The molecular identification and thermal attributes of forensically important blowflies (Diptera: Calliphoridae: Chrysomya)

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The molecular identification and thermal attributes of forensically important blowflies (Diptera: Calliphoridae: *Chrysomya*)

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Declaration

I, Leigh Alden Nelson, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced and acknowledged. This document has not been submitted for qualifications at any other academic institution.

Leigh Alden Nelson
November, 2008

The fly heeds not death; eating is all to him.

African proverb

The forensically important *Chrysomya rufifacies* female (left) and male (right) on pig carrion.
Photo: L.A. Nelson

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Abstract

Forensic entomology applies the study of arthropods associated with carrion, in terms of species succession and development rates, to determine the minimum time since death, or postmortem interval (PMI). Correct species identification is crucial, as the rate of larval development can vary substantially between species. The identification of forensically important blowflies of the genus *Chrysomya* (Diptera: Calliphoridae) may be hampered by their close morphological similarities, especially as immatures. DNA-based approaches, such as those investigated here, have the capacity to be useful for the identification of forensic entomological evidence in cases where morphological characters are unreliable.

In this study, two DNA regions were investigated as potential candidates for the identification of the nine *Chrysomya* species in Australia: (1) the second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA) and (2) the cytochrome oxidase I (COI) DNA 'barcode' region. The ITS2 region was assessed by sequence comparison, which identified five restriction enzymes (DraI, BsaXI, BciVI, AseI and HinfI) that were able to differentiate most members of the genus by polymerase chain reaction (PCR) restriction fragment length polymorphism (PCR-RFLP). The closely related species pairs *Chrysomya latifrons* + *Chrysomya semimetallica* and *Chrysomya incisuralis* + *Chrysomya rufifacies* could not be separated by restriction profile analysis, but the latter could be separated using the size differences resulting from amplification of the entire ITS region. Identical restriction profiles were generated from eight *Ch. incisuralis* specimens, suggesting low intraspecific ITS2 variation within this species. Phylogenetic analysis of the ITS2 sequence, which is a possible means by which species could be identified, proved successful for the identification of the majority of *Chrysomya* species.

The COI 'barcode' region resolved all nine *Chrysomya* species as reciprocally monophyletic, following a neighbour-joining (NJ) analysis of the Kimura two-parameter distances. Mean intraspecific and interspecific sequence divergences were 0.097% and 6.499%, respectively. The hybrid status of one specimen was confirmed

following subsequent ITS2 sequence analysis. In another instance, this nuclear region was used to verify four cases of specimen misidentification that had been highlighted by the COI analysis. The COI DNA barcode was successful in identifying *Chrysomya* species from the east coast of Australia. The ability of the barcode to identify two *Chrysomya nigripes* specimens from Thailand shows potential for this method to be expanded to other blowfly genera and continents. This result confirmed previous successes with COI as a genetic barcode for species identification and comparisons at the intra- and interspecies levels.

Together with correct species identification, thermodevelopment data of blowfly species are vital for the estimation of the PMI. The close morphological and molecular similarities among *Chrysomya* species led to speculation as to whether members of this genus shared similar developmental profiles. The aim was to establish whether genetically closely related species would share similar developmental profiles. This would permit the application of developmental data to a number of closely related species, including those for which thermodevelopmental studies are lacking. If Australian *Chrysomya* were found to share developmental profiles, identification of the blowfly specimen to a level beyond genus may not be necessary, or at least it may not be necessary to distinguish morphologically similar sister species. The experimental design employed in this study sets it apart, to date, from other published larval development studies. Nowhere else have the developments of such closely related blowfly species been compared. As the species were collected from the same geographical location, the effects of acclimation and population-level genetic variation were not variables in this study. The experimental conditions in this study were virtually identical, which enabled direct comparisons to be made among the species. This study established that the sister species *Ch. megacephala* and *Ch. saffranea* differed significantly in their developmental profiles, as well as compared with the more distantly related *Ch. rufifacies*. Because of this, genetic distance was not considered to be a useful factor for predicting thermodevelopment profiles of closely related species within a genus, and highlighted the necessity for correct species identification.

List of Abbreviations

%	percent
±	plus or minus
ACT	Australian Capital Territory
ANOVA	analysis of variance
approx.	approximately
C.	*Calliphora*
Ch.	*Chrysomya*
cm	centimetre
COI	cytochrome oxidase subunit I
COII	cytochrome oxidase subunit II
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	east
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
g	gram
GTR + I + Γ	general time reversible model with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution
GTR	general time-reversible model
h	hour
i.e.	that is
ITS	internal transcribed spacer
kb	kilobase
km	kilometre
L	litre
ml	millilitre
mM	millimolar

mtDNA	mitochondrial DNA
N	number
ng	nanogram
NSW	New South Wales
ºC	degrees Celcius
P	probability
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. comm.	personal communication
Pfu	*Pyrococcus furiosus*
PMI	post-mortem interval
Qld	Queensland
rDNA	ribosomal DNA
S	south
SE	standard error
sp.	Species (singular)
spp.	Species (plural)
UV	ultraviolet
V	Volts
Vic.	Victoria
vs.	versus
V/V	on a volume per volume basis
µl	microlitre
µM	micromolar

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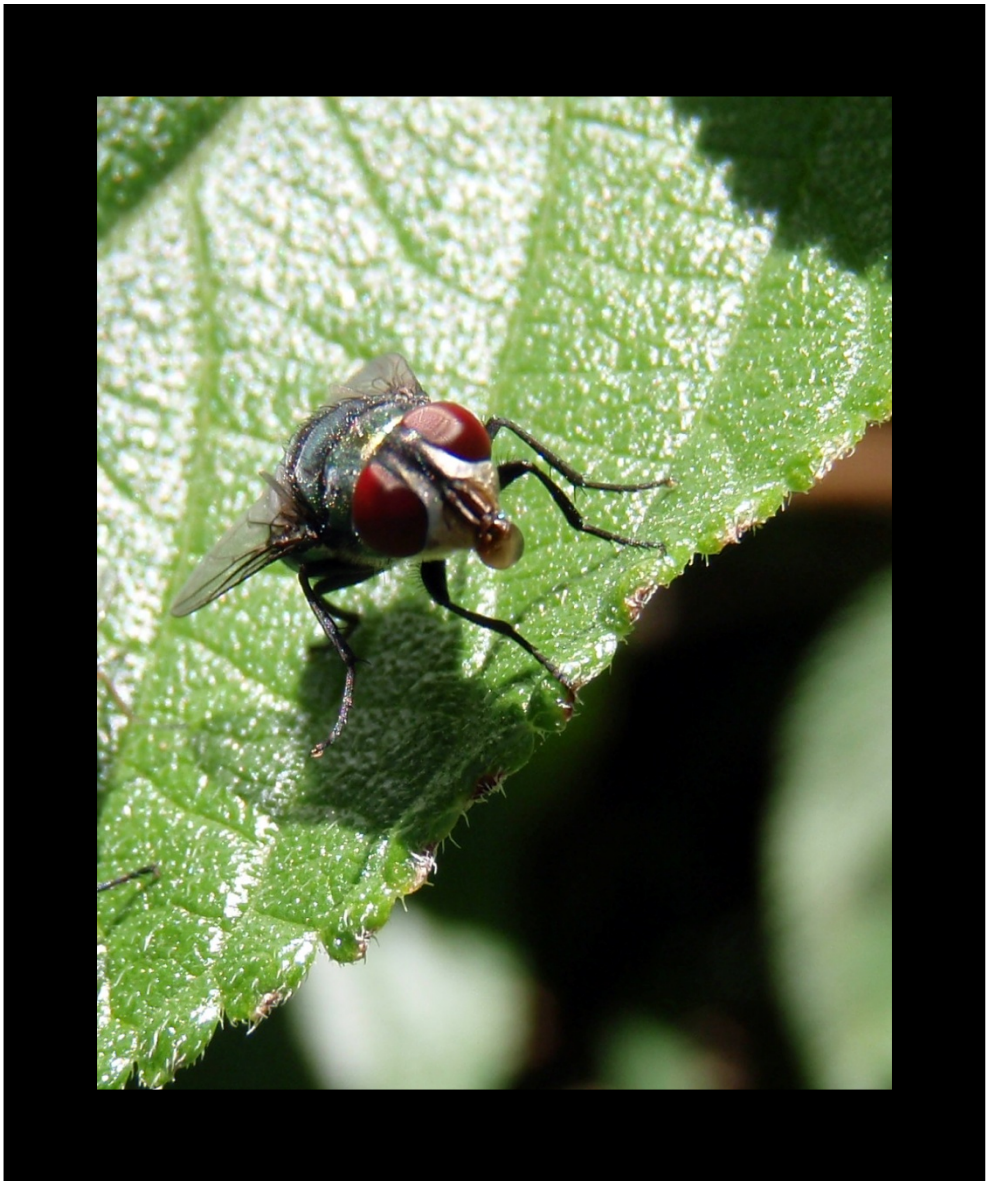


Female *Chrysomya rufifacies* resting on foliage above carrion.
Photo: L.A. Nelson

Chapter 1. General Introduction

1.1. Introduction to forensic entomology

A carcass goes through a series of biological, chemical, and physical changes as it decomposes. Decomposing carcasses represent a nutrient-rich temporary microhabitat and food source for a broad array of organisms from bacteria and fungi to insects and vertebrates (Erzinçlioglu, 1996). These organisms are attracted by specific states of decay, with particular species colonising a carcass for a limited period, producing a predictable faunal succession. Since the work of Mégnin (1894), the succession of arthropods on decaying carrion has been described extensively (e.g. Bornemissza, 1957; Payne, 1965; Early & Goff, 1986; Anderson & VanLaerhoven, 1996; Kirkpatrick & Olson, 2002; Watson & Carlton, 2003). Carrion succession is a classical subject in ecology, although more recent studies have emphasised observations applicable to criminal investigations (Sukontason *et al.*, 2001; Wolff *et al.*, 2001; Centeno *et al.*, 2002; Archer, 2003).

An established field of the forensic sciences, forensic entomology applies the study of arthropods associated with carrion to determine the minimum time since death, or postmortem interval (PMI). In some cases, forensically important arthropods may provide clues as to the place or manner of death (e.g. rural, urban, indoors or outdoors; wound type and location) or if the body has been moved (e.g. Greenberg, 1991; Benecke, 1998). Forensic entomology is intricately associated with the scientific fields of medical entomology, insect taxonomy, and forensic pathology (Byrd & Castner, 2001a). Forensic entomology rests upon a foundation of scientific principles that have been accepted by the courts as admissible evidence (Greenberg & Kunich, 2002). Although occasionally collected from human corpses (e.g. Rodriguez *et al.*, 1983; Carvalho *et al.*, 2000), invertebrate succession data have mainly come from experiments on pig carcasses (e.g. Payne, 1965; Archer, 2002; Kirkpatrick & Olson, 2002; Joy *et al.*, 2006). The domestic pig (*Sus scrofa* Linnaeus) is an animal model frequently utilised for forensic entomological studies because they (a) have been shown to attract a comparable insect fauna (Schoenly *et al.*, 2007), (b) display

similar decomposition patterns to those of human corpses, (c) are available in various sizes comparable to those of humans of different ages, (d) are relatively inexpensive and easy to obtain, and (e) do not illicit public objections to their use (Catts & Goff, 1992).

Two lines of entomological evidence are used in PMI determination. In the first, the time required for an insect to attain an observed developmental stage can be extrapolated backwards to estimate the time of oviposition, and hence, the approximate minimum time since death (von Zuben *et al.*, 1998; Jenson & Miller, 2001). The second approach takes advantage of the predictable succession of arthropod species commonly observed on a wide variety of carrion, including flies (Diptera), beetles (Coleoptera), moths and butterflies (Leipidoptera), and wasps and ants (Hymenoptera) (Wells & LaMotte, 1995; Amendt *et al.*, 2004). The decay rates of human cadavers have been shown to relate directly to these faunal successional patterns (Rodriguez *et al.*, 1983). Forensic entomology is based on the knowledge of the succession sequence and the life-cycles of these species.

The identification of carrion-breeding species is the first step undertaken by the forensic entomologist in an attempt to estimate the PMI (Benecke, 1998; Wells & LaMotte, 2001). As flies often lay eggs that hatch before the experts arrive at the crime scene, the time of oviposition is only an approximation and can become one of the pivotal points in a trial (Greenberg & Kunich, 2002). Correct species identification is crucial, as the rate of larval development can vary substantially between species (Wells *et al.*, 2001). Consequently, misidentification could lead to inaccurate estimation of the PMI. The estimation of the PMI may be a deciding factor in the determination of the guilt of an accused person in a court of law (Byrd & Allen, 2001b).

The leading role in the biological decay of a carcass is played by representatives of the order Diptera (flies), including the families Calliphoridae, Muscidae and Sarcophagidae. Second in importance are members of the order Coleoptera (beetles) (Putman, 1983; Marchenko, 2001). Blowflies (Diptera: Calliphoridae) are the most

numerous and well-studied component of cadaver entomofauna (e.g. O'Flynn, 1983; Benecke, 1998; Bharti & Singh, 2003; Grassberger *et al.*, 2003; Tenorio *et al.*, 2003; Amendt *et al.*, 2004). Since the carrion-breeding blowfly species rarely deposit eggs on a live host, the estimated age of a larva is therefore the estimated minimum PMI (von Zuben *et al.*, 1998; Jenson & Miller, 2001; Wells & King, 2001). Body temperature and other physical conditions such as rigor mortis cease being applicable indicators of the PMI after about 72 hours (Anderson & VanLaerhoven, 1996). Since blowflies are often the first to arrive at and invade a carcass, they provide one of the most accurate methods for revealing the PMI after this period. A thorough description of the history and development of forensic entomology is given by Benecke (2001) and Greenberg and Kunich (2002).

1.2. Forensically important blowflies (Diptera: Calliphoridae)

The Calliphoridae (blowflies) are a large family of the Diptera, with over a thousand species from approximately 150 genera recognised worldwide (Byrd & Castner, 2001c). The lifecycles of the various blowfly species are somewhat similar (Putman, 1983) (Fig. 1.1). The main difference is whether the species is oviparous (egg-laying), or ovoviviparous and consequently viviparous (eggs hatch prior to or directly after being deposited). Blowfly genera that have been found to be the most significant primary insects attending a carcass after death include *Calliphora* and *Chrysomya* (Levot, 2003). Others, such as *Lucilia*, and the family Sarcophagidae (flesh flies), are less frequently recovered, but are nonetheless important. After the blowflies and flesh flies, members of the family Muscidae are the next most noteworthy insect group involved in carcass decomposition, and are generally present during the later stages (Arnaldos *et al.*, 2005).

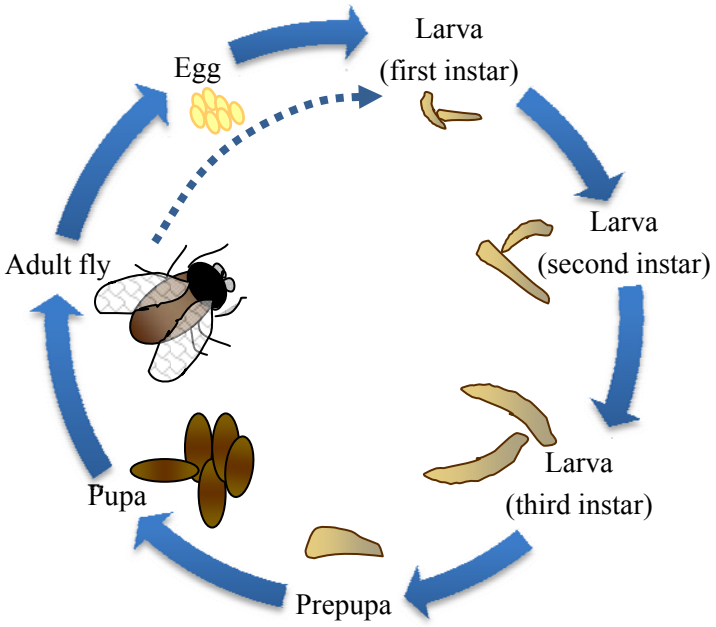


Figure 1.1. Generalised lifecycle of the blowfly. The adult fly lays eggs (up to ~300) which hatch approximately 6-24 h later. Some flies (e.g. *Sarcophaga* sp. and *Calliphora augur*) lay live young directly onto the feeding substrate (dotted arrow). The first-instar larva moults approximately 24 h later. This second-instar larva moves around in the maggot mass, moulting 24 h later to become the third-instar larva, which has increased in size. About two days later, the pre-pupa larva stops feeding and migrates away from the feeding substrate to find a suitable pupation site (usually in soil). It transforms into a pupa four days later. The pupa resides within the puparium, in which it undergoes metamorphosis to form the adult fly approximately ten days later. Upon emergence from the pupa, the adult fly feeds on a protein source, and then mates; eggs are laid a few days later.

Knowledge of the blowfly lifecycle enables the PMI to be calculated retrospectively, based on the period required for a given species to reach the development stage that had been collected from the decomposing remains. For the PMI to be valid, it is necessary to have data on the rate of development of a particular species, at temperatures experienced by the larvae that were growing on the corpse. Larval growth rates have been shown to increase positively with temperature, between upper and lower developmental thresholds (e.g. Davies & Ratcliffe, 1994; Byrd & Butler, 1997; Grassberger & Reiter, 2002b; Donovan *et al.*, 2006). Larval thermodevelopmental data are typically obtained from studies carried out under laboratory conditions. The need to predict insect development time has led to various mathematical models that describe the effect of temperature on larval development. Of these models, the forensic entomologist typically uses two general types: the degree-day summation method and the nonlinear temperature inhibition model (Byrd

& Allen, 2001a). The PMI estimation by these methods can be complicated by factors such as delayed colonisation (e.g. due to a wrapped and/or buried corpse; Anderson, 2001), the occurrence or otherwise of nocturnal oviposition (e.g. Greenberg, 1990; Singh & Bharti, 2001; Amendt *et al.*, 2007), and characteristics of the environment such as temperature, wind, rainfall and humidity (Campobasso *et al.*, 2001). Additionally, the understanding of factors influencing larval development is incomplete, and the applicability of laboratory data is somewhat restricted to 'ideal' or artificial conditions, not frequently encountered in casework. For these reasons, the availability of accurate developmental data for commonly encountered blowfly species is of utmost importance.

The application of blowflies in PMI estimation has been hampered by the difficulties associated with correct species identification at all developmental stages (eggs, larvae, pupae and adults) (Catts, 1992; Benecke, 1998). The identification of blowfly larvae often relies upon the recognition of subtle variations in morphological features, such as the cephalopharyngeal skeleton and microtubercles, which requires specialised taxonomic knowledge (Erzinçlioglu, 1996; Wallman, 2001b; Ratcliffe *et al.*, 2003; Wells & Stevens, 2008). For some species, identification is feasible by collecting larvae from the corpse and raising them to adulthood, but the time needed for this could delay an investigation. Even then, species confirmation may involve the dissection and inspection of the genitalia (especially the aedeagus in males), a difficult task for those lacking specialised taxonomic training. There are cases where insect larvae may die before identification is possible, and others where only fragments of insect evidence are available for analysis (e.g. the puparia, dead or decomposed adults or larvae) (O'Flynn, 1983; Stevens & Wall, 2001; Schroeder *et al.*, 2003; Siriwattanarungsee *et al.*, 2005). Although these incomplete samples can sometimes be identified to family, it is much more difficult to identify species (Malgorn & Coquoz, 1999). For these reasons, it would be useful if forensic laboratories could implement DNA typing of forensic insect specimens.

1.2.1. Genus Chrysomya

Blowflies of the genus *Chrysomya* are considered to be of profound forensic importance for determination of the PMI, due to their rapid colonisation of corpses, extensive oviposition and abundance in many habitats (Byrd & Butler, 1997). Representatives from this genus constitute 44% of the blowfly species present in murder or suspicious death cases investigated in New South Wales, Australia (Levot, 2003). *Chrysomya* species can be distinguished from other calliphorids by "the combination of a ciliated stem vein, a convex occiput and a thoracic squama with hairs in the center of the upper suface" (Wells & Kurahashi, 1996). While members of this genus are often present as primary colonisers, they are particularly stimulated to oviposit by distinctive chemical cues produced by maggots of other genera (e.g. *Calliphora*), and as such may also be present as secondary colonisers (Fuller, 1934; Norris, 1965; Byrd & Castner, 2001c). This behaviour is due to the predatory nature of *Chrysomya* larvae, particularly *Chrysomya rufifacies* (Macquart), which feed on, repel and often totally displace the primary larvae due to competition (Faria & Godoy, 2001; Bharti & Singh, 2002). The heavily sclerotised spines and processes on third-instar 'hairy maggots' of *Ch. rufifacies* may enhance their ability to capture and restrain their prey (Baumgartner, 1993).

Twenty eight *Chrysomya* species are known from the Oriental, Australasian and Oceanian regions (Wells & Kurahashi, 1996). With the exception of *Chrysomya saffranea* (Bigot) and *Chrysomya latifrons* (Malloch), all seven *Chrysomya* species known from Australia (and included in this study) are also found in other countries, particularly within the Asian region (Kurahashi, 1989). The identification of several species within this genus is hampered by their similar morphologies, even as adults (Wells & Kurahashi, 1996; Wallman, 2001a). Some members of this genus can only be distinguished on the basis of subtle morphological differences such as pigmentation (Fig. 1.2). Molecular data suggest very close genetic relationships among some members of the genus *Chrysomya* in Australia (Wallman *et al.*, 2005). The morphological similarity of species of this genus, especially their immatures, makes a DNA-based approach to their identification advantageous.

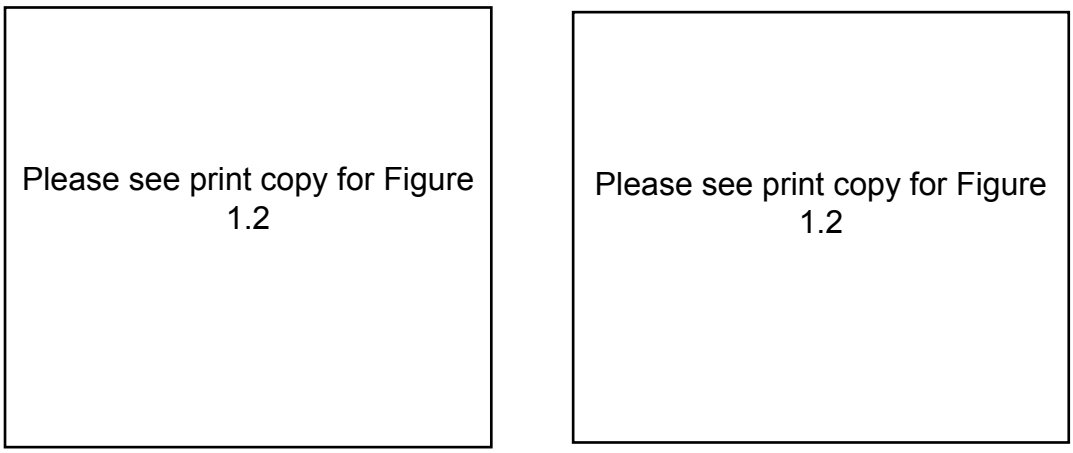


Figure 1.2. Photographs of two members of the genus *Chrysomya*; *Chrysomya latifrons* (left) and *Chrysomya semimetallica* (right), illustrating the high degree of adult morphological similarity among some members of this genus. Arrows identify two areas of subtle morphological variation (A, notopleuron; B, katepisternum) that can be used to distinguish the two species. *Ch. latifrons* exhibits darker colouration than *Ch. semimetallica* in both of these areas. Photos: J.F. Wallman.

1.3. DNA analysis in entomology

Prior to the development of DNA-based technologies, protein markers, especially allozymes, were the main molecular marker employed for entomological investigations (e.g. Krafsur, 1993; Taylor *et al.*, 1996; Wallman & Adams, 1997; 2001). Compared to protein markers, greater levels of polymorphism can be detected by examining the DNA of insects directly, including point mutations within each codon as well as insertions or deletions (Loxdale & Lushai, 1998). Additionally, DNA is not influenced by environmental factors or the developmental stage of an organism, as are allozymes (Parker *et al.*, 1998). DNA markers have been developed to distinguish closely related insect species and to infer phylogenetic relationships (e.g. Haymer *et al.*, 1992; Gallego & Galián, 2001; Kengne *et al.*, 2003; Otranto *et al.*, 2003). A variety of DNA-based methods, each with its own advantages and disadvantages, have been developed to evaluate the genetic divergence of closely related arthropod species, a thorough discussion of which is given by Loxdale and Lushai (1998). The most widely sequenced regions in insect systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) (Caterino *et al.*, 2000).

1.3.1. Mitochondrial DNA

The most thoroughly studied sequences have been those of the mitochondrial genes. In animals, mitochondrial genomes are maternally inherited, small (15 to 20 kb in length), circular, and encode 37 genes: two ribosomal RNAs (for small- and large-subunit rRNA), 13 protein coding genes, and 22 transfer RNAs (Boore, 1999) (Fig. 1.3). These genes can potentially be rearranged in many combinations, the order of which is often used to investigate the evolution of organisms and their genomes. Animal mitochondrial genomes also have a large noncoding region that contains elements that control the replication and transcription of the genome. This region, being rich in adenine and thymine nucleotides in insects, is known as the 'A+T-rich region' or 'control region' (Zhang & Hewitt, 1997). As a molecular marker, mtDNA has many advantages: (a) it is present in multiple copies per cell, thereby making the genes more easily detected than single-copy chromosomal genes, (b) it generally evolves faster than nuclear DNA, making it useful for studies at the species level, and (c) it is a mosaic molecule with different regions evolving at different rates, facilitating primer design and providing solutions for questions at various taxonomic levels (Zhang & Hewitt, 1996; Hoy, 2003).

Some molecular-based techniques, mostly employing mtDNA, have been proposed to identify forensically important blowflies (e.g. Malgorn & Coquoz, 1999; Wells & Sperling, 1999; Wallman & Donnellan, 2001; Wells et al., 2001; Harvey et al., 2003a; Harvey et al., 2003b; Ratcliffe et al., 2003; Chen et al., 2004; Zehner et al., 2004; Saigusa et al., 2005). The majority of this research has examined subunit I and/or II of mitochondrial cytochrome oxidase (e.g. Malgorn & Coquoz, 1999; Wells & Sperling, 1999; Wallman & Donnellan, 2001; Wells et al., 2001; Harvey et al., 2003a; Schroeder et al., 2003; Chen et al., 2004).

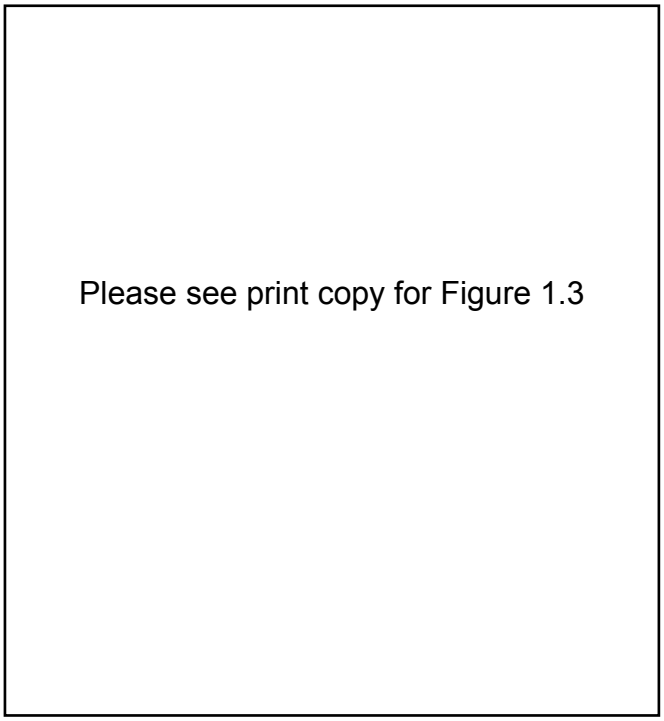


Figure 1.3. Map of mitochondrial DNA of the fruit fly *Drosophila yakuba* (16,019 nt). Genes for tRNAs (hatched areas) are denoted by the one-letter code for their corresponding amino acids. Protein-coding genes are denoted COI, COII, and COIII for the genes encoding subunits 1, 2 or 3 of *cytochrome c oxidase* and Cyt b for the *cytochrome b* gene. The ND genes encode subunits of the NADH dehydrogenase system. The origin of replication (O) within the A+T-rich region and the direction of replication (R) are shown. Arrows indicate the direction of transcription of each coding region (After Wolstenholme & Clary, 1985).

However, there are various difficulties associated with using mtDNA, including length and sequence heteroplasmy, a non-constant molecular clock, non-neutrality, rapid lineage sorting and nuclear mitochondrial-like sequences, or pseudogenes (Zhang & Hewitt, 1996; Holland & Parsons, 1999; Caterino *et al.*, 2000; Bensasson *et al.*, 2001). The non-coding regions of a genome are under the least evolutionary constraint and therefore are most informative for species identification purposes (Loxdale & Lushai, 1998). Unfortunately, apart from the 'control region' (approximately 1000 bp in length), the mitochondrial genome is highly economised, containing few sections of non-coding DNA (Lunt *et al.*, 1998). The 'control region' itself is characterised by features that limit its usefulness for evolutionary and species identification purposes (Zhang & Hewitt, 1997). In contrast, the nuclear genome represents a much greater source of non-coding DNA (Zhang & Hewitt, 2003), including the transcribed spacer regions of ribosomal DNA (see 1.3.2). Therefore,

although it is popular to use mtDNA regions for identification purposes and to study the evolutionary relationships of closely related species, it is of immense interest to find additional informative regions in the nuclear DNA (Lin & Danforth, 2003). Because the evolution and phylogenetic utility of the mitochondrial genome has already received considerable attention in the literature (e.g. Moritz *et al.*, 1987; Holland & Parsons, 1999), and its use in identifying forensically important blowflies has been demonstrated (e.g. Wells & Sperling, 1999; Wallman & Donnellan, 2001; Wells *et al.*, 2001), the remaining focus of this section will be on the nuclear ribosomal DNA, which has already been investigated for the identification of forensically important Diptera (Calliphoridae, Muscidae and Sarcophagidae) (Ratcliffe *et al.*, 2003).

1.3.2. Nuclear ribosomal DNA

Rapidly evolving genes and nucleotide regions are considered useful for comparisons of closely related taxa (Brower & DeSalle, 1994; Alvarez & Hoy, 2002). The nuclear ribosomal DNA genes are one such set of DNA markers. The eukaryotic nuclear genome is typically composed of hundreds or thousands of copies of rDNA genes in tandemly repeated transcriptional units separated from each other by intergenic spacers (IGS) (Long & Dawid, 1979; Elder & Turner, 1995). Each repeated unit contains the conserved 18S, 5.8S and 28S coding genes which are in turn separated by two non-conserved, noncoding internal transcribed spacer (ITS) regions – ITS1 and ITS2, respectively (Williams *et al.*, 1988; Brower & DeSalle, 1994) (Fig. 1.4). Another non-coding region, the external transcribed spacer (ETS), precedes the 18S gene. Universal polymerase chain reaction (PCR) primers, designed to anneal to the highly conserved genes (18S, 5.8S and 28S) flanking the ITS regions (Fig. 1.4), enable amplification of the spacers due to their relatively small size (typically 600-1000 bp) and high copy number of rDNA repeats – attributes which enhance the amplification of PCR product from low-concentration DNA templates (Appels *et al.*, 1980; Loxdale & Lushai, 1998; Morris & Mound, 2004).

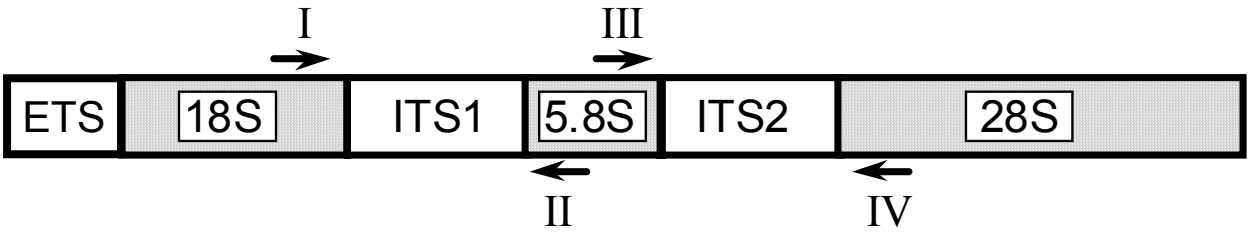


Figure 1.4. Schematic diagram of a single repeat unit of rDNA which consists of the conserved 18S, 5.8S and 28S rDNA genes (shaded areas), two highly variable internal transcribed spacers (ITS) (unshaded areas) and the external transcribed spacer (ETS) (not to scale). Intergenic spacers (IGS) (not shown) separate the repeated transcriptional units from each other. Roman numerals indicate the conserved regions to which amplification primers anneal.

