A Molecular Study of the Forensically Important Calliphoridae (Diptera): Implications and Applications for the future of Forensic Entomology

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Bachelor of Science/Bachelor of Arts;
Bachelor of Science Honours (Zoology)

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of
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This thesis is dedicated to
my parents,
Dave and Leonie,
and husband, Michael.
Your faith, love and endless support
have made this possible.
Thank you
SUMMARY

A common application of forensic entomology is the estimation of post-mortem interval (PMI). This is most frequently estimated from the age of calliphorid specimens collected from a corpse, and in many cases it is the immature stages that are encountered. A critical step in the estimation of PMI is the accurate identification of insects to species level, with misidentification potentially resulting in the application of unsuitable developmental data and therefore inaccuracy in the resulting estimate.

Identification has long been attempted on a morphological basis, but complicated by the lack of larval keys to the Calliphoridae, limited diagnostic features in immature stages and the poor preservation of specimens. Standard practice in forensic entomology is the rearing of immatures collected from the corpse through to the more distinctive adult stages, however this process is time-consuming and may be hindered where specimens die during rearing. Furthermore, many cases are presented for forensic entomologist as an afterthought and specimens are already preserved. Consequently, a new approach to the identification of calliphorids is sought which will overcome the problems of the morphological and rearing methods.

This thesis considers the DNA-based identification of forensically important calliphorids, building upon work already contributed to the field, considering important implications of these studies and extending the utility of DNA in calliphorid studies. Prior to the commencement of this study, previous work had often consisted of small-scale studies of locality-specific calliphorid species, or focused on a particular genus or subset of species. This study contributes greatly to the currently small body of data generated for species of global importance.

Critical to any DNA-based identification technique is the selection of a region of DNA to provide distinction at the necessary taxonomic level. In the case of calliphorids, the ability to distinguish between species is vital, however the collection of data displaying population level variation is also desirable. Species such as Chrysomya rufifacies may show behavioural or biological variation that may be indicative of the presence of cryptic species. DNA may display genetic variation between cryptic forms, making population level variation an important secondary factor in the selection of a region of DNA for calliphorid distinction.
The cytochrome oxidase I (COI) gene has been widely used in studies of forensically important calliphorids. This thesis studied the COI gene of individuals representing 27 species across 22 countries, forming the most comprehensive global study of the forensically important calliphorids to date. Analysis was performed using phylogenetic techniques, whereby relationships between individuals could be graphically represented and used to infer evolutionary relationships between taxa. This study showed the ability of COI to successfully distinguish between calliphorid species with the exception of *Ch. megacephala/Ch. saffranea* and *Calliphora stygia/C. albifrontalis* complexes. On an intraspecific level, both *Lucilia cuprina* and *Ch. rufifacies* displayed intraspecific variation, suggesting the need for critical study of the status of these two species.

As a secondary diagnostic tool for the complexes not resolved using the COI gene, both the nuclear ribosomal DNA (rDNA) multigene family and the mitochondrial control region were assessed. The non-coding rDNA internal transcribed spacers (ITS) were shown to successfully separate *C. stygia* and *C. albifrontalis*, however the control region was not able to distinguish between *Ch. megacephala* and *Ch. saffranea* despite indications from previous studies. Limited intraspecific variation was observed over these regions despite their non-coding nature and therefore lack of functional constraint. It is concluded that further taxonomic consideration of *Ch. megacephala*, *Ch. saffranea*, *Ch. rufifacies* and *L. cuprina* is warranted.

The culmination of this study is the consideration of applications of molecular data to forensic entomology. A sequence-specific priming (SSP) technique is presented for the identification of the forensically significant calliphorids of Australia and New Zealand, along with a new method for the extraction and storage of calliphorid DNA samples using Whatman FTA cards. These techniques will potentially improve the efficiency and accuracy of identification in the estimation of PMI using calliphorids.

The use of calliphorid DNA is not limited to PMI estimation, but may also be applied to museum studies. DNA was extracted from pupal casings from 300 year old mummified corpses, however difficulty was encountered in amplifying the DNA reproducibly. This illustrates however, the wide-ranging implications of the calliphorid sequence data gathered in this study.
This thesis makes a significant contribution to the consideration of the status of some global calliphorid species. The new technique presented for identification of Australian and New Zealand species is the culmination of an important body of data that will ultimately contribute to the strong foundation of forensic entomology and our future accuracy, efficiency and utility as a routine investigative tool.
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THESIS STRUCTURE
This thesis is presented as a series of scientific papers prefaced with brief literature reviews. These papers are either published, under review or in the process of being submitted to leading international journals. Details of papers contained within each chapter and my contribution to each are detailed below. I was responsible for the experimental design, data collection and writing of each publication, with the exception of Appendix 1, which I was responsible for the preparation of the paper.

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Chapter 3:

Chapter 4:

Chapter 5:
Chapter 6:
**ML Harvey**, MW Mansell, S Gaudieri & IR Dadour. Extraction of DNA from calliphorid puparia: extending the utility of forensic entomology. In Preparation. Contribution: 95%

Chapter 7:

**Harvey, M.L.,** Dadour, I.R. & Gaudieri, S. A new approach to the rapid identification of forensically significant calliphorids of Australia and New Zealand, In preparation. Contribution: 85%

Supporting Publications in Appendices:

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<table>
<thead>
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<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>adenine</td>
<td></td>
</tr>
<tr>
<td>Acephalic</td>
<td>lacking an external head skeleton</td>
</tr>
<tr>
<td>Apodous</td>
<td>lacking legs</td>
</tr>
<tr>
<td>Aristate</td>
<td>pouch-like antenna with lateral bristle</td>
</tr>
<tr>
<td>Calypter</td>
<td>scale-like structure between the base of the wing and the haltere of a fly</td>
</tr>
<tr>
<td>Coarctate</td>
<td>pupae where last larval stage is contained within the larval skin</td>
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<tr>
<td>COI</td>
<td>cytochrome oxidase I</td>
</tr>
<tr>
<td>Cryptic species</td>
<td>a group of species that satisfy the definition of species but are morphologically indistinguishable</td>
</tr>
<tr>
<td>Cytosine</td>
<td></td>
</tr>
<tr>
<td>dNTP</td>
<td>dinucleotide triphosphates</td>
</tr>
<tr>
<td>Eucephalic</td>
<td>with a distinct head capsule</td>
</tr>
<tr>
<td>Filiform</td>
<td>thread-like antennae</td>
</tr>
<tr>
<td>Guanine</td>
<td></td>
</tr>
<tr>
<td>Halteres</td>
<td>Club-like structures modified from the hind-wings, present throughout the Diptera and important for balance and stabilisation during flight</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>Hemicephalic</td>
<td>with an incomplete head capsule</td>
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<tr>
<td>Indel</td>
<td>insertion/deletion event</td>
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<td>IGS</td>
<td>intergenic spacer</td>
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<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<tr>
<td>NOR</td>
<td>nucleolus organiser region</td>
</tr>
<tr>
<td>Ovoviviparity</td>
<td>Production of eggs that hatch within the mother</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PMI</td>
<td>post-mortem interval</td>
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<tr>
<td>Ptilinum</td>
<td>fluid-filled structure that everts from the front of the fly head to facilitate emergence from puparium</td>
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<tr>
<td>rDNA</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<td>SMRS</td>
<td>specific mate recognition system</td>
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<tr>
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<td>Mouthparts adapted for sucking or clinging</td>
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General Introduction
Forensic entomology is one of the primary tools used for the estimation of postmortem interval (PMI), utilising insects to estimate time since death. The Calliphoridae (blowflies) are the most important insects to colonise a body following death, and these flies, and their application to the forensic sciences, will be the main focus of this thesis.

Insect genetics have become an important facet of forensic science in the last decade, applying DNA-based technologies to entomological evidence to increase the applications, accuracy and efficiency of forensic entomology in a legal context. This thesis will evaluate the molecular approach to insect evidence, using the data gathered to consider both entomological and forensic issues concurrently.

This chapter will introduce the calliphorids and their importance in forensic entomology, complications in the field to date, and the use of DNA to resolve some critical issues in the development of the field.

1.1 Diptera: The Flies
The order Diptera is a diverse insect taxon composed of more than 86,000 species worldwide (Byrd & Castner, 2001). Flies are among the most biologically and morphologically diverse insect groups, as evident from their ubiquitous presence in nature. Their ability to colonise almost any environment makes them an enormously successful taxon.

The flies are characterised morphologically by the presence of a single pair of membranous, mesothoracic wings, with hind wings reduced to club-like structures referred to as halteres. These modified appendages are used for stabilisation in flight. The mesothorax is well-developed to accommodate this single pair of functional wings, while the pro- and metathoraces are reduced in size.

Mouthparts are generally suctorial, but may vary greatly across the taxon. The Diptera includes such diverse insects as the piercing-sucking mosquitoes and the rasping-tearing tabanids, and consequently considerable modifications to mouthpart structure are evident in the group.
Dipteran larvae are generally elongate and cylindrical with indistinct segmentation, and most frequently apodous. The integument features rows of microscopic spines that are taxonomically important. The head morphology is generally characterised as euccephalic, hemicephalic or acephalic, and forms an important character in the identification keys.

The order Diptera is divided into two distinct suborders, the Nematocera and the Brachycera. These suborders are distinguished on the basis of adult morphology, with the Nematocera bearing long, filiform antennae and a delicate, slender body form. The Brachycera are more robust individuals with short, generally aristate antennae (Zborowski & Storey, 1995; Colless & McAlpine, 1996).

The Brachycera is further divided into two divisions, the Orthorrhapha and the Cyclorrhapha. The Cyclorrhapha are considered the more highly evolved of the two divisions, with coarctate pupae (larvae pupate in the last larval skin), and adults emerge from the pupal casing through the pumping of a fluid-filled ptilinum which following emergence is evidenced by the presence of a ptilinal fissure in adults (Colless & McAlpine, 1996). Of particular interest within the Brachycera is the forensically significant family Calliphoridae- the blowflies.

1.2 The Calliphoridae: The Blowflies

This large family of flies contains more than 1000 described species (Byrd & Castner, 2001). Adult calliphorids display enormous diversity in biology, and are generally attracted to moist substrate where they feed upon nectar, honeydew and the products of decomposition. Their ability to colonise a variety of nutrient-rich yet largely undesirable materials makes it no surprise that they have adapted to colonise carrion, making them an enormously important group in the decomposition process.

Adult calliphorids are medium-sized flies (Figure 1.1a), often brightly coloured in metallic blue, green, black or bronze. Their metallic shimmer may be reduced to a dull sheen by a covering of fine dust or powder (Byrd & Castner, 2001), frequently seen in the endemic Australian *Calliphora*. The bright metallic colouring, while characteristic, is not exclusive to the Calliphoridae and is occasionally visible in the closely-related Muscidae (house flies) and should therefore not be used as the sole diagnostic character.
Adults possess well-developed lower calypters, with the forewing M1 vein strongly bent forward (Figure 1.1b) (Colless & McAlpine, 1996).

Figure 1.1 (a) Morphology of an adult calliphorid showing robust nature and single pair of developed wings and (b) characteristic forewing of a calliphorid with vein M1 bent strongly forward (from Colless & McAlpine, 1996).

The larval calliphorid form is elongate, 8-23mm in length for mature larvae, apodous and cream in colour. The terminal segment possesses six or more cone-shaped tubercles around the perimeter, surrounding the taxonomically significant posterior spiracles. The spiracles, adapted for breathing, feature spiracular slits that slant towards the centre of
the larva, characterising them from the closely-related Sarcophagidae (flesh flies) with their outward or downward slanting slits (Byrd & Castner, 2001).

Diversity in adult morphology and biology makes adult calliphorids perfectly suited to exploit a wide variety of habitats. This is evident in the adaptation to ovoviviparity in the group, whereby immatures hatch from eggs within the body of their mother, being deposited live and ready to colonise. This enables the larvae to immediately exploit an ephemeral food source once deposited, as shown in the forensically important Western Australian calliphorid Calliphora dubia, the blue-bodied blowfly. Several calliphorids are also associated with myiasis of live individuals, infesting a wide variety of vertebrates (Rognes, 1997; Sukontason et al., 2005).

Many species of calliphorid are found on carrion, in particular members of the genera Lucilia (= Phaenicia) (green bottle flies), Calliphora (blue bottle flies), Chrysomya and Cochliomyia (screwworm flies). Female calliphorids are highly adept at locating carrion and arrive within minutes of death, making them the most important insects in the field of forensic entomology.

The remainder of this chapter will address the importance of calliphorids in forensic entomology, and the importance of molecular techniques to the field. The current applications of DNA to the field will be introduced in exploring the present interface of molecular entomology and forensic science.

1.3 Forensic Entomology: Historical Background

Forensic entomology is broadly defined as the interaction of insects and other arthropods with legal matters (Hall, 1990). Although the field has come of age as a science only in the last 25 years, it is a field with a long history.

The first documented case of forensic entomology took place in 13th century China. In “The Washing Away of Wrongs” (as translated by McKnight, 1981), Chinese criminalist Sung Tz’u reported a case in which insects were used to identify a murderer. A murder was committed by slashing, and all villagers were ordered to bring their sickles to a single location. The sickles were laid on the ground, and flies were attracted
to a single sickle, presumably responding to traces of tissue and blood. On this basis, the owner of the sickle was connected to the crime and he broke down and confessed.

Francesco Redi (1668, as told in Hall, 1990) studied the colonisation of rotting meat by flies. He concluded that the generally accepted “spontaneous generation” of maggots from meat was actually a result of infestation by adult flies, based on observations of rotting meat whether open to, or protected from, fly activity. This represented a significant breakthrough in our understanding of insect involvement in decomposition.

The first documented Western case of forensic entomology involved Bergeret in France, in 1855 (Hall, 1990; Benecke, 2001a). Bergeret investigated the death of an infant whose body was located behind a mantle. Through analysis of the insect assemblage found in association with the corpse, he concluded the baby had died two years previously, therefore exonerating the current occupants of the premises and casting suspicion on earlier tenants. This case represented the first analysis of insect succession to estimate time since death, and introduced the concept of a predictable entomological succession following death.

J.P. Megnin was responsible for raising awareness in both medical and legal areas of the potential of entomology for use in forensic investigations in the late 1800s (Hall, 2001). Megnin characterised eight stages of decomposition and the insects associated with them, which served as the basis for further related research by other scientists. Useful contributions to the field were subsequently made by Leclercq and Nuorteva (Smith, 1986).

Many researchers contributed to the development of the field after initial recognition of the information able to be obtained from insects at crime scenes. Particularly important were the works of Payne et al. (1968) on insect succession on pig carcasses. From the Australian perspective, Bornemissza (1957) collected useful data on the succession of arthropods on carrion.

It is perhaps in the last 25 years that the number of researchers and practitioners has proliferated in the field. Following Smith’s “A Manual of Forensic Entomology”
(1986), research in the field has expanded and forensically oriented entomological articles appear in the scientific journals with greater frequency from 1991 onwards.

1.4 Applications of Forensic Entomology: The Contemporary Scene

The potential applications of forensic entomology are numerous, encompassing any situation that may involve an interaction between insects and other arthropods, and the law. Therefore, the utility of the field is categorised under three separate headings: urban, stored-product and medicolegal entomology (Lord & Stevenson, 1986 as cited in Hall, 1990).

1.4.1 Urban Forensic Entomology

Urban forensic entomology generally concerns the interaction of insects with man-made structures and other aspects of human society. This may include the infestation of buildings by termites and cockroaches (Hall, 1990), and the breeding of flies in livestock and similar facilities (Hall, 2001).

1.4.2 Stored-Product Forensic Entomology

The stored-product aspect of forensic entomology involves the infestation of stored commodities by insects. Infestations may include the harvesting and storage of crops and subsequent invasion by an insect pest, or domestic invasion of kitchen products by insects. This aspect also encompasses the infestation of food sold by retailers to the public (Hall, 2001), which may result in prosecution and substantial fines.

1.4.3 Medicolegal Entomology

Undoubtedly the most widely recognised aspect of forensic entomology is the medicolegal aspect. Frequently featured as the main forensic investigative application of entomology in fiction, television and film, this is the area that the majority of research is directed towards. It frequently centres on the investigation of violent crimes.

The main applications include:

- Determination of time and location of human death (Hall, 1990)
- Determination of circumstances surrounding death (Hall, 1990; Roeterdink et al., 2004,)

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• Cases involving neglect and abuse, generally of the very old or very young, or pets (Lord, 1990; Goff et al., 1991; Benecke, 2001b; Anderson & Huitson, 2004)

• Evidence of postmortem corpse relocation: an area may be characterised by its faunal assemblage (Benecke, 1998a; Hwang & Turner, 2005)

1.5 Estimation of time since death
The use of insects in the estimation of PMI is based upon knowledge of the locality-specific faunal assemblage of carrion. Insects are attracted to carrion in a predictable sequence, colonising when the physical condition of the carrion is most suited to their needs. As the body progresses through the stages of decomposition, from flesh to bloat, decay, dry and skeletal stages, the odours emitted by the corpse change (Anderson, 2001), reflecting the physical changes in the body. The odours vary in attractiveness to different insects, and as the body decomposes and various resources are depleted new insect taxa will colonise, being more suited to the current decompositional stage. These insect taxa reflect the physical changes in the body and are therefore predictable and useful in estimation of PMI.

The estimation of PMI relies on thorough knowledge of the predictable sequence in which insects will arrive at a body, in conjunction with temperature dependent, species-specific developmental data and documentation of local environmental conditions during the period the body was exposed. Among the predominant insects in a corpse succession are the Calliphoridae (blowflies), and their role in decomposition is an important one.

1.6 Insect Succession on Carrion
The life cycle of a corpse-inhabiting calliphorid species is shown in Figure 1.2. In a general corpse succession, the first insects to be attracted to the body are the blowflies (Diptera: Calliphoridae). Female flies possess a unique ability to detect the odour of this valuable yet ephemeral resource, and may arrive within minutes of death (Anderson, 2001). Within a few hours of death, during daylight hours, females will deposit large numbers of eggs or live larvae around facial, genital or anal orifices, or wounds, if present. Following the hatching of eggs, larvae will begin to feed upon the by-products
of decomposition, secreting enzymes and disseminating bacteria that facilitate the consumption of the soft tissues of the corpse (Lord, 1990).

Figure 1.2 Generalised life cycle of an oviparous (egg-laying) calliphorid in a forensic context (from http://www.silcom.com/~vector/flies.html). The larval stage, while represented by a single stage in this figure, is composed of three larval instars each punctuated by moulting of the exocuticle.

Larval growth is rapid, yet punctuated by a series of moults dictated by the restrictive larval cuticle. Calliphorid larvae will moult twice during their larval development, with the intermittent stages referred to as first, second and third instar respectively. During each moult the larval cuticle is shed, facilitating further growth in the subsequent instar. In the late second and third instars larvae form highly exothermic masses of large numbers of larvae (Wells & Lamotte, 2001).

At the cessation of feeding, larvae will leave the corpse seeking sheltered sites in soil or under nearby objects to pupate. Larvae purge their alimentary canals prior to pupation. The third instar larval cuticle then becomes loosened and hardened, forming the more robust encasement which protects the pupa inside. Over a period of days to weeks, the pupa undergoes metamorphosis to adult form, and will emerge from the casing as an adult fly.

While calliphorids are usually the first insects to arrive following death, numerous other taxa may be present during the decomposition process. Sarcophagidae (flesh flies), Muscidae (house flies) and Piophilidae are among the dominant dipteran fauna, while beetles such as members of the Staphylinidae (rove beetles) and Histeridae (clown
beetles), Cleridae (checkered beetles), Dermestidae (skin beetles), Silphidae (carrion beetles) and Trogidae (hide beetles) may be present, either as predators of necrophagous insect species, or feeding on skin, hair and clothing remnants. Recently, the fauna inhabiting the soil beneath the corpse has become a topic of interest for its potential in estimation of PMI (Dadour & Harvey, Appendix 2).

Familiarity with the order of succession exhibited in various circumstances in a given locality is vital in estimation of PMI (Anderson, 2001). The presence of a particular assemblage of taxa inhabiting the carrion allows the forensic entomologist to estimate the minimum amount of time required for the corpse to have become suitable for these insects, and for their development to a particular life stage.

In late decomposition stages when the time elapsed since death is considerable, the entomologist relies upon the presence of a particular set of insects to characterise the period of time since death. In these late stages the estimates are generally provided as broader timeframes, due to the difficulty in factoring the effect of environmental variables precisely over a long period, and the lack of useful data for late colonising species.

During the early stages of decomposition, particularly through the fresh, bloat, and active decay stages, the entomologist is able to provide the narrowest timeframes for PMI. It is in these stages that calliphorid larvae are the predominant corpse inhabitants, removing the soft tissues of the corpse, and as early colonisers of the corpse their age is able to be estimated using developmental and temperature data. According to Morris & Dadour (2005), blowflies are currently the only insects able to assist in the provision of an accurate PMI.

In such cases, it is generally the immature calliphorid stages that are collected from the corpse. These must be accurately identified to species level, and in the case of larvae, their length measured. Recent studies indicate that width may be an equally useful measurement in the future for use with developmental data (Day & Wallman, 2006). Developmental data describing the growth of immature stages at a variety of temperatures may then be used in conjunction with relevant temperatures and rainfall for the crime scene area. Given the species identification of the specimen, and the
stage/length of the individual, the minimum time required for the insect to reach its current size can be estimated, and therefore the minimum time since death. A complication to this age estimation is the collection of pupae, which may be difficult to age once preserved. Dissection and study of stage of development have been used, while recent studies (Ames et al., 2006b; Zehner et al., 2006) have investigated the use of molecular approaches such as measuring gene expression to indicate stage of development. The collection of pupae therefore remains a problem where preservation prevents rearing.

The estimation of PMI based on insect development is affected by numerous variables that may influence the decomposition, and therefore the role of insects on the body. The estimation of PMI therefore relies on knowledge of the effect each variable may have on insect succession. Factors such as shade, season, rainfall, coverage, burial and hanging may affect which insects colonise the body, and when they arrive. Consequently, research has focuses on a number of variables in recent years, including:

- Drugs and alcohol: drugs and/or alcohol in the bloodstream may affect calliphorid development and numerous studies have considered their effects (Campobasso et al., 2004; O’Brien & Turner, 2004; Tabor et al., 2005)
- Marine or aquatic cases: the occurrence of carrion in a body of water facilitates colonisation by aquatic insects, and in some cases terrestrial concurrently (Hobischak & Anderson, 2002; Anderson & Hobischak, 2004; Sukontason et al., 2005)
- Arson: the effects of the burning of carrion on insect succession (Anderson, 2005)
- Burial: the effect of burying a body on reducing accessibility for insects (Bourel et al., 2004)

1.7 The Problem: Identification

Critical to the estimation of PMI is the correct identification of insects (Zehner et al., 2004; Morris & Dadour, 2005). Incorrect identification may result in the application of inappropriate developmental data in estimation of insect age, a significant problem considering the great variability in growth rates between (and within) various taxa (Higley & Haskell, 2001; Stevens et al., 2002) The relatively similar species C. vicina and C. vomitoria, of the same genus, may both be found on carrion, however there
developmental rates vary greatly. At 26.7°C, *C. vicina* requires 508 hours to complete egg to adult emergence, while *C. vomitoria* requires 854 hours (Kamal, 1958). An error in identification may allow these specimens to be misidentified, and irrelevant data to be applied in their age estimation. Potentially, this may result in a difference of several days in the ultimate PMI estimate. Such errors result in erosion of credibility of the expert, and potentially, contribute to a possible miscarriage of justice. Most forensic entomologists will therefore employ the expertise of a taxonomist specialised in the particular taxon of interest to ensure accuracy for subsequent court proceedings.

The enormous diversity of insects, and particularly of fly species, makes accurate identification a challenging task for the entomologist (Benecke, 1998b). It is generally the immature calliphorid stages that are collected in early decomposition and require identification, and these are among the hardest to identify. Any diagnostic characters for a species are generally less distinct in immature stages making morphological identification problematic (Hall, 1990; Catts & Goff, 1992; Sperling *et al.*, 1994; Benecke 1998b).

In particular, larvae of very closely related species may be extremely difficult to identify, such as the sister species *Chrysomya rufifacies* and *Chrysomya albiceps* (Tantawi & Greenberg, 1993). Common characters used in larval identification include the comparative morphology of mouth-hooks and features of the posterior spiracles (Figure 1.3), easily obscured in damaged and poorly preserved specimens.

**Figure 1.3** Morphological characters used in the identification of the common Australian calliphorid species (a) *Calliphora stygia* (b) *Calliphora augur* and (c) *Lucilia cuprina*. O = oral sclerite; M = mouth hook; B = button; P = peritreme (from Morris & Dadour, 2005)
There are also few keys to the immature stages of forensically important calliphorid species available, and those available only cover the most commonly observed fly species (Morris & Dadour, 2005). While several keys do exist for specific localities (e.g. Wallman, 2001; Wells et al., 1999; van Emden, 1954), each locality has its own distinct assemblage of calliphorid species present in the corpse fauna, making the development of such keys a complex task.

Adult flies can also appear very similar morphologically, even to the trained eye of the dipteran taxonomist. Tantawi & Greenberg (1993) investigated the identification of the forensically important Ch. rufifacies and Ch. albiceps. These species are generally considered to be biologically equivalent and had long occupied geographically separate distributions, but the development of an overlap zone has complicated the identification process. The distinction of the species is generally based on the presence of a prostigmatic bristle in Ch. albiceps, yet Tantawi & Greenberg (1993) note that certain Ch. rufifacies individuals also exhibit this character, making confident identification difficult.

Furthermore, adult identification may often depend on characters not easily observable, highly subjective or only present in one sex. For example:

“8 (9) Hypopygium of male big and rather prominent, shining green. 6-11mm……………………………L. caesar” (Zumpt, 1965)

In this couplet from a key to adult calliphorids, only male flies are able to be distinguished, making the identification of a female fly impossible. The subjective nature of identification is visible with the key character in distinction of the morphologically similar Lucilia cuprina and L. sericata, where identification is based upon the variable character of “fore-femur black or dark bluish metallic” as compared to “fore-femur bright metallic green” (Zumpt, 1965). The perception of colour variation is highly subjective, and the actual colour of the structure in question may be affected significantly by the method of preservation.

The successful morphological identification of specimens depends largely on the correct collection and preservation of them. This relies on suitably trained individuals being responsible for the collection and initial processing of insects from the crime scene. Unfortunately, the forensic entomologist is not always present at the scene, and
evidence may reach them in a sub-optimal state. In particular, specimens may be damaged during collection, storage or transportation, meaning that important diagnostic characters for physical identification may be missing (Malgorn & Coquoz, 1999).

**Rearing**

Rearing immature stages through to adult stage is generally conducted by forensic entomologists to aid in identification. Adult flies are usually more distinct than immatures due to their diversity of colour and form, and therefore more easily identifiable. Rearing also may provide useful information as to the age of the insects when collected in egg or pupal stage, where size is not a useful measure (Bourel et al., 2003). Following collection of a sample of immatures from a corpse, half of these are suitably preserved and stored, and the other half are reared through to adulthood.

While the rearing of individuals is standard practice among forensic entomologists, it is not always possible. In situations where the entomologist does not attend the scene, or samples are passed on for analysis as an afterthought some time following discovery of the corpse, specimens will generally have been stored in ethanol and rearing is impossible. Specimens may also die when rearing is attempted, possibly as a result of parasitism by parasitoid Hymenoptera (Wallman & Adams, 2001). Furthermore, in cases where a PMI estimate is required quickly, rearing may delay the process for days to weeks. In the interest of the preservation of integrity of the evidence for subsequent court proceedings, it is also desirable to present evidence in the form in which it is collected. Rearing alters the form of the evidence creating a potential difficulty for juries in understanding the continuity of the evidence. In addition, rearing in a laboratory situation where other insects are already present further increases the risk of contamination of the sample with non-relevant insects (Morris & Dadour, 2005).

**1.8 Cryptic Species**

Morphological identification, whether from immature or reared individuals, has a significant potential pitfall. Cryptic species complexes may prove a significant threat to the accuracy of PMI estimation. Cryptic species occur where two or more species may appear morphologically indistinguishable, however they are distinctly separate, reproductively isolated groups with their own biological niche. Their role in the corpse
succession may differ markedly, as may their rates of development, yet they may be easily confused in identification leading to inaccuracy in PMI estimation.

Cryptic species have been encountered in numerous insect taxa. The mosquitoes (Culicidae) have been reported to contain several cryptic species complexes not reliably separated using morphological characters (Singh et al., 2004; Wilkerson et al., 2004; Cook et al., 2005). This has necessitated the development of alternate means of identification.

Given the numerous limitations inherent to the current morphological and rearing-based identification techniques, an alternate technique is needed for routine use in the identification of insects associated with carrion. This may potentially act as a complement to and safeguard for the traditional morphological and rearing approaches to identification, and in addressing the status of some forensically important species.

Common to any study that considers species status is the importance of the concept of species chosen. Numerous definitions exist for the term species, and each impacts differently on the inclusiveness of the grouping. These will be considered further in Chapter 4.

1.9 Alternate Approaches to Identification

In the study of forensically important dipterans and their medically significant related taxa, a wide variety of approaches have been suggested for providing identification, distinguishing populations and deriving data for consideration of such issues as species status. There are several criteria for consideration in the selection of a new method for the identification of calliphorids in forensic entomology.

A new technique for identification should:

- Be fast in providing identification between all species of forensic importance in a given area, facilitating the efficient provision of PMI estimates to parties seeking the opinion of an entomologist
- Be accurate and form a robust confirmation of the identity of an insect
- Be applicable to all life stages of the calliphorid
• Overcome the limitations of the current morphological and rearing approaches, and therefore provide identification from damaged, poorly preserved and dead specimens

An additional consideration in the development of an identification technique is its potential to provide further information about the insects studied. The elucidation of species status from data derived from forensic specimens will contribute to confirmation of some of the basic species assumptions made in this field.

In studying the species status of forensically significant calliphorids, data must obviously be gathered across the geographical distribution of the taxon in question. This population level data provides useful insight into the genetic processes at work in a species, the level of variation within the species, and therefore the possible validity of the species status, making the collection of data with intraspecific potential desirable.

Tantawi & Greenberg (1993) noted that the species status of *Chrysomya rufifacies* and *Ch. albiceps* requires review if these species are to be used in forensic investigations. As previously discussed, they are morphologically similar species. Originally displaying distinct distributions but now sympatric, they have been shown to produce fully fertile offspring in bidirectional crosses (Ullerich, 1963 cited in Tantawi & Greenberg, 1993). Obviously, under a recognitionist concept of species, *Ch. albiceps* and *Ch. rufifacies* have ceased to exist as separate species.

Similarly, *Ch. rufifacies* itself displays variation across its own distribution. This species displays variable behaviour in its corpse preference, in some localities representing a primary species, arriving shortly after death, and in other localities the species is secondary, arriving once the body is already colonised by other species and the decomposition process is underway.

Population data can also provide suggestion of postmortem movement of a body, indicating when a body may have been relocated based on some form of biological variation in the insects present. This may potentially redirect investigations to a particular geographical area, focussing valuable investigative resources and increasing efficiency.
A variety of methods have been suggested to address the issue of calliphorid identification for forensic purposes, and provide data for study of some of the critical issues mentioned. Such methods have included scanning electron microscopy as an alternate approach to morphological identification, allozyme electrophoresis, cuticular hydrocarbon compositions and DNA-based techniques.

**Scanning electron microscopy (SEM)**

Calliphorid eggs may prove particularly problematic for identification. Their very small size makes observation of potentially diagnostic characters difficult. Greenberg & Singh (1995) used scanning electron microscopy (SEM) to identify individuals, but found several confounding factors: first, conspecific interpopulation variability; second, high similarity between eggs of congeneric species and third, similarity between certain species of different genera. Climate, season and weather were also cited as important factors influencing egg morphology. In addition, this morphological approach is still subject to error where cryptic species are encountered (Hill & Crampton, 1994).

**Allozyme Electrophoresis**

Allozymes have been used successfully in studies of numerous insect taxa, showing value in identification as well as studies of species status (Ramirez & Chi, 2004). Allozyme analysis studies the migration of proteins under the effect of an electric field and provides a cost-effective method of producing identification and data for evolutionary studies. Allozymes were employed by Wallman & Adams (1997; 2001) in a systematic study of the Australian carrion-breeding *Calliphora*. The data successfully distinguished between all species.

Allozymes are proteins that are under environmental influence and not always expressed throughout the entire life of an organism, thus reducing their discriminatory power (Sperling *et al.*, 1994). They are also more difficult to handle than other target molecules such as DNA due to their increased susceptibility to degradation (Hoy, 1994). In addition, allozyme analysis is a comparative analysis requiring control samples of known identity to be run with each analysis, necessitating the storage of numerous control samples (Wallman & Adams, 2001).
The main limitation of the use of allozyme electrophoresis in forensic entomology, however, is the necessity for immature specimens to be provided to the entomologist either alive or freshly frozen (Wallman & Adams, 2001). Situations where specimens are received in such a condition are rare due to the absence of the entomologist at the crime scene, or consultation of an entomologist as an afterthought when evidence is already preserved in ethanol. Allozyme analysis can be discounted on these grounds alone for its potential utility as a diagnostic technique.

Cuticular Hydrocarbon Composition

At a population level, Byrne et al. (1995) investigated the use of cuticular hydrocarbon composition of adult calliphorids to determine whether postmortem relocation of a corpse could be detected on this basis. While they found the technique to be useful, there are numerous limitations to such an approach. Where immatures are collected, these must be reared to adulthood for cuticular hydrocarbon analysis. The composition of the cuticle is also affected by the diet and the age of the insect, inducing greater variability and uncertainty to the analysis. These limitations undermine the accuracy required from a new diagnostic tool.

DNA-based Techniques

Deoxyribonucleic acid (DNA) holds enormous promise as the basis for a diagnostic technique for use in forensic entomology. This molecule is the most basic chemical form and genetic blueprint for the development of an individual, and contains copious information for the investigation of an organism. It has been widely used in the entomological world in systematic, identification and population genetics studies.

There are numerous advantages to the use of DNA as a tool of interspecific distinction and intraspecific insight. These include:

- The relative stability of the molecule (Phillips & Simon, 1995): DNA is a robust molecule capable of long-term preservation in a variety of storage situations
- DNA content remains constant throughout all life stages of an organism irrespective of environmental conditions (Hill & Crampton, 1994)
- DNA analysis provides data of high information content for manipulation in subsequent evolutionary analyses, and provides information at a more empirical level than morphological analysis alone
In a forensic entomological context, DNA-based techniques hold significant value for a number of reasons. The ability to distinguish the immature stages of forensically important calliphorids, particularly those with significant morphological similarity, is of enormous potential for increasing the accuracy and efficiency of PMI estimation. The ability to provide identification without rearing of immatures and from samples that have been preserved following collection is important in aiding the entomologist to accurately estimate PMI in previously difficult situations.

DNA also holds particular value in the context of egg and pupal samples. Eggs, once preserved, may be extremely difficult to identify without the use of SEM. Empty pupal casings may remain at a crime scene for long periods, depending on environmental conditions. These puparia may provide the entomologist with an indication of the minimum PMI based on the time from egg to adult emergence, in cases where the time elapsed since death is not long. Casings have been reported to persist intact for as long as 2000-2500 years (Teskey & Turnbull, 1979), yet have generally been of little interest to crime scene investigators.

In situations where the body has been *in-situ* for many years, pupal casings may provide a useful indication of seasonality of death based on the assemblage of fly species present on the body. This may be significant in criminal and historical contexts. Casings may often be identified to species or at least genus level based on morphology, as the coarctate nature of the pupae means that physical characters from the last larval instar are retained on the puparium. However, puparia become weathered when exposed to the environment and may fragment and lose important diagnostic characters. The use of DNA to identify puparia collected from human remains would significantly extend the utility of forensic entomology beyond its current timeframe of value.

DNA has been employed in forensic entomology for diagnostic purposes since 1994, with the publication of Sperling *et al.*’s landmark paper (1994). The next section will review the current applications of DNA-based techniques in forensic entomology, with a view to the direction of the current study.
1.10 DNA: Forensic Entomological Applications to Date

The success of DNA in providing useful interspecific distinction is owed largely to the advent of the polymerase chain reaction (PCR). This technique was invented by Kary B. Mullis in 1985 and revolutionised the field of molecular biology.

