However, Wells & Kurahashi (1994) removed all larvae and killed them in boiling 70% ethanol. Wolff *et al*. (2001) appear to have simply preserved their samples in 70% ethanol.

Preservation of entomological evidence becomes even more important if the DNA analysis of the maggot found on dead human body is to be attempted because some preservation methods suitable for storing maggots for microscopic examination may not be suitable for keeping DNA intact. Colder temperatures are often used as a preservation strategy as they help to reduce or eliminate bacterial growth and enzymatic activity. Some researcher reported that ethanol solutions may initially reduce the recovery of DNA from maggot, but there will not be further declined at longer storage times. Maggots kept at a cooler environment helped physical preservation as well as DNA preservation (Linville *et al*., 2004).

As with other DNA containing biological evidence, steps should be taken to prevent bacterial growth and the enzymatic degradation of DNA. The success of PCR reactions was determined using 1% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were detected under a UV illuminator and the gel

photos are exhibited under the Appendix D. Some amplification was repeated if amplified DNA was present, but there was failure to obtain a sequence. This failure was associated with a low amount of PCR product indicated by a weak band in the agarose gel.

DNA molecular analyses for all the maggot samples preserved in 70% ethanol and kept at -4°C, were successful. However, for maggot samples kept without any preservative solutions (70% ethanol) at -4°C, induced some loss of DNA, DNA recovery was reduced and amplification of DNA was less successful than in maggots stored in 70% ethanol. The method on how the maggot samples were preserved also influences the results of the DNA molecular analysis. This is because when the DNA molecular analysis results of *Chrysomya megacephala* and *Chrysomya rufifacies* were examined, the maggot samples which had been preserved in 70% ethanol shows a better results compared to maggot samples which had not been preserved in 70% ethanol.

The sequences of DNA which was obtained from this study will be kept as a reference for DNA sequences in the DNA barcoding. As a result, the entomological evidence that has been obtained from the crime scene for the immature stages of the blow fly can be recognized easily and in a shorter period. This saves the time that has been used to rear the immature stages of the blow fly to the 3rd instar or adult stage for the identification process.

Usually, for the process of molecular identification, after the DNA sequences had been obtained, phylogentic tree has to be reconstructed to determine the species of the blow fly. Besides that the DNA sequences that has been obtained has to be aligned with published data of blow fly species from other countries where this process consumes a longer time (Meyer & Paulay, 2005).

When entomological evidence is obtained from crime scene whether in the form of immature stages or distorted features and the morphological identification cannot be conducted therefore, the process of species identification using molecular analysis will be conducted. After that, upon obtaining the DNA sequences, the DNA sequences will be compared with the DNA sequences from the database of DNA barcoding. These will indirectly expediate the process of species identification in the field of forensic entomology.

Therefore it is recommended that more research to be done where more blow fly species will be processed using the molecular method for each stage of the blow fly life cycle and then the DNA sequences that have been obtained can be kept as reference sequences. The knowledge of local fauna is very useful in forensic investigations because data from other regions, which may have both different environmental and faunal characteristics, may not provide a sufficient degree of accuracy (Arnaldos *et al*., 2004).

The presence of more blow fly species in the DNA barcoding will allow the DNA sequences in DNA barcoding to be used more efficiently. Wells *et al*. (2007) highlighted that DNA-based species identification must be based on a database comprising replicate samples from a wide geographic range. To materialize the system of DNA barcoding for a more systematic blow fly species identification, will take more time and require an expert system of integrated information where species names and their DNA barcodes are coupled to data of life cycle and geographic distributions. Therefore, the DNA barcoding that had been obtained from this research can be used as a starting step to realize the DNA barcoding for blow flies species in the field of forensic entomology in Malaysia.

The Barcode of Life project was proposed to promote DNA barcoding as a global standard for sequence-based identification of eukaryotes. In 2004, this project was formally initiated by the establishment of the Consortium for the Barcode of Life (CBOL), which aims to develop a standard protocol for DNA barcoding and to construct a comprehensive DNA barcode library. Species descriptions using barcodes based on type specimens will become more common and important in the near future (Jinbo *et al*., 2011).

5.4 GENERAL DISCUSSION

There are many networks such as the European Association of Forensic Entomology (EAFE), American Board of Forensic Entomology (ABFE) and the North American Forensic Entomology Association (NAFEA), developed by forensic entomology experts to broaden our knowledge on insects and how they can be beneficial to crime and civil investigations. Recently, the creation of Malaysian Association of Forensic Entomology (MAFE) was one of the efforts made by Malaysian entomologists to upgrade the field of forensic entomology in Malaysia.

Forensic entomology in Malaysia is expanding and rapidly developing. The younger generation is becoming more aware of this field and the research in institutions of higher learning are emphasizing more in-depth studies in forensic entomology, while creating more graduates who are experts in the field. Nevertheless the low usage or the application of forensic entomology in the area of forensic investigation by the crime scene police officers remains a great challenge. Entomological evidence that is found at crime scene is still overlooked by a minority of crime scene police officers. At this moment, maggots or larvae found on dead human bodies will normally be collected by the pathologists who conduct the post-mortem examination to be sent to entomologists to determine the PMI.

Some of the crime scene police officers collect the entomological evidence from dead human body during crime scene investigations to determine the PMI. There are also some crime scene police officers who ignore the entomological evidence found on dead human body during crime scene investigations. This does not mean that the crime scene police officers are doing this intentionally, as unfortunately the crime scene police officers are not exposed widely in the area of forensic entomology. Crime scene police officers must find a way to resolve this problem and must be committed to apply forensic entomology in the police investigations. More exposure and professional training must be given to crime scene police officers in the field of forensic entomology and the importance of it in the field of crime investigations. In addition to that, Forensic Laboratory of Royal Malaysia Police has to make sure that all crime scene police officers do not disregard or avoid any entomological evidence found during crime scene investigations.

The crime scene police officers are the first personnel to be exposed to entomological evidence during crime scene investigations. Therefore it is advisable that Forensic Laboratory of Royal Malaysia Police set up a unit of forensic entomology in their laboratory to investigate the entomological evidence. Furthermore, this action will increase the image and the credibility of the police in their forensic science investigation.

Species identification in the field of forensic entomology is extremely important. This is due to the fact that accurate species identification will give accurate estimation of PMI. The estimation of a more accurate PMI will assist in the police investigation in many ways. The primary focus of forensic entomology is the establishment of the PMI using insect life cycles. A good forensic entomologist will give detectives an estimate, to the day or even the hour, when the body was first colonized by insects. Investigators

compare this estimate with witness accounts of when the victim was last seen alive. Questions such as; where was the victim between when he was last seen and when insects first invaded his corpse? Was he alive or was the body hidden somewhere? These are questions often asked and answers are expected.

Entomological evidence can also help determine the circumstances of abuse and sexual assault. Victims that are incapacitated (bound, drugged or otherwise helpless) often have associated fecal and urine soaked clothes or bed dressings. Such material will attract certain species of flies that otherwise would not be recovered. Their presence can yield many clues to both ante-mortem and post-mortem circumstances of the crime.

Currently, it is now possible to use DNA technology not only to help determine insect species but to recover and identify the blood meals taken by blood feeding insects. The DNA of human blood recovered from the digestive tract of an insect can place suspects at a known location within a definable period of time and recovery of the victims' blood can also create a link between perpetrator and suspect. The insects recovered from decomposing human remains can be a valuable tool for toxicological analysis. The voracious appetite of the insects on corpse can quickly skeletonise the remains. In a short period of time the fluids (blood and urine) and soft tissues needed for toxicological analysis disappear. However, it is possible to recover the insect larvae and run standard toxicological analyses on them as with human tissue. Toxicological analysis can be successful on insect larvae because their tissues assimilate drugs and toxins that accumulate in human tissue prior to death (Bourel *et al*., 2001; Gagliano-Candela & Aventaggiato, 2001; DiZinno *et al*., 2002)

It is recommended that maggots found on dead human bodies during crime scene investigations are better if one uses both morphological and molecular methods to

ensure accurate species identification. This is because the determination of blow flies species found at crime scene is important in determining the PMI.

In general, forensic science uses the natural sciences to be applied in investigating crime cases. This is also a fact for the field of entomology. As a university subject classic entomology was gradually replaced by modern molecular aspects as DNA barcoding (Hajibabaei *et al*., 2007). Several methods have been proposed for species identification from DNA sequence data (Meier *et al*., 2006). Tree-based method was focused (Hebert *et al*., 2003a, 2003b), in which the emphasis is placed on finding monophyletic groups. Tree-based methods have recently been shown to be particularly effective at avoiding false-positive results (Ross *et al*., 2008).

Actually the database of mitochondrial DNA sequences for different insect stages which was preserved with and without 70% ethanol was used as references data for maggot samples found in crime scene to get a faster result. For example the DNA barcoding for the blow fly should be established like the Forensic DNA Databank Malaysia, established by Forensic Laboratory of Royal Malaysia Police force for the purpose of identifying any 'living or deceased person'.

Other than that, a method on rearing the blow flies in the laboratory is also discussed. This can be used extensively as a guideline in rearing blow flies in Forensic Laboratory of Royal Malaysia Police. Forensic entomology cases require appropriate collection and continuity of insect evidence as with any other evidence recovered from a crime scene. Ideally, an entomology expert was available to carry out these duties leading to the later analysis and interpretation of insect evidence. In the real world, the responsibility of first collection and continuity of potential insect evidence often lies with the crime scene police officers.

The guidelines in Appendix F are specifically designed for crime scene police officers to provide 'bare bones' guidelines for police investigators in Forensic Laboratory of Royal Malaysia Police in covering their most immediate needs and tasks in collecting and preparing insect evidence to be passed to a forensic entomologist. With an idea of what is needed and why, then one can make use of the resources at hand. With proper training and wide application at the crime scene, it is hoped that species identification process for maggot or larvae that are found on dead human bodies can be estimated more accurately and indirectly this can give a more accurate PMI estimation. To sum up, although some ground was covered in the field of forensic entomology in the last 25 years, a lot of work still needs to be done. Moreover, even if the field is considered to be global, more scientists in each geographical region should work on producing region-specific data that can be used in criminal investigations.

CHAPTER 6
CONCLUSION

6.0 CONCLUSION

The interest in forensic entomology is expanding rapidly across the world. It is believed that education will be an effective and comprehensive approach to spreading the knowledge and awareness of forensic entomology. Forensic entomology can be promoted and improved with knowledge and awareness among Malaysians through curricula development, relevant policies, programmes and training. It is hoped that the increased knowledge and awareness may eventually contribute to the growth of forensic entomology.

The importance of species identification process in forensic entomology cannot be denied. The correct species identification for maggots or larvae that are found on dead human bodies during crime scene investigations will be the core in determining the estimation of PMI. Mitochondrial DNA nucleotide sequence data are a promising tool for identification of the forensically important blow flies' species, certainly within Malaysia and apparently for most parts of the world. Although molecular methods are very useful especially if it is necessary to identify small fragments of insect material or very young larvae they should be used together with the conventional methods. The latter are faster, cheaper and moreover are the basis for molecular species identification.

The DNA barcoding had been created for two blow fly species where it is only used as a reference sample for the process of species identification for maggot samples found in a crime scene. DNA identification of Calliphoridae species has the potential to be extremely useful, but it is at a relatively early stage of research. DNA molecular identification is currently only used to enhance confidence in traditional morphological

identification. Research in this area has not yet progressed far enough for DNA identification to stand alone. Therefore more effort is needed, involving more researchers in the field of forensic entomology in the effort to realize the existence of more DNA barcoding for multiple gene regions for blow fly species likely to be encountered in Malaysia.

DNA barcoding holds promise for the use of Caliphoridae species in death investigations in Malaysia. The initial data suggest that accurate DNA-based identification of these species is relatively straightforward, otherwise almost unidentifiable by the non-specialist. However, effective use would require an expert system of integrated information where species names and their DNA barcodes are coupled to data of life cycle and geographic distributions, as are already available for some dipteran families overseas (Byrd & Castner, 2001). The present study is a first step towards the development of such a system in Malaysia.

Entomologists must always work within a reasonable time frame for the age estimations of insects. This is because entomologists deal with organisms in natural systems and therefore account for the biological variation that occurs in these systems. Exact estimations that pinpoint the time of death may never be possible. With continued research in the field of forensic entomology, entomologists can develop a better understanding of such variation and can allow for better predictions of the period of insect activity and, potentially, the amount of time elapsed from death of the organism to insect colonization. In the end, such an understanding of this variation could lead forensic entomologists closer to providing estimates of the true PMI.

Other than that, the area of forensic entomology promises a new employment opportunity to new graduates. Technology in forensics is developing very fast and it is widely accepted by the judiciary system. Furthermore, many government and private

agencies in this new era of technology are looking for more graduates from the science field.

6.1 RECOMMENDATIONS

The application of entomology to forensic science, in spite of its great potential, continues to be only an occasional exercise in Malaysia. There are several reasons for this issue. Data about the carrion fauna in different climatic and geographical regions of this vast country is virtually less-existent. Because of lack of awareness and study, entomological investigations are not carried out as a matter of routine in crime scene investigations in our country and therefore a body of knowledge is not being built up based on accumulated experience from actual cases.

There is absolute dearth of works dealing with various kinds of biological information about the fauna of decay, which is prerequisite before any conclusions can be drawn from the available entomological data (Singh *et al*., 1999). Knowledge from physiology and ecology of insects (Reibe & Madea, 2010) can be coupled with genetic tools (Reibe *et al*., 2009) to improve both basic research and application in real case work. As a consequence, the potential value of entomology to forensic science has not been fully realized and little active research work is being undertaken in Malaysia.

Workers like Smith (1973), Nuorteva (1977), Erzinclioglu (1983,1985,1986), Meek *et al*. (1983), Lord & Burger (1984), Keh (1985), Smith (1986), Catts & Haskell (1990), Goff (1993), Byrd & Castner (2000) and Goff (2000) have repeatedly stressed on routine entomological investigations in criminal cases and for separate entomological sections in government forensic laboratories. It cannot be overemphasized that only by the adoption of these measures, will this subject be able to develop satisfactorily. In the mean time, however, there is much that can be done by crime scene police officers,

pathologists in mortuaries and entomologists in universities and other research organizations.