Ribosomal DNA has been employed as a molecular marker in phylogenetic and evolutionary studies for a wide variety of both closely and distantly related organisms, including the insects (e.g. Appels *et al.*, 1980; Paskewitz *et al.*, 1993; Vogler & DeSalle, 1994; Bayer *et al.*, 1996; Clemente *et al.*, 2002; Carvalho *et al.*, 2004). The distribution of conserved sequences within the rDNA repeat unit is correlated with rRNA function (Gerbi, 1986). As a consequence of encoding parts of the functional ribosome, the 18S, 5.8S and 28S regions are under much higher evolutionary constraint than the ITS. While these more conserved coding sequences are valuable for studying the systematics of higher taxonomic levels of insects, the ITS regions are most suitable for genus and species comparisons due to their comparably rapid rates of evolutionary divergence (Pelandakis & Soliqnac, 1993; Newton *et al.*, 1998). Although transcribed to form part of the precursor RNA, the ITS regions do not directly contribute to the construction of a ribosome due to their subsequent cleavage and removal from this molecule (Gerbi, 1986; Parkin & Butlin, 2004). The evolutionary importance of the ITS regions is evidenced by the occurrence of tracts of highly conserved ITS sequence throughout the eukaryotes (Brown *et al.*, 1972; Walton *et al.*, 1999a; Schultz *et al.*, 2005). Such regions may be essential to the formation of secondary structures characteristic of the ITS regions, and selection pressures may control the amount of mutation tolerated in each rDNA copy (van der Sande *et al.*, 1992). While the exact function of the ITS regions remains unclear, it is believed that their hydrogen-bonded secondary structures may provide the recognition and docking signals required for the processing reactions of the precursor mRNA (Gerbi, 1986).

Most repetitive sequences, such as the rDNA genes, have been found to evolve by concerted evolution, a complex molecular process which results in greater sequence similarity within individuals and species than between species. Such interspecies variability was first demonstrated by Brown *et al.* (1972) for the frog sister species *Xenopus laevis* and *Xenopus mulleri*. Relatively rapid rates of mutation can occur within the ITS regions, and comparisons between the regions of some closely related species have revealed numerous insertions and/or deletions (indels) scattered within extensive tracts of conserved sequence (Hackett *et al.*, 2000; Douglas & Haymer, 2001). It is because of the observed ITS divergence level that these regions have proved useful for comparing closely related insect species, subspecies or populations that otherwise show little genetic divergence (e.g. Unfried & Gruendler, 1990; Vogler & DeSalle, 1994; Sang *et al.*, 1995; Douglas & Haymer, 2001; Gallego & Galián, 2001; Murrell *et al.*, 2001; von der Schulenburg *et al.*, 2001; Kengne *et al.*, 2003; Ratcliffe *et al.*, 2003; Thomson *et al.*, 2003; Dumont *et al.*, 2005). Through the study of multigene families, the phenomenon and mechanisms of concerted evolution continue to be described (e.g. Elder & Turner, 1995; Liao, 1999; Vollmer & Palumbi, 2004). It is believed that the process maintains sequence integrity between the rDNA repeat units through mechanisms such as gene conversion, gene amplification and unequal crossover between repeated units (Brown *et al.*, 1972; Arnheim, 1983; Hillis & Dixon, 1991; Fritz *et al.*, 1994; Elder & Turner, 1995).

A prerequisite for the utilisation of a marker for species identification is that its interspecies variation should be considerably greater than its intraspecific variation (Campbell *et al.*, 1995). While the multicopy nature of rDNA makes it particularly easy to amplify, this same characteristic may also introduce obstacles: although it is generally accepted that concerted evolution acts to homogenise the sequences of the multiple rDNA genes within a species, several studies have highlighted the occurrence of varying levels of intraspecific variation (e.g. Ferris *et al.*, 1993; Navarro & Weaver, 2004). For instance, Paskewitz *et al.* (1993) found levels of intraspecific variation in the ITS2 region of the Afrotropical *Anopheles gambiae* (Diptera: Culicidae) species complex of between 0.1 and 0.4%. In similar studies, Campbell

(1995) detected levels of up to 0.9% in *Strongylus* (Nematoda) species, while Schlötterer *et al.* (1994) found intraspecific variation to be less than 0.05% in ITS regions of *Drosophila melanogaster* (Diptera: Drosophilidae). However, other studies have found complete species-wide homogeneity of ITS sequences, suggesting that concerted evolution was operating efficiently in those species, for example trematodes (Trematoda: Fasciolidae; Adlard *et al.*, 1993), spider mites (Acari: Tetranychidae; Navajas *et al.*, 1998), *Timarcha* leef beetles (Coleoptera: Chrysomelidae; Gómez-Zurita *et al.*, 2000), and *Anopheles* spp. (Diptera: Culicidae; Kampen *et al.*, 2003; Marrelli *et al.*, 2005). It is apparent that the levels of ITS sequence variation observed are in no way predictable or constant across a group of related taxa (Ferris *et al.*, 1993; Tang *et al.*, 1996). For sequence homogenisation to occur, concerted evolution must exceed the rate of appearance of new variants (Campbell *et al.*, 1997; Beebe *et al.*, 2000). One theory explaining intraspecific variation is that there is reduced gene flow among different populations of a single species, which would limit the spread and fixation of mutations within a population (Navajas *et al.*, 1998; Onyabe & Conn, 1999).

Another criterion for the use of a marker in species identification is that intraindividual variation should be negligible, or at most, much lower than interspecific variation. In addition to intraspecific variation, inefficient homogenisation among rDNA repeats can lead to intraindividual ITS variation, or heteroplasmy, among the tandemly repeated gene units within a single specimen (e.g. Paskewitz *et al.*, 1993; Vogler & DeSalle, 1994; Tang *et al.*, 1996; Fenton *et al.*, 1998; Newton *et al.*, 1998; von der Schulenburg *et al.*, 2001; Leo & Barker, 2002; Parkin & Butlin, 2004). Consequently, not all repeats are necessarily identical, even though concerted evolution is believed to maintain sequence integrity between these units (e.g. Hillis & Dixon, 1991). Intraindividual variation is thought to occur when concerted evolution is not fast enough to homogenise repeats in the face of high rates of mutation (Campbell *et al.*, 1997). The ITS region has been found to evolve up to two and a half times faster than the mitochondrial cytochrome oxidase I (COI) gene in mites (Acarina: Tetranchyidae: *Tetranychus urticae*) (Navajas *et al.*, 1998), suggesting that concerted evolution would have to be a very efficient process to

homogenise all ITS repeats within an individual, let alone a species. One theory suggests that the occurrence of intraindividual polymorphisms represents the transition period when a new mutation has arisen in one rDNA copy and is beginning to spread to other copies in the genome (Parkin & Butlin, 2004). Although most data support the theory of concerted evolution, it is not known whether the mechanisms operate in exactly the same fashion for repetitive noncoding DNA, such as the internal transcribed spacers (Liao, 1999). It is apparent that the processes of this phenomenon are poorly understood, and that inadequate homogenisation can lead to the occurrence of intraspecific and in some cases intraindividual ITS variation in some species (e.g. Vollmer & Palumbi, 2004).

If concerted evolution does operate efficiently, unambiguous sequences can be obtained by direct sequencing of PCR products, suggesting that intraindividual sequence variation is rare or nonexistent (e.g. Brower & DeSalle, 1994; Murrell *et al.*, 2001; Congdon *et al.*, 2002). Extensive intraindividual variation generally produces characteristically illegible electropherograms that are indicative of multiple sequence types (Onyabe & Conn, 1999; Hackett *et al.*, 2000). In such cases, direct sequencing will either reveal the sequence of the most abundant copy of the repeat unit (in which case intraindividual variation will be overlooked) or will fail entirely due to mixed signal from different rDNA copies when one form does not predominate. When multiple rDNA copies are equally abundant, electropherograms appear to contain staggered, superimposed sequences due to competition among the ITS variants (Morris & Mound, 2004). The existence of intraindividual sequence polymorphism can be established by cloning the ITS region and sequencing several clones from the same individual (Cornel *et al.*, 1996; e.g. Tang *et al.*, 1996; Fenton *et al.*, 1998). However, while cloning can give an indication of the existence of heteroplasmy, it cannot reveal the relative abundances of the different ITS copies within the individual unless multiple clones are sampled. Studies that have identified intraindividual variation typically examine only a couple of clones per individual (e.g. Mukwaya *et al.*, 2000, who examined one clone only), and this is still a very small sample of potential ITS sequences considering some insects have around 700 copies

of rDNA (Alvarez & Hoy, 2002). Therefore, it is necessary to sequence sufficient clones per specimen to gauge the abundance of different ITS variants.

There has been contradictory evidence on the level of intraindividual variation found within taxa, suggesting that this is by no means predictable or consistent across all species, and that the extent of variation should be scrutinised for each species being studied (e.g. Schlötterer *et al.*, 1994). In their study of black flies (Diptera: Simuliidae), Tang *et al.* (1996) found intraindividual ITS1 variation to be higher than that for ITS2. Some authors have failed to identify intraindividual ITS variation despite cloning, suggesting that concerted evolution had homogenised the numerous repeats sufficiently for its use in phylogenetic analysis for the group of taxa in question (e.g. Toda & Komazaki, 2002; Young & Coleman, 2004; Marrelli *et al.*, 2005). Other authors have found ITS intraindividual variation to be so high as to render the region unsuitable for population studies (e.g. ITS2, Leo & Barker, 2002). Inconsistent levels of intraindividual variation across a species have been attributed to differences in population level processes such as gene flow and genetic drift (Onyabe & Conn, 1999). The occurrence of both intraspecific and intraindividual variation has important implications for the use of the rDNA region as markers in phylogenetic studies. Unless adequate sampling of ITS copies within individuals from multiple geographic populations is carried out, caution should be used when making phylogenetic inferences or using it as a diagnostic tool, especially among closely related species. Further research to establish the molecular details of concerted evolution is certainly required, especially given the attention this region has received for insect identification purposes (e.g. Campbell *et al.*, 1995; Thomson *et al.*, 2003; Song *et al.*, 2008).

1.4. DNA-based techniques for species identification

1.4.1. PCR-RFLP analysis

While DNA sequencing analysis enables the detection of high levels of sequence polymorphism, the method can be impractical for routine identification due to the prerequisite of a DNA sequencing facility (Abdullahi *et al.*, 2004). An alternative method of species identification involves PCR amplification of the DNA region, followed by restriction fragment length polymorphism (RFLP) analysis (e.g. Malgorn & Coquoz, 1999; Barber *et al.*, 2000; Kampen *et al.*, 2003; Ratcliffe *et al.*, 2003). A PCR product is first generated and then digested to completion using one or more restriction endonucleases that recognise specific DNA sequence motifs, usually four to six bases in length (Loxdale & Lushai, 1998). Sequence differences can either introduce additional restriction sites or eliminate sites. Consequently, the procedure can yield an array of DNA fragments unique to a particular species, the pattern of which can be visualised over UV-light, using ethidium bromide-stained agarose gels (Walton *et al.*, 1999a). This method can provide information on windows of sequence variation without the expense of sequencing every individual.

PCR-RFLP methods have been developed to hasten the identification of a number of insect species. For example, Sperling *et al.* (1994) differentiated three blowfly species (Calliphoridae: *Phormia regina, Phaenica sericata* and *Lucilia illustris*) by PCR-RFLP of part of the mitochondrial COI gene. The screwworm flies *Cochliomyia hominivorax* and *Cochliomyia macellaria* (Diptera: Calliphoridae) were also characterised by PCR-RFLP of mitochondrial DNA (Lessinger *et al.*, 2000; Litjens *et al.*, 2001). A similar method was utilised by Schroeder *et al.* (2003) for the differentiation of calliphorid larvae (*Lucilia sericata, Calliphora vicina* and *Calliphora vomitoria*) on human corpses using COI and COII regions of the mitochondrial DNA. The PCR-RFLP method has been developed for the identification of some cryptic mosquito species, due to a demand for the prompt and reliable recognition of malaria vectors present in a given area (e.g. Mukabayire *et al.*,

1999; Walton *et al.*, 1999b; Hackett *et al.*, 2000; Favia *et al.*, 2001; Gentile *et al.*, 2001; Beebe *et al.*, 2002; Fanello *et al.*, 2002; Kengne *et al.*, 2003).

The ITS region appears to be a reliable marker for PCR-RFLP identification of species within entomopathogenic nematode genera (Campbell *et al.*, 1995; Hung *et al.*, 1996; Newton *et al.*, 1998; Pamjav *et al.*, 1999), while the bark beetle forest pests *Tomicus destruens and Tomicus piniperda* (Coleoptera: Scolytidae) have been differentiated by PCR-RFLP of the ITS2 region (Gallego & Galián, 2001). Abdullahi *et al.* (2004) used ITS PCR-RFLP as an efficient method to distinguish cassava-associated populations of whiteflies (Hemiptera: Aleyrodidae: *Bemisia tabaci*), while Toda and Komazaki (2002) and Moritz *et al.* (2002) used the same procedure to identify thrips (Thysanoptera: Thripidae). The method has also been developed for the rapid detection of fruit fly (Diptera: Tephritidae) species for quarantine purposes (Armstrong *et al.*, 1997), to determine genetic differences between populations of New Zealand flatworms (Platyhelminthes: Geoplanidae) (Dynes *et al.*, 2001), and for the identification of medically important freshwater snails (Mollusca: Planorbidae) (Carvalho *et al.*, 2001). Recently, PCR-RFLP of the ITS regions has been used to identify a select group of morphologically similar Diptera (Calliphoridae, Muscidae and Sarcophagidae) larvae that are of forensic importance (Ratcliffe *et al.*, 2003). These findings suggest that the ITS regions might be sufficiently variable to construct unique identification markers of a number of carrion-breeding blowflies, including Australian members of the genus *Chrysomya.*

PCR-RFLP is a preferred identification technique in many cases, as it is reproducible, straightforward, efficient and relatively inexpensive (Brunner *et al.*, 2002). Since the rDNA unit is repetitive in nature, the ITS regions can be readily amplified from any blowfly life cycle stage, and therefore have considerable potential to be employed as a sensitive laboratory tool to identify immatures, including eggs, as well as adults. Although PCR-RFLP can be developed for species identification without prior determination of the sequence (e.g. Ratcliffe *et al.*, 2003), this approach has its limitations as the presence of polymorphisms can go undetected. Although every nucleotide is detected with DNA sequencing, only the restriction sites present in a

particular sequence are assessed by RFLP analysis. A potential problem with an RFLP technique is that a single point mutation within a species or population can completely alter the restriction profile of a specimen, possibly leading to an incorrect identification. To ensure that diagnostic restriction sites within a population are fixed, it is imperative that the occurrence of intraspecific variation is established. DNA sequencing gives a much better indication of the level of sequence variation between species, and allows for accurate prediction of restriction sites without the need for extensive screening. This will ultimately minimise the possibility of species misidentification.

1.4.2. Phylogenetic analysis

Due to the existence of intraspecific variation within taxa, some workers suggest that sequence-based identification should be the preferred technique as this takes advantage of all sequence information from a particular species (e.g. Brunner *et al.*, 2002). Phylogenetic analysis is a method of species identification which exploits the maximal information content of a sequence. The technique can, in principal, be utilised to identify forensic entomological specimens, and has been investigated for the identification of blowflies using mitochondrial DNA (e.g. Wells *et al.*, 2001; Wells & Sperling, 2001; Wells, 2002; Zehner *et al.*, 2004). This method of identification involves the inclusion of sequence data from an unidentified specimen in a phylogenetic analysis of the same region from identified specimens of the same taxonomic group (Brunner *et al.*, 2002). Identification is then made based on the statistical confidence of the grouping of the unidentified specimen with an identified specimen within the database (Wells *et al.*, 2001; Brunner *et al.*, 2002).

Specimen identification using suitable gene region(s) in phylogenetic analysis will work provided that the species of the unidentified specimen is represented in the database (Wells & Sperling, 2001). To ensure a successful identification is made, it is vital for the reference databases to be as complete and relevant as possible to the local fly fauna. One of the advantages of a phylogeny-based identification system is that potential intraspecific variation can be easily incorporated into the analysis by

expanded taxon sampling, unlike PCR-RFLP analysis where the existence of such variation can invalidate the results. Provided that there is no overlap between intraspecific and interspecific variation, a 'divergence factor' can be decided upon, whereby all unidentified sequences within this tolerance level are accepted as being from a particular species (Wells *et al.*, 2001). Even if the specimen is forced to group with a close relative due to incomplete representation of species in the database, the phylogenetic analysis may still lead to useful forensic inferences. For instance, a specimen may group with relatives that share similar developmental profiles, which may still aid in the estimation of the PMI. Genetic relatedness might also prove useful for extrapolating developmental times for species that have not been studied in the laboratory.

1.4.3. DNA barcoding

The larger genetic variability that exists between species (compared to 'within species' variation) has long been exploited to develop molecular identification systems for select groups of taxa (e.g. Campbell *et al.*, 1995; Armstrong *et al.*, 1997; Kengne *et al.*, 2003). A 'DNA barcode' is any short sequence of DNA (400-800 bp) that is sufficiently variable to function as a unique species identifier. DNA barcodes have been employed for complex groups such as nematodes and mosquitoes (e.g. Floyd *et al.*, 2002; Besansky *et al.*, 2003). To be of use as a DNA barcode, a DNA sequence must (1) contain significant species-level genetic variability and divergence, (2) possess conserved flanking sequences for developing universal primers for amplification of the barcode region in numerous taxa, and (3) have a short sequence length to facilitate rapid amplification and sequencing of the region from samples of all qualities (Hebert *et al.*, 2003; Stoeckle, 2003).

Recently, there has been a suggestion to standardise the method and DNA region of choice for the identification of all animals on Earth (Hebert *et al.*, 2003). A 658 bp region of the gene encoding the first subunit of mitochondrial cytochrome oxidase (COI) has been shown to be suitable for the identification of a range of taxa including protozoa (Hymenostomatida) (Lynn & Strüder-Kypke, 2006), gastropods (Mollusca)

(Remigio & Hebert, 2003), springtails (Collembola) (Hogg & Hebert, 2004), butterflies (Lepidoptera) (Hebert *et al.*, 2004a), birds (Hebert *et al.*, 2004b), mayflies (Ephemeroptera) (Ball *et al.*, 2005) and fish (Ward *et al.*, 2005). While the COI DNA barcode (hereafter referred to as the 'DNA barcode') may not provide deep phylogenetic resolution, it provides enough phylogenetic signal to identify sequences, and hence species (Schander & Willassen, 2005). The proponents of COI DNA barcoding envisage the construction of a universally accessible, curated database comprising all animal COI sequences, which will then serve as 'the basis for a global bioidentification system for animals' (Hebert *et al.*, 2003). The Consortium for the Barcode of Life (CBOL; http://barcoding.si.edu/) was launched in 2004 and contains more than 160 organisations from 50 nations. The need for a barcode database motivated development of the Barcode of Life Data System; a sophisticated barcode repository, workbench and bioinformatics platform for species identification and discovery (www.barcodinglife.org; Ratnasingham & Hebert, 2007).

The benefits of DNA barcoding to species identification and discovery have been presented (e.g. Hebert & Gregory, 2005; Savolainen *et al.*, 2005). It is believed that a standardised approach to species identification would consolidate global research efforts and encourage data exchange (Armstrong & Ball, 2005). A DNA barcoding system would be enormously beneficial for critical identifications, including those species with medical, ecological, economic or environmental significance (e.g. Besansky *et al.*, 2003; Scicluna *et al.*, 2006). DNA barcoding has been shown to be valuable for insect surveillance and quarantine purposes, and displays potential as a tool for unified and coordinated global pest species detection (Ball *et al.*, 2005; Ball & Armstrong, 2006). In addition to species identification, the construction of a barcode database could expose novel DNA barcodes that may indicate provisional new species (Hebert *et al.*, 2004a).

The DNA barcoding concept has received extensive attention in the media (e.g. The Economist, 2004; Wade, 2004; Amos, 2005; Leahy, 2005) and other pertinent publications (e.g. Pennisi, 2003; Holmes, 2004; Roach, 2005; Holloway, 2006). Although the concept has received criticism (e.g. Will & Runbinoff, 2004; Ebach &

Holdrege, 2005; Wheeler, 2005), barcode advocates believe that this resistance is due to misconceptions (Hebert & Gregory, 2005). While the ability to assign unknown specimens to already characterised species is conceivable, the use of the barcode as a species discovery tool is more contentious (e.g. Sperling, 2003; Smith, 2005; Will *et al.*, 2005). Concerns have been expressed regarding the capacity of a single DNA sequence to discriminate all animal species (e.g. Moritz & Cicero, 2004; Will & Runbinoff, 2004). Barcoding advocates maintain that the technique would be successful for the majority of animal taxa, and that it would be used as a tool to recognise potential new species whose status could only be resolved with further studies (Hebert *et al.*, 2003; Schindel & Miller, 2005). Although proponents of DNA barcoding insist that the method is not intended to resolve taxonomic relationships (e.g. Schindel & Miller, 2005), opponents argue that the system will place excessive reliance upon DNA rather than morphology, which will in turn promote the generation of inferior phylogenies (e.g. Will & Runbinoff, 2004). There is concern that DNA barcoding will compete with traditional taxonomy for both students and funding (e.g. Ebach & Holdrege, 2005), although this has been refuted (Gregory, 2005; Hebert & Gregory, 2005).

Morphology-based identifications of specimens can often be particularly challenging, requiring the services of expert taxonomists whose numbers are limited and declining (Besansky *et al.*, 2003; Tautz *et al.*, 2003; Ball & Armstrong, 2006). It has been suggested that a DNA barcoding approach would free these experts from routine identifications (Tautz *et al.*, 2002; Hebert & Gregory, 2005). A DNA-based identification system has the capacity to identify all life stages, including morphologically cryptic species and those displaying phenotypic plasticity (e.g. Carew *et al.*, 2005). Additionally, the sensitivity of a PCR-based system enables the amplification of DNA from very small pieces of tissue; DNA barcoding has already proven successful with dried museum specimens up to 23 years old (Hebert *et al.*, 2004a). Barcoding is said to increase the speed, objectivity and efficiency of species identifications (Meyer & Paulay, 2005). A system such as this would facilitate a more standardised approach to the identification of entomological evidence, and would provide the opportunity to build a DNA sequence database that could link geographic

origin data with specific voucher specimens. If successful, a DNA barcoding approach could therefore have significant benefits for forensic entomology, particularly when (1) specimens are difficult to identify morphologically (eggs, larvae and partial or damaged specimens) and (2) taxonomic expertise is not readily accessible.

1.5. Conclusions

The utilisation of forensic insect evidence in PMI estimation is reliant upon rapid and accurate species identification. The multitude of forensically important species represents a substantial obstacle to streamlined PMI estimation. Molecular markers are powerful tools that can be valuable alternatives to traditional morphological methods of identification. DNA-based identification of forensically important blowflies is potentially valuable in terms of speed, versatility, reliability and simplicity. In terms of speed, the sensitivity of DNA-based techniques enables the identification of material from any stage of the life cycle, thus not requiring the lengthy and risky rearing of larvae to adulthood (Marrelli *et al.*, 2005). In terms of versatility, PCR amplification techniques are effective for screening material insufficient for morphological identification, including fragmented insect remains and preserved specimens (Malgorn & Coquoz, 1999). Reliability is also improved, given adequate population sampling. Finally, in terms of simplicity, the identification procedure can be performed by any trained laboratory worker, as comprehensive knowledge of morphology is not required. Identification by direct comparison of sequences (either by complete sequencing, PCR-RFLP or DNA barcoding) has the potential to be efficient and cost-effective. For example, a PCR-RFLP approach has the ability to identify entomological specimens in approximately six hours in a standard laboratory (Ratcliffe *et al.*, 2003). Such molecular techniques can reduce turnaround times for the processing of entomological evidence and, perhaps, expedite the identification of a suspect.

1.6. Aims

1. ***To develop a reliable molecular method/s for streamlined identification of Australian blowflies***

Hypothesis: All Australian *Chrysomya* species can be distinguished diagnostically by DNA methods.

Many studies have proposed molecular methods for identifying forensically important blowflies, but few have examined Australian species. No studies have focused entirely on the identification of Australian blowflies of the genus *Chrysomya*. In order to investigate molecular methods for identification of members of this genus, three analytical approaches will be assessed:

i. Profile polymerase chain reaction restriction fragment polymorphism (PCR-RFLP) of the ITS2 region (Chapter 2);
ii. Evaluate phylogenetic-based identification using ITS2 sequences (Chapter 2);
iii. Cytochrome oxidase I (COI) 'DNA barcoding' (Chapter 3);
    a. Evaluate the barcoding approach (in general), and
    b. Test the utility of the approach for identification of Australian members of the genus *Chrysomya.*

2. ***To study the thermal attributes of members of this genus*** **(Chapter 4)**

Hypothesis: Some Australian *Chrysomya* species share similar thermodevelopmental profiles.

Studies concerning the developmental profiles of Australian *Chrysomya* are currently lacking. Together with correct species identification, this information is vital for the estimation of the postmortem interval. In addition, previous studies have revealed close morphological similarities and low genetic divergences among Australian *Chrysomya* (e.g. Wallman *et al.*, 2005). Consequently, there is speculation as to

whether members of this genus share similar developmental profiles. This study will provide important developmental data for three Australian *Chrysomya* species to:

i. Contribute crucial data for estimation of the PMI by forensic entomologists;
ii. Establish whether very closely related species (determined from genetic analyses) differ in their growth rates;
iii. Determine whether one set of developmental data can be applied to a group of closely related flies, and
iv. Ascertain whether growth rates for one species can be inferred from data already obtained from a close relative.

If Australian *Chrysomya* are found to share developmental profiles, identification of the blowfly specimen to a level beyond genus may not be necessary, or at least it may not be necessary to distinguish morphologically similar sister species. Conversely, if developmental profiles are found to differ significantly among the species, the need for correct species identification is imperative.

1.7. Thesis format

Since most of this thesis has already been published, each chapter has been written as a journal article. The general format remains this way except for a few modifications to make the chapters consistent and understandable. Consequently, some repetition exists between the General Introduction and the introduction of each chapter, as well as between the General Conclusions and the discussion of each chapter. There is also some repetition among chapters of the methodology used. The title page of each chapter details publication information. All references have been compiled to form one chapter at the end of the thesis.

Two female *Chrysomya rufifacies* resting in a tree above carrion.
Photo: L.A. Nelson

Chapter 2. Identification of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2)

This chapter is slightly modified from the paper:

Nelson, L.A., Wallman, J.F. and Dowton, M. (2008) Identification of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2), *Forensic Science International,* 177: 238–247.

Contributions: JFW and MD designed research; LAN performed research; LAN analysed the data; LAN wrote the paper.

Please note that this work was first started in the year preceding the PhD.

2.1. Introduction

The identification of invertebrate species found in association with a corpse is the first step undertaken by the forensic entomologist in an attempt to estimate the postmortem interval (PMI) (Wells & LaMotte, 2001). It is important that the identification process is both rapid and accurate. The use of blowflies (Diptera: Calliphoridae) in PMI estimation has been hampered by difficulties associated with correct species identification at all developmental stages (eggs, larvae, pupae and adults) (Catts, 1992; Benecke, 1998). The genus *Chrysomya* may represent a large component of the blowfly species present in cases of murder or suspicious death (e.g. Levot, 2003). The identification of several species of this genus is hampered by their similar morphologies, even as adults (Wells & Kurahashi, 1996). Molecular data also suggest very close genetic relationships among some members of the genus *Chrysomya* in Australia (Wallman *et al.*, 2005), but offer a potentially less ambiguous approach to their identification.

One method of molecular species identification involves polymerase chain reaction amplification (PCR) of a DNA region followed by restriction fragment length polymorphism (RFLP) analysis. The complete internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) has been found to be a reliable marker for PCR-RFLP identification of many species, including economically important fruit flies (Armstrong *et al.*, 1997) and, more recently, morphologically similar dipteran larvae of forensic importance (Ratcliffe *et al.*, 2003). These findings suggest that the ITS regions might be sufficiently variable to construct unique identification markers for a number of carrion-breeding blowflies, including Australian members of the genus *Chrysomya.*

Another method of species identification applying molecular data uses phylogenetic analysis – sequence data from an unidentified specimen is analysed using the same gene region as identified specimens of the same taxonomic group (Brunner *et al.*, 2002). The identity of the unknown specimen is deduced from its pairing with its closest relative within a phylogenetic tree. This method has been investigated for the

identification of forensically important blowflies using mitochondrial DNA (e.g.Wells *et al.*, 2001; Wells & Sperling, 2001; Wells, 2002). It is possible that the ITS regions of Australian *Chrysomya* species may contain sufficient interspecies sequence variation to facilitate species identification using this method.

DNA-based identification of forensically important blowflies is potentially a valuable alternative to traditional morphological methods, providing speed, versatility, reliability and simplicity. In terms of speed, the sensitivity of DNA-based techniques enables the identification of material from any stage of the life cycle, thus not requiring the lengthy and risky rearing of larvae to adulthood (Marrelli *et al.*, 2005). In terms of versatility, PCR amplification techniques are effective for screening material insufficient for morphological identification, including fragmented insect remains and preserved specimens. Reliability is also improved, because, given adequate population sampling, taxon identification is more certain, especially given the difficulties of identifying blowfly immatures. Finally, in terms of simplicity, the identification procedure can be performed by any trained laboratory worker, as comprehensive knowledge of morphology is not required. A PCR-RFLP approach has the ability to identify entomological specimens in approximately 6 h in a standard laboratory (Ratcliffe *et al.*, 2003), without the need to access a sequencing facility as required by other methods (e.g. Wells *et al.*, 2001), which can speed up turnaround times for the processing of entomological evidence and, perhaps, expedite the identification of a suspect.

The present study extends the work of Wallman *et al.* (2005) on Australian blowflies. However, instead of investigating the phylogenetic relationships among all carrion-breeding species using a multi-gene approach, we investigate the utility of a single, more variable marker for differentiating closely related blowflies within a single genus. We assess the applicability of the ITS2 region of rDNA, using PCR-RFLP, for the differentiation of all species of *Chrysomya* known from Australia, and evaluate the potential usefulness of this region for the phylogenetic identification of these species. With the exception of *Ch. saffranea* and *Ch. latifrons*, all *Chrysomya* species

included in this study are also found in other countries, particularly within the Asian region (Kurahashi, 1989).

2.2. Materials and Methods

2.2.1. Specimens

Adult *Chrysomya* specimens were collected from locations along the east coast of Australia and identified morphologically by JFW (Table 2.1). The specimens were collected directly into absolute alcohol, in which they were stored. Voucher specimens, stored in ethanol at 4°C, are maintained in the Diptera Collection in the School of Biological Sciences, University of Wollongong.

2.2.2. DNA Extraction

Two legs from each fly were found to be sufficient for the extraction of genomic DNA, which was performed by a "salting out" protocol (Sunnucks & Hales, 1996). The DNA was resuspended in 50 µl of sterile distilled water and stored at 4°C.

2.2.3. PCR amplification

Initially, the entire ITS region containing both ITS1, 5.8S and ITS2 plus primer flanking sequence was amplified as a single PCR product using primers 1975F (5'-TAACAAGGTTTCCGTAGGTG-3') and 52R (5'-GTTACTTTCTTTTCCTCCCCT-3') (Ratcliffe *et al.*, 2003) (Sigma Genosys, Sydney, Australia), with the aim of sequencing both ITS1 and ITS2. Additionally, two primers, designated L1 (5'-RRCGGTGGATCACTCGGCTC-3') and L2 (5'-AYCCAYGAGCCGAGTGATCC-3') (Sigma Genosys) were designed to anneal to the 5.8S gene to prime internal sequencing reactions for ITS2 and ITS1, respectively. These primers were selected based on comparison of conserved regions of the 5.8S gene in flies downloaded from

GenBank, especially from *Lucilia illustris, Calliphora vicina* and *Drosophila melanogaster* (accession numbers AF498038, AF498026 and U20145, respectively).