PCR was designed to amplify an exponential number of copies of target DNA from a specific organism, creating a large volume of target DNA for use in subsequent molecular procedures. In a forensic context PCR is particularly important, as the starting amount of material may be relatively small (e.g. a hair, a drop of blood) and, depending on the conditions to which it has been exposed, DNA may be relatively degraded. PCR enables a small amount of starting material to be increased to a level suitable for further manipulation.

Within forensic entomology, an important facet of molecular research has been the extraction of ingested host DNA from the alimentary canal of immature calliphorids (Carvalho et al., 2005, Appendix 2). This unique application has involved studies of host DNA ingestion, while relying on interspecific calliphorid studies for the identification of the hosts themselves. This approach may be employed to identify the species of animal host upon which the larvae have fed, but at this stage identification of a particular individual from larval stomach contents remains complicated by the degradation of host DNA by digestive enzymes.

There are numerous techniques that have been proposed for the development of an interspecific distinction tool for the forensically significant calliphorids. The majority have been based on DNA sequencing and subsequent phylogenetic analysis, however other techniques have been suggested. A large number of studies of forensic calliphorids and the techniques they have utilised are summarised in Table 1.1. While the majority of these papers specifically address the interspecific distinction of calliphorid species, some have been listed as they address forensically important calliphorid species in a non-forensic context but still have relevance and application in the context of this study.
Table 1.1 Summary of literature investigating the molecular-based identification of a variety of forensically significant calliphorid species

<table>
<thead>
<tr>
<th>Technique Used</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random Amplified Polymorphic DNA (RAPD)</td>
<td>Stevens &amp; Wall (1996); Stevens &amp; Wall (1997a); Benecke (1998b)</td>
</tr>
<tr>
<td>Polymerase Chain Reaction-Restriction Fragment Length (PCR-RFLP) of mtDNA</td>
<td>Sperling et al. (1994); Narang &amp; DeGrugillier (1995); Taylor et al. (1996); Gleeson &amp; Sarre (1997); Malgorn &amp; Coquoz (1999); Lijens et al. (2001); Schroeder et al. (2003)</td>
</tr>
<tr>
<td>PCR-RFLP of nuclear DNA (internal transcribed spacers)</td>
<td>Ratcliffe et al. (2003)</td>
</tr>
<tr>
<td>Sequencing of nuclear ribosomal RNA (rRNA) genes 28S</td>
<td>Stevens &amp; Wall (2001); Stevens et al. (2002); Stevens (2003)</td>
</tr>
<tr>
<td>Sequencing of mitochondrial Ribosomal RNA (rRNA) genes 12S</td>
<td>Stevens &amp; Wall (1996); Stevens &amp; Wall (1997a); Stevens &amp; Wall (1997b); Lessinger et al. (2000); Junqueira et al. (2004)</td>
</tr>
<tr>
<td>Sequencing of mitochondrial cytochrome b gene</td>
<td>Hall et al. (2001)</td>
</tr>
<tr>
<td>Sequencing of mtDNA cytochrome oxidase I + II (COI + COII) regions</td>
<td>Sperling et al. (1994); Gleeson &amp; Sarre (1997); Malgorn &amp; Coquoz (1999); Wells &amp; Sperling (1999); Vincent et al. (2000); Lessinger et al. (2000); Wallman &amp; Donnellan (2001); Wells &amp; Sperling (2001); Stevens et al. (2002); Harvey et al. (2003a,b); Schroeder et al. (2003); Stevens (2003); Junqueira et al. (2004); Wells, Lunt &amp; Villet (2004); Saigusa et al. (2005); Ames et al., (2006a)</td>
</tr>
<tr>
<td>Sequencing of mtDNA Control Region</td>
<td>Stevens &amp; Wall (1997a); Lessinger et al. (2000); Lessinger &amp; Azeredo-Espin (2000); Lessinger et al. (2004); Junqueira et al. (2004)</td>
</tr>
</tbody>
</table>
The value of the techniques employed in the studies from Table 1 will be discussed further in this section.

(a) Random Amplified Polymorphic DNA (RAPD)

RAPDs are a useful technique designed for the detection of sequence polymorphisms in organisms where previous nucleotide sequence data is not available. RAPDs are based on random priming using short primers of arbitrary sequence that act as both forward and reverse primers in the PCR reaction. The primers produce a series of bands that may be visualised using agarose gel electrophoresis, and each of the products represent a single genetic locus (Hill & Crampton, 1994). Individuals may effectively be “profiled” on the basis of the presence and absence of products of particular size.

The obvious advantage in RAPDs is their potential for use with organisms for which little sequence data has been gathered. In the case of the calliphorids, where many species are yet to be considered, this is of particular relevance. The use of multiple primers to gather a large body of RAPD data may then contribute to the identification of species, sub-species and cryptic species (Black, 1993).

RAPDs do, however, hold several limitations. Non-genetic variation has been noted in the analysis of the progeny of controlled matings, indicating some artifacts in priming. The co-migration of products of equal size complicates analysis (Hoy, 1994), and homologous loci may therefore be difficult to identify (Palumbi, 1996).

Perhaps the greatest limitation in the use of RAPDs is the issue of reproducibility. RAPDs are affected by minute changes in reaction parameters, including changes in the thermocycler used. This makes the reproduction of identical results in independent laboratories using different equipment problematic. Benecke (1998) suggests that this is not an issue following tests on independent thermocyclers, however numerous authors have debated the reliability issue (Perez et al., 1998; Fernandez et al., 2003).

Harvey (2000) found RAPDs difficult to reproduce reliably, even when concurrent identical reactions were conducted. While RAPDs are useful in producing results quickly from individuals not previously studied, their use in casework relies on the development of a suitable database of forensically relevant species. The nature of the
profile produced and the potential for the appearance of novel, non-genetic variation may reduce their application to exclusionary purposes only. Despite the obvious advantages of RAPDs, their applicability and utility in forensic casework is not convincing.

(b) Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP is a useful technique harnessing the advantages of PCR with the use of enzymes to produce characteristic profiles for individuals based on recognition of specific nucleotide sequences (Edwards, 1998). Under this technique, PCR is used to amplify large amounts of template from a particular target region. Enzymes are added to the template, designed to cleave the DNA at particular recognition sites based on nucleotide sequence. The template is then electrophoresed to reveal fragments of varying sizes, depending on the location of restriction sites, and profiles may be used in distinction and study of different genetic lineages (Harrison, 1989).

PCR-RFLP is obviously more specific than RAPDs, and requires some previous nucleotide data for species so that appropriate restriction enzymes may be selected. A range of enzymes may be required to produce the necessary bands, and this may increase the cost associated with the technique. As several different enzymes may be used concurrently, a variety of conditions may be required making the use of sub-optimal buffers and conditions for one enzyme, in order to facilitate another enzyme, a limitation on the technique. The major disadvantage to the technique is the need for sequence data, and until suitable population level data can be gathered and species status confirmed for relevant species, a technique that considers nucleotide data directly is preferable. The culmination of such sequence data in the identification of informative restriction sites and ultimately, a diagnostic assay based on the PCR-RFLP technique does however hold great promise.
(c) Sequencing of Nuclear DNA: the RNA Genes and Spacers

Ribosomes are cellular organelles found in the cytoplasm of the cell, and in eukaryotes, also in the mitochondria. The ribosomes are responsible for the production of polypeptide chains and are synthesised in the nucleus as two subunits (four in insects), and are composed of ribosomal RNA (rRNA) and proteins.

The nuclear genes encoding the ribosomes, and the non-coding regions that separate them are often of interest to systematic biologists. The nuclear rDNA genes are arranged as shown in Figure 1.4. The 18S, 5.8S and 28S subunits are the rDNA genes encoding for ribosomal production. The internal transcribed spacers (ITS1 and ITS2) are non-coding regions of DNA, often of interest in phylogenetic studies due to their lack of functional constraints and therefore increased scope for variation. The entire arrangement is referred to as a multigene family located within the nucleolus organiser regions (NORs) and is tandemly repeated in hundreds to thousands of copies.

Figure 1.4 Arrangement of nuclear rDNA genes and spacers (from Muir & Schlotterer, 1999). The arrowed region refers to the tandem repetition of the rDNA arrangement. The abbreviations 18S, 5.8S and 28S represent the ribosomal coding gene subunits, and the external transcribed spacer (ETS), internal transcribed spacers (ITS) and intergenic spacer (IGS) are non-coding introns.

Such multigene families form useful targets for phylogenetic study due to the array of evolutionary rates present in them. The nuclear origin of this region means that the mutation rate is generally considered to be lower than mitochondrial DNA, and therefore in species where evolutionary divergence has occurred more recently, the rDNA region of the mitochondrial DNA may be more useful (Hillis et al., 1996). The nuclear rDNA genes and their intervening spacers have, however, shown promise in the
study of closely related species, particularly within the Culicidae. Within these regions, length differences have been used with great success in the distinction of species not reliably separated using morphology (Chen et al., 2003) and appear to hold promise for use in forensic entomology.

(d) Sequencing of the Mitochondrial Cytochrome Oxidase Genes

Mitochondrial DNA (mtDNA) makes a useful target for use in forensic entomology studies. Mitochondria are self-replicating organelles found in the cytoplasm of eukaryotic cells. They are unique in possessing their own ribosomes, maternally-inherited DNA and transfer RNA (tRNA) molecules. The mtDNA is a circular molecule, composed of a variety of non-coding regions (Figure 1.5). The use of mtDNA in the study of the forensic calliphorids is advantageous for several reasons:

- Relatively higher copy number than nuclear DNA, facilitating extraction from very small amounts of starting material: this is particularly advantageous in forensic studies where small amounts of material may be available for testing, and destruction of an entire exhibit is not desirable
- Ease of extraction and manipulation (Hoy, 1994)
- Low number of insertions or introns (Moritz et al., 1987): this ensures relative conservation of size and structure and simplifies the amplification process
- Large number of previous studies creating universal primers as a starting point for mitochondrial analysis
Mitochondrial DNA is often assumed to evolve at a faster rate than nuclear DNA, and therefore provide greater scope for variation (Hillis et al., 1996). Zhang & Hewitt (1997) state however that insect nuclear and mitochondrial DNA evolve at similar rates, and therefore there is unlikely to be significant difference in evolutionary rates between the two forms.

The cytochrome oxidase I (COI) gene has been studied extensively in entomology, providing distinction in numerous taxa. The COI plays an important role in the cell as the terminal catalyst in the mitochondrial respiratory chain (Lunt et al., 1996). It is involved in electron transport as well as translocation of protons across the cell membrane. It stands among the largest of the protein-coding genes in the insect mtDNA, and with its conserved and variable regions has been a popular subject of evolutionary studies. It is also well conserved throughout metazoan organisms, allowing for the use of universal primers. The region consists of some 1500 base pairs in the calliphorids, making it a target of a realistically amplifiable size, assuming limited degradation in the template.
Sequencing of DNA is an effective means to obtain large amounts of material of high information content. Other techniques may be more cost-effective, but sequencing produces the empirical data upon which all other analyses depend. It is the confirmation of the underlying chemical nature of the individual reflected in results from other molecular procedures and morphological analyses.

From a phylogenetic perspective, sequencing provides the biologist with useful data for the construction of evolutionary relationships, analysis of variation and relatedness, and reflection on the genetic processes driving the evolution and therefore variation reflected in the organisms. In a forensic context, a diagnostic technique needs to be robust and accurate, and sequencing appears to be the most useful technique to gather the required nucleotide data upon which a more efficient and cost-effective means of distinction can be based. The cytochrome oxidase genes appear to be a useful target for such sequencing, and the popularity of the region amongst molecular forensic entomologists appears a testament to its utility.

(e) Sequencing of the Mitochondrial Control Region

The benefits of sequencing for deriving data of high information content were discussed above. The control region, however, differs considerably from the cytochrome oxidase genes.

The control region, also referred to as the D-loop or A+T-rich region, is the largest non-coding region of DNA in the insect mitochondrial genome. It is generally regarded to mutate quickly due to the lack of functional constraints. While this holds significant promise for location of intraspecific variation, it provides issues for higher level taxonomic studies as phylogeny cannot be ascertained without ambiguity where the risk of base saturation exists (Lunt et al., 1996). In such situations, the number of changes in a position cannot be determined, for example the presence of thymine (T) in a position may be considered ancestral, and thus presence of a T would be identified as no change, while over time the position may have experienced transition between adenine (A) and T numerous times. Thus a potentially high number of substitutions occurring in that site.
overtime may be significantly underestimated and rapid mutation and evolution undetected.

The control region is highly variable in both size and sequence and may contain tandem repetition associated with heteroplasmy (Zhang & Hewitt, 1997). Contrary to popular opinion though, Zhang & Hewitt (1997) suggest the control region may have limited utility in inter- and intraspecific studies. Given the directional mutation pressure and high A+T content of the control region (up to 96%), variation at the nucleotide level is limited, and complicated by the incidence of heteroplasmy. The authors suggest this region may actually evolve no faster than nuclear non-coding sequences.

Many authors now suggest that the insect mtDNA does not, in fact, evolve as quickly as insect nuclear DNA. This raises an interesting issue in molecular forensic entomology: can nuclear non-coding regions actually provide greater within species insight than the much promoted mitochondrial control region?

### 1.11 Analysis: Molecular Systematics

The merits of DNA sequencing in providing data with high information content have already been discussed. Page & Holmes (2005) state that the value of DNA sequences is in the detail they provide about an organism- “the instructions for how each working part should be assembled and operated”. While the data may hold tremendous value, the method of analysis is critical to exploiting its full potential.

Molecular systematics is the use of DNA sequences to construct the phylogenetic relationships between individuals. The use of complex algorithms and models to explore the similarity and difference between sequences allows the relatedness of any two individuals to be inferred, forming hypotheses as to the ancestry of taxa, their hypothetical ancestors and their evolution over time.

The majority of molecular studies into the forensically important Calliphoridae have been molecular systematic studies, inferring phylogenies to determine the relatedness of individuals and therefore graphically depict the relationships between species. The utility of particular DNA regions to provide data that reflects the divergence of species
is assessed using phylogenetic methods, allowing individuals to be characterised on the basis of the presence of particular character states throughout members of their taxon.

Phylogenetic analysis depends strongly on the selection of appropriate methods and models of reconstruction of the relationships between taxa. The relationships constructed represent the evolutionary relationships between the taxa, and their divergence from hypothesised common ancestry. Dean & Ballard (2004) state that a common problem with modern phylogenetics is that evolution and ancestry are inferred from a single tree created using the most parsimonious method of evolution. The majority of phylogenetic methods assume the fastest evolution of individuals possible, potentially misrepresenting important evolutionary steps, particularly evident where DNA sequence data is the basis for analysis and base saturation may complicate phylogenetic analysis. It is vital, therefore, that the DNA region chosen for analysis reflects an appropriate level of variation for the taxonomic level in question and serves as an accurate record of substitution and therefore, potentially, evolution.

In the context of forensic entomology, phylogenetic analysis provides a visual depiction of the relevant species, and therefore an evaluation of the utility of a DNA region to reflect the relatedness of individuals from a particular species. In more variable regions, intraspecific variation may also provide an insight into the relationships between conspecific populations, and therefore consideration of potential speciation events. Such events may also be reflected in the behaviour of divergent populations, having implications for the estimation of time since death where a particular behaviour is generally assumed to be common to an entire taxon. Phylogenetic analysis therefore provides a useful means of processing high volumes of data and displaying results in a visual and meaningful manner.

1.12 Aims of Thesis
As discussed in this chapter, the issue of identification in forensic entomology is an area which requires immediate study. This study aims to examine some of the critical issues with a view to evaluating the current application of DNA to forensic calliphorids and new techniques to improve the accuracy and efficiency of the field and verify some basic assumptions.
The COI region of mtDNA has been widely promoted in forensic entomology for interspecific distinction, however the majority of studies have considered the fauna of small, defined localities or a single species of interest. Few studies have considered species of global importance, assessing species status and considering intraspecific variation. In this study, the value of the COI region to forensic entomology will be evaluated through the study of the COI of calliphorids from around the world, with a primary focus on species significant in Australia, addressing the following questions:

- Is the COI region of mtDNA useful in interspecific distinction for calliphorid species of forensic importance on a global scale?
- Does the COI region allow inference relating to status of these calliphorid species? Is there intraspecific variation capable of providing population level information?

The nuclear rDNA multigene family and the non-coding mtDNA control region are two regions that have been largely neglected in forensic entomology applications, but provide a useful insight into the evolutionary processes of the calliphorids. These regions represent two non-coding regions of DNA providing a useful contrast between nuclear and mtDNA. Given the assertion that mtDNA evolves faster than nuclear DNA, but suggestion that this is not so in insects, it seems appropriate to question:

- Do these regions hold some value in inferring the status of some forensically relevant calliphorids not resolved using the COI gene?
- Can non-coding DNA regions provide greater intraspecific variation than the COI region, which currently dominates molecular forensic entomology?

The culmination of this data will ultimately result in the development of a robust, efficient and accurate method for the identification of forensic calliphorids. The consideration of important species status and population level issues based on nucleotide variation will be considered through phylogenetic analysis, with the overall questions to be answered in this thesis being:

- Is COI the most appropriate choice for interspecific distinction, or is there more to be learned from the rDNA or control regions?
• Can a simple, efficient and accurate identification technique be developed based on the data in this study to eliminate the necessity for expensive and time-consuming sequencing?
• What does the phylogenetic analysis developed from sequence data gathered in this study tell us about the species status and population genetics of these species?
• What implications do DNA studies have for the future of forensic entomology?

This thesis will address the above questions in the form of scientific papers. Chapters 2, 3 and 4 will evaluate the utility of the COI gene in terms of interspecific distinction and the assessment of pertinent taxonomic issues. In chapter 5, the mitochondrial control region and ribosomal spacer units will be considered for their value in the resolution of taxonomic issues arising from study of the COI gene of forensically important calliphorids. Chapter 6 will address the extension of molecular-based identification to include fly puparia in both contemporary and historical contexts. In chapter 7, data collected in this thesis will culminate in the development of a molecular-based identification protocol for routine use in forensic entomological applications. The thesis concludes with a discussion of findings presented in the various studies, and implications for the future of forensic entomology.
CHAPTER 2
Mitochondrial DNA
Cytochrome Oxidase I gene:
Potential for Distinction between some Forensically Important fly species (Diptera) in Western Australia
The selection of a genomic region for a molecular study depends largely on the nature of the information required. Regions that exhibit low substitution rates are useful for the inference of higher level phylogenetic relationships, while hypervariable regions prove more useful for the investigation of lower level and often population level studies (Hwang & Kim, 1999). However, mutation and substitution rates vary between taxa, complicating the selection of an appropriate region of DNA for investigation at a specific taxonomic level.

The first important choice in the selection of a region is between nuclear and mitochondrial DNA (mtDNA). MtDNA is often the molecule of choice in molecular studies for a variety of reasons, not least for its comparatively higher mutation rate than nuclear DNA (Hwang & Kim, 1999). The insect mtDNA is a circular molecule (Figure 1.5), generally between 14 to 17kb. Variation in the mtDNA occurs primarily as a result of single nucleotide polymorphisms (SNPs), length variation occurring in non-coding regions as a result of inserted or deleted repeats, or by the replication and insertion of some portion of the mtDNA (Hwang & Kim, 1999). The ease of isolation, high copy number relative to nuclear DNA and lack of recombination make the mtDNA a useful target for molecular study (Lunt et al., 1996). Relative conservation in gene order also facilitates molecular study of the mtDNA (Goldenthal et al., 1991). MtDNA also contains both conserved and variable regions, reflecting the variation in evolutionary rate across the molecule (Crozier et al., 1989). However, in contrast to other taxa, the nuclear DNA of flies (Drosophila spp.) evolves at the same rate as mtDNA (Moritz et al., 1987; Zhang & Hewitt, 1997).

The major non-coding region, known as the control or A+T rich region is often utilised in population level studies due to relatively high variation, often length variation resulting from tandem repetition in the sequence (Lunt et al., 1997). This region is not under strict functional constraints, however it does play a role in the initiation of replication and transcription (Moritz et al., 1987).

Protein-coding mitochondrial genes such as the cytochrome oxidase I (COI) gene are commonly chosen for lower level phylogenetic studies due to their relatively conserved nature across taxa and therefore ability to be amplified using universal primers. This facilitates the collection of data on taxa not previously studied. In addition, the mitochondrial protein-coding genes display a high rate of substitution in third codon
positions, often synonymous changes resulting in no amino acid change. This makes nucleotide sequence data a useful subject for study of lower taxonomic levels, while the amino acid sequence is of greater use in higher level analyses (Hwang & Kim, 1999).

Table 2.1 provides a summary of the general usefulness of commonly studied regions of DNA at specified taxonomic levels. It is evident from this table that the non-coding nuclear intergenic spacer (IGS) and internal transcribed spacer (ITS) regions, and the non-coding mitochondrial control region are most useful for low level taxonomic level studies, as a result of the high variability in these areas. The protein coding mtDNA genes hold the most promise for use with species level studies, an important focus of this study.

**Table 2.1** Taxonomic levels at which some commonly studied nuclear and mitochondrial regions of DNA may be useful in inferring phylogenies (Hwang & Kim, 1999)

<table>
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<tr>
<th>Region</th>
<th>Kingdom</th>
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The bold lines indicate mainly applicable categorical levels of each molecular marker or gene region while the dot lines indicate less frequently applicable categorical levels.

In a forensic context, the region of DNA chosen for identification of insects needs to provide distinction at the species level, and as Table 2.1 indicates, the NADH dehydrogenase (ND) genes and cytochrome oxidase (CO) genes provide useful targets. The CO genes have been investigated extensively in the Insecta and, therefore, provide a convenient array of universal primers as a starting point for sequencing. In a study of the COI gene of the Muscidae, Sarcophagidae, Calliphoridae and Drosophilidae, the
COI gene was shown to display high nucleotide sequence conservation across all four families (De Oliveira et al., 2005), making this gene useful in distinction of both the forensically significant Calliphoridae and Sarcophagidae. Hwang & Kim (1999) further indicate that the COI is the most conserved of the CO genes, providing greater likelihood that universal primers will successfully amplify the forensically important calliphorids.

Variation at the subspecies and population level of the calliphorids in this study would also be desirable. This may be obtained from the nuclear spacer regions (IGS and ITS), the control region, or possibly the ND and CO genes (Table 2.1). The interspecific distinction, as the main goal of this study, appears most likely to be successfully obtained from the CO genes and therefore the COI was chosen as the target region, with possible population level variation to be evaluated.

The COI encoding region of mtDNA has been a greatly utilised region in identification and phylogenetic studies within the Insecta. Numerous studies have capitalised on the potential of the region to infer relationships across a diversity of taxa, including Lepideoptera (Sperling et al., 1999), Hymenoptera (Crozier et al., 1989; Diptera (Gleason et al., 1998; O’Grady et al., 1998; Wells et al., 2001b; Martin et al., 2002; Otranto et al., 2003; Noel et al., 2004; Lyra et al., 2005) and Hemiptera (Sunnucks & Hales, 1996).

The COI gene was therefore chosen as the target region for this study. At the commencement of this study in 2000, the basic ability to identify the locality specific carrion fauna had been established in several studies. Sperling et al. (1994) had displayed the ability to distinguish a number of species using restriction fragment length polymorphisms (RFLP) based on the COI region. Gleeson & Sarre (1997), Malgorn & Coquoz (1999), Wells & Sperling (1999) and Vincent et al., (2000) had all confirmed the potential of the COI.

This study served as a pilot investigation of the utility of the COI region to distinguish between calliphorid species commonly observed on carrion in Western Australia. The aims of the study were to determine:

- Can the COI of forensically important calliphorids from Western Australia be amplified using universal primers?
• Is it possible to distinguish these species based on sequence data for the COI gene?
• What population level information can be gained for these species based on the COI gene?
• What potential does the COI hold for the distinction of a wider range of forensically significant calliphorids?

This study was commenced as an honours project in 2000. The technique for amplification was optimised during the honours year. In 2001 the study was continued as part of the current PhD study, with sequencing performed and analysis conducted. The study is published in the current form as:

Abstract

Forensic entomology requires the fast and accurate identification of insects collected from a corpse for estimation of the postmortem interval (PMI). Identification of specimens is traditionally performed using morphological features of the insect. Morphological identification may be complicated however by the numerical diversity of species and physical similarity between different species, particularly in immature stages. In this study, sequencing was performed to study the mitochondrial DNA (mtDNA) as the prospective basis of a diagnostic technique. The sequencing focused on a section of the cytochrome oxidase I encoding region of mtDNA. Three species of calliphorid (blow flies) commonly associated with corpses in Western Australia, *Calliphora dubia*, *Chrysomya rufifacies* and *Lucilia sericata*, in addition to specimens of *Calliphora augur* and *Chrysomya megacephala* were studied. Phylogenetic analysis of data revealed grouping of species according to genus. The DNA region sequenced allowed identification of all species, providing high support for separation on congeneric species. Low levels of variation between some species of the same genus however indicate that further sequencing is required to locate a region for development of a molecular-based technique for identification.

*Key words*: Calliphorid; Forensic entomology; Cytochrome oxidase I; Mitochondrial DNA
1. Introduction

Forensic entomology involves the study of the interaction of insects and other arthropods with legal matters (Hall, 1990). An important contribution made by the forensic entomologist is the estimation of the time since death or postmortem interval (PMI). Insects collected from a corpse must be identified accurately (Morris, 1993), their age estimated and relevant developmental data applied to approximate the PMI.

Accurate estimation of the PMI requires accurate identification of insects, as misidentification may result in the application of incorrect developmental data. Identification of the individuals may be complicated by many factors, which include the diversity of adult fly species, the particular larval life stage collected and the collection of dead insects only. Immatures are most frequently collected, yet their diagnostic characters may be difficult to recognise in these stages and there are few comprehensive larval keys to Australian species. Immatures, therefore, may require rearing to adult stage for accurate identification, a time-consuming process. Furthermore, specimens may be killed before presentation to the entomologist, or damaged specimens may lack diagnostic characters. In many legal systems, the maintenance of integrity of evidence for presentation in court proceedings is of high importance (Tilstone, 1993), and integrity may be regarded by some judicial parties as being compromised where rearing has occurred, as it involves a change in the form of the evidence.

To overcome difficulties associated with identification by morphological features more appropriate techniques need to be developed, largely to augment the morphological data already established (Simon, 1990). These techniques also need to provide more rapid identification, as early identification may be crucial for the successful conclusion of a case (Morris, 1993; Sperling et al., 1993). Identification should also be possible from any life stage of the insect and any method of preservation.

DNA based techniques appear promising due to the durability and stability of the DNA molecule. The techniques can also overcome difficulties associated with morphological identification where specimens are damaged and diagnostic characters are lacking. In addition, molecular techniques potentially provide a more rapid identification as DNA can be extracted from any stage and subjected to further protocols immediately.
In order to develop a suitable diagnostic test for use in forensic entomology, species-specific molecular markers need to be identified for interspecific distinction. DNA sequencing produces data of high information content and allows both intra- and interspecific comparison, and these species-specific markers are used for identification purposes. Sequencing of the mitochondrial region encoding the cytochrome oxidase I (COI) gene is particularly useful in evolutionary studies, population genetics and systematics due to the relatively high degree of variation in the region (Malgorn & Coquoz, 1999). Generally mtDNA has a higher mutation rate than nuclear DNA and, therefore, an increased chance of generating species-specific markers. In addition, mtDNA may be isolated more easily than nuclear DNA (Harrison, 1989), which is clearly advantageous to forensic studies where specimens may be incomplete or in poor condition.

The dipteran family Calliphoridae are of immense importance forensically as they are generally the first arthropods to locate and oviposit onto a corpse. Previous studies of the Calliphoridae have also focused on sequencing of the mitochondrial region encoding the COI gene (Sperling et al., 1993; Malgorn & Coquoz, 1999; Benecke & Wells, 2000; Wallman & Donnellan, 2001).

This study involved sequencing to examine the potential of DNA for use in the identification of insects in forensic investigations. The sequence variation within the COI gene of the mitochondrial genome of several species was selected for examination. Western Australian individuals of *Calliphora dubia* (Macquart), *Chrysomya rufifacies* (Macquart) and *Lucilia sericata* (Meigen) were sequenced over a region of 278 base pairs. In addition, specimens of *Chrysomya megacephala* (Fabricius) and *Calliphora augur* (Fabricius) from other Australian states were included. From this data both intra- and interspecific sequence diversity were considered to form inferences about the relationships between the species, for the development of an identification technique. These species were selected because of their importance in death investigations in Western Australia (Morris & Dadour, 2005).

2. Materials and Methods

2.1 DNA Extraction

DNA was extracted using the phenol-chloroform extraction technique of Taylor *et al.* (Taylor *et al.*, 1996), with the proteinase K incubation step reduced from three to two
hours. Third instar larvae, pupae and adults were used. Specimens of *L. sericata* (four specimens), *C. dubia* (five specimens) and *Ch. rufifacies* (three specimens) were obtained from laboratory colonies maintained by regular addition of new individuals from populations over a 1600 km$^2$ area of southwestern Australia. In addition, specimens from Queensland and Victoria were included. These were three individuals of *Ch. rufifacies*, and a single individual of each of *L. sericata*, *Ch. megacephala* and *C. augur*.

### 2.2 Primers

A region of the COI gene was amplified using the primers C1-J-2495 (5’ CAG CTA CTT TAT GAG CTT TAG G3’) and C1-N-2800 (5’ CAT TTC AAG CTG TGT AAG CAT C3’). The primer sequences were taken from previous studies of the Calliphoridae (Sperling *et al.*, 1993; Wells & Sperling, 1999).

### 2.3 Polymerase chain reaction (PCR)

Final concentrations in a 25 µl PCR reaction mix were: 1.5 units Taq polymerase (Promega), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$ (Promega 10x PCR Reaction Buffer), 200µM dNTPs and 25 pM each of the forward and reverse primers (Genset Pacific Oligos). Between 50-150 ng of template DNA, quantitated using 260/280nm spectrophotometry, was used in each reaction successfully, with 100 ng optimal. Reactions were conducted on a Corbett Research FTS-960 thermal cycler. Cycling conditions were 35 cycles of: 92°C for 1 min, 48°C for 1 min and 72°C for 2 min, followed by storage at 4°C in the original PCR mix.

### 2.4 Sequencing

DNA was sequenced both forward and reverse for all specimens. In addition, a sequence for the COI of *Ch. rufifacies* was obtained from the public DNA database (Genbank accession number AF083658).

Sequencing was conducted using ABI PRISM Big Dye Terminator sequencing kits and an ABI 373 (Applied Biosystems) sequencer.

Sequence chromatograms were edited and discrepancies between forward and reverse sequences resolved using Sequence Navigator (v1.01, Applied Biosystems), and the
resultant sequences aligned using ClustalW (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Comparison of the sequences was performed to identify intra- and interspecific nucleotide differences.

Phylogenetic systematic analysis is the study of the relationships between different groups of organisms, and is thus based on similarity and difference in characters chosen (see Hillis & Moritz, 1990 for further phylogenetic definition). A neighbour-joining tree using the Tamura-Nei (Tamura & Nei, 1993) model of nucleotide substitution was constructed using the MEGA package. Maximum parsimony analysis was also conducted to calculate the tree based on the least number of evolutionary steps. 500 bootstrap replications were performed. Bootstrap values are calculated by repeated random sampling of the data to provide an indication of the confidence limits for a particular grouping.

3. Results

The sequence analysis was based on 278 base pairs. Sequences are deposited in the DNA Databank of Japan (consecutive accession numbers AB080249-AB080260).

Fig. 2.1 displays the neighbour-joining tree constructed from sequence data. Invariant positions in the sequence were removed and the remaining variant positions numbered relative to the original sequence to indicate the number of substitutions supporting the divergence of the taxa (Table 2.2). The neighbour-joining tree was identical to the tree obtained using maximum parsimony methods. Three distinct congeneric clusters were formed based on the sequence data. High bootstrap values support the three nodes. Bootstrap values provide an indication of the percentage support for a grouping by randomly re-sampling the data.

The two species of the genus Chrysomya were grouped with high bootstrap support. At species level, specimens of *Ch. rufifacies* formed a single cluster with 100 percent support. Within the *Ch. rufifacies* clade considerable variation is evident, with Australian individuals showing a small amount of differentiation from the American individual for which a Genbank sequence was obtained. The Queensland individuals showed some divergence from each other, but no variation correlating with geographical separation from the Western Australian individuals.
The specimens of *L. sericata* were grouped together with 99 percent bootstrap support, yet the one Queensland individual displayed considerable variation from the Western Australian individuals. The long branch lengths support the division between the two locations, with all Western Australian individuals being identical.

The *Calliphora* individuals clustered together with a value of 100, but with low support for the separation of *C. augur* and *C. dubia*. Three substitutions separated the two species over this region, compared with the 20 substitutions separating the congeneric *Ch. megacephala* and *Ch. rufifacies*. The *C. dubia* individuals displayed a small degree of intraspecific variation, each having one or two polymorphisms distinguishing them from the other individuals.
4. Discussion

The high support for the congeneric grouping of species illustrates the potential of the COI for use in interspecific distinction. The ability to clearly distinguish between these three forensically prominent genera based on such a small region provides a strong indication of the possible utility of a larger region of the COI.

Separation of *Ch. megacephala* and *Ch. rufifacies* was highly supported, making this region useful for identification of these species. The intraspecific variation within *Ch. rufifacies* does, however, require further consideration.

Individuals of *Ch. rufifacies* are reported to be morphologically identical across the entire distribution of the species, yet there are behaviourally distinct populations displaying life history differences. In Hawaii the species is a primary strike species locating corpses soon after death (Baumgartner, 1993), yet in Australia it is described as a secondary species (Morris, 1993). Contrary to the claim that *Ch. rufifacies* is a secondary species in Australia, on the eastern coast in Queensland the species is considered primary all year round, and on the south-eastern coast in New South Wales is primary in summer and secondary in winter (pers. comm. I. Dadour). The morphological similarity yet behavioural variation indicates the possible presence of cryptic species, or possibly a habitat shift as a reaction to ecological competition with other primary strike species in certain areas. Alternatively, some genetic switch may be influencing the behavioural variation occurring across the distribution, affected by seasonality. The implication of such species complexes is that the different species within a complex may each have different developmental histories and thus an inappropriate set of developmental data may be applied, making a PMI estimate inaccurate.

The preliminary results obtained in this study indicate a small amount of variation between individuals, both on national and international scales. In particular, the Queensland individuals appear to display a degree of variation. Sampled from the Brisbane area in south-east Queensland, this variation is surprising in comparison with the Western Australian individuals which show less variation. *Ch. rufifacies* is suggested to be introduced to new regions via shipping and aircraft (Baumgartner,
1993), indicating the possibility of multiple introductions of *Ch. rufifacies* to the Brisbane region by such means.

The variation within *L. sericata* again reflects the geographical separation of populations. The species is also introduced by similar means to *Ch. rufifacies*, supporting the relatively high level of variation between the two populations.

The difficulty in distinction between *C. augur* and *C. dubia* based on COI sequence data was also reported by Wallman & Donnellan (2001). These two endemic Australian species are closely related and further sequencing is required to separate them.

Ultimately, more field colonies need to be sampled and sequenced to confirm the degree of intraspecific variation for each species. In addition, the results of this study indicate the importance of sampling over a wider geographic range, as geographically separated populations displayed considerable divergence, with the exception of the aforementioned *Ch. rufifacies* populations. Sequencing of geographically isolated populations will allow confirmation of species status, ensuring cryptic species are not overlooked as may be the case under the current morphological approach to identification. Sequence data is also required from a variety of populations to ensure that nucleotide changes identified are species-specific, and not only common to isolated populations. This will result in accurate identification with confidence that a particular marker or group of markers within the sequence are characteristic and diagnostically useful.

5. Conclusions

It was anticipated that this study would identify polymorphisms that could potentially be exploited as the bases of molecular techniques for identification. Sequencing this region of mtDNA has provided data that appear to indicate the potential of mtDNA in the development of an identification protocol for use in forensic entomological studies.

The 278 base pair region of the COI sequenced in this study displays the potential of mtDNA for use as a discriminatory tool in forensic investigations. A larger region of the COI will need to be sequenced for the separation of closely related species, such as *C. augur* and *C. dubia*, yet the results of this study do indicate the value of the COI in general. Further sequencing of an increased number of specimens over a wider
distribution will allow the resolution of species status and development of a simpler and more reliable technique for identification of insects in forensic investigations.