Based on the findings of the present study, some issues and challenges pertaining to forensic entomology particularly in Malaysia are highlighted as follows:

i) The main obstacle in forensic entomological research is the choice of a research substitute for human cadavers, but the choice of carcass will depend upon the exact purpose of the research.

ii) Adult insects have been studied taxonomically, but their larvae are poorly known. The larvae and puparia of the species belonging to families Calliphoridae, Sarcophagidae and others are in particular need of taxanomic study. Such studies are needed because very often in forensic cases identification has to be done on the basis of larvae alone, and in such cases, valuable information remains locked away because of lack of knowledge. There is essential need for nationwide education and training particularly among the crime scene police officers on morphological identification of forensically important blowflies.

iii) Corpse-associated insects may yield information on chemical contaminants present in the corpse before death. This is because maggots feeding on the tissue can accumulate certain substances and thus are more sensitive to tests. Experimental work needs to be undertaken for studying the transfer of various kinds of poisons from a dead body into the maggots that have been feeding upon it.

iv) In certain indoor cases, the corpse may be discovered only after the maggots have finished feeding and left to pupate. In these cases it is not possible to

estimate time of death by examination of larvae but structural and morphological changes in pupae or pupae cases should, in principle, enable a useful estimate to be reached. This field has hardly been explored.

v) Many kinds of clues can be obtained during actual crime scene case investigations. This type of knowledge cannot be gained from research under experimental conditions.

vi) It is a known fact that, maggot activity raises the temperature of the corpse, but little is known of the relationship between this temperature and the ambient temperature. This requires investigation particularly.

vii) Do not overlook the possibility that larvae and pupae could be found at some distance from the body. This is seldom investigated.

viii) The lack of insect activity can be important; sampling that considers the presence and absence of entomological evidence must form part of sampling strategy.

ix) If an entomologist cannot attend the crime scene, do not forget to pass on as much evidence as possible by way of photography and written description of the scene.

x) Establish a National Reference Lab for Forensic Entomology in which molecular method is routinely applied for fly species identification.

xi) Build up a National DNA Database for forensically important blow flies.

A thorough understanding of insects and their habits should enable entomologists to contribute in great measure to the reconstruction of events of a death scene. In all cases, whether small or large in scale, knowledge of the distribution, biology and

behaviour of insects found at a crime scene can provide information on when and how crime was committed. It cannot be overemphasized that a visit by the entomologist to the scene of crime would yield much more information than it is possible to glean from samples collected and submitted to entomologist by a non-entomologist.

It is hoped that, before too long, entomology will become a fully incorporated branch of forensic science in Malaysia. Future research will enhance forensic entomology as a genuine, quantitative scientific discipline and improve the quality and accuracy of case reconstructions made (Hall, 2000). In conclusion, the PMI estimation by entomological techniques is a useful forensic tool and should be widely applied in crime scene investigation in Malaysia.

SCHOLARLY CONTRIBUTIONS

List of publications

Kavitha, R., Nazni, W.A., Tan, T.C., Lee, H.L. & Sofian Azirun, M. (2012). Molecular identification of fly maggots recovered from corpses during death scene investigation in Malaysia. *Asia Pacific Journal of Molecular Biology & Biotechnology* **20**(2):73-82.

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List of accepted manuscripts

Kavitha, R., Nazni, W.A., Tan, T.C., Lee, H.L. & Sofian Azirun, M. (2013). Recovery of forensically important entomological specimens from human cadavers in Malaysia (2005-2010). Journal of Forensic and Legal Medicine. In press. (*ISI-Cited Publication*).

Kavitha, R., Tan, T.C., Lee, H.L., Nazni, W.A. & Sofian Azirun, M. (2013). DNA typing of Calliphorids collected from human corpses in Malaysia. Tropical Biomedicine. In press (*ISI-Cited Publication*).

Kavitha, R., Tan, T.C., Lee, H.L., Nazni, W.A. & Sofian Azirun, M. (2013). Molecular identification of Malaysian *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) using life stage specific mitochondrial DNA. Tropical Biomedicine. In press (*ISI-Cited Publication*).

List of submitted manuscript

Kavitha, R., Tan, T.C., Lee, H.L. & Sofian Azirun, M. (2013). Awareness of Forensic Entomology among Malaysia: A Knowlwdge, Attitude, Practice Study. Submitted to Forensic Science Policy & Management: An International Journal on 20/2/2013.

List of conference presentations

Kavitha, R., Lee, H.L., Nazni, W., Chen, C.D. & Sofian Azirun, M. (2009). Forensic Entomology in Peninsular Malaysia: Case Studies. Paper presented at the 45th Annual Scientific Seminar of Malaysian Society of Parasitology & Tropical Medicine on 18 & 19 March 2009 at Grand Seasons Hotel, Kuala Lumpur, Malaysia.

Kavitha, R., Tan, T.C., Lee, H.L. & Sofian Azirun, M. (2011). Discovery of a new species of blowfly from crime scene investigation in Malaysia. Paper presented at the 47th Annual Conference of the Malaysian Society of Parasitology & Tropical Medicine on 3 & 4 March 2011 at IMU Bukit Jalil, Kuala Lumpur, Malaysia.

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APPENDIX A: QUESTIONNAIRE

Soalan Kaji Selidik Untuk Mengkaji Kesedaran Dan Pengetahuan Terhadap Bidang Forensik Entomologi Di Malaysia

Questionnaire To Study The Awareness And Knowledge About Forensic Entomology In Malaysia

1. Forensik Entomologi adalah satu bidang yang mengkaji serangga yang dijumpai di mayat manusia. Apakah tahap pemahaman anda tentang bidang Forensik Entomologi?

   Forensic Entomology is a study about insects found on dead bodies. What is your degree of understanding about Forensic Entomology?

   1. □ Tinggi
      High

   2. □ Sederhana
      Medium

   3. □ Rendah
      Low

   4. □ Tidak tahu
      No idea

2. Adakah anda tahu bahawa bidang kaji serangga yang dimaksudkan dalam bidang Forensik Entomologi termasuk telur lalat, lalat (larva), pupa, lalat dewasa, kepompong dan serangga-serangga lain seperti kumbang?

   Do you know that the study of insect in Forensic Entomology includes study of the egg, the larvae, the pupa, the adult, the empty puparium (skin of pupa) and other insects like beetle?

   1. □ Ya
      Yes
      Jika Ya, dari mana anda mengetahuinya,
      If Yes, from where did you get the information,

      1.1 □ Kawan, ……
      Friends

      1.2 □ Komunikasi Multimedia …….
      Mass Media

      1.3 □ Pengetahuan Profesional……..…
      Professional Knowledge

      1.4 □ Lain-lain……..
      Others

   2. □ Tidak tahu
      No idea

3. Adakah anda tahu bahawa ulat yang dijumpai di mayat manusia asalnya daripada telur lalat dan boleh digunakan untuk menentukan masa kematian?

   Do you know that maggots (larvae) found on a dead human body are originated from the flies and can be used to determine the postmortem interval (time of death)?

   1. □Ya
      Yes
      Jika Ya, dari mana anda mengetahuinya,
      If Yes, from where did you get the information,

      1.1 □ Kawan,……
      Friends

      1.2 □ Komunikasi Multimedia ……..
      Mass Media

      1.3 □ *Pengetahuan Profesional*……
      Professional Knowledge

      1.4 □ Lain-lain……
      Others
   2. □Tidak
      No idea

4. Pernahkah anda mengutip ulat-ulat atau serangga-serangga lain yang dijumpai di mayat manusia untuk membantu siasatan?

   Have you ever collect the maggots (larvae) or any other insects found on a dead human body to assist in investigations?

   1. □Ya,
      Yes,

      Jika Ya,
      If Yes,
      1.1 □ Teknik yang anda gunakan untuk mengutip ulat,……
      What are the techniques you used to collect the maggots?

      1.2 □ Di bahagian badan yang mana ulat itu dikutip,……
      Which part of the body the maggots were collected from

      1.3 □ Pengetahuan Profesional,……
      Professional Knowledge

      1.4 □ Lain-lain,……
      Others
   2. □ Tidak
      No

   3. □ Kadang kala
      Sometimes

   4. □ Bergantung kepada pegawai atasan
      Depends on senior officers

5. Adakah anda tahu bahawa ulat atau larva yang dijumpai di mayat manusia boleh juga digunakan untuk menentukan punca kematian dan/atau tempat kematian dan/atau bahagian badan yang mengalami luka yang teruk dan sebagainya?

Do you know that the maggots (larvae) found on a dead human body can be used to determine the cause of death of a person and/or the surrounding area of the crime and/or the position of the wounds in the body?

1. □ Ya
   Yes
   Jika Ya, dari mana anda mengetahuinya,
   If Yes, from where did you get the information,

   1.1 □ Kawan,……
   Friends

   1.2 □ Komunikasi Multimedia ……..
   Mass Media

   1.3 □ *Pengetahuan Profesional*……
   Professional Knowledge

   1.4 □ Lain-lain……
   Others

2. □ Tidak tahu
   No idea

6. Adakah anda tahu bahawa lalat dapat mengesan mayat manusia dalam tempoh masa kurang daripada 24 jam bergantung kepada keadaan persekitaran?

Do you know that flies can locate the dead human body within 24 hours, depending on the surrounding area where the dead body is found?

1. □ Ya
   Yes

   Jika Ya, dari mana anda mengetahuinya,
   If Yes, from where did you get the information,

   1.1 □ Kawan,……
   Friends

   1.2 □ Komunikasi Multimedia ……
   Mass Media

   1.3 □ *Pengetahuan Profesional*……
   Professional Knowledge

   1.4 □ Lain-lain……
   Others

2. □ Tidak
   No idea

7. Adakah anda selalu menjumpai mayat manusia yang dikerumuni oleh ulat (larva)?

   Do you *always encounter a dead human body infested with maggots (larvae)?*

   1. □ Ya,
      Yes,

      Jika Ya,
      If Yes,

      1.1 □ Selalu
      Always

      1.2 □ Satu kes mayat dengan ulat dalam tempoh masa seminggu
      One dead human body infested with maggots every week

      1.3 □ Satu kes mayat dengan ulat dalam tempoh masa sebulan
      One dead human body infested with maggots every month

   2. □ Jarang
      Seldom

   3. □ Tidak
      No

8. Adakah anda pernah menjumpai kepompong kosong semasa menyiasat tempat kejadian yang melibatkan mayat manusia?

   Have you ever found an empty puparium (skin of pupa) in a crime scene during investigation or during postmortem?

   1. □Ya,
      Yes,

      Jika Ya,
      If Yes,

      1.1 □ Selalu
      Always

      1.2 □ Satu mayat manusia dengan kepompong kosong dalam tempoh masa seminggu
      One dead human body with an empty puparium every week

      1.3 □ Satu mayat manusia dengan kelompong kosong dalam tempoh masa sebulan
      One dead human body with an empty puparium every month

   2. □ Jarang
      Seldom

   3. □ Tidak
      No

9. Adakah anda tahu bahawa larva atau ulat yang dijumpai di mayat-mayat manusia telah membantu untuk menyelesaikan penyiasatan polis di negara-negara lain yang mengaplikasikan penggunaan Forensik Entomologi dalam proses penyiasatan kes bunuh?

Do you know that the maggots or larvae found on a dead human body can assist in crime scene investigations in other countries which apply Forensic Entomology to assist in murder cases?

1. □ Ya,
   Yes,

   Jika Ya daripada siapa anda mengetahuinya,
   If Yes from whom did you know from,

   1.1 □ Kawan,……
   Friends
   1.2 □ Komunikasi Multimedia ……
   Mass Media

   1.3 □ *Pengetahuan Profesional*……
   Professional Knowledge
   1.4 □ Lain-lain……
   Others
2. □ Tidak tahu
   No idea

10. Adakah soal selidik ini membantu ini memperkenalkan kepada anda tentang bidang kajian serangga iaitu forensik entomologi dan fungsi-fungsinya serta kepentingannya dalam membantu penyiasatan kes polis?

Does this questionnaire introduce to you Forensic Entomology - the study of insects in assisting crime scene investigations?

1. □ Ya
   Yes

   If Yes, 1.1 □ Pendedahan tentang forensik entomologi yang pertama
   First time exposure to Forensic Entomology

   1.2 □ Peningkatan sebanyak 25% tentang bidang Forensik Entomologi
   25% increase about Forensic Entomology field

   1.3 □ Peningkatan sebanyak 50% tentang bidang Forensik Entomologi
   50% increase about Forensic Entomology field

   1.4 □ Peningkatan sebanyak 75% tentang bidang Forensik Entomologi
   75% increase about Forensic Entomology field

2. □ Pengetahuan Profesional
   Professional Knowledge

3. □ Tidak
   No

BUTIR-BUTIR PERIBADI

PERSONAL PARTICULARS

1. NAMAPENUH:…….
   *FULL NAM*E

2. NO.KAD
   PENGENALAN:……..**.....**
   IDENTITY CARD NUMBER

3. UMUR:……..**..**
   AGE

4. ALAMAT
   RUMAH:…….**.....**
   HOUSE ADDRESS…….**.....**

5. ALAMATTEMPATBEKERJA:……
   OFFICEADDRES……...

6. JAWATAN:…….……
   POSITION

7. PANGKAT:…….…….**.....**
   RANK

8. TAHAP PENDIDIKAN:…….……..
   ACADEMIC QUALIFICATION

9. PENGALAMAN BEKERJA (TAHUN):…….……...
   WORKING EXPERIENCE (YEARS)

10. KEKERAPAN MENYIASAT KES BUNUH SEPANJANG TEMPOH PERKHIDMATAN:……
    FREQUENCY OF INVESTIGATING INTO MURDER CASES DURING PERIOD OF SERVICE

TARIKH:…… TANDATANGAN:…….....…….

DATE *SIGNATURE*

Soalan Kaji Selidik Untuk Mengkaji Kesedaran Dan Pengetahuan Terhadap Bidang Forensik Entomologi Di Malaysia

Questionnaire To Study The Awareness And Knowledge About Forensic Entomology In Malaysia

1. Berapakah jenis spesies lalat yang anda tahu dalam bidang Forensik Entomologi?

   How many types of flies do you know in the field of Forensic Entomology?

   1. ☐ Satu spesies; nyatakan…….

      One species; please state here……

   2. ☐ Dua spesies; nyatakan……..

      Two species; please state here……....

   3. ☐ Lebih daripada tiga spesies;nyatakan……...

      More than three species; please state here……..

   4. ☐ Tidak tahu

      No idea

   5. ☐ Lain-lain;……..

      Others;…….

2. Sebagai seorang individu yang terlibat dalam bidang Forensik Entomologi bolehkah anda mengenalpasti apakah jenis spesies sesuatu lalat?

   Since you are involved in Forensic Entomology field, can you identify the types of the fly species?

   1. ☐ Ya,

      Yes,

      Jika Ya, bagaimana anda mengenalpastinya;…….

      If Yes, how do you identify them;……...

   2. ☐ Meminta bantuan pensyarah untuk proses identifikasi

      Seek the help from the lecturers to identify them

   3. ☐ Boleh, sikit-sikit

      Can, a bit

   4. ☐ Tidak tahu

      No idea

   5. ☐ Lain-lain;…….

      Others;……..

3. Dalam proses pembelajaran anda atau kajian anda apakah proses yang digunakan untuk mengidentifikasikan spesies sesuatu lalat dan apakah kelebihan kaedah tersebut dalam proses mengenalpasti spesies lalat?

In your studies or research, what types of process do you use to identify the fly species and what are the advantages of the related identification process?

1. □ Identifikasi morfologi;……

   Morphological identification;……

2. □ Identifikasi DNA;……

   Molecular DNA identification;……

3. □ Bantuan Pensyarah;……

   Help from the lecturers;……

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……..