Amplification of both the entire ITS region and ITS2 alone was carried out as follows: each 20 µl PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% TritonX-100, deoxynucleotide triphosphates (dNTPs) (25 µM each of dATP, dCTP, dGTP and dTTP), 1.25 mM $MgCl_2$, 0.4 µM of each primer and 0.75 U *Taq* DNA polymerase (Promega, Madison, WI). Amplification of the entire ITS region required the addition of 2.5 mU of *Taq Pfu* (*Pyrococcus furiosus*) (Promega). The entire ITS region of *Ch. flavifrons*, which could not be amplified by this procedure, was amplified as follows: each 20 µl reaction mixture contained 2 µl OptiBuffer™ (Bioline, Luckenwalde, Germany), 3.75 mM $MgCl_2$, dNTPs (62.5 µM each of dATP, dCTP, dGTP and dTTP), 0.4 µM of each primer and 1.6 U BIO-X-ACT™ Long DNA Polymerase (Bioline). All reaction mixtures contained 0.5 µl DNA extract, replaced by 0.5 µl H_2O in the negative controls.

PCR reactions were carried out on an automated Palm-CyclerII (Corbett Research, Sydney, Australia). The temperature cycle for amplification of the entire ITS region consisted of an initial denaturation step at 95˚C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing across a temperature gradient of 45-65°C for 30 s, and elongation at 68°C for 3 min. The last cycle was followed by 10 min incubation at 68°C to complete any partially synthesised strands. The ITS2 temperature cycle consisted of an initial denaturation step at 94˚C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing across a temperature gradient of 45-65°C for 30 s, and elongation at 72°C for 2 min. The last cycle was followed by 5 min incubation at 72°C to complete any partially synthesised strands. All PCR products were visualised by agarose gel electrophoresis and stored at 4°C. PCR products were purified by polyethylene glycol (PEG) precipitation (Lis, 1980) prior to restriction digestion and sequence analysis.

Table 2.1. Voucher codes, collection data and accession numbers for the Australian *Chrysomya* species from which DNA was extracted.

Species	Voucher	GenBank accession number	Sex	Collection locality	Date collected
Ch. flavifrons (Aldrich)	LN7	DQ310494–DQ310497	♀	Kuranda, Qld (16°49′S, 145°38′E)	22 January 2002
Ch. latifrons (Malloch)	LN2	DQ310492	♀	Halfway Creek, NSW (30°14′S, 153°06′E)	17 April 2001
Ch. megacephala (Fabricius)	LN5	DQ310488	♂	Karuah, NSW (32°38′S, 151°57′E)	19 April 2001
Ch. nigripes Auberton	LN6	DQ310489	♀	Kuranda, Qld (16°49′S, 145°38′E)	22 January 2002
Ch. rufifacies (Macquart)	LN8	DQ310487	♂	Kuranda, Qld (16°49′S, 145°38′E)	22 January 2002
Ch. saffranea (Bigot)	LN9	DQ310490	♀	Kuranda, Qld (16°49′S, 145°38′E)	22 January 2002
Ch. semimetallica (Malloch)	LN1	DQ310493	♀	Halfway Creek, NSW (30°14′S, 153°06′E)	17 April 2001
Ch. varipes (Macquart)	LN3	DQ310491	♀	Karuah, NSW (32°38′S, 151°57′E)	19 April 2001
Ch. incisuralis (Macquart)	LN4	DQ310486	♀	Halfway Creek, NSW (30°14′S, 153°06′E)	18 April 2001
Ch. incisuralis (Macquart)	JW26	—	♀	Halfway Creek, NSW (30°14′S, 153°06′E)	18 April 2001
Ch. incisuralis (Macquart)	JW133	—	♀	Halfway Creek, NSW (30°14′S, 153°06′E)	18 April 2001
Ch. incisuralis (Macquart)	JW28	#	♀	Way Way State Forest, NSW (30º48′S, 152º55′E)	18 April 2001
Ch. incisuralis (Macquart)	JW29	—	♀	Coffs Harbour, NSW (30º18′S, 153º07′E)	16 April 2001
Ch. incisuralis (Macquart)	JW46	—	♀	Kuranda, Qld (16°49′S, 145°38′E)	21 January 2002
Ch. incisuralis (Macquart)	JW70	#	♂	Tinaroo Falls, Qld (17º10′S, 145º33′E)	23 January 2002
Ch. incisuralis (Macquart)	JW63	—	♂	Coranderrk Bushland Reserve, Vic. (37º40′59″S, 145º31′04″E)	15 February 1999

A dash (—) indicates specimens from which ITS2 was not sequenced. A hash (#) indicates specimens whose ITS2 sequences were identical to *Ch. incisuralis* voucher LN4, Qld = Queensland; NSW = New South Wales; Vic. = Victoria.

2.2.4. DNA Sequencing

Sequencing reactions were performed with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Version 3.1) (PE Applied Biosystems, Foster City, CA) using the Palm-CyclerII (Corbett Research). PCR primers were used to initiate the sequencing reactions, which were set up and purified according to the manufacturer's instructions. Sequencing reaction products were then separated using the Automated ABI PRISM™ 377 Sequencer (PE Applied Biosystems) or the 3130x Genetic Analyzer (PE Applied Biosystems). Due to difficulties encountered with direct sequencing for *Ch. flavifrons*, the purified ITS2 PCR product was cloned using the pGEM-T Easy Vector System (Promega) and transformed into *Escherichia coli* strain JM109 competent cells. Four clones were sequenced from this species using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Version 3.1) (PE Applied

Biosystems) with the vector primers M13F (5'-GGCCGCGGGAATTCGATT-3') and M13R (5'-CGAATTCACTAGTGATT-3').

DNA sequences were confirmed and edited manually using BioEdit Sequence Alignment Editor (Version 6.0.7; Hall, 1999). Sequences from each species were aligned using CLUSTAL W (Thompson *et al.*, 1994) with the default settings. The boundaries of ITS2 were deduced by comparisons with rDNA sequences of dipteran species including *Calliphora vicina* (AF498025), *Chrysomya albiceps* (CAL551433) and *Lucilia illustris* (AF498038). Nucleotide sequences reported in this paper have been submitted to GenBank and their respective accession numbers are indicated in Table 2.1.

2.2.5. Phylogenetic analysis

Phylogenetic analyses were performed by Bayesian inference (MrBayes, v3.1; Huelsenbeck & Ronquist, 2001), as it estimates the substitution model and rate parameters directly from the data. The general time reversible model (command: nst = 6; Tavaré, 1986), with variable sites assumed to follow a discrete gamma distribution (command: rates = gamma; Yang, 1994) (GTR + Γ) was selected as the best-fit model of nucleotide substitution (MrModeltest v2.2; Nylander, 2004). Because MrModeltest does not assess whether rates should be allowed to vary in different lineages (command: covarion = yes; Penny *et al.*, 2001), we assessed this using Bayes factors, as described in Nylander *et al.* (2004), wherein the likelihood of trees is compared with and without the additional covarion parameter. For the ITS2 analysis, Bayes factors indicated a significant improvement in likelihood when rates were allowed to vary within a lineage, and therefore covarion = yes was included in the model. In some analyses, sequence data from three mitochondrial gene regions (ND4-ND4L, COI and COII) were included (Wallman *et al.*, 2005). These data were divided into nine partitions, each partition representing a different coding position for each of the three regions. The mitochondrial dataset was analysed using a general time reversible model with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (GTR + I + Γ; Yang, 1994). For combined

analyses, substitution and rate parameters were estimated separately for each partition (command: unlink; Ronquist & Huelsenbeck, 2003).

For each analysis, two independent runs were performed. Each Markov chain Monte Carlo (MCMC) process was set so that four chains, three heated and one cold, ran simultaneously. We conducted runs for 1 million generations, with trees being sampled every 100 generations yielding a total of 10,000 trees for analysis. Independent analyses indicated that "stationarity" (or "burnin": lack of improvement in maximum likelihood scores) was reached at no later than 50,000 generations; thus, the first 500 trees were discarded from each analysis as the burnin, and the remaining trees were used to generate a 50% majority consensus tree. Posterior probabilities were estimated by counting the proportion of trees that recovered a particular group after the burnin period. Three non-*Chrysomya* species were included in all analyses, with *Hemipyrellia fergusoni* designated as the outgroup.

2.2.6. ITS2 restriction digestion

Restriction enzyme nomenclature and recognition sites are reported as recommended by Roberts *et al.* (2003). ITS2 PCR products were digested separately with the restriction enzymes Dra I (recognition sequence: TTT↓AAA), BsaX I [recognition sequence: (9/12)ACNNNNNCTCC(10/7)], BciV I [recognition sequence: (5/6)GGATAC], Ase I (recognition sequence: AT↓ TAAT) (New England Biolabs, Ipswitch, MA) and Hinf I (recognition sequence: G↓ANTC) (Promega). Approximately 80 ng of PCR product was digested for 2.5-3.0 h at 37°C in a total of 10 µl using between 1.0 and 2.5 units of each restriction enzyme. Samples without endonuclease were included as controls. Restriction products were resolved in 2% w/v agarose-TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0) gels at 80 V, stained with ethidium bromide and photographed over UV light. Size comparisons were made to molecular size standards (250 ng of EcoR I/Hind III-digested lambda DNA [Promega] or 600 ng of Hyperladder II [Bioline]).

2.3. Results

2.3.1. Amplification of the entire ITS region

The entire ITS region from all *Chrysomya* species investigated in this study was amplified without difficulty, with the exception of *Ch. flavifrons*. Despite an extensive optimisation schedule, this target was difficult to amplify using the conventional reaction conditions. Amplification of the fragment was finally achieved using the BIO-X-ACT™ Long DNA Polymerase Kit (Bioline). The PCR fragments produced from each species varied in size, being approximately 1010 bp (*Ch. varipes*), 1036 bp (*Ch. incisuralis* [LN4]), 1120 bp (*Ch. flavifrons*), 1150 bp (*Ch. rufifacies*), 1181 bp (*Ch. semimetallica*), 1213 bp (*Ch. latifrons*), 1281 bp (*Ch. megacephala*), 1354 bp (*Ch. nigripes*) and 1354 bp (*Ch. saffranea*) (Fig. 2.1). These fragments are of similar size to those reported by Ratcliffe *et al.* (2003) for forensically important Diptera (1200-1500 bp). Although minor bands can be observed, especially from the amplification of *Ch. flavifrons* (lane 3), it appears that the major fragments generated from most species were of different sizes. The uniquely sized fragments allow tentative identification of most species analysed here, with the exceptions of distinguishing *Ch. latifrons* from *Ch. semimetallica,* and *Ch. nigripes* from *Ch. saffranea*, due to ITS fragment size similarities.

In order to assess intraspecific size variation, the ITS region was amplified from eight *Ch. incisuralis* specimens collected from six geographical populations (Table 2.1). The PCR fragments produced from each of these specimens were identical in size, with all fragments being approximately 1000 bp (data not shown). The fragment size similarity within *Ch. incisuralis* suggests that there is less intraspecific ITS size variation than there is interspecific size variation in this species.

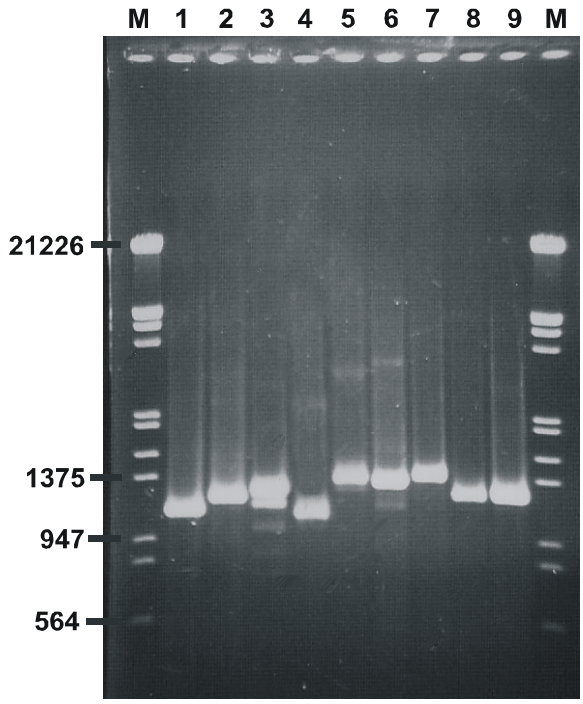


Figure 2.1. Agarose gel of PCR fragments containing the entire ITS region amplified from Australian members of the genus *Chrysomya.* Lanes 1 to 9 are fragments amplified from *Ch. incisuralis* (1), *Ch rufifacies* (2), *Ch. flavifrons* (3), *Ch. varipes* (4), *Ch. nigripes* (5), *Ch. megacephala* (6), *Ch. saffranea* (7), *Ch. latifrons* (8) and *Ch. semimetallica* (9). M = EcoR I/Hind III-digested lambda DNA molecular standards; bars indicate sizes (bp) of selected molecular standards.

2.3.2. Amplification of ITS2

Initial experiments were directed at amplifying the entire ITS region with the aim of sequencing ITS1 and ITS2 to investigate the level of interspecific variation in this region, and to identify restriction sites. Sequencing reactions yielded high quality sequencing data from the ITS2 primers but not from the ITS1 primers, with ITS1 electropherograms displaying overlapping peaks and high background noise. These sequencing difficulties shifted the focus of the study to the ITS2 region alone, which was amplified separately. The amplification generated fragments of approximately 560 bp in length for all *Chrysomya* species (data not shown). Amplification from *Ch. flavifrons* (particularly the entire ITS region) gave several shadow bands suggesting the presence of multiple rDNA sequence variants in this species. The ITS2 products

were subsequently used for direct sequencing, or in the case of *Ch. flavifrons*, for cloning prior to sequencing, and for restriction analysis.

2.3.3. ITS2 sequences from *Chrysomya* species

Ribosomal DNA sequences were generated for the ITS2 of all nine Australian *Chrysomya* species (Fig. 2.2). Sequencing confirmed that the ITS2 fragments were of similar, and in some cases identical, lengths for all species, ranging from 430 bp (*Ch. megacephala*) to 449 bp (*Ch. nigripes*). *Chrysomya favifrons* is represented by the consensus sequence from four clones. Prior to this, incomprehensible sequence reads from *Ch. flavirons* suggested the presence of intraindividual variation. To assess this, four ITS2 clones from *Ch. flavifrons* were sequenced in both directions. Several nucleotide differences were detected, including two base substitutions and variable length within a poly-T region (Fig. 2.2). The four cloned sequences possessed between 0 and 0.23% variation within the 454 ITS2 nucleotides that were compared.

The multiple sequence alignment exposed a substantial degree of variation between species, with base substitutions, insertions and deletions all being present. Sequence divergences ranged from just 0.23% between *Ch. latifrons* and *Ch. semimetallica*, to 11.82% between *Ch. nigripes* and *Ch. semimetallica* (Table 2.2). Tracts of conserved sequence were followed by highly variable regions, the latter being potentially useful for species identification purposes.

Table 2.2. Pairwise sequence divergences (calculated using the Kimura-two-parameter distance model) between Australian members of the genus *Chrysomya* based on differences in nucleotide sequences of ITS2.

		1	2	3	4	5	6	7	8	9
1	*incisuralis*	—								
2	*rufifacies*	0.0218	—							
3	*megacephala*	0.0851	0.1024	—						
4	*nigripes*	0.0900	0.0899	0.0955	—					
5	*saffranea*	0.0899	0.1072	0.0046	0.1005	—				
6	*varipes*	0.0762	0.0761	0.0791	0.0814	0.0814	—			
7	*latifrons*	0.0814	0.1032	0.0774	0.1107	0.0718	0.0979	—		
8	*semimetallica*	0.0890	0.1084	0.0775	0.1182	0.0718	0.1030	0.0023	—	
9	*flavifrons*	0.0801	0.0854	0.0783	0.0931	0.0805	0.0287	0.0964	0.1015	—

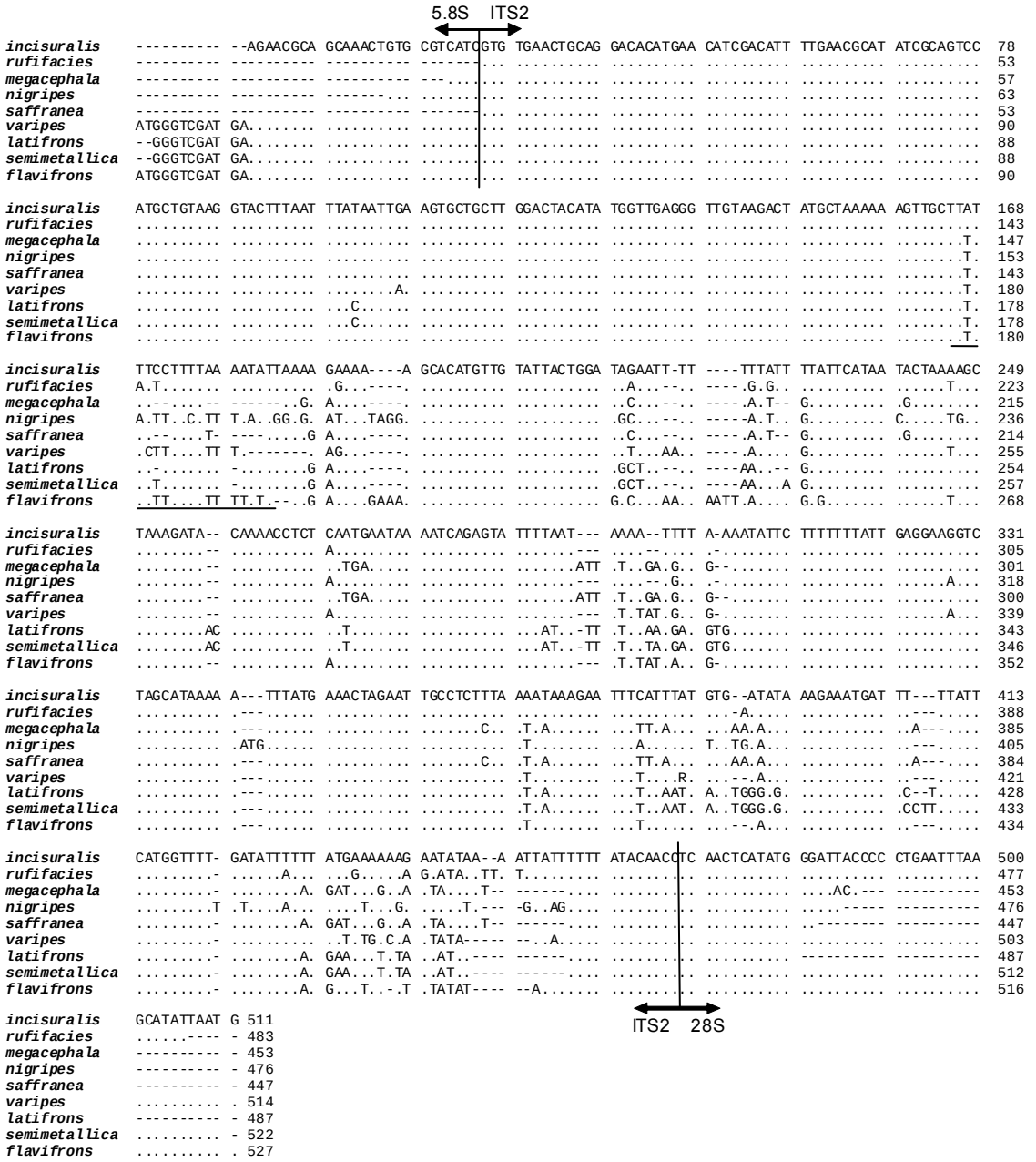


Figure 2.2. Alignment of ITS2 sequences of all Australian members of the genus *Chrysomya*. Where a nucleotide is identical to the topmost species (*incisuralis*), identity is indicated with a full stop (.); hyphens (-) are introduced to achieve maximum alignment (insertions/deletions) and nucleotide symbols follow IUB codes (R: G/A). The region of variable T-length among *Ch. flavifrons* clones is underlined.

2.3.4. Assessment of geographical variation in ITS2 sequences of *Ch. incisuralis*

Inspection of sequence data obtained from three *Ch. incisuralis* specimens (LN4, JW28 and JW70) revealed complete ITS2 sequence identity. This is of significance as the specimens were sampled from populations separated by a minimum of approximately 100 km (between specimens LN4 and JW28) and a maximum of approximately 1690 km (between specimens JW28 and JW70). This intraspecific sequence homogeneity supports the theory that interspecific ITS variation is considerably larger than the intraspecific variation (Navajas *et al.*, 1998).

2.3.5. Restriction analysis

Using BioEdit (Hall, 1999), the ITS2 sequence data were assessed for the presence of unique restriction sites that could be exploited for PCR-RFLP analysis. This process identified five restriction enzymes (Dra I, BsaX I, BciV I, Ase I and Hinf I) that could differentially digest the Australian *Chrysomya* species (Table 2.3). The identification of these five restriction enzymes was later confirmed using the program PCR-RFLP Designer (available at http://webdev.sitacs.uow.edu.au/research/honours/users/ajs91/).

The restriction digestions yielded fragments whose combined approximate sizes corresponded to those of the undigested fragments. Negative (no enzyme) controls revealed PCR products of approximately 560 bp in size for all species (Fig. 2.3). Although restriction digestions followed the manufacturer's protocols, undigested ITS2 fragments were faintly visible in some digestions. Experimentation with incubation times and restriction enzyme concentrations did not reduce the amount of undigested product remaining. For this reason, it was believed that undigested product was due to minor rDNA sequence polymorphism at the restriction site within an individual fly. This was not seen to be a problem with the identification system, as the amount of undigested product remaining was always negligible.

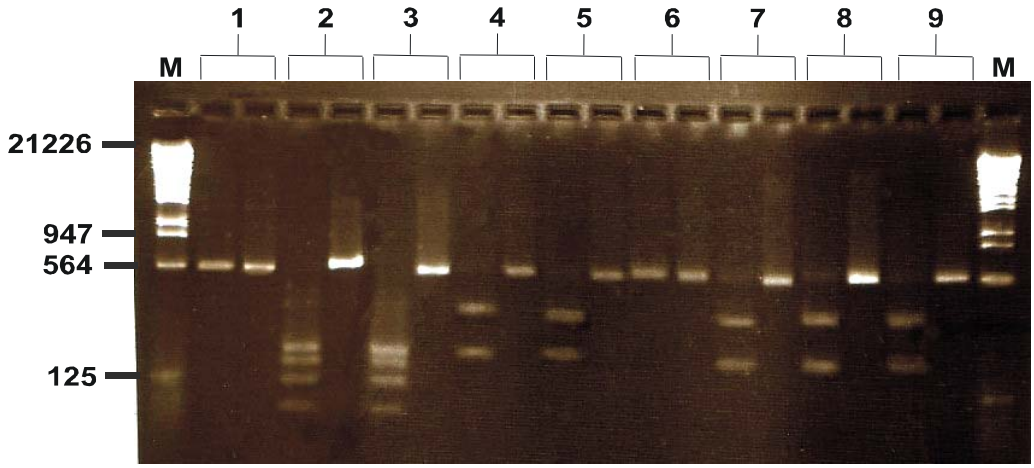


Figure 2.3. An example of a 2% agarose gel showing PCR-RFLP profiles obtained following digestion of the rDNA ITS2 with Dra I. Numbers 1-9 refer to digested (left lane) and undigested (right lane) ITS2 fragments from *Ch. megacephala* (1), *Ch. incisuralis* (2), *Ch. rufifacies* (3), *Ch. nigripes* (4), *Ch. varipes* (5), *Ch. flavifrons* (6), *Ch. saffranea* (7), *Ch. semimetallica* (8) and *Ch. latifrons* (9). M = EcoR I/Hind III-digested lambda DNA molecular standards; bars indicate sizes (bp) of selected molecular standards.

2.3.6. Digestion with Dra I

ITS2 sequence data indicated that *Ch. saffranea, Ch. varipes, Ch. nigripes, Ch. latifrons* and *Ch. semimetallica* should have one Dra I restriction site, *Ch. incisuralis* and *Ch. rufifacies* should have three Dra I sites, while *Ch. megacephala* and *Ch. flavifrons* lack Dra I sites. Digestion of the ITS2 products from all *Chrysomya* species produced two fragments for the species with one restriction site (approx. 210 and 350 bp) and four fragments for the species with three restriction sites (approx. 65, 125, 165 and 210 bp), while those with no restriction sites remained undigested (approx. 560 bp) (Fig. 2.3).

Table 2.3. Calculated restriction fragment sizes (bp) following digestion of PCR-amplified ITS2 region of Australian *Chrysomya* species with five restriction enzymes. Cut sites are those within the ITS2 region only, while fragment sizes are "as appearing on gel", i.e. including flanking sequence and primers.

Chrysomya species	Restriction enzymes									
	Dra I		Hinf I		BsaX I		BciV I		Ase I	
	Cut site	Fragment size (bp)	Cut site	Fragment size (bp)	Cut site	Fragment size (bp)	Cut site	Fragment size (bp)	Cut site	Fragment size (bp)
flavifrons	-	568	-	568	-	568	-	568	201	310 258
incisuralis	152 278 343	209 164 126 65	-	564	-	564	-	564	-	564
latifrons	151	353 208	384	441 120	-	561	-	561	-	561
megacephala	-	551	-	551	-	551	172	322 229	-	551
nigripes	155	358 212	-	570	194 164	319 221 30	-	570	-	570
rufifacies	152 277 342	209 167 125 65	-	566	-	566	-	566	-	566
saffranea	153	345 210	-	555	-	555	175	323 232	-	555
semimetallica	152	357 209	387	444 122	-	566	-	566	-	566
varipes	155	343 212	-	555	-	555	-	555	-	555

2.3.7. Digestion with other restriction enzymes (Hinf I, BsaX I, BciV I and Ase I)

Sequence data revealed the presence of a single Hinf I restriction site for *Ch. semimetallica* and *Ch. latifrons*. Digestion of ITS2 products with Hinf I produced two fragments (approx. 120 and 440 bp) from these species only. Sequence analysis identified two unique BsaX I restriction sites in the ITS2 sequence of *Ch. nigripes.* Due to the close proximity of the two BsaX I restriction sites (30 bp), only the two larger digestion products were visible by agarose gel electrophoresis (approx. 220 and 320 bp). A single recognition site for BciV I was identified within the ITS2 sequences of *Ch. saffranea* and *Ch. megacephala*. Digestion with this enzyme yielded two

fragments from each of these species (approx. 230 and 320 bp). A unique single restriction site for Ase I was identified within the ITS2 sequence of *Ch. flavifrons*, and digestion with this enzyme revealed two fragments from this species (approx. 260 and 300 bp).

The differential cutting abilities of the five restriction enzymes facilitated the generation of a PCR-RFLP protocol to identify members of the genus *Chrysomya* (Fig. 2.4). Following DNA extraction and amplification of the ITS2 region from an unidentified fly specimen, the fragment can be digested using the restriction enzyme Dra I. This enzyme is able to narrow species identification considerably, resulting in either no digestion (*Ch. megacephala* and *Ch. flavifrons*), digestion at three sites (*Ch. incisuralis* or *Ch. rufifacies*) or at one site (*Ch. saffranea, Ch. varipes, Ch. latifrons, Ch. semimetallica* or *Ch. nigripes*). The outcome of this step facilitates the selection of the subsequent restriction enzymes needed for identification. For instance, if two bands were observed after the Dra I digestion, further restriction analysis would be conducted using the enzymes Hinf I, BsaX I and BciV I.

The protocol has the ability to differentiate between the closely related species pair *Ch. saffranea* and *Ch. megacephala,* despite their divergence being only 0.46%. The procedure is able to narrow the identification to either *Ch. semimetallica* or *Ch. latifrons*, or to either *Ch. incisuralis* or *Ch. rufifacies.* Although it is not too surprising that *Ch. semimetallica* and *Ch. latifrons* cannot be separated by restriction analysis with a sequence divergence of only 0.23%, it is unfortunate that unique restriction sites do not exist for the differentiation of *Ch. incisuralis* and *Ch. rufifacies,* as their ITS2 sequences differ by a much larger 2.18%. However, *Ch. incisuralis* and *Ch. rufifacies* can be distinguished by amplification of the entire ITS region (approx. 1036 bp and 1150 bp, respectively), which can be differentiated by electrophoretic separation (as seen in lanes 1 and 2 of Fig. 2.1). Unfortunately, this same method cannot be utilised to distinguish *Ch. semimetallica* and *Ch. latifrons* as readily, as their ITS fragment sizes are very close (approximately 1230 bp) (lanes 8 and 9, Fig. 2.1). Further identification of the abovementioned species may be made possible by the design of species-specific primers to amplify unique PCR fragments.

In a subsequent analysis, the ITS2 fragments of eight specimens of *Ch. incisuralis*, collected from a total of six different locations (Table 2.1), were digested with the restriction enzyme Dra I, which has three sites within the ITS2 of this species. No variation in the size of the undigested PCR product or in the restriction patterns produced was detected. This is of practical significance as it suggests that there should be minimal RFLP pattern differences within species, a phenomenon that would otherwise complicate species identification using this method.

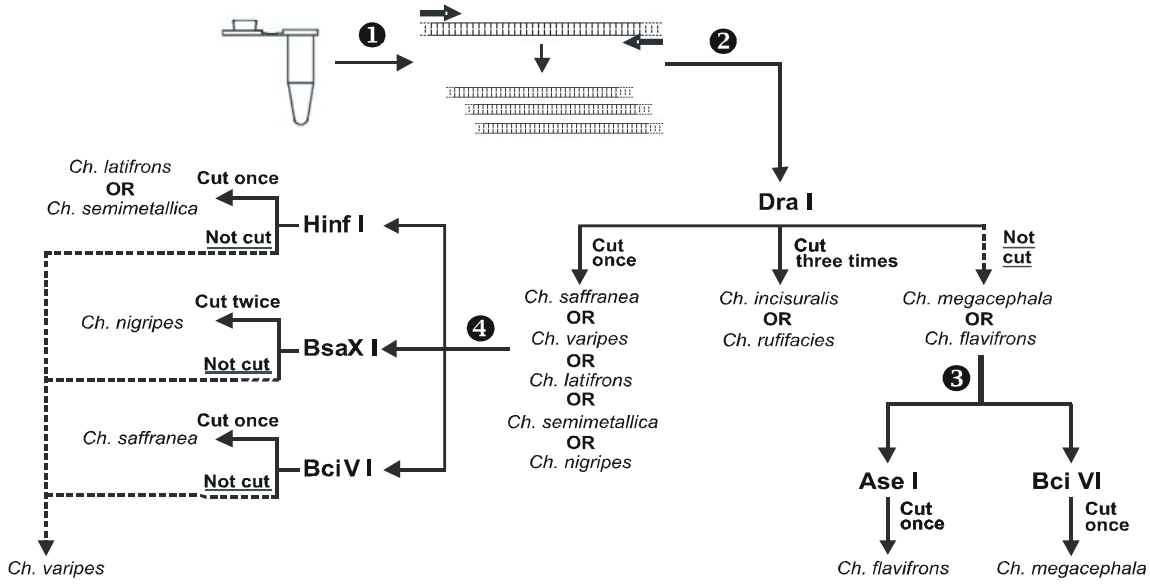


Figure 2.4. PCR-RFLP protocol for the identification of Australian members of the genus *Chrysomya* using the restriction enzymes Dra I, Hinf I, BsaX I and BciV I and Ase I. ❶ DNA is extracted from the specimen and the ITS2 region is PCR amplified using a combination of primers L1 and 52R. ❷ The PCR product is digested using Dra I, revealing a range of digestion products, the sizes of which can be visualised by electrophoretic separation. ❸ If no digestion occurs, further analysis takes place with the restriction enzymes BciV I and Ase I. ❹ If the PCR product was cut once only, additional analysis is undertaken using the restriction enzymes Hinf I, BsaX I and BciV I in turn until identification is made.

2.3.8. Phylogenetic analysis

As an alternative method for species identification, the sequence data from an unidentified specimen could be included in a phylogenetic analysis of the same sequence region from identified specimens. Provided the correct identity of the unknown specimen is contained within the reference taxa in the analysis, the unknown specimen will group with its closest relative on the tree (Wells *et al.*, 2001; Brunner *et al.*, 2002). On this basis, we examined whether phylogenetic analysis of ITS2 could be used to identify *Chrysomya* species from Australia.