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**Fig 2.1** Neighbour joining tree displaying relationships between *L. sericata*, *C. augur*, *C. dubia*, *Ch. megacephala*, and *Ch. rufifacies* based on partial sequence of COI. Bootstrap values indicate support for nodes. The bar indicates 0.02 substitutions per site.
Table 2.2 Nucleotide substitutions for variant positions in partial sequences obtained for *Ch. rufifacies*, *Ch. maegacephala*, *L. sericata*, *C. augur* and *C. dubia* numbered relative to the entire sequence obtained

| **Ch. rufifacies (Qld)** | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. rufifacies (Qld)** | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. rufifacies (WA)**  | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. rufifacies (WA)**  | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. rufifacies (WA)**  | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. rufifacies (AF03658)** | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. megacephala (Qld)** | T A T A T T T T A A C A C T T C T A T T A T A T C A T T T |
| **Ch. rufifacies (Qld)** | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **L. sericata (Qld)**   | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **L. sericata (Qld)**   | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **L. sericata (WA)**    | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **L. sericata (WA)**    | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **L. sericata (WA)**    | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **L. sericata (WA)**    | C T A G T T T T T A A C A C T T C T A T T A T A T C A T T T |
| **C. augur (Vic)**     | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. augur (Vic)**     | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **C. dubia (WA)**      | C T C G A T C C T T T T T T T A A T C A T T T C A C T C T T |
| **Ch. rufifacies (AF03658)** | A C T A A T A A C A G T A T A C C T T C T T C C T C C G T |
| **Ch. rufifacies (Qld)** | A C T A A T A A C A G T A T A C C T T C T T C C T C C G T |
| **Ch. rufifacies (Qld)** | A C T A A T A A C A G T A T A C C T T C T T C C T C C G T |
| **Ch. rufifacies (Qld)** | A C T A A T A A C A G T A T A C C T T C T T C C T C C G T |
| **Ch. rufifacies (Qld)** | A C T A A T A A C A G T A T A C C T T C T T C C T C C G T |
| **Ch. rufifacies (Qld)** | A C T A A T A A C A G T A T A C C T T C T T C C T C C G T |
| **L. sericata (Qld)**  | A T T A C T G A A T A G T C T A C C T C A T A C T C C G C T |
| **L. sericata (Qld)**  | A T T A C T G A A T A G T C T A C C T C A T A C T C C G C T |
| **L. sericata (Qld)**  | A T T A C T G A A T A G T C T A C C T C A T A C T C C G C T |
| **L. sericata (Qld)**  | A T T A C T G A A T A G T C T A C C T C A T A C T C C G C T |
| **L. sericata (Qld)**  | A T T A C T G A A T A G T C T A C C T C A T A C T C C G C T |
| **L. sericata (Qld)**  | A T T A C T G A A T A G T C T A C C T C A T A C T C C G C T |
| **C. augur (Vic)**     | T T T T G G A A A A C A T A A T A T A T A T A T T C C C C G T |
| **C. dubia (WA)**      | T T T T G G A A A A C A T A A T A T A T A T A T T C C C C G T |
| **C. dubia (WA)**      | T T T T G G A A A A C A T A A T A T A T A T A T T C C C C G T |
| **C. dubia (WA)**      | T T T T G G A A A A C A T A A T A T A T A T A T T C C C C G T |
| **C. dubia (WA)**      | T T T T G G A A A A C A T A A T A T A T A T A T T C C C C G T |
GENERAL DISCUSSION
This study evaluated the potential of a small region of the COI gene to provide
distinction between some species of forensic importance in Western Australia, based on
a set of universal PCR primers suggested in the calliphorid literature.

The universal primers C1-N-2800 and C1-J-2495 proved useful for the amplification of
278bp of the COI gene, displaying their utility for application to multiple genera and
strong potential for use with previously unstudied species. This is of particular
importance in localities where data is being gathered on forensically significant, novel
calliphorid species.

The region was shown to successfully distinguish between *Ch. megacephala*, *Ch.
rufifacies*, *L. sericata* and *C. dubia*, all common calliphorid species on carrion in
Western Australia. The separation of the sister species *C. dubia* and *C. augur* did not,
however, receive strong bootstrap support, perhaps as a result of the limited size of the
region of DNA sequenced. *C. augur* is not found in Western Australia and distinction
from *C. dubia* is not pertinent for Western Australian cases, however in areas where
both species are found, or where evolutionary relationships are being inferred between
the species, the issue of the molecular identification of these species must be addressed.
Population level variation over this region was limited.

The collection of a body of sequence data suitable for species distinction and
consideration of species issues necessitates the sequencing of specimens from
geographically separated, conspecific populations. Multiple individuals should be
sampled from each populations to ensure that genetic markers chosen for populations
and species are present throughout all insects to be identified. It is equally important
that, where possible, insects sequenced originate from wild populations as opposed to
laboratory colonies subject to inbreeding and consequently genetic isolation.

The COI does appear to hold significant value for the distinction of forensically
important calliphorid species, however future studies will need to consider a larger
fragment of the COI. The extension of the region will potentially increase the number of
informative sites present in the sequences and provide stronger support for the
separation and identification of some closely related species, and possibly intraspecific variation for consideration of potential cryptic species complexes.
CHAPTER 3:
The Distinction of Forensically Important calliphorids of southern Africa & Australia: Utility of 1167bp of the Cytochrome Oxidase I Gene
The potential of the cytochrome oxidase I (COI) gene to distinguish between forensically important calliphorid species within Western Australia was confirmed in the previous chapter. The study illustrated, however, the need to increase the size of the region sequenced to provide more sequence data and greater scope for informative variation.

Living organisms of the world are frequently divided according to zoogeographic regions, reflective of the location and environmental conditions observed in that region. The zoogeographic regions are illustrated in Figure 3.1. Neighbouring regions may share faunal taxa, resulting from similarity in environment and the transport of fauna as a result of close proximity.

![Figure 3.1 The zoogeographic regions of the world (Swanson, 2005)](image)

Some forensically important calliphorid species such as *Chrysomya megacephala* and *Lucilia sericata* are relatively cosmopolitan species. The presence of such species across a number of biogeographical regions illustrates their adaptive ability and raises several important questions such as: (1) from where did they originate? (2) How did they become spread across such a wide and biogeographically diverse distribution? (3) Is the adaptation to biologically diverse habitats reflected by morphological and/or behavioural variation? (4) What implication does this variation have for forensic entomology? and (5) Do these variable forms of a species represent distinct species in their own right?
The Afrotropical and Australasian regions are neighbouring zones representing their own distinct fauna, yet both regions were originally derived from Gondwana Land. Their common historical biogeography is also reflected in the sharing of some common species, such as coastal lizards found in both Australasia, and Eastern Africa and Madagascar (Rocha et al., 2006). Under theories of vicariance biogeography, plate tectonics are thought to explain the occurrence of common taxa on neighbouring, or historically neighbouring continents (Shaw, 2001; de Queiroz, 2005). Likewise, theories of oceanic dispersal, that is, dispersal across oceans by a variety of mechanisms may be credited as the reason for shared taxa between continents (de Queiroz, 2005). Whatever the reason, the occurrence of shared faunal taxa makes the study of the genetic relatedness between common and closely-related species a useful exercise in the assessment of species status.

Forensic entomology has been an important investigative tool in South Africa in recent years, and as time passes is becoming more prominent. The Supreme Court of South Africa first accepted forensic entomological evidence in 1995 (Mervyn Mansell, personal communication). The science is now supported there by two prominent practitioners, despite considerable amounts of casework (Mervyn Mansell, personal communication). The application of molecular-based identification techniques would be a useful way to accelerate the estimation of post-mortem interval (PMI) by eliminating the need to rear all immature insects to adult stage for identification. However, the molecular status of forensically important southern African calliphorids has generally been neglected in the literature, with forensic research directed towards decompositional studies, mainly on large animal carcasses (Louw & van der Linde, 1993) or studies relating to the behaviour and biology of specific calliphorid species (Laurence, 1988). Therefore a molecular survey of the forensically important calliphorid species would be an important contribution to the advancement of forensic entomology as a routine investigative tool in South Africa.

The sequencing of cosmopolitan species has obvious benefits in the consideration of population level variation and possible species complexes. However, there are some sister species pairings that also warrant verification of their separation based on molecular data.
The sister species *Ch. rufifacies* and *Ch. albiceps* are forensically significant calliphorids that were thought for a long time to be synonymous (Azeredo-Espin & Lessinger, 200). The species long occupied separate distributions, with *Ch. rufifacies* commonly found throughout the Australasian and Oriental regions, and *Ch. albiceps* mainly Palaeartic (Tantawi & Greenberg, 1993). In the last 25 years, however, the distributions have expanded significantly. *Ch. albiceps* has expanded throughout the Afrotropical region. *Ch. rufifacies* is now found in the Nearctic region, and with the introduction of *Ch. rufifacies* to Costa Rica, and *Ch. albiceps* to Brazil, the species are have now been reported to overlap in the New World (Wells & Sperling, 1999). The New World includes American continents and adjacent islands, while the Old World refers to Europe, Asia and Africa. The comparison of *Ch. rufifacies* and *Ch. albiceps* makes an important molecular comparison, given their high morphological similarity, similarity in biological niches and forensic relevance, and impending sympatry (Tantawi & Greenberg, 1993; Wells & Sperling, 1999). In addition, the morphologically similar species *Ch. megacephala* and *Ch. marginalis* are both present in Africa with impending sympatry, (Erzinclioglu, 1990) making their accurate identification an important consideration.

The common biogeographical origin of Australia and southern Africa, and thus the shared nature of some species and presence of closely related species makes comparison with some Australian species a useful exercise for examining levels of genetic variation within and between calliphorid species. With the exception of the study from Chapter 2 of this thesis, just one study (Wallman & Donnellan, 2001) had explored the fauna of Australia. Wallman & Donnellan reported success in distinguishing the majority of Australian forensically important species, and recorded difficulty only in separating closely-related endemic species from the genus *Calliphora* based on 639bp of the COI gene, illustrating a need for further study of the Australian fauna using a larger region of this gene.

The following study aimed to increase the size of the COI region sequenced, based on success in the preliminary study (Chapter 2). The specific questions addressed included:

- Does the use of a larger amount of COI sequence data improve resolution of the separation between forensically important species?
- What level of variation is observed over this region between conspecific populations from the Afrotropical and Australasian regions?
• Does the phylogeny obtained from the data reflect the shared historical biogeography of southern Africa and Australia?

• What implication does the expansion of the COI region sequenced hold for the identification of species?

Specimens were collected and obtained firsthand from other researchers during 12 months spent at the University of Pretoria in South Africa, with some work performed at Rhodes University in Grahamstown. This study has been published in the following format:

Abstract.

One major aspect of research in forensic entomology is the investigation of molecular techniques for application to the accurate identification of insects. Studies to date have addressed the corpse fauna of many geographical regions, but generally neglected the southern African calliphorid species. In this study, forensically significant calliphorids from South Africa, Swaziland, Botswana and Zimbabwe and Australia were sequenced over an 1167 base pair region of the COI gene. Phylogenetic analysis was performed to examine the ability of the region to resolve species identities and taxonomic relationships between species. Analyses by neighbour-joining, maximum parsimony and maximum likelihood methods all showed the potential of this region to provide the necessary species-level identifications for application to postmortem interval (PMI) estimation, however higher level taxonomic relationships did vary according to method of analysis. Intraspecific variation was also considered in relation to determining suitable maximum levels of variation to be expected during analysis. Individuals of some species in the study represented populations from both South Africa and the east coast of Australia, yet maximum intraspecific variation over this gene region was calculated at 0.8%, with minimum interspecific variation at 3%, indicating distinct ranges of variation to be expected at intra- and interspecific levels. This region therefore appears to provide southern African forensic entomologists with a new technique for providing accurate identification for application to estimation of PMI.

Key words. Calliphoridae, blowflies, Africa, mitochondrial DNA, forensic entomology
Introduction

Forensic entomology has been an important investigative tool for many years, particularly through its use in court trials in providing an estimation of postmortem interval (PMI) in homicide cases. This application of entomology to investigations demands great accuracy in PMI estimations, resulting in significant research addressing this issue.

The correct identification of specimens is a critical prerequisite in the estimation of PMI using insects, but this may be difficult using the traditional morphology-based approach (Prins, 1982; Wallman, 2001). Several studies have addressed this issue by using DNA sequences to identify insects, most choosing to use mitochondrial DNA (mtDNA) as the basis for sequencing (Sperling et al., 1994; Malgorn & Coquoz, 1999; Wallman & Donnellan, 2001; Wells & Sperling, 2001; Harvey et al., 2003). These studies have revealed the potential for the use of mtDNA in providing more accurate identifications for the estimation of PMI.

The majority of literature in the field of forensic entomology has addressed the corpse fauna of the United States, Europe, Britain and Australia, but generally neglected Africa. In southern Africa, forensic entomology is being increasingly incorporated into death investigations (Mervyn Mansell, pers. obs.). To date, southern African research has focused largely on the succession of insects on corpses (Louw & van der Linde, 1993), and a few studies have considered the succession on animal carcasses that may be applied to human corpse succession (Meskin, 1986; Braack & De Vos, 1987; Ellison, 1990). Despite increased interest in forensic entomology, DNA-based identification still remains a curiosity rather than an application in southern Africa. This is largely a result of the small amount of genetic data collected on the forensically significant species. A robust typing method requires a large body of data to ensure that characters used to distinguish species are represented among all populations of a species, and consequently to consider the intraspecific variation that may be observed across the distribution of a species. The usefulness of such information becomes evident as several African insects associated with carcasses have now been identified from South America (Lawrence, 1986).
mtDNA is recognized as being useful for evolutionary study due to a relatively higher mutation rate than nuclear DNA (Hoy, 1994), and also the presence of both conserved and variable segments. Evolutionary studies of forensically important Calliphoridae using phylogenetic techniques allow visualization of relationships between species, based on levels of similarity in sequence data. Such relationships are useful to elucidate, as they often reflect the morphological and behavioural discrepancies observed in the field and provide a greater understanding for entomologists of potential pitfalls in data. This may be in the discovery of species complexes, or simply in the phylogenetic confirmation of the genetic separation of two highly similar species over which species status may be questioned.

The potential of the cytochrome oxidase I (COI) encoding region of mtDNA has been shown to be useful for identification in many studies (e.g. Sperling et al., 1994; Malgorn & Coquoz, 1999). Various segments of this region have been sequenced in different studies, ranging from 278 base pairs to the entire COI gene. This study considered the use of an 1167bp region of the COI gene for identification of forensically important calliphorids in southern Africa. The region encompassed the 278bp segment used by Harvey et al. (2003) and the 639 sites used by Wallman & Donnellan (2001) to provide successful distinction with the Australian corpse fauna.

Flies from Zimbabwe, Swaziland, Botswana, Zambia and South Africa were sequenced and phylogenetic analyses used to construct evolutionary relationships between them. Species sequenced were Chrysomya albiceps (Wiedemann), C. megacephala (Fabricius), C. putoria (Wiedemann), C. marginalis (Wiedemann), C. inclinata Walker and Lucilia sericata (Meigen). Forensically significant Australian species from the sub-families Chrysomyinae and Luciliinae were also included, in order to test intraspecific variation for species also found in southern Africa. Specimens of C. rufifacies (Macquart), C. varipes (Macquart) and L. cuprina (Wiedemann), were also included, along with the muscid Hydrotaea rostrata (Robineau-Desvoidy). Hydrotaea rostrata was included primarily for its forensic relevance, but also as an outgroup. While a sarcophagid species may have formed a more suitable outgroup, there were no sequences available of sufficient length and thus a more distantly related muscid was chosen. The Calliphorinae, while commonly found on corpses in Australia, are only represented by Calliphora croceipalpis (Jaennicke) and C. vicina (Robineau-Desvoidy) in southern Africa and were not included in this analysis. The potential of the COI
encoding region for use in identification of flies for PMI estimation based on insects is discussed.

**Materials and Methods**

**Samples**

Adult flies were used in this study as adult morphological characters allowed more rapid and accurate identification to species level than larval characters. Specimens were identified using keys and characters in Zumpt (1965). Origins of specimens are displayed in Table 3.1, and geographical locations in Figure 3.2. Flies were trapped in Zimbabwe, Swaziland, Botswana, Zambia and South Africa, and Australian specimens were taken from laboratory colonies or trapped using liver-baited traps. Specimens were preserved in 70% ethanol and refrigerated.

**DNA Extraction**

Extraction was performed using a Chelex 100 (BioRad) technique modified from Hunt (1997). An incision was made under the left wing of each specimen, and flight muscles removed and macerated. In a few specimens flight muscles had degraded and consequently, a single wing of the fly was used for extraction. The remainder of the specimen was then stored in ethanol and refrigerated, as a voucher specimen. The muscle or wing was then frozen using liquid nitrogen and ground to a fine powder using micropestles in 1.5mL eppendorf tubes. 100µL of a 5% solution of Chelex was added to the ground material, vortexed, and incubated for 15 minutes on a 95°C heat block. Following incubation, the sample was vortexed for 5 seconds, and centrifuged for 3 minutes at maximum speed. The supernatant was removed and stored at -20°C.

**PCR Conditions and Purification of PCR Products**

The primers used amplified a region of approximately 1270 base pairs. Primers were C1-J-1718 (5’-3’ GGAGGATTTGGAAATTGATTAGTTCC) and TL2-N-3014 (5’-3’ TCCAATGCACTAATCTGCCATATTCA) (Simon *et al.*, 1994).

The PCR reaction mix consisted of: 1 X PCR buffer (Biotools), 200µM dNTPs (Biotools), 1.5mM MgCl₂, 25pm each primer and 3µl template DNA, and water added to a total volume of 50µL. A Perkin Elmer GeneAmp PCR System 2400 thermocycler was used. The program began with a 1 minute 30 second 94°C denaturation period,
followed by 36 cycles of: 94°C for 22 seconds, 48°C for 30 seconds and 72°C for 1 minute 20 seconds. A final extension period of 1 minute at 72°C was used, followed by storage at 4°C. Electrophoresis of PCR products was performed using 1.5% agarose gels with ethidium bromide staining. Products were purified using the High Pure PCR Product Purification Kit (Roche), with elution in 50μL of water as opposed to the elution buffer supplied by the manufacturer.

**Sequencing**

Sequencing reactions were performed using the ABI PPRISM Big Dye Terminator 3.0 Sequencing Kit (Perkin Elmer) under recommended cycling conditions, but with the annealing temperature lowered to 48°C. The external primers were C1-J-1718 and TL2-N-3014, and the internals were C1-J-2183 (5’-3’ CAACATTTATTTTGATTTTTTGG) and C1-N-2329 (5’-3’ ACTGTAATATATGATGAGCTCA) (Simon *et al*., 1994). Sequences were compiled using the ABI 3100 and ABI 377 systems. Analysis was performed using Sequence Navigator software (v1.01, Applied Biosystems), Chromas v1.43 (http://trishul.sci.gu.edu.au/~conor/chromas.html) and DAPSA (University of Cape Town). Phylogenetic analysis was performed using MEGA 2.0 (Kumar *et al*., 2001) and PAUP*4.0 (Swofford, 2002) software packages. Sequences were submitted to GenBank (see Table 3.1 for accession numbers).

**Analysis**

Both neighbour-joining and maximum parsimony techniques were used for analysis to compare the results of both distance and discrete methods. Sequences were tested for purifying selection using the Z-test option in MEGA. Neighbour-joining analysis was performed using the Tamura-Nei model of substitution and bootstrapping (n=500) conducted using MEGA. A heuristic maximum parsimony search was conducted and bootstrapped (50 replicates). The maximum likelihood analysis was performed using the HKY85 model of substitution with starting trees derived from parsimony, and the transition/transversion ratio and gamma shape parameter estimated. Parsimony and likelihood analyses were conducted in PAUP*. *H. rostrata* was the outgroup in all analyses.
Results

Calliphorid sequences from 41 flies were successfully sequenced and aligned with that of one muscid individual. The sequences corresponded to positions 1776-2942 of *Drosophila yakuba* (GenBank accession number NC_001322). No insertions or deletions were identified within the aligned sequences. Of 1167bp analysed, 287 of these positions were variant and of these, 211 were parsimony-informative characters.

The nucleotide composition showed much higher frequencies of adenine (A) and thymine (T) at 40% and 30% of total nucleotide composition as compared to 15% cytosine (C) and 15% guanine (G). The sequences were also tested for purifying selection using a Z-test, comparing the relative abundance of synonymous and non-synonymous substitutions, indicating that in general purifying selection appears to have acted on the sequences with synonymous changes far exceeding non-synonymous substitutions.

Pairwise divergence between species was calculated and is expressed as percentages in Table 3.2. Variation was calculated based on an average computed from all individuals of the species. *Hydrotaea rostrata*, a muscid included as an outgroup, displayed 12.83-15.7% variation from all calliphorid species. The lowest interspecific variation was between the two sister species pairings of *L. sericata* and *L. cuprina* with 3%, and *C. rufifacies* and *C. albiceps* at 3.5%. At a higher level, the sub-families Chrysomyinae and Luciliinae displayed greater than 8% variation across all species.

The tree resulting from neighbour-joining analysis is shown in Figure 3.3. Maximum parsimony analysis returned 20 possible trees, all reflecting identical topologies. The consensus tree was computed, and the bootstrapped version resulted in the same tree as gained using neighbour-joining. Maximum likelihood analysis also returned a similar tree (not shown). Likelihood analysis was performed using the HKY85 model in PAUP*, using unequal base frequencies. The gamma distribution shape-parameter and transition/transversion ratio were estimated from the data as 0.11567 and 2.42, respectively.

The trees produced using both the distance and the discrete methods of analysis yielded very similar topologies. The sub-family status was well supported, with the Luciliinae displaying 100% support in all analyses, and the species of the Chrysomyinae also
grouping together, but with lower support. In almost all analyses, individuals of each species received bootstrap support of 100%, indicating the strong basis for species distinction. The major discrepancy between the results came in the grouping of *C. varipes* with the closely related *C. rufifacies* and *C. albiceps* under likelihood, yet with the other *Chrysomya* species under neighbour-joining and maximum parsimony. However, when the likelihood trees were bootstrapped, *C. varipes* reverted to grouping with the other *Chrysomya* species.

*C. rufifacies* and *C. albiceps* formed a single grouping with strong support, but separated into two distinct species groups. The morphologically similar *C. putoria* and *C. inclinata* also formed a separate grouping, and *C. varipes* was a comparatively isolated group within the Chrysomyinae. *L. sericata* and *L. cuprina* also separated to form species groupings with full bootstrap support.

Intraspecific variation was relatively low. Maximum values observed between individuals of each species are displayed in Table 3.3. The highest level was observed in *C. varipes* at 0.8%, in individuals collected from one location and presumably members of a single population. *C. putoria* individuals from Botswana and Zambia displayed 0.6% variation. Despite representatives from multiple countries in the *C. megacephala*, *C. albiceps* and *L. sericata* samples, very little intraspecific variation was observed.

*C. marginalis* showed a small separation between the Pretoria (urban) and other specimens. Similar division was shown between *C. megacephala* from Brisbane (east coast), and the Perth (west coast) and African individuals.
Discussion

The high adenine and thymine frequencies observed in the sequences are often characteristic of insect mtDNA, including the Diptera (Lewis et al., 1995; Bernasconi et al., 2000). Considering that the sequences are protein coding, purifying selection to minimize non-synonymous changes within the sequence is not surprising. The outgroup was quite distant from the calliphorids, as expected since the Muscidae and Calliphoridae are representative families from different superfamilies.

In searching for a possible diagnostic tool for use in forensic investigations, the method selected needs to be robust. In molecular-based identification, a specimen may be identified by similarity and difference in the sequence when compared to other individuals. Such a method will be a useful identification tool only if a reference sample from the same species is present in the sequence database, necessitating thorough knowledge of all species likely to be attracted to decomposing organic matter in a region (Wells & Sperling, 2001). In the absence of an appropriate reference sample, the individual will simply group with the most closely matched reference sample. It is consequently useful to have indications of levels of interspecific and intraspecific variation to be expected in making an identification based on sequence data, so that pairwise distances may be used as a confirmation of validity to support both the morphological and molecular-based analysis conducted. Defined levels of intra and interspecific variation that can be expected in identifications will provide such a confirmation.

Morphologically, calliphorids are generally simple to identify to sub-family level, and the molecular data here support the separation of the Chrysomyinae and Luciliinae. C. rufifacies and C. albiceps, and L. cuprina and L. sericata, are recognized as being difficult to distinguish morphologically (Stevens & Wall, 1996; Wells & Sperling, 1999), and over this 1167bp region displayed 3.5% and 3% variation respectively. The maximum intraspecific variation was 0.8%, showing a distinct separation between individuals of different populations of a species, and a separate species altogether. This supports the finding of Wells & Sperling (2001) of interspecific divergence of ≥3%, and intraspecific divergence of ≥1% in a number of forensically significant calliphorid
species. Thus calculation of pairwise difference between individuals should provide a useful indication of the validity of a grouping in an analysis.

The phylogenetic analyses by all three methods produced similar results, all supporting the sub-family separation and distinct clustering of individuals with conspecific individuals. The strong bootstrap support through all techniques indicates the robust nature of the region for use in providing distinction between species.

The change in the position of *C. varipes* within the trees, according to technique, involves grouping with either the *C. rufifacies/C. albiceps* group, or the other *Chrysomya* species. Neighbour-joining and maximum parsimony phylogenies concur on the latter positioning of the species, perhaps indicating this grouping to be the most probable. The change in the position of the species under likelihood analysis, when bootstrapped on separate occasions, indicates the instability of the likelihood phylogeny. Therefore, while likelihood analysis supports the general topology, and supports the 100% bootstrap support for all species clusters, the phylogeny has been excluded. It is likely that the discrepancy under this method is a product of taxon sampling, and that species necessary to place *C. varipes* in a phylogeny with some stability using this technique have been excluded. The *Chrysomya* species sequenced were chosen for their forensic significance in particular regions, and consequently, species of importance in constructing a phylogeny may have been overlooked. A stable position for *C. varipes* might otherwise be found by extending the region sequenced to gain more data or by sequencing another gene.

The low intraspecific variation across several countries and two continents for some of the species indicates the value of the mtDNA region for interspecific distinction, and the need for a region of greater variability for population level studies. It would be advantageous to have the ability to identify the geographical origin of a fly, particularly in locating the place of death when a body has been shifted, based on changes in the insect DNA from flies from both the place of killing and place of disposal. *Chrysomya megacephala* and *L. sericata* were both sequenced from Australia and Africa and showed little variation between populations. Ironically, the largest amount of intraspecific variation was observed in *C. varipes* individuals from the same population. A new region of DNA is clearly required for population level study, perhaps the hyper-

From a southern African perspective, this study provides a strong foundation for molecular-based identification of forensically important flies. While comparison with Australian individuals has shown the necessity of further work to study intraspecific variation, the data have shown the robust nature of this mtDNA region to provide species identifications. Levels of inter and intraspecific divergences appear clearly defined. Population level studies will perhaps allow identification of the geographical provenance of flies in investigations, as well as any possible subspecies that may complicate identification. Further studies will also need to consider other forensically significant species in southern Africa, such as Calliphora croceipalpis and C. chloropyga. COI sequence data appear to provide a strong basis for species identification, and will prove an invaluable contribution to forensic entomology as an investigative tool in southern Africa.
Acknowledgements

This study was funded by the Rotary Foundation of Rotary International in the form of an Ambassadorial Scholarship to MLH. Thanks are extended to Prof. Clarke Scholtz and Jenny Edrich at the University of Pretoria in South Africa for supervision, facilities and technical assistance; Nicola Lunt from the Department of Zoology and Entomology at Rhodes University, South Africa for technical assistance; and Silvana Gaudieri from the Centre for Forensic Science at the University of Western Australia for help in analyses.
### Table 3.1 Individuals included in study with locality and accession number

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<th>Accession Number</th>
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<tr>
<td></td>
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<td></td>
<td>Snake Island, Botswana</td>
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<td>---------------------------</td>
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<td>AB112863</td>
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<td>(Muscidae)</td>
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Table 3.2 Pairwise divergence between species expressed as a percentage of 1167 base pairs

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<th>H. rostrata</th>
<th>C. rufifacies</th>
<th>C. albiceps</th>
<th>C. inclinata</th>
<th>C. putoria</th>
<th>C. megacephala</th>
<th>C. marginalis</th>
<th>C. varipes</th>
<th>L. sericata</th>
<th>L. cuprina</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. rostrata</td>
<td>13.5</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C. rufifacies</td>
<td>12.8</td>
<td>3.5</td>
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<tr>
<td>C. albiceps</td>
<td>15.7</td>
<td>8.8</td>
<td>8.1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>C. inclinata</td>
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<td>7.7</td>
<td>7</td>
<td>3.6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C. putoria</td>
<td>14.9</td>
<td>7.3</td>
<td>6.6</td>
<td>5.1</td>
<td>4.9</td>
<td></td>
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<tr>
<td>C. megacephala</td>
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<td>7.5</td>
<td>7.8</td>
<td>5.7</td>
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<td>7.3</td>
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<td>8.7</td>
<td>9.9</td>
<td>3</td>
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<tr>
<td>L. cuprina</td>
<td>10.1</td>
<td></td>
<td>9.4</td>
<td>9.5</td>
<td>8.5</td>
<td>8.7</td>
<td>9.9</td>
<td>3</td>
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</table>
Table 3.3 Maximum intraspecific variation for each species expressed as a percentage of 1167 base pairs (species represented by a single individual omitted)

<table>
<thead>
<tr>
<th>Species</th>
<th>% variation within species</th>
</tr>
</thead>
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<td>C. rufiacies</td>
<td>0.1</td>
</tr>
<tr>
<td>C. albiceps</td>
<td>0.2</td>
</tr>
<tr>
<td>C. putoria</td>
<td>0.6</td>
</tr>
<tr>
<td>C. megacephala</td>
<td>0.3</td>
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<tr>
<td>C. marginalis</td>
<td>0.3</td>
</tr>
<tr>
<td>C. varipes</td>
<td>0.8</td>
</tr>
<tr>
<td>L. sericata</td>
<td>0.1</td>
</tr>
<tr>
<td>L. cuprina</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure 3.2** Map of southern Africa showing locations sampled (map modified from MapSource WorldMap Version 2.00, 1999)
Figure 3.3 Neighbour-joining tree with branch lengths and bootstrap support for calliphorid species of forensic importance
GENERAL DISCUSSION

This study clearly illustrates the advantage of increasing the size of the COI region sequenced in distinguishing forensically important species. The increased amount of sequence data provided greater scope for variation, particularly in the separation of some closely-related species such as *Ch. albiceps* and *Ch. rufifacies*.

The comparison of conspecific individuals from the Afrotropical and Australasian regions did not reflect any particular genetic separation between populations. This may be attributed to unsuitability of the COI gene for this application, or perhaps reflect the movement of individuals between the two continents, and therefore facilitation of gene flow between otherwise allopatric populations. This may only be addressed through the sequencing of other DNA regions, and the consideration of more widespread populations of cosmopolitan taxa using the COI gene.

This study has clearly illustrated the potential for distinction of some forensically important calliphorids based on the COI gene. The comparison of southern African and Australian calliphorids showed the limited intraspecific variation within many species, yet demonstrated clearly the robust nature of the region for genetically separating all species.

The global study of calliphorids with wide distributions is a critical step in verifying species status, a basic assumption of forensic entomology. The collection of molecular data for these species will allow analysis of their status and contribute to the future accuracy and efficiency of this field.
CHAPTER 4:
A Global Study of Forensically Significant Calliphorid Species
The ability to identify forensically significant calliphorids from 1167bp of cytochrome oxidase I (COI) data was unequivocally established in the previous chapter. Species were identified with strong separation between species and uniformity within species, forming the foundation for robust identification of the calliphorids of interest in this study.

The conserved nature of the calliphorid COI gene is particularly advantageous in the study of species status and distinction. The selective pressures acting to maintain the strong similarity between populations create a platform for the development of a diagnostic assay based upon a region of DNA with uniformity amongst conspecific individuals. The slow evolving rate of the COI, while advantageous for species level studies, has limited use above family level in higher taxonomic studies (Howland & Hewitt, 1995; Bernasconi et al., 2000). Cook et al. (2005) also found very high interspecific and very low intraspecific variation across the COI gene of Aedes mosquitoes in West Africa, and conclude this attribute makes the COI an ideal target for robust species level identification. The absence of indels or repeat units maintains uniformity in length of the region creates a conserved region for study, with variation based solely on single nucleotide polymorphisms (SNPs).

Species Concepts
Forensic entomology, as indicated in Chapter 1, depends on the accurate identification of specimens where PMI is to be estimated based on species-specific fly development rates. The existence of species complexes poses a threat to accurate identification due to the possibility of variation between sibling species in behaviour and development. Insight into the status of forensic species is therefore sought from a genetic perspective in this study, by way of sequencing geographically isolated populations.

Fundamental to the assessment of species status and any biological study is the issue of species concepts. Species have been defined in many ways using a variety of criteria that govern the inclusiveness of the concept, and are the basis for how nature is divided into orderly categories (Cracraft, 2000). The concept applied determines the criteria that define the grouping, and therefore influences which individuals are included, meaning that populations considered conspecific under one concept may be different species under another. This obviously has implications for the current study in assessing species
status. Also important, and dependent on the concept subscribed to, is how speciation occurs and new species are formed and the mechanisms that contribute to their divergence (e.g. Gray & Cade, 2000). A few of the commonly accepted species concepts will be considered here in defining species for this study. This discussion is by no means exhaustive, given the plethora of species concepts proposed in the literature.

The Morphological concept of species delineates species on the basis of physical characters. This concept, based on α-taxonomy, has been commonly applied in the initial stages of identification and description of new species, particularly in entomology (Cracraft, 2000). While the prospect of intraspecific variation is acknowledged under the concept, it cannot be fully considered and described where only a single specimen is available for study. This concept is subject also to the problem of morphological similarity and undetected cryptic species, where morphologically identical but biologically distinct specimens are unwittingly grouped together. Genetic data in particular has challenged the morphological concept of species in recent years.

The Biological Species Concept or isolation concept has been one of the most accepted views, with species defined as groups of interbreeding natural populations, reproductively isolated from other such groups (Mayr, 2000). Many issues have been debated regarding the validity of this concept. Isolating mechanisms proposed as leading to speciation under this concept involve pre- and post-mating processes, arising in sympathy, and not applicable where individuals are already geographically isolated, therefore neglecting the classic allopatric model of speciation (Templeton, 1989). More importantly, there is difficulty in asserting the potential of populations to interbreed, and therefore issues with allopatric populations and their taxonomic status. In the current study the consideration of allopatric populations is of vital importance, making the use of this concept in its strictest form difficult. When viewed from the perspective of the potential of these populations to interbreed as opposed to the isolating mechanisms at work, the concept becomes more useful. Mayr (2000) states that every geographically isolated population is a potential “incipient species”, and that the challenge is in inferring how far they have already progressed on their way to speciation. Therefore, morphologically and behaviourally they may appear separate, but may still mate to provide fertile hybrids, illustrating that speciation is in progress but not complete and they remain conspecific (Pinkster, 1983).
The Biological Species Concept was countered with the Recognition Concept of species (Paterson, 1985) defining species as the most inclusive group of biparental organisms sharing a common specific mate recognition system (SMRS), enabling them to identify and mate with conspecific mates. The focus under this concept is on the facilitation of reproduction, as opposed to the prevention of reproduction under the Biological Species Concept (Templeton, 1989). This concept placed emphasis on pre-mating isolating mechanisms between individuals, as opposed to the Biological concept with its focus on reproductive isolation by way of production of hybrids, incompatibility of males and females, or both (Dobzhansky, 1935). While both of these concepts form useful arguments for species as inclusive groupings of individuals with the ability to interbreed with conspecific individuals, neither concept addresses the issue of asexual organisms, making them of limited applicability in many biological systems.

The evolutionary concept views species from a phylogenetic perspective, with species defined as an individual lineage of individuals or ancestral descendant populations distinct from other lineages (Wiley, 1978). The similar, but modified Phylogenetic concept of species views species as an “irreducible (basal) cluster of organisms that is diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent” (Cracraft, 1989). These closely related concepts both cater for a phylogenetic approach to species assessment, with conflict at the level of species delineation. The phylogenetic concept considers basal groupings, providing scope for infinite numbers of species to be defined based on undefined levels of variation at the terminal nodes of phylogenetic trees, as opposed to the more broad classification of “lineages” that may include an ancestor and its descendants. Both of these concepts suffer from the problem of defining acceptable levels of intraspecific variation, allowing distinction between species and conspecific populations (Templeton, 1989). Likewise, they do not deal with the processes that contribute to speciation, but with the cohesion of the groups resulting from this process.