   Others;…….

4. Apakah motif utama proses pembelajaran anda berkenaan dengan bidang Forensik Entomologi?

What is the main motive in your studies or research related to the Forensic Entomology field?

1. □ Mengenalpasti jenis spesies lalat

   To identify the types of fly species

2. □ Menentukan masa kematian

   To determine the postmortem interval (PMI)

3. □ Menentukan proses sucession lalat

   To determine the insect succession

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……...

   Others;…….

5. Apakah masalah yang selalu anda hadapi dalam proses pembelajaran dan penyelidikan yang berkaitan dengan bidang Forensik Entomologi?

What are the problems that you always encounter in your studies and research related to the Forensic Entomology field?

1. □ Masalah untuk mengenalpasti spesies lalat

   Problem in identifying the fly species

2. □ Masalah yang berkaitan dengan proses analisa DNA lalat

   Problems in analysing the fly DNA

3. □ Kekurangan sumber maklumat yang berkaitan dengan Forensik Entomologi

   Lack of information on Forensic Entomology

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……..

   Others;…….

6. Apakah sebab utama yang menyebabkan anda memilih Forenisk Entomologi sebagai bidang penyelidikan anda atau sebagai salah satu topik yang penting dalam proses pengajian anda?

What is the main reason for you to choose Forensic Entomology as your research field or as an important subject for your studies?

1. □ Minat

   Enthusiasm

2. □ Dorongan untuk mengetahui dengan lebih mendalam tentang bidang Forensik Entomologi

   Motivation to get to know more about Forensic Entomology

3. □ Pengaruh daripada kawan dan pensyarah

   Influence from friends and lecturers

4. □ Subjek wajib

   Compulsory subject

5. □ Lain-lain;……..

   Others;…….

7. Pada pendapat anda, bagaimanakah kedudukan atau status bidang penyelidikan Forensik Entomologi di negara kita?

In your opinion, what is the position and status of Forensic Entomology field in our country?

1. □ Masih di tahap awal dan tidak ada harapan untul berkembang secara meluas

   Still in the early stage without hope for improvement

2. □ Sistem pendidikan negara patut memainkan peranan yang penting dalam mengalakkan lebih ramai pelajar membuat penyelidikan dalam bidang Forensik Entomologi

   Department of education in our country have to play an important role to encourage more students to research Forensic Entomology

3. □ Mempunyai masa depan yang cerah untuk berkembang maju

   Will continue to move forward and develop fully into a discipline by itself

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……..

   Others;……..

8. Apakah sumbangan anda dalam memajukan bidang Forensik Entomologi di negara kita?

What is your contribution to improving the Forensic Entomology field in our country?

1. □ Memperkenalkan bidang Forensik Entomologi kepada pelajar-pelajar lain

   More publicity to introduce Forensic Entomology to other students

2. □ Membuat lebih banyak kajian yang berkaitan dengan bidang Forensik Entomologi

   Do more research related to Forensic Entomology

3. □ Menyertai seminar yang diadakan di dalam dan luar negara dan membuat pembentangan kertas kerja untuk memperkenalkan hasil kajian anda dan membincangkan isu-isu yang berkaitan dengan bidang Forensik Entomologi

   Take part in the seminars which are held in our country and overseas to introduce your research findings and to discuss important issues related to Forensic Entomology?

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……..

   Others;…….

9. Pada pendapat anda, apakah sumbangan yang boleh diperolehi melalui perkembangan dan kemajuan bidang Forensik Entomologi di negara kita?

In your opinion, what are the contributions from Forensic Entomology field in our country?

1. □ Membantu dalam penyiasatan kes bunuh dan menetukan masa kematian

   Involvement in murder case investigations and to determine the postmortem interval, (PMI)

2. □ Membolehkan negara kita berdiri sama tinggi dengan negara-negara lain yang maju dalam bidang Forensik Entomologi

   To make our country proud by representing Malaysia when working together with other countries well known in the practice of Forensic Entomology

3. □ Menwujudkan peluang pekerjaan yang baru

   More career opportunity

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……..

   Others;…….

10. Pada pendapat anda, jabatan kerajan yang mana lebih memerlukan bantuan dari unit penyelidikan Forensik Entomologi di negara kita?

    In your opinion, which government departments need the services of the Forensic Entomology researchers more in our country?

1. □ Jabatan Polis

   Police Department

2. □ Jabatan Hospital

   Hospital Department

3. □ Pusat Pengajian Tinggi / Pusat Penyelidikan

   Higher Learning Institutions / Research Centres

4. □ Tidak tahu

   No idea

5. □ Lain-lain;……..

   Others;……..

TERIMA KASIH KE ATAS KESUDIAN ANDA UNTUK MENJAWAB SOAL SELIDIK INI

THANK YOU VERY MUCH FOR ANSWERING THIS QUESTIONNAIRE

APPENDIX B: SOCIO DEMOGRAPHIC FACTORS FOR THE FIRST 10 QUESTIONS

Socio demographic factors	Frequency (%)		X^2	p value
	Number (N)	Percentage (%)		
1. Forensic Entomology is a study about insects found on dead bodies. What is your degree of understanding about Forensic Entomology?				
• High	34	8.5		
• Medium	171	42.5		
• Low	141	35.1		
• No idea	56	13.9		
• High	205	51.0	0.329	0.327
• Low	197	49.0		
2. Do you know that the study of insect in Forensic Entomology includes study of the eggs, the larvae, the pupa, the adult, the empty puparium (skin of pupa) and other insects like beetle?				
Yes,				
• Friends	27	8.0		
• Mass Media	85	25.3		
• Professional Knowledge	171	50.9		
• Others	53	15.8		
• No	66	16.4		
• Yes	336	83.6	0.108	0.446
• No	66	16.4		
3. Do you know that maggots (larvae) found on a dead human body are originated from the flies and can be used to determine the post-mortem interval (time of death)?				
Yes,				
• Friend	29	7.9		
• Mass Media	82	22.3		
• Professional Knowledge	192	52.3		
• Others	64	17.4		
• No	35	8.7		
• Yes	367	91.3	0.003	0.552
• No	35	8.7		

4. Have you ever collect the maggots (larvae) or any other insects found on a dead human body to assist in investigations? • Yes • Sometimes • Depends on senior officers • No	 68 9 12 313	 16.9 2.2 3.0 77.9		
• Yes • No	89 313	22.1 77.9	16.681	0.0001
5. Do you know that the maggots (larvae) found on a dead human body can be used to determine the cause of death of a person and/or the surrounding area of the crime and/or the position of the wounds in the body? Yes, • Friends • Mass Media • Professional Knowledge • Others • No	 15 59 168 46 114	 5.2 20.5 58.3 16.0 28.4		
• Yes • No	288 114	71.6 28.4	1.003	0.193
6. Do you know that flies can locate the dead human body within 24 hours, depending on the surrounding area where the dead body is found? Yes, • Friends • Mass Media • Professional Knowledge • Others • No	 22 54 179 48 99	 7.3 17.8 59.1 15.8 24.6		
• Yes • No	303 99	75.4 24.6	0.512	0.288

7. Do you always encounter a dead human body infested with maggots (larvae)? Yes, • Always • One dead human body infested with maggots every week • One dead human body infested with maggots every month • Seldom • No	 17 32 23 127 203	 23.6 44.4 31.9 31.6 50.5		
• Yes • Seldom	72 330	17.9 82.1	17.695	0.0001
8. Have you ever found an empty puparium (skin of pupa) in a crime scene during investigation or during post-mortem? Yes, • Always • One dead human body with an empty puparium every week • One dead human body with an empty puparium every month • Seldom • No	 2 13 13 62 312	 7.1 46.4 46.4 15.4 77.6		
• Yes • Seldom	28 374	7.0 93.0	7.348	0.010
9. Do you know that the maggots or larvae found on a dead human body can assist in crime scene investigations on other countries which apply Forensic Entomology to assist in murder cases? Yes, • Friends • Mass Media • Professional Knowledge • Others • No idea	 16 174 104 26 82	 5.0 54.4 32.5 8.1 20.4		
• Yes • No	320 82	79.6 20.4	2.316	0.088

10. Does this questionnaire introduce to you Forensic Entomology – the study of insects in assisting crime scene investigations? Yes, • First time exposure to Forensic Entomology • 25% increase about Forensic Entomology field • 50% increase about Forensic Entomology field • 75% increase about Forensic Entomology field • Professional Knowledge • No	106 169 46 20 45 61	31.1 49.6 13.5 5.9 11.2 15.2		
• Yes • No	341 61	84.8 15.2	1.092	0.194

APPENDIX C: SOCIO DEMOGRAPHIC FACTORS FOR THE SECOND 10 QUESTIONS

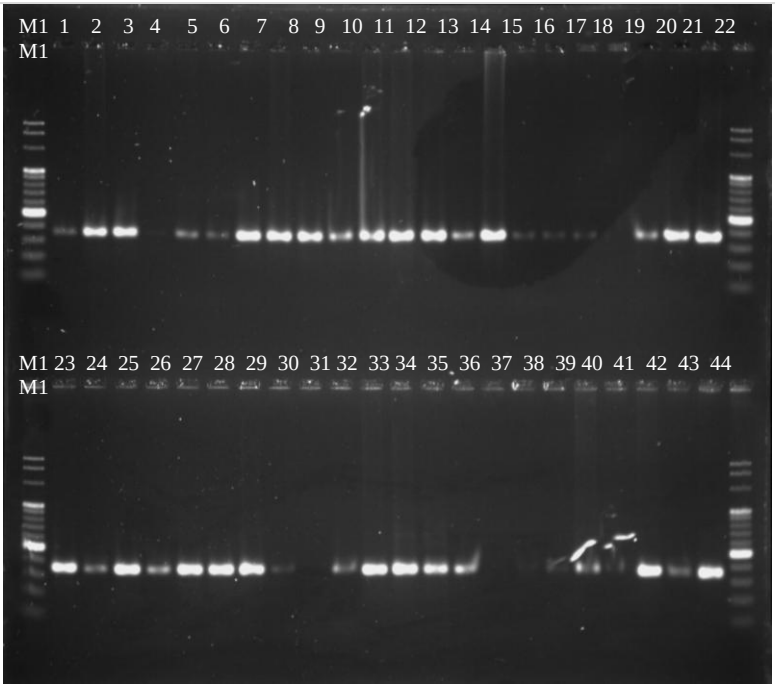
Socio demographic factors	Frequency (%)		X^2	p value
	Number (N)	Percentage (%)		
1. How many types of flies do you know in the field of Forensic Entomology?				
• One species	17	10.8		
• Two species	13	8.2		
• More than three species	78	49.4		
• No idea	44	27.8		
• Others	6	3.8		
• At least one species or more	108	68.4	0.004	0.683
• No idea	50	31.6		
2. Since you are involved in Forensic Entomology field, can you identify the types of the fly species?				
Yes,				
• If yes, how do you identify them	35	22.2		
• Seek the help from the lectures to identify them	39	24.7		
• Can, a bit	52	32.9		
• No idea	30	19.0		
• Others	2	1.3		
• Yes	126	79.7	0.324	0.495
• No	32	20.3		
3. In your studies or research, what types of process do you use to identify the fly species and what are the advantages of the related identification process?				
• Morphological identification	91	57.6		
• DNA Molecular identification	4	2.5		
• Help from the lecturers	33	20.9		
• No idea	27	17.1		
• Others	3	1.9		
• Yes	128	81.0	0.717	0.529
• No	30	19.0		

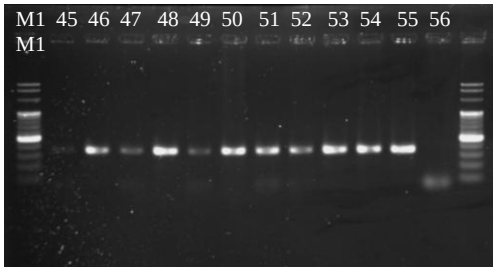
4. What is the main motive in your studies or research related to the Forensic Entomology field?				
• To identify the types of fly species	26	16.5		
• To determine the post-mortem interval (PMI)	85	53.8		
• To determine the insect succession	19	12.0		
• No idea	19	12.0		
• Others	9	5.7		
• Yes	130	82.3	5.024	0.081
• No	28	17.7		
5. What are the problems that you always encounter in your studies and research related to the Forensic Entomology field?				
• Problem in identifying the fly species	51	32.3		
• Problems in analysing the fly DNA	9	5.7		
• Lack of information on Forensic Entomology	57	36.1		
• No idea	31	19.6		
• Others	10	6.3		
• Yes	117	74.1	2.638	0.165
• No	41	25.9		
6. What is the main reason for you to choose Forensic Entomology as your research field or as an important subject for your studies?				
• Enthusiasm	30	19.0		
• Motivation to get to know more about Forensic Entomology	49	31.0		
• Influence from friends and lecturers	16	10.1		
• Compulsory subject	38	24.1		
• Others	25	15.8		
• Yes	133	84.2	0.704	0.406
• No	25	15.8		

7. In your opinion, what is the position and status of Forensic Entomology field in our country? • Still in the early stage without hope for improvement • Department of education in our country have to play an important role to encourage more students to research Forensic Entomology • Will continue to move forward and develop fully into a discipline by itself • No idea • Others	 18 64 63 11 2	 11.4 40.5 39.9 7.0 1.3		
• Moving forward • No idea	145 13	91.8 8.2	2.553	0.228
8. What is your contribution to improving the Forensic Entomology field in our country? • More publicity to introduce Forensic Entomology to other students • Do more research related to Forensic Entomology • Take part in the seminars which are held in our country and overseas to introduce your research findings and to discuss important issues related to Forensic Entomology • No idea • Others	 40 47 29 29 13	 25.3 29.7 18.4 18.4 8.2		
• Yes • No	116 42	73.4 26.6	2.518	0.173
9. In your opinion, what are the contributions from Forensic Entomology field in our country? • Involvement in murder case investigations and to determine the post-mortem interval, (PMI). • To make our country proud by representing Malaysia when working together with other countries which well known in the practice of Forensic Entomology	 90 23	 57.0 14.6		

• More career opportunity • No idea	38 7	24.1 4.4		
• At least one contribution • None	151 7	95.6 4.4	6.034	0.128
10. In your opinion, which government departments need the services of the Forensic Entomology researchers more in our country?				
• Police Department • Hospital Department • Higher Learning Institutions/ Research Centres • No idea • Others	78 30 42 4 4	49.4 19.0 26.6 2.5 2.5		
• At least one department • None	150 8	94.9 5.1	5.084	0.145