Strong posterior probability support (approximately 1.00) indicated close relationships between some species pairs (*Ch. incisuralis* and *Ch. rufifacies*, *Ch. varipes* and *Ch. flavifrons*, *Ch. latifrons* and *Ch. semimetallica*, *Ch. megacephala* and *Ch. saffranea*) in the Bayesian analysis (Fig. 2.5). The position of *Ch. nigripes* was less consistently recovered. The short branch lengths between *Ch. megacephala* and *Ch. saffranea,* as well as between *Ch. semimetallica* and *Ch. latifrons*, indicate a high degree of sequence similarity between these pairs, which corresponds with that observed in the pairwise sequence divergence analysis (Table 2.2). The close genetic relationships of the abovementioned species are supported by studies that have examined morphological features of members of this genus (Wells & Kurahashi, 1996; Wallman, 2001a).

Some of the relationships indicated by the ITS2 analysis were consistent with a more extensive analysis of mitochondrial ND4-ND4L, COI and COII sequences reported by Wallman *et al.* (2005) for the same group of taxa. These included the close genetic relationships between *Ch. megacephala* and *Ch. saffranea*, *Ch. latifrons* and *Ch. semimetallica, Ch. rufifacies* and *Ch. incisuralis,* and *Ch. varipes* and *Ch. flavifrons.* However, the ITS2 analysis placed *Ch. nigripes* as sister to *Ch. incisuralis* and *Ch. rufifacies*, while the analysis of Wallman *et al.* placed it as sister to a clade comprising *Ch. varipes, Ch. flavifrons, Ch. megacephala* and *Ch. saffranea*. To examine whether differences between the ITS2 and mitochondrial trees were due to the small size of the ITS2 dataset (494 aligned ITS2 characters vs. 3029 aligned

mitochondrial characters), and/or the reduced taxon sampling in the ITS2 dataset (9 taxa in the ITS2 analysis vs. 17 taxa in the mitochondrial analysis), a combined analysis was conducted incorporating mitochondrial data for each population for which ITS2 sequences had been determined (Wallman *et al.*, 2005).

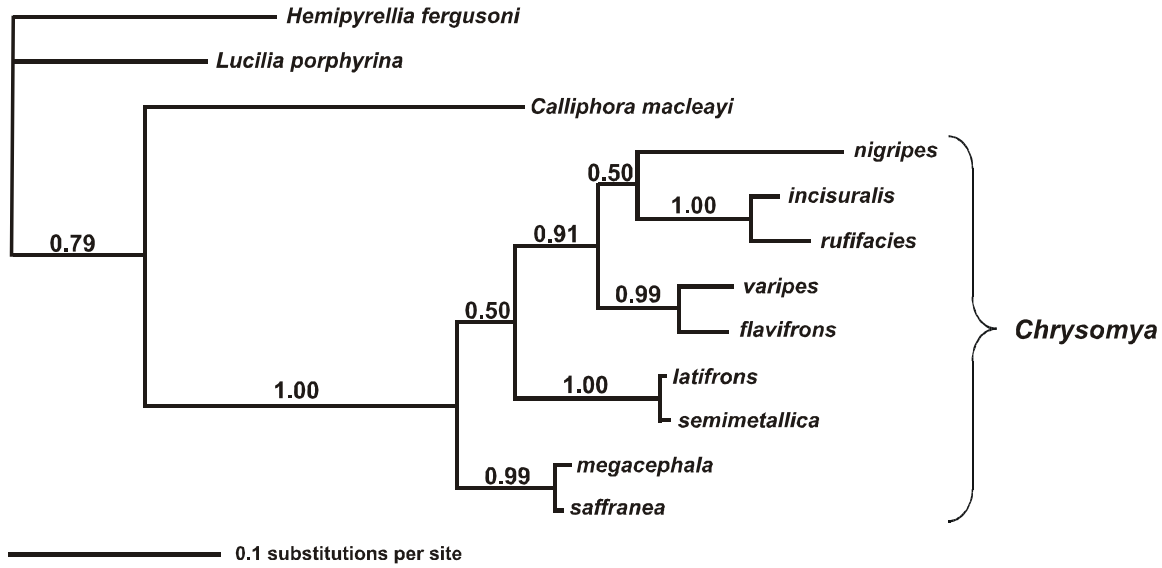


Figure 2.5. Most likely tree of relationships among Australian members of the genus *Chrysomya* species based on Bayesian analysis of ITS2 sequences. Analyses were conducted using MrBayes (v3.1), specifying *Hemipyrellia fergusoni* as the outgroup. Numbers over the branches represent posterior probabilities.

Analysis of the combined ITS2 and mitochondrial dataset recovered the tree shown in Fig. 2.6. Although there are many similarities between this tree and the recently published mitochondrial tree, particularly between sister species (e.g. *Ch. latifrons* + *Ch. semimetallica*, and *Ch. varipes* + *Ch. flavifrons* were recovered in both), there remain differences. For example, the mitochondrial dataset recovers *Ch. incisuralis* + *Ch. rufifacies*, as does ITS2 alone (Fig. 2.5), while the combined analysis recovers *Ch. incisuralis* and *Ch. rufifacies* as a grade (Fig. 2.6). The position of *Ch. nigripes* also varies, with its position differing in each of the three analyses (ITS2 alone, mitochondrial dataset, and ITS2 combined with mitochondrial data).

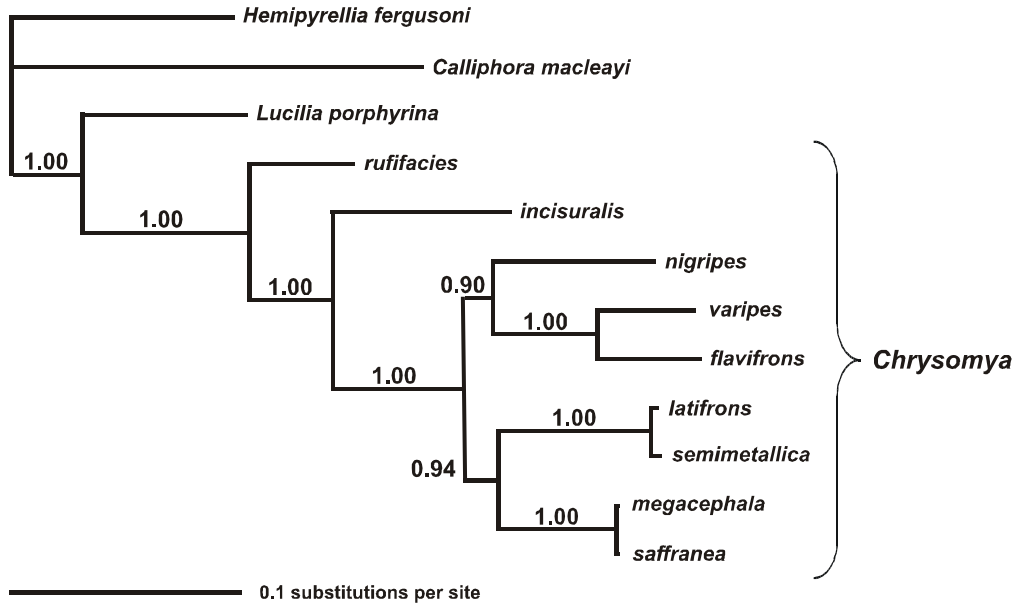


Figure 2.6. Most likely tree of relationships among Australian member of the genus *Chrysomya* species based on Bayesian analysis of combined ITS2, ND4-ND4L, COI and COII sequences. Analyses were conducted using MrBayes (v3.1), specifying *Hemipyrellia fergusoni* as the outgroup. Numbers over the branches represent posterior probabilities.

We were particularly concerned that the extensive taxonomic sampling in the mitochondrial dataset of Wallman *et al.* was resolving relationships more reliably than the restricted ITS2 dataset. To investigate this, the mitochondrial dataset of Wallman *et al.* was reanalysed after pruning some taxa from it. For example, if only one specimen of *Ch. rufifacies* was included, it was recovered as sister to all other species of *Chrysomya* (i.e. as in Fig. 2.6), while it was recovered as sister to *Ch. incisuralis* when all four representatives of *Ch. rufifacies* were included, as reported by Wallman *et al.* (2005). We conclude that the differences between the relationships recovered by Wallman *et al*. (2005) and those based on ITS2 reported here are most likely due to the increased taxonomic sampling by Wallman *et al.* (2005).

These differences, together with the low posterior probabilities for some branches of the ITS2 tree, suggest that ITS2 alone is unable to reliably resolve phylogenetic relationships among *Chrysomya* species and therefore is insufficient for their phylogeny-based identification. Our analyses suggest that an ITS2 phylogeny-based

identification of *Chrysomya* species would require much-expanded taxonomic sampling and/or additional mitochondrial sequence data.

2.4. Discussion

In this study, we assessed the rDNA ITS2 region as a potential marker for the identification of *Chrysomya* species from Australia. Sequence data from ITS2 revealed interspecific divergences that could be useful for species identification. Intraspecific sequence variation was assessed in three *Ch. incisuralis* specimens, collected from geographically distinct populations. The lack of ITS2 sequence variation among these specimens suggests that the homogenisation of ITS2 in *Ch. incisuralis* is efficient, being more rapid than the generation of new variants. While intraspecific ITS2 variation was not detected for *Ch. incisuralis*, its occurrence in other species cannot be ruled out. For this reason, the existence of intraspecific sequence variation should be investigated further, particularly for those species also found outside Australia.

With the exception of *Ch. flavifrons,* unambiguous ITS2 sequences were generated from all species without difficulty, suggesting that intraindividual variation was negligible in these species. Although the relative abundances of each ITS2 sequence variant were not assessed for *Ch. flavifrons* (only four clones were sequenced), the finding suggests that inefficient homogenisation of rDNA repeats is occurring within individuals of this species. Levels of intraspecific and intraindividual variation fluctuate unpredictably among taxa, suggesting that the extent of both should be scrutinised for each taxon under study (Paskewitz *et al.*, 1993; e.g. Schlötterer *et al.*, 1994).

Differentiation between the closely related species *Ch. incisuralis* and *Ch. rufifacies,* based on ITS fragment size, could be improved by the use of a higher resolution separation, such as a polyacrylamide gel system. An enhanced separation technique could possibly be employed to facilitate ITS size-based identification of Australian *Chrysomya*, which has been achieved for sibling species of other Diptera (e.g. Tang *et*

al., 1996; Kampen *et al.*, 2003). While RFLP and ITS size variation was not able to separate *Ch. latifrons* and *Ch. semimetallica* in this study, the very close morphological and genetic relationship between these species may indicate similar developmental profiles that would not require further differentiation to be made for the purposes of PMI estimation (Wallman & Donnellan, 2001). However, this is speculation and would need to be tested using developmental data collected for both species under various temperature regimes (see Chapter 4).

In accordance with the identical ITS2 sequences of three *Ch. incisuralis* specimens, the restriction patterns produced using Dra I with eight *Ch. incisuralis* specimens did not vary. As Dra I cuts the ITS2 of *Ch. incisuralis* three times, there were more opportunities for polymorphisms to be detected than with a less frequently cutting enzyme. These results suggest that the level of nucleotide variation is fairly constant within this species, and is independent of geographical origin (e.g. Newton *et al.*, 1998). The lack of restriction pattern variation within a species is in agreement with most previous studies (e.g. Rinaldi *et al.*, 2005) but not all, such as that of Clemente *et al.* (2002), who detected restriction pattern variation in the grasshopper *Dichroplus elongatus* at both intrapopulation and interpopulation levels.

A potential limitation of a PCR-RFLP technique is that a single point mutation within a species can introduce additional restriction sites or remove them. The effect of such mutations could completely alter the restriction profile of a specimen, which could lead to an incorrect identification. Similarly, a specimen could be misidentified if it belonged to a genus other than *Chrysomya* because of the specificity of the PCR-RFLP protocol that we describe.

Pending expansion of our PCR-RFLP approach to other genera of forensically important blowflies, more certain identification can only be achieved using DNA sequencing, because it takes advantage of the maximal information contained within a sequence from a particular species (e.g. Brunner *et al.*, 2002). Phylogenetic or 'tree-based' analysis is one such method of species identification that uses all the information contained within a sequence. An advantage of this method is that

intraspecific variation (such as that noted by Wallman *et al.* (2005) between populations of *Ch. rufifacies*) can be incorporated easily into the analysis by expanded taxon sampling, therefore increasing the likelihood of a correct identification. This avoids problems arising as a result of intraspecific variation, which, in PCR-RFLP analysis, can invalidate the results. As with any diagnostic method, specimen identification by phylogenetic analysis would only work provided the reference database is complete for, and relevant to, the local fly fauna (Wells & Sperling, 2001). This would help ensure reciprocal monophyly for each species within the gene region of interest, the importance of which has recently been highlighted by Wells *et al*. (2006). Identification would then be made based on the statistical confidence of the grouping of the unidentified specimen with an identified specimen within the database (Nielsen & Matz, 2006). The technique can, in principal, identify forensic entomological specimens (Wells *et al.*, 2001; Wells & Sperling, 2001).

One of the objectives of this study was to examine the utility of the ITS sequences as phylogenetic markers for members of the genus *Chrysomya.* Although the original aim was to use sequence data from ITS1 and ITS2, extensive sequencing difficulties with ITS1 forced the use of ITS2 alone for the remaining analyses. This resulted in reduced sequence data and therefore a lower number of phylogenetically informative characters. Phylogenetic analyses using ITS2 data were not as successful at resolving the deeper relationships among the *Chrysomya* as the analysis of the mitochondrial dataset conducted by Wallman *et al.* (2005). The mitochondrial analysis is considered more reliable due to the larger sequencing dataset and increased taxon sampling. However, the main purpose of the study of Wallman *et al.* (2005) was to elucidate the deeper phylogenetic relationships among Australian carrion-breeding blowflies, not expressly for the identification of members of a single genus. For the purposes of species identification, sequencing of multiple gene regions is costly in time and money, and could be avoided by the identification of a single appropriate gene region.

A comparison of ITS2 sequence data with the regions sequenced by Wallman *et al.* (2005) reveals that ITS2 sequence divergences outperform the individual mitochondrial regions in 32 cases out of the 36 (89%) pairwise comparisons made

(data not presented). The ITS2 region displays higher pairwise sequence divergences than the region of COI employed by Wallman *et al.* (2005), which is frequently employed in other identification studies, in 35 cases out of the 36 (97%) pairwise comparisons made (data not presented). While this study offers a preliminary investigation into the potential application of ITS2 for blowfly species identification, further replication is needed to ensure reproducibility and suitability of the region for this purpose. Expanded taxon sampling with ITS2, and the continued investigation of other gene regions, should identify a suitable candidate sequence for phylogeny-based species identification of these flies. Additionally, this study should ideally be expanded to include other blowfly taxa, thus enhancing the usefulness of this method as a tool in forensic entomology.

Staring into the eyes of a female *Chrysomya megacephala*.
Photo: L.A. Nelson

Chapter 3. Use of the COI 'barcode' for identification of forensically and medically important blowflies

This chapter is slightly modified from the paper:

Nelson, L.A., Wallman, J.F. and Dowton, M. (2007) Using COI barcodes to identify forensically and medically important blowflies, *Medical and Veterinary Entomology*, 21: 44-52.

Contributions: JFW and MD designed research; LAN performed research; LAN analysed the data; LAN wrote the paper.

3.1. Introduction

Recently, there has been a suggestion that the method and DNA region of choice for the identification of all animals on Earth should be standardised – the so-called "DNA barcoding" approach (Hebert *et al.*, 2003). This approach utilises a 658 bp region of the gene encoding the first subunit of mitochondrial cytochrome oxidase (COI), corresponding to nucleotide positions 1490 – 2198 of the *Drosophila yakuba* mitochondrial genome (Clary & Wolstenholme, 1985) (Fig. 3.1). This region has been shown to be suitable for the identification of a range of taxa including gastropods (Mollusca) (Remigio & Hebert, 2003), springtails (Collembola) (Hogg & Hebert, 2004), butterflies (Lepidoptera) (Hebert *et al.*, 2004a), birds (Hebert *et al.*, 2004b), mayflies (Ephemeroptera) (Ball *et al.*, 2005) and fish (Ward *et al.*, 2005). The proponents of COI DNA barcoding envisage the construction of a universally accessible, curated database comprising all animal COI sequences, which will then serve as "the basis for a global bioidentification system for animals" (Hebert *et al.*, 2003). The many benefits of DNA barcoding for species identification and discovery have been discussed (e.g. Hebert & Gregory, 2005), although the concept continues to be hotly debated (e.g. Wheeler, 2005). It is believed that a standardised approach to species identification would consolidate global research efforts and be beneficial for the identification of those species with medical, economic or environmental significance (Armstrong & Ball, 2005). In addition to species identification, the construction of a barcode database could expose novel DNA barcodes that may indicate provisional new species (Hebert *et al.*, 2004a).

A DNA barcoding approach may be useful for the identification of taxa for which the use of morphology, or the association of different life stages, is problematic. For these reasons, the utility of the COI barcode sequence for the identification of blowflies of the genus *Chrysomya* (Diptera: Calliphoridae) was tested. Some members of this genus are causative agents of myiasis in humans and domestic animals (Hall & Wall, 1995). They have also been identified as vectors of disease among people exposed to conditions of poor sanitation and nutrition (Maldonado & Centeno, 2003). Additionally, *Chrysomya* species constitute a significant proportion of the blowfly

species present in cases of murder or suspicious death (e.g. Levot, 2003). The identification of species found in association with a corpse is one of the first steps undertaken by the forensic entomologist in an attempt to estimate the postmortem interval (PMI) (Wells & LaMotte, 2001). The application of blowflies in PMI estimation has been hampered by difficulties associated with correct species identification at all developmental stages (eggs, larvae, pupae and adults) (e.g. Catts, 1992), particularly when only fragments of insect evidence (e.g. puparia, dead or decomposed larvae or adults) are available for analysis (Stevens & Wall, 2001). Blowfly identification requires specialist taxonomic knowledge, often relying upon the recognition of subtle variations in morphological features such as the cephalopharyngeal skeleton and microtubercles in immatures, and the dissection and inspection of the genitalia in adults (e.g. Wallman, 2001b). Some adult members of the genus *Chrysomya* can only be reliably distinguished on the basis of subtle morphological differences such as pigmentation. The morphological similarity of *Chrysomya* species, especially their immatures, makes a DNA-based approach to their identification advantageous. An assortment of molecular methods have been investigated for the identification of blowfly species, utilising a range of nuclear (e.g. Ratcliffe *et al.*, 2003) and mitochondrial (e.g. Wallman & Donnellan, 2001; Wells *et al.*, 2001) DNA regions. Analysis of the COI barcoding region in *Chrysomya* will not only offer insight into its utility for the identification of blowflies of forensic and medical importance, but will also further evaluate the capacity of the COI barcode as a global identification system for all animals.

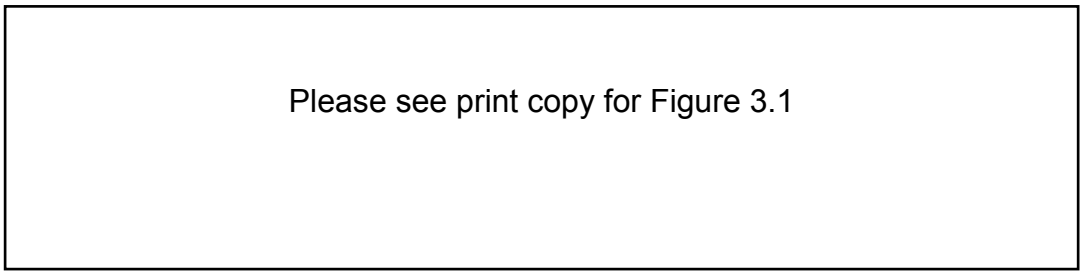


Figure 3.1. The complete COI gene with location of the 658 bp COI 'barcode' region, corresponding to nucleotide positions 1490 – 2198 of the *Drosophila yakuba* mitochondrial genome (Clary & Wolstenholme, 1985). The 'barcode' region is represented by *Ch. semimetallica* sequenced in this study; regions of COI sequenced in previous studies are shown for comparison: *Ch. semimetallica* sequenced by Wallman *et al.* (2005) and *Ch. chloropyga* sequenced by Wells *et al.* (2004). Numbers in brackets refer to accession numbers.

3.2. Materials and Methods

3.2.1. Specimens

Specimens were collected from locations along the east coast of Australia and identified morphologically by JFW (Table 3.1; Fig. 3.2). The specimens were collected directly into absolute alcohol, and are maintained at 4°C in the Diptera Collection in the School of Biological Sciences, University of Wollongong. Where possible, specimens selected for molecular analysis were from a range of geographic locations. In addition, two *Chrysomya nigripes* Auberton specimens from Chiang Mai, Thailand, provided a preliminary test-case for the ability of the COI barcode to distinguish species from other continents.

Table 3.1. Specimen information, Genbank accession numbers and collection localities for the Australian *Chrysomya* species studied, and three calliphorid outgroups.

Species	Voucher ID	GenBank accession no. COI and (ITS2)	Collection locality
Ch. flavifrons (Aldrich)	LN7	DQ647333	Kuranda, Qld (16°49′S, 145°38′E)
	JW47	DQ647331	Kuranda, Qld (16°49′S, 145°38′E)
	JW65	DQ647334	Tinaroo Falls, Qld (17º10′S, 145º 33′E)
	JW95	DQ647332	Mt Stuart, Qld (19°21′S, 146°47′E)
Ch. latifrons (Malloch)	LN2	DQ647347 (DQ310492)	Halfway Creek, NSW (30°14′S, 153°06′E)
	JW19	DQ647345 (EF071960)	Halfway Creek, NSW (30°14′S, 153°06′E)
	JW19.2	DQ647344 (EF071961)	Halfway Creek, NSW (30°14′S, 153°06′E)
	JW183	DQ647382 (EF071962)	Mt Keira, NSW (34°20′S, 150°51′E)
	JW190	DQ647381 (EF071963)	Cambewarra, NSW (34°49′S, 150°34′E)
Ch. megacephala (Fabricius)	LN5	DQ647352 (DQ310488)	Karuah, NSW (32°38′S, 151°57′E)
	JW25	DQ647350 (EF071964)	Hornsby Heights, NSW (33°39′S, 151°05′E)
	JW97	DQ647351 (EF071965)	Mt Stuart, Qld (19°21′S, 146°47′E)
	JW100	DQ647353 (EF071966)	Kuranda, Qld (16°49′S, 145°38′E)
Ch. nigripes Aubertin	LN6	DQ647355	Kuranda, Qld (16°49′S, 145°38′E)
	JW67	DQ647354	Tinaroo Falls, Qld (17º10′S, 145º33′E)
	JW98	DQ647356	Mt Stuart, Qld (19°21′S, 146°47′E)
Ch. rufifacies (Macquart)	LN8	DQ647360	Kuranda, Qld (16°49′S, 145°38′E)
	JW71	DQ647358	Tinaroo Falls, Qld (17º10′S, 145º33′E)
	JW93	DQ647359	Mt Sampson, Qld (27°24′S, 152°39′E)
	JW94	DQ647357	Mt Stuart, Qld (19°21′S, 146°47′E)
	JW161	DQ647361	Black Mountain, ACT (35°16′S, 149°06′E)
Ch. saffranea (Bigot)	LN9	DQ647367 (DQ310490)	Kuranda, Qld (16°49′S, 145°38′E)
	JW45	DQ647364 (EF071967)	Kuranda, Qld (16°49′S, 145°38′E)
	JW56	DQ647366 (EF071968)	Kuranda, Qld (16°49′S, 145°38′E)
	JW66	DQ647368 (EF071969)	Tinaroo Falls, Qld (17º10′S, 145º 33′E)
	JW96.2	DQ647363 (EF071970)	Mt Stuart, Qld (19°21′S, 146°47′E)
	JW96.3	DQ647362 (EF071971)	Mt Stuart, Qld (19°21′S, 146°47′E)
	JW107	DQ647365 (EF071972)	Kuranda, Qld (16°49′S, 145°38′E)
Ch. semimetallica (Malloch)	LN1	DQ647373 (DQ310493)	Halfway Creek, NSW (30°14′S, 153°06′E)
	JW32	DQ647372 (EF071973	Halfway Creek, NSW (30°14′S, 153°06′E)
	JW42	DQ647349 (EF071974)	Kuranda, Qld (16°49′S, 145°38′E)
	JW42.2	DQ647346 (EF071975)	Kuranda, Qld (16°49′S, 145°38′E)
	JW51	DQ647348 (EF071976)	Kuranda, Qld (16°49′S, 145°38′E)
	JW51.2	DQ647343 (EF071977)	Kuranda, Qld (16°49′S, 145°38′E)
	JW75	DQ647369 (EF071978)	Kuranda, Qld (16°49′S, 145°38′E)
	JW104	DQ647374 (EF071979)	Kuranda, Qld (16°49′S, 145°38′E)
	JW105	DQ647371 (EF071980)	Kuranda, Qld (16°49′S, 145°38′E)

Table 3.1. continued

Species	Voucher ID	GenBank accession no. COI and (ITS2)	Collection locality
	JW132	DQ647370 (EF071981)	Coffs Harbour, NSW (30º 18'S, 153º 07'E)
Ch. varipes (Macquart)	LN3	DQ647379	Karuah, NSW (32°38'S, 151°57'E)
	JW15	DQ647377	Myall Lakes National Park, NSW (32°25'S, 152°22'E)
	JW92	DQ647376	Mt Sampson, Qld (27°24'S, 152°39'E)
	JW108	DQ647380	Kuranda, Qld (16°49′S, 145°38′E)
	JW136	DQ647375	Coranderrk Bushland Reserve, Vic. (37º 40'59''S, 145º 31'04''E)
	JW164	DQ647378	Black Mountain, ACT (35°16'S, 149°06'E)
Ch. incisuralis (Macquart)	LN4	DQ647342	Halfway Creek, NSW (30°14'S, 153°06'E)
	JW26	DQ647338	Halfway Creek, NSW (30°14'S, 153°06'E)
	JW133	DQ647340	Halfway Creek, NSW (30°14'S, 153°06'E)
	JW28	DQ647339	Way Way State Forest, NSW (30º 48'S, 152º 55'E)
	JW29	DQ647335	Coffs Harbour, NSW (30º 18'S, 153º 07'E)
	JW46	DQ647337	Kuranda, Qld (16°49′S, 145°38′E)
	JW70	DQ647336	Tinaroo Falls, Qld (17º 10' S, 145º 33'E)
	JW63	DQ647341	Coranderrk Bushland Reserve, Vic. (37º 40'59''S, 145º 31'04''E)
Ch, saffranea/Ch. megacephala hybrid	JW96	-	Mt Stuart, Qld (19°21′S, 146°47′E)
Calliphora ochracea Schiner	JW6	DQ647328	Halfway Creek, NSW (30°14'S, 153°06'E)
Hemipyrellia fergusoni Patton	JW1	DQ647329	Halfway Creek, NSW (30°14'S, 153°06'E)
Lucilia porphyrina (Walker)	JW3	DQ647330	Halfway Creek, NSW (30°14'S, 153°06'E)

COI, cytochrome oxidase I; ITS2, second ribosomal internal transcribed spacer; Qld, Queensland; NSW, New South Wales; ACT, Australian Capital Territory; Vic, Victoria.

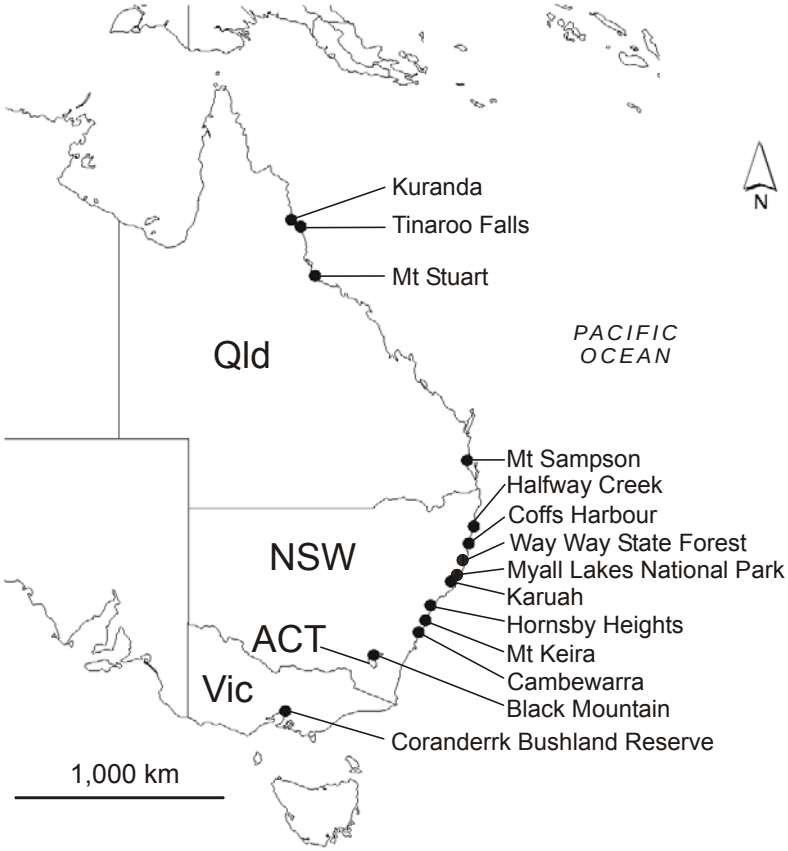


Figure 3.2. Map of locations on the east coast of Australia from which specimens in this study were collected. States are abbreviated as follows: Qld = Queensland, NSW = New South Wales, ACT = Australian Capital Territory, Vic = Victoria.

3.2.2. DNA Extraction, amplification and sequencing

Genomic DNA was extracted from either the flight muscle or from two legs of each fly by a 'salting out' protocol (Sunnucks & Hales, 1996). The DNA was resuspended in 50 µl of sterile distilled water and stored at 4°C. The COI barcoding region was amplified using primers LCO1490-L (5'-GGTCWACWAATCATAAAGATATTGG-3') and HCO2198-L (5'-TAAACTTCWGGRTGWCCAAARAATCA-3'), which are slightly modified forms of the primers designed by Folmer *et al.* (1994). For confirmation of the identities of some specimens, (all *Chrysomya latifrons* (Malloch), *Chrysomya semimetallica* (Malloch), *Chrysomya saffranea* (Bigot) and *Chrysomya megacephala* (Fabricius)) the second ribosomal internal transcribed spacer (ITS2)

region was amplified and sequenced using primers L1 (5'-RRCGGTGGATCACTCGGCTC-3') and 52R (5'-GTTACTTTCTTTTCCTCCCCT-3'; Ratcliffe *et al.*, 2003). Each 20 µl PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton®X-100, deoxynucleotide triphosphates (dNTPs) (25 µM each of dATP, dCTP, dGTP and dTTP), 1.25 mM $MgCl_2$, 0.4 µM of each primer and 0.75 U *Taq* DNA polymerase (Promega, Madison, WI). All reaction mixtures contained 0.5 µl DNA extract, except for the negative controls. The PCR temperature cycles, carried out on a Palm-Cycler™ II (Corbett Research, Sydney, Australia), consisted of an initial denaturation step at 94˚C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing across a temperature gradient of 45-65°C for 30 s, and elongation at 72°C for 2 min. The last cycle was followed by 5 min incubation at 72°C to complete any partially synthesised strands. All PCR products were visualised by agarose gel electrophoresis to identify the optimum annealing temperature (generally 45-47°C). These amplicons were treated with ExoSAP-IT® (GE Healthcare, Buckinghamshire, England) according to the manufacturer's instructions, to degrade residual primers and unincorporated dNTPs, and stored at 4°C.

Sequencing reactions were performed on ExoSAP-IT®-treated PCR product with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Version 3.1) (PE Applied Biosystems, Foster City, CA) using the Palm-Cycler™ II (Corbett Research). PCR primers were used to initiate the sequencing reactions, which were set up according to the manufacturer's instructions. Cycling conditions were thirty cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Individual reactions (12 µl) were transferred to fresh 1.5 ml tubes containing 74 µl of precipitation buffer (68% ethanol, 3.4 mM EDTA, pH 8.0 and 81 mM sodium acetate, pH 5.2). The mixtures were incubated at room temperature for 15 min and then spun at 16,000 x *g* for 15 min. The supernatant was discarded and 180 µl ice-cold 70% ethanol pipetted gently onto the pellet. The tubes were spun for a further 5 min after which the supernatant was discarded completely and the samples air-dried. Sequencing reaction products were then separated using the 3130x Genetic Analyzer (PE Applied Biosystems).