Templeton (1989) sought to overcome the limitations of the Biological, Recognition and Evolutionary concepts of species with the development of the Cohesion concept. Species are defined under this concept as “the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms” (Templeton, 1989). Species identification relies on locating these cohesive mechanisms in operation without first having some criteria to define the species exhibiting the
“phenotypic cohesion” (Endler, 1989). Furthermore, the concept abandons recognition of the evolutionary lineage of the individuals (Mishler & Budd, 1990).

From a molecular entomological perspective, the phylogenetic/evolutionary concepts are most likely to apply given the phylogenetic methods used in this study to assess species status. These are the most commonly applied concepts in taxonomic studies (Templeton, 1989). It is fair to state, however, that considerable overlap exists between concepts and that the isolating/recognition mechanisms central to other concepts are likely to be reflected in the basal units represented in the phylogenies, given the reasonable assumption that the genetic data is foundational to the morphological, behavioural and biological variation that groups and distinguished the individuals and thus species. Molecular data contributes greatly to the identification of variation on a fundamental level, however the assertion of the presence of isolating mechanisms and shared SMRS is problematic based purely on molecular data.

Levels of Variation: Defining Boundaries
Of interest in considering the status of species from an evolutionary genetic perspective is the degree of genetic variation between lineages (Ayala et al., 1974). The levels of variation acceptable at intra- and interspecific boundaries are, however, difficult to determine. In cases of recent divergence, reproductive isolation may be incomplete and speciation is in process, and processes such as introgression, particularly in mitochondrial DNA (Harrison et al., 1987) may complicate the identification of divergent populations as being “good” species (Powell et al., 1999; Besansky et al., 2003).

Evolutionary rates vary across genes, taxonomic level and between taxa themselves, making the designation of acceptable variation levels a movable task. Much of the species assessment from a molecular perspective within the Diptera is focused on the medically significant mosquitoes (Culicidae). Cook et al. (2005) recorded interspecific levels between Aedes species of up to 17.7% over the COI gene, with the intraspecific variation levels at less than 2%. In Chapter 3, limited variation was observed between conspecific individuals, with the highest level at 0.8% between Chrysomya varipes individuals from a common population. Maximum levels of 0.8% intraspecific variation and minimum 3.0% variation have been observed across the calliphorid COI gene in duplicate studies across varying calliphorids taxa (Wells & Sperling; 2001; Harvey et
al., 2003b), and may represent potential boundaries for the assessment of species status based on data from this gene.

While culicid data correlates nicely with variation levels from the calliphorids, studies of the closely related Oestridae question the utility of the COI in defining species. Otranto et al. (2003) identified intraspecific divergence of up to 1.59% across the COI gene of 18 oestrid species, with interspecific variation starting at just 0.7% between very closely related species. This illustrates the overlap of expected within and between species variation level, confounding the expected percentages observed in culicid and calliphorid studies.

The ability to define thresholds of expected variation upon which to assign species affinity, and identify novel species is a complex and dangerous practice. This relies on the appointment of a predetermined level of variation expected both within and between species, and has attracted heated debate in the molecular identification literature (Moritz & Ciccer, 2004; Meyer & Paulay, 2005). An underlying assumption of such an approach is that of monophyletic clustering of conspecific individuals, and paraphyly and polyphyly are recorded to occur in up to 23% of metazoan species, generally as a product of gene choice and incomplete lineage sorting (Funk & Omland, 2003).

A thorough understanding is also required of the basic taxonomy of species before thresholds may be applied, and given the infancy of the genetic barcoding approach with calliphorids, the use of thresholds as a definitive diagnosis of species must be cautioned for the moment. However, levels of variation and phylogenetic relationships provide important indications of potential speciation events, and are therefore useful tools for recognising taxa requiring further study.

As mentioned, the choice of gene may significantly affect the result of any phylogenetic study. Shaw & Danley (2003) explain that different genes may simultaneously reflect differential gene flow, necessitating careful consideration of the gene chosen in any study. They explain that porous species boundaries may occur where species are living in sympatry, but differential gene flow is occurring. Some genes will experience divergent evolution, while the hybridising of the species facilitates homogenisation of other genes, maintaining gene flow. Therefore different genes will differentially reflect
the genetics of different species, affecting the assessment of species status and potentially arriving at different conclusions with different genes.

The COI was chosen for this study as a region with potential for both inter- and intraspecific distinction. While interspecific variation has been confirmed, variation at the population level has been limited. The possible reasons for low levels of variation between populations are numerous. The species sequenced may have diverged so recently as to limit the intraspecific variation over this region, or functional constraints over the gene may be significantly limiting observable changes. The low variation may be attributed to homogenisation, as a result of gene flow between presumed allopatric populations as flies may be transported between otherwise separated locations (Baumgartner, 1993). The COI gene has been useful in revealing cryptic species in other insect taxa, reflecting behavioural and geographical patterns within taxa (Scheffer et al., 2004) and may hold potential for insight into the status of calliphorid taxa.

**Global Study: Looking to the Future of Forensic Entomology**

As a science which is locality-specific, forensic entomology generally only considers local populations of forensically significant calliphorid species. Molecular studies of the forensic calliphorids have thus generally focused on the study of individuals from a specific locality, gathering data for the distinction of specimens encountered in that region (e.g. Malgorn & Coquoz, 1999; Sperling et al., 1994; Wallman & Donnellan, 2001; Harvey et al., 2003a; Ratcliffe et al., 2003; Schroeder et al., 2003; Saigusa et al., 2005). Such locality-limited studies are cost-effective, and generally address the calliphorid fauna most likely to be encountered in casework, yet there are several important reasons to geographically widen the molecular study of forensic calliphorids.

The majority of a forensic entomologist’s cases will be centred within his or her main locality, and their familiarity with species in that area, and genetic data gathered for that locality will equip them adequately for such cases. Yet it is likely that cases may extend beyond urban boundaries to isolated areas where genetic differences between conspecific populations may challenge the ability of the entomologist to molecularly identify specimens with confidence. Therefore a widened genetic database for species will facilitate use of data outside the main area of practice, extending the potential for the use of this science beyond urban centres.
In addition, many calliphorid species of forensic significance have expanding distributions. Species such as *Chrysomya rufifacies* have considerably expanded their distribution in recent years (Baumgartner, 1993), displacing native species and altering the predictable corpse succession. With this spread has come reported variable behaviour across the distribution, from primary behaviour in the United States (Baumgartner, 1993) to secondary and seasonally affected behaviour in Australia (Harvey *et al.*, 2003a). Such behaviour is widely recorded, nor published, and therefore must be treated with caution. It has not been explored previously using either molecular or morphological study, but holds enormous implications for forensic entomology.

Cosmopolitan species such as this are frequently assumed to constitute solid species, and not complexes. However the existence of complexes may lead to inaccuracy in PMI estimation where data from isolated populations, and potentially differentiated populations, is applied to sibling species that have undergone undetected speciation. Therefore is it important that the fundamental assumption of species status in this field now be addressed for species of widespread importance.

Few studies of calliphorids across extended distributions have been attempted. Vincent *et al.* (2000) considered 6 species commonly found in Europe, yet individuals were all collected from a single location and allopatric variation was not considered. Stevens & Wall (2001) likewise studied calliphorid species from Britain and Europe, but isolated populations were not sampled. Wallman *et al.* (2005) studied 34 species from Australia however for many species just one individual was included, and consideration of cosmopolitan species sampled from throughout the world is required for the assessment of species status. Specific taxa have been more frequently studied from a global perspective, for example Stevens & Wall (1996) have thoroughly considered *Lucilia cuprina* and *L. sericata* from many global populations. Wells & Sperling (2001) have adopted a more widespread approach to molecular study of the Chrysomyinae, yet no more than two individuals were considered for any species.

The following study therefore stands among the most thorough molecular surveys of the forensically significant calliphorids undertaken to date. Data was gathered for 91 individuals from 27 species, with 22 countries represented. A further 28 sequences from the publicly available genetic database (NCBI) were included for comparative purposes.
This study addressed several issues:

- The COI gene has been shown (Ch 3) to be useful in distinguishing species from neighbouring geographical regions: does this also extend to species of forensic importance from isolated geographical regions?
- Do cosmopolitan species such as *Ch. rufifacies*, *L. sericata* and *L. cuprina* display intraspecific variation across their distributions and if so, what implication may this have for their species status?
- Does the expansion of the size of the COI region used provide greater distinction between closely related *Calliphora* species not easily distinguished by Wallman & Donnellan (2001)?
- What insight does the COI gene provide into the status of species such as *Ch. rufifacies*, *Ch. putoria*/*Ch. chloropyga* and *L. cuprina*? What are the implications for the use of these species in forensic entomological investigations?

The following study is currently under review in this form:

Abstract
A proliferation of molecular studies of the forensically significant Calliphoridae in the last decade has seen molecule-based identification of immature and damaged specimens become a routine complement to traditional morphological identification as a preliminary to the accurate estimation of post-mortem intervals (PMI), which depends on the use of species-specific developmental data. Published molecular studies have tended to focus on generating data for geographically localised communities of species of importance, which has limited the consideration of intraspecific variation in species of global distribution. This study used phylogenetic analysis to assess the species status of 27 forensically important calliphorid species based on 1167 base pairs of the COI gene of 119 specimens from 22 countries, and confirmed the utility of the COI gene in identifying most species. The species *Lucilia cuprina*, *Chrysomya megacephala*, *Ch. saffranea*, *Ch. albifrontalis* and *Calliphora stygia* were unable to be monophyletically resolved based on these data. Identification of phylogenetically young species will require a faster-evolving molecular marker, but most species could be unambiguously characterised by sampling relatively few conspecific individuals if they were from distant localities. Intraspecific geographical variation was observed within *Ch. rufifacies* and *L. cuprina*, and is discussed with reference to unrecognised species.

Keywords.
Calliphoridae, forensic entomology, blowflies, COI, cox1, intraspecific variation
Introduction

The advent of DNA-based identification techniques for use in forensic entomology in 1994 (Sperling et al., 1994) saw the beginning of a proliferation of molecular studies into the forensically important Calliphoridae. The use of DNA to characterise morphologically indistinguishable immature calliphorids was quickly recognised as a valuable molecular tool with enormous practical utility. Numerous studies have since addressed the DNA-based identification of calliphorids (Malgorn & Coquoz, 1999; Stevens & Wall, 2001; Wallman & Donnellan, 2001; Harvey et al., 2003a, b). 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 (Stevens & Wall, 1997) and the mitochondrial control region (Stevens & Wall, 1997). The majority of molecular studies, however, have used the cytochrome oxidase I (COI or cox1) encoding region of mitochondrial DNA (mtDNA) (Sperling et al., 1994; Malgorn & Coquoz, 1999; Vincent et al., 2000; Wallman & Donnellan, 2001; Harvey et al., 2003b).

The COI gene holds enormous utility for species identification. Lying within the mitochondrial genome, it has the advantages of easy isolation, higher copy number than its nuclear counterparts, and conserved sequence and structure across taxa yet sufficient scope for variation and therefore identification. COI has been well studied in the Insecta, with its utility for distinction between closely related species of Diptera demonstrated by the large number of COI studies of species complexes in the Culicidae (e.g. Garros et al., 2005).

Calliphorid molecular taxonomic studies have focused largely on sequencing of the COI gene and have illustrated the ability to successfully distinguish between a wide variety of forensically important species (Sperling et al., 1994; Malgorn & Coquoz, 1999; Vincent et al., 2000; Wallman & Donnellan, 2001; Harvey et al., 2003b). The main limitation to the use of COI sequence data has been the inability to distinguish between some closely related species of the genus Calliphora. Wallman et al. (Wallman & Donnellan, 2001; Wallman et al., 2005) found difficulty in separating the C. augur/C. dubia, C. stygia/C. albifrontalis and C. hilli/C. varifrons species pairs, creating difficulty in geographical regions where these sister species overlap. Our first aim was to contribute to solving such problems by enlarging the available data set.
A number of species of forensic utility are relatively cosmopolitan, such as *L. cuprina*, *L. sericata*, *Ch. rufifacies*, *Ch. megacephala* and *C. vicina*. However, forensic entomology is a locality-specific science. Molecular studies are generally directed to the specific fauna found in a region, with little attention to intraspecific genetic variation. Specimens from new localities may not exactly match published DNA sequences, and it then becomes important to be able to assign an identification based on partial rather than perfect matching. Our second aim was therefore to take a more global view of genetic variation in blowflies, by using conspecific specimens from geographically distant localities to estimate the range of variation found in various recognised species.

Specimens that are similar to, but do not lie within the known range of genetic variation of a recognised species may represent previously-unsampled geographical variation of that species, another species recognised by systematics but not yet sampled for DNA, or taxonomically unrecognised cryptic species that have not yet been sampled for DNA. Our third aim was to present DNA sequences for species in the second category, to diminish the ambiguity of imperfect matches.

The occurrence of cryptic species, which appear morphologically the same as named species but differ in their behaviour, development or other biology, may contribute to significant error in PMI estimates. For example, *Ch. rufifacies* is a fly with a widespread distribution displaying variable behaviour in different localities (Harvey et al., 2003b), and Wallman et al. (2005) have recently indicated the possibility of cryptic species within *Ch. rufifacies* in Australia. Our fourth aim was to seek evidence of cryptic species by estimating the amount of genetic variation found within and between recognised species and identifying exceptions to the general trends.

This study gathered data on a variety of forensically significant calliphorids across their geographical distributions and assessed the potential for the COI gene to provide distinction between the species. Sequencing of 1167 base pairs of the mitochondrial COI gene was conducted and phylogenetic analysis used to represent the relationships between the taxa. A total of 47 new sequences are supplied, along with the 44 sequences from Harvey et al. (2003b), and 28 relevant sequences obtained from the publicly available DNA database at www.ncbi.nlm.nih.gov. This study therefore considered 119 flies from 28 species and 22 countries.
Materials and Methods

Samples
FLies were obtained from a variety of locations, either trapped by the authors using liver-baited traps or kindly supplied by peers. They were identified using traditional morphological characters. Specimens used in this study are listed in Table 4.1.

DNA Extraction
DNA was extracted from the flight muscles of specimens using a DNEasy Tissue Kit (Qiagen) according to manufacturer’s instructions, with an overnight incubation step.

Amplification
Approximately 1270bp of the COI gene was amplified using the primers C1-J-1718 (5’- 3’ GGAGGATTTGGAAATTGATTAGTTCC) and TL2-N-3014 (5’-3’ TCCAATGCACTAATCTGCCATATTA) (Simon et al., 1994). For the amplification of some species, TL2-N-3014 proved problematic and therefore a degenerate primer TL2-N-3014MOD (5’-3’ TCCATTGCACATACTGCCATATTA) was designed based on the sequence of Chrysomya chloropyga (accession number AF352790), and used to amplify a number of individuals for which amplification was not achieved with the original reverse primer.

The PCR reaction mix composed of: 1 X PCR buffer (Biotools; Fisher Biotec), 200µM dNTPs (Biotools; Fisher Biotec), 1.5mM MgCl₂, 25pM each primer, 1 unit of Taq polymerase (Biotools; Fisher Biotec), 10-150ng of template DNA, and water added to a total volume of 50µL. Reactions were performed on Perkin Elmer GeneAmp PCR System 2400 and Applied Biosystems GeneAmp PCR System 2700 thermocyclers. Cycling conditions were: 90 seconds 94°C denaturation, followed by 36 cycles of: 94°C for 22 seconds, 48°C for 30 seconds and 72°C for 80 seconds. A final extension period of 1 minute at 72°C was used, followed by holding at 4°C. Products were visualised using 1.5% agarose gels with ethidium bromide staining and UV transillumination.

PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen), according to manufacturer’s instructions.
**COI Sequencing**
Sequencing reactions were performed using the ABI PRISM Big Dye Terminator 3.0 or 3.1 Sequencing Kit (Perkin Elmer), according to the manufacturer’s protocol. Cycling conditions for the sequencing reactions were as per the manufacturer’s recommendations, but the annealing temperature was lowered to 48°C. Individuals were sequenced using the external primers C1-J-1718 and TL2-N-3014 or (TL2-N-3014MOD, where appropriate), and the internal primers C1-J-2183 (5’-3’ CAACATTTATTTTGGTTTTTG) and C1-N-2329 (5’-3’ ACTGTAATATATGAGCTCA).

**Sequence Analysis**
Sequences were visualised using Chromas v1.43 (http://trishul.sci.gu.edu.au/~conor/chromas.html), and alignments and editing conducted using DAPSA (Harley, 1996). Sequences were submitted to DDBJ (accession numbers in Table 4.1). Additional COI sequences of some relevant calliphorids were obtained from the publicly available DNA database (Genbank) at www.ncbi.nlm.nih.gov (Table 4.1).

Phylogenetic analyses were performed using MEGA 3.0 (Kumar et al., 2001), PAUP*4.0 (Swofford, 2002) and MrBayes v.3.0b4 (Huelsenbeck & Ronquist, 2001). MEGA was used to calculate of pairwise distances and to conduct the Z-test for purifying selection within the sequences. MEGA was employed in the distance analysis using the neighbour-joining method with the Tamura-Nei model of substitution and 500 bootstrap replications. Base frequencies, transition/transversion ratio and the gamma shape parameter were estimated from the data using PAUP, and analyses were performed using MrBayes v.3.0b4. These Bayesian inference analyses were conducted using one cold and three hot chains, and the INVGAMMA model. Analyses were run for 1,500,000 generations, sampling every 100 generations. The likelihood scores from every 100 generations was plotted to evaluate when stationarity had been reached. From the plots, it appeared that the burn-in phase was complete by 50,000 generations. However, the first 1000 trees were excluded as burn-in, this exclusion being considered to be conservative. Posterior probabilities (PP) were calculated from the remaining trees by means of a majority rule consensus tree produced using PAUP.

A further analysis was conducted using 13 individuals of *Ch. rufifacies*, using MEGA to perform neighbour-joining analysis with 500 bootstrap replications. *Hydrotaea rostrata*
was the assigned outgroup in all analyses, with the exception of the *Ch. rufifacies* distance analysis.

**Results**

A total of 47 individuals were sequenced and aligned over 1167 base pairs of the COI gene. A further 72 additional sequences were obtained from Genbank for comparative purposes. The sequences correspond to positions 1776-2942 of the *Drosophila yakuba* mitochondrial genome (accession number NC_001322). No insertions or deletions were located over this region. Of the 388 variable positions identified, 318 of these were considered parsimony-informative.

The base frequencies within this region were estimated as: adenine (A) 33.9%, cytosine (C) 13.3%, guanine (G) 7.5% and thymine (T) 45.2%. The transition/transversion ratio was 2.99. The Z-test for purifying selection indicated that synonymous changes within the sequences were far greater than non-synonymous changes, and that purifying selection had occurred.

**Identification**

The muscid outgroup, *Hydrotaea rostrata*, was clearly separated from the calliphorids in the Bayesian Inference tree (Figure 4.1). Furthermore, the calliphorid species were correctly assigned to the subfamilies Chrysomyinae, Luciliinae or Calliphorinae, with each of the sub-families monophyletic. Good posterior probability (PP) support for a sister-group relationship between the Calliphorinae and Luciliinae was obtained, but support for a monophyletic Chrysomyinae was weak.

The genus-level arrangement accurately reflected the affiliations of the species, and was supported by PP values above 75%.

At the species level, all of the species has PP support of over 94% except *Ch. megacephala* (83%), *L. sericata* (81%), and *Ch. putoria* (69%). Most specimens were accurately assigned to their respective species (Figure 4.1). The exceptions were *C. stygia* and *C. albifrontalis*, which were intermingled; *Ch. megacephala*, which formed a paraphyletic grade with respect to a monophyletic (*C. rufifacies + C. albiceps*) and a monophyletic *Ch. saffranea* (Figure 4.1) because of two Malaysian specimens
(AY909052 and AY909053); and *Lucilia cuprina*, which formed two distinct clades that were collectively paraphyletic with respect to *L. sericata* (Figure 4.1).

One clade of *L. cuprina* consisted of individuals from Australia, Senegal and Uganda, while the other represented Taiwan, Thailand and Hawaii. The latter group were more probably related to the *L. sericata* clade than to conspecific individuals from the other clade.

**Intraspecific variation**

Where it could be calculated, all values for intraspecific variation (Table 4.2) fell below 0.8%, with the exception of *L. cuprina* and *Ch. rufifacies*. *Chrysomya rufifacies* showed two well-supported subgroups (Figure 4.1). A separate neighbour-joining analysis of 722bp of COI data for this species allowed an analysis including more individuals from the public databases. A radial tree (Figure 4.2) effectively illustrated the subdivision within the cluster, with Malaysian and Taiwanese individuals forming one group distinct from a second group of individuals from Australia and the United States. Pairwise comparison indicated that when considering the species as two clades, one containing the Malaysian and Taiwanese individuals, and the remaining individuals as a group, 0.94% variation was observed.

Pairwise calculation indicated a relatively high value of 3.94% variation across the two clades of *L. cuprina*. However, variation within the two clades was much smaller (Table 4.2). Unfortunately, insufficient sequences were available for a more detailed analysis.

Within each species, individuals from the same locality were intermixed with specimens from other sites (Figure 4.1), and no geographical patterns were obvious.

**Interspecific variation**

Levels of interspecific variation between calliphorid species varied from 0.23 – 13.34% (Table 4.3). Species pairs such as *Ch. rufifacies/Ch. albiceps* and *Ch. chloropyga/Ch. putoria* were separated by 3.64 and 2% respectively. *Calliphora dubia* and its sister species *C. augur* were clearly differentiated, yet displayed only a 1.27% difference. *Lucilia sericata* differed from *L. cuprina* by 2.8%, yet only 0.93% from the Asian clade. In general, species were separated by a level of at least 3%, with the exception of some closely related pairings. *Chrysomya megacephala* and *Ch. safranea* differed by only 0.23%.
Discussion
At the nucleotide level, the high A+T bias of the blowfly COI gene reflects the general composition of insect mtDNA. The 79.1% A+T composition is higher than that of the COI gene in representatives of the genera *Locusta* (Orthoptera), *Anopheles* (Diptera), *Drosophila* (Diptera) and *Apis* (Hymenoptera) (Lunt et al., 1996). The confirmation of purifying selection having acted over the sequences was not surprising, given the coding nature of the region and critical functional constraints over the proteins. This promises well for the future of COI as an identification tool for blowflies.

Identification of taxa
This study showed that the COI successfully distinguished all but four out of 27 species, even when conspecific specimens were drawn from well-separated parts of the geographical distributions. Genera were clearly separated and the subfamilial arrangement reflected morphological findings commonly reported in most taxonomic literature, with the Luciliinae and Calliphorinae grouping together and *Cochliomyia* positioned basally within the Chrysomyinae.

The subfamily Chrysomyinae contains the genera *Cochliomyia* and *Chrysomya*, which separate clearly in this analysis. *Cochliomyia hominivorax* is largely a pest of livestock, involved in myiasis. Its counterpart, *Co. macellaria*, is also of forensic importance, and the morphological similarity of the species makes their molecular identification of particular interest. Litjens et al. (2001) report the potential occurrence of the two species in the same myiasis, making their distinction important for any forensic or medical entomologist in areas where the species are sympatric. These species were successfully distinguished using sequence data from the COI gene.

The sister species *Ch. putoria* and *Ch. chloropyga* are Afrotropical blowflies associated with latrines and carrion respectively. The two were long treated as synonymous despite evidence of ecological and reproductive separation (Wells et al., 2004; Rognes & Paterson, 2005). Despite recent recognition of their status as distinct species (Rognes & Paterson, 2005), the morphological characters used to distinguish them are of little use with eggs and early instars (Wells et al., 2004). Wells et al. (2004) sequenced the two species over 593bp of the COI gene, and maximum parsimony analysis determined *Ch. chloropyga* to be paraphyletic with respect to *Ch. putoria*. In this study, with twice as
many characters, both species were monophyletic. Apparently the increased size of the dataset contributed valuable distinguishing nucleotide information. Our results confirm the identity of *Ch. putoria* in South America (Figure 4.1).

The sister species *Ch. rufifacies* and *Ch. albiceps* are particularly distinct, despite their morphological similarity. This is in line with an estimate that the *rufifacies* lineage is at least 4 million years old (Wallman *et al.*, 2005). These species were particularly distinct from the rest of their genus, lying at the end of an unusually long branch. They are usually placed together as the sister clade to the rest of their genus (e.g. Wallman *et al.*, 2005), and their position in Figure 1 may be the cause of the low PP support for *Chrysomya* in our analysis.

*Chrysomya megacephala* and *C. saffranea* are generally regarded as being morphologically very similar but ecologically distinct. In this study, their maximum interspecific sequence variation was only 0.33% even though COI is a relatively fast-evolving gene. By comparison, variation within *C. megacephala* was 0.34% (Table 2). Wallman *et al.* (2005) compared one individual each of *Ch. saffranea* (Queensland) and *Ch. megacephala* (New South Wales) and reported only 0.4% variation between them across a variety of regions, including 822bp of the COI gene. It may be that the mtDNA regions sequenced to date, which include 3008bp from the COI, COII, ND4 and ND4L genes (2005), are not useful in distinguishing these species, perhaps as a result of relatively recent speciation: they are estimated to have originated within the last million years (2005). Since this is probably insufficient time to complete lineage sorting, it is not a surprise that *C. megacephala* is paraphyletic with respect to *C. saffranea*. Funk & Omland (2003) have assessed that 23% of metazoan species are not molecularly monophyletic. Fortunately, the very low variation in *C. saffranea* makes this species sufficiently distinctive to be identifiable. As discussed already, the position of the (*Ch. rufifacies* + *Ch. albiceps*) clade within *C. megacephala* is probably an artefact, and does not affect the identifiability of any of these species.

The remaining species of the genus *Chrysomya* that have been sequenced are uncontentiously identifiable (Figure 4.1).

The genus *Calliphora* is well-represented by a clade of endemic species in the Australasian region, but it is very difficult to separate some of these based on molecular
data. Using four genes, Wallman et al. (2005) had difficulty separating members of the C. hilli group (including C. varifrons), C. augur/C. dubia and C. stygia/C. albifrontalis. In this study C. hilli and C. varifrons were clearly distinguished with 4.94% interspecific variation. It appears that the three additional genes used by Wallman et al. (2005) obscured the signal from the COI gene, but our result may equally be due to the increase in size of the COI region we used.

Like the previous pair of species, C. stygia and C. albifrontalis are morphologically similar flies that speciated less than a million years ago (2005), and appear to be present together in certain locations (Wallman & Donnellan, 2001). The COI data in this study did not successfully distinguish between the two species, which are mutually polyphyletic. New regions of DNA such as the internal transcribed spacer (ITS) regions are more useful in distinguishing such closely related species (Harvey, unpublished data).

The sister species Calliphora augur and C. dubia also formed monophyletic clades, although the latter received no PP support (Figure 4.1). Wallman et al. (2005) found a comparable pattern of PP support, and estimated the pair to have diverged just over a million years ago. These results imply that lineage sorting of COI in blowflies may take about two million years, and that a faster-evolving molecular marker is needed for younger species.

The sister species L. sericata and L. cuprina are morphologically similar, yet on the basis of this data are separated quite convincingly. High support was obtained for L. sericata as distinct from L. cuprina.

Geographical variation
The molecular taxonomic facet of forensic entomology generally accumulates genetic data for a DNA region suitable for identifying all species of forensic importance in a specific locality. The global genetic status of the species is generally of little relevance, but in the larger picture it may be vital to ensuring that the basic assumptions of forensic entomology are sound. Our results show that geographical variation was not more pronounced in species sampled from larger geographical distributions. For example Ch. albiceps and Ch. megacephala show levels of intraspecific variation comparable with C. augur, C. stygia and C. chloropyga (Table 4.2). Furthermore, no geographical pattern of
relationship was evident in most species (Figure 4.1), except *Ch. rufifacies* and *L. cuprina*. Part of the explanation for this may be that most carrion-breeding flies are synanthropic, and are spread by human activities. This is illustrated by the occurrence of European species like *C. vicina* in Tasmania and *L. sericata* in New Zealand, and the African species *Ch. putoria* in South America (Figure 4.1). This is reassuring for the use of COI sequences in identification of blowflies, because it implies that most of the variation within a species can be captured by relatively localised samples.

**Cryptic species**

The geographical structuring of the *C. rufifacies* and *L. cuprina* clades is linked with unusually high intraspecific variation that is suggestive of the presence of currently unrecognised taxa.

*Chrysomya rufifacies* is a species of great forensic importance. Primarily a tropical Australasian and Oriental fly, the species now inhabits areas of the United States (Baumgartner, 1993). The behaviour of *Ch. rufifacies* is variable across its distribution. In the United States the species is a primary carcass coloniser (Baumgartner, 2003), while in Australia it varies from primary (Queensland) to primary in summer and secondary in winter (south-eastern coast of New South Wales) to secondary all year round (Western Australia) (Wallman & Donnellan, 2001). Such variable behaviour may be a result of ecological interaction with other carrion-colonising species, with *Ch. rufifacies* occupying variable positions in the decompositional succession based on the behaviour of other species. It may also be that a species displaying a wide geographic distribution and variable behaviour might also display correlated genetic variation.

Based on data from four mtDNA genes from four individuals from four Australian states, Wallman *et al.* (2005) suggested that there are two sibling species within *Ch. rufifacies* in Australia, one in the south-east and one in the north-east. Our Western Australian individuals seem to be part of the south-east taxon. However, combining the COI data from both studies (Figure 4.2) showed that the Western Australian, Tasmanian and United States individuals at most constitute only one slightly variable clade (Table 4.2). Our analyses (Figures 4.1 & 4.2) provide evidence that the Malaysian and Taiwanese individuals formed a separate cluster from the Australian/American clade. Clearly, the evidence for cryptic species within Australia must come from the three other genes sequenced by Wallman *et al.* (2005). Given the seminal nature of current
molecular studies of the Calliphoridae, it is risky to form conclusions about species status from a single-gene phylogeny and limited sampling, but the occurrence of an Asian clade and an Australian/American clade is also indicated by preliminary nuclear internal transcribed spacer (ITS) data (Harvey, unpublished data).

*Lucilia cuprina* is classified into two subspecies, *L. c. cuprina* (Wiedemann) and *L. c. dorsalis* Robineau-Desvoidy (Norris, 1990). The former inhabits the New World, Asia, Indonesia, and Oceania, while the latter is Afrotropical and Australasian (Stevens & Wall, 1996; Montgomery, 1990). The two are found to readily interbreed in the laboratory, and hybrid populations are suggested to exist in areas of Australia (Norris, 1990). Norris (1990) indicated that all specimens from Western Australia, Northern Territory, Victoria, South Australia and Tasmania were of *L. c. dorsalis*, and that this subspecies was also established in New Zealand. *Lucilia c. cuprina* has been reported from northern Queensland, as has the occurrence of hybrid populations showing features of both subspecies from Queensland and New South Wales.

Based on the published distributions, the observation of two paraphyletic clades of *L. cuprina* in this study could be explained by genetic variation between subspecies. The clade from Australia and Africa would represent *L. c. dorsalis*, and the Pacific individuals *L. c. cuprina*. Wallman et al. (2005) suggested that, based on four mitochondrial genes sampled from three specimens of *L. cuprina*, their Western Australian *L. cuprina* may be *L. c. dorsalis*, while their New South Wales and Queensland individuals represented *L. c. cuprina*. They rightly indicated that, given the history of hybrid populations on the east coast of Australia, this cannot be concluded with certainty, but suggest that *cuprina* and *dorsalis* lineages are separate species, an interpretation supported by the paraphyly found in their analyses and our. Our results (Figure 1) imply that if this is the case, *L. c. dorsalis* is the sister taxon to the sibling species *L. sericata* and *L. c. cuprina*. In our study the Townsville, Queensland individual appears to be *L. c. dorsalis*, so that it is likely that both taxa are resident in Queensland and extensive sampling and study is required, given the tendency of the variants to interbreed.

The Hawaiian individuals make an interesting point for consideration. Stevens & Wall (1996) used RAPD data and 12S rRNA gene sequences to evaluate the status of *L. cuprina*. Based on their RAPD data, their Hawaiian individuals appeared to be of *L. c.*
cuprina, while their Australian, New Zealand and African individuals were L. c. dorsalis. In contrast, their 12S rRNA data grouped the Hawaiian individuals most closely with L. sericata, as we found in this study. Stevens & Wall (1996) concluded that the Hawaiian individuals were either L. c. cuprina, or perhaps reflected a new L. cuprina x sericata hybrid. Stevens et al. (2002) found the 28S rRNA gene placed their Hawaiian individuals with the main L. cuprina grouping, while the COI and COII analysis again grouped these closer to L. sericata. They tentatively interpreted the result to reflect a L. cuprina x sericata hybrid produced by crossing of a female L. sericata and a male L. cuprina, facilitating the maintenance of the maternally inherited L. sericata mitochondrial genotype. The absence of individuals in the study from any expected L.c.cuprina regions pre-empts conclusions regarding subspecies. The identification of the Townsville individual as having L.c.cuprina morphology must be treated with caution, given the reported existence of hybrid populations of the two subspecies in that area (Norris, 1990).

Based on morphology (Norris, 1990), our L. cuprina individual from Thailand was concluded to represent L.c.cuprina. Stevens et al. (2002) further indicate their Hawaiian individuals to bear L.c.cuprina morphology. Given the data in the current study, it appears likely that the clustering with Thai and Taiwanese individuals reflects the status of the Hawaiian individuals as L. c. cuprina, congruent with the taxon traditionally recognised as the dominant Asian form. Study of the Thailand and Taiwanese individuals using a nuclear gene may provide evidence to the contrary.

**Conclusions**

The molecule-based identification of calliphorids relies on the location of unique stretches of DNA sequence that are common to (at least sub-sets of) all members of the chosen taxon, yet distinct from all other taxa. This necessitates extensive sequencing of conspecific individuals to verify the robust nature of markers chosen as species identifiers. This study initiates the sequencing of intercontinental conspecific populations, and indicates that most of the variation within a species may be adequately captured by samples of as few as ten geographically distant conspecific individuals. This may be partly attributed to the high mobility of blowflies.

Some species show more intraspecific variation than others, and the discovery of an outlying sample must address the question of whether it is an extreme example, or if it
represents an otherwise unsampled species. It is tempting to seek an empirical heuristic threshold (Moritz & Cicero, 2004; Meyer & Paulay, 2005), but the differentiation of species on predetermined thresholds of genetic divergence relies on the degree of overlap between intraspecific variation and interspecific divergence. While thresholds of intraspecific variation and interspecific divergence have been suggested in other studies to delimit species boundaries and infer novel species, this is a fraught practice where taxa are undersampled (Meyer & Paulay, 2005), as they are in the Calliphoridae. Moritz & Cicero (2004) state that threshold overlap is greater where a large proportion of closely related species are included, such as the Australian Calliphora species. The application of thresholds may result in errors as frequent as 20% (Meyer & Paulay, 2005) when the basic taxonomy of the individuals has yet to be determined. Harvey et al. (2003b) and Wells & Sperling (2001) reported levels of 0.8% maximum intraspecific variation and 3% minimum interspecific divergence in the Calliphoridae, but in this study these thresholds overlap, cautioning against such a practice in calliphorid studies. Phylogenies such as those presented in this study may provide an inference of possible species issues, but no reliable conclusions may be reached without thorough morphological and biological study and species status should not be assessed solely on a molecular basis.

This study has again illustrated the enormous potential for the use of the COI gene for distinguishing between forensically significant calliphorid species, considering these species from a global perspective and linking allopatric populations of species often considered in isolation. This study has supported the existence of sibling species within L. cuprina, and illustrated the potential unsuitability of the COI gene for distinction between young species like Ch. saffranea and Ch. megacephala, and C. stygia and C. albifrontalis, probably as a result of incomplete lineage sorting. This may necessitate the adoption of a secondary assay for distinction of such species, perhaps the ITS regions.