APPENDIX D: GEL PHOTOS





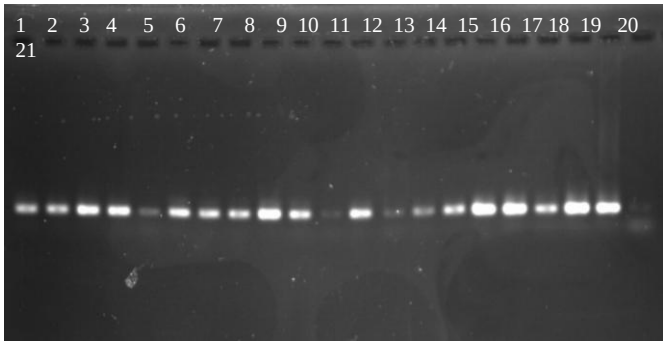
1: Sample 16
2: Sample 5ii
3: Sample 26
4: Sample 6i
5: Sample 6ii
6: Sample 43
7: Sample 22
8: Sample 7ii
9: Sample 23
10: Sample 4ii
11: Sample 7i
12: Sample 8
13: Sample 4i
14: Sample 27
15: Sample 5i
16: Sample 13
17: Sample 48
18: Sample 10ii
19: Sample 10i
20: Sample 1i
21: Sample 3i
22: Sample 29

23: Sample 25ii
24: Sample 1ii
25: Sample 21
26: Sample 2ii
27: Sample 2i
28: Sample 25i
29: Sample 3ii
30: Sample 18
31: Sample 24
32: Sample 30
33: Sample 31i
34: Sample 33
35: Sample 11
36: Sample 47
37: Sample 17ii
38: Sample 20
39: Sample 28
40: adult Ch.mega without ethanol
41: empty pupa Ch.mega without ethanol
42: adult Ch.mega in ethanol
43: empty pupa Ch.mega in ethanol
44: adult Ch.rufi without ethanol

45: empty pupa Ch.rufi without ethanol
46: adult Ch. rufi in ethanol
47: empty pupa Ch. rufi in ethanol
48: egg Ch.rufi without ethanol
49: 1st instar Ch.rufi without ethanol
50: 2nd instar Ch.rufi without ethanol
51: 3rd instar Ch. rufi without ethanol
52: pupa Ch.rufi without ethanol
53: egg Ch.rufi in ethanol
54: 1st instar Ch.rufi in ethanol
55: pupa Ch.rufi in ethanol
56: Negative control
M1: Perfect 100 bp DNA Ladder (EURx)

PCR products of partial COI (Batch 1)

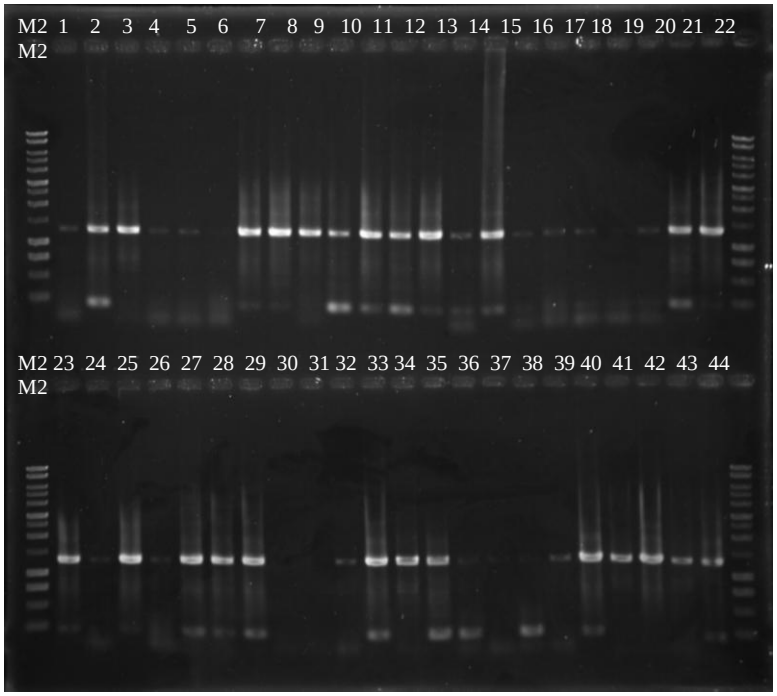
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

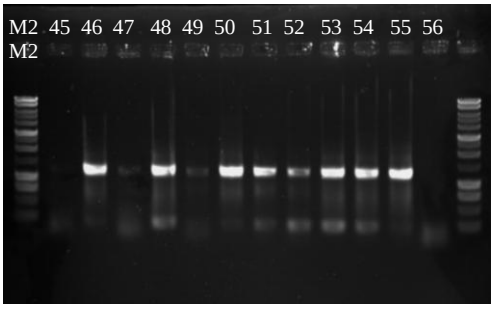


PCR products of partial COI (Batch 1) - Repeat

1: Sample 16
2: Sample 6i
3: Sample 6ii
4: Sample 43
5: Sample 13
6: Sample 48
7: Sample 10ii
8: Sample 10i
9: Sample 1ii
10: Sample 18
11: Sample 24
12: Sample 30
13: Sample 17ii
14: Sample 20
15: Sample 28
16: empty pupa Ch.mega without ethanol
17: empty pupa Ch.mega in ethanol
18: empty pupa Ch.rufi without ethanol
19: empty pupa Ch.rufi in ethanol
20: 1st instar Ch.rufi without ethanol
21: Negative control

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

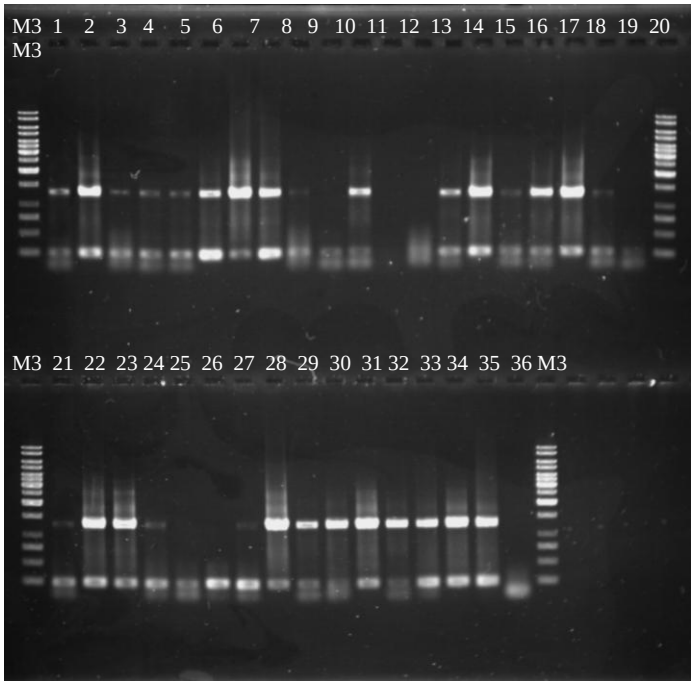




1: Sample 16	23: Sample 25ii	45: empty pupa Ch.rufi without ethanol
2: Sample 5ii	24: Sample 1ii	46: adult Ch.rufi in ethanol
3: Sample 26	25: Sample 21	47: empty pupa Ch.rufi in ethanol
4: Sample 6i	26: Sample 2ii	48: egg Ch.rufi without ethanol
5: Sample 6ii	27: Sample 2i	49: 1st instar Ch.rufi without ethanol
6: Sample 43	28: Sample 25i	50: 2nd instar Ch.rufi without ethanol
7: Sample 22	29: Sample 3ii	51: 3rd instar Ch.rufi without ethanol
8: Sample 7ii	30: Sample 18	52: pupa Ch.rufi without ethanol
9: Sample 23	31: Sample 24	53: egg Ch.rufi in ethanol
10: Sample 4ii	32: Sample 30	54: 1st instar Ch.rufi in ethanol
11: Sample 7i	33: Sample 31i	55: pupa Ch.rufi in ethanol
12: Sample 8	34: Sample 33	56: Negative control
13: Sample 4i	35: Sample 11	M2: Perfect Plus 1kb DNA Ladder (EURx)
14: Sample 27	36: Sample 47	
15: Sample 5i	37: Sample 17ii	
16: Sample 13	38: Sample 20	
17: Sample 48	39: Sample 28	
18: Sample 10ii	40: adult Ch.mega without ethanol	
19: Sample 10i	41: empty pupa Ch.mega without ethanol	
20: Sample 1i	42: adult Ch.mega in ethanol	
21: Sample 3i	43: empty pupa Ch.mega in ethanol	
22: Sample 29	44: adult Ch.rufi without ethanol	

PCR products of COII (Batch 1)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

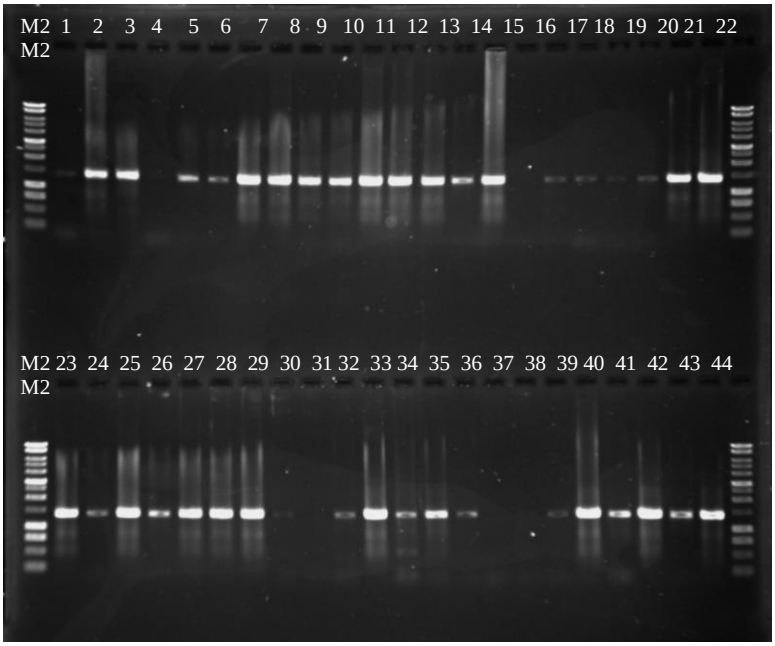


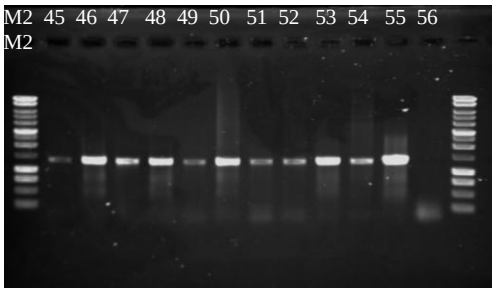
1: Sample 16
2: Sample 5ii
3: Sample 6i
4: Sample 6ii
5: Sample 43
6: Sample 4ii
7: Sample 7i
8: Sample 8
9: Sample 27
10: Sample 13
11: Sample 48
12: Sample 10ii
13: Sample 10i
14: Sample 1i
15: Sample 3i
16: Sample 1ii
17: Sample 2ii
18: Sample 3ii
19: Sample 18
20: Sample 24

21: Sample 30
22: Sample 31i
23: Sample 11
24: Sample 47
25: Sample 17ii
26: Sample 20
27: Sample 28
28: adult Ch.mega without ethanol
29: empty pupa Ch.rufi without ethanol
30: empty pupa Ch.rufi in ethanol
31: egg Ch.rufi without ethanol
32: 1st instar Ch.rufi without ethanol
33: pupa Ch.rufi without ethanol
34: egg Ch.rufi in ethanol
35: 1st instar Ch.rufi in ethanol
36: Negative control
M3: 1kb DNA Ladder (Fermentas)

PCR products of COII (Batch 1) - repeat

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

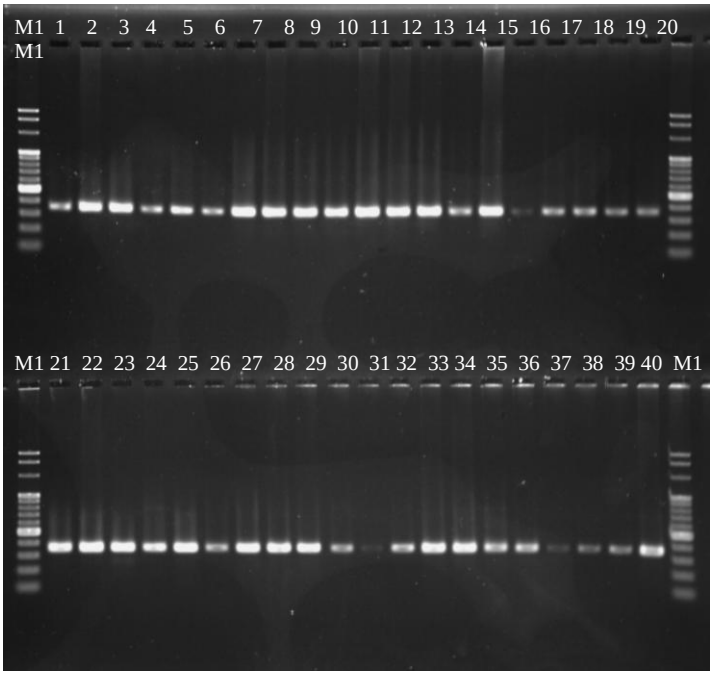


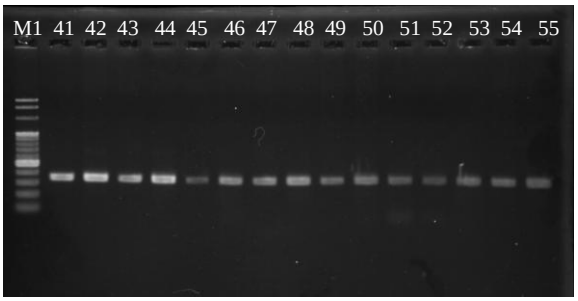


1: Sample 16	23: Sample 25ii	45: empty pupa Ch.rufi without ethanol
2: Sample 5ii	24: Sample 1ii	46: adult Ch.rufi in ethanol
3: Sample 26	25: Sample 21	47: empty pupa Ch.rufi in ethanol
4: Sample 6i	26: Sample 2ii	48: egg Ch.rufi without ethanol
5: Sample 6ii	27: Sample 2i	49: 1st instar Ch.rufi without ethanol
6: Sample 43	28: Sample 25i	50: 2nd instar Ch.rufi without ethanol
7: Sample 22	29: Sample 3ii	51: 3rd instar Ch.rufi without ethanol
8: Sample 7ii	30: Sample 18	52: pupa Ch. rufi without ethanol
9: Sample 23	31: Sample 24	53: egg Ch.rufi in ethanol
10: Sample 4ii	32: Sample 30	54: 1st instar Ch.rufi in ethanol
11: Sample 7i	33: Sample 31i	55: pupa Ch.rufi in ethanol
12: Sample 8	34: Sample 33	56: Negative control
13: Sample 4i	35: Sample 11	M2: Perfect Plus 1kb DNA Ladder (EURx)
14: Sample 27	36: Sample 47	
15: Sample 5i	37: Sample 17ii	
16: Sample 13	38: Sample 20	
17: Sample 48	39: Sample 28	
18: Sample 10ii	40: adult Ch.mega without ethanol	
19: Sample 10i	41: empty pupa Ch.mega without ethanol	
20: Sample 1i	42: adult Ch.mega in ethanol	
21: Sample 3i	43: empty pupa Ch.mega in ethanol	
22: Sample 29	44: adult Ch.rufi without ethanol	