3.2.3. DNA sequence analysis

DNA sequences were confirmed and edited manually using BioEdit Sequence Alignment Editor (Version 6.0.7; Hall, 1999) and ChromasPro (Version 1.33; Technelysium Pty Ltd, available online at www.technelysium.com.au/ChromasPro.html). Mitochondrial COI and nuclear ribosomal ITS2 sequences reported in this paper have been submitted to GenBank and their respective accession numbers are indicated in Table 3.1. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) with the default settings. Some data analysis was undertaken via the workbench of the Barcode of Life Data (BOLD) Systems Management and Analysis System (www.boldsystems.org; Ratnasingham & Hebert, 2007).

Nucleotide sequence divergences were calculated using the Kimura-two-parameter (K2P) distance model (Kimura, 1980). A bootstrap (1000 replicates) neighbour-joining (NJ) analysis (Saitou & Nei, 1987), performed in PAUP* (Version 4.0b10; Swofford, 2001), provided a graphic display of the patterns of divergence among the species. Three non-*Chrysomya* species (*Calliphora ochracea* Schiner, *Hemipyrellia fergusoni* Patton and *Lucilia porphyrina* (Walker) (Diptera: Calliphoridae)) were included as outgroups.

To assess whether the phylogenetic analyses were robust to changes in the analytic approach, Bayesian analyses were also performed, using MrBayes (Version 3.1; Huelsenbeck & Ronquist, 2001). In the first analysis the data were unpartitioned. In the second, the data were divided into three partitions representing the first, second and third codon positions of COI. For the unpartitioned analysis, the general time reversible model (command: nst = 6) (Tavaré, 1986), with rate variation allowing a proportion of invariable sites (command: rates = propinv) (GTR + I + Γ; Gu *et al.*, 1995; Waddell & Penny, 1999), was selected as the best-fit model of nucleotide substitution (MrModeltest v2.2; Nylander, 2004). For the partitioned analysis, the general time reversible model with variable sites assumed to follow a discrete gamma distribution (command: rates = gamma) (GTR + Γ; Yang, 1994) was selected as the

best-fit model of nucleotide substitution (MrModeltest v2.2). Substitution and rate parameters were estimated separately for each partition (command: unlink). For each analysis, four independent runs were performed. Each Markov chain Monte Carlo (MCMC) process was set so that four chains, three heated and one cold, ran simultaneously. Runs were conducted for 1 million generations, with trees being sampled every 100 generations, yielding a total of 10,000 trees for analysis per run. Independent analyses indicated that 'stationarity' (or 'burnin': lack of improvement in maximum likelihood scores) was reached at no later than 150,000 generations; thus, the first 1,500 trees were discarded from each analysis as the burnin, and the remaining trees were used to generate a 50% majority consensus tree. Posterior probabilities were estimated by counting the proportion of trees that recovered a particular group after the burnin period. The three non-*Chrysomya* species were included in all Bayesian analyses, with *Calliphora ochracea* designated as the outgroup.

3.3. Results and Discussion

3.3.1. Amplification and sequencing of the COI barcode region

The COI region proved straightforward to amplify and sequence. A 658 bp fragment of the COI gene was sequenced from all 56 Australian specimens representing all nine Australian *Chrysomya* and three calliphorid outgroups. In addition, two Thai specimens of *Ch. nigripes* were amplified and sequenced. Well defined electropherogram peaks and the absence of stop codons indicated that coamplification of nuclear pseudogenes did not occur (Zhang & Hewitt, 1996). In accordance with previous work (e.g. Hebert *et al.*, 2003), the sequences aligned with ease due to the absence of insertions and deletions. Nucleotide composition showed an AT bias within *Chrysomya* (mean A = 30.4%, T = 38.1%, C = 15.8%, G = 15.6%).

The mean within species and within genus K2P divergences (0.097% and 6.499%, respectively; Table 3.2) for *Chrysomya* are similar to those reported by Smith *et al.*

(2006) for parasitoid flies (Diptera: Tachinidae) (0.170% and 5.781%, respectively) and those reported by Hajibabaei *et al.* (2006) for three families of Lepidoptera (0.35% and 5.007%, respectively). The highest interspecific sequence divergence for *Chrysomya* existed between *Chrysomya incisuralis* (Macquart) and *Ch. varipes* (mean = 9.166%, range = 9.078 – 9.254%). Lower COI sequence divergences existed between the species pairs (in descending order of interspecific divergence) *Ch. incisuralis* and *Chrysomya rufifacies* (Macquart); *Ch. varipes* and *Chrysomya flavifrons* (Aldrich); *Ch. latifrons* and *Ch. semimetallica*; and *Ch. megacephala* and *Ch. saffranea* (Table 3.3). No intraspecific COI sequence variation was detected in either *Ch. latifrons* or *Ch. megacephala.* Mean intraspecific and interspecific COI sequence divergences differed by more than an order of magnitude for *Chrysomya* (Table 3.2), although there was overlap in the range of the divergences. This overlap is not due to shared barcode sequences among different species, but rather to intraspecific variation in some species exceeding interspecific variation between other closely related species pairs: this is attributable to the low sequence divergence (mean = 0.480%) between *Ch. megacephala* and *Ch. saffranea* (Table 3.3), and the high intraspecific sequence divergence encountered for *Chrysomya varipes* (Macquart) (mean = 0.295%, range = 0 – 0.612%).

Table 3.2. Summary of genetic divergences (using the K2P model) of 9 species (52 sequences) within the genus *Chrysomya* in Australia.

Chrysomya species	n	Distance (%)			
		Minimum	Mean	Maximum	SE
Ch. flavifrons	4	0.000	0.076	0.152	0.076
Ch. incisuralis	8	0.000	0.065	0.152	0.075
Ch. latifrons	5	0.000	0.000	0.000	0.000
Ch. megacephala	4	0.000	0.000	0.000	0.000
Ch. nigripes	3	0.000	0.101	0.152	0.072
Ch. rufifacies	5	0.000	0.091	0.152	0.075
Ch. saffranea	7	0.000	0.043	0.152	0.069
Ch. semimetallica	10	0.000	0.115	0.305	0.097
Ch. varipes	6	0.000	0.295	0.612	0.162
Comparisons within:					
Species		0.000	0.097	0.612	0.119
Genus		0.458	6.499	9.254	1.864

n, number of specimens

Table 3.3. Percentage sequence divergences (K2P) between selected sister *Chrysomya* species for the cytochrome oxidase I (COI) barcode region.

	Minimum	Mean	Maximum	SE
Ch. saffranea vs. *Ch. megacephala*	0.458	0.480	0.612	0.167
Ch. latifrons vs. *Ch. semimetallica*	1.231	1.387	1.543	0.099
Ch. flavifrons vs. *Ch. varipes*	5.058	5.238	5.558	0.135
Ch. incisuralis vs. *Ch. rufifacies*	6.696	6.764	6.865	0.083

SE, standard error

Extremely low sequence divergences between sister species and among species complexes are believed to be indicative of their recent origin (Funk & Omland, 2003; Tautz *et al.*, 2003). Instances of low sequence divergence between closely related species pose significant challenges for barcoding, especially when ancestral polymorphisms are retained, which can lead to the occurrence of shared barcode sequences (e.g. Hajibabaei *et al.*, 2006). If the level of barcode variation is not taken into account in such instances, confidence in identification could be undermined (Armstrong & Ball, 2005). Where closely related species cannot be separated due to shared barcode sequences and morphological similarity, additional analysis of other genes, possibly of nuclear origin, would be required (Hebert *et al.*, 2003).

Comprehensive taxonomic sampling is particularly essential for taxa with an extensive geographical range. We are confident, due to the number of specimens included in the analysis, that we adequately sampled the levels of intraspecific variation for *Chrysomya* from the east coast of Australia. The overlap between the intraspecific and interspecific sequence divergences should not be problematic for the identification of specimens from this genus collected along the east coast, as they should group reliably with their correct species on a tree (Meyer & Paulay, 2005). There did not appear to be a correlation between geographic distance and COI sequence divergences for the specimens examined in this study. Indeed, no intraspecific divergence was detected for *Ch. megacephala*, despite specimens originating from geographically distinct populations (distances ranging from approximately 306 km to 1870 km). This was further confirmed by examining the

COI sequences of the two Thai *Ch. nigripes* specimens, which displayed a mean percentage divergence of 0.28 from the Australian *Ch. nigripes* specimens collected approximately 9,500 km away. Varying levels of intraspecific divergence were encountered within other species, but these did not appear to reflect the geographic proximity of populations from which specimens were sampled. Although this study did not find a convincing relationship between geographic distance and sequence divergence, this has been noted in the past (e.g. Ball *et al.*, 2005) and further investigations will be required for those species with transcontinental distributions.

3.3.2. Neighbour-joining analysis of COI barcode sequences

The purpose of this study was to investigate whether the COI barcode provided sufficient resolution to identify blowflies of the genus *Chrysomya.* The NJ analysis confirmed this, and supported results of previous studies that have found the COI barcode to be an effective tool for identification purposes (e.g. Hebert *et al.*, 2003). All *Chrysomya* species were resolved as reciprocally monophyletic groups, despite low COI divergences between some sister species (Fig. 3.3). While the COI barcode region alone is not intended to be used to resolve taxonomic relationships, it appears to contain enough phylogenetic signal to delineate close relationships within *Chrysomya* from the east coast of Australia. As a test of the power of the COI barcode, the two Thai *Ch. nigripes* specimens were included with all Australian species in a subsequent neighbour-joining analysis. They formed a monophyletic group with the Australian *Ch. nigripes* specimens (data not shown). This result suggested that we had adequately sampled intraspecific COI variation from the east coast of Australia, and provided encouraging evidence about the utility of the COI barcode to delineate members of this genus in Australia, and possibly other regions in the world.

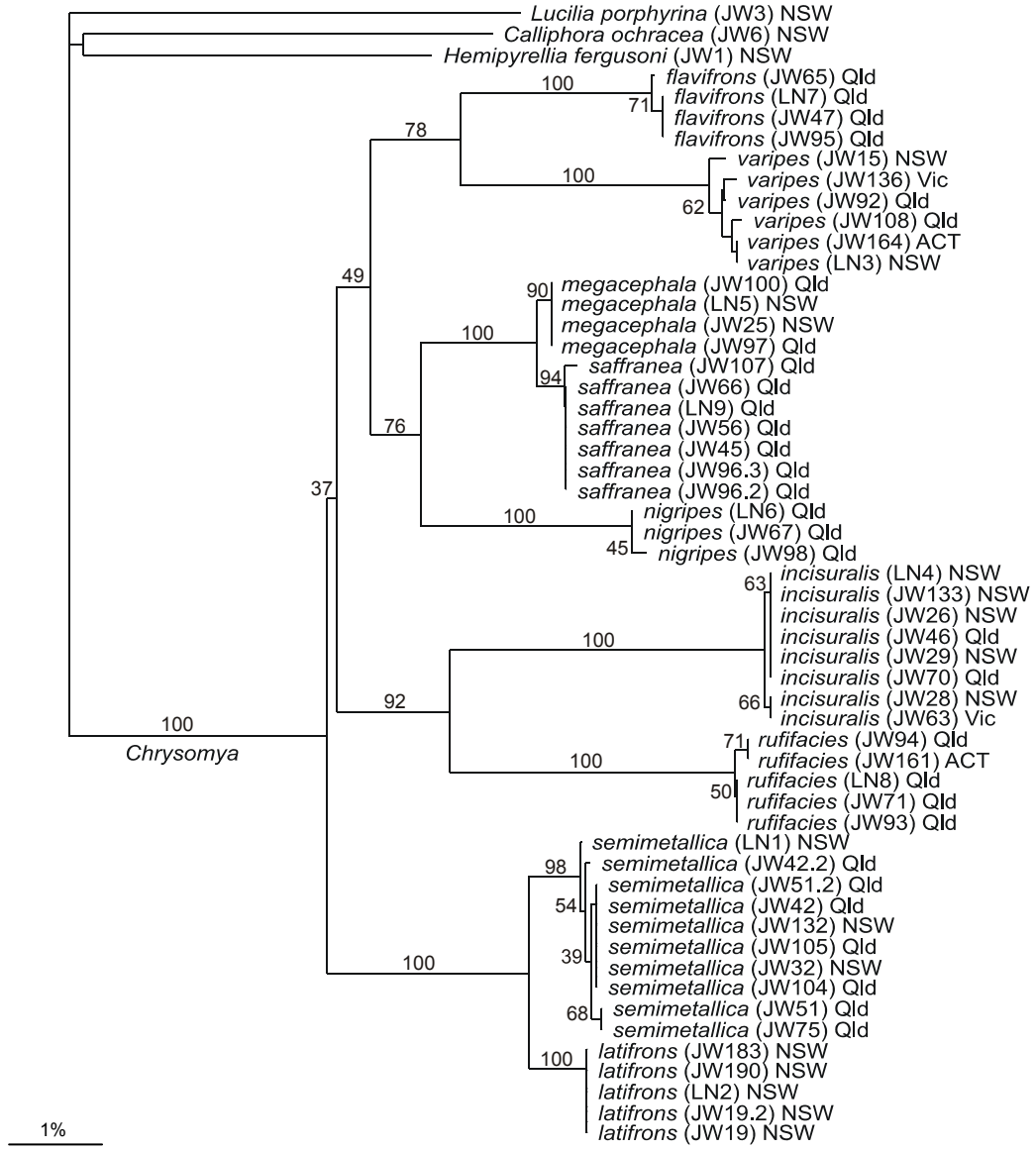


Figure 3.3. NJ tree of K2P distances for 55 COI sequences from all nine Australian *Chrysomya* and three calliphorid outgroups. Numbers above branches refer to bootstrap proportions among 1000 bootstrap replicates. Specimen voucher codes referred to in Table 3.1 are shown in parentheses following species names. Collection locations are summarised to state, and are abbreviated as follows: Qld = Queensland, NSW = New South Wales, Vic = Victoria, ACT = Australian Capital Territory.

Some of the relationships denoted by the COI barcode region were consistent with a more extensive analysis of mitochondrial ND4-ND4L, COI and COII sequences reported by Wallman *et al.* (2005) for the same group of taxa. These included the close genetic relationships between *Ch. megacephala* and *Ch. saffranea*, *Ch. latifrons* and *Ch. semimetallica, Ch. rufifacies* and *Ch. incisuralis,* and *Ch. varipes* and *Ch. flavifrons.* The close genetic relationships between the species pairs *Ch. megacephala*

and *Ch. saffranea*, and *Ch. latifrons* and *Ch. semimetallica*, are supported by studies that have examined morphological features of members of this genus (Wells & Kurahashi, 1996; Wallman, 2001a). The NJ analysis of the COI barcode region placed *Chrysomya nigripes* Aubertin as sister to *Ch. saffranea* and *Ch. megacephala,* while the more comprehensive analysis of Wallman *et al.* (2005) placed it as sister to a clade comprising *Ch. varipes, Ch. flavifrons, Ch. megacephala* and *Ch. saffranea*.

3.3.3. Comparison of methods of COI barcode analysis

Although the COI barcode is not designed to resolve phylogenetic relationships, it must reliably distinguish reciprocally monophyletic groups in order to delineate species. The NJ method has been promoted as the analysis tool of choice for the construction of barcoding databases, due to its advantage of speed and its performance when sequence divergences are low (Hebert *et al.*, 2003; Ball *et al.*, 2005). However, a comparison of tree building methods is vital during the development of the barcoding method, particularly since the suitability of this method for species delineation has been questioned in the past (e.g. DeSalle *et al.*, 2005). In some cases, an oversimplified or inadequate phylogenetic analysis may fail to distinguish reciprocally monophyletic groups, whereas an analysis that more realistically models the history of molecular evolution for the COI gene may perform better (e.g. Whelan *et al.*, 2001; Sullivan & Joyce, 2005). To assess this, we compared the NJ tree with trees generated from Bayesian analyses of the COI data. Both tree-building methods recovered each *Chrysomya* species as a monophyletic group (Bayesian data not shown).

3.3.4. ITS2 sequence analysis for selected species

Two complications were encountered early in this study. In the first, four specimens which had been preliminarily identified in the field as *Ch. latifrons* (JW42, JW42.2, JW51 and JW51.2, from Kuranda, Qld) were recovered with *Ch. semimetallica* in the COI NJ tree. The second complication concerned a specimen (JW96) which was identified morphologically as *Ch. saffranea,* but was recovered with its closest

relative, *Ch. megacephala*, in the NJ tree. These issues raised concerns of (1) an inadequate phylogenetic analysis, (2) an inability of COI to resolve these species, or (3) incorrect specimen identification, due to the significant morphological similarities shared by each set of sister species. Because of these close relationships, the possibility of high levels of intraspecific variation, perhaps due to retained ancestral polymorphisms, and hybridisation, were considered (Hajibabaei *et al.*, 2006). To investigate these concerns, (1) the COI data were reassessed using a Bayesian analysis, (2) the ITS2 region was analysed from all *Ch. latifrons*, *Ch. semimetallica, Ch. megacephala* and *Ch. saffranea* specimens, and (3) further morphological examinations were undertaken. The ITS2 region was shown previously to distinguish members of the genus *Chrysomya,* the results of which appear in Chapter 2.

The Bayesian analyses of the COI dataset did not alter or improve the delineation of species boundaries established by the NJ tree. Detailed morphological examination, coupled with ITS2 and COI analysis, confirmed that the four *Ch. semimetallica* specimens had been misidentified as *Ch. latifrons*, and that this was the cause of the apparent misplacement of the specimens in the NJ tree. Distinguishing these sister species is particularly challenging due to their close morphological similarity. The identifications of the abovementioned specimens were modified accordingly for the final analysis. The ITS2 sequence analysis revealed no intraspecific sequence variation for *Ch. latifrons* and *Ch. semimetallica*, and an interspecific divergence of 1.345%. The ITS2 interspecific divergence is comparable to that for COI (mean = 1.387%). The detection of misidentified specimens reinforces the utility and sensitivity of a DNA-based identification system in circumstances where morphological characters are either unreliable, or require specialised taxonomic scrutiny.

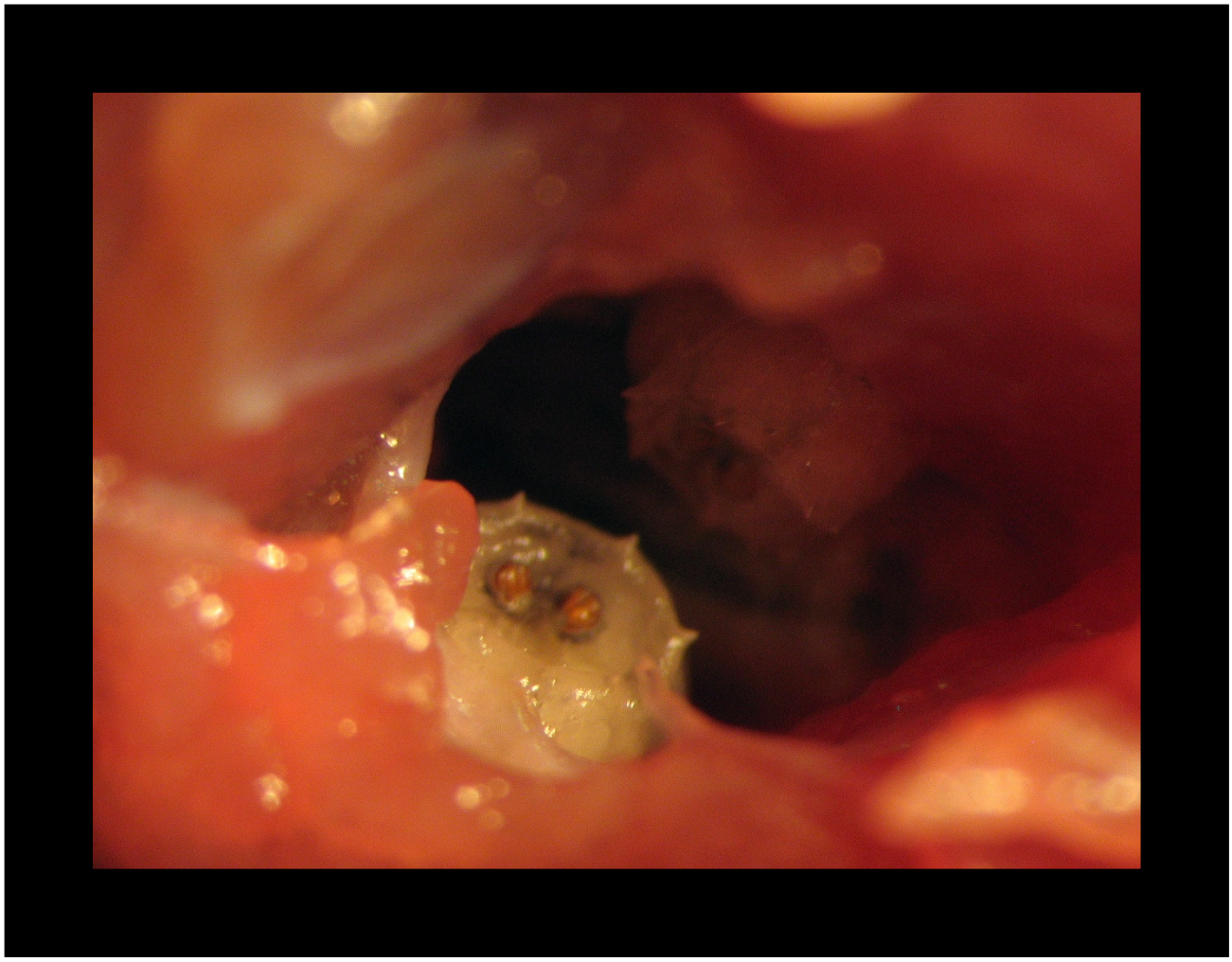
The ITS2 sequence of specimen JW96 was the same as five of the seven *Ch. saffranea* specimens, while differing from *Ch. megacephala* ITS2 by two base changes and three indels. The ITS2 sequence of specimen JW96 therefore agreed with the morphological identification and placed specimen JW96 as *Ch. saffranea.* Together with the morphological evidence, the conflicting species assignment by the

nuclear and mitochondrial DNA suggested that the specimen was a hybrid resulting from the mating between a female *Ch. megacephala* and a male *Ch. saffranea*, due to the maternal contribution of mitochondrial DNA. Due to the suspected hybrid status of this specimen, it was excluded from further investigations. The ITS2 sequence analysis revealed a lack of variation within this region for *Ch. megacephala*, and a low level of sequence variation for *Ch. saffranea* (mean = 0.107%, range = 0 – 0.464%). Overall, the mean interspecific ITS2 sequence divergence between *Ch. megacephala* and *Ch. saffranea* was 0.462% (range = 0.461 – 0.465%), which is comparable to the COI interspecific divergence (mean = 0.480%).

Although considered less likely, an alternative explanation for the disagreement between the nuclear and mitochondrial sequences for JW96 could be the retention of a shared ancestral polymorphism by this specimen (e.g. Funk & Omland, 2003). However, hybridisation is believed to be possible between such closely related species as *Ch. megacephala* and *Ch. saffranea* (J.F. Wallman, pers. comm.), which exhibited the lowest interspecific COI divergence within *Chrysomya* (mean COI divergence = 0.480%, excluding *Ch. saffranea* specimen JW96). Wallman *et al.* (2005) found a similarly low (0.403%) mitochondrial DNA sequence divergence between these species. Furthermore, ITS2 sequence analysis of the *Ch. megacephala* and *Ch. saffranea* specimens in this study confirmed their close genetic relationship (mean interspecific ITS2 divergence = 0.462%, excluding specimen JW96). The suspected occurrence of hybridisation among flies was also noted by Smith *et al.* (2006) for parasitoid tachinids (Diptera: Tachinidae), based on sequence analysis of the COI barcode region and the first ribosomal internal transcribed spacer (ITS1). The discovery of hybrid specimens draws attention to a limitation of any identification system that is based on a single character (Stevens *et al.*, 2002). Were it not for the combined molecular evidence given by COI and ITS2, along with morphological examination, barcode analysis would have identified the specimen as *Ch. megacephala.* The inclusion of sequence data from more than one DNA region would therefore enhance confidence in DNA-based identifications (Matz & Nielsen, 2005). The need for re-examination of the misplaced specimens in this study highlights the importance of a voucher collection for all members of a barcode database.

3.4. Conclusion

This study has found that a COI barcode identification system would be suitable for the identification of *Chrysomya* species from the east coast of Australia. The COI barcode region proved straightforward to amplify and sequence, which would facilitate the rapid generation of a barcode database and subsequent identifications of specimens. While the COI barcode provided adequate resolution to separate Australian *Chrysomya* species, this study demonstrated the use of a nuclear gene, ITS2, in providing extra information in cases of uncertain specimen identifications. The multitude of fly species potentially encountered in forensic cases represents a substantial obstacle to streamlined PMI estimation. A technique that could aid the prompt and accurate identification of specimens of all life stages, or fragments thereof, would be enormously beneficial for the utilisation of forensic insect evidence. Based on the results for *Chrysomya*, it is foreseeable that DNA barcoding could be effective for the identification of other blowfly taxa. Further investigations should confirm this feasibility, and establish the reliability of the technique for routine application in forensic cases and other circumstances featuring flies of applied importance.



Posterior spiracles of a *Chrysomya megacephala* larva visible as it burrows into kangaroo mince.
Photo: L.A. Nelson

Chapter 4. Thermal attributes of *Chrysomya* (Diptera: Calliphoridae) species

4.1. Introduction

The term 'forensic entomology' encompasses all areas of entomology as applied to the law. However, the term is most commonly associated with its involvement in cases of murder or suspicious death, where it can provide a minimum time since death, or post-mortem interval (PMI). The PMI can be estimated in two ways: from the predictable succession of arthropod colonisation of a corpse (the most important being the Diptera) and by the age of maggots, or fly (Diptera) larvae, present on a corpse (mostly of the family Calliphoridae; Schoenly *et al.*, 1991; Anderson & VanLaerhoven, 1996; El-Kady, 1999). The latter is based on the application of existing developmental data for the species of blowfly present together with available temperature measurements (Sukontason *et al.*, 2008). These estimates are usually made using thermal summation models (such as the degree-day method) or isomorphen and/or isomegalen diagrams (Grassberger & Reiter, 2001; Higley & Haskell, 2001; Greenberg & Kunich, 2002). The initial PMI estimate can then be amended to address scene-specific characteristics, such as insect accessibility to the body (Byrd & Castner, 2001c). As the size and developmental stage of the blowfly larvae collected in a case provide important indicators for the estimation of the PMI, factors which affect these variables must be considered by the forensic entomologist.

As ectotherms, the rates of most physiological processes in insects are highly dependent on temperature (Heinrich, 1981; Wall *et al.*, 1992; Byrd & Castner, 2001b; Grassberger & Frank, 2003). Blowfly development is positively correlated with increasing temperatures (within developmental thresholds), producing a sigmoidal relationship (Sukontason *et al.*, 2008) (Fig. 4.1). Unlike most insects, however, blowfly larvae have partial control over their microenvironment. It has been shown that when larval numbers are plentiful, a maggot-mass effect can elevate temperatures up to 20°C above ambient (Cianci & Sheldon, 1990). The larvae thermoregulate by

moving in and out of the mass (Slone & Gruner, 2007). The maggot-mass effect increases metabolic rates and food processing efficiency, accelerates developmental rates, provides a competitive advantage, and mitigates the effects of low temperature conditions (Casey, 1981; Cianci & Sheldon, 1990; Catts, 1992; Turner & Howard, 1992; Greenberg & Tantawi, 1993; VanLaerhoven, 2008). While temperature is the most important environmental variable influencing the rate of larval development, the duration of larval growth varies according to the species, mainly on genetic bases (Campobasso *et al.*, 2001). Tarone and Foran (2006) explained that an understanding of the influence of each of these factors is best achieved by altering one of them while keeping the others constant. Some studies have demonstrated larval growth to be plastic with respect to rearing conditions (Wells & Kurahashi, 1994; Tarone & Foran, 2006; 2008). For example, variable growth rates have been demonstrated for larvae raised under constant temperatures on different feeding substrates (e.g. Green *et al.*, 2003; Kaneshrajah & Turner, 2004; Day & Wallman, 2006a). Larval densities and competition have been found to influence developmental times and larval fecundity in some species (Goodbrod & Goff, 1990; Kheirallah *et al.*, 2007).

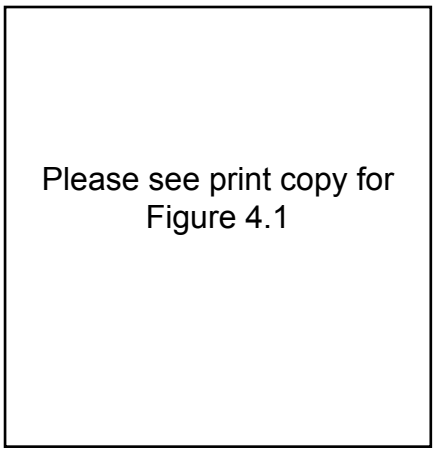


Figure 4.1. The generalised thermal development curve showing a curvilinear relationship at low and high temperatures and a linear relationship between these extremes. After Byrd and Castner (2001b).

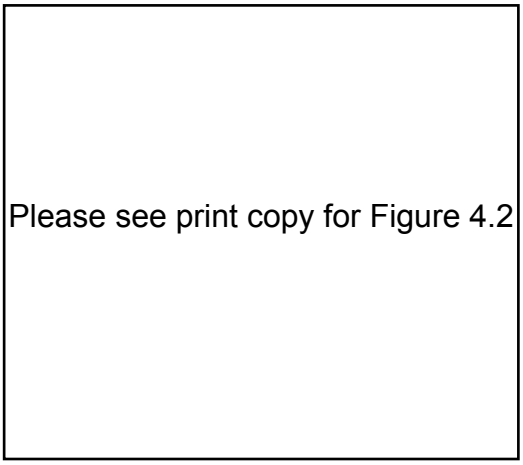
Laboratory studies provide the forensic entomologist with a developmental time-scale for a particular species. Developmental stage and body length are the most common parameter for estimation of the age of immature blowflies (e.g. Byrd & Butler, 1996; 1997; Anderson, 2000; Byrd & Allen, 2001b; Grassberger & Reiter, 2002a; Day & Wallman, 2006b). This approach is well supported by the literature and is admissible in court (Tarone & Foran, 2008). Despite this, different growth rates have been reported for the same species (Kamal, 1958; Greenberg, 1991; Wells & Kurahashi, 1994; Anderson, 2000; Grassberger & Reiter, 2001). The application of larval length for PMI estimation is further complicated by these data not being available for all species that may be encountered in a forensic case. This situation is exacerbated by the requirement of some thermal summation methods for an input of developmental data for a species from a minimum of six constant temperatures (Richards & Villet, 2008). The limited data that are available have typically been generated using laboratory techniques that have not been standardised. Because the environmental conditions and genetic backgrounds of the flies vary so greatly, the ability to directly compare developmental studies is restricted. The variable nature of published blowfly development data, especially the non-standardised laboratory studies on which PMI estimates are based, necessitates a more reliable method by which developmental data can be utilised (Tarone & Foran, 2006).

Determination of the fly species, as well as age, is mandatory for the use of entomological evidence in forensic investigations (El-Kady, 1999; Song *et al.*, 2007). However, the use of blowflies in PMI estimation is complicated by difficulties associated with correct species identification at all developmental stages (eggs, larvae, pupae and adults) (e.g. Catts, 1992), particularly when only fragmented or degraded insect evidence are available for analysis (Stevens & Wall, 2001). Traditional morphology-based identification typically requires specialised taxonomic knowledge. While morphological identification is often possible, DNA-based identification may be required to separate morphologically indistinguishable species or specimens for which morphological characters are absent (Dawnay *et al.*, 2007). Various DNA techniques have been proposed to identify forensically important blowflies (e.g. Sperling *et al.*, 1994; Malgorn & Coquoz, 1999; Wells & Sperling, 1999; Wallman &

Donnellan, 2001; Wells *et al.*, 2001; Harvey *et al.*, 2003a; Harvey *et al.*, 2003b; Ratcliffe *et al.*, 2003; Chen *et al.*, 2004; Zehner *et al.*, 2004; Saigusa *et al.*, 2005; Nelson *et al.*, 2007; Song *et al.*, 2007; Nelson *et al.*, 2008). However, considerably little is known about the amount of genetic variation existing in blowfly populations, so this method is regarded as a complement to, rather than a substitute for, standard taxonomy-based identification of species (El-Kady, 1999; Nelson *et al.*, 2007). The close morphological and genetic relationship of some species questions whether they differ sufficiently in their thermobiologies to warrant their discrimination for forensic purposes (Wallman & Donnellan, 2001). There has been a suggestion that developmental rates of some species could be deduced from their phylogenetic position relative to other species whose developments have been characterised (Wallman *et al.*, 2005).