Sequencing of individuals from a wide variety of localities, and thus the pooling of data from various researchers, will greatly contribute to the taxonomy of calliphorids on a global scale. Molecular forensic entomology, while in its infancy, has increased the ability to identify unknown immature calliphorids. It is the extensive sampling of populations and more species that will ultimately contribute to the increased relevance of the field to the courtroom.
Acknowledgements
We thank numerous people for their generous donation of flies for this study: Geoff Allen, Dallas Bishop, Amoret Brandt, M. Lee Goff, Ana Carolina Junqueira, Gary Levot, Nicola Lunt, Mervyn Mansell, Nolwazi Mkize, Carol Simon, Tony Postle, Cameron Richards, K. Sukontason, NCBI and Ruxton Villet. Jennifer Roy provided technical assistance. Some field work was funded by Rhodes University grants to MHV. MLH was supported in part by Rotary International, and the American Australian Association.
Table 4.1 Individuals used in this study, listed with locality of origin and GenBank accession number, and publication data where identified from another publication.

“New sequence” indicates sequences have not been published elsewhere and have been submitted to the public databases, with release pending publication of this study.

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*New sequence indicates sequences have not been published elsewhere and have been submitted to the public databases, with release pending publication of this study*
Figure 4.1 Bayesian inference tree constructed from 1167bp of COI data. Posterior probabilities are indicated on nodes. Ch=Chrysomya, C=Calliphora, Co= Cochliomyia and L=Lucilia
Figure 4.2 Radial neighbour-joining tree based on 1167bp of COI data for *Chrysomya rufifacies* individuals from a variety of localities
Table 4.2 Maximum intraspecific variation expressed as a percentage of the total of 1167 base pairs of COI data.

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### Table 4.3

Calculated raw interspecific distances using a neighbour-joining approach with Tamura-Nei model of substitution

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**Note:** Each row represents the distance between two species, with the first species in the left column and the second species in the second column. The distances are calculated using the Tamura-Nei model of substitution.
GENERAL DISCUSSION

This study forms one of the most thorough global studies of forensically significant calliphorids using molecular techniques to explore taxonomic status. It has raised important issues for further consideration to ensure the sound taxonomic status of some key species.

The COI gene was shown to have use in the distinction of species across extended distributions, revealing important, species-specific substitutions to be used in robust distinction assays. The species sequenced showed strong conservation of sequence across their distributions, and generally, distinction from other closely related species.

Intraspecific variation was identified in two key species: Ch. rufifacies and L. cuprina. Multiple haplotypes were revealed in these species, indicating the existence of possible species complexes, however the functional constraints over this gene may be limiting the extent of observable variation. This may account for the lack of variation between populations of many other species.

The expansion of the COI region sequenced provided greater clarity in the distinction of the closely related C. augur/C. dubia and C. hilli/C. varifrons sister species pairings, however C. stygia and C. albifrontalis were unable to be distinguished over this region. In addition Ch. megacephala was paraphyletic with respect to Ch. saffranea. It is suggested that this is a product of incomplete lineage sorting given relatively recent divergence, and an alternate region must be sought for molecular distinction of these species.

This study clarified the status of some important species pairs such as Ch. chloropyga and Ch. putoria, but raised important issues about the status of Ch. rufifacies, and L. cuprina. The results of this study indicate the importance of further investigation using morphological, behavioural and other molecular techniques to ascertain their taxonomic status.

The nuclear ribosomal genes and interleaving non-coding spacer regions have been used successfully in the study of cryptic taxa in the Anopheles species complexes (Hackett et al., 2000; Gentile et al., 2001). Combined nuclear and mitochondrial approaches have
been very useful in the study of numerous species groups (O’Grady et al., 1998; Sharpe et al., 2000; Clark et al., 2001; Martin et al., O’Grady & Kidwell, 2002) and may hold promise as an extension to the COI gene work in this study. The nuclear genes in *Drosophila* have been shown to evolve as fast as mitochondrial genes (Zhang & Hewitt, 1997). Alternately, the non-coding mitochondrial control region may provide greater distinction given the reduction in functional constraints relative to the COI gene.

The COI gene has formed a useful foundation in identifying critical issues to be further investigated. The inability to identify some species based on the COI gene has been observed in this study, and further study must consider the location of an alternate gene or region that will resolve these issues.
CHAPTER 5:
A Preliminary Investigation
into the use of Alternate Regions
of DNA to Complement COI-based
distinction of
Forensically Significant Calliphorids
The ability to successfully distinguish between numerous forensically significant calliphorids species using the COI gene was established in earlier chapters of this work. In particular, the applicability of the gene to the study of genetic variation between allopatric, conspecific populations of some cosmopolitan species was confirmed in the previous chapter.

Two closely-related species pairs, *Calliphora stygia/Calliphora albifrontalis* and *Chrysomya megacephala/Chrysomya saffranea* proved difficult to separate based on COI data, indicating unsuitability of the COI gene for this purpose, possibly as a result of recent divergence and consequently, incomplete lineage sorting. In the same study, *Ch. rufifacies* and *Lucilia cuprina* both displayed intraspecific variation, indicating the importance of further study to confirm sound species status. Additional molecular data from alternate regions of DNA are required to further investigate the status of these species.

In selecting an alternate region for DNA sequencing, it is vital the region reflect observable variation at the relevant taxonomic level. The distinction of closely related species and detection of cryptic species is the desired outcome of this study, necessitating selection of a region evolving more quickly than the COI gene and therefore reflecting greater nucleotide variation at this taxonomic level.

In the mtDNA, the non-coding control region may hold promise. As a non-coding region, it was originally thought that animal mtDNA evolved more rapidly (5-10 times faster) than single copy nuclear DNA due to the lack of functional constraint over its evolution (Avise, 1986; Zhang & Hewitt, 1997). While this is true for many vertebrates, including mammals, in insects this may not be true. The substitution rates of insect nuclear and mtDNA are generally similar (Harrison, 1989), suggested to be a result of the much faster mutation rate of the insect nuclear genome (Sharp & Li, 1989).

The control region has a high A+T content (generally over 85%) and appears to be under strong directional mutation pressure maintaining this nucleotide bias and consequently, a low substitution rate (Zhang & Hewitt, 1997). This may limit the evolutionary information
obtainable from the region, given the possibility of base saturation. Of particular utility in
the distinction of taxa is the size variability of the control region, often a product of tandem
repetition under concerted evolution. This makes length-based identification a possibility
and this would overcome the need for sequencing, however it is important to ensure the
band amplified is in fact the desired target before such an approach can be employed.
Structural rearrangement may also provide useful comparisons at higher taxonomic levels
with rearrangement of the flanking tRNA genes relative to the control region being taxon
specific (Zhang & Hewitt, 1997).

Two main control region structure groups are recognised among the insects (Zhang &
Hewitt, 1997). The calliphorid control region appears to be a Group 1 control region,
containing a highly conserved region and a more variable region featuring considerable
variation in sequence and length (Monnerot et al., 1990). Zhang & Hewitt (1997) conclude
that the insect mtDNA may not be the best target choice where variability is of primary
importance. Caccone et al. (1994) found that the control region of some mosquito species
was less variable than the third codon positions of the mitochondrial ND4 and ND5 genes.
However, it is concluded by Zhang & Hewitt (1997) that Group 1 control regions such as
that in the calliphorids may be of utility in intraspecific studies, where the highly variable
domain may provide informative variation.

Despite limitations in the evolutionary rate of the control region, several studies have
exploited this region. Lessinger et al. (2004) found considerable variation between the
calliphorid species Ch. megacephala, Ch. chloropyga and Ch. albiceps, indicating potential
for the use of the region to distinguish between some of the species in this study. In
particular, the Ch. megacephala/Ch. saffranea complex may be separated using control
region data. PCR-RFLP of the control region has also yielded useful information about the
population structure of the calliphorid Cochliomyia hominivorax, indicating potential for
intraspecific diversity in other calliphorid species.

Given the similar mutation rates between nuclear and mtDNA, the selection of a non-
coding nuclear region for study of closely related species holds great promise. The
ribosomal DNA (rDNA) consists of a multigene family tandemly repeated in the nuclear
DNA of eukaryotes. The coding regions, entitled subunits, are interleaved with the non-coding spacer regions, commonly utilised in the study of intra- and interspecific variation. The gene order within the family is: external transcribed spacer (ETS), 18S coding gene, internal transcribed spacer 1 (ITS1), 5.8S coding gene, second internal transcribed spacer (ITS2), 28S coding region and intergenic spacer (IGS).

The ribosomal spacer regions have been used extensively in the study of closely-related and cryptic species within the insects with strong resolving power. Studies of species complexes in the Anopheles genus have frequently utilised sequence data from the ITS1 and ITS2 regions (e.g. Gentile et al., 2001). The ITS2 region has been reported to evolve 2.5 times faster than the COI in Tetranychus species (Navajas et al., 1998), an indication that the region holds greater scope for variation between closely related species.

Baffi & Ceron (2002) reported the utility of the ITS1 region in separating sibling species within a Drosophila complex. In general though, few studies have considered the ITS1 region, perhaps due to its considerable size and the reported presence of repeat regions hampering DNA sequencing (Sharpe et al., 2000). The ITS2 has been more widely employed than the ITS1, displaying considerable interspecific variation and therefore scope for distinction within species complexes. The ITS2 may be highly variable on an interspecific level, with reliable alignment of sequences occasionally difficult or impossible (Phuc et al., 2003). The scope for variation between species does, however, provide scope that the closely related species pairing in this study may be distinguished using the ITS2 region.

A multi-region approach to calliphorid status has the potential to combine the resolving power of several genes, providing greater insight than from a solitary region (Azeredo-Espin & Lessinger, 2006). Given the respective advantages of both nuclear and mtDNA, a combined approach to species distinction has the potential to significantly improve the resolution of relationships and improve the reliability of phylogenetic analyses (Maddison, 1997). O’Grady et al. (1998) found the combined resolution of relationships between members of a Drosophila saltans species group was greatly increased using combined analysis of the nuclear ITS1 and alcohol dehydrogenase genes, along with the
mitochondrial COI and COII genes. The combined approach also has been utilised by several other authors (Clark et al., 2001; O’Grady & Kidwell, 2002; Chen et al., 2003). Sharpe et al. (2000) utilised a combined ITS2 and COII approach to identify the presence of two cryptic species within an Anopheles complex. Martin et al. (2002) combined the mitochondrial cytochrome B and COI genes with the nuclear globin 2b gene, and found that the nuclear gene resolved the relationships between four Chironomus species most fully, while mtDNA grouped individuals by continent of origin and not species.

This study aimed to address critical issues arising from the study of the COI gene in previous chapters by considering alternate DNA regions. The main aim was to provide a means for separation within the Ch. megacephala/Ch. saffranea and C. stygia/C. albifrontalis complexes, or alternately, infer whether synonymy appears possible. The potential species complex within Ch. rufifacies was also considered. Data from the control region and ITS regions was assessed for potential to resolve taxonomic anomalies revealed in the COI data, and make some general inferences about the comparative rates of evolution in these genes. In addressing these issues, hypotheses about the status of these species may be formulated with greater certainty.

The specific points addressed were:

- What can the ribosomal ITS regions contribute to the molecular distinction of calliphorid species, particularly within the C. stygia/C. albifrontalis complex identified using the COI gene?
- Does the successful distinction between Chrysomya species by Lessinger et al. (2004) using the mitochondrial control region extend to the distinction of Ch. megacephala from Ch. saffranea?
- Does the non-coding nature of the rDNA spacers provide further support for the haplotypes of Ch. rufifacies revealed using the COI gene?
- Do these non-coding regions provide greater scope for variation than the coding COI gene?
- Does the data support the status of these species, or is further taxonomic revision warranted?
Lucilia cuprina was not addressed in this study due to difficulty in obtaining specimens from both haplotypes identified using the COI gene.

The following study is under review in the following form:

Abstract

A major area of research in forensic entomology has been the molecular-based identification of blowflies (Calliphoridae) found in association with carrion. This complement to the traditional morphologically-based process has contributed to the efficiency and accuracy of identification and subsequent post-mortem interval (PMI) estimation. Most studies have utilised the mitochondrial cytochrome oxidase I (COI) gene in providing distinction, however there are limitations in this technique. Our previous work has revealed the existence of *Chrysomya megacephala/Ch. safranea* and *Calliphora stygia/C. albifrontalis* species complexes based on COI data, preventing individuals within these complexes from being assigned to their correct species. This study is a preliminary investigation into the use of two alternate DNA regions to provide the necessary distinction within species complexes. The two regions evaluated were non-coding regions of nuclear and mitochondrial DNA. The nuclear ribosomal DNA was utilised for the potential of the internal transcribed spacers (ITS) to distinguish between calliphorid species and to provide distinction between haplotypes of *Ch. rufifacies* inferred from COI data. Despite difficulty in the alignment of portions of sequence, seven calliphorid species were able to be distinguished based on this region, most notably *C. stygia* and *C. albifrontalis*. Limited variation was observed within *Ch. rufifacies*, preventing further conclusions relating to the status of this species. In addition, the mitochondrial control region was assessed for its potential to provide insight into the *Ch. megacephala/Ch safranea* complex, over 339bp of data revealing no species specific markers useful for this purpose and limited variation. This study provides a new region for distinction of *C. stygia* and *C. albifrontalis*, and discusses the potential of the rDNA spacers and mtDNA control region for use in a multigene approach to forensic entomology.

Keywords

Calliphorid, mtDNA, ITS, rDNA, *Chrysomya, Calliphora, Lucilia*
Introduction
Forensic entomology largely centres around the estimation of the minimum time since death in cases of unnatural death. This estimation relies on the age determination of insects, most commonly fly larvae, and is often complicated by difficulties in identification of the morphologically similar larval blowfly stages. Molecular technologies have been widely researched for their potential application in forensic entomology in recent years to improve efficiency and accuracy of insect identification. A variety of techniques have been proposed for use in increasing the accuracy and efficiency of the field. However, techniques have largely centred around sequencing of DNA for the high information content of the data gained and potential for simultaneous distinction and phylogenetic analysis.

Fundamental to any molecular study is the selection of a region of DNA informative at the relevant taxonomic level. Sequencing studies in forensic entomology have most commonly utilised mitochondrial DNA (mtDNA), frequently the cytochrome oxidase I, either using sequence data for distinction (Malgor & Coquoz, 1999; Wells & Sperling 1999; Vincent et al., 2000; Wallman & Donnellan, 2001; Wells & Sperling, 2001; Harvey et al., 2003a, b, Chapter 4) or polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) (Sperling et al., 1994; Schroeder et al., 2003).

The COI has proven successful in distinction of forensically important calliphorids species. Localised studies have generally illustrated the potential to distinguish regional forensic calliphorids fauna using the COI gene. Harvey et al. (Chapter 4) showed in their global study of forensic calliphorids the utility of the COI on a cross-continental scale, however they also highlighted some important issues. Difficulty was shown in the distinction of the endemic Australian species Calliphora stygia from the closely related C. albifrontalis, as with the separation of Chrysomya megacephala from the morphologically similar Ch. saffranea. The study also highlighted intraspecific variation within Ch. rufifacies and Lucilia cuprina. Two haplotypes were highlighted within each of these species, raising questions over their accurate identification.

The inability to separate C. stygia from C. albifrontalis and Ch. megacephala from Ch.
saffranea necessitates the location of a new region of DNA to further distinguish individuals from these complexes. In addition, the *Ch. rufifacies* and *L. cuprina* species issues should be addressed using a multi-region approach to ascertain the true nature of the variation between the individuals.

**Control Region**

Popular in vertebrate studies, the control region is generally less commonly included in insect molecular work. The reasons for this are numerous, including difficulty in amplification and DNA sequencing due to a high A+T content and the presence of secondary structures (Azeredo-Espin & Lessinger, 2006). More importantly though, while the control region is generally perceived to be among the most rapidly evolving genes in the vertebrates, this does not hold true for insects (Zhang & Hewitt, 1997). The insect control region evolves at a rate similar to nuclear DNA (Harrison, 1989). Lessinger & Azeredo-Espin (2000) identified the control region as containing two regions, a conserved (A) region and a variable (B) region. They describe the B domain as evolving without selective pressure, besides adherence to an A+T bias, and therefore as the hypervariable region.

A number of studies have sequenced the control region of calliphorids important in myiasis, many of which have dual forensic significance (Lessinger *et al.*, 2000; Litjens *et al.*, 2001; Junqueira *et al.*, 2004). Lessinger *et al.* (2004) studied *Ch. megacephala, Ch. chloropyga* and *Ch. albiceps*, locating significant interspecific variation within the duplicated part of the control region, and a series of conserved sequence blocks within the conserved region. Lessinger & Azeredo-Espin (2000) suggest that the control region may have limited utility in resolving phylogenetic relations between taxa. However, the high variability displayed in the duplicated region could not be aligned with confidence between three *Chrysomya* species (Lessinger *et al.*, 2004), and suggests that it may be useful in distinction of cryptic taxa within the *Chrysomya*. 
Ribosomal DNA (rDNA)

The ITS1 region has been used successfully in many insect studies, and appears to hold greatest promise in interspecific studies. Baffi & Ceron (2002) distinguished two Drosophila sibling species on the basis of this region, using the size of the region alone as a diagnostic assay. The ITS1 region may prove difficult to work with though, as the large size of the region and presence of a repeat unit in many taxa hampers amplification and sequencing efforts (Sharpe et al., 2000).

The ITS2 region has also been used successfully in insect studies, however the high variability observed in this region may make interspecific alignment difficult, and occasionally impossible. Intraspecific studies have focused on this region frequently, distinguishing lepidopteran haplotypes and resolving phylogenies (Birch et al., 1994). Studies of cryptic species in the mosquitoes have also utilised the ITS2 region, discriminating within species complexes (Marinucci et al., 1999). Several studies (Manomani et al., 2001; Phuc et al., 2003) have exploited the interspecific and low intraspecific variation of this region with the development of a sequence specific priming (SSP) technique for fast, PCR-based distinction between sibling species. Length variation between species over this region is an important diagnostic feature (Clark et al., 2001), and generally length differences in the genome result from insertion and deletion events (indels) of repeat units (Nardon et al., 2005). Variation between species may be considerable, with Phuc et al. (2003) indicating inability to align ITS2 interspecific sequences on account of significant variation, while no intraspecific variation was located in Anopheles minimus individuals.

Hackett et al. (2000) used the ITS2 region for distinction between two Anopheles species. Length variation of the region was sufficient for distinction, and sequence data identified the presence of a cryptic taxon. A combined ITS1 and ITS2 study by Gentile et al. (2001) successfully distinguished cryptic taxa within Anopheles gambiae. They note however that the tandem repetition of the rDNA gene family may result in variation within an individual, by way of heterozygosity or variation in different copies of the gene family, a potential issue when utilising this region of DNA.
**Combined Mitochondrial and Nuclear Approach**

Stevens (2003) considered calliphorids of importance in myiasis using a multigene approach, employing the COI/COII and nuclear 28S rRNA gene to elucidate relationships between species, which disagreed largely with morphological analyses. From a forensic perspective, Ratcliffe *et al.* (2003) employed PCR-RFLP of the ITS regions for the distinction of forensically important calliphorids, muscids and sarcophagids, highlighting the high interspecific variation yet low intraspecific variation as useful attributes of the ITS regions.

This study is a preliminary assessment of the value of some alternate regions of DNA in addressing critical issues revealed from recent studies using COI data (Harvey *et al.*, Chapter 4). A survey of genetic variation over the nuclear rDNA spacer regions of several calliphorids species was conducted, and illustrated the ability to distinguish *C. albifrontalis* from *C. stygia* based on this region. The potential to distinguish *Ch. megacephala* from *Ch. saffranea* is explored using the mitochondrial control region, given the success of Lessinger *et al.* (2004) in revealing variation between *Chrysomya* species. The rDNA spacer regions have provided great insight into cryptic species complexes in the Culicidae, and in this study were therefore assessed for utility in distinction of the *Ch. rufifacies* haplotypes inferred from COI data. The study therefore complements the use of the COI gene in providing identification of forensically significant calliphorid species.

**Methods**

Samples used in the study were from a variety of locations, and are listed in Table 5.1. Extractions were performed according to the manufacturer’s protocol using the QIAGEN DNeasy Kit, with products eluted in 50µL of water and stored at -20°C. DNA was extracted from the flight muscles of adult flies, which were removed through an incision in the right side of the thorax of the flies. These were ground using micropestles in microcentrifuge tubes prior to the incubation step of the protocol.

**rDNA Amplification**

The rDNA ITS regions were amplified for individuals of several species as an indication of interspecific variation and potential for distinction within complexes. A number of *Ch.*
rufifacies individuals were further sequenced in an attempt to locate intraspecific variation. Individuals sequenced are shown in Table 5.1. PCR reactions were conducted in 25µl final volumes. The PCR reaction mix consisted of: 1 X PCR buffer (Fisher Scientific), 200µM dNTPs (Fisher Scientific), 1.5mM MgCl₂, 25pM each primer (MWG Biotech), 1 unit Taq polymerase (Fisher Scientific) and 3ng of template DNA. The primers used were 1975F and 52R from Ratcliffe et al. (2003) (Table 5.2). Cycling conditions were: 94°C for 2 min; followed by 25 cycles of: 94°C for 1 minute, 56°C for 1 minute and 72°C for one minute, and a final hold at 4°C on a Perkin Elmer Cetus.

Between 5 and 8 identical 25µl PCR reactions were conducted for each individual, and products pooled. This ensured a high quantity of the correct template for sequencing. Individuals were sequenced using the two external primers, 1975F and 52R, as well as two internal primers modified from Ji et al. (2003). The internal primer sequences were CAS5p8sBldMod (modified from CAS5p8sBld), and CaS5p8sFcMod (modified from CaS5p8sFc) (Table 5.2).

Control Region Amplification
The control region was amplified for individuals of *Ch. megacephala* and *Ch. saffranea* as shown in Table 5.1. PCR conditions were: 1 X PCR buffer (Fisher Scientific), 200µM dNTPs (Fisher Biotec), 2.5mM MgCl₂, 25pM each primer (Geneworks), 1.5 units Taq polymerase (Fisher Scientific) and 1ng template DNA. Cycling conditions were: 30 cycles of: 94°C for 30 seconds, 45°C for 1 minute and 60°C for two minutes, followed by an additional round of 94°C for 30 seconds, 45°C for 1 minute and 60°C for ten minutes, and a final hold at 4°C. Primers used were SR-J-14776 and CMEG A (Lessinger et al., 2004). Initially, nested PCR was attempted using the external primers SR-J-14233 and TM-N-193, and internal primers T1-N-24 and SR-J-14776 (Lessinger & Azeredo-Espin, 2000). Sequencing efforts were complicated by the presence of the duplicated *trnI* gene within which the primer T1-N-24 anneals, also found by Lessinger et al., 2004). New primers were therefore selected for this study, and sequences are listed in Table 5.2.

Sequencing
Purification of PCR products was performed using the QiaQuick PCR Purification Kit.
(Qiagen). Sequencing was performed using the ABI PRISM Big Dye Terminator 3.1 Sequencing Kit (Perkin Elmer), according to the manufacturer’s protocol. Cycling conditions for the sequencing reactions were as per the manufacturer’s recommendations. Sequences were analysed using Chromas v2.31 (www.techneleysium.com.au/chromas.html) and DAPSA (University of Cape Town). Phylogenetic analysis was performed using MEGA 3.0 (Kumar et al., 2001), with alignments using ClustalW.

Results

Interspecific rDNA Variation

The primers amplified a fragment of approximately 1400-1500bp, with some length difference between species. Primer 1975F was not useful in sequencing for most species, encountering repeat regions early in the ITS1 region and terminating. Consequently, the sequences presented here begin after this repeat region, yielding 1350bp of data. *Chrysomya megacephala* was only able to be sequenced over the ITS1 due to the occurrence of a poly-T region in the ITS2 that terminated sequencing efforts, while the *C. dubia* ITS2 contained a poly-T repeat limiting data collected.

Alignment with confidence proved difficult in parts of both the ITS1 and ITS2 regions. The 5.8S gene was however highly conserved, with one substitution in *C. albifrontalis* relative to the other species, and the ITS regions appeared relatively conserved in the areas flanking the 5.8S. Greatest difficulty was experienced in alignment of *Lucilia sericata* and *Ch. megacephala* with the *Calliphora* individuals over the ITS1.

Figure 5.1 shows the alignment of the latter part of the ITS1 and 5.8S gene, where alignment could be preformed with confidence. The relatively high genus level similarity is clear, with few differences between the *Calliphora* species and strong similarity between *Ch. rufifacies* and *Ch. megacephala*. The closely related species *C. augur* and *C. dubia* are identical over this region, while *C. stygia* and *C. albifrontalis* do vary. The ITS1 therefore appears relatively conserved within genera, but variable between genera. The ITS2 displayed high interspecific variation with difficulty aligning species with confidence. Numerous indels were present, necessitating manual alignment of sequences with limited confidence in alignment of many areas of sequence.
Calliphora stygia/Calliphora albifrontalis

The forward external primer 1975F did not sequence successfully in either *C. stygia* or *C. albifrontalis*, resulting in the diminished amount of data obtained. In *C. stygia*, a poly-A repeat 76 bases into the sequence may have resulted in some slippage, and was followed shortly by a 15bp repeat, and subsequent sequence deterioration.

The ITS1 proved difficult to align early in the sequence and therefore the alignment was shortened to feature sequence aligned with confidence. There is however, scope for identification based on the ITS1 given the considerable variation observed. The 5.8S gene was highly conserved as expected, with a single substitution distinguishing the species. The ITS2 featured six indel events, in addition to several substitutions, and was useful in distinguishing the two species. The sequence alignment between *C. stygia* and *C. albifrontalis* over a portion of this region is shown in Figure 5.2.

Chrysomya rufifacies

The rDNA fragment amplified from *Ch. rufifacies* was approximately 1500bp in length, and no length variation was observed between individuals. The sequence analysed was cut to approximately 970bp as the forward 1975F primer proved problematic in sequencing due to the presence of a poly-A region approximately 150bp into the ITS1 region. This poly-A repeat consisted of at least 17 consecutive A nucleotides, complicating sequencing and therefore was excluded from the study. Sequencing using the internal reverse primer CAS5p8sBldMod was similarly terminated encountering a poly-T repeat. It is suggested, given the reduced length of sequence data obtained, that these two repeat regions were separated with up to 350bp of intervening sequence which was unable to be obtained in this study. Following this repeat, approximately 435bp of ITS1 data was collected, the 5.8S ribosomal subunit with 63bp, and 460bp of ITS2 sequence.

Limited intraspecific variation was observed in *Ch. rufifacies*, and is displayed in Figure 5.3. A 5bp indel was detected in the individuals from Thailand, positioned shortly following the ITS1 poly-A repeat. Two isolated nucleotide substitutions were also identified in the Thai individuals. No variation was observed in the 5.8S or ITS2 regions.
Control Region Variation

*Chrysomya megacephala/Chrysomya saffranea*

The duplicated region (amplicon 1) of the control region of three individuals of *Ch. megacephala* and two individuals of *Ch. saffranea* was amplified in a single fragment of approximately 1000bp. The forward primer SR-J-14776 provided approximately 720bp of sequence data for each species, however the primer CMEGA failed in all sequencing attempts. As a consequence, the data presented has been sequenced in the forward direction only.

A possible case of heteroplasmy was observed in the control region of a Broome individual of *Ch. saffranea*. This was not reflected in the other individual from the same population, and requires confirmation before conclusions are made about this finding. Limited variation was observed to distinguish the two species from each other over this region, with no single substitution over 339bp common to a single species (Figure 5.4).

Discussion

The mitochondrial control region and nuclear rDNA spacers were investigated for their potential to support the previously established COI-based identification of forensically important calliphorids. Harvey *et al.* (Chapter 4) revealed several potential difficulties in the use of the COI gene in distinction within the *Ch. megacephala/Ch. saffranea* and *C. stygia/C. albifrontalis* complexes.

The rDNA was successfully amplified in species across three genera, however sequencing was complicated by the presence of repeat regions in most species. Repeats were most frequently poly-A or poly-T sequences, and Sharpe *et al.* (2000) similarly reported the occurrence of repeat units as a complicating factor in ITS1 sequencing. The length variation between species was observed on the basis of acrylamide electrophoresis but not quantified using sequence data given the difficulty in obtaining complete data for the region. There exists potential, however, that length variation may provide a rapid means of distinction between closely related species. This requires further study as sequence variation was the focus of this study.
The conservation of the 5.8S gene was expected given the coding nature of this region. The difficulty in alignment of the spacer regions reflects the variation present over these regions and their potential value in species distinction. The ITS1 was highly variable between genera, complicating alignment. The ITS2 is more commonly utilised in insect molecular studies however alignment of different species may be difficult or impossible (Phuc et al., 2003), and in this study parts of the ITS2 could not be aligned with confidence. The use of the ITS2 has generally been limited to the study of cryptic and sibling species where distinction within taxa and complexes is required, and the variation observed in this preliminary study certainly supports the use of the ITS2 for such purposes. Phylogenetic study of species level relationships across genera is unlikely to be possible using ITS2 data given the subjectivity of alignment. The region does appear to hold promise for distinction of the complexes arising from Harvey et al. (Chapter 4).

*Calliphora stygia/Calliphora albifrontalis*

*Calliphora stygia* and *C. albifrontalis* are closely related endemic Australian blowflies found commonly associated with carrion. Their high morphological similarity and polyphyly when studied over the COI gene makes their distinction difficult, and they are suggested to have overlapping distributions, making their distinction critical.

The rDNA data clearly illustrates the potential for distinction between these species. Numerous indels and substitutions provide the basis for the development of a length-based PCR assay or a sequence specific priming (SSP) technique for the distinction of these species. This will necessitate the collection of sequence data from populations across their distributions, as intraspecific variation has not been considered in the current study. However, studies in other insect taxa have reported little to no variation within species over the spacer regions (Phuc et al., 2003), and intraspecific study of *Ch. rufifacies* in this study revealed limited variation. The spacer regions appear to be suitable for the distinction of these species from the complex identified using the COI gene.
**Chrysomya rufifacies**

Based on the success of the ITS2 region in providing insight into species complexes in other insect taxa, the rDNA spacer regions were hypothesised to be potentially useful in providing further distinction between haplotypes of *Ch. rufifacies* identified using the COI gene (Harvey et al., Chapter 4). Problematic to the study of the ITS1 region was the occurrence of a poly-A repeat region that terminated sequencing efforts. This region may potentially represent a source of informative variation, given that the number of repeats may vary between haplotypes.

No variation was observed within the sequenced fragment of the ITS2 despite success in distinction of cryptic species in other insect taxa. The five base pair indel and two substitutions distinguishing the Thai individuals may potentially reflect haplotype variation, but this would require further sequencing of individuals from the populations used by Harvey *et al.* (Chapter 4) for confirmation. It appears that variation in the nuclear spacer regions is limited in this species.

COI variation was greater than rDNA spacer variation, perhaps reflecting an increased evolutionary rate in the coding mtDNA as compared to non-coding nuclear DNA. Preliminary results of the duplicated region of the *Ch. rufifacies* mitochondrial control region (unpublished) have also revealed limited variation. Moriyama & Powell (1997) reported considerably higher synonymous substitution rates in *Drosophila* mitochondrial genes than nuclear DNA, and this study appears to support a higher general mitochondrial substitution rate. Substitutions observed in control region sequence data were not constant throughout individuals from common populations, indicating they are not good population markers. It is possible that an alternate approach such as microsatellites may potentially hold value in elucidation of the status of *Ch. rufifacies*. It must be considered, however, that there may exist little or no genetic differentiation between haplotypes if divergence is recent and speciation is in progress. Molecular data may not provide the solution to the taxonomic status of this species.
Chrysomya megacephala/Chrysomya saffranea

Given the successful distinction of three Chrysomya species using the mtDNA control region (Lessinger et al., 2004), this region was assessed for ability to separate a Ch. megacephala/Ch. saffranea species complex. The control region was amplified using the primer CMEG A, designed based on the Ch. megacephala sequence (Lessinger et al., 2004). This primer was not useful in sequencing of Ch. megacephala and Ch. saffranea in this study, indicating that an alternate primer may be required for sequencing in both sense and antisense directions.

There was limited variation observed, and no substitution was present as a species marker useful in distinction. Similar to the unpublished Ch. rufifacies data, substitutions were present in individuals only and may be a result of base saturation given the extremely high A+T bias of the mitochondrial control region, or may simply be an indicator of the limited variation over this region. The species are estimated to have diverged in the last million years, and this may account for low variation as a result of incomplete lineage sorting (Wallman et al., 2005). The relationship between these species is unlikely to be elucidated based on the control region, and the nuclear rDNA spacers are suggested as a future region for investigation. Until this can be resolved, these species must be considered part of a complex unable to be reliably separated.

This study has investigated alternate regions for the investigation of some species complexes revealed from sequencing of the COI gene. The rDNA spacers have been shown to have great utility in interspecific distinction, particularly useful in the separation of the morphologically similar C. stygia and C. albifrontalis. Intraspecific variation over this region was limited, providing little insight into the potential Ch. rufifacies complex. However, this conservation of sequence increases the value of the region as a species distinction tool. The Ch. megacephala/Ch. saffranea complex was not able to be separated based on the mtDNA control region, supporting the reported limited variation observed in other insect taxa over this region (Zhang & Hewitt, 1997) despite the non-coding nature of this area. Both the Ch. megacephala/Ch. saffranea complex and Ch. rufifacies should be further studied using alternate regions to elucidate their status and contribute to the strong identification platform already offered by the COI gene.
Acknowledgements
MH was supported as a Sir Keith Murdoch Fellow of the American Australian Association during the completion of part of this work at the University of Tennessee Knoxville. Thanks are extended to Prof Richard Jantz, Prof Karla Matteson for providing facilities and equipment, lab staff at University of Tennessee Medical Center Knoxville Developmental Genetics lab for technical assistance, and K. Sukontason, M. Lee Goff, D. Bishop, M. Villet, T. Postle and G. Levot for flies supplied for this study.
**Table 5.1** List of specimens used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Region Studied</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysomya rufifacies</em></td>
<td>Chiang Mai University, Thailand (n=2) (lab colony)</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Perth, Australia (n=4)</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>20km North of New Norcia, Western Australia</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Oahu, Hawaii</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Darwin, Australia</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Wallaceville, New Zealand (n=2)</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Kaitoke, New Zealand</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Knoxville, Tennessee</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td><em>Chrysomya megacephala</em></td>
<td>Honolulu, Hawaii</td>
<td>Control region</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Honolulu, Hawaii</td>
<td>rDNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sydney, Australia</td>
<td>Control region</td>
<td>X</td>
</tr>
<tr>
<td><em>Chrysomya saffranea</em></td>
<td>Broome, Western Australia (n=2)</td>
<td>Control region</td>
<td>X</td>
</tr>
<tr>
<td><em>Calliphora albifrontalis</em></td>
<td>20km North of New Norcia, Western Australia</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td><em>Calliphora augur</em></td>
<td>Sydney, Australia</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td><em>Calliphora stygia</em></td>
<td>Kaitoke, New Zealand</td>
<td>rDNA</td>
<td>X</td>
</tr>
<tr>
<td><em>Lucilia sericata</em></td>
<td>Perth, Australia</td>
<td>rDNA</td>
<td>X</td>
</tr>
</tbody>
</table>
Table 5.2 Primer sequences for rDNA and mitochondrial control region reactions conducted in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1975F</td>
<td>5’TAACAAGGTTTCCGTAGGTG3’</td>
<td>rDNA gene family; external primer</td>
</tr>
<tr>
<td>52R</td>
<td>5’GTTAGTTTCTTTTCTCCCTCCT3’</td>
<td>rDNA gene family; external primer</td>
</tr>
<tr>
<td>CAS5p8sBldMod</td>
<td>5’ATATGCGTTCAAAATGTCGATGTTCA3’</td>
<td>rDNA gene family; internal sequencing primer</td>
</tr>
<tr>
<td>CaS5p8sFcMod</td>
<td>5’TGAACATCGACATTTTGAACGCATAT3’</td>
<td>rDNA gene family; internal sequencing primer</td>
</tr>
<tr>
<td>SR-J-14776</td>
<td>5’GCTGGCACGAATTTTGGCTC3’</td>
<td>mtDNA control region</td>
</tr>
<tr>
<td>CMEG A</td>
<td>5’ATGATATTTCTTAACCTGGATT3’</td>
<td>mtDNA control region</td>
</tr>
</tbody>
</table>

* Primers are indicated as internal or external, with internal sequencing primers used only for sequencing from the original PCR product
Figure 5.1 Interspecific variation in part of the ITS1 and 5.8S between individuals of *C. albifrontalis* (CA), *C. stygia* (CS), *C. augur* (CG), *C. dubia* (CD), *Ch. rufifacies* (CR) and *Ch. megacephala* (CM). Shading denotes 5.8S gene. Dashes indicate indels, dots identity and an asterisk indicates a conserved site amongst the species.
Figure 5.2 Variation between *C. albifrontalis* and *C. stygia* in a portion of the ribosomal DNA ITS2 region. Dashes indicate indels, dots identity and an asterisk indicates a conserved site amongst the species.
Tennessee  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Nedlands, Perth  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Wallaceville, NZ  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Kaitoke, NZ  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Kaitoke, NZ  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Darwin  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Hawaii  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Nedlands, Perth  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
New Norcia, WA  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Perth outskirts  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Perth outskirts  AAAGGCAAAATAAAAACTT  TATGTGGCTATATTATATATTTTTAATGTGTTC
Thailand  AGAGGCAAAATAAAAACTTATATTATGTGGCTATATTATATTTTTAATGTGTTC
Thailand  AGAGGCAAAATAAAAACTTATATTATGTGGCTATATTATATTTTTAATGTGTTC

Figure 5.3 Variation between individuals of *Ch. rufifacies* in the ITS1 region of nuclear DNA
Figure 5.4 Variation over 339bp of the duplicated region of the mitochondrial control region in individuals of *Ch. megacephala* (CM) and *Ch. saffranea* (CS). Dashes indicate indels, dots identity and an asterisk indicates a conserved site amongst the species.
GENERAL DISCUSSION

This study aimed to resolve some critical issues identified in the Chapter 4 study of forensically important calliphorids using the COI gene. Distinction within the *Ch. megacephala/Ch. saffranea* and *C. stygia/C. albifrontalis* complexes was addressed, in addition to investigation of a region to provide further insight into the haplotypes of *Ch. rufifacies* identified using the COI gene.