PCR products of complete COI (Batch 1)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

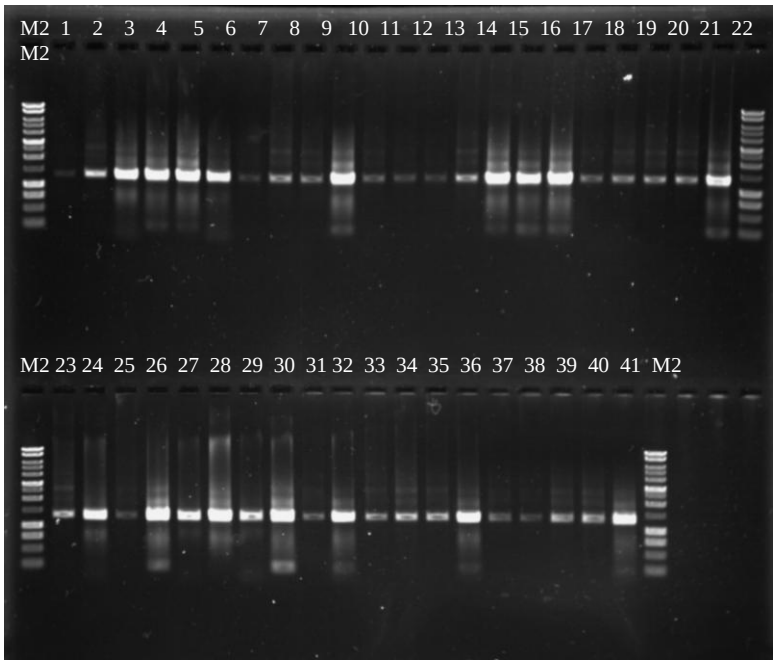




1: Sample 16	23: Sample 25ii	45: empty pupa Ch.rufi without ethanol
2: Sample 5ii	24: Sample 1ii	46: adult Ch.rufi in ethanol
3: Sample 26	25: Sample 21	47: empty pupa Ch.rufi in ethanol
4: Sample 6i	26: Sample 2ii	48: egg Ch.rufi without ethanol
5: Sample 6ii	27: Sample 2i	49: 1st instar Ch.rufi without ethanol
6: Sample 43	28: Sample 25i	50: 2nd instar Ch.rufi without ethanol
7: Sample 22	29: Sample 3ii	51: 3rd instar Ch.rufi without ethanol
8: Sample 7ii	30: Sample 18	52: pupa Ch.rufi without ethanol
9: Sample 23	31: Sample 24	53: egg Ch.rufi in ethanol
10: Sample 4ii	32: Sample 30	54: 1st instar Ch.rufi in ethanol
11: Sample 7i	33: Sample 31i	55: pupa Ch.rufi in ethanol
12: Sample 8	34: Sample 33	M1: Perfect 100 bp DNA Ladder (EURx)
13: Sample 4i	35: Sample 11	
14: Sample 27	36: Sample 47	
15: Sample 5i	37: Sample 17ii	
16: Sample 13	38: Sample 20	
17: Sample 48	39: Sample 28	
18: Sample 10ii	40: adult Ch.mega without ethanol	
19: Sample 10i	41: empty pupa Ch.mega without ethanol	
20: Sample 1i	42: adult Ch.mega in ethanol	
21: Sample 3i	43: empty pupa Ch.mega in ethanol	
22: Sample 29	44: adult Ch.rufi without ethanol	

Purified PCR products of partial COI (Batch 1)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

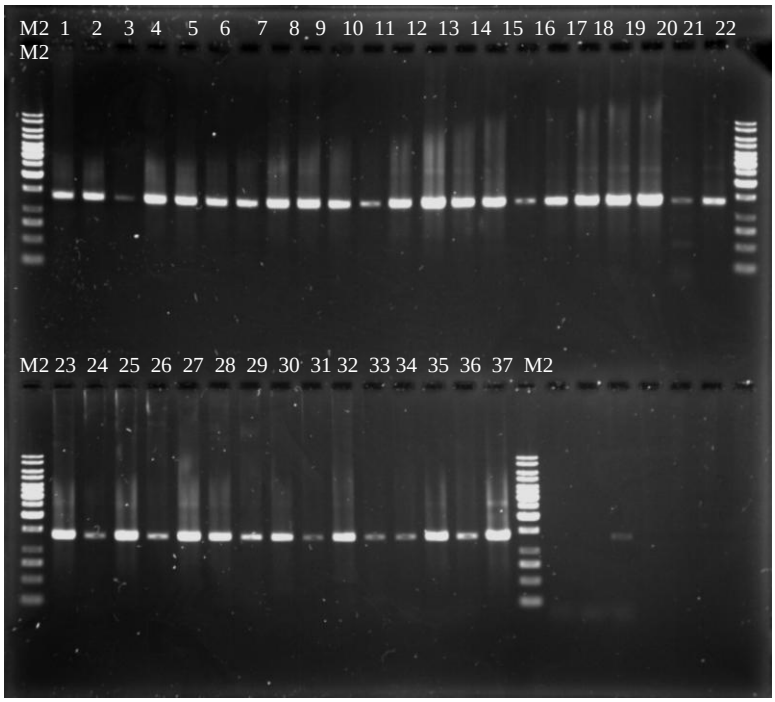


1: Sample 16
2: Sample 5
3: Sample 26
4: Sample 22
5: Sample 7ii
6: Sample 23
7: Sample 4ii
8: Sample 7i
9: Sample 8
10: Sample 4i
11: Sample 5i
12: Sample 48
13: Sample 1i
14: Sample 3i
15: Sample 29
16: Sample 25ii
17: Sample 21
18: Sample 2ii
19: Sample 2i
20: Sample 25i
21: Sample 3ii
22: adult Ch.mega without ethanol

23: Sample 31i
24: Sample 33
25: Sample 11
26: adult Ch.mega without ethanol
27: empty pupa Ch.mega without ethanol
28: adult Ch.mega in ethanol
29: empty pupa Ch.mega in ethanol
30: adult Ch.rufi without ethanol
31: empty pupa Ch.rufi without ethanol
32: adult Ch.rufi in ethanol
33: empty pupa Ch.rufi in ethanol
34: egg Ch.rufi without ethanol
35: 1st instar Ch.rufi without ethanol
36: 2nd instar Ch.rufi without ethanol
37: 3rd instar Ch.rufi without ethanol
38: pupa Ch.rufi without ethanol
39: egg Ch.rufi in ethanol
40: 1st instar Ch.rufi in ethanol
41: pupa Ch.rufi in ethanol
M2: Perfect Plus 1kb DNA Ladder (EURx)

Purified PCR products of COII (Batch 1)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

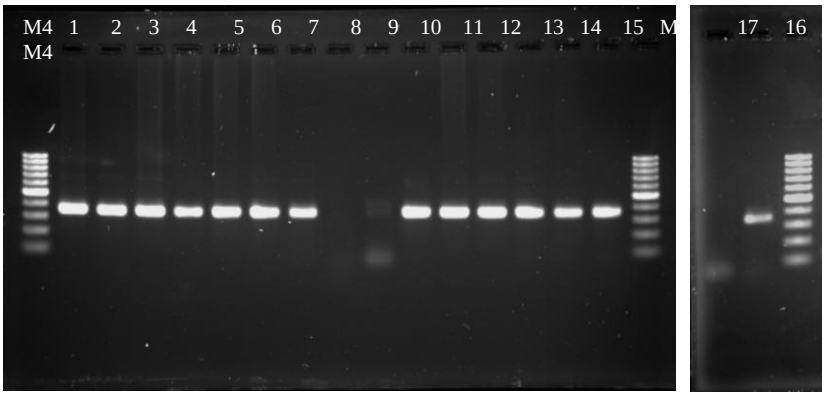


1: Sample 5ii
2: Sample 26
3: Sample 6ii
4: Sample 22
5: Sample 7ii
6: Sample 23
7: Sample 4ii
8: Sample 7i
9: Sample 8
10: Sample 4i
11: Sample 27
12: Sample 3i
13: Sample 29
14: Sample 25ii
15: Sample 21
16: Sample 2ii
17: Sample 2i
18: Sample 25i
19: Sample 3ii
20: Sample 31i

21: Sample 33
22: Sample 11
23: adult Ch.mega without ethanol
24: empty pupa Ch.mega without ethanol
25: adult Ch.mega in ethanol
26: empty pupa Ch.mega in ethanol
27: adult Ch.rufi without ethanol
28: adult Ch.rufi in ethanol
29: empty pupa Ch.rufi in ethanol
30: egg Ch.rufi without ethanol
31: 1st instar Ch.rufi without ethanol
32: 2nd instar Ch.rufi without ethanol
33: 3rd instar Ch.rufi without ethanol
34: pupa Ch.rufi without ethanol
35: egg Ch.rufi in ethanol
36: 1st instar Ch.rufi in ethanol
37: pupa Ch.rufi in ethanol
M3: 1kb DNA Ladder (Fermentas)

Purified PCR products of complete COI (Batch 1)

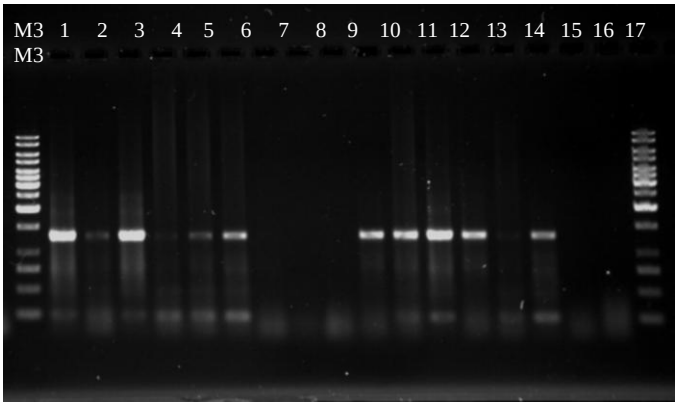
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

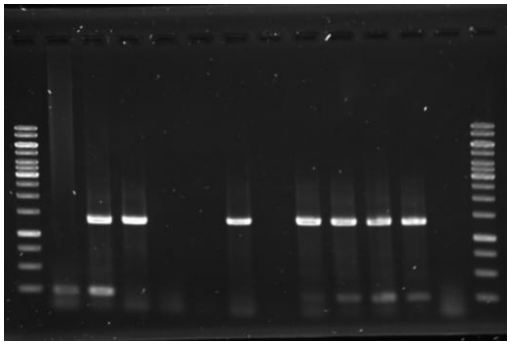


1: pupa Ch.mega without ethanol
2: egg Ch.mega without ethanol
3: pupa Ch.mega in ethanol
4: 1st instar Ch.mega without ethanol
5: 3rd instar Ch.mega without ethanol
6: 2nd instar Ch.mega without ethanol
7: Sample 34
8: Sample 35
9: Sample 9
10: Sample 19
11: 3rd instar Ch.mega in alcohol
12: 2nd instar Ch.mega in alcohol
13: 1st instar Ch.mega in alcohol
14: egg Ch.mega in alcohol
15: Sample 15i
16: Sample 32
17: Negative control-COI
M4: 100bp DNA Ladder (Fermentas)

PCR products of partial COI (Batch 2)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

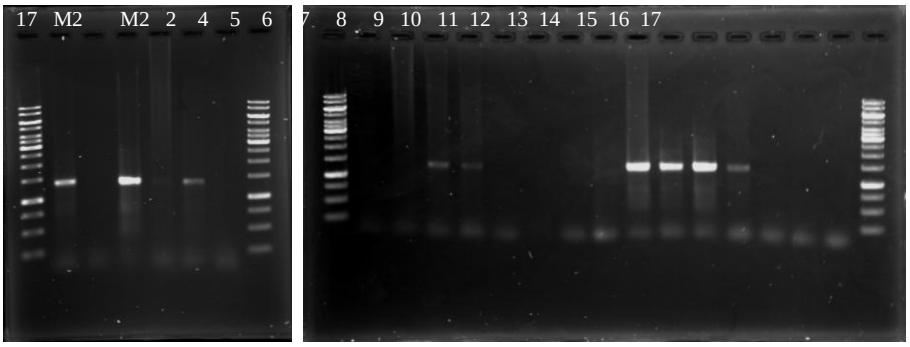




1: pupa Ch.mega without ethanol
2: egg Ch.mega without ethanol
3: pupa Ch.mega in ethanol
4: 1st instar Ch.mega without ethanol
5: 3rd instar Ch.mega without ethanol
6: 2nd instar Ch.mega without ethanol
7: Sample 34
8: Sample 35
9: Sample 9
10: Sample 19
11: 3rd instar Ch.mega in alcohol
12: 2nd instar Ch.mega in alcohol
13: 1st instar Ch.mega in alcohol
14: egg Ch.mega in alcohol
15: Sample 15i
16: Sample 32
17: Negative control-COII
M3: 1kb DNA Ladder (Fermentas)

PCR products of COII (Batch 2)

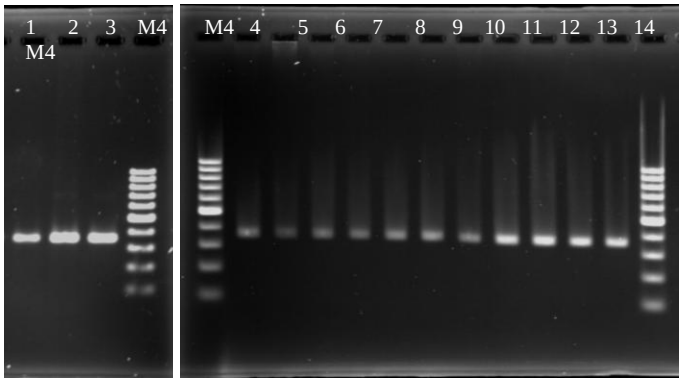
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



1: pupa Ch.mega without ethanol
2: egg Ch.mega without ethanol
3: pupa Ch.mega in ethanol
4: 1st instar Ch.mega without ethanol
5: 3rd instar Ch.mega without ethanol
6: 2nd instar Ch.mega without ethanol
7: Sample 34
8: Sample 35
9: Sample 9
10: Sample 19
11: 3rd instar Ch.mega in alcohol
12: 2nd instar Ch.mega in alcohol
13: 1st instar Ch.mega in alcohol
14: egg Ch.mega in alcohol
15: Sample 15i
16: Sample 32
17: Negative control-TL
M2: Perfect Plus 1kb DNA Ladder (EURx)

PCR products of complete COI (Batch 2)