In this study, the principal aim is to establish whether closely related blowflies of the same genus vary significantly in their larval developmental profiles. Attempts were made to control for any population-specific genetic variation among the fly species being investigated, as well as among the methods employed. Australian members of the genus *Chrysomya*, from which the species investigated in this study belong, have been characterised morphologically and molecularly (Wallman, 2001a; Wallman *et al.*, 2005; Nelson *et al.*, 2007; 2008). With the exception of *Chrysomya latifrons* (Malloch) and *Chrysomya incisuralis* (Macquart), the other seven Australian *Chrysomya* species have wide distributions, particularly within the Asian region (Kurahashi, 1989; Wells & Kurahashi, 1994; Sukontason *et al.*, 2008). Developmental rates for some members of this genus have been reported, but no such studies have been recorded in Australia. Some *Chrysomya* species in Australia display very similar morphologies, even as adults (Fig. 4.2), and low genetic distances indicate recent divergences (Wallman *et al.*, 2005). The two closest relatives, sister species *Chrysomya saffranea* (Bigot) and *Chrysomya megacephala* (Fabricius), were selected for study here along with the hairy maggot blowfly, *Chrysomya rufifacies* (Macquart), a more distantly related member of this genus. These species were chosen on the basis of molecular analyses which revealed very low sequence divergences between *Ch. saffranea* and *Ch. megacephala*, and yet a relatively large genetic distance of these

species from *Ch. rufifacies* (Table 4.1). These species could also be sourced from the same locality, which was favoured to avoid any local adaptation or acclimation that may exist for each species (Higley & Haskell, 2001; Donovan *et al.*, 2006).



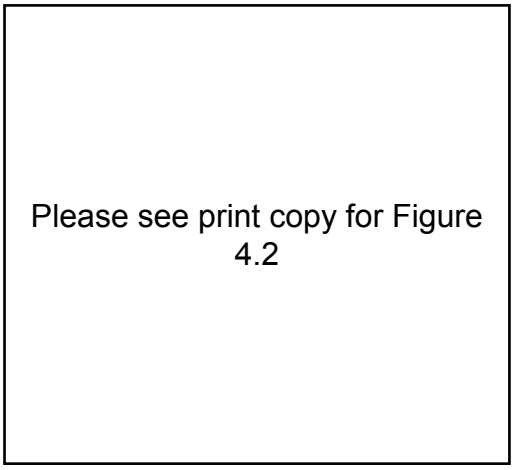


Figure 4.2. Sister species *Chrysomya saffranea* (left) and *Chrysomya megacephala* (right) display considerable morphological similarity, differing primarily in the coloration of the supravibrissal and subvibrissal setulae, the general vicinities of which are indicated by arrows (Wallman, 2001a). Photos: J.F. Wallman and L.A. Nelson.

Table 4.1. Mean percentage sequence divergences (Kimura two-parameter) by DNA region between sister species *Ch. megacephala* and *Ch. saffranea*, and their divergence from *Ch. rufifacies*.

Please see print copy for Table 4.1

This study provides developmental data for these three Australian *Chrysomya* species at three temperatures. These data are used to establish whether related species (as determined from genetic analyses) differ significantly in their growth rates. Of particular interest is whether one set of developmental data can be applied to a group of closely related flies. If two very closely related species display identical developmental profiles, their taxonomic separation may not be necessary for the purposes of PMI estimation. Indeed, if all members of a particular genus have identical developmental profiles, identification of the blowfly specimen to a level beyond genus may not be necessary for this purpose. If this is the case, efforts should be concentrated on determining the thermobiological preferences for forensically important species under standardised conditions with less emphasis placed on distinguishing related species by DNA identification. If larval development is shown to be species-specific, the need for correct species identification is crucial and warrants further examination.

4.2. Materials and Methods

4.2.1. Choice of species

Chrysomya rufifacies, Ch. megacephala and *Ch. saffranea* were selected for detailed study. Previous trapping had established their sympatry and abundance near Cairns, Qld, Australia (J.F. Wallman, pers. comm.). A comparison of the development of very closely related species (*Ch. saffranea* and *Ch. megacephala*) with that of a less closely related member of the same genus, *Ch. rufifacies*, would provide insight into the effect of genetic distance on developmental variation. These species have been recorded in crime scene samples in Australia (J.F. Wallman, pers. comm.; Levot, 2003). In addition, these species could be cultured in the laboratory successfully.

4.2.2. Fly cultures

Adult *Ch. rufifacies, Ch. megacephala* and *Ch. saffranea* were collected near Cairns, northern Queensland, Australia (16.893884 S, 145.752558 E). Flies were collected by sweep net from a piglet carcass used as an attractant. Females were identified in the field and placed in individual glass tubes containing a small amount of kangaroo mince and cotton wool. Cotton wool was previously found to encourage oviposition in these species. This may be due to tactile stimuli, similar to the fur of a carcass, which would provide a protected site in which to oviposit (Monzu, 1977). The adults were preserved in absolute ethanol for further molecular and morphological identification in the laboratory, and are maintained at 4°C in the Diptera collection at the School of Biological Sciences, University of Wollongong. The larvae of each female were maintained separately until her identity was confirmed. Adult identifications were established morphologically according to Wallman (2001a). The identities of *Ch. saffranea* and *Ch. megacephala*, which share considerable morphological similarity, were confirmed further by analysis of the mitochondrial cytochrome oxidase I 'barcode' region, following the protocol of Nelson *et al.* (2007). For some specimens, the ribosomal second internal transcribed spacer (ITS2) was sequenced according to Nelson *et al*. (2008).

Once identified, the adults of the F_0 generation of each species were pooled and maintained in separate plastic cages (300 × 500 × 250 mm) with fly mesh lids. Cultures were kept in the rearing room of the Forensic Entomology Research and Analysis Laboratory, University of Wollongong, at 25±3°C with a photoperiod of 12:12 (light:dark) h that included a 15 min 'dusk' transition period of low light between each phase. Each culture was provided with granulated raw sugar and water *ad libitum*, and kangaroo mince for ovary maturation and oviposition.

4.2.3. Larval thermogenesis

Larvae feeding in a mass can elevate their growing temperatures considerably above ambient air temperature, leading to increased developmental rates compared to those

expected from ambient temperatures alone (VanLaerhoven, 2008). The lower threshold of larval density needed to generate heat sufficient to override ambient fluctuations was not known for the species under investigation. Therefore, the number of larvae for each species that could be used per replicate without generating additional heat was investigated prior to the conduct of the temperature experiments. Numbers of larvae investigated were 0, 10, 20, 50 and 100. Freshly laid eggs were counted and placed on approximately 120 g of kangaroo mince. Each treatment was repeated in triplicate at a constant temperature of 25±0.2°C under a 12:12 h light cycle in an Axyos incubator (Brisbane, Australia) fitted with a Eurotherm 2604 temperature-control unit (Quantum Scientific, Australia) set at 60% relative humidity. Each replicate contained three temperature loggers (iButtons, accuracy ±1.0°C, resolution 0.5°C; Maxim Integrated Products Inc., Sunnyvale, CA, USA) inserted into the feeding substrate to record temperatures experienced by the growing larvae. Additional temperature loggers were positioned within the incubator to record any variation present. Intermittent water spraying of the feeding substrates prevented desiccation. The replicates were left in the incubator until pupation had commenced. At the completion of each time point, the replicates were removed and the temperature data from the loggers downloaded.

4.2.4. Growth under different temperatures

The flies used in these experiments were between two and ten generations after being removed from their natural source populations. Inbreeding was not believed to affect the fecundity, longevity and activity of the flies (Mackerras, 1933). Flies were allowed to oviposit on kangaroo mince placed in cages. Eggs were collected within 1 hour of the first observation of oviposition. This was designated as the beginning of development (0 h), as it closely approximates the start of the biological clock used in PMI estimates (Catts & Goff, 1992; Sukontason *et al.*, 2008). Clumps of approximately 50 eggs (clump size determined from prior experimentation) were separated and placed onto excess (~120 g) kangaroo mince contained within 100 ml disposable polystyrene weigh boats. An earlier study determined this number of maggots comprising each replicate negated temperature increases caused by a maggot

mass effect. The weigh boats were placed into 1 L plastic rearing containers with ventilated fine mesh lids, on top of ~3 cm wheaten chaff which provided a dry shelter for pupation. To ensure sufficient larval measurements could be made (while avoiding a maggot-mass effect), it was necessary to prepare separate replicates. Four replicates per species (at each time point) were placed randomly into a temperature-controlled cabinet at one of four temperature regimes (20, 25, 30 and 35±0.2°C) under a 12:12 h light cycle. For the 25°C temperature regime, the Axyos incubator (25±1.0°C) was used with 60±5% relative humidity. For the remaining temperature regimes, a Thermoline incubator was used (Model TRI 396-1-SD, Thermoline Scientific, Australia). For this incubator, a beaker of water provided a relative humidity of 60±4%. Temperature and humidity within each cabinet were monitored with data loggers (iButtons and Tinyview TV-1500, Gemini Data Loggers Pty Ltd, Australia).

Rearing containers were left undisturbed until an allocated time had elapsed. Time points were every 24 h from oviposition until at least 95% of each cohort had pupated. Pupation was characterised by failure of the larvae to elongate and move in response to being disturbed. For each 24-hourly time point, careful dissection of the feeding substrate ensured all larvae were recovered from the mince. Larvae were killed by immersion in just-boiled water, dried with paper towel and preserved in 80% EtOH. Larval lengths were measured to the nearest 0.1 μm using a Leica $MZ7_5$ dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany) and Motic Images Plus 2.0 ML software (Motic China Group Company Ltd., Xiamen, China). Previous research (unpublished data) had shown that there was no significant difference between measurements made with digital callipers and the Motic software. Body length was measured as the distance, viewed laterally, between the most distal parts of the head and the last abdominal segment. Larval instars were determined by examination of the posterior spiracles. The proportions of first-, second-, and third-instar larvae and pupae were noted for each sample.

4.2.5. Temperature preferences

Larvae can regulate their own temperatures by behavioural positioning within the maggot mass. Developmental rates of blowflies at their preferred temperature can be used to calculate accelerated larval development caused by maggot-mass formation (Byrd & Butler, 1996). To determine the temperature preferences of newly-emerged feeding larvae of the three species, an experiment was set up utilising an existing temperature gradient apparatus which provided a range of temperatures from approximately 16-57°C (Fig. 4.3). The apparatus consisted of a copper tray, one end of which was attached to a heating element fixed at a constant temperature of 60°C, while the other end was connected to an insulated box containing ice at 0°C. The copper tray was filled with a 50:50 (V/V) mixture of kangaroo mince and hydrated water saving crystals (Yates® Waterwise Water Storing Crystals, Orica Australia Ltd Pty) to a depth of 1 cm, which provided a feeding substrate for the larvae that would not desiccate at the high temperatures experienced at the heated end of the gradient. The tray was inserted into a transparent Perspex tube with foam plugs at either end, for insulation and to prevent the escape of larvae. Prior to the addition of any larvae, the kangaroo mince mixture was allowed to equilibrate for 1 h to the opposing temperatures at each end of the gradient.

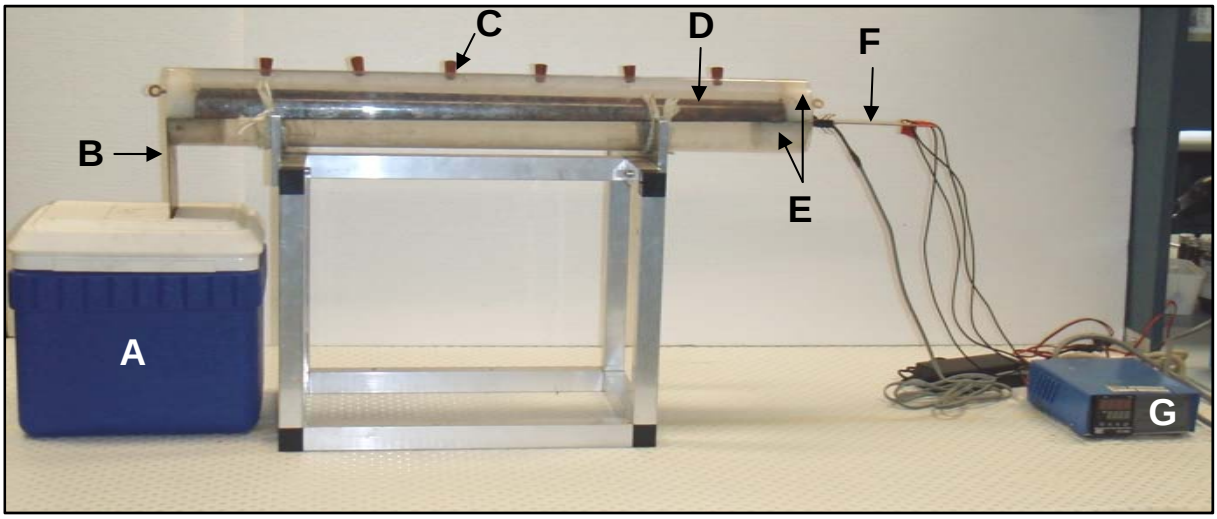


Figure 4.3. Temperature gradient apparatus used for larval temperature preference experiments. A, insulated ice box; B, copper sheet; C, plugged holes 100 mm apart for temperature measurement; D, copper tray containing feeding substrate; E, foam plugs (at both ends); F, heating element; G, temperature control box.

Eggs were obtained on kangaroo mince from individual cultures and monitored hourly until hatching. For each experiment, 30 newly-hatched first-instar larvae were randomly selected and distributed evenly along the length of the copper tray at 100 mm intervals. Larvae were left for 24 h, after which time their positions were recorded. Temperatures were measured at 100 mm intervals along the copper tray using a digital temperature probe (Model HI 93510N, Hanna Instruments, Australia). The temperature preferences of the larvae were then inferred. All larvae were recovered from the copper tray at the conclusion of each experiment, which was repeated in triplicate for each species. An additional control experiment was conducted for each species, where all experimental conditions were identical to those described, except that no temperatures were applied to either end of the copper tray (i.e. the experiment was conducted at room temperature, at an average of 21ºC).

4.2.6. Larval survivorship

Early pilot studies established that kangaroo mince had a tendency to desiccate readily at the heated end of the copper tray (Fig. 4.3), changing the composition of the feeding substrate markedly. The addition of the water storage crystals counteracted this problem, but their effect on the larvae was unknown. A separate experiment was therefore conducted to assess the effect of the water storage crystals on larval survivorship. For each species, 30 newly-hatched larvae were placed on either 120 g kangaroo mince or 120 g of a 50:50 (V/V) mixture of kangaroo mince and water storage crystals. Four replicates per species were produced and left for 24 hours at 20.2±2°C after which time all maggots were killed as previously described. In this experiment, survivorship was calculated as the number of larvae that survived from egg to approximately first-instar on the growth medium – the approximate time frame investigated for the temperature preference experiments.

4.2.7. Data and statistical analyses

Data from iButtons were downloaded with iButton Viewer software (iButton-TMEX Runtime Environment Version 3.12, Maxim Integrated Products Inc.); data from the

Tinyview TV-1500 were downloaded with Tinytag Explorer (Version 4.4.183, Gemini Data Loggers Pty Ltd). These data were imported into Excel® (Microsoft Corporation, Redmond, WA, USA).

Statistical analyses were performed using JMP (Version 5.1; SAS Institute Inc., Cary, USA). In all tests, a 5% level of significance was used. Survivorship was assessed using the Student's *t*-test, comparing numbers of surviving larvae grown on kangaroo mince alone with those grown on kangaroo mince containing water storage crystals. These comparisons were repeated for each species. There were three replicates of 30 larvae each for each food substrate. While there was not a linear relationship between temperature and distance along the temperature gradient, a plot of the inverse of temperature (ºC) versus distance along the gradient showed a strong linear relationship. The line of best fit relating inverse of temperature to distance was used to predict the temperature at a certain distance. Larval temperature preferences were calculated by inference from this standard curve (Appendix 1). For larval growth experiments at three temperatures, the mean larval lengths of different species at the same time point and temperature were compared with one-way analysis of variance (ANOVA). Tukey-Kramer HSD was used to determine which species differed.

4.3. Results

4.3.1. Species identification

The specimens were identified by adding their COI sequences to an existing barcode database for this genus (Nelson *et al.*, 2007). This analysis confirmed morphological identifications in all but two instances: two *Ch. saffranea* specimens were resolved paraphyletic to a clade comprising *Ch. megacephala* and *Ch. saffranea.* The two specimens showed a COI divergence of 0.15% between them, with a mean divergence of 0.40% and 0.54% from all other *Ch. saffranea* and *Ch. megacephala* specimens, respectively. The 0.15% is at the high end of the intraspecific divergence noted previously for *Ch. saffranea* of 0.043% (range 0.00-0.152%, Nelson *et al.*, 2007). To

investigate this further, the ITS2 regions were sequenced from these specimens. Both ITS2 sequences were identical. Sequence analysis revealed ITS2 divergences of 0.29% and 0.47% from *Ch. saffranea* and *Ch. megacephala* specimens, respectively. While the specimens showed the lowest divergence from *Ch. saffranea*, their larvae were nonetheless excluded from the fly cultures.

4.3.2. Larval thermogenesis

Temperature data recorded within the Axyos cabinet showed a distinct diurnal fluctuation despite its constant temperature setting. The temperature decreased by an average of 0.75°C overnight and exceeded the 25°C set temperature on average by 0.25°C during the day (data not shown). This fluctuation was attributable to the poor insulation of both the cabinet and the building within which the cabinet was housed. Despite this, the diurnal fluctuation was observed to be consistent and did not show any deviation beyond that reported. Most interestingly, the temperatures recorded within the feeding substrates, including the 0 larvae control, were consistently an average of 1°C lower than those recorded within the cabinet. This phenomenon was assumed to be due to evaporative cooling of the moist feeding substrate. The 0, 10, 20, 50 and 100 larvae treatments displayed no detectable deviations from the temperatures recorded from the control (data not shown). To remain cautious, 50 larvae per replicate were considered a suitable number for the temperature development studies that followed.

4.3.3. Growth under different temperatures

Replicates for a single time point, temperature and species were pooled as no significant difference was observed among these ($P>0.05$). Eggs occluded (hatched) between 6 and 24 h after oviposition at all temperatures except 20ºC, where eggs occluded between 24 and 48 h. Considerable difficulties were experienced when rearing larvae of all species, particularly *Ch. saffranea*, at 20°C. High mortalities, considerably stunted larvae and high variability in larval lengths were produced; therefore this temperature was believed to be too cool for adequate growth to take

place. Consequently, data collected at 20°C were not considered reliable and were excluded from further analyses.

As sampling was conducted at 24-hourly intervals, large changes in the proportions of first-, second- and third-instar larvae and pupae were observed for each species at each time point (Figs 4.6 – 4.8). For instance, for *Ch. megacephala* at 25°C, 100% of the 24 h sample comprised first-instar larvae, and 100% of the 48 h sample comprised second-instar larvae (Fig. 4.6). The transition between these stages could be revealed by more frequent sampling during this period. The transition from first- to second-instar larvae at 25°C was slowest for *Ch. rufifacies,* with 38% of the 48 h sample still consisting of first-instar larvae. First- and second-instar larvae were both observed from 24 h for all species at 30 and 35°C (Figs. 4.7 and 4.8, respectively). The third-instar larval stage lasted the longest in all species. At 25°C, third-instar larvae were first recorded at 72 h for *Ch. megacephala* and *Ch. saffranea,* and at 96 h for *Ch. rufifacies.* This stage lasted several days after the first observations of pupation for these species at 25°C (Fig. 4.6). At 30 and 35°C, third-instar larvae were observed from 48 h. A significant difference in the proportions of the larval stages was observed at this time ($P<0.05$). This is particularly notable for *Ch. saffranea* at 35°C, where the sample consisted wholly of third-instar larvae while the other species displayed a large proportion of second-instar larvae (Fig. 4.8). The transition from third-instar larvae to pupae for *Ch. rufifacies* at 25 and 30ºC appeared to be slower than the other two species, with a much larger overlap of the two stages during this period (Figs. 4.6 and 4.7). A significant difference in the larval stages was seen at 168 h at 25°C ($P<0.05$), with *Ch. rufifacies* third-instar larvae comprising 100% of the sample while the majority of larvae for the other two species had pupated (Fig. 4.6). A similar difference was observed at 96 h at 35°C ($P<0.05$), where almost all *Ch. rufifacies* comprised third-instar larvae and the majority of *Ch. saffranea* larvae had pupated (Fig 4.8). At 25°C, pupation was first observed at markedly different times for the different species; 144 h (*Ch. saffranea*), 168 h (*Ch. megacephala*), and 192 h (*Ch. rufifacies*). In comparison, at 30°C and 35°C, pupation was first observed at 96 h for all species. Effectively, the 5-10°C increase in temperature from 25°C sped up the onset of pupation by 48, 72 and 96 h for *Ch. saffranea, Ch. megacephala* and *Ch.*

rufifacies, respectively. The decrease in time to pupation with increase in temperature was expected. Many *Ch. rufifacies* larvae were observed pupating within or very close to the feeding substrate, while larvae of the other two species preferentially chose the chaff as their pupation site.

As expected, the growth profiles showed a decrease in third instar larval length until pupation (Fig. 4.4). After the maximum length was achieved at each temperature, (after 120, 72, and 72 h, respectively; Table 4.2) there was a distinct decline in the numbers of larvae available for analysis due to the progression from third-instar to the pupal stage. Because of this, data from later time points were not suitable for statistical comparisons of larval lengths. There was no distinct trend in the differences between the species; analysis revealed significant differences in larval lengths for each species at the majority of time points for each temperature (Fig. 4.4). This was particularly the case for 30°C, where all measurements were significantly different. There were a few cases where significant differences in length were not detected between two species, but where the two species in question together differed significantly from the third. This occurred for 96 h at 25°C where *Ch. megacephala* and *Ch. rufifacies* differed significantly from *Ch. saffranea*, and for 120 h when *Ch. megacephala* and *Ch. saffranea* differed significantly from *Ch. rufifacies*. This was also observed for 24 h at 35°C where *Ch. megacephala* and *Ch. saffranea* differed significantly from *Ch. rufifacies.* There was no obvious trend in the size order for each species for each temperature examined, although *Ch. saffranea* appeared to be larger than the other species at the majority of time points compared.

Table 4.2. Maximum larval lengths (µm) of *Chrysomya* species grown at constant temperatures of 25, 30 and 35°C on kangaroo mince.

	Maximum larval length (µm) reached at constant temperatures					
	25°C		30°C		35°C	
***Chrysomya* species**	mean	SE	mean	SE	mean	SE
Ch. saffranea	15,086	90	15,215	113	13,221	82
Ch. megacephala	14,922	115	13,434	153	13,754	61
Ch. rufifacies	12,427	70	13,237	76	14,683	72

SE, standard error

Inspection of the data at all three temperatures reveals what appears to be a rightward shift of the growth patterns for *Ch. megacephala* and *Ch. rufifacies* relative to the pattern for *Ch. saffranea* (Fig. 4.4). At 30ºC, *Ch. saffranea* changes in length from being the largest of all three species at 72 h to the smallest at 96 h. This suggests that *Ch. saffranea* reached its maximum size ahead of the other two species and is already starting to shrink (pupation process) at the following time point. Compared to 25 ºC, the data for 30 and 35ºC are somewhat limited due to the rapid development of the species at these temperatures, rendering relatively few larval length time points for analysis after 96 and 72 h, respectively. It is apparent that more frequent sampling could enhance these data. For instance, at 35°C, it is quite feasible that *Ch. saffranea* attains its maximum length some time between 48 and 72 h, but that the present sampling regime was unable to reveal this.

As a whole, these results do not support the hypothesis that more closely related species share more similar growth rates; as *Ch. megacephala* and *Ch. saffranea* showed no significant similarity for each temperature investigated, compared with their similarity with *Ch. rufifacies*. The only conclusion that can be drawn from this set of data is that growth rates appear to be species-specific and influenced by factors other than genetic relatedness. While most of the mean lengths differed significantly for each species at each time point, examination of data points for the three species at each temperature revealed overlapping lengths at each time point. For instance, the largest difference in mean lengths was seen at 35ºC at 48 h, but examination of the spread of the lengths at this point reveals considerable overlap in the sizes of larvae among the species (Fig. 4.5). This overlap in larval sizes is even more apparent at time points for which the difference in the means is lower, for example at 25ºC at 72 h (Fig. 4.5).

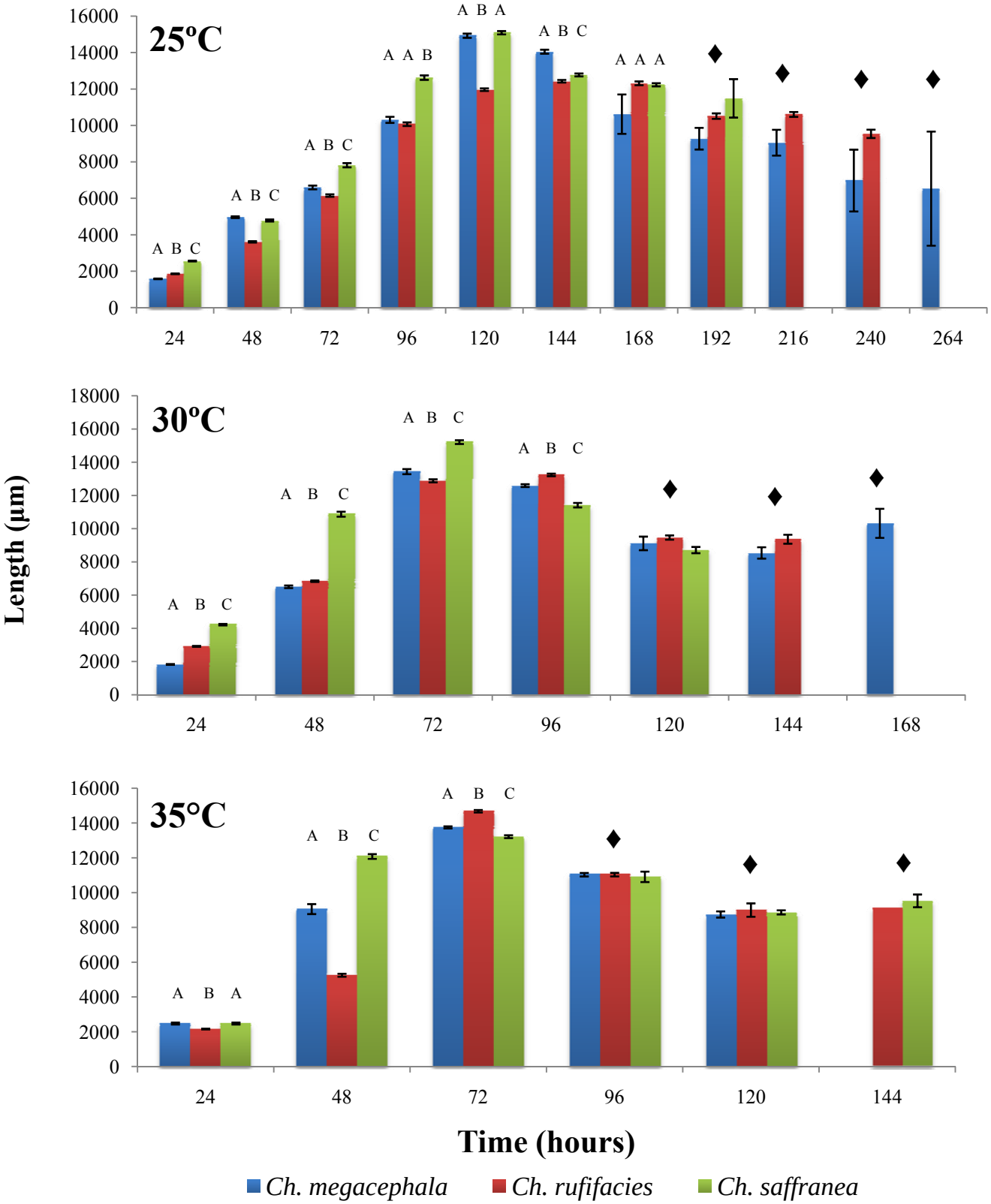


Figure 4.4. Mean larval lengths of *Ch. megacephala*, *Ch. rufifacies* and *Ch. saffranea* measured at 24-hourly intervals, growing at constant temperatures of 25, 30 and 35°C under a 12:12 (light:dark) h photoperiod. Bars are ± standard error. The same letter connecting species at a given time point and temperature denote nonsignificant differences in mean lengths. ♦ = insufficient larvae remaining in sample for valid statistical comparison to be made.

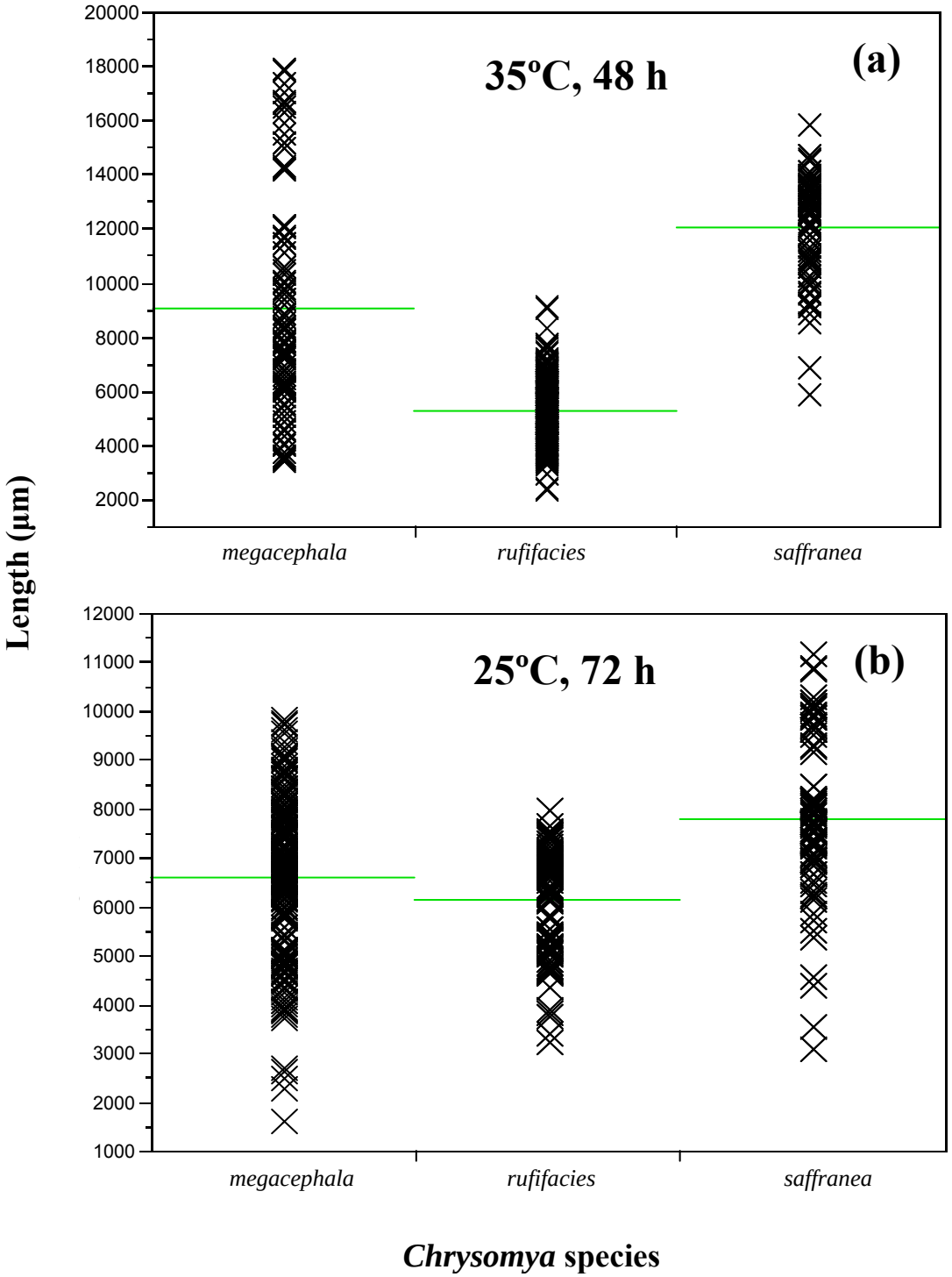


Figure 4.5. Examples of the variation in larval lengths of *Ch. megacephala*, *Ch. rufifacies* and *Ch. saffranea* larvae at two times and temperatures, where means (green lines) were significantly different. (a) The 35ºC, 48 h sample displayed the largest difference in means, although closer inspection reveals a considerable overlap in larval lengths. (b) This overlap is even more marked at 25ºC, 72 h, where the difference in means is much smaller.