The rDNA spacers proved useful in the interspecific distinction of seven calliphorid species despite difficulty in aligning regions of the ITS1 and ITS2 with confidence. Conservation of the 5.8S gene sequence and the spacer sequence immediately flanking this gene provided useful variation. The high variation makes alignment and subsequent phylogenetic analysis using these regions unlikely on an inter-generic level.

*Calliphora stygia* and *C. albifrontalis* were distinguished based on the ITS sequences, however further population sampling is vital to verify the robust nature of the species markers observed. The spacer regions appear likely to provide a useful secondary diagnostic technique where identification to complex level based on COI data leaves a possibility of two species identities. The mitochondrial control region, however, did not distinguish between *Ch. megacephala* and *Ch. saffranea*, perhaps attributable to the limited size of the region used or incomplete lineage sorting. These species may potentially be distinguished based on the rDNA spacer regions.

The rDNA data yielded little intraspecific variation in *Ch. rufifacies*. Based on previous studies of cryptic species, it was anticipated that this region may prove suitable for identifying possible cryptic species, however, perhaps as a result of the limited number of individuals and their respective origins this did not occur. It is possible that recent divergence and current speciation is not yet reflected in the spacer regions. Further taxonomic consideration is required for this species.

Given the ease of amplification and sequencing of the COI gene, this region appears the most useful for routine diagnostic work. The non-coding regions investigated in this study
did not yield the increased intraspecific variation expected given the reduction in functional
constraint over the sequence, however they appear to hold value for the distinction of
closely-related species in complexes not resolved using COI data, reflecting the faster
evolving nature of these non-coding regions. It is difficult to draw any conclusions relating
to the evolutionary rates of calliphorid nuclear and mitochondrial DNA based on this study
and the small amount of control region data analysed. The limited data gathered in this
preliminary study illustrates, however, strong species distinction over nuclear non-coding
regions, and supports their use as a secondary diagnostic tool in forensic entomology.
CHAPTER 6:
Practical Application of DNA-based Calliphorid Identification:
Forensic Cases and Museum Artefacts
The accumulation of sequence data is an important foundation to the routine use of molecular techniques for identification in forensic entomology. This data constitutes a database for comparison with unknown individuals by way of phylogenetic analysis, and therefore graphical representation of the relatedness between individuals.

The primary application of sequence data is in the identification of specimens as a preliminary step to the estimation of post-mortem interval (PMI). Becoming more routinely used worldwide as a complement to the traditional morphological and rearing approaches to identification, molecular techniques have contributed greatly to the efficiency and accuracy of forensic entomology as an investigative tool.

There are further potential applications of this sequence data. The identification of insects from crime scenes may contribute not only to PMI estimation, but to the identification of specimens that may indicate post-mortem movement of remains. Species may be highly specific in their choice of habitat, and their discovery in an atypical habitat may provide investigators with a new avenue of investigation. For example, Lucilia sericata is largely an urban species within Australia, while its sister species Lucilia cuprina is generally a rural species found in the vicinity of livestock where it is an agent of myiasis (Montgomery, 1990). The identification of L. cuprina individuals on human remains in an urban habitat therefore serves as an important indication that remains may have been relocated from a primary crime scene in a rural habitat.

The use of insects to identify geographic provenance has other important applications. In particular, molecular entomological techniques may apply to the location of the origin of museum artefacts where documentation is lacking but insect remnants remain.

Calliphorid puparia are extremely resilient insect remnants that may persist in the environment for hundreds of years after the adult fly has emerged. Nystrom et al. (2005) reported calliphorid puparia from a burial in Chachopoya, Peru, from a site occupied from ca. AD 800 to A.D. 1532. Faulkner (1986) discovered 800 year old invertebrate remains in association with human remains in Pacatnamu, Peru, including calliphorid puparia. These
puparia may be used to reconstruct historical events surrounding a death, determine seasonality and possibly contribute to determination of provenance of artefacts removed from their origin without documentation. The advanced weathering of these puparia may prevent accurate identification, and therefore DNA techniques hold promise for overcoming morphological limitations. Such techniques are faced by the numerous difficulties associated with the amplification of ancient DNA, including inhibition of amplification and degradation of template. The viability of DNA from historical puparia may be limited significantly by its age and preservation conditions.

In a forensic context, the presence of puparia serves as an indication that a species has progressed through its entire immature development on remains, and may therefore provide an estimate of minimum PMI. The seasonality of some calliphorid species serves as a useful indication, however, of the season of colonisation of remains, and consequently when puparia are collected some years from the time of death they may help to narrow the season of death significantly. They may, however, be considerably weathered as a result of exposure to the environment and therefore difficult to identify. The use of molecular techniques to identify these insect remnants to species level therefore holds significant forensic value, in addition to the historical aspect.

This chapter considers the application of DNA-based identification sequence data and techniques to the identification of forensically important calliphorids. The two sections presented reflect the utility of molecular-based identification in both historical and contemporary situations. The first presents an effort to identify puparia of historical origin. In the second, the use of DNA sequencing is illustrated in cases where preserved insect material was provided for the purpose of PMI estimation.

The specific questions addressed in this study were:

- Can DNA be extracted from contemporary puparia in sufficient quantity and quality to provide identification?
- Is DNA able to be extracted from historically significant puparia of considerable age? If so, is it viable in PCR for the identification of specimens?
• Can the sequence data amassed in previous chapters be used to provide identification of calliphorids in forensic cases?

This chapter consists of a paper currently in preparation, followed by two examples of cases where DNA-based identification has been utilised. Details of the paper are:

Abstract

Forensic entomology is frequently used in the estimation of post-mortem interval, particularly in the early stages of decomposition when immature insects are numerous. Following the consumption of the soft tissues empty puparia may persist for hundreds of years at a scene due to their resilient nature. The literature illustrates their value in determining seasonality of death and reconstructing events surrounding death, but such applications rely on accurate identification of the puparia which may prove difficult if they are highly weathered or fragmented. This study examines the potential for the DNA-based identification of calliphorid puparia, reporting a technique for the extraction and amplification of DNA from both contemporary and older puparia. The technique was refined to extract DNA from puparia recovered from 300 year old mummified corpses of unknown origin in reconstructing the calliphorid species assemblage present in their associated insect fauna, and ultimately providing a tentative geographic origin for the corpses. The extraction and amplification of DNA from older material must consider the critical issues of DNA degradation, and possible PCR inhibition by co-extracted substances. DNA was successfully extracted from contemporary and older material in this study. Amplification was not reproducible, and was complicated by degradation of template, and possible inhibition. This study shows difficulty in the amplification of aged puparia, but illustrates the potential for the use of molecular techniques with contemporary puparia.

Keywords: puparia, DNA extraction, Calliphoridae, pupal casing, forensic entomology
Introduction

The forensic entomologist is frequently called upon to estimate post-mortem interval in death investigations. The use of locality specific successional data may be combined with local weather conditions to estimate the minimum period of time the carrion fauna may have been present. In many investigations the body may be discovered while soft tissues remain and insects are still active, and the use of species-specific developmental data may assist in the estimation of time since death. But when the necrophages, their predators, parasites and corpse omnivores that form part of the predictable and useful successional data depart, the forensic entomologist may be left only with empty fly puparia.

The discovery of puparia may be useful as an indication of the fly species that were present on the body during decay, but following the emergence of flies from the puparia the age of the remaining casings cannot be determined. To measure age based upon physical weathering relies upon knowledge of the many environmental factors such as sun, moisture, edaphic properties and climate, and would vary greatly between locations.

Puparia may persist at a death scene long after all other entomological or biological evidence has disappeared. Their highly resilient nature has seen them preserved in 16th-century cesspits at St Saviourgate, York (McCobb & Briggs, 2004) present as sub-fossils, partially replicated in calcium phosphate. Furthermore, they possess tremendous value in extending the utility of forensic entomology in death investigations beyond the traditional timeframe for which entomologists are generally consulted.

Nystrom et al. (2005) utilised palaeoentomology to aid in the reconstruction of mortuary behaviour concerning a mummy bundle from Chachopoya, Peru. This site was occupied from ca. AD 800 to A.D. 1532, and insect material, mainly composed of empty calliphorid puparia was recovered from remains. A combined anthropological and entomological approach allowed a hypothetical situation to be developed for the injury and wrapping sequence of the body. The presence of hymenopteran parasitoids in the sample, and identification of their emergence holes in empty puparia, also contributed significantly.
Gilbert & Bass (1967) utilised puparia as an indication of the seasonality of Arikara burials aged at 130-160 years old. The presence of the puparia of calliphorid and sarcophagid flies found only between late March and mid-October aided in reconstructing the timing of the burials.

Nuorteva (1987) similarly promoted the use of puparia as an indicator of the seasonality of death, utilising puparia of *Phormia terranovae*, the single common blowfly species in Finland during spring, as an indicator of a winter or spring death. This may represent an important piece of information in any investigation where time has elapsed since death and PMI interval estimation is scant. Archer & Elgar (2003) caution determination of seasonality on the basis of absence of seasonally specific taxa however, due to the cryptic nature of insect remnants at a scene. Soil beneath and surrounding the body should also be searched for the presence of other species that may indicate an alternate time frame (1987). The use of puparia also assumes that larvae fed upon the corpse, and did not migrate from a nearby alternate source, and thus the area should be searched for other remains.

Faulkner (1986) reported the discovery of 800 year old invertebrate remains in association with human remains at a depth of 2.75m at Pacatnamu, Peru. The material was well preserved with dipteran puparia represented, and the author notes the absence of the anterior ends of the puparia, indicating successful emergence of the adult stage. Faulkner used the invertebrate evidence to support the notion that remains initially decomposed on the surface and were later buried, yet reports difficulty in identification of one taxon of flies present amongst the puparia recovered. Teskey & Turnbull (1979) report the recovery and identification of dipteran puparia from pre-historic graves at a site dated between 2000-2500 years ago. The fragile and fragmented puparia were able to be sorted using configuration of posterior spiracles, yet species identification required the better preserved specimens be softened in detergent, and later cleaned using an ultrasonic cleaner. Morphological identification could then be performed.

Morphological identification of puparia is certainly possible, though may prove very difficult (Siriwattanarungsee et al., 2005; Archer & Elgar, 2003). Third instar larvae retain the last larval cuticle as they pupate, meaning that many larval characters are preserved as
part of the puparium. This is particularly evident in the case of the distinctive species *Chrysomya rufifacies* (the hairy maggot blowfly) where the fleshy larval processes are identifiable on puparia. Amorim & Ribeiro (2001) utilised the texture of the puparia surface, conspicuousness of perispiracular region tubercles and the distances between peritremes in the postspiracular region to distinguish between puparia of *Chrysomya putoria*, *C. megacephala* and *Cochliomyia macellaria*. Such characters facilitate identification from relatively recent puparia, yet those that have remained exposed at a scene for some time may have become considerably weathered and such characters may not be suitable. Furthermore, fragmentation and disintegration of puparia may occur over time reducing the potential to locate specimens bearing multiple diagnostic characters.

Nystrom *et al.* (2005) record the identification of puparia to family level only, due to the poor condition of the material recovered. While material may be well-preserved, anterior ends of puparia are frequently absent and in sub-optimal conditions, considerable weathering may have occurred. This may obscure diagnostic characters and hinder the identification process, making the development of an alternate means of identification desirable. Scanning electron microscopy (SEM) has proven useful in distinguishing puparia of forensically important species (Siriwattanarungsee, 2005) but still relies on adequate preservation of the puparia.

Molecular techniques are widely employed in forensic entomology in identification of the immature stages of forensically important species. DNA forms a useful diagnostic tool due to its relative stability, ability to be obtained from damaged and fragmented specimens, efficiency in providing identification and overcoming the limitations of the traditional yet time-consuming rearing and morphological identification. The majority of studies have focused on the identification of the Calliphoridae, generally employing sequencing of the cytochrome oxidase I (COI) encoding region of mitochondrial DNA (mtDNA) (Harvey *et al.*, 2003a, b; Sperling *et al.*, 1994; Wallman & Donnellan, 2001).

The extraction and amplification of DNA from insect material recovered from contemporary cases has been widely researched and documented, yet has largely focused on the living insect material collected from a scene and rarely the insect remnants, such as
empty puparia, that may remain at a scene long after the soft tissues of the corpse are consumed. DNA has been successfully extracted and amplified from contemporary empty puparia (Vincent et al., 2000; Malgorn & Coquoz, 1999), however there has been no documentation of a technique for use with older samples.

In amplification of older samples, there are two major issues of concern. First, DNA degrades over time, with the rate of degradation dependent on numerous environmental factors. This degradation may result in DNA becoming highly fragmented with few longer target copies for amplification. Second, in older material substances that inhibit the enzyme activity in PCR may be co-extracted with DNA from the sample, reducing the efficiency of the PCR reaction. Numerous substances such as heme or the reagents used to prepare template DNA may inhibit PCR (Creader, 1996), also tannins, humic acids and fulvic acids are common soil-derived PCR inhibitors (O’Rourke et al., 2000), and the exact identity of the inhibiting components of many PCR systems remain unknown. In older samples, the problem of PCR inhibition must be addressed.

This study examines the potential to extract and amplify DNA from puparia for subsequent identification, with a view to addressing a practical issue. Samples were obtained from mummified corpses currently situated in a museum collection. The individuals became naturally mummified in a cave environment some 300 years ago in an unknown location. A technique was assessed for ability to extract and amplify DNA from the puparia for identification of the assemblage of fly species found associated with the corpses, with a view to identifying their geographical provenance. A technique was developed for use in conjunction with both contemporary and the older samples, and critical technical issues and applications are discussed.

2. Materials and Methods

2.1 Samples and Storage

Aged puparia were obtained from a European museum. These samples contained few intact puparia, with the majority considerable fragmented. Contemporary puparia were taken from a laboratory colony of Calliphora dubia, collected within a few weeks of adult emergence. These puparia were stored dry in phials, frozen at -20°C.
2.2 Preparation of Puparia for Extraction

Contemporary puparia were reasonably clean due to their laboratory origin, however field collected puparia were coated with dirt, and contained remnants of parasitoid material and other insect material accumulated over time.

Samples were initially examined using a dissecting microscope and any large pieces of foreign material either in or on the casing removed manually using forceps. Puparia were then placed in double distilled water (ddH₂O) and inverted repeatedly for 2-3 minutes, removing the majority of contaminating material yet preserving the delicate structure of older casings. They were then removed and dried on a heat block at 56˚C to facilitate grinding in the initial extraction step. All steps were mirrored in a tube designated as the negative extraction control, to ensure no contamination was introduced during the extraction process. This sample was treated in an identical manner to pupal material, through to and including the amplification stage.

2.3 DNA Extraction

The extraction was performed using the DNEasy Tissue Kit (Qiagen) according to the manufacturer’s instructions with some modifications. Where possible, extraction steps were conducted in a fume hood. Puparia were washed by agitation in sterile water prior to extraction. Pupal casings were placed in 1.5ml microcentrifuge tubes, and ground using a sterile micropestle until a coarsely ground material was produced. Lysis buffer and Proteinase K were added as per the manufacturer’s protocol, and incubated for three hours at 56˚C. Overnight incubation was also trialled. At the elution step, 100µl of elution buffer was added, and samples eluted at room temperature for 10 minutes prior to centrifuging.

Extraction was tested using a variety of amounts of starting material. For the aged material, a single puparium (approximately 1.4mg) was utilised, 3 puparia, and 25mg (approximately 10-12 puparia), the suggested amount of starting material under the QIAGEN protocol. Contemporary extractions were performed using a single puparium.
2.4 DNA Visualisation and Quantitation
Samples were electrophoresed on a 2% agarose gel at 120V. DNA was visualised using ethidium bromide staining and UV transillumination. Quantitation was performed using a NanoDrop ND-1000 spectrophotometer.

2.5 Amplification
Extracts were utilised in PCR to confirm the presence of DNA and ascertain its viability for amplification and other procedures. The region of DNA amplified was the COI encoding region of mtDNA. Fragments of four sizes (220, 320, 650 and 1270bp) were amplified to infer DNA quality. Primer sequences are listed in Table 6.1. The primer sequence differs for TL2-N-3014 from previous papers; this new primer sequence is a modified form with the fifth position altered to accommodate sequence variation in this priming site in a variety of calliphorids. Three RAPD primers were also tested (REP1R, Primer 808 and Primer 1) from Benecke (1998b) using the conditions from the same study, with the aim of amplifying smaller products to confirm DNA viability and ascertain possible levels of degradation based on profiles produced.

PCR reaction mixes consisted of: 1 X PCR buffer (Fisher Scientific), 2.5mM MgCl₂ (Fisher Scientific), 200µM dNTPs (Applied Biosystems), 25pM each primer, 2 units Taq polymerase (Fisher Scientific), 5µL 5% bovine serum albumin (BSA) and water added to a total volume of 50µL. Amount of DNA template varied. PCR reactions were also spiked with contemporary DNA to test for the presence of inhibitors.

Cycling conditions for the 220bp and 320bp fragments were taken from Harvey et al. (2003a), and for the two other primer pairs from Harvey et al. (2003b). Reactions were performed using an Applied Biosystems GeneAmp PCR System 2700.

2.6 Purification & Sequencing
PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen) and sequenced using the ABI Prism BigDye Terminator v3.1 Sequencing Standard kit (Applied Biosciences) according to manufacturers’ instructions.

3. Results

3.1 Extraction

The extraction protocol successfully yielded DNA from all aged samples with 25mg starting material. A total of 17-36ng/µl of template was reproducibly extracted from these samples. A study was made to determine whether yield was increased by extended incubation. Results were inconsistent from both single and three casing extractions, with 0-7ng/µl being released, with close to 50% of extractions not yielding any detectable template. Samples with 25mg starting material yielded 50-180ng/µl of DNA. In testing the contemporary casings, DNA was reproducibly extracted from individual puparia. A single contemporary puparium yielded approximately 200ng/µl of high quality DNA, with a 260/280 ratio between 1.81 and 2.0.

Critical to the extraction process was the initial grinding step. Material was best ground when perfectly dry, and extractions were only successful when a powder consistency was achieved. In cases where the puparium was left in larger pieces, very low yield was obtained.

3.2 Amplification

Amplification of samples proved a useful indication of the degree of degradation of template DNA. DNA from a single aged puparium was submitted to PCR in concentrations up to 140ng per PCR reaction, however no product was amplified. The addition of higher amounts of template from the 25mg extractions was successfully amplified, with a minimum of 100ng of template DNA required. Amplification was successful only in the presence of BSA. In Figure 6.1, the results of contemporary puparia amplification are displayed. Fragments were amplified up to 1270bp, using 25ng of starting template.
Figure 6.2 displays the results of experiments conducted to determine whether inhibition was responsible for the lack of amplification in samples extracted from aged puparia. Spiking with contemporary DNA template resulted in amplification, although producing a slightly weaker product. This illustrates that while inhibition may have occurred on some scale, it was not the main prohibitive factor in these reactions. Degradation of template was more likely the cause for PCR failure. RAPD primers did not amplify any product with aged sample, while smeared products were observed with contemporary DNA.

3.3 Sequencing
Samples from contemporary puparia were successfully purified and sequenced. Sequences obtained were of high quality, and were identified following analysis and comparison against known individuals. Two aged 25mg samples were amplified, and verified. These were the only samples detectably amplified from aged puparia, and did not represent a species studied in the particular laboratory at any time, therefore negating contamination issues. The aged samples of unknown species affinity were identified as *Chrysomya chloropyga* from phylogenetic alignment with a sequence database for global calliphorids (Harvey *et al.*, Chapter 4).

**Discussion**
Puparia represent a valuable yet under-utilised source of information at crime scenes and in the study of historically significant artefacts and sites. The ability to identify these insect remnants and use the biology of identified species to provide valuable information is an important issue with the potential to extend the utility of forensic entomology far beyond its current applications and traditional period of utility.

The pre-extraction examination and washing of puparia was largely a precaution designed to reduce the potential for inhibition by soil-derived compounds such as humic acids, fulvic acids and tannins, as samples appeared coated with dirt. These compounds may be inhibitory in PCR at low concentrations. O’Rourke *et al.* (2000) suggest that phenol chloroform extraction techniques be used for the removal of such phenolic compounds. In this study, Qiagen spin-column based extraction was preferred for a number of reasons,
including reduced toxicity of reagents, efficient template recovery and removal of inhibitors, and reduced handling of the sample and thus less potential for contamination.

Carvalho et al. (2005) addressed the washing of calliphorid larvae for removal of potential external contamination prior to extraction of ingested DNA from the larval alimentary tract. They determined that water washes, with vortexing, were effective in cleaning the larvae. In this study the same methodology was employed, however the fragile nature of the puparia made vortexing potentially destructive and thus rapid, repeated inversion was preferable yet effective.

This study has shown the ability to extract DNA from material of considerable age. Critical to this process was the thorough grinding of material, facilitating the lysis of cells and release of larger amounts of DNA. The amount of material used in extractions was another critical issue. Ultimately, extraction from a single puparium is desirable, to ensure there is no mixing of template from different species. However, a single aged puparium did not yield sufficient template, and was unable to be amplified. The extraction kit manufacturer’s recommendation of 25mg for animal tissue was proved optimal in this study.

The recovery of high molecular weight DNA from contemporary casings confirmed the utility of the extraction technique with puparia. However, DNA extracted from older samples was unable to be visualised on gels despite a high yield, as confirmed by spectrophotometry, presumably due to the highly degraded nature of the template. Such degradation of older samples provides a challenge in the amplification and subsequent manipulation of the template from these samples.

For several reasons, the chosen target for amplification was a region of mtDNA, for several reasons. In ancient DNA studies, where highly degraded template is frequently encountered, mtDNA is often the molecule targeted for amplification (Capelli et al., 2003) due to the presence of hundreds of copies in each cell, as opposed to the single copy of nuclear DNA (O’Rourke et al., 2000). The chance of successful amplification is increased by prospectively more copies of template. In addition, mtDNA, and specifically the COI encoding region, have been the focus of many studies of the forensically important
calliphorids, allowing a significant body of sequence data to be accessed for comparison and the exploitation of previously optimised primer pairs and PCR protocols.

Amplification of DNA from individual casings was unsuccessful, while the 25mg extractions were amplifiable. It is suggested that the 25mg extractions provided more copies of longer template fragments than single puparia where aged template was concerned. Template from a single contemporary puparium was sufficient for amplification, showing the high quality of template present.

The degradation of template in this study was tested using three universal primer pairs producing COI fragments of varying size, of 320, 650 and 1270bp. The successful amplification of DNA from contemporary puparia confirms the high quality of this template, as seen in gel visualisation of the extract. The inability to amplify DNA from aged puparia is attributed to the degradation of the sample, and may be a result of the conditions of preservation of the casings. The design of primers to amplify a smaller fragment may result in successful amplification.

Critical to any PCR reaction is the amount of DNA template added. Contemporary DNA was amplified with just 25ng of template, while results were only obtained with 100ng and higher of older template, presumably due to the intense degradation of such samples. Aged templates are often affected by either inhibition, degradation or a combination of both of these factors. A common approach to a PCR influenced by inhibitors is to dilute the template to a level where amplification is possible, however this also potentially reduces the number of starting copies of template of sufficient size for amplification of the target fragment. Additives such as BSA, betaine and DMSO may also help to overcome the action of inhibitors. Spiking of reactions with contemporary DNA showed that inhibitors did not prevent amplification, although their effects may have been somewhat countered by the addition of BSA. In this study, it became apparent that the lack of intact template was the most likely limiting factor.

The authenticity of results in studies of ancient DNA are of vital importance, as with forensic DNA. Authentication is difficult, relying on negative controls and comparison with
contemporary reference samples. Capelli et al. (2003) comment that in studies of ancient DNA, there is rarely comparison between multiple aged samples, but more frequently aged and contemporary samples. There will exist genetic distance between the ancient molecule and the contemporary reference molecule, meaning that allowance must be made in comparisons for variation within a taxon over time. In addition, the stochastic nature of contamination, as promoted by the high sensitivity of PCR conditions used under protocols for ancient DNA amplification, makes the unintended amplification of contemporary reference samples routinely used in the laboratory a significant risk. The separation of working areas for these samples and temporal separation of their processing reduces such risk. Verification of results by separate labs, cloning of products and the confirmation of uniqueness of ancient samples as compared to routinely used samples will also aid in confirmation of authenticity of results.

In the context of the geographic origin of the specimens, the identification of Ch. chloropyga has great importance. This species has an African affinity, and so indicates the mummified bodies may have originated in this area. The identification of other species from the samples will provide a larger faunal assemblage to perhaps narrow the origin further.

This study has clearly illustrated the potential for the identification of calliphorid puparia based on DNA. This may increase the utility of puparia in cases where significant time has elapsed since death. DNA has been successfully extracted from 300 year old puparia, however reproducible amplification was not able to be achieved in this study as a result of template degradation. It is possible that DNA from puparia of significant age may still be amplifiable in other situations where preservation is optimal and exposure to DNAses and other sources contributing to degradation are excluded. This may only be confirmed by study with puparia of various ages, and verification of ability to extract and identify template in a number of independent laboratories. It is anticipated that the ability to extract and amplify DNA from puparia for molecular-based identification will contribute greatly to both forensic and historical studies.

Acknowledgements
MH wishes to acknowledge the University of Pretoria, Clarke Scholtz and Jennifer Roy for facilities and technical assistance, and Rotary International for an Ambassadorial Scholarship facilitating her study at the University of Pretoria where part of this study was conducted.
Table 6.1 Primer pairs used to amplify the three fragments tested in this study

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer &amp; Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>220bp COI</td>
<td>TY-J-1460 (5’ TACAATTTATCGCTAAACTTCAGCC 3’) C1-N-1687 (5’ CAATTCCAAATCCTCCAATTAT 3’)</td>
<td>Sperling et al., 1994; Wells &amp; Sperling, 1999</td>
</tr>
<tr>
<td>320bp COI</td>
<td>C1-J-2495 (5’ CAGCTACTTTATGAGCTTTAGG 3’) C1-N-2800 (5’ CATTTCAGCTGTGTAAGCATC 3’)</td>
<td>Sperling et al., 1994; Harvey et al., 2003b; Harvey et al., 2003a</td>
</tr>
<tr>
<td>650bp COI</td>
<td>UEA7 (5’ TACAGTTGGAATAGACGTTGATAC 3’) TL2-N-3014 B (5’ TCCAWTGCACTAATCTGCCATATA 3’)</td>
<td>Simon et al., 1994; Harvey et al., 2003b</td>
</tr>
<tr>
<td>1270bp COI</td>
<td>C1-J-1718 (5’ GGAGGATTTGGAAATTGATTAGTTCC 3’) TL2-N-3014 B (as above)</td>
<td>Simon et al., 1994; Harvey et al., 2003b</td>
</tr>
</tbody>
</table>
Figure 6.1 Amplification of contemporary DNA extracted from a single pupal casing. Fragments are approximately 320 (lane 2), 650 (lane 3) and 1270bp (lane 4) respectively of the COI gene. The DNA ladder is in lane 1.
Figure 6.2 Effect of spiking aged samples with contemporary DNA template to test for inhibition of amplification. Lane 1: DNA ladder, lane 2: negative control, lane 3: 120ng template from aged samples, lane 4: 100ng template from aged samples with 60ng contemporary DNA, lane 4: 150ng template from aged samples with 60ng contemporary DNA, lane 6: 60ng contemporary DNA.
Case Histories
The collection of sequence data has facilitated the molecular identification of calliphorid larvae in actual forensic cases where the estimation of PMI is critical. DNA sequencing has been utilised in casework to establish species identity of several larvae over the duration of this thesis. Two cases are discussed below as evidence of the utility of sequencing and the benefit of DNA in identification.

Case 1
Three preserved calliphorid larvae were supplied by police, collected from a crime scene in the Northern Territory, Australia. Two individuals were second instar larvae, the third a third instar larva. DNA was extracted from the middle third of the larvae utilising the QIAGEN DNEasy Tissue Kit, and anterior and posterior portions of each larva retained as they contained the physical diagnostic characters of the larvae required for morphological confirmation.

Sequencing was conducted over the 278bp region of the COI gene described in Chapter 2. Sequences were analysed and imported into MEGA 3.0 (Kumar et al., 2001), and neighbour-joining analysis utilised to provide identification. The phylogenetic tree obtained is shown below in Figure 6.3. The three unknown individuals were clustered with Calliphora dubia individuals, and alignments showed 99% similarity with this species. It was concluded on both morphological and molecular grounds that these individuals were C. dubia.
Figure 6.3 Neighbour-joining tree showing alignment and clustering of unknown individuals with *Calliphora dubia* individuals (CD). Other abbreviations: WD=Wallman & Donnellan (2001), CAUGUR= *C. augur*, CR=Chrysomya rufifacies, LS=Lucilia sericata. Numbers indicate bootstrap values (n=500). Scale bar indicates number of substitutions per site. Tree was constructed during the early stages of study when limited data had been collected.

Case 2
A single first instar calliphorid larva was obtained from Ravensthorpe, Western Australia. DNA was extracted from this larva using the QIAGEN DNEasy Tissue Kit, and amplified over 1167bp of the COI gene using the protocol from Chapter 3. Analysis was performed using MEGA 3.0 (Kumar *et al.*, 2001). The resulting neighbour-joining tree is shown in Figure 6.4. The sample (UNKNOWN) is clearly identified as *C. dubia*. 
Figure 6.4 Neighbour-joining tree displaying the relationship of the unknown individual to other sequences in our database over 1167bp of COI data. Letters following species names (A-F) indicate multiple individuals. Numbers indicate bootstrap values (n=500). Scale bar indicates number of substitutions per site.
GENERAL DISCUSSION

This chapter aimed to address the use of sequence data accumulated in previous chapters in some practical applications requiring the identification of calliphorid species. This involved the identification of puparia for historical and museum purposes, and larvae for the estimation of PMI in forensic cases.

The ability to extract, amplify and sequence viable DNA from contemporary puparia was demonstrated, allowing their accurate identification and application in estimating PMI and determining issues such as seasonality of death in cases of extended PMI. While DNA was able to be extracted from 300 year old calliphorid puparia, this template was unable to be amplified reproducibly. While it was originally thought this may have been a result of inhibition by co-purified contaminants in DNA extracts, this was shown to most likely be a result of extreme degradation of the DNA from the aged puparia. It is possible puparia preserved in more suitable conditions over time may contain better preserved DNA and be amplifiable.

The identification of immature calliphorids for use in casework has been shown in numerous cases to be successfully achieved by means of DNA sequencing and subsequent phylogenetic analysis. While this remains a useful tool, it is likely that in the future, estimates of statistical reliability of this technique may be required when presented as evidence in court. In addition, the development of a new diagnostic technique that circumvents the need for laborious and expensive sequencing is beneficial to the development of forensic entomology as an important tool in the estimation of PMI.
CHAPTER 7:
A New Approach to Extraction, Storage and Identification of DNA from Insects for Forensic Entomological Applications
The preceding chapters of this thesis have addressed the gathering of DNA sequence data to address several issues in the identification and species status of forensically important calliphorids. The assessment of species status based on this data and the identification of potential species complexes is of fundamental importance in the assurance of several basic assumptions in forensic entomology. The primary aim of this thesis, however, is the distinction of forensically important calliphorids.

The sequence data gathered over the COI gene and rDNA spacers in previous chapters distinguished all species, with the exception of the *Chrysomya megacephala/Ch. saffranea* complex which was complicated by the Malaysian *Ch. megacephala* individuals (Chapter 4). Sequence data and subsequent phylogenetic analysis form a useful diagnostic assay, and certainly form the “gold standard” for identification of calliphorids. The value of this method of identification was illustrated in the case reports presented in the previous chapter.

Sequencing is expensive, however, and may be time-consuming. Efficiency in sequencing is largely dependent on the equipment and facilities available to the forensic entomologist. In situations where sequences are processed off-site delays can be considerable, impeding identification of insects and subsequent estimation of PMI.

The accumulation of a large body of calliphorid sequence data has facilitated the development of a sequence-based diagnostic technique. Sequence-based techniques have the benefit of being highly specific as compared to techniques based on arbitrary priming such as RAPDs. In previous forensic entomological studies, sequence data has often been exploited in the development of polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLPs) (Narang & Degrugillier, 1995; Taylor *et al*., 1996; Sperling & Wells, 1994; Malgorn & Coquoz, 1999; Ratcliffe *et al*., 2003; Schroeder *et al*., 2003; Ames *et al*., 2006). Under this technique, restriction sites are identified in sequence data and used to cleave PCR products in predictable sites. Cleaved products are visualised on gels and fragment sizes used to identify species. In areas where several species are present a number of enzymes may be required for distinction with sub-optimal conditions for
particular enzymes, requiring different conditions and possibly involving expensive enzymes that must be stored and utilised infrequently, depending on case loads.

In considering the identification problems associated with the calliphorids, a useful parallel can be drawn to the mosquitoes (Diptera: Culicidae). Of medical and veterinary importance, the mosquitoes are often identified using molecular techniques and therefore much of the molecular literature relating to the Diptera focuses on this group. Many culicid studies have utilised the high information content sequence data to develop sequence specific priming (SSP) techniques (Manonmani et al., 2001; Fettene et al., 2002; Kampen et al., 2003; Phuc et al., 2003; Noel et al., 2004). SSP involves the use of primers specifically designed to anneal to a target species, using sequence data to ensure mismatches in the 3’ end of the primer in non-target species. The technique has the advantage of involving a single PCR assay followed by electrophoresis, eliminating the need for restriction enzymes or any further manipulation. This is an efficient technique for diagnostic purposes, and holds enormous promise for forensic entomology. This chapter will address the development of an SSP technique for Australian and New Zealand calliphorid species.

Of critical importance in any molecular assay is the extraction of high quality DNA in sufficient quantity for use in PCR. Many techniques have been used for extraction of DNA from calliphorids, including phenol: chloroform, Chelex, DNAzol and commercial extraction kits. These techniques all require that DNA extracts be frozen for storage following extraction, leaving them susceptible to degradation and desiccation. It is desirable that samples be stored following identification, in case further analysis should be required at a later date. Whatman have developed FTA cards, a method for the extraction and long-term storage of DNA samples. Samples are able to be stored at room temperature and transported without refrigeration, facilitating the portability of samples between labs. FTA cards have not been utilised with calliphorids to date, and require optimisation for use with insect samples from all life stages.