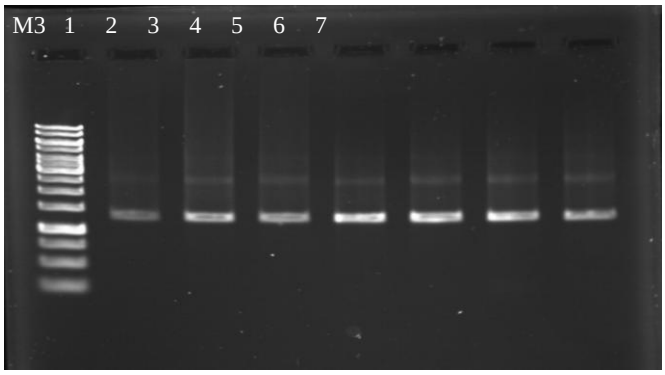
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



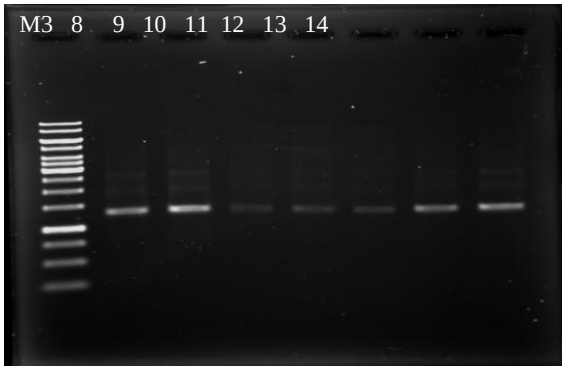
1: pupa Ch.mega without ethanol
2: egg Ch.mega without ethanol
3: pupa Ch.mega in ethanol
4: 1st instar Ch.mega without ethanol
5: 3rd instar Ch.mega without ethanol
6: 2nd instar Ch.mega without ethanol
7: Sample 34
8: Sample 19
9: 3rd instar Ch.mega in alcohol
10: 2nd instar Ch.mega in alcohol
11: 1st instar Ch.mega in alcohol
12: egg Ch.mega in alcohol
13: Sample 15i
14: Sample 32
M4: 100bp DNA Ladder (Fermentas)

Purified PCR products of partial COI (Batch 2)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



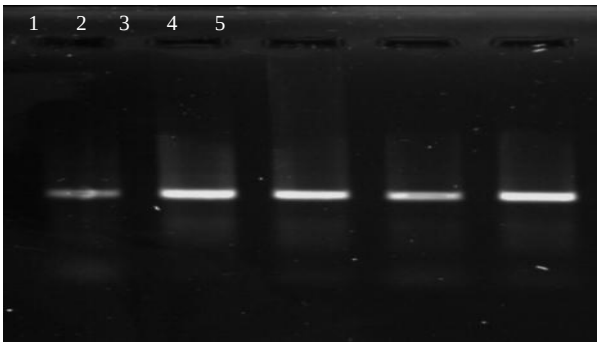
1: 2nd instar Ch.mega without ethanol
2: Sample 34
3: Sample 19
4: 2nd instar Ch. mega in ethanol
5: 1st instar Ch.mega in ethanol
6: egg Ch.mega in ethanol
7: Sample 15i
M3: 1kb DNA Ladder (Fermentas)



8: pupa Ch.mega without ethanol
9: pupa Ch.mega in ethanol
10: 3rd instar Ch.mega without ethanol
11: 2nd instar Ch.mega without ethanol
12: 3rd instar Ch.mega in ethanol
13: 2nd instar Ch.mega in ethanol
14: 1st instar Ch.mega in ethanol
M3: 1kb DNA Ladder (Fermentas)

Purified PCR products of COII (Batch 2)

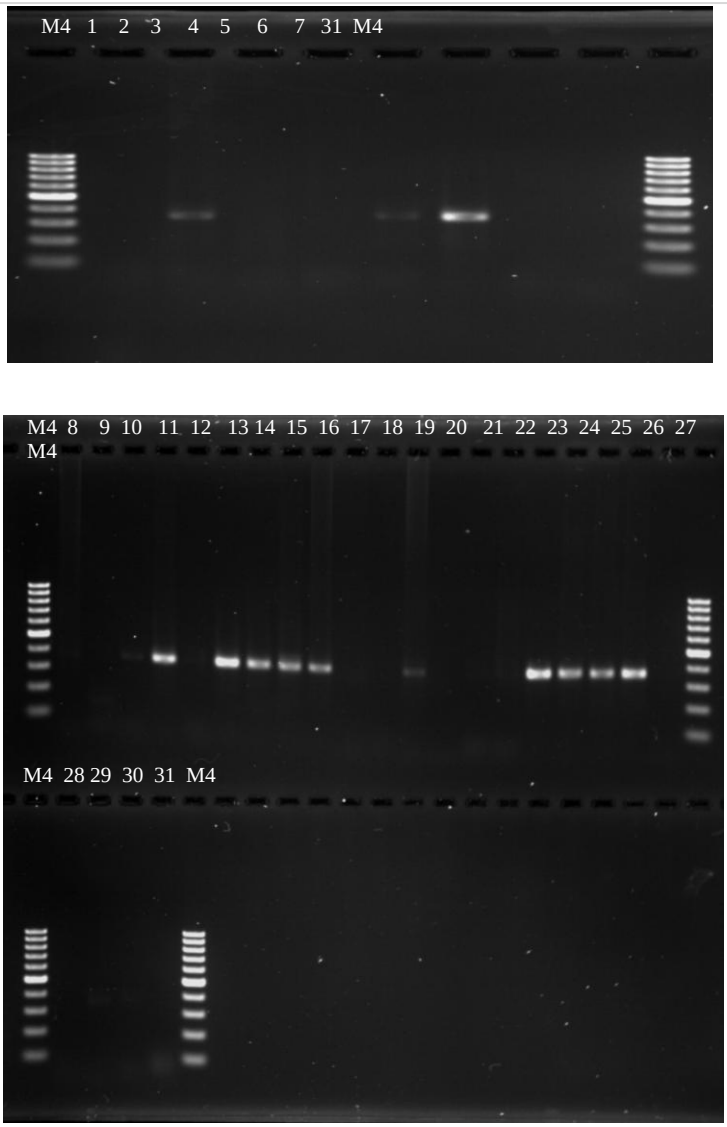
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



1: pupa Ch.mega without ethanol
2: pupa Ch.mega in ethanol
3: 3rd instar Ch.mega in ethanol
4: 2nd instar Ch.mega in ethanol
5: 1st instar Ch.mega in ethanol

Purified PCR products of complete COI (Batch 2)

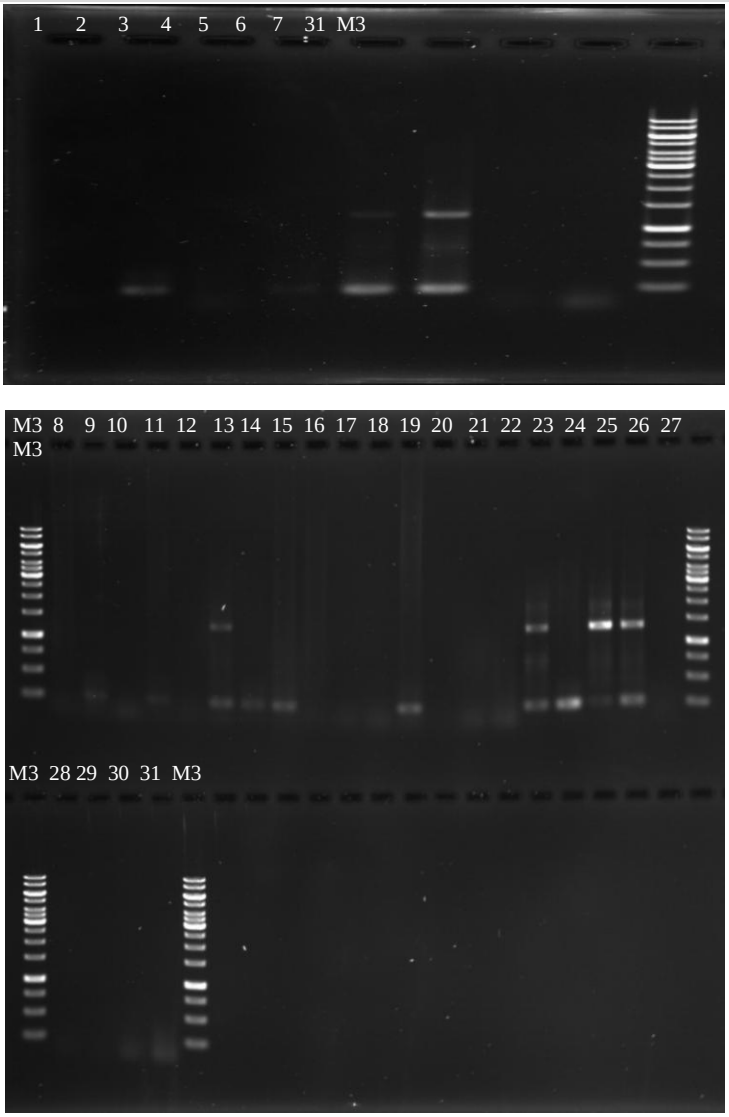
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



1: Sample 9
2: Sample 19
3: Sample 35
4: Sample 34
5: Sample 15i
6: Sample 15ii
7: Sample 32
8: Sample 36
9: Sample 37
10: Sample 38
11: Sample 39
12: Sample 40
13: Sample 41
14: Sample 42
15: Sample 12
16: Sample 44
17: Sample 45
18: Sample 46
19: Sample 14
20: Sample 17i
21: Negative control-COI
22: Negative control-COI
23: Sample 49
24: Sample 50
25: 2nd instar Ch. rufi in ethanol
26: 3rd instar Ch.rufi in ethanol
27: empty pupa Ch.rufi in ethanol
28: empty pupa Ch.rufi without ethanol
29: empty pupa Ch.mega in ethanol
30: empty pupa Ch.mega without ethanol
31: Negative control-COI
M4: 100bp DNA Ladder (Fermentas)

PCR products of partial COI (Batch 3)

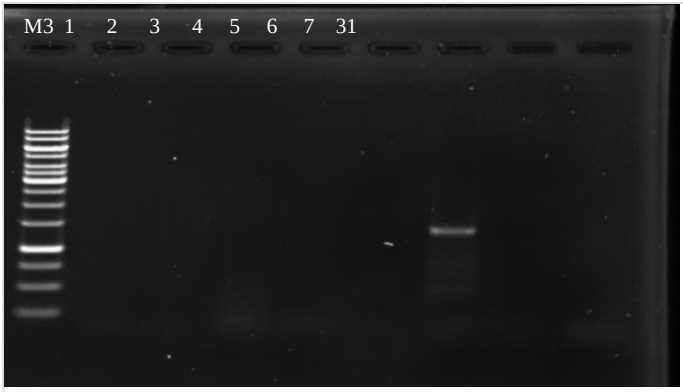
*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

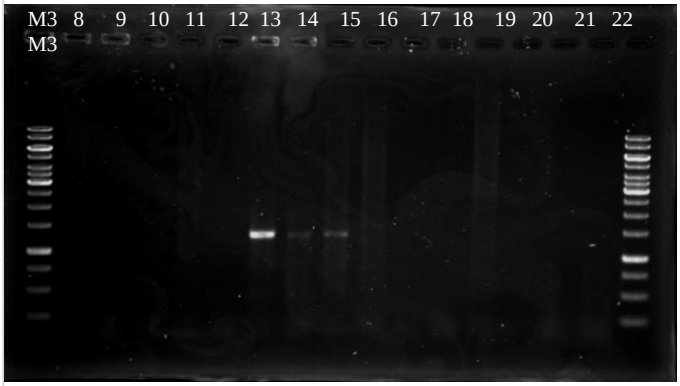


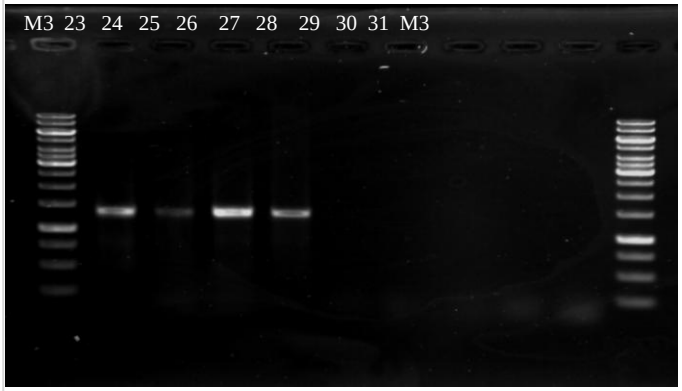
1: Sample 9
2: Sample 19
3: Sample 35
4: Sample 34
5: Sample 15i
6: Sample 15ii
7: Sample 32
8: Sample 36
9: Sample 37
10: Sample 38
11: Sample 39
12: Sample 40
13: Sample 41
14: Sample 42
15: Sample 12
16: Sample 44
17: Sample 45
18: Sample 46
19: Sample 14
20: Sample 17i
21: Negative control-COII
22: Negative control-COII
23: Sample 49
24: Sample 50
25: 2nd instar Ch. rufi in ethanol
26: 3rd instar Ch.rufi in ethanol
27: empty pupa Ch.rufi in ethanol
28: empty pupa Ch.rufi without ethanol
29: empty pupa Ch.mega in ethanol
30: empty pupa Ch.mega without ethanol
31: Negative control-COII
M3: 1kb DNA Ladder (Fermentas)

PCR products of COII (Batch 3)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



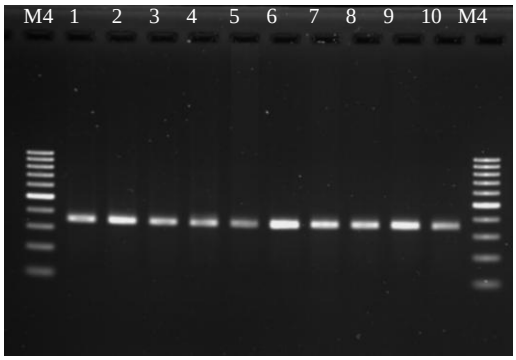




1: Sample 9
2: Sample 19
3: Sample 35
4: Sample 34
5: Sample 15i
6: Sample 15ii
7: Sample 32
8: Sample 36
9: Sample 37
10: Sample 38
11: Sample 39
12: Sample 40
13: Sample 41
14: Sample 42
15: Sample 12
16: Sample 44
17: Sample 45
18: Sample 46
19: Sample 14
20: Sample 17i
21: Negative control-TL
22: Negative control-TL
23: Sample 49
24: Sample 50
25: 2nd instar Ch. rufi in ethanol
26: 3rd instar Ch.rufi in ethanol
27: empty pupa Ch.rufi in ethanol
28: empty pupa Ch.rufi without ethanol
29: empty pupa Ch.mega in ethanol
30: empty pupa Ch.mega without ethanol
31: Negative control-TL
M3: 1kb DNA Ladder (Fermentas)