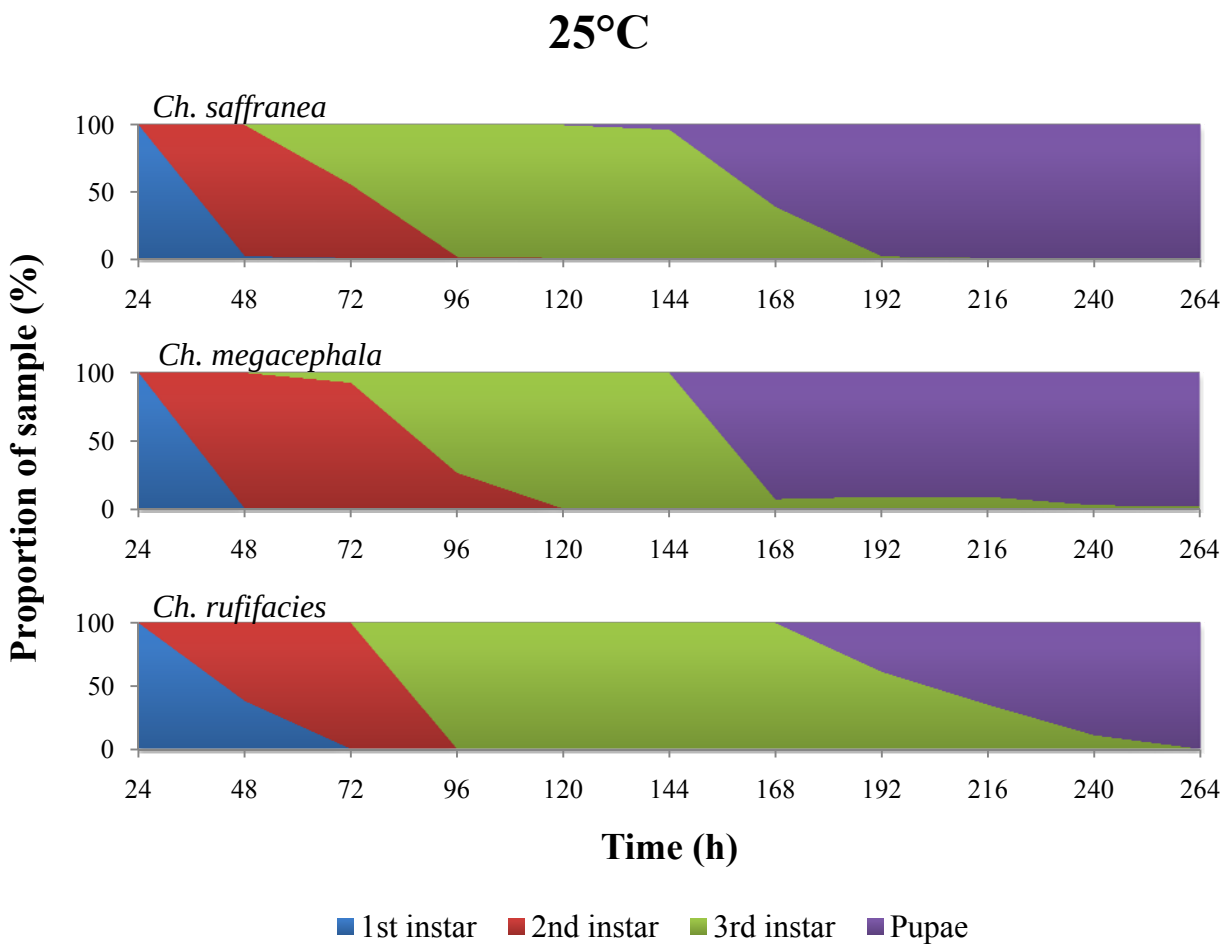


Figure 4.6. Proportions of *Ch. megacephala*, *Ch. rufifacies* and *Ch. saffranea* immature (first, second and third instars and pupae) observed per time point (hours since egg laying) when grown at 25°C. Example: A vertical line drawn at 36 h for *Ch. saffranea* reveals that approximately 50% of the larvae are first instar and 50% are second instar at this time.

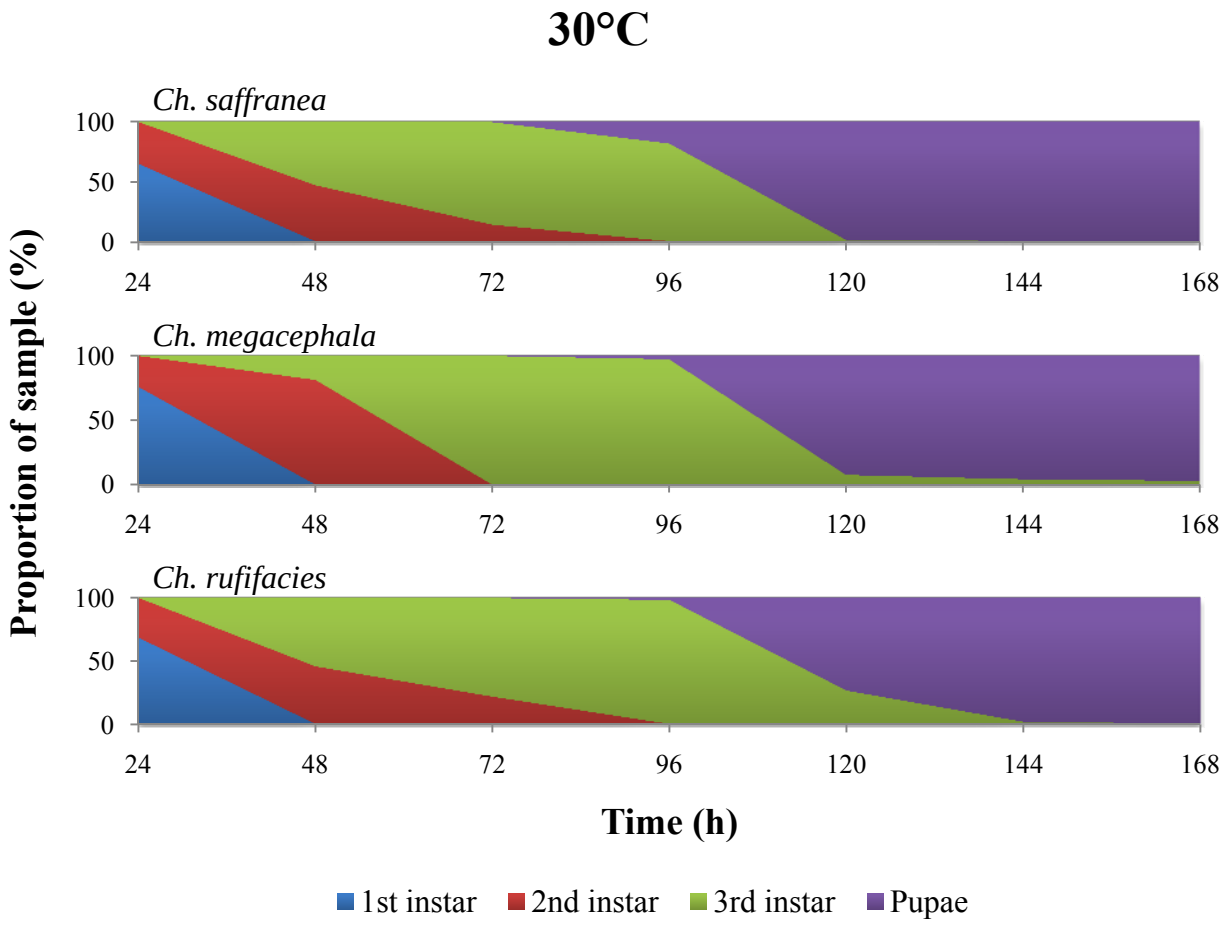


Figure 4.7. Proportions of *Ch. megacephala*, *Ch. rufifacies* and *Ch. saffranea* immature (first, second and third instars and pupae) observed per time point (hours since egg laying) when grown at 30°C.

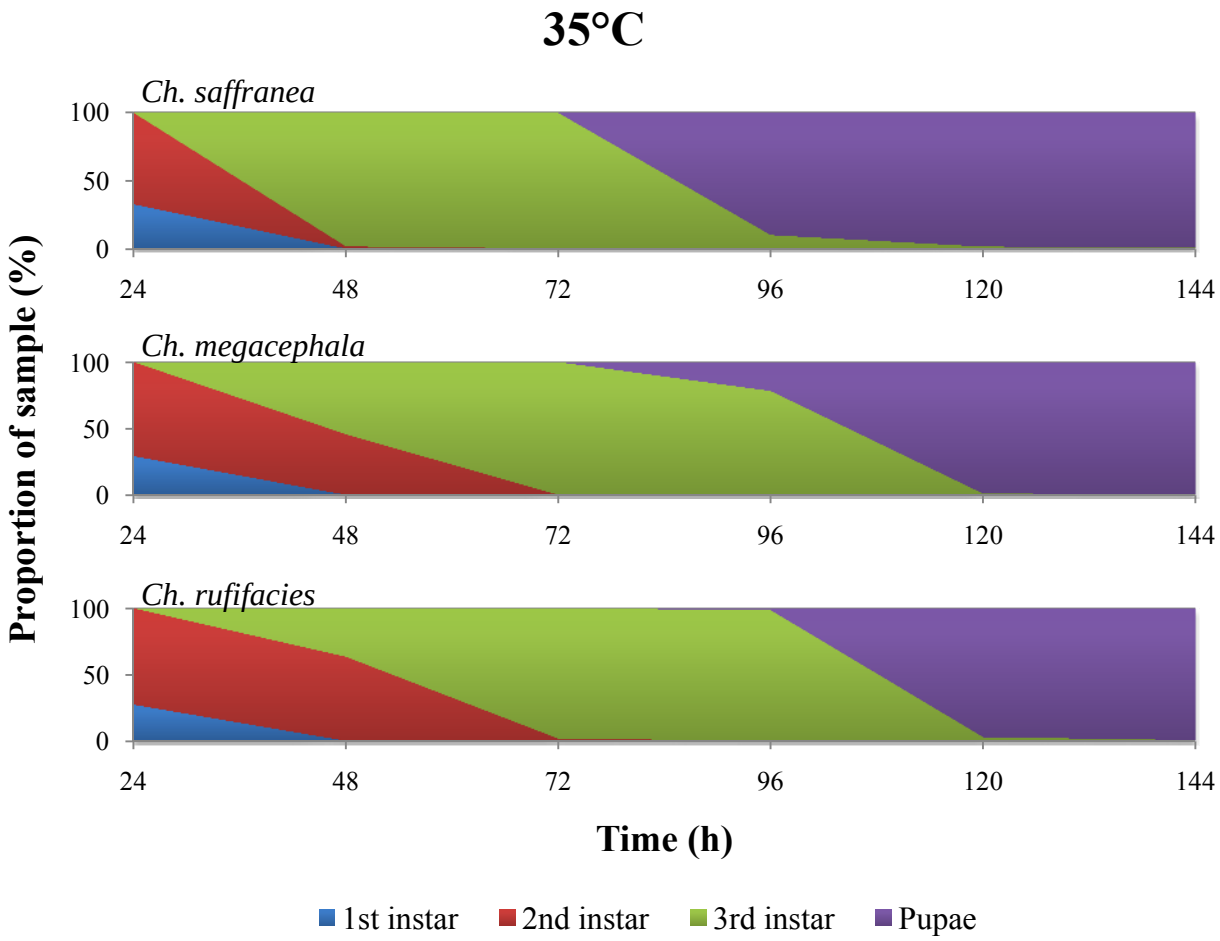


Figure 4.8. Proportions of *Ch. megacephala*, *Ch. rufifacies* and *Ch. saffranea* immature (first, second and third instars and pupae) observed per time point (hours since egg laying) when grown at 35°C.

4.3.4. Temperature preferences and larval survivorship

Initial observation of the survivorship of larvae fed on kangaroo mince alone versus a 50:50 (V/V) mixture of kangaroo mince and water storage crystals appeared to suggest a slightly higher survival on kangaroo mince without crystals. However, closer inspection of the data revealed the difference to be an average of less than one larva per treatment. For all species, there was no statistically significant difference in survivorship on kangaroo mince alone or on a 50:50 (V/V) mixture of kangaroo mince and water storage crystals (P>0.05) (Fig. 4.9). Consequently, these data were combined to give mean survival rates of 65.4, 67.1 and 70% for *Ch. megacephala*, *Ch. saffranea* and *Ch. rufifacies*, respectively, to be used as predicted survival rates in the temperature gradient experiment (below). There was no significant difference between survival rates among the species (P>0.05). These data indicated the expected percentage of larvae that would be recovered from the copper tray at the conclusion of the temperature preference experiments.

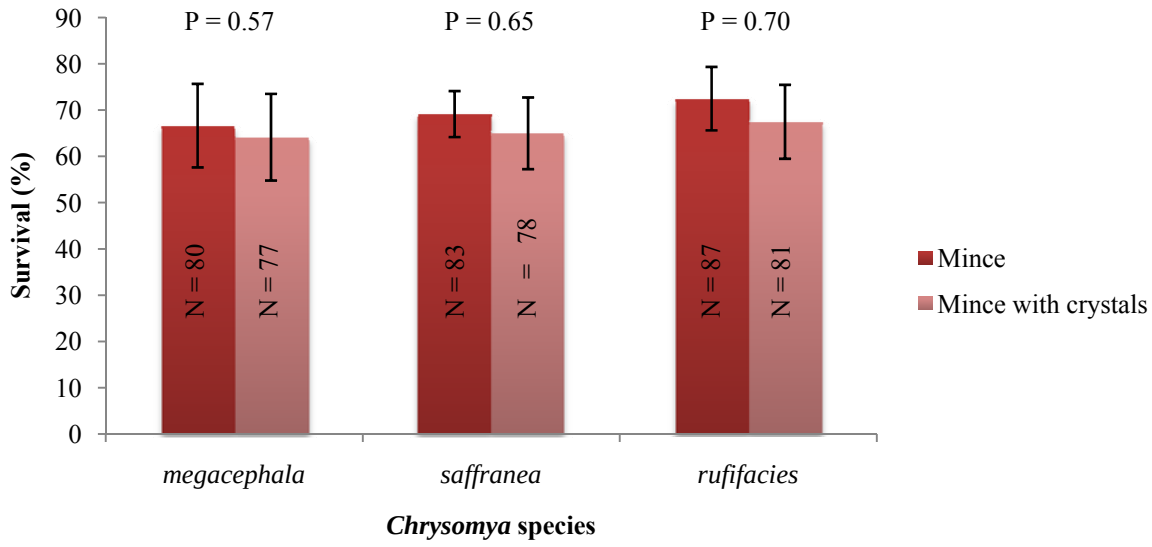


Figure 4.9. Mean percentage survival of *Chrysomya* larvae grown on kangaroo mince alone ('mince') or a 50:50 (V/V) mixture of kangaroo mince with water storage crystals ('mince with crystals') at 20±2°C over 24 h. No significant difference was detected between treatments and species (P>0.05). N, total number of larvae surviving out of 120 for each treatment. Bars are ± standard error.

The mean survival for each species from the temperature gradient experiments was calculated as the percentage of larvae that were recovered from across the feeding substrate at the conclusion of the experiment. The mean survivorship for each species was 51.1, 71.1 and 81.1% for *Ch. megacephala*, *Ch. saffranea* and *Ch. rufifacies*, respectively (Fig. 4.10). The only significant difference in survivorship on the temperature gradient was that between *Ch. megacephala* and *Ch. rufifacies* (P = 0.022). However, the survivorships noted here for each species were not significantly different to those predicted by the initial study of survivorship (above), where no gradient temperature was applied (P>0.05).

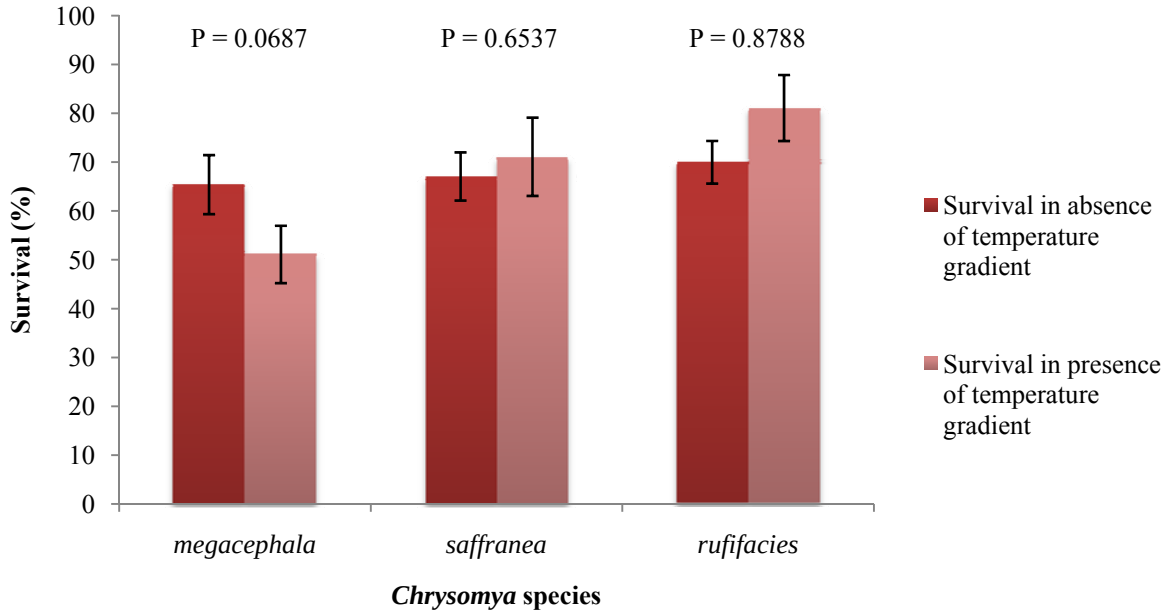


Figure 4.10. Mean percentage survival of *Chrysomya* larvae after 24 hours on a temperature gradient (approx. 16-57°C) comprising a 50:50 (V/V) mixture of kangaroo mince with water storage crystals and predicted survival based on growth under constant temperatures (20.2±2°C). No differences were detected between predicted and actual survivals for a given species at the 0.05 significance level. The survivals for *Ch. megacephala* and *Ch. rufifacies* on the temperature gradient differed significantly. Bars are ± standard error.

The mean temperatures (± SE) preferred by each species, as determined by inference from the standard curve (Appendix 1), were 38.9± 0.44, 39.2± 0.26) and 34.1± 0.16°C for *Ch. megacephala*, *Ch. saffranea* and *Ch. rufifacies*, respectively (Fig. 4.11). There was no significant difference between the temperature preferences of *Ch. megacephala* and *Ch. saffranea* (P = 0.3947). However, these temperatures were both

significantly different to the mean temperature preferred by *Ch. rufifacies* larvae (P<0.0001 for *Ch. rufifacies* compared with each of *Ch. megacephala* and *Ch. saffranea*). The control experiment indicated that, with a lack of heating or cooling applied to either end of the gradient, maggots dispersed randomly along the length of the copper tray.

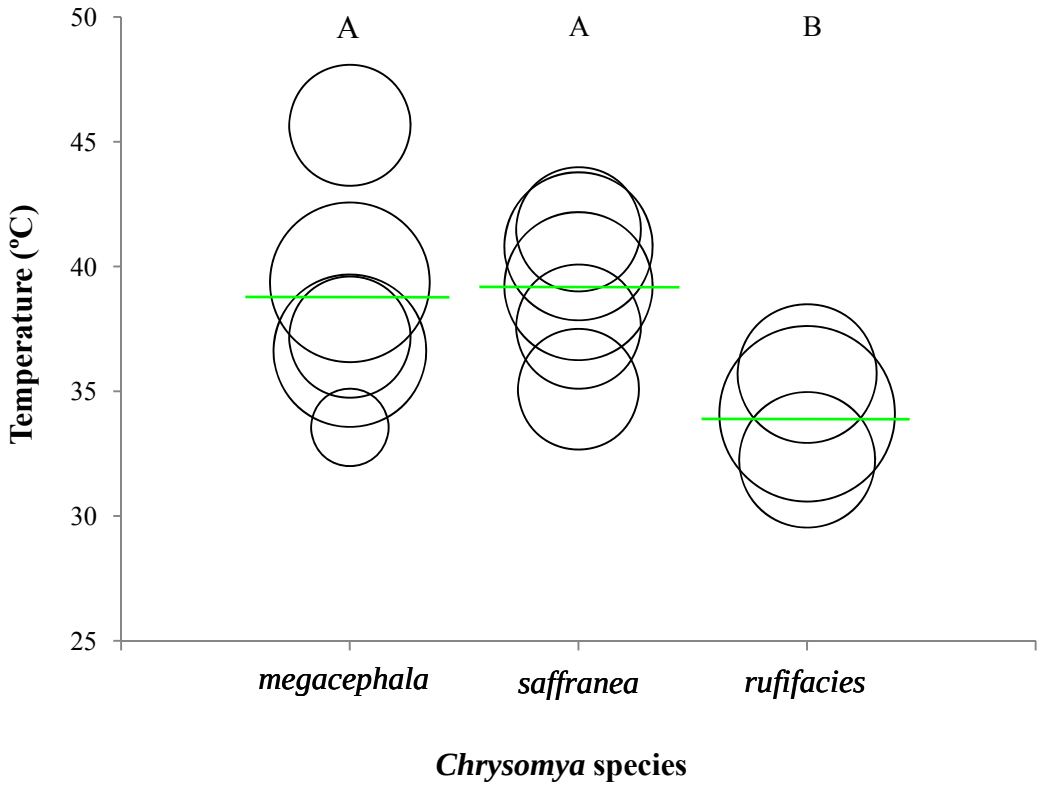


Figure 4.11. Distributions of *Chrysomya megacephala*, *Ch. rufifacies* and *Ch. saffranea* larvae after 24 hours feeding on a temperature gradient (approx. 16-57°C) comprising a 50:50 (V/V) mixture of kangaroo mince with water storage crystals. Larvae were distributed evening along the gradient and left to migrate. Circles represent groups of larvae at a particular temperature. The temperature is indicated by the centre of each circle, and the area is proportional to the number of larvae present. Mean temperatures are indicated in green; identical letters above measurements indicate non-significant differences between species.

4.4. Discussion

4.4.1. Species identifications

The identities of the *Chrysomya* specimens collected in the field were confirmed by molecular analyses, with the exception of the two 'mystery' specimens for which conclusive identifications were not possible. Because the DNA sequences of these two specimens differed substantially from both *Ch. megacephala* and *Ch. saffranea* (and indeed all other *Chrysomya* species contained within the databases used) they may be a different species altogether. Therefore, their larvae were not included in the cultures that were used for developmental experiments. This was necessary, as one of the principal aims of this study was to measure the effect, if any, of genetic distance on developmental profiles. Further sequence analysis could reveal the true identities of these specimens. Alternatively, it may be the case that the extent of intraspecific variation existing in *Ch. megacephala* and *Ch. saffranea* has not been encompassed by the COI 'barcode' and ITS2 databases employed for these identifications.

The low interspecific genetic differences noted between *Ch. megacephala* and *Ch. saffranea* suggest that these species have only recently diverged (Wallman *et al.*, 2005). The close relationship of these species was reinforced further by the identification of a suspected *Ch. megacephala/Ch. saffranea* hybrid caught in Cairns (Nelson *et al.*, 2007). The extent and abundance of such hybridisation is not known, but its occurrence led to questions regarding the status of these two species as separate entities. Understanding of the genetic bases for speciation in general is still limited (Wu, 2001). One of the definitions of species is that they are reproductively separated from each other by 'isolating mechanisms', including genetic traits that prevent interbreeding (Coyne & Orr, 1998). However, some consider sexual isolation to be the last step in speciation (Dobzhansky, 1935). It is therefore possible that these species are still undergoing the final stages of speciation and have not yet reached equilibrium. Very little is known about the population ecology of these two species in Australia. The thermal data presented here support the theory of species-specific

development, indicating that a more thorough investigation of these two species is required.

4.4.2. Larval densities

It has been shown that larval population density is related to the increase in heat generated relative to ambient temperatures (Marchenko, 2001; Slone & Gruner, 2007). In many studies, the ambient temperatures (e.g. the set-points of temperature-controlled cabinets) are reported, but the temperatures actually experienced by the growing larvae are neglected (e.g. Greenberg & Tantawi, 1993; Byrd & Butler, 1997; Ireland & Turner, 2006). In order to compare growth of all three species at constant temperatures, it was necessary to conduct a pilot study investigating larval densities to ensure that larvae were actually growing at the temperatures to which they were being exposed. The 1°C temperature drop (relative to ambient) recorded within the feeding substrates in these experiments was an incidental observation, and may be a common, yet unreported, occurrence in other developmental studies. Such an occurrence should be taken into account when producing larval development data for use in PMI estimations. The larval numbers investigated in the pilot study did not raise the substrate temperature above ambient, suggesting that an excess of 100 larvae per 120 g kangaroo mince would be required to achieve this. This is consistent with the findings of Kheirallah *et al.* (2007) who showed that 170 larvae per 120 g of 'minced meat' were required to raise the temperature of the feeding substrate above ambient. The conservative use of 50 larvae per 120 g of feeding substrate was decided upon for this study. While this is an unrealistic situation in comparison with that likely to exist in an actual carcass or dead body, it was necessary to minimise the uncontrolled variables for the sake of this study (O'Flynn, 1983). Indeed, approximately 200 larvae can arise from just one oviparous blowfly (Mackerras, 1933).

Although the aim of this study was to eliminate any maggot-mass effect, the literature suggests that it is vital to consider this when estimating the PMI based on larval development. Increased larval density can decrease the developmental time due to higher temperatures, the accumulation of digestive enzymes, and the physical mixing

and perforation of the food source which facilitates feeding. For instance, a temperature elevation of 20°C above ambient, due to a maggot-mass effect, corresponds to a four-fold increase in metabolic rate as opposed to that estimated from ambient temperature data (Turner & Howard, 1992). This is of particular importance given that air temperatures (obtained from the scene or nearby weather stations) are typically employed for PMI estimates (Archer, 2004), which rarely take into account the effect of increased temperatures experienced by the growing larvae (O'Flynn, 1983; Cianci & Sheldon, 1990; Turner & Howard, 1992; Anderson & VanLaerhoven, 1996; Campobasso *et al.*, 2001). Conversely, insufficient larval numbers have been shown to be detrimental to larval survival, due to decreased feeding efficiency from inadequate secretion of digestive enzymes (dos Reis *et al.*, 1999).

Another important factor that needs to be considered is the effect of inter-species competition on larval development (Williams & Richardson, 1984; Kheirallah *et al.*, 2007). There is substantial evidence that blowfly larvae are negatively influenced by competition for food, leading, for example, to a reduction in size, increased mortality and undersized adults (Ireland & Turner, 2006). There is an added dynamic of competition from predatory and cannibalistic *Ch. rufifacies* larvae (Goodbrod & Goff, 1990). It is highly likely that more than one blowfly species will be recovered from a body, therefore the effects of inter-species competition need to be taken into account (Levot, 2003). Ignoring these effects on developing larvae would inevitably lead to an inaccurate PMI estimate. It is evident that further studies are required on the effect of larval density and competition, and should be focused on mimicking conditions likely to be experienced by growing larvae.

4.4.3. Larval development

The three species in this study were straightforward to rear in the laboratory, provided a protein source was available immediately after emergence. However, problems maintaining other members of this genus have been experienced, particularly *Ch. incisuralis* (pers. obs.). The length of time from oviposition to larval hatching (6-24 h)

experienced here corresponded to that reported previously for *Chrysomya* species (Sukontason *et al.*, 2008). The changes in length measurements over time, recorded in this study, were also in keeping with that recorded elsewhere (Levot *et al.*, 1979). The exponential increase in larval size in just a few days is necessary to accommodate the rapidly expanding crop and intestine, which is facilitated by the plasticity of the cuticle and the two moults during this time (Greenberg, 1991). The third instar was the longest stage in duration for all species, indicating that it is the larval form most likely to be encountered in forensic cases (Greenberg, 1991; Wallman, 2001b). The latter portion of the larval development profiles, during which the larvae have finished feeding, shows the reduction in length typical of this stage, where growth has stopped (Williams & Richardson, 1984; Greenberg, 1991). At this point, larval size is no longer a useful criterion for age due to the reversal of body size and larger variance in weight and length (Wells & Kurahashi, 1994; Tarone & Foran, 2008). There is also the probability of high variability in the post-feeding stage, and blowfly larvae can delay pupation if conditions are sub-optimal (Wells & Kurahashi, 1994; Byrd & Butler, 1997). For these reasons, it is evident that attention should be focused on understanding length variation in the earlier portions of larval growth, where it is a more useful measure of larval age.

The influence of temperature on the development of these species was exemplified by comparing the proportions of immature stages comprising each sample. The onset of pupation was brought forward considerably by raising the temperature from 25 to 30ºC. A similar reduction in the time to pupation was not seen when the temperature was raised from 30 to 35ºC, indicating that these temperatures are close to the thermal optima for these species. The reduction in time required to complete development gives an obvious competitive advantage to blowfly larvae in utilising the finite resource of a carcass (Heinrich, 1996). The responses of these species to temperature demonstrate their adaptive success as carrion breeders in consuming this transient food source efficiently and rapidly (Levot *et al.*, 1979). The tendency of *Ch. rufifacies* to pupate on or close to the food substrate, observed in this study, has been noted previously (Norris, 1965; Greenberg, 1991; Baumgartner, 1993). This behavioural trait is believed to be a survival mechanism unique to this species (J.F. Wallman, pers.

comm.), made possible by the spines which are characteristic of the 'hairy maggot', and are present on the surface of the puparium (Sukontason *et al.*, 2008). This textured surface renders the puparium somewhat like a plant burr, which cannot be removed easily by predators from the hair and other remains of the dead animal in which the larvae were developing.

The high mortalities and general poor performance of larvae at 20°C was not expected. Cairns (the region from which the fly populations originated) is tropical, experiencing fairly uniform temperatures throughout the year (Australian Bureau of Meteorology, 2008). Midsummer temperatures range from 23-31°C, while midwinter temperatures range from 18-26°C. Interestingly, the average minimum temperature only falls below 20°C between May and September, with the lowest mean minimum of 17°C being recorded in July. During these months, it is possible that the activity of *Chrysomya* species present in this region declines or that the species disappears altogether, as happens in southern Australia. It has been reported that members of this genus have temperature tolerances that correspond with their tropical distribution (Baumgartner, 1993). However, it is expected that larval development remains possible during the cooler months due to the maggot-mass effect in infested carrion in the field. This would maintain high temperatures, overcoming unfavourable weather conditions and extending the breeding season (Greenberg & Tantawi, 1993; Slone & Gruner, 2007). Indeed, maggot-masses have been observed to be active when air temperatures are as low as -4°C (Greenberg, 1991). The maggot-mass effect was deliberately avoided in the present study, but may be required for adequate development of larvae at this temperature. Future work is required to examine the dynamics of the maggot-mass, especially for studies generating data relevant to PMI estimation.