This chapter represents the assimilation of sequence data into a diagnostic assay for routine use in forensic entomology in Australia and New Zealand. This is supported by the
optimisation of the FTA protocol for use with calliphorid samples. Together, these two studies provide a new procedure for the extraction, identification and storage of DNA from forensic entomology cases. The specific aims of the studies are:

- To optimise the Whatman FTA protocol for use with calliphorid samples to facilitate the storage and transport of samples
- To develop a new identification technique utilising the SSP format for the rapid identification of calliphorids from Australia and New Zealand
- To introduce the SSP technique as a new method for the routine distinction of calliphorids in forensic entomology

This chapter is composed of two important studies. The first proposes a new method for the extraction and storage of DNA from calliphorids in forensic entomology. This study has been published in the following form:


The second paper describes an SSP assay developed for the efficient and accurate identification of forensically important calliphorids of Australia and New Zealand. This paper has been prepared for submission to Forensic Science International in the following format:

An alternative for the Extraction and Storage of DNA from Insects in Forensic Entomology

Abstract
An important area of recent research in forensic entomology has been the use of insect DNA to provide identification of insects for fast and accurate estimation of time since death. This requires DNA to be extracted efficiently and in a state suitable for use in molecular procedures, and then stored on a long-term basis. In this study, Whatman FTA™ cards were tested for use with the Calliphoridae (Diptera). In particular, testing examined their ability to effectively extract DNA from specimens, and store and provide DNA template in a suitable condition for amplification using the polymerase chain reaction (PCR). The cards provided DNA that was able to be amplified from a variety of life stages, and thus appears to be of sufficient quality and quantity for use in subsequent procedures. FTA cards therefore appear suitable for use with calliphorids, and provide a new method of extraction that is simple and efficient and allows for storage and transportation without refrigeration, consequently simplifying the handling of DNA in forensic entomological cases.

Keywords
Forensic science, forensic entomology, calliphorid, DNA, Whatman FTA
Introduction

The utility of DNA in forensic entomology has become a major area of research in recent years. Researchers have firmly established the potential to characterise insects of forensic importance to species level on the basis of specific regions of DNA (Sperling et al., 1994; Benecke, 1998; Harvey et al., 2003a, b).

Forensic entomologists most commonly employ DNA based techniques for use with calliphorids, or blowflies, due to their general ubiquity at crime scenes, and their unique ability to be among the first insects to locate a corpse following death (Byrd & Castner, 2001). This makes them particularly important, as they most frequently begin the predictable insect succession on a corpse, and are useful in estimation of time since death. Generally it is the immature stages that require DNA based identification due to the high level of morphological similarity between species, but adults may also require analysis.

The application of DNA based technologies to any field requires the development and optimisation of protocols suitable for the extraction and long-term storage of samples. An extraction technique should preferably be simple, efficient, provide DNA of sufficient quality and quantity to be utilised in subsequent procedures, and ideally, involve non-hazardous reagents. Storage issues include the long-term viability of the sample, preserved in a state suitable for reanalysis should such a request arise.

Traditionally the extraction methods utilised in this field have included Chelex based techniques and variations on phenol: chloroform extraction (Sperling et al., 1994; Benecke, 1998; Harvey et al., 2003b). Samples are subsequently frozen for long-term storage. These techniques provide DNA suitable for use in subsequent procedures, however the hazardous nature of the phenol: chloroform reagents is obviously disadvantageous. Techniques that improve the extraction, storage and handling of DNA, whether through cost, labour or safety are obviously useful alternatives to be considered.

FTA™ cards (Whatman BioSciences) provide an ideal alternative for the extraction and storage of samples. The technique involves application of a sample to the card, and the FTA-treated paper lyses cells within the sample and immobilises the released DNA. The
sample dries, and a disc may be punched from the card and prepared through a series of washes to be used directly in the polymerase chain reaction (PCR). Cards may be stored at room temperature.

The cards have been utilised for forensic purposes in the collection and processing of DNA samples (Whatman, 2004). Other applications have included use with corals (Crabbe, 2003), and also DNA extraction from saliva from beetle quid chewers, where inhibitors in the sample created difficulties for subsequent DNA amplification but were eliminated using the FTA™ method (Salvador & De Ungria, 1997).

The cards hold several specific advantages for use in this field if proven suitable for use with entomological samples. They provide a fast and simple method of preparing the sample, and reduce chances of contamination as liquid samples are no longer being handled but small discs of paper. Transportation of samples, either between entomologists or simply from one location to another does not require refrigeration, and long-term storage issues such as freezer space and potential desiccation of samples become less pertinent.

This study tested the ability of the cards to produce DNA of sufficiently high quality and quantity to be employed in PCR. The main criteria assessed were the overall ability to be amplified, potential for use with a variety of insect life stages, and ability to amplify products of considerable size to ensure samples are not significantly degraded during the extraction process. Species tested in this study were all members of the dipteran family Calliphoridae: *Chrysomya megacephala* (Fabricius), *Chrysomya rufifacies* (Macquart), *Lucilia sericata* (Meigen), *Calliphora dubia* (Macquart) and *Cochliomyia hominivorax* (Coquerel). Various life stages were employed, and conclusions drawn as to the potential application of FTA™ cards in this field.
Materials and Methods

Insects used in the study were collected from the field and represented a variety of calliphorid species. These species were *Chrysomya megacephala*, *Chrysomya rufifacies*, *Lucilia sericata*, *Calliphora dubia* and *Cochliomyia hominivorax*. Adult specimens were stored in 70% ethanol; larvae and pupae were first subjected to 30 seconds in boiling water then stored in 70% ethanol.

Preparation of the insect material for application to the FTA™ cards varied according to the life stage used. Flight muscles of adult flies were removed through a small incision in the right side of the thorax, and the remaining insect kept as a voucher specimen. In the case of pupae, the organism was removed from the casing, and the pupal casing itself retained as a voucher. For larval specimens, both second and third instar larvae were used, generally with the middle third of the insect being selected and the morphologically characteristic anterior and posterior segments retained for any further verification.

Specimens were ground in 100µl 1X TE buffer (10mM Tris-HCl, 1mM EDTA, Fisher Scientific), pH 7.5 using a micropestle in a 2mL tube until the solution was partially homogenised. Following grinding, the sample was applied to an FTA card and allowed to dry for a minimum of one hour.

To verify the successful lysis of cells, and binding and extraction of DNA, samples were prepared for amplification using PCR. Discs of both 1.2mm and 2.0mm diameter were prepared to determine the optimal sized disc for use with calliphorid samples. The manufacturer’s protocol was followed for the extraction from the cards. Two washes were found to be necessary using the FTA™ Purification reagent (Whatman BioSciences), and two washes using 1X TE buffer.

Larval, pupal and adult samples were all prepared, and tested using PCR. To confirm the quality, and therefore low degradation of the sample during preparation, PCR reactions were performed to amplify fragments of varying sizes. The fragments amplified all lie
within the cytochrome oxidase I (COI) encoding region of mitochondrial DNA (mtDNA). These were approximately 320, 650 and 1270 base pairs in size and are listed in Table 7.1.

PCR reaction mixes consisted of: 1 X PCR buffer containing 1.5mM MgCl₂ (Fisher Scientific), 200µM dNTPs (Applied Biosystems), 25pM each primer, 1 unit Taq polymerase (Fisher Scientific) and water added to a total volume of 50µL. Cycling conditions for the 320bp fragment were taken from Harvey et al. (2003a), and for the two other primer pairs from Harvey et al. (2003b).

Electrophoresis of PCR products was performed using 1.5% agarose gels stained with ethidium bromide.
Results

Discs were prepared from multiple individuals from several calliphorid species, and subjected to PCR. All samples were successfully amplified, including larval, pupal and adult samples. PCR products were clear, intense and of the expected product size when viewed on agarose gels.

Several samples were tested to determine the optimal disc size for use in reactions. Discs were trialled in concurrent reactions and results verified through repetition, and amplified bands were greater in intensity when template was provided in the smaller (1.2mm) disc size.

To ensure the overall quality of the template, and thus minimal degradation of the sample during the lysis, binding and washing processes, PCR reactions amplifying various sized products were conducted. Figure 7.1 displays the resulting products, with 320, 650 and 1270 base pairs successfully amplified from a variety of species and life stages.
Discussion

This study shows the potential for use of FTA™ cards for forensic entomological applications. The protocols used prepared DNA in a suitable condition for use in subsequent molecular procedures.

The ability to amplify samples from a variety of calliphorid life stages is desirable, as any stage may be collected from a scene and require analysis in its current stage. Immature and adult stages vary markedly in morphology, and thus the physical and chemical composition of the stages may also vary. The cards successfully lysed cells from both immatures and adult flight muscles, and provided DNA in a state suitable for use in PCR. Any potential inhibiting compounds were therefore successfully removed.

The optimal disc size suggested in the Whatman protocol was the larger, 2.0mm disc. The PCR products produced with the smaller disc were generally more intense in this study, perhaps indicating that sufficient DNA is lysed from the samples for use of a smaller disc. The larger discs may be providing considerably more template to the reactions, and thus reducing the efficiency of the PCR system.

The ability to amplify a fragment of up to 1270 base pairs from the template prepared using the cards indicates that the DNA is of sufficient quality to be used in molecular procedures. Further testing would be required to confirm amplification of larger fragments, but it appears that the DNA being extracted is of considerable quality and should not prove difficult to manipulate in other procedures.

Obvious advantages of these cards include storage issues, as samples are easily stored in a stable form on a piece of card, eliminating the need for freezing of samples. This also reduced the chances of both contamination and desiccation of a sample. Samples are also transportable at room temperature, and most infectious agents are considered to be deactivated on contact with the card, thus removing potential biohazards (Whatman, 2004).
Whatman FTA™ cards therefore provide DNA of a suitable quality and quantity for use in forensic entomology, and hold several advantages over traditional methods of extraction and storage used in this field.

**Acknowledgements**

This work was undertaken during a study period spent in the Department of Anthropology, University of Tennessee Knoxville. Thanks are extended to Prof Richard Jantz, Prof Karla Matteson for providing facilities and equipment, and lab staff at University of Tennessee Medical Center Knoxville Developmental Genetics lab for technical assistance. Associate Professor Ian Dadour and the Centre for Forensic Science at the University of Western Australia for support and assistance in preparation of this manuscript.
Table 7.1 Primer pairs used to amplify the three fragments tested in this study

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Fragment Size</th>
<th>Primer &amp; Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>320bp</td>
<td>C1-J-2495 (5’ CAGCTACTTTATGAGCTTTAGG 3’)</td>
<td>Sperling et al., 1994; Harvey et al., 2003a,b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1-N-2800 (5’ CATTCCAAGCTGTGTAAGCATC 3’)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>650bp</td>
<td>UEA7 (5’ TACAGTTGGAATAGACGTTGATAC 3’)</td>
<td>Simon et al., 1994; Zhang &amp; Hewitt, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TL2-N-3014 (5’ TCCAATGCACATCTGCATATTA 3’)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1270bp</td>
<td>C1-J-1718 (5’ GGAGGATTTGGAATTTGATTAGGTCC 3’)</td>
<td>Simon et al., 1994; Harvey et al., 2003b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TL2-N-3014 (as above)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7.1 Picture of 1.5% agarose gel showing fragments of 320, 1270 and 650 base pairs successfully amplified using discs prepared from FTA cards. Lane 1 DNA ladder in 100bp increments to 1000bp and then a single band at 1500bp; lane 2 320bp, lane 3 1270bp, lane 4 650bp.
A new approach to the rapid identification of forensically significant calliphorids of Australia and New Zealand

Abstract
The molecular-based identification of forensically important calliphorids has become an almost routine facet of forensic entomology, as a complement to the traditional morphological and rearing approaches. Identification is generally based on sequencing of the cytochrome oxidase I (COI) gene of the mitochondrial DNA (mtDNA), with analysis and formal identification based on phylogenetic methods. Sequencing, though the most accurate and desirable approach, is an expensive and often time-consuming exercise. Many studies have sought to overcome the necessity of sequencing with the development of restriction assays based on COI sequence data. Sequence-specific priming (SSP) is a useful diagnostic technique frequently employed in identification of the morphologically similar Culicidae. Based on a PCR matrix of primers designed on the basis of sequence data specifically to amplify chosen taxa, SSP may potentially identify samples based on a single PCR assay. This study addressed the identification of nine calliphorid species and two species complexes commonly found in association with carrion in Australia and New Zealand. Primers were designed based on 1167bp of COI sequence data, and optimised for the development of a key to identification based on the simple presence and absence of products of expected size with seven specific primers. This is the first SSP assay published for use with the calliphorids, and represents a novel approach to increasing the accuracy and efficiency of identification and subsequent post-mortem interval (PMI) estimation.

Keywords
Forensic entomology; calliphorid; diagnostic assay; SSP
Introduction

Research in forensic entomology frequently addresses issues in the identification of insects collected from crime scenes, particularly identification of the various life stages of the Calliphoridae (blowflies). Calliphorids are generally the first insects to locate a body following death, and are therefore the most frequently encountered and most useful members of the corpse assemblage in the estimation of the post-mortem interval (PMI). Critical to this process is the correct identification of the calliphorids collected, and the traditional morphological approach is hindered by lack of larval keys, poor preservation of specimens and inability to rear through to adult stage for more reliable distinction.

Sukontason et al. (2004) have promoted staining techniques in the identification of calliphorid eggs, allozymes were used by Wallman & Adams (2001) in association with calliphorid larvae adults, and Figarola et al. (2001) suggested the use of antibody-based enzyme-linked immunosorbent assays (ELISA) for identification of the calliphorid Cochliomyia hominivorax. These techniques have largely been overshadowed by the benefits of the polymerase chain reaction (PCR) and its ability to provide identification from small amounts of poorly preserved tissue from any life stage.

Molecular-based identification has become an important facet of forensic entomology, largely utilising sequencing of the mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene. Numerous authors have sequenced this gene to gather data for sequence-based identification, often from phylogenetic inference (Sperling & Wells, 1994; Malgorn & Coquoz, 1999; Harvey et al., 2003a, b, Harvey et al., Chapter 4). Sequencing provides data of high information content that can be analysed using phylogenetic analysis, a convenient means to identify unknown individuals by comparison to a database of known individuals. Sequencing is expensive however, and depending on the facilities available at an institution, may be time-consuming where sequencing reactions cannot be completed on-site. Many authors have proposed alternate approaches to identification in an effort to circumvent the expense, workload and time involved in sequencing.

DNA-based assays for use with calliphorids have commonly utilised a PCR-based restriction fragment length polymorphism (PCR-RFLP) approach, identifying suitable
enzymes from sequence data and using profiles of cleaved fragments to identify species (Narang & Degrugillier, 1995; Taylor et al., 1996; Sperling & Wells, 1994; Malgorn & Coquoz, 1999; Ratcliffe et al., 2003; Schroeder et al., 2003; Ames et al., 2006). This approach is useful, but may require an array of expensive enzymes run under different conditions to identify an insect where numerous species are present in a locality. Random amplified polymorphic DNA (RAPD) is a useful technique for amplifying individuals for which little or no sequence data has previously been gathered, and was suggested by Benecke (1998) as a tool for identification of calliphorids. The use of RAPDs has been questioned for the reproducibility and reliability across labs of species-specific profiles, and the technique suffers from some inherent problems. These include the appearance of novel bands complicating analysis, difficulty in identifying homologous loci (Palumbi, 1996) and the co-migration of fragments complicating scoring (Hoy, 1994).

Looking to other insect taxa, there have been some useful techniques employed for molecular-based identification. Culicid studies addressing the development of diagnostic assays are plentiful, given the medical and veterinary significance of the mosquitoes. Sequence-specific priming (SSP) techniques are particularly useful and several studies have reported the successful use of these with mosquito taxa (Manonmani et al., 2001; Fettene et al., 2002; Kampen et al., 2003; Phuc et al., 2003; Noel et al., 2004). SSP involves the development of sequence-specific primers designed from sequence data to amplify a particular taxon, allowing a rapid, PCR-based assay for distinction based on the presence or absence of a fragment of expected size. This capitalises on the data gathered from sequencing studies while improving the efficiency of the identification process.

This study considered the identification of a number of species of forensic significance from Australia and New Zealand. Seven primers were selected for their potential to identify eight individual species and three species complexes based on a PCR matrix. The optimised assay is described, along with examples of the application of this technique to unknown individuals in a blind test to illustrate its utility in interspecific distinction.
**Materials and Methods**

Sequence data for 1167bp of the COI gene gathered in Harvey *et al.* (Chapter 4) was viewed using the phylogenetic program MEGA 3.0 (Kumar *et al.*, 2001). Specific substitutions that distinguished groups of individuals were chosen as the 3’ position of priming sites, and primers designed.

Fly DNA was obtained from adult specimens. DNA was obtained from the flight muscles of the specimens using a DNEasy Tissue Kit (Qiagen) according to manufacturer’s instructions. These individuals had been previously sequenced over the COI gene to confirm their species affinity.

Primers were optimised, and the final PCR reaction mix consisted of: 1 X PCR buffer (Fisher Biotec), 200µM dNTPs (Fisher Biotec), 1.5mM MgCl₂, 25pM each primer, 1 unit of Taq polymerase (Fisher Biotec), 5µl 5% BSA, 10-150ng of template DNA, and water added to a total volume of 50µL. Reactions were performed on a BioRad iCycler. Cycling conditions were: 90 seconds 94°C denaturation, followed by 36 cycles of: 94°C for 22 seconds, variable annealing temperature (see Table 2) for 30 seconds and 72°C for 1 minute 20 seconds. A final extension period of 1 minute at 72°C was used, followed by holding at 4°C. Products were visualised using 2% agarose gels with ethidium bromide staining and UV transillumination.

Thirteen species specific reverse primers were designed to be used in combination with the forward COI primer C1-J-1718 (Simon *et al.*, 1990). Of these thirteen primers, four were modified with the addition of a second mismatched base at the 3’ end of the primer to minimise annealing and subsequent extension in non-target species. Seven primers were selected for their ability to distinguish between species, and their sequences are shown in Table 7.2.
Results

The design and optimisation of primers resulted in the modification of several primers from their initial form, and the rejection of others for their unsuitability in providing distinction between taxa. A total of 22 primers were tested, and seven chosen for their suitability in distinction. The identification of an individual using this technique is therefore based upon nine reactions, inclusive of negative and positive controls.

Annealing temperatures varied from primer to primer, despite attempts to match them closely during primer design. Adjustments were required to obtain desired specificity. The matrix of required annealing temperatures, expected product sizes and key for diagnosis of species identity are displayed in Table 7.3. The addition of BSA significantly increased specificity of the primers.

Closely related species such as *Calliphora hilli* and *C. varifrons* proved difficult to separate, given the low number of substitutions available for primer design. The design of primers using the COI gene is complicated by the relatively high A+T content, significantly lowering the expected melting temperature ($T_m$) of some primers.

The multiplexing of reactions to include an internal control for each reaction proved difficult, and therefore the assay was limited to the test primers for their specificity and distinctive ability. Figures 7.2 and 7.3 display examples of assays conducted for an individual of *C. dubia*, and an individual of *Ch.megacephala/Ch.saffranea*, showing the ability to use the matrix to reach an identification based on positive and negative results.
Discussion
This study illustrates the potential use of the SSP technique in forensic entomology. The ability to distinguish eight species and three complexes based on ten PCR reactions eliminates the need for subsequent sequencing or restriction with enzymes, both popular techniques in interspecific distinction.

It should be noted that some reactions were shown during optimisation to amplify a visible product in reactions designated as expected negatives in the matrix (Table 7.3). It is important to note from this table the expected product size- in situations where non-specific amplification was recorded this was most often amplification of a much smaller fragment than the target band and therefore not the required product. This problem was generally resolved during optimisation, however product size should always be checked to ensure the amplified product is most likely the desired target.

An obvious limitation is the inability to distinguish between species pairings in the three complexes in this study. *Calliphora stygia* and *C. albifrontalis* are unable to be reliably distinguished based on the COI gene (Harvey *et al.*, Chapter 4) and therefore a secondary assay is required for their distinction. Harvey (unpublished data) has shown the potential for distinction based on the nuclear ribosomal DNA spacer regions, ITS1 and ITS2. Sequencing of these regions may be a useful secondary assay where an individual is identified to the *C. stygia/C. albifrontalis* complex using the current SSP assay.

*Chrysomya megacephala* and *Ch. saffranea* are able to be distinguished based on COI data, however some Malaysian *Ch. megacephala* individuals have shown greater affinity to *Ch. saffranea* in COI analyses (Harvey *et al.*, Chapter 4). Consequently, it was decided in this study to provide a conservative identification to a complex of these species, and overcome any potential error from taxonomic issues between these species. Harvey *et al.* (Chapter 4) also showed the distinct presence of haplotypes within *L. cuprina*, seemingly representing the subspecies *L. c. cuprina* and *L. c. dorsalis*. Given the greater similarity of *L. c. cuprina* to *L. sericata* based on COI data, identification of *L. cuprina* as a single
species within this assay was difficult. These species were therefore treated as a complex, with distinction possible based on sequencing of the COI gene as in Harvey et al. (2003b).

An important component of an SSP assay is the incorporation of an internal PCR control to each reaction. This will be a fragment that amplifies to confirm the viability of the DNA and other reagents. While a positive control has been incorporated as a ninth reaction in this assay, an internal control in each reaction is more useful. This will involve multiplexing, and this study sought to propose and verify the utility of specific primers before optimisation of a multiplex reaction. In this way, the SSP technique may be accepted by others and developed for their own locality specific calliphorid taxa. In addition, multiplex reactions may be developed using multiple SSP primers to reduce the overall number of reactions required, so that the currently required 10 reactions (inclusive of controls) may be reduced to 5 or less, ideally.

An obvious limitation in any sequence-based diagnostic assay, including RFLP and SSP, is the potential for misidentification based on nucleotide substitution. Substitutions within a priming site may result in the non-recognition of the target site, affecting the result. Under SSP, this necessitates the collection of intraspecific data to ensure priming sites remain applicable. The selection of an appropriate DNA region upon which to base the assay is also vital, and in this study the COI gene was chosen both for the large body of sequence data already gathered over this region, but also the highly conserved nature of the gene, yet with scope for distinction.

This study illustrates the potential for molecular identification of forensically important calliphorids based on a PCR assay. While this provides a rapid identification of species, sequencing remains the definitive method of identification and is an important means of confirmation. This SSP assay does, however, provide a new method for the efficient and accurate identification of forensically significant calliphorids.
Table 7.2 Primer sequences for ten sequence specific primers.

<table>
<thead>
<tr>
<th>Primer Name and Position Number</th>
<th>Sequence (5’-3’)</th>
<th>Size of Expected Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSP1 (217)</td>
<td>GGTATTCGGTCAAAAGTTACA</td>
<td>217</td>
</tr>
<tr>
<td>SSP2B (456)</td>
<td>ATTCTTGRCTAAATAATATGTG</td>
<td>456</td>
</tr>
<tr>
<td>SSP3 (1045)</td>
<td>CAATWGAAATWGAATTACG</td>
<td>1045</td>
</tr>
<tr>
<td>SSP4B (523)</td>
<td>TCCTAATARACCAATAGCTAGG</td>
<td>523</td>
</tr>
<tr>
<td>SSP5B (1101)</td>
<td>CTAAAACTTTCTCAAAYAATAC</td>
<td>1101</td>
</tr>
<tr>
<td>SSP10 (247)</td>
<td>GCAGTAATAACTACAGATCAT</td>
<td>247</td>
</tr>
<tr>
<td>SSP12 (700)</td>
<td>CCTAAAGCTCATAAAGTAGCA</td>
<td>700</td>
</tr>
<tr>
<td>SSP13 (580)</td>
<td>GCTCGAGTATCTACATCTATA</td>
<td>580</td>
</tr>
</tbody>
</table>
### Table 7.3 Matrix of PCR products expected for identification*

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2B</th>
<th>3</th>
<th>5B</th>
<th>10</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td>52</td>
<td>54</td>
<td>58</td>
<td>54</td>
<td>58</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td><strong>Product Size (bp)</strong></td>
<td>217</td>
<td>456</td>
<td>1045</td>
<td>1101</td>
<td>247</td>
<td>700</td>
<td>580</td>
</tr>
<tr>
<td>C. dubia</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ch. megacephala/Ch. saffranea</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>L. sericata/L. cuprina</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>C. albifrontalis/C. stygia</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Ch. rufifacies</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. hilli</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. varifrons</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch. varipes</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. ochracea</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. vicina</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. augur</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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</tbody>
</table>

*An X denotes a positive product of the specified fragment size*
Figure 7.2 PCR matrix for identification of *C. dubia*. The DNA ladder is indicated in the first lane, then SSP primers in ascending numerical order labelled on the gel, and negative control and positive controls (C1-J-1718 and TL2-N-3014) marked. Positive results of the correct target size are shown with primers 1, 2B, 10, 12 & 13.
**Figure 7.3** PCR matrix for identification of *Ch. megacephala/Ch.saffranea*. The DNA ladder is indicated in the first lane, then SSP primers in ascending numerical order labelled on the gel, and negative control and positive controls (C1-J-1718 and TL2-N-3014) marked. Positive results of the correct target size are show with primers 2B, 10, 12 & 13.
GENERAL DISCUSSION

This chapter aimed to address the improved processing of forensic entomological samples. A new method was proposed for the extraction, storage and transport of DNA from entomological samples, and DNA sequence data compiled in previous chapters was developed into a preliminary assay for the rapid identification of forensically important calliphorids of Australia and New Zealand.

Whatman FTA cards were optimised for the successful extraction of high quality DNA suitable for amplification of fragments of at least 1200bp in size. These cards provide a useful method for fast DNA extraction, the transport of samples between labs without the need for refrigeration, and samples may be stored long-term at room temperature. This reduces the chance of desiccation of samples and maintains template suitable for use in the event of re-analysis being requested. Further testing of the cards later in time along with exposure to a variety of different storage conditions and treatments may prove useful in confirming their utility.

The SSP assay proposed, though in its preliminary stages, represents a useful alternative to the currently utilised sequencing. RFLPs have also been suggested, and hold potential for use in identification. SSPs provide a simple, PCR-based assay negating the need for a subsequent digestion step and the use of potentially expensive enzymes, however there are many variables that may impact the success of such assays. SSPs obviously require development with species encountered in each locality, sequencing of a large number of conspecific individuals to ensure the robustness of markers selected, and optimisation on individual thermocyclers to adequately assess the variability encountered between different laboratories and equipment.

Extraction using FTA cards, combined with an SSP approach to identification provide a useful methodology for the molecular-based analysis of calliphorids. It is anticipated that these techniques will be applicable to forensic entomology worldwide, and will greatly enhance the accuracy and efficiency of this field.
CHAPTER 8:
CONCLUSIONS
The main objective of this thesis was to study the forensically important calliphorids using molecular techniques. This included evaluation of current applications of DNA-based techniques to calliphorid identification and make basic inferences relating to species status of common taxa. In considering the limitations and issues currently facing molecular identification, new approaches were made to overcome these difficulties and extend the utility and applications of current calliphorid identification studies.

The cytochrome oxidase I (COI) gene has been widely utilised in identification of forensically significant calliphorid species (Sperling et al., 1994; Malgorn & Coquoz, 1999; Harvey et al., 2003a), and in this study has been confirmed to have great utility in interspecific distinction. This thesis presents a thorough global study of forensic calliphorids using COI sequence data, showing the highly conserved nature of the region on an interspecific level. This low variation makes the COI useful in studying allopatric populations of calliphorid taxa, and provides a robust basis for the development of diagnostic assays based on COI markers likely to be constant regardless of location.

The low interspecific variability of the COI was exploited in identifying haplotypes of Chrysomya rufifacies, potentially representing cryptic species. The sub-species of Lucilia cuprina, L. c. cuprina and L. c. dorsalis were also supported based on the COI gene. The COI gene was therefore shown to be potentially informative at the cryptic species level. Furthermore, this study also questioned the ability of the COI to gain population level variability, and it can be concluded that limited population level variation is observed over this region.

The COI gene was unable to distinguish complexes of Calliphora stygia/C. albifrontalis and Ch. megacephala/Ch. saffranea, perhaps a result of recent divergence or gene choice. This raises issues as to its utility with a limited group of closely related species, and necessitated the evaluation and adoption of an alternate region of DNA.
Investigation into the utility of the nuclear rDNA spacers (ITS1 and ITS2) and mitochondrial control region aimed to evaluate the suitability of these non-coding regions in addressing issues unresolved from the COI gene. The mitochondrial control region proved difficult to amplify and sequence, making it a problematic target for routine application. Over a small area of this region, limited interspecific variability was observed and intraspecific variability representative of population affinity was not identified. The ribosomal spacers, however, were able to distinguish between closely related species, displaying numerous indels and substitutions. On an intraspecific level, there was very limited variability over these spacers within Ch. rufifacies, a preliminary indication that the desired ability to characterise populations based on sequence data may not be possible based on these non-coding areas.

The limited data collected over these non-coding regions prohibits any strong conclusions relating to the comparative evolutionary rates of mitochondrial and nuclear DNA. It is interesting to note, however, that strong interspecific distinction was observable in a coding mitochondrial gene (COI) and a non-coding nuclear region, but not observed between two species over a non-coding mitochondrial gene. The collection of further calliphorid sequence data over these DNA regions may contribute to a greater understanding of the evolutionary rates of coding vs. non-coding and mitochondrial vs. nuclear DNA regions.

Despite difficulty in distinguishing a limited number of calliphorid complexes, the COI gene provides the most useful DNA region studied to date for the identification of most species. While the rDNA spacers appear to hold value for insight into some closely related species, the COI gene has proved useful in many localities and on a global scale has importance in revealing relationships between species and forming inferences about their status. It is proposed from this thesis that the COI gene should remain the gene used routinely in the molecular identification of calliphorids in forensic entomology.

The application of COI DNA sequence data was illustrated in Chapter 6, where case studies clearly showed the importance of this data in identification of immature specimens collected from actual cases. Furthermore, the importance of molecular techniques for calliphorid identification have been extended in this thesis to puparia.
The use of puparia extends the utility of forensic entomology over a longer period following death, and in application to museum and historical samples represents a novel foray into molecular palaeoentomology involving calliphorids.

The collection of data for the identification of 27 calliphorid species has culminated in the development of a sequence specific priming (SSP) technique for the Australian and New Zealand calliphorid corpse fauna. In combination with the Whatman FTA technique optimised in Chapter 7, this provides a useful new approach to the more efficient and accurate identification of forensically important calliphorids. The adoption of this technique to the locality-specific fauna of areas currently utilising forensic entomology holds value for simple PCR-based identification and overcomes other time-consuming and expensive approaches.

The future of molecular-based identification depends on the accumulation of further data for the consideration of the status of some species of widespread importance. The COI gene will contribute significantly to assessment of species and their relationships. In seeking intraspecific variation in these calliphorid taxa, microsatellites or single nucleotide polymorphisms (SNPs) may hold greater value than the relatively conserved regions studied in this thesis. However, taxonomic status will only be validly confirmed where a combined morphological, molecular and biological approach is adopted.

This thesis has addressed the molecular-based identification of forensically important calliphorids. The inferences about species status and new applications proposed in this study have contributed greatly to the understanding of the relationships between forensic taxa, their status, and their application in forensic entomology, while considering the value of alternate DNA regions to provide greater clarity to the field. The study represents a rare consideration of molecular forensic entomology on a global scale, addressing issues fundamental to the use of calliphorids in PMI estimation. It is anticipated that this thesis and its individual studies will have significant implications for the future of the molecular-based identification of calliphorids, contributing to increased accuracy and efficiency in the estimation of post-mortem interval.


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APPENDICES
APPENDIX 1:
Isolation and detection of ingested DNA from the immature stages of
Calliphora dubia
(Diptera: Calliphoridae)
Abstract
The forensic entomologist frequently bases time since death estimation on fly larvae. In some cases the food source on which these larvae have completed their development may be questionable, and requires verification to ensure the accuracy of estimation. Ingested DNA may be isolated from the alimentary canal of immature insects to determine the nature of alimentary contents. Previous studies have confirmed the ability to extract ingested DNA from third instar blowfly larvae. This study considered the potential to detect ingested DNA from immature stages of the blue bodied blowfly *Calliphora dubia* (Macquart) that had fed on sheep liver. Individuals from early first instar larvae through until day three pupae were decontaminated, and DNA isolation and detection trialled by amplification of the sheep satellite I region. Fragments of 197bp and 87bp were successfully isolated and detected in all ages through until day two pupae, with detection unsuccessful on day three of the pupal stage. This study presents a suitable protocol for the isolation and detection of ingested DNA from immature stages of *C. dubia*.
Introduction

Forensic entomology is most frequently recognised in the medicolegal context for its utility in the estimation of minimum time since death, referred to as postmortem interval (PMI). This estimation is frequently based upon the minimum age of the fly larvae recovered from the corpse, as immature fly stages are the insects most commonly collected from a corpse (Castner 2001). It is the Calliphoridae, or blowflies, that are generally first to colonise the corpse. Their larvae often represent the oldest larvae to have developed on the body, and the oldest larvae that can be utilised in the estimation of the PMI (Schoenly et al. 1996).

Estimation of PMI based upon the age of larvae collected from a corpse assumes that these larvae have developed entirely on this resource. Generally, other signs of decomposition, on and around the corpse, will justify this assumption (Wells et al. 2001). In some cases however, other fly attracting substrates are present in the vicinity of the corpse, raising questions as to the resource on which individuals may have completed their development.

Situations where host confirmation is necessary include the discovery of a corpse in a garbage disposal area where other food scraps are present, or a corpse located in a bushland area where alternate animal carcasses may provide suitable development sites for fly larvae. Larvae may have fed on food scraps or other carcasses before moving onto the corpse. Larvae may leave a resource either naturally, that being undisturbed, for example when they have ceased to feed and move away in search of sites for pupation. Alternatively, they may be disturbed on their original food source and move elsewhere. Estimating PMI based upon the oldest larvae at a scene may therefore be flawed, as such larvae may have developed upon a substrate external to the corpse.

Consequently, it is necessary for the forensic entomologist to have methods suitable for confirming the food source on which larvae have completed their development, particularly in situations where this may be disputed. Material present in the digestive system of the insect must be identified.
DNA, a molecule which is present in all biological fluids, is more resistant to environmental degradation than other biological molecules such as proteins (Benecke & Wells 2001) and therefore makes a suitable subject for use in host detection. Molecular biological techniques have been used to identify the sources of blood meals consumed by hematophagous insects such as mosquitoes (Coulson et al. 1990; Gokool et al. 1993; Kreife & Kempfer, 1999) and crab lice (Lord et al. 1998). Human DNA isolated from beetles that had fed on human skeletonised remains (Dizinno et al. 1995) and mosquito DNA present in the beetle alimentary canal (Zaidi et al. 1999) has also been identified.

Critical in host detection is the quality and quantity of host DNA present in the alimentary canal of the insect. Importantly, the isolation method used to obtain host DNA from the larvae should not result in further degradation of the DNA, and should provide a sufficient quantity of DNA which can be detected and used in subsequent molecular procedures. Following isolation, a detection method is required that is sensitive enough to detect low quantities of poor quality, partly degraded host DNA, yet specific enough to detect only host template and not the insect’s own DNA.

Polymerase chain reaction (PCR) is a useful technique for amplifying very low quantities of target DNA into larger amounts suitable for detection, and the advent of PCR has thus significantly enhanced the sensitivity and specificity of DNA detection (Lord et al. 1998). Wells et al. (2001) successfully isolated and amplified host DNA from third stage larvae of the blowfly Cynomyopsis cadaverina. Their study neglected, however, to consider the possibility of surface contamination of the larvae. Immatures may move across a surface without actually feeding, and thus it is vital to ensure any host DNA detected was in fact ingested by the insect and came from inside the alimentary canal. In a later study, Linville & Wells (2002), determined washing in 20% bleach prior to extraction to be a suitable decontamination method, an issue that will be discussed in this study.

This study focused on the blue bodied blowfly, Calliphora dubia (Macquart). This species is a primary species in Western Australia (Morris 1993), commonly found on bodies shortly following death. The females are larviparous, laying fully developed
first instar larvae. The study examined the potential for isolation and detection of host DNA from the alimentary canal throughout the immature stages of *Calliphora dubia*.

**Materials and Methods**

**Sampling and Preservation**

*Calliphora dubia* adults were obtained from a laboratory colony. Trays of fresh sheep liver with a few droplets of sheep blood were placed in cages of flies for one hour, and larvae were deposited on the liver. Sheep liver was used in this study due to the constraints involved in utilising human tissue. Following oviposition, 300-500 larvae were transferred onto a foam tray containing 1 gram of fresh sheep liver per larva. The foam tray was placed into a container one third filled with sand and covered with a mesh lid. The container was placed into a cabinet maintained at a constant temperature of 24°C and the liver was kept moist with a few sprays of water daily.