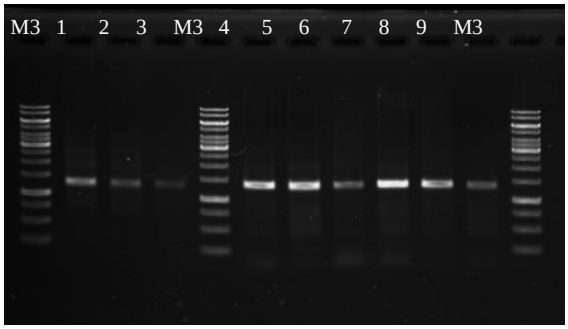
PCR products of complete COI (Batch 3)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*



1: Sample 15ii
2: Sample 39
3: Sample 41
4: Sample 42
5: Sample 12
6: Sample 44
7: Sample 49
8: Sample 50
9: 2nd instar Ch. rufi in ethanol
10: 3rd instar Ch.rufi in ethanol
M4: 100bp DNA Ladder (Fermentas)

Purified PCR products of partial COI (Batch 3)



1: Sample 15ii
2: 2nd instar Ch. rufi in ethanol
3: 3rd instar Ch.rufi in ethanol
4: Sample 15ii
5: Sample e 41
6: Sample 49
7: Sample 50
8: 2nd instar Ch. rufi in ethanol
9: 3rd instar Ch.rufi in ethanol
M3: 1kb DNA Ladder (Fermentas)

Purified PCR products of COII and complete COI (Batch 3)

*Note:Ch.mega = *Chrysomya megacephala*
Ch.rufi = *Chrysomya rufifacies*

APPENDIX E: AMPLIFICATION RESULTS FOR ANALYSIS ON MITOCHONDRIAL DNA FOR COI (PARTIAL AND COMPLETE) AND COII GENES

Sample	Proses	Partial COI		COII		Complete COI		Quantity	$A_{260/280}$	Concentration (ng/ul)
		F	R	F	R	F	R			
1i	PCR	√		F		F		3	-	-
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
1ii	PCR	√		F		F		3	-	-
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
2i	PCR	√		√		√		3	-	-
	SEQ.	√	√	ESL	ESL	√	√	6		
	ALIGN.	√		-		√		2		
2ii	PCR	√		√		√		3	-	-
	SEQ.	√	√	ESL	ESL	√	√	6		
	ALIGN.	√		-		√		2		
3i	PCR	√		√		√		3	-	-
	SEQ.	√	√	ESL	ESL	√	√	6		
	ALIGN.	√		-		√		2		
3ii	PCR	√		√		√		3	-	-
	SEQ.	√	√	ESL	ESL	√	√	6		
	ALIGN.	√		-		√		2		
4i	PCR	√		√		√		3	-	-
	SEQ.	√	√	√	√	√	√	6		
	ALIGN.	√		√		√		3		
4ii	PCR	√		√		√		3	-	-
	SEQ.	√	√	ESL	ESL	√	√	6		
	ALIGN.	√		-		√		2		
5i	PCR	√		√		√		3	1.90	43.37
	SEQ.	√	√	√	ESL	√	√	6		
	ALIGN.	√		√		√		3		
5ii	PCR	√		√		F		3	-	-
	SEQ.	√	√	ESL	F	-	-	4		
	ALIGN.	√		-		-		1		
6i	PCR	√		F		F		3	-	-
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		

6ii	PCR	√		F		F		3	1.46	18.38			
	SEQ.	√	√	-	-	-	-	2					
	ALIGN.	√		-		-		1					
7i	PCR	√		√		√		3	-	-			
	SEQ.	√	√	√	ESL	√	√	6					
	ALIGN.	√		√		√		3					
7ii	PCR	√		√		√		3	-	-			
	SEQ.	√	√	√	√	√	√	6					
	ALIGN.	√		√		√		3					
8	PCR	√		√		√		6	-	-			
	SEQ.	√	√	√	ESL	√	√	3					
	ALIGN.	√		√		√		3					
9	PCR	F		F		F		3	1.26	4.68			
	SEQ.	-	-	-	-	-	-	0					
	ALIGN.	-		-		-		0					
10i	PCR	√		F		F		3	-	-			
	SEQ.	√	√	-	-	-	-	2					
	ALIGN.	√		-		-		1					
10ii	PCR	√		F		F		3	-	-			
	SEQ.	√	√	-	-	-	-	2					
	ALIGN.	√		-		-		1					
11	PCR	√		√		√		3	-	-			
	SEQ.	√	√	ESL	F	√	√	6					
	ALIGN.	√		-		√		2					
12	PCR	√		F		F		3	1.99	25.68			
	SEQ.	F	F	-	-	-	-	2					
	ALIGN.	-		-		-		0					
13	PCR	F		F		F		3	-	-			
	SEQ.	-	-	-	-	-	-	0					
	ALIGN.	-		-		-		0					
14	PCR	F		F		F		3	2.01	15.68			
	SEQ.	-	-	-	-	-	-	0					
	ALIGN.	-		-		-		0					
15i	PCR	√		√		F		3	1.12	4.11			
	SEQ.	√	√	ESL	ESL	-	-	4					
	ALIGN.	√		-		-		1					

15ii	PCR	√		F		√		3		
	SEQ.	√	√	ESL	ESL	√	√	6	1.14	7.08
	ALIGN.	√		-		√		2		
16	PCR	√		√		F		3		
	SEQ.	√	√	ESL	F	-	-	4	1.67	20.60
	ALIGN.	√		-		-		1		
17i	PCR	F		F		F		3		
	SEQ.	-	-	-	-	-	-	0	2.55	2.27
	ALIGN.	-		-		-		0		
17ii	PCR	F		F		F		3		
	SEQ.	-	-	-	-	-	-	0	-	-
	ALIGN.	-		-		-		0		
18	PCR	√		F		F		3		
	SEQ.	√	√	-	-	-	-	2	1.53	36.16
	ALIGN.	√		-		-		1		
19	PCR	√		√		F		3		
	SEQ.	√	√	ESL	F	-	-	4	2.42	11.35
	ALIGN.	√		-		-		1		
20	PCR	F		F		F		3		
	SEQ.	-	-	-	-	-	-	0	-	-
	ALIGN.	-		-		-		0		
21	PCR	√		√		√		3		
	SEQ.	√	√	√	√	√	√	6	-	-
	ALIGN.	√		√		√		3		
22	PCR	√		√		√		3		
	SEQ.	√	√	√	√	√	√	6	1.59	22.08
	ALIGN.	√		√		√		3		
23	PCR	F		F		F		3		
	SEQ.	-	-	-	-	-	-	0	-	-
	ALIGN.	-		-		-		0		
24	PCR	F		F		F		3		
	SEQ.	-	-	-	-	-	-	0	1.66	122.79
	ALIGN.	-		-		-		0		
25i	PCR	√		√		√		3		
	SEQ.	√	√	ESL	ESL	√	√	6	-	-
	ALIGN.	√		-		√		2		

25ii	PCR	√		√		√		3	-	-
	SEQ.	√	√	√	√	√	√	6		
	ALIGN.	√		√		√		3		
26	PCR	√		√		√		3	-	-
	SEQ.	√	√	√	√	√	√	6		
	ALIGN.	√		√		√		3		
27	PCR	√		F		√		3	-	-
	SEQ.	√	√	-	-	√	√	4		
	ALIGN.	√		-		√		2		
28	PCR	√		F		F		3	-	-
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
29	PCR	√		√		√		3	-	-
	SEQ.	√	√	√	√	√	√	6		
	ALIGN.	√		√		√		3		
30	PCR	√		F		F		3	1.77	21.47
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
31i	PCR	√		√		√		3	-	-
	SEQ.	√	√	ESL	ESL	√	√	6		
	ALIGN.	√		-		√		2		
31ii	PCR	F		F		F		3	-	-
	SEQ.	-	-	-	-	-	-	-		
	ALIGN.	-		-		-		0		
32	PCR	√		F		F		3	1.07	15.72
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
33	PCR	√		√		√		3	-	-
	SEQ.	√	√	√	√	N	N	6		
	ALIGN.	√		√		-		2		
34	PCR	√		√		F		3	1.90	11.30
	SEQ.	√	√	ESL	F	-	-	4		
	ALIGN.	√		-		-		1		

35	PCR	F		F		F		3	1.77	14.48
	SEQ.	-	-	-	-	-	-	0		
	ALIGN.	-		-		-		0		
36	PCR	F		F		F		3	2.14	18.78
	SEQ.	-	-	-	-	-	-	0		
	ALIGN.	-		-		-		0		
37	PCR	F		F		F		3	1.81	6.98
	SEQ.	-	-	-	-	-	-	0		
	ALIGN.	-		-		-		0		
38	PCR	√		F		F		1	1.89	5.98
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
39	PCR	F		F		F		3	2.01	8.79
	SEQ.	-	-	-	-	-	-	0		
	ALIGN.	-		-		-		0		
40	PCR	F		F		F		3	3.05	2.72
	SEQ.	-	-	-	-	-	-	0		
	ALIGN.	-		-		-		0		
41	PCR	√		F		√		2	1.20	6.12
	SEQ.	√	√	-	-	√	√	4		
	ALIGN.	√		-		√		2		
42	PCR	√		F		F		1	2.02	5.58
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
43	PCR	√		F		F		3	1.50	21.83
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
44	PCR	√		F		F		3	0.98	2.07
	SEQ.	√	√	-	-	-	-	2		
	ALIGN.	√		-		-		1		
45	PCR	F		F		F		3	1.60	11.37
	SEQ.	-	-	-	-	-	-	0		
	ALIGN.	-		-		-		0		
46	PCR	F		F		F		3	3.00	3.97
	SEQ.	-		-	-	-	-	0		
	ALIGN.	-		-		-		0		

47	PCR	√		F		F		3	-	-			
	SEQ.	√	√	-	-	-	-	2					
	ALIGN.	√		-		-		1					
48	PCR	√		F		F		3	-	-			
	SEQ.	√	√	-	-	-	-	2					
	ALIGN.	√		-		-		1					
49	PCR	√		F		√		2	0.92	1.74			
	SEQ.	√	√	-	-	√	√	4					
	ALIGN.	√		-		√		2					
50	PCR	√		F		√		2	1.93	7.18			
	SEQ.	√	√	-	-	√	√	4					
	ALIGN.	√		-		√		2					

Note: √ = done; ESL = early signal loss; N = noise data; F = failed; - = not performed; PCR = Polymerase Chain Reaction; SEQ = sequencing; ALIGN. = Alignment.

STANDARDS AND GUIDELINES IN FORENSIC ENTOMOLOGY

Forensic entomology cases require appropriate collection and continuity of insect evidence as with any other evidence recovered from a crime scene. Ideally, an entomology expert is hands on to carry out these duties leading to the later analysis and interpretation of insect evidence. In the real world, the responsibility of first collection and continuity of potential insect evidence often lies with the crime scene police officers. In turn, they must pass on the evidence to the forensic entomologist. These guidelines are specifically for forensic crime scene police officers. The main purpose of this Standard Operating Procedure (SOP) is to encourage a high level of competency in the field of forensic entomology, to promote and establish common standards of practice, especially with respect to the collection and preservation of entomological evidence and corresponding temperature data and this SOP has been altered from the article with the title of 'Best practice in forensic entomology standards and guidelines' which has been published in International Journal of Legal Medicine by Dr. Jens Amendt from Institute of Forensic Medicine, University of Frankfurt. The first and most important stage of the procedure in forensic entomology involves careful and accurate collection of insect evidence at the scene. This involves knowledge of the insect behavior, therefore it is best performed by an entomologist. Unfortunately, the entomologist is often not called to the crime scene so the evidence is dependent on accurate collection by the crime scene police officers.

Entomological evidence is likely to be collected by forensic investigators including personnel from the office of Medical Examiner, crime scene police officers or pathologists. In order to ensure the accuracy of the collection of entomological evidence at scene or at autopsy, the following concepts and procedures should be observed by forensic investigators. A systematic, quality-assured approach should also exist for collection, preservation and even packing and transport of entomological samples, not only to prevent contamination or destruction of evidence and to guarantee the chain of custody but also because forensic entomology deals with living organisms which should be treated with care.

EQUIPMENTS, TOOLS AND PRESERVATIVES

The collection of entomological evidence at the crime scene, especially from the dead human bodies, requires the wearing of protective clothing mainly to avoid any contamination of the scene with fibers or other materials from the investigators. In particular, it is strongly recommended that forensic entomology practitioners wear overalls, gloves and shoe covers or boots. For the collection of insects and additional information at the scene, the following equipment's are recommended:

a) Tool box

b) Protocol sheets for writing down what specimens were collected, when and where

c) Dark graphite pencil or pen with waterproof and alcohol-proof ink (do not use standard inks, because they will dissolve in wet surroundings or if splashed with ethanol)

d) Labels

e) Fine and medium forceps (with different levels of spring tension for collecting adults and the more fragile immature insects)

f) Spoons for collecting maggots

g) Fine paintbrush for collecting eggs (after moistening the brush)

h) Vials and storage boxes of different sizes for preserving living and dead insects

i) Shovel or trowel for taking soil and leaf-litter samples and searching for buried larvae/pupae

j) Robust plastic or paper (double bagged) bags for soil samples and leaf litter

k) Sawdust or tissue paper for handling eggs and living larvae in vials or storage boxes

l) Thermometer for measuring the body and ambient temperatures, as well as the larval mass temperature

m) Ethanol (70%) for storing specimens

n) Camera or video for picture documentation (photographic evidence should include a measurement scale)

o) Material for sealing the samples (sticker or sealing wax)

p) Plastic container with ventilation can be used for storing living insect samples

q) Handheld insect capture net for catching flying insects, if necessary

r) Temperature data longer for measuring the scene temperature at least for 30 days period after body recovery

s) Measuring tape

METHODS FOR COLLECTING ENTOMOLOGICAL EVIDENCE

Samples of insects of all stages should be collected from different areas of the body, from the clothing and from the soil or carpet. Insects will often congregate in wounds and in and around natural orifices. If collecting specimens that are already dead, regardless of whether they are adult or immature, store them in 70% ethanol. Use 70% ethanol to preserve specimens and for health and safety reasons and because tissues are less well-preserved for morphological and molecular identification, do not use water, formalin/formaldehyde or other preservatives.

Flies can be found as:

i) Eggs (in egg masses usually)

ii) Larvae or maggots (in range of sizes)

iii) Pupae and/or empty cases

iv) Adults

If collecting living specimens:

Eggs: Fly eggs are laid in batches and normally laid in or near dark, moist orifices of the body, such as the ears, nose, eye lids, mouth or genitalia. They may also be laid in folds of skin behind the ears, in joint creases, or on clothing which has absorbed body fluid exudates. So it is important that all sides of the body are examined and it may be necessary to attend the post mortem to check further for insects, if the body is fully clothed or has been wrapped in something. The individual clumps of eggs can be collected with a child's paint brush dipped in water or with forceps and carefully placed in a container without any food.

Half should be preserved in 70% ethanol. The rest should be placed in a vial with a little damp tissue paper to prevent dehydration and provided with beef liver prior to be receiving by entomologist. Make sure there is tissue or sawdust present if liver is added, to prevent drowning during putrefaction of liver and use ventilated tubes and jars. Newly emerged maggots can escape through holes, so a paper towel held over the top of the vial with a rubber band is excellent, as long as the vial stays upright.