Approximately twenty larvae were sampled at each sample period, extending 11 days from oviposition until the third day of pupation. Sample details are displayed in Table I. Larvae were killed in boiling water then preserved in 70% ethanol and stored at 4°C. A small pin hole was made in pupal cases using a sterile pin prior to preservation. All specimens were boiled in 10ml of water and placed into 10 ml tubes of ethanol. These high volumes of boiling water and ethanol were used to dilute out surface contaminating DNA.

**Decontamination and DNA Isolation**

Carvalho (2003) trialled a number of techniques for the removal of surface contamination from the cuticle of fly larvae, and concluded, converse to Linville & Wells (2002), that water washing was the most successful technique. The DNA yielded in the subsequent isolation step was less degraded than DNA obtained following decontamination with bleach.

Each insect was placed into a 1.5 ml centrifuge tube containing high pure water. Volumes were determined according to the size of the specimen. For smaller specimens (first and second stage larvae) 500 ul of high pure water was used, while for larger specimens (third stage larvae, post-feeding larvae and pupae) 1 ml of high pure water was added. The tubes containing the smaller specimens were vortexed for
30 seconds and those containing larger specimens were vortexed for 1 minute. After vortexing the specimen was transferred into a fresh tube containing water and again vortexed. This was repeated three times.

A study of techniques for isolation of host DNA from the alimentary canal of immature fly stages (Carvalho 2003) found the phenol:chloroform technique to provide DNA of sufficient quality and quantity for subsequent detection using PCR. The Qiagen technique proved similarly successful, however phenol:chloroform was selected for its cost efficiency.

Treatment was according to larval size. For smaller larvae (first and second stage larvae) 500 ul volumes were used, and for the larger third stage feeding, post-feeding larvae and pupae, 1 ml volumes were used. One volume of TE buffer (pH 7.3) was added to a 1.5 ml centrifuge tube, 0.2 ug Proteinase K was added and the tubes incubated at 55 °C overnight. An equal volume of phenol was added to each tube and the tubes inverted prior to centrifuging at 11 000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a new centrifuge tube containing two volumes of chloroform. The tubes were inverted and centrifuged for 5 minutes. The supernatant was then transferred into new tubes containing two volumes of chloroform, inverted and centrifuged for 10 minutes. The supernatant was removed to new centrifuge tubes and an equal volume of ice-cold isopropanol added. The tubes were inverted and kept at – 20 °C overnight for DNA precipitation. The tubes were subsequently centrifuged for 10 minutes and the isopropanol removed. The pellet was washed with 70% ethanol and allowed to briefly air dry, and then resuspended in TE buffer (pH 7.3) varying between 10 ul and 100 ul, depending on the amount of original starting material.

**Amplification**

PCR was performed to amplify host DNA to a detectable level. The PCR protocols were optimised by Carvalho (2003). Two fragments were amplified to determine the level of degradation of the host DNA isolated. These fragments were 197 base pairs (bp) and 87bp respectively.
Sequences for primers are shown in Table II. Primer sequence OAST2 for sheep satellite I DNA was obtained from Chikuni et al. (1994). Primer OAST3 was designed to anneal four bases downstream of OAST1 from Chikuni et al. (1994) to overcome a problem with non-specific binding. Primers Int1 and Int2 were designed to bind internal to the region amplified by OAST3 and OAST2 primers, thus amplifying a smaller region of sheep satellite I DNA. Primers S1 and S2 were designed internal to Int1 and Int2 primers. All sheep primers were tested against fly DNA to ensure specificity during amplification of host DNA.

External primers OAST3 and OAST2 amplify a product of 370 bp. Internal primers Int1 and Int2 produce a band of 197 bp, and S1 and S2 primers amplify a product 87 bp in length.

197bp fragment

Nested PCR was carried out with a final volume of 10 ul, with 1 ul of DNA template and 9 ul of PCR master mix containing dinucleotide triphosphates (dNTPs), MgCl₂, forward and reverse primers and PlatinumTaq Polymerase™ (Gibco). The PCR master mix was made up as 10 ml volumes containing 20 ul of 100 mM dCTP, dGTP, dTTP and dATP (Boehringer Manheim), 300 ul of 50 mM MgCl₂ (Gibco), 100 ul of 20 mg/ ml BSA (Gibco) and 8280 ul ultra pure water (Biotech). Platinum Taq Polymerase™ (5U/ul) was added to the PCR mix at a concentration of 10 ug/ml, along with 1ug/ml of OAST2 and OAST3, the external primers in the first round of PCR. In the second round, 3ul of 1ug/ml of each internal primer, Int1 and Int2 were used. PCR was carried out in the same PTC- 100 Programmable Thermal Controller (MJ, or equivalent). Negative controls containing no DNA were run parallel with each PCR, together with positive controls spiked with a sheep DNA extract.

The first round of PCR consisted of 95 °C for 5 minutes, followed by 25 cycles of 93 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute. In the second round, the annealing temperature was reduced to 50°C and only 20 cycles were performed. All reactions were stored at 4°C.

87bp fragment
Double standard PCR was performed using primers S1 and S2. PCR reaction mixes and cycling conditions were the same, however the second round of PCR was performed at an annealing temperature of 60°C, and 7µg/ml of each primer was used in each reaction.

Visualisation
The resulting products were electrophoresed at 30 or 40 volts for 2 to 3 hours on a 2% w/v agarose gel (NA Pharmacia) in TAE. Following electrophoresis, gels were stained in ethidium bromide and visualized under UV transillumination.

Results
The nested PCR successfully amplified ingested sheep DNA from a range of immatures. As figure I shows, sheep DNA was detected in all stages from the first larval sample (1.1) through to day 2 pupae (pu2). No sheep DNA was detected from day 3 pupae (pu3).

Double standard PCR was employed to amplify the ingested sheep DNA using newly designed primers S1 and S2 (Figure II). These primers were designed to amplify a smaller region than Int1 and Int2, and amplified the host DNA to detectable levels in all stages from the first larval sample (1.1) through until the second day pupae (pu2). Again, ingested host DNA was unable to be detected from the third day pupal samples (pu3).

![Figure I Detection of sheep DNA using nested PCR, with primers OAST2 and OAST3 in the first round, and Int1 and Int2 in the second round: Sheep DNA](image-url)
was detected in all fly stages except pu3. Lane 1: specimen 1.1; lane 2: specimen 1.2; lane 3: specimen 2.1; lane 4: specimen 3.1; lane 5: specimen 3.3; lane 6: specimen pre1; lane 7: specimen pre3; lane 8: specimen pu1; lane 9: specimen pu2; lane 10: specimen pu3; lane 11: negative water control; lane 12: positive sheep control.

Figure II Detection of a shorter sheep DNA fragment using double standard PCR and primers S1 and S2: Sheep DNA was detected in all stages except for pu3. Lane 1: specimen 1.1; lane 2: specimen 2.1; lane 3: specimen 3.2; lane 4: specimen pre1; lane 5: specimen pre3; lane 6: specimen pu2; lane 7: specimen pu3; lane 8: negative water control; lane 9: positive sheep control.

Discussion
A critical issue in the isolation of ingested DNA from insects is the removal of surface cells and DNA from the exterior of the individual. This important initial step affects the ability to detect host DNA, ensuring the detected DNA has actually been ingested, and also requiring that this decontamination step does not damage the ingested DNA by the entrance into the alimentary canal of reagents.

Linville & Wells (2002) and Carvalho (2003) both studied methods for surface decontamination. Carvalho’s success with the water washing technique was not shared by Linville & Wells (2002), presumably as a result of the increased amount of agitation enhancing the removal of contaminating cells from the insect surface in Carvalho’s method. Carvalho (2003) showed that the ability of isolated DNA to be amplified was affected by the pre-treatment bleach washes, perhaps attributable to the use of 70% bleach as compared to 20% used by the other authors. However, the water
washing technique is obviously preferable, eliminating any potential for additional artifactual degradation due to the bleach washes.

In this study, DNA was successfully isolated from the alimentary canal of insects. The isolation technique is obviously an important consideration as ingested DNA must be isolated in both sufficient quality and quantity suitable for amplification by PCR. Linville & Wells (2002) successfully isolated ingested DNA from an alimentary canal dissected from the insect. The authors indicate this to be advisable as the remainder of the insect may be preserved for taxonomic purposes, which is a valid point. However, dissection of small larvae can be a delicate and laborious task and requires considerable experience. It is therefore desirable that a chosen isolation method is able to provide accurate results when material other than the alimentary canal is used. A critical consideration in use of a larger amount of the insect tissue is the specificity of the PCR primers, to ensure only ingested DNA is targeted.

Ingested DNA was successfully detected from larvae that had only recently been deposited on sheep liver, through to pupae in their second day of this pupal lifestage. This result was found with fragments of both 197bp and 87bp, indicating that by the third day of pupal development the ingested DNA had obviously degraded to a size somewhat smaller than 87bp. The degree of degradation in these samples could be tested by design of new primers to amplify yet smaller fragments and could be combined with the use of a more sensitive detection technique such as Maldi-TOF.

Larvae will feed until their crops are fully distended and receptors in the wall of the alimentary canal signal for them to stop feeding (Davies and Friend, 1995). In third instar post-feeding larva, the crop downsizes dramatically. The alimentary canal of the fly larva contains digestive enzymes which breakdown its food materials into monomers (Greenberg 1991; Gullan & Cranston 1994). In addition, the larvae empties their alimentary canal contents through peristaltic constriction of the alimentary canal (Robertson 1936). Greenberg (1991) showed that the crop empties as fast as the larva ages during the first 40 hours after crop engorgement or fully fed larva. Greenberg (1991) also observed that the crop empties more slowly after these 40 hours until pupation takes place. This suggests that the crop, although visually empty still contains food materials. Therefore, this may provide an explanation as to
why host DNA is still present at detectable levels in the alimentary canal of pupae at day two (pu2). However by day three, the material has either been eliminated, degraded to pieces smaller than 87bp, or is perhaps present in such low copy number by this stage that the PCR is not sensitive enough to detect it.

This study clearly shows that ingested DNA can be detected in the immature stages of C. dubia through to the second day of pupal development. Design of new primers amplifying smaller fragments would perhaps allow detection in later stages of development. The decontamination, isolation and amplification methods developed in this study have provided a valuable method for the detection of host DNA in corpse-feeding larvae. This technique will facilitate verification of the resource on which individuals taken from a crime scene have developed, and contribute to the accuracy of forensic entomology as an investigative tool.

Acknowledgements
CFS, Curtin etc

References

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Table I: Sampling times and name given to each sample

<table>
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<th>Day</th>
<th>Insect stage</th>
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<td>5</td>
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<tr>
<td>2</td>
<td>2nd</td>
<td>23</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>3rd</td>
<td>47</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>3rd</td>
<td>72</td>
<td>3.2</td>
</tr>
<tr>
<td>5</td>
<td>3rd</td>
<td>95</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>Prepupa</td>
<td>119</td>
<td>Pre1</td>
</tr>
<tr>
<td>7</td>
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<td>143</td>
<td>Pre2</td>
</tr>
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</tr>
<tr>
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<td>Pu2</td>
</tr>
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<td>11</td>
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Table II Sequences for primers used for amplification and detection of host DNA

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>OAST3</td>
<td>5'-GGTGTAATTAGCCTCGAGAAGCCT-3' *</td>
</tr>
<tr>
<td>OAST2</td>
<td>5'-AAGCATGACATTGCTGCTAAGTTC-3'</td>
</tr>
<tr>
<td>Int1</td>
<td>5'-AACCTCCTGATTTTCTTGAG-3'</td>
</tr>
<tr>
<td>Int2</td>
<td>5'-TGGAGAGGAATCCTCAGGTT-3'</td>
</tr>
<tr>
<td>S1</td>
<td>5'-GGTCCCCATCGTAAGTCGAGAACA-3'</td>
</tr>
<tr>
<td>S2</td>
<td>5'-AGAGTCCCCCGAGCAAATCGAATG-3'</td>
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APPENDIX 2:
The Role of Invertebrates in Terrestrial Decomposition: Forensic Applications
Running title: Decomposition by invertebrates

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Summary/Abstract
Soil type is particularly important in the decomposition of buried carrion, and is affected by numerous variables such as climate, vegetation, soil temperature and moisture and drainage. As a consequence soil type affects the type of vegetation found in an area, which invariably affects the invertebrate fauna. This review shows the dearth of research on carrion-related soil invertebrates. Unfortunately numerous invertebrate groups have been neglected as possible predictors of post mortem interval; however the limited studies that have been published demonstrate their potential to help solve crimes.

Keywords
Invertebrates, arthropods, forensic, decomposition, taphonomy
The decomposing corpse provides an ephemeral, yet nutrient-rich substrate that can be inhabited by a wide variety of organisms. As the bacteria begin the processes of cell breakdown, fermentation and putrefaction, at the other end of the spectrum the large, scavenging animals begin to play a significant role in the consumption of the soft tissues of the corpse (Smith 1986). This in association with the smaller, more abundant invertebrate fauna are the major factors responsible for a large part of the decompositional process of the exposed corpse.

Carrion itself forms a lucrative substrate for invertebrate colonisation, however in outdoor situations the interface between this substrate and the underlying soil, and the soil itself, also form attractive environments for certain organisms. The seepage of nutrient-rich fluids into the soil beneath the corpse significantly alters the microenvironment, affecting the inhabitant fauna. The arthropod assemblage may thus be considered to be affected by and reflective of the decomposition of the corpse, and may therefore have some potential in contribution to forensic investigation (Bornemissza, 1957).

The invertebrate assemblage associated with carrion is composed mainly of insects (Smith, 1986). The role of insects in the decomposition process has become a well-recognised area of forensic investigation, with forensic entomology commonly employed in homicide investigations (Morris and Dadour 2005).

The use of invertebrates other than carrion-dwelling insects, particularly the soil fauna, has received little attention in a forensic context. Goff & Catts (1990) state that there are a wide variety of arthropods that may be found associated with a corpse, and may be indicative of the stage of decomposition, but not all are equally useful in estimation of PMI. This may be attributed to the lack of research on groups other than the most abundant carrion inhabitants, the flies and beetles.

The potential of invertebrates found in the soil beneath a corpse and how this might contribute to forensic investigations is considered in this chapter.
The Invertebrates

The invertebrates are a diverse group of organisms classified by the absence of a backbone in all taxa. The invertebrates include sponges, molluscs, a variety of worm groups and arthropods, and their enormous variability in morphology is reflected by their diversity in general biology and habitat.

Certainly the most diverse invertebrate group is the phylum Arthropoda. This phylum contains at least 750,000 species, greater than three times more species than all other described animal species together (Ruppert & Barnes, 1994). This group includes the crustaceans, insects, spiders, mites, scorpions, centipedes and millipedes, and includes species with both terrestrial and aquatic affinities.

Forensically, the insects have been the main invertebrate focus. A wide variety of insects are attracted to carrion and are responsible for the consumption of much of the soft tissues of a corpse, and are thus the most frequently encountered organisms on carrion. The ability of this group to locate and colonise carrion is exploited in the field of forensic entomology.

Forensic entomology

Forensic entomology is broadly defined as the interaction of insects and other arthropods with legal matters. The field includes a wide variety of applications, encompassing any situation that may involve an interaction between insects and other arthropods, and the law. The applications are generally categorised as urban, stored-product and medicolegal forensic entomology (Lord & Stevenson, 1986).

The urban aspect generally deals with the effect of insects on man-made structures or other facets of human society. This may include the infestation of buildings by arthropod pests (Hall, 1990), and the breeding of flies in livestock facilities (Hall, 2001). The stored-product facet concerns the infestation of stored commodities by insect pests, or domestic invasion of kitchen products by insects. This also encompasses the infestation of food sold by retailers to the public (Hall, 2001).

Undoubtedly the most widely recognised aspect of forensic entomology is the medicolegal aspect. Frequently featured as the main forensic investigative application...
of entomology in fiction, television and film, this is the area that the majority of research is directed towards. Applications of this facet are wide-ranging and include the investigation of cases involving neglect by carers, generally in the elderly or very young (Benecke, 2001), investigation of unexplained cases of death or the causation of traffic accidents as a result of insect involvement (Hall, 2001).

The most recognised applications of medicolegal entomology however, and perhaps forensic entomology as a whole, involve the estimate of postmortem interval (PMI) and circumstances surrounding a death.

**Succession**
The estimation of PMI is based upon knowledge of the locality-specific succession of insects occurring on a corpse following death. This predictable succession may be used, in conjunction with data describing the temperature-dependent developmental data for carrion frequenting insects, to estimate PMI based on the minimum amount of time required for insect development.

The use of successional data in estimation of PMI assumes that following death, an orderly and predictable succession of insect species occurs on a corpse. Insects are generally the first organisms to locate a body following death. The insects may be classified as adventive or incidental species, simply visiting by chance and having little forensic relevance (Goff & Catts, 1990); predators that may feed on other species that have already colonised the body, or necrophages that feed from the body itself and are most useful in estimation of PMI.

As the body progresses through the stages of decomposition, from flesh to bloat, decay, dry and skeletal stages, the odours emitted by the corpse change (Anderson, 2001), reflecting the physical changes in the body. The odours vary in attractiveness to different insects, and as the body decomposes and various resources are depleted, new insect types will colonise, being more suited to the current decompositional stage. These insect taxa reflect the physical changes in the body and are therefore predictable and useful in estimation of PMI.
Flies (Diptera) and beetles (Coleoptera) are the insects most frequently collected from corpses (Lord, 1990), and these are consequently the focus of most forensic invertebrate research and applications. The blowflies (Diptera: Calliphoridae) are usually the first insects to arrive following death. Female flies will deposit eggs or live larvae around orifices or wound sites on the corpse, and larvae will secrete enzymes and bacteria, facilitating consumption of the soft tissues of the corpse. Larvae will feed through three stages of growth (instars) each punctuated by the moulting of their size-restricting cuticle, enabling further growth. At the cessation of feeding, larvae will pupate in soil, clothing or beneath surrounding objects, and following a period of metamorphosis, the adult fly emerges. The empty pupal casings may persist in soil for many years.

The arrival of blowflies, and subsequently their larvae, is followed quickly by the arrival of the flesh flies (Diptera: Sarcophagidae), other carrion flies (diptera musciidae), and predaceous beetle species such as rove beetles (Coleoptera: Staphylinidae), carrion beetles (Silphidae), clown beetles (Histeridae), skin beetles (Dermestidae) and checkered beetles (Cleridae). A variety of other fly families may be found in association with the body, and hide beetles (Trogidae) and larvae of some of the above beetle groups may feed on carrion itself, often on remains of hair, skin and clothing in late decomposition (Smith 1986).

Current Research

Current research focuses largely on above ground successional studies, developmental rate studies, and other factors that impact on PMI estimation. DNA-based studies are becoming more common, facilitating the use of DNA for identification of insects and increased accuracy and efficiency (Harvey et al., 2003; Wallman & Donnellan, 2005). Studies have also concerned the possible characterisation of human DNA ingested by blowfly larvae (Carvalho et al, in press; Wells et al., 2001).

Deaths occur in a variety of situations, clothed, unclothed, the corpse may be wrapped following death (Goff, 1992), covered with vegetation, burned (Avila & Goff, 1998), submersed in water, hung or buried. Each of these circumstances alters the decompositional environment in some way, and defines the environment within which carrion-related organisms will interact.
While the body itself forms the primary decompositional site, the beneath it may be equally important. Corpses located in outdoor environments on a terrestrial surface create an interface within which soil fauna and carrion-dwelling organisms interact. The interactions in this zone are affected by soil type, vegetation, decomposition of the corpse and a variety of environmental factors. Apart from the work by Bornemissza (1957) and Lundt (1964) the succession of insects in this interface, and within the soil itself, has been largely overlooked in the literature and the forensic implications have yet to be considered.

**The Soil/Corpse Interface**

The decomposing corpse in a terrestrial environment alters the substrate beneath it. This initiates a series of changes in vegetation and fauna, beginning a succession of arthropods affected by the decomposing carrion above.

Bornemissza (1957) observed the greatest effect of the decomposing corpse on the soil beneath to occur during the black putrefaction and butyric fermentation stages. Fluid seepage contributes to development of a crust of hair, plant matter and the uppermost soil layer beneath the body. During fermentation, the decomposition fluids released from the body, along with the waste products excreted by the insects feeding on the body, combine to kill the plants beneath the body and the soil fauna, altering the microenvironment. Anderson & VanLaerhoven (1996) found that vegetation under, and around the body for 20-30cm was killed by fluids released during active and advanced decay stages, and number of arthropod species in the soil was reduced from 30 before carrion placement to two species, 15 days following placement in British Columbia.

During the decay stage the soil beneath the carrion may become disturbed to a depth of approximately one inch by the action of arthropods, particularly dipteran larvae, burrowing (Reed, 1958). Decomposition fluids and associated arthropods are reported to affect the soil to a depth of 14 cm, with most effect in the upper soil layers. The area directly beneath the body, the “carrion zone” serves as a decompositional zone occupied by carrion dwellers, distinct from a surrounding area of approximately 10cm, which provides an “intermediate zone” of both carrion, and regular soil-
dwelling invertebrates (Bornemissza, 1957). 10-20 cm away from the body, the soil fauna is typical of general litter dwelling fauna, but perhaps the size of these zones may be dependent on the size of the carrion, as Bornemissza’s work was based on guinea pigs and human decomposition may produce greater amounts of fluid.

Payne (1965) indicated that in South Carolina, once carrion reached the dry stage of decomposition, there began an overlap of carrion and soil-dwelling insects on and around the corpse. Bornemissza (1957) recorded that one year following the placement of carrion, the soil arthropod assemblage had not yet returned to its pre-decompositional state. The soil-surface and litter dwelling arthropods took longer to recolonise, as compared to subterranean taxa. The crust formed by hair, vegetation and fluids persisted long after placement, and was most pronounced beneath oral and anal regions where seepage is greatest. Restoration of the soil community occurred only following heavy rains. Anderson & VanLaerhoven (1996) similarly observed that 271 days following death, the vegetation and soil fauna had not returned to normal. This alteration to the soil may perhaps be informative in PMI estimation some time following death.

A variety of insects associated with soil beneath a corpse are discussed herein. The presence of many of these invertebrates is recorded for a variety of locations, but the timing of their arrival and departure varies markedly. This may be attributed to edaphic and climatic factors, or simply to the particular species involved at a location. The discussion is centred around each type of organism in a more generalised sense and variation between geographic localities is inevitable.

Class Arachnida

Mites (Acari)

Mites are commonly associated with soil under the corpse in late decomposition stages (Goff & Catts, 1990, p.43; Smith, 1986, p.165; Payne, 1965; Johnson, 1975). Anderson & VanLaerhoven (1996) recorded mites as the most abundant invertebrates beneath surface carrion.

Goff and Catts (1990) indicate several mite groups of significance. The Macrochelidae are phoretic on beetles, including the sylphid *Necrophorus* species,
they attach themselves to flies and are transported to the body in early decomposition. They leave the beetle, and may be found feeding mostly on fly eggs (Gibbs & Stanton, 2001). Anderson & VanLaerhoven (1996) observed macrochelid abundance in soil to increase over time in the presence of carrion, and Reed (1958) similarly observed a great abundance around carrion.

The soil-dwelling mites of the Cunaxidae are predatory mites living in the soil beneath remains, feeding on insects and their larvae associated with the remains. The Winterschmidtiiidae and Acaridae may be found towards the end of decay, feeding on fungus and detritus (Goff & Catts, 1990). Bornemissza (1957) also recorded the activity in late decay of tyroglyphid mites consuming any remaining skin and completely skeletonising remains.

The presence of particular mite groups varies markedly with location. Bornemissza’s (1957) study in Perth, Australia found gamasid mites during active decay and as the remains began to dry, with tyroglyphid mites following in the dry stage. He cites Kühnelt however, for the presence of mites only in the final stages of decomposition in Europe. This may be indicative of geographical behavioural variation, caused by climatic or edaphic factors.

**Spiders (Araneae)**

Spiders may be collected from remains where they may be predaceous upon other corpse-inhabiting arthropods (Goff & Catts, 1990; Smith, 1986; Reed 1958). They may be encountered beneath carrion capturing prey, but as general predators with unpredictable arrival time at carrion are unlikely to be useful in estimation of PMI.

**Millipedes**

Millipedes are commonly found in the moist environment beneath a body (Byrd & Castner, 2001; Goff & Catts, 1990). They are generally recognised as being plant feeders, but have been reported to frequent carrion in many studies, generally in the dry stage of decomposition. Smith (1986) cites several studies as reporting the presence of *Cambala annulata* (Say) during active decay.

**Class Insecta**
Springtails (Collembola)
Springtails are small insects commonly recognised by their springing behaviour when disturbed in leaf litter or soil. They are considered to be mainly plant feeding with some species consuming decomposing animal material (Goff & Catts, 1990) in damp soil and leaf litter habitats. The environment created by the seepage of fluids from the decomposing corpse provides springtails with a damp and nutrient-rich habitat.

During active decay, however, the seepage of large amounts of decomposition products into the soil may alter springtail numbers. In Perth, Australia, Bornemissza (1957) recorded significantly lower numbers during active decay when fluids were being released, and during the formation of a crust beneath the body. The subterranean springtails were entirely absent. Anderson & VanLaerhoven (1996) reported the return of springtails to the soil beneath carrion 94 days following placement of the body in British Columbia, with populations increasing to higher abundances by day 111 than prior to placement of the carrion.

Springtails have also been recorded from graves, and surface exposed carrion in other localities, but are often herbivorous species which appear to have little relevance in estimation of PMI.

Silverfish (Thysanura)
The silverfish, commonly found as household and stored-product pests, may be found in association with remains in the drier stages of late decomposition (Goff & Catts, 1990).

Cockroaches (Blattodea)
Cockroaches may be found feeding upon the decomposing remains (Goff & Catts, 1990; Payne, 1965).

Ants (Hymenoptera)
Ants are frequently associated with carrion, but their significance is difficult to determine. They are often located living within the soil beneath or close to surface carrion, making it unclear whether they are simply adventive, or may actually be predictable members of the succession beneath carrion.
Bornemissza (1957) recorded ants from soil beneath the carrion feeding on the carrion itself. Similarly, Payne (1965) observed ants feeding around body orifices, and recorded their greatest activity to occur during night time hours. Ants are often responsible for the removal of immature insects from carrion, particularly fly larvae (Johnson, 1975). It is suggested by Fuller (1934) however, that the presence of ants is not predictable, as carrion equally proximate to ant nests in her study were not always colonised. Ants are therefore likely to be adventive, and while seasonality and nesting sites may be informative in PMI estimation, their arrival at carrion may not be overly useful in estimation of PMI.

**Earwigs (Dermaptera)**

Earwigs, inhabiting the soil beneath carrion, have been reported to feed on carrion (Bornemissza, 1957).

**Flies (Diptera)**

Following the cessation of feeding in the third larval stage, fly larvae will leave the body and pupate in soil, clothing or under nearby objects. Bornemissza (1957) noted that the soil structure beneath a corpse was significantly altered by the activity of the burrowing larvae, as well as other carrion-related insects. The discovery of larvae, pupae or empty puparia may be important in the estimation of PMI, given relevant sets of developmental data for significant dipteran species.

The piophilid, or cheese-skipper species *Piophila casei* is reported to pupate in soil around carrion after cessation of feeding (Fuller, 1934) and thus may be encountered.

**Beetles (Coleoptera)**

Beetles are important members of the carrion assemblage, useful in estimation of PMI. They are often voracious predators of dipteran larvae on carrion, and arrive following colonisation by the larvae during the fresh stage with numbers increasing during bloat (Johnson, 1975). Once the initial necrophages have left the corpse, PMI estimation may require use of other taxa that persist beyond soft tissue consumption at a crime scene, such as the Coleoptera (Kulshrestha & Satpathy, 2001). A number of
studies have specifically focused on the carrion-frequenting Coleoptera (e.g. Shubeck, 1970; Easton, 1965; Kauffman, 1937a, b, c).

The Silphidae, or carrion beetles possess a variety of life histories, from development in decomposing vegetation to predation, and a number of species have been observed to feed on decomposing animal material (Byrd & Castner, 2001; Leblanc & Strongman, 2002). The burying beetles of the genus *Necrophorus* capture and bury small animals, which they subsequently lay their eggs upon. Steele (1927) reported *Necrophorus orbicollis*, *N. tomentosus*, *Silpha americana* and *S. novaboracensis* all to prey upon dipteran larvae on carrion. Other species will make impressions in decomposing flesh and lay their eggs in these impressions; however it is the *Necrophorus* species that will primarily be located within the soil. They may be encountered in leaf litter beneath, and around the body.

Putman (1978) observed *Necrophorus* species frequenting rodent carcasses in England, with the greatest number during summer and autumn. The author notes, however, that the carrion was rarely buried by the beetles, with burials attempted, but not completed. Adults were found on carcasses as early as 24 hours following placement, presumably preying upon blowfly larvae. Reed (1958) similarly observed no burials by *Necrophorus* spp., but did encounter them beneath carrion in Tennessee.

The Staphylinidae, or rove beetles, may be found in the surface layer of soil. They are voracious maggot predators, actively chasing maggots below the soil surface (Smith, 1986). These beetles may arrive shortly following colonisation of the corpse by blowfly larvae, with peak numbers under and around the corpse observed just prior to, and at the time of larval migration from the corpse (Putman, 1978).

Clown beetles, of the Histeridae, are predators in both adult and larval stages, preying predominantly on fly larvae. They may be found in upper soil layers, and are found as predators on the body between bloat to dry stages of decomposition (Byrd & Castner, 2001). Payne & King (1969) record the occurrence of these beetles in the soil/carrion interface during daylight hours, with feeding activity occurring at night. The Cleridae (checkered beetles) are similarly predators in larval and adult stages and may be associated with upper soil layers.
The Scarabaeidae (scarab or dung beetles) of the subfamily Scarabaeinae, and also the Geotrupidae (also dung beetles) frequent dung or carrion, which is generally rolled into a ball and eggs laid in the ball prior to burial (Byrd & Castner, 2001). The egg hatches and larvae develop on this nutrient-rich ball, and may therefore be found in the soil beneath a body. The scarab and dung beetles construct tunnels in soil beneath carrion (Smith, 1986; Payne & King, 1969).

Payne (1965) observed adult scarab beetles arriving as early as the bloat stage in South Carolina. During nocturnal hours they emerged from soil and fed on carrion, remaining in tunnels they constructed beneath carrion during the daylight hours.

A variety of other beetle groups may be associated with carrion. Payne & King (1969) record the occurrence of Hydrophilidae underneath surface carrion. In addition, many beetle groups usually found dwelling in leaf litter or other decomposing organic matter have been collected from surface carrion. Leiodid beetles, usually found in decaying vegetative matter, were found on carrion, as were the fungus-feeding orthoperids and the vegetation-dwelling anthicids (Payne & King, 1969). Reed (1958) also recorded the presence of Leptodiridae species, frequently recovered from decomposing vegetative matter, gathering around body openings, underneath carrion or in nearby leaf litter. In the same study, Trogidae species, both adult and immature, were observed in soil under carrion.

**Deeper down: Invertebrates on Buried Bodies**

The burial of a body alters the decomposition process significantly from surface decomposition, particularly with relation to scavenging, temperature and insect colonisation (Fiedler & Graw, 2003). The insects generally colonising a body laid on the surface are inhibited, as are the airborne bacteria (Smith, 1986). The depth, soil type and nature of the burial are obviously important factors (Lundt, 1964).

The most important effect of burial on decomposition is the increase in time required for biomass reduction, relative to exposed carrion (Smith, 1986). Smith (1986) suggests blowflies, responsible for the majority of biomass reduction on carrion, are excluded from the corpse at a depth of just 2.5cm. However, Simpson & Strongman
(2002) report the occurrence of the blowfly *Cynomyopsis cadaverina* on carrion buried at a depth of 30cm. Rodriguez & Bass (1985) also observed Sarcophagidae (flesh fly) and blowfly larvae on burials at a depth of one foot, as did VanLaerhoven & Anderson (1999). In the latter study, adult flies were observed attempting to move through surface soil cracks and reach the body, particularly following heavy rain. Eggs were also laid on the surface following rain, and on hatching, larvae moved down the soil cracks. Blowflies would therefore be unexpected at depths greater than this, and presence of larvae at greater depths may suggest burial occurred some time following death, allowing flies access to the body.

In cases where exhumation occurs a considerable time after burial, the activity of blowflies on the carrion may be indicated only by the presence of empty puparia in soil. Puparia may persist for hundreds of years following death. Certain fly species are seasonal, and the presence of their puparia may be a useful indicator as to the seasonality of a death, or burial (Gilbert & Bass, 1967).

Burial does not preclude invertebrates from accessing the corpse. In a burial situation, certain insects such as the flies *Muscina* spp. (family Muscidae), and *Morphotheria kerteszi* (family Heliomyzidae) will lay eggs on the soil surface, and following hatching, larvae will burrow through the soil to the carrion (Smith, 1986). Other adult insects will burrow down through the soil to oviposit directly onto carrion, such as the Staphylinidae (Coleoptera), and Phoridae (Diptera). Adults of the phorid *Conicera tibialis* will burrow up to 2m below the surface to oviposit on a body (Bourel *et al*., 2004). Cheese skipper larvae (Piophilidae) have been recorded on buried carrion in rural areas of Nova Scotia (Simpson & Strongman, 2002), and Sphaeroceridae (Diptera) may also be abundant on buried carrion (Bourel *et al*., 2004).

Beetles may also be found on buried carrion. Payne & King (1969) discovered a carabid beetle species *Anillinus fortis* Horn, associated with buried carrion. Simpson & Strongman (2002) also reported the sylphid *Necrophila americana*, and some histerid, carabid and staphylinid species on buried carrion in rural areas of Nova Scotia, but not in urban areas. VanLaerhoven & Anderson (1999) report the occurrence of many beetle species, including silphids, on buried carrion, but suggest
these beetles to be unreliable in a successional sense as they are generalised predators and their arrival is not predictive.

A thorough knowledge of the succession occurring on buried carrion is required if it is to be employed in PMI estimation. As with surface carrion, a predictable succession may be used to estimate PMI, and in cases where dipteran larvae are still present, the use of temperature dependent developmental data may be applied. The use of such data obviously requires consideration of season and temperature. VanLaerhoven & Anderson (1999) determined soil temperature to be a better predictor of internal temperature of buried carrion than ambient temperature, and suggest use of soil temperature for estimation of insect development.

VanLaerhoven & Anderson (1999) recorded the occurrence of some species on buried carrion to be predictable, and suggest that burial successions can be used in estimation of PMI. This obviously requires further studies in each specific locality where such data is to be employed. The variability in species present and differences in behaviour and developmental rates makes the development of a generalised successional database impractical, as with the current successional data for above ground carrion.

Conclusions

The type of soil is an important factor in decomposition (Bornemissza, 1957). Soil type affects the type of vegetation found in an area, and consequently the invertebrate fauna. The properties of a soil and its potential for drainage would be assumed to also affect the faunal assemblage found beneath carrion. Soil type is particularly important in the decomposition of buried carrion (Simpson & Strongman, 2002), as affected by numerous variables such as climate, vegetation, soil temperature and moisture (Motter, 1898) and drainage (Rodriguez & Bass, 1985).

Anderson & VanLaerhoven (1996) concluded from their studies that soil fauna and its succession may be very useful in estimation of PMI, being useful for a longer time following death than the surface carrion assemblage alone. This review has shown that there has been limited study of carrion-related soil invertebrates. Studies in the relevant literature indicate the presence of a few groups to occur in soil beneath
carrion at distinct and possibly predictable stages of decomposition, but there are numerous invertebrate groups still neglected.

The use of the soil succession in PMI estimation will require development of successional databases for locations where it is to be applied. This will involve consideration of all invertebrate groups associated with carrion, and selection of groups that are truly predictable in their arrival at a corpse in relation to decompositional stage. Numerous studies have identified groups both beneath the body, and associated with burials, that may be useful in PMI estimation, but the successional data required to apply such observations is still lacking. Invertebrates play an important role in terrestrial decomposition, and further study will provide a new method for estimation of PMI and open new frontiers in forensic entomology.

References
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