Maggots: Larvae will be located as the body is searched for eggs. They too tend to be in the orifices, such as the eyes, ears, nose and so on, including any wounds which were made on the body. Collect different sizes of maggots. Maggots will be found crawling on or near the remains and may be in maggot masses. The masses generate heat. The larvae should be collected from each site in batches of 20–30 per jar, so that no additional heat is generated during transit.

More than one collection jar per infestation site may be needed. The first instar is the smallest and most vulnerable of the three larvae stages and the larvae, if sampled at this stage, it can easily die. It is necessary, therefore to protect them from drying out when collecting and culturing these larvae from a corpse at a crime scene. If a larvae mass is noted, it should be photographed and the mass temperature should be taken, prior to the location being sampled. The temperature of every maggot mass should be taken at each site on the body and labelled which maggots come from a particular mass, so that this can be taken into consideration when calculating the crime scene thermal history.

If possible, store the majority of individuals of each sample in vials under controlled conditions (temperature or humidity known); cool temperatures are ideally at room temperature in Malaysia. The vials should allow entry of air but prevent the escape of maggots. They should be lined with coarse sawdust or tissue paper to absorb fluids produced by the maggots. The living samples should be transferred to an expert for rearing within 24 hours. Label containing information as shown below, should be included inside and outside the collecting jars used at a crime scene.

Crime Scene No.:
Officer in charge:
Collector:
Date:
Item No.:
Location and description:

Label for the colleting jar

Kill the remaining specimens with hot (60°C), but not boiling water, by immersion for 30 seconds is ideal to achieve best preservation. Boiling water will cause darkening of the specimen. Afterwards pour off the water and rinse once with ethanol before storing them in a vial with 70% ethanol. All procedures should be undertaken as soon after collection as possible and with all times recorded. If hot water is not available at the scene, the larvae should be killed as soon as possible at the laboratory or the use of hot water could be replaced by killing the larvae in a deep freezer (ideally -20°C) for 30 minutes. Do not insert living larvae directly into ethanol because they will darken within a few days due to putrefaction and they will also shrink. Shrinkage will make the estimation of real length difficult and can hide some morphological characters.

Pupae: Fly pupae are usually found some distance from the body. The third instar post feeding larvae stages migrate and can be found in soil 3 to 5 cm below the soil surface, in pockets, under carpets, in leaf litter or in any nooks and crannies which are available in buildings. An organized search strategy should be used to do this. The best method is to search on a grid of a metre apart over a 36 square metre area

surrounding the body, if it is not in a house. This is a slow, time-consuming activity in which the soil should be sampled at the intercepts of the grid, using a trowel to a depth of 10 cm. The soil may need to be sieved over a tray or it can be hand-searched.

If the pupae are still on the body then either there may have been some restriction to larvae migration or a particular species of insect is indicated. Pupae changes colour from white to dark brown over time, so all pupae, of whatever colour, should be collected. The pupae collected from crime scene are placed in a container with a moist paper towel and suitably labelled. They do not require feeding but should be taken back to the laboratory for identification. The pupae should be cultured through to emergence if at all possible, so that species identification can be confirmed. The empty puparium case should also be retained as additional evidence. Those which do not hatch provide the examples of preserved specimens from the scene. If possible, transfer pupae for rearing within 24 hour, otherwise, store each sample in vials under controlled known temperature and humidity conditions. Cool temperatures are ideally at room temperature in Malaysia. The lid of the vials should be punched with small holes to allow entry of air.

Maintain living insect samples under known temperature conditions

Adults: Flying insects present at the scene should be collected first using a net, before hand-collecting any specimens from the body. This is because they are most easily captured using a net and may disappear if disturbed. Insects such as beetles, which are visible on the surface of the body, can be collected by hand-picking and placed in individual labelled containers. Live adults should be killed by placing into a vial which is then put into a freezer (ideally set at -20°C) for 30 minutes. Afterwards, store the dead specimens in 70% ethanol. If facilities are available for pinning adult specimens, then this can be done, because it often simplifies identification. Adults that are newly emerged from their puparium should only be killed after allowing the wings to fully harden and the colouring to develop.

Insect remnants: Remnants such as pupae skins, empty puparium or beetle faeces, which document previous insect activity and presence, should be stored, when completely dehydrated, in dry conditions in vials or in 70% ethanol.

Beetles: Can be found as adults, larvae or grubs, pupae and also as cast skins. All stages are equally important. They move fast and are often found under the body and in and under clothing. They can be placed in vials with some air. They only need to be fed if it will be more than 24 hrs before they reach an entomologist. If necessary they can be fed with extra maggots. They are cannibalistic so should not be placed in the same vial.

Other insects: Other insects may be present. If you are not sure whether it is an insect, collect it anyway and place in a vial.

Other samples: Soil and leaf litter samples will also be useful. About a coffee can size of soil from under or very near the body is useful. If the soil below the body is extremely wet, it is better to collect the soil from near the remains.

WHERE TO COLLECT THE ENTOMOLOGICAL EVIDENCE

It is fundamental to note that the collection of insects and other arthropods from a crime scene may interrupt the remains. Therefore, the forensic entomologist or the crime scene police officers during collection should contact the investigative police officer and make proper planning for the collection of entomological evidence. Upon deciding on the method chosen, utmost care should be taken during insect collection so that the remains are not disturbed as much as possible. Before collections are made, notes should be taken as to the general habitat, ambient weather conditions, and location of the dead body. Observations should also be done to describe the microhabitat that is surrounding the dead body.

On the corpse at the death scene or during autopsy

It is essential that collection of insect evidence and temperature data from the dead body requires the cooperation of the crime scene police officers or the forensic pathologist, depending on the person in charge at the scene or at the autopsy. One must be careful that nothing is moved or taken from the deceased without specific authorization from the relevant authority. In every case, extreme caution should be exercised when using forceps or other tools for collecting insects to avoid inadvertently inflicting postmortem artifacts on the deceased.

Sites for sampling should include:

i) The natural orifices and eyes

ii) Traumatic wounds

iii) At the corpse-substrate interface and under the body

iv) In the pleats of clothes and pockets, shoes and socks

v) From the carpet, bag or material in which the body might have been wrapped

vi) From the plastic body bag in which the corpse or the remains have been enclosed for transport to the place of autopsy and storage

vii) Collect from everywhere on the corpse, not only from the locations of most concentrated insect activity

viii) Collect specimens of every shape and size of larvae or pupae, using different vials for different types and sizes. Try to collect specimens of every types and sizes

ix) Sample size will vary depending on the number of larvae found, but as a guide, it should range from all the larvae, where fewer than 100 are available, to 1-10% of the larvae, where thousands are available

If adult insects present:

i) Obviously recently emerged blowflies, which are recognized by a silvery appearance and shriveled wings, with the behavior of creeping or running on the ground

ii) Dead flies or beetles lying on the ground or on the window sills

iii) If possible, collect flying insects with an insect net

Around the deceased at the crime scene

It is strongly recommended to collect entomological specimens from the surrounding area before removal of the remains and also from directly under and in close proximity to the remains after removal. One has to search the area away from the body intensively up to at least 2m and even further 10m, depending on the circumstances for insects that might have dispersed from the body. Take a control sample at a distance from the body, beyond the range at which insects are likely to have dispersed from the body to obtain a background level of insect abundance. It is especially essential to look under any stones, rocks and fallen logs outside and under carpets, pillows or skirting boards to ensure that insects are not missed.

Outdoor scene

It is advisable to take soil samples up to 2m away from the corpse, from more than one compass point up to a depth of at least 10cm or even more depending on the circumstances and if available also collect some leaf litter or other soil-covering detritus.

Indoor scene

Larvae can leave the corpse and disperse widely before pupation and could, therefore, be found in different rooms. Collect the most matured insect specimens that have developed on the corpse (adults, pupae, post-feeding and feeding larvae, or eggs) and remnants such as empty fly puparium or beetle exuviae.

MICROCLIMATIC CONDITIONS AND ECOLOGICAL FEATURES OF THE SCENE

i) Describe the conditions of the corpse (position, sunshine or shade, clothes, wounds) using protocol sheets and photographs or video.

ii) Describe the crime scene in detail using protocol sheets and photographs or video.

iii) Collect temperatures: ambient, body, ground surface, soil (up to 10cm depth) and of any larval masses.

iv) Request weather data for the general area of discovery of the body from the nearest meteorological station for the period from last sighting of the deceased up to discovery of the dead body.

v) If possible and depending on the availability of the crime scene, the temperature for one month should be recorded with a temperature data logger at the position of the corpse. It is preferable that these measurements be made hourly using an electronic data logger. If this is no longer possible, daily maximum and minimum temperature records should be obtained.

vi) Temperature inside larval masses could be 10 to 20°C above ambient temperature, therefore one should consider that the heat generated from larval aggregations could prevent the cooling effect of the body in refrigeration units.

vii) Only use reliable temperature data to estimate a time of death based on the developmental stage of the insects.

TRANSPORTATION AND STORAGE OF THE CORPSE

If entomological samples were collected at the scene, it is very important to the forensic entomological investigation that the environmental condition of the dead body be recorded throughout the process of transportation and storage until autopsy is conducted. The dead body is usually transported in a body bag and kept in cold storage. It is imperative that temperature during transportation and storage be recorded and available to the forensic entomologist.

DOCUMENTATION AND THE CHAIN OF CUSTODY

i) Document the name of the instructing authority and principal contact and the time and type of approach

ii) Document date and time of sample collection

iii) Specify the whole sample clearly with a single code (number and name), this code will be your reference to the case in the future and it has to be placed on any sample

iv) Label each vial you use and note the position of sampling (head, leg and carpet) on a protocol sheet

v) Seal each sample to guarantee chain of custody

vi) Name of person who collected the specimen

PROCESSING ENTOMOLGICAL EVIDENCE AT THE LABORATORY

It is strongly recommended to send the insect evidence to an expert in forensic entomology, who is familiar with local species and in processing the specimens at the laboratory. In general, the laboratory should use operating procedures that ensure that all necessary samples and information are documented in a way that can be clearly traced in audit.

Arrival of specimens at the lab

If the samples were not collected personally, make sure to document the following upon arrival of the samples:

i) Who is the instructing authority and what is the time and type of approach?

ii) When and who received the samples?

iii) How the samples were transported (parcel post or courier)?

iv) In what condition were the samples received (did some larvae obviously die during the storage or transport? Were there any seals? If yes, were they whole or damaged)?

v) Specify the whole sample clearly with a single code (number and/or name), this code will be the reference of the case in the future and it has to be placed on every sample.

vi) All of these parameters and data must be mentioned in the report.

vii) Label any storage box and vial with the specific code before removing the seal or opening the containers.

viii) Check the name on the form (Hospital form) if it tally's with the name on the specimen bottle.

ix) Decide if specimens should be reared up to the adult stage. If yes, what quantity of specimens will be reared to the adult stage?

x) The specimen is sent to the correct institution.

xi) Storage of specimens must be under lock and key all the times.

Rearing immature stages

Any living, immature specimen that is not going to be killed should be placed immediately into a rearing chamber. Rear all stages under conditions of temperature and humanity that are controlled and recorded.
This means that:

i) This is done ideally by using a certified and calibrated incubator with constant temperatures or at least by maintaining a careful documentation of the temperature patterns by using a certified temperature data logger inside the incubator.

ii) If an incubator is not available, it is advisable as a minimum measure to rear larvae at ambient temperature, to be able to document the remaining part of the life cycle of the samples and to aid identification, because adults are easier to be identified than those in the immature stages. To ensure a careful documentation of the temperature patterns, for example, with the use of a certified thermometer.

iii) Monitoring larval growth should be done to ensure successful emergence of adults and to document the respective dates of moulting, pupation and hatching.

Treatment of soil and leaf-litter samples

Because a different stage of development shows different levels of mobility, the following is recommended:

i) Examine the samples visually by spreading them on a specially prepared area, such as a framed panel on the bench. Here, the main targets for search are immobile pupae and living larvae for further rearing. A sieve and water flotation can sometimes be helpful to sort soil samples, depending on the type of soil and the type of particulate matter, including leaf litter.

ii) If you are not able to examine the soil samples immediately, seal and store them in refrigerator (~4°C) to avoid further development of immature stages and the growth of mould or fungus.

Preparation and identification of insects

It goes beyond the scope of the present paper to give an introduction into the morphological features of necrophagous insects and their terminology. Valuable introductions exist but of course each geographic region has its own characteristic fauna and very often, an appropriate identification key, which enables the investigator to identify the insects relevant to their case.

i) Label any identified or examined specimen, microscope slide or other sample with the laboratory code for the individual case and with the code of the specific vial. It must be clear, without any ambiguity, which individuals were the basis for the analysis. This will guarantee that a possible future specialist will be able to identify the specific sample on which the analysis was based.

ii) If you are not able to examine and identify the insects, immature or adult, in an appropriate manner, keep them well-labelled in a vial filled with 70% ethanol.

iii) Storage in ethanol ensures that a later DNA analysis for identification is still possible. This may be necessary if a morphological identification is not possible or where morphological determinations have to be confirmed in complex situations (in the case of so-called sister or sibling species, which are morphologically similar or identical, especially in the young larval stages).

iv) Identify immature specimens (especially larvae) using reliable identification keys and establish the most matured adults available in the sample. For larvae, record the stage of development (first, second, third, post-feeding, pre-pupae).

Protocol sheet for the collection of entomological evidence

Collected by: _____

Date/Time: _____

Case No.:_____

Specifications

Age_____ Sex_____ Height_____
Weight_____

Position: Buried ☐ (estimated depth :_____) above ground ☐

Lying ☐ Hanging ☐ (in contact with the ground) ☐

In water ☐

Remarks:_____
_____

Clothing: entirely ☐ Partial ☐ Naked ☐

Body covered ☐ with _____

Remarks:_____
_____

Degree of decomposition: fresh ☐ early decomposition ☐

Advanced decomposition ☐ skeletonization ☐

Bloated ☐

Remarks:_____
_____

Evidence of scavenger*:_____

Wounds*:_____

Scene of death

Outdoor: Forest ☐ Field ☐ Pasture/Grassland ☐ Shrubbery ☐

Public park ☐ on grass/soil ☐ on sealed ground ☐ pond/lake/river ☐

Building: Storehouse ☐ Barn/Stable ☐ Dwelling house ☐ Cottage ☐

Ground (carpet, parquet, etc.):_____

Which room: _____ Heated: ☐ Air condition: ☐

Open window: ☐

Miscellaneous (e.g car):_____

Remarks:_____
_____

Temperature

Record the air temperature at the crime scene for 30 days after the discovery of the body.

NOTES:

- Collect around 20 to 30 maggots for specimens
- A fairly large sample should be collected, representative of the complete range of maggot sizes present
- The sample will be arranged into 2 different containers, one container contains 70% ethanol and another container, empty ventilated container with food for breeding.

Collected by : _____

Date/Time : _____

Case No. :_____