The exact experimental conditions adopted by other researchers are unknown, thereby making differences between other studies and the present one difficult to assess. The mean sizes of *Ch. rufifacies* larvae at 25ºC were considerably smaller than those reported by Byrd and Butler (1997). This discrepancy could be related to the different experimental conditions utilised by these authors, including continuous light, a pork

food medium and flies sourced from another continent (North America). The mean sizes of *Ch. megacephala* larvae at 25, 30 and 35ºC were considerably smaller overall than those reported by Nishida *et al.* (1986), working with a Japanese population, although the experimental conditions of this study are unknown. However, the development times of *Ch. rufifacies, Ch. megacephala* and *Ch. saffranea* correspond with those reported by O'Flynn (1983) for Australian populations.

Although the mean lengths per species differed significantly for the majority of times and temperatures examined in this study, the spread of larval lengths (at a specific time and temperature) suggests that growth data of one species could be substituted by those of another. A common practice in developmental studies is to select the largest individuals from the sample to measure (e.g. Byrd & Butler, 1996; Byrd & Butler, 1997; 1998; Grassberger & Reiter, 2001; Greenberg & Kunich, 2002; Sukontason *et al.*, 2008). The justification for this approach is that the largest maggots represent the oldest individuals present in a sample, and are most likely to have originated from the first egg laying episode. This practice has been criticised due to the high variability in larval lengths for a given age, a phenomenon confirmed by this study. The natural variation in larval size for each age clearly warrants further investigation as to whether selecting only the largest individuals is a valid procedure for the generation of growth curves to be used for PMI estimations.

Standardised larval killing and preservation methods were employed throughout this study, which allowed direct comparisons of larval lengths for each species, temperature and time point. However, killing and preservation methods have been questioned in the past, as different protocols have been shown to affect the measurable larval length (Tantawi & Greenberg, 1993; Day & Wallman, 2008). For the purposes of PMI estimation, it is therefore important that larvae be killed and preserved by the same method as those from which developmental growth curves were generated. Alternatively, the possibility of length alteration by another killing and preservation technique needs to be taken into account when making age inferences from existing developmental data. It has been suggested that dry weight is less affected than body length by different preservation methods (Wells & Kurahashi,

1994). Also, it has been reported that instar data are more effective than size for estimating the age of young larvae (Wells & Kurahashi, 1994).

The validity of constant temperature experiments, given the naturally fluctuating diurnal temperature patterns, has been questioned (Greenberg, 1991). Constant temperature studies are unnatural representations of larval development for two reasons: (1) ambient temperatures usually follow a sinusoidal diurnal fluctuation, and (2) larval temperatures usually fluctuate much more and are consistently much higher (20-27°C more) than ambient temperatures (Greenberg, 1991; Turner & Howard, 1992). These factors have important implications for the application of laboratory-generated developmental data to PMI estimation. It has been suggested that developmental data generated from a constant temperature could be applied to diurnal temperature conditions, as long as the mean temperature values are comparable (Byrd & Butler, 1997). However, mixed results have been obtained from constant versus alternating temperatures in the laboratory (e.g. Introna *et al.*, 1989; Davies & Ratcliffe, 1994; Byrd & Butler, 1996). Some workers believe that laboratory studies under constant temperatures may not depict larval development accurately (Greenberg & Tantawi, 1993), but may be applicable to indoor forensic situations where environmental factors are fairly constant (Catts, 1992).

The typical medium used for maggot development studies has been ovine, porcine or bovine liver (Levot *et al.*, 1979; Williams & Richardson, 1984; Wall *et al.*, 1992; Wells & Kurahashi, 1994; Anderson, 2000; Grassberger & Reiter, 2001; 2002b; Ames & Turner, 2003). The use of liver as a feeding substrate seems to be related to its ease of availability, low cost and uniform consistency. However, its use has been questioned in the past, and some studies have identified poor larval growth on liver compared to other food substrates (e.g. Kaneshrajah & Turner, 2004; Day & Wallman, 2006a). This is important because the data on maggot development are frequently applied to the estimation of the PMI – where maggots have been feeding on a much wider array of tissues. In this study, kangaroo mince was utilised as it was found to be reliable in terms of its consistency and quality; it was easily measured, and the *Chrysomya* maggots were previously found to respond poorly to liver (ovine

and bovine) as a feeding substrate (pers. obs.). The components of kangaroo mince (protein 21.6 % w/w, moisture 75.3 % w/w, fat 0.6 % w/w, ash 1.3 % w/w, cholesterol 23.2 mg/100g, sodium 41.7 mg/100g, potassium 308.4 mg/100g, energy 409 kJ/100g; Luddenham Pet Meats, pers. comm.) were also believed to be more representative of an entire body, comprising more than one tissue type. Due to the differential growth observed for larvae on various tissue types, location on the corpse from where larvae are collected should be recorded along with other evidence (Lord & Burger, 1983; Day & Wallman, 2006a; Ireland & Turner, 2006). Caution should be exercised in the application of developmental data based on only one type of food substrate (Kaneshrajah & Turner, 2004). It is also necessary to scrutinise carefully the differences in growth rates within a species that may be due to the substrate on which the larvae fed.

4.4.3. Larval temperature preferences

The larval temperature preference experiments showed *Ch. rufifacies* to prefer a temperature approximately 4.8°C lower than *Ch. megacephala* and 5.1°C lower than *Ch. saffranea* (Fig. 4.11). This was an interesting result given that *Ch. rufifacies* has previously been shown to tolerate higher temperatures, and has even been implicated in elevating them (Williams & Richardson, 1984). However, this corresponded closely to the preferred temperature of 35.1±0.4ºC for this species shown by Byrd and Butler (1997) after 24 h on a very similar gradient device. The temperature preference (gradient) experiment was the only component of this study to reveal a higher similarity between the sister species *Ch. megacephala* and *Ch. saffranea* than with that of *Ch. rufifacies.* Further investigation into the temperature preferences of these species, and the preferences of other forensically important genera, is warranted.

The survivorships for all three species on the temperature gradient were low, but not significantly different to those predicted by the earlier studies for both food substrates (Figs 4.9 and 4.10). It is possible that larvae deposited at the two ends of the temperature extremes (approximately 16 and 57°C) died before they could reposition themselves at a more biologically tolerable temperature along the gradient. The

Chrysomya maggots formed distinct masses on the temperature gradient, which was different to the more sparsely distributed pattern observed for two *Calliphora* species that have been investigated on the temperature gradient previously (A. Johnson, unpublished data). Thermal optima, such as those established by these gradient experiments, reveal important information regarding the temperature preferences of growing larvae. Because larvae possess the ability to thermoregulate within the maggot-mass, experiments such as this one indicate the likely temperatures experienced by larvae collected from a mass on a corpse at a death scene. Such data enhance the accuracy of the PMI estimate by enabling adjustments to be made for the presence of maggot-masses.

4.4.5. Comparing growth of *Chrysomya* species

The production of growth curves is typical of studies that provide thermodevelopment data for the purpose of PMI estimation (e.g. Grassberger & Reiter, 2002a; Sukontason *et al.*, 2008). The growth data generated here were required only to establish whether the lengths of species under investigation differed significantly at various time points throughout their development. While it may be tempting to fit growth curves to these data, any curve-fitting approach involves its own assumptions and arbitrary modelling choices (Wells & LaMotte, 2001). It was not the purpose of this study to investigate methods to model larval development, which continue to be described (von Zuben *et al.*, 1998; Richards & Villet, 2008; Tarone & Foran, 2008), as do methods to compare growth curves (e.g. Wang, 2000). For these reasons, curves were not fitted to the data.

The experimental design employed in this study sets it apart (to date) from other published larval development studies. The developments of such closely related blowfly species have not previously been compared. As the species were collected from the same geographical location, the effects of acclimation and population-level genetic variation were not variables in this study. The experimental conditions in this study were virtually identical, which enabled direct comparisons to be made among the species.

After examining the developmental profiles for the three *Chrysomya* species investigated, questions remained regarding the practical application of growth data for PMI estimation. The hypothesis was that members of a genus would share similar enough developmental profiles to allow inferences to be made for a species of the same genus for which detailed developmental studies were lacking. Although all three sets of data were found, on the whole, to differ significantly, a large spread of larval lengths was seen for each species at each time point and temperature. Unless a large sample of larvae was obtained from a case, it appeared likely that growth data could be interchanged for these species. To investigate the development of this genus further, comparison data from another genus had to be examined. In New South Wales, the genera *Calliphora* and *Chrysomya* are reported in 66 and 44% of forensic entomology cases, respectively (Levot, 2003). There are many reported cases where a *Calliphora* species occurred together with a *Chrysomya* species, and 52% of the time this was *Ch. rufifacies* with *C. augur* (Levot, 2003). To put the *Chrysomya* growth data in perspective with another genus, it seemed logical to compare them with data for another forensically significant species, *C. augur*. Although some aspects of experimental protocol varied between the present study and that of Day (2006), many other aspects remained similar. A comparison was made of the growth data of the *Chrysomya* species at three temperatures with equivalent data from Day (2006) (Appendix 2). This comparison revealed much smaller *Chrysomya* lengths relative to those of *C. augur*, which were all significantly different at comparable times since oviposition ($P<0.05$). One difference which could contribute to the more rapid growth and larger sizes of *C. augur* larvae is that these flies are ovoviviparous, depositing live larvae directly onto the food source. This competitive advantage eliminates the extra time required by egg-laying species for occlusion, enabling larval growth immediately. Despite this, considerable differences can be seen between the *Chrysomya* and *Calliphora* data. When viewed in this context, it would be realistic to expect that, with more detailed data, confidence limits could delineate a more general *Chrysomya* growth curve that could be used to infer the ages of these species compared to those of *Calliphora*. Such a concept could have important applications given that these two genera are often found together in forensic cases, where the ages of the larvae of both species would be required for PMI estimation. The extent to

which this would be of any practical use would need to be tested. The effect of competition between these species would also need to be investigated.

4.4.6. Conclusions

Blowfly evolution shows a strong selection for rapid location and consumption of the transient food sources that comprise their main breeding medium (Greenberg, 1991; Kheirallah *et al.*, 2007). Several factors have been identified that could influence variations in fly development observed in the literature, including larval densities, photoperiod (constant versus alternating), temperature regime (constant versus fluctuating), relative humidity, food source, and geographic origin of the population (Wigglesworth, 1965; Sukontason *et al.*, 2008). It is likely that population-specific factors influence the growth rates of species. This is highlighted by the variation in growth rates that have been noted for geographical races of the same species (El-Kady, 1999). Concerns have been expressed regarding the application of development data collected for one population of flies when applied to another population of the same species (Erzinçlioglu, 1983). It is therefore imperative to investigate growth rates of species from different regions in order to evaluate the reliability of such data (El-Kady, 1999). Drugs and toxins can also influence development; for example, larvae raised on tissues containing cocaine experience considerably accelerated growth rates, enough to alter the PMI estimate by up to 24 h (Goff *et al.*, 1989). The effects of all of these factors on development require separate investigation.

It is apparent that genetic closeness does not afford the forensic entomologist the luxury of inferring a general developmental profile for closely related species. It is for this reason that correct species identification and application of the corresponding developmental data are imperative for an accurate PMI estimation. Failure to recognise and distinguish species could lead to miscalculation of the PMI. Future research required in this area includes replicated growth rate data under a variety of conditions and temperatures. These experiments should be conducted under prescribed, standardised conditions to enable direct comparisons among populations and species. There is a need for laboratory experiments to be supplemented with data

collected in the field and from casework. Such studies would enable the evaluation of laboratory-collected data in terms of its applicability to actual casework. It is imperative that laboratory studies produce data that are of practical utility for the estimation of the PMI.

***Chrysomya megacephala* eggs on kangaroo mince.**
Photo: L.A. Nelson

Chapter 5. General Conclusions

This study provides insight into the genetic relationships and thermobiologies of Australian members of the genus *Chrysomya,* a forensically important group of blowflies. This work builds upon that of Wallman *et al.* (2005), contributing to the understanding of blowfly relationships on the Australian continent. In particular, the DNA analyses provide useful data for establishing a DNA-based identification system for forensically important species. The investigation of DNA barcoding for this genus contributes to the worldwide effort of 'barcoding life', while also highlighting the importance of considering additional, possibly nuclear, DNA markers (such as ITS2) for species identification purposes. Although close genetic relationships were observed among members of this genus, thermodevelopmental profiles of three *Chrysomya* species were found to differ significantly, and were not correlated with genetic closeness. However, preliminary analysis (Appendix 2) suggests that the development of *Chrysomya* species is significantly different from *Calliphora* species, possibly permitting the use of more generalised growth curves from each genus for the estimation of larval ages.

5.1. Investigation of molecular methods for streamlined identification of Australian blowflies

5.1.1. Utility of the ITS2 region for PCR-RFLP and phylogenetic-based identification

The rDNA ITS regions were assessed as potential markers for the identification of *Chrysomya* species from Australia. Early results detected considerable sequencing difficulties for ITS1, and forced the use of ITS2 alone for the remaining analyses. The findings revealed evidence of inefficient homogenisation of rDNA repeats within individuals of some species. The ITS2 fragment sizes and the PCR-RFLP technique were not able to separate the sister species *Ch. semimetallica* and *Ch. latifrons*. While there was no evidence of intraspecific ITS2 sequence variation that would complicate identifications using the PCR-RFLP technique, this is a genuine concern for any

technique that only measures restriction sites. Because of the specificity of the PCR-RFLP protocol that we describe, a specimen could be misidentified (false-positive) if it belonged to a genus other than *Chrysomya*. Pending expansion of the PCR-RFLP approach to other genera of forensically important blowflies, more certain identification can only be achieved using DNA sequencing, because it takes advantage of the maximal information contained within a sequence from a particular species (e.g. Brunner *et al.*, 2002). Such a method can incorporate intraspecific variation through expanded taxon sampling, which would otherwise invalidate PCR-RFLP results. The results presented here showed that ITS2 sequence divergences among *Chrysomya* species are higher than the individual mitochondrial regions sequenced by Wallman *et al.* (2005), therefore providing a useful platform from which to develop future identification studies.

5.1.2. Evaluation of the COI DNA barcode

The COI DNA barcode was successful in identifying *Chrysomya* species from the east coast of Australia. The ability of the barcode to identify two *Ch. nigripes* specimens from Thailand shows potential for this method to be expanded to other geographical locations and additional *Chrysomya* species. This study confirmed previous successes with COI as a genetic barcode for species identification and comparisons at the intra- and interspecies levels, that is, its ease of detection due to the high copy number coupled with its ready amplification, a suitable rate of mutation, and ease of sequencing and alignment.

Any DNA-based identification method relies upon the reciprocal monophyly of species at the DNA region being used. As such, the issue of delineating species boundaries becomes important. There is substantial literature on the theory and methodology of phylogenetic inference and hypotheses of species concepts, but there is little on the methods for delimiting species boundaries (Sites & Marshall, 2003). Consequently, species boundaries are best predicted through a combination of phylogenetic analysis, consideration of additional biological evidence, and evaluation with taxonomic expertise (Lipscomb *et al.*, 2003; Wheeler, 2005). DNA barcoding

has received most of its criticism from the suggestion that it may be a useful tool for recognising species by a standard percent or 'threshold' sequence divergence. Although a standard percent sequence divergence has been shown to be suitable for species delineation in some taxa (2%, or ten times the average intraspecific difference; e.g. Hebert *et al.*, 2003; Monaghan *et al.*, 2005), this suggestion has been denounced due to the overlap between intra-and interspecific sequence variation frequently encountered (e.g. Funk & Omland, 2003; Moritz & Cicero, 2004; Cognato, 2006).

Low sequence divergences between species in this study confirmed that the sequence divergence thresholds advocated by other studies would fail to recognise Australian species of this genus as separate. This study also revealed an overlap in the ranges of genetic variation noted within and between species, where COI variation within one species (*Ch. varipes*) exceeded that between other species (*Ch. megacephala* and *Ch. saffranea*). The low sequence divergences between some species (namely the sister species pairs *Ch. saffranea+Ch. megacephala* and *Ch. latifrons+Ch. semimetallica*) are indicative of their relatively recent origin (Funk & Omland, 2003; Tautz *et al.*, 2003). For such situations, expanded taxon sampling would determine the stability of the barcode sequence within each species, therefore enhancing confidence in identifications (Armstrong & Ball, 2005). Conversely, cases of deep COI divergences among individuals that had been assigned to single species may be useful in indicating previously unrecognised or cryptic species (Ward *et al.*, 2005; Smith *et al.*, 2007). Although hybrid specimens are said to be rare in nature, the identification of a suspected *Ch. megacephala/Ch. saffranea* hybrid specimen by a combination of COI and ITS2 analysis highlights a case where DNA barcoding will not work (Ball *et al.*, 2005). In cases of young species pairs and hybrid specimens, a secondary independent molecular marker, such as ITS2 used here, may be required to establish or confirm identifications in forensically important species as it has for other Diptera (Stevens *et al.*, 2002; Smith *et al.*, 2007).

The acceptance and adoption of a single identification method for species within a region, or globally, would enhance the accuracy of and confidence in identifications

of forensically important species. Although this study did not find a convincing relationship between geographic distance and sequence divergence, this phenomenon has been noted in the past and maybe observed if sampling was expanded to geographically diverse locations (e.g. Ball *et al.*, 2005). The creation of a global database would incorporate any intraspecific variation existing for each species worldwide. Such a database would permit the detection of new forensic indicator species arriving in an area, given the expanding geographic ranges of blowfly species (e.g. Baumgartner & Greenberg, 1984; Grassberger *et al.*, 2003; Siddig *et al.*, 2005; Tomberlin *et al.*, 2006; Williams & Villet, 2006). For such a database to be developed it would be essential to maintain rigorous standards of record keeping and voucher maintenance. Caution would need to be exercised to detect and prevent the amplification of nuclear pseudogenes, and to carefully assess the ability of the DNA barcode to identify species in accordance with established taxonomy.

The success of the COI barcode for identification of Australian *Chrysomya* species cannot be guaranteed for all blowflies. Indeed, Whitworth *et al.* (2007) reported the inability of part of the COI region to distinguish blowflies of the genus *Protocalliphora* (Diptera: Calliphoridae), due to the non-monophyly of many of the species at the mitochondrial level. This was attributed to introgressive hybridisation associated with infection by *Wolbachia,* cytoplasmically inherited endosymbiotic bacteria found in the reproductive tissues of some invertebrates. Although *Protocalliphora* blowflies are not forensically important, given that between 15 and 75% of insect species harbour *Wolbachia*, identifications at the species level based on mtDNA may not be possible for all taxa (Hurst & Jiggins, 2005). However, it has been stated that such introgressions are limited to very closely related species capable of hybridisation, suggesting that barcoding at a higher taxonomic rank should remain possible (Baudry *et al.*, 2003). Such cases necessitate the use of nuclear DNA markers or a combined approach to species identification, possibly utilising complimentary techniques such as microsatellite analysis (Wells & Stevens, 2008).

5.2. Thermal attributes of *Chrysomya* species

The morphological similarities and close genetic relatedness between members of the genus *Chrysomya* promoted the hypothesis that the developmental profiles of some of the species may also be similar. This could have significant implications for the estimation of the PMI: if closely related species share similar developmental profiles, the absolute identification of those species beyond a predetermined taxonomic level may not be necessary. This study established that the sister species *Ch. megacephala* and *Ch. saffranea* differed significantly in their development, as well as compared with the more distantly related *Ch. rufifacies.* Because no significant similarity was detected between the developments of the sister species compared with *Ch. rufifacies*, genetic distance, using ITS2 and the COI barcode, was not considered to be a useful factor for predicting thermodevelopmental characteristics of closely related species within a genus. Clearly, factors other than genetic closeness, estimated here using the ITS2 and COI regions, are responsible for the differences in development profiles observed among species.

5.3. Recommendations and future research

Since publication of Chapter 2 (Nelson *et al.*, 2008), further geographic sampling was carried out for Chapter 3 (Nelson *et al.*, 2007). Due to the promising results from ITS2, it would be ideal to incorporate these additional samples into the existing data set for a further assessment of ITS2. The results of this study indicate that a concurrent sequence analysis of ITS2 and COI could provide a much more robust diagnostic tool than COI barcoding alone. Future research should encompass an expansion of the molecular methods describes herein to other forensically important fly species, and an evaluation of the identification procedure in casework.

Some of the factors contributing to the inconsistencies in thermodevelopmental characteristics reported are most likely due to differences in rearing conditions among studies, such as humidity, rearing media and larval densities (Baumgartner, 1993). However, this study was unique in that the only variable factor was the species being

studied: all other conditions were virtually identical. Future research is required to investigate the influence of the abovementioned factors on the development of forensically important species, particularly with the aim to characterise the effects of such factors on different species. Such research would ideally investigate the development of geographically disparate populations of the same species, to establish whether separate populations differ at all in their thermal responses, which would be important for PMI estimates (unpublished data). It is evident from the literature that competition among species, particularly with the predatory *Ch. rufifacies*, can influence larval development and lead to heavy mortalities (e.g. Goodbrod & Goff, 1990; Baumgartner, 1993; dos Reis *et al.*, 1999; Green *et al.*, 2003; D'Alberto, 2004; Ireland & Turner, 2006). A better understanding of the influence of competition will enable more accurate estimations of larval age, and hence, the PMI. Future work is also required to understand the dynamics of the maggot mass, to (1) observe the temperatures produced by the mass, (2) determine how these dynamics affect larval growth rates, and (3) incorporate influences of the maggot mass into the current methods of calculating the postmortem interval.

As with all forensic entomological studies, the data generated are only as good as their practical applicability to casework. It would be interesting to test whether the differences in *Chrysomya* larval sizes at the times and temperatures examined herein are significant in a practical sense as well as a statistical one. That is, given the variability (overlap) of the species' larval lengths at a given time and temperature, would it make a significant difference which *Chrysomya* growth data were used to correctly estimate the ages of a sample of *Chrysomya* larvae collected from a case? This question is justified, as the forensic entomologist is often presented with a much smaller sample of larvae than those for which such development data are produced. Comparison of the *Chrysomya* data with comparable data collected for *Calliphora* revealed significant differences in lengths at corresponding temperatures and time points. These preliminary data suggests that it may be possible to generate generalised genus-specific growth curves that could be useful as an initial tool for ageing *Calliphora* and *Chrysomya* larvae collected from a crime scene. Such a concept may appear to oversimplify the data, but could have practical use for species for which

developmental profiles have not been collected. Knowledge of the temperatures at crime scenes is less precise than those from controlled-temperature experiments, and these temperatures are subject to much larger extremes and fluctuations. Given the limited availability of published studies on the effects of microclimate, maggot masses and larval competition, it can be safely stated that this lack of knowledge necessitates numerous assumptions and therefore a high degree of error in the estimation of the PMI by entomological evidence. As such, the forensic entomologist is obliged to favour more conservative estimates of the PMI when providing his or her expert opinion. Only through continuing research will methods for species identifications improve, and the dynamics of larval growth and development be understood. Ultimately, this will improve the confidence of a PMI estimation based on entomological evidence.

Chrysomya saffranea **pupae at various stages of hardening.**
Photo: L.A. Nelson

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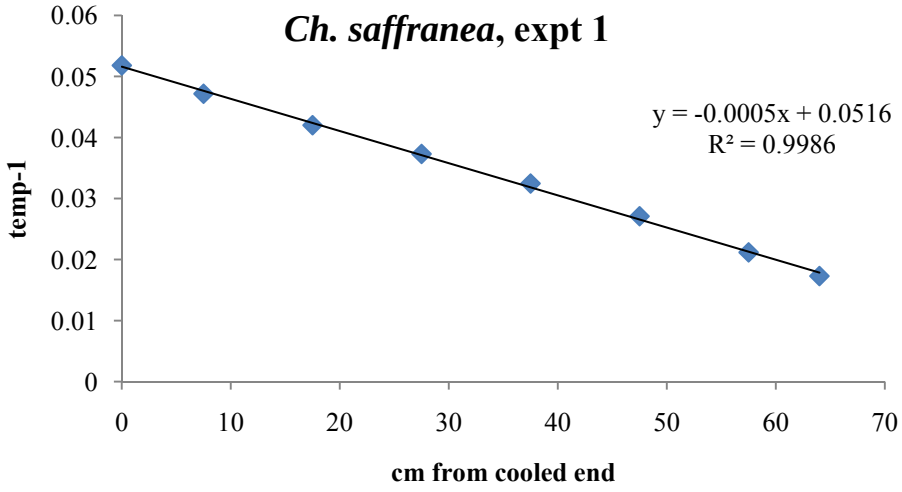
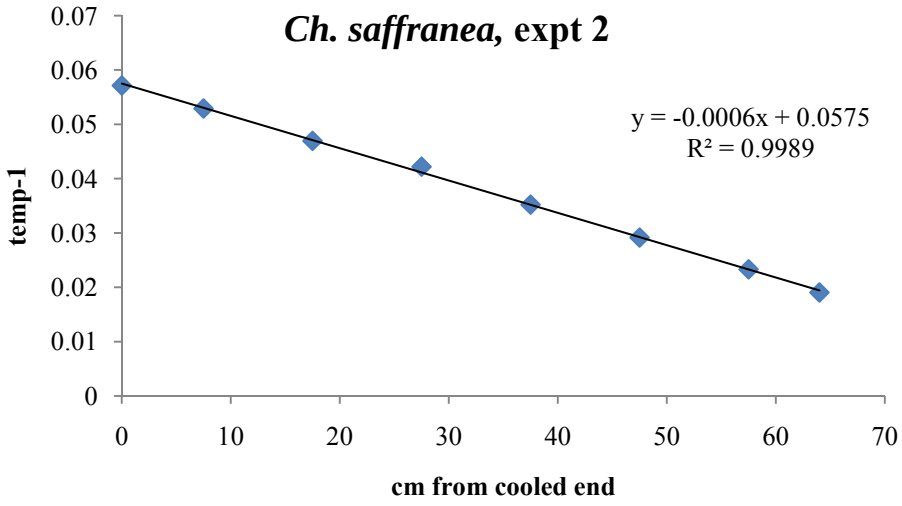
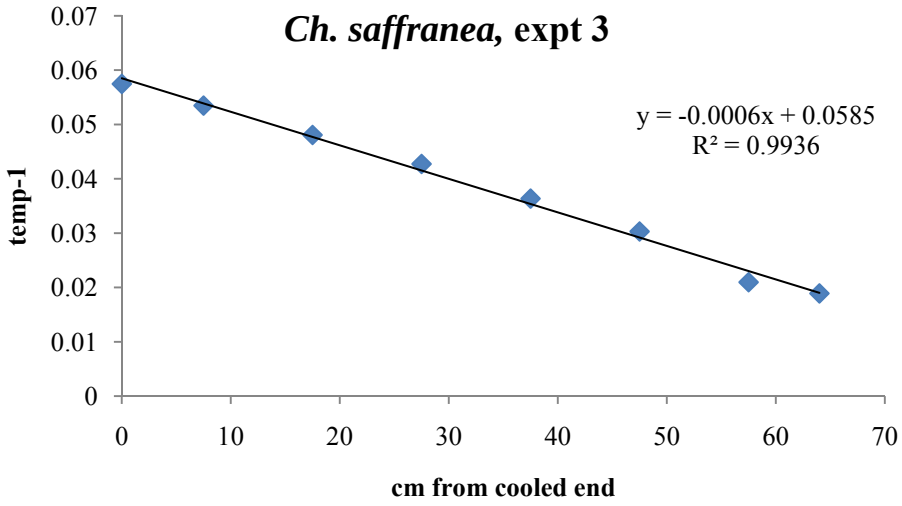
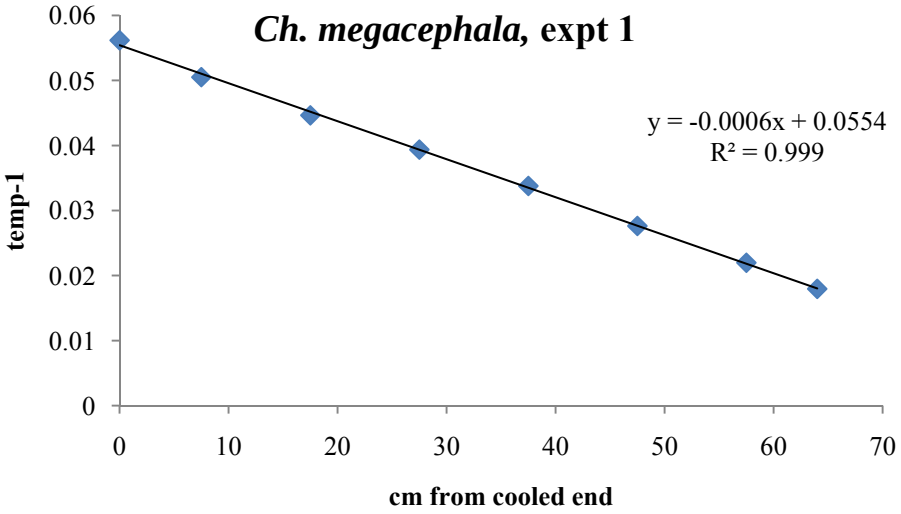
Appendix 1. Standard curves for inference of preferred temperatures

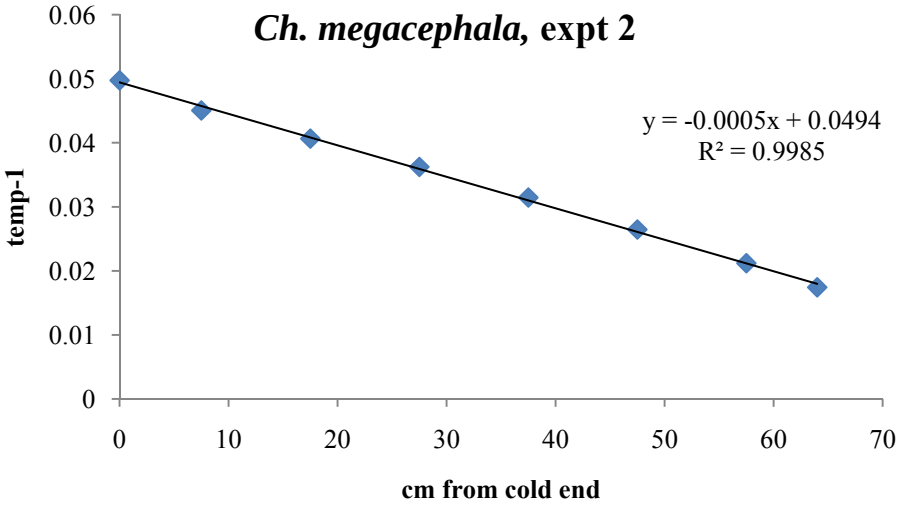
Standard curves used for inference of preferred temperatures from three experiments each for *Ch. megacephala, Ch. saffranea* and *Ch. rufifacies.* The curves were generated by plotting the inverse of the temperature in degrees Celsius ('temp^{-1}') recorded at various points along the length of the copper gradient tray ('cm from cooled end').

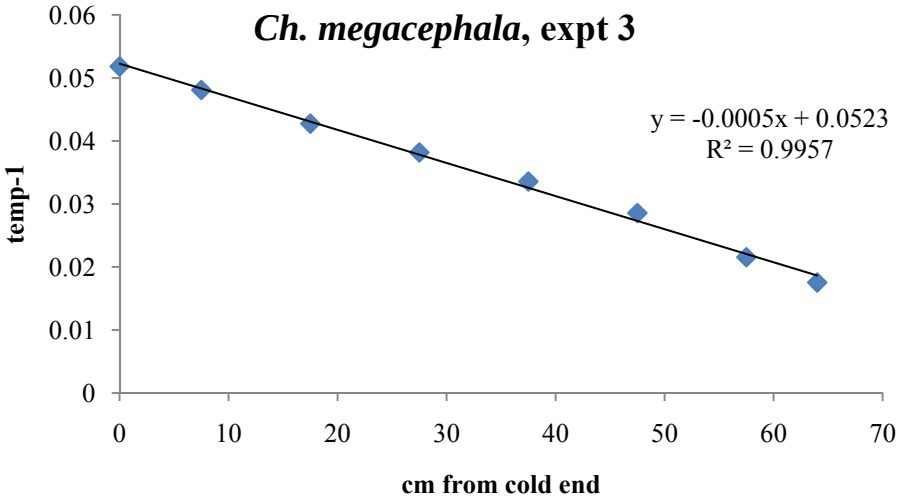

Ch. saffranea, expt 1
y = -0.0005x + 0.0516
R² = 0.9986
temp-1
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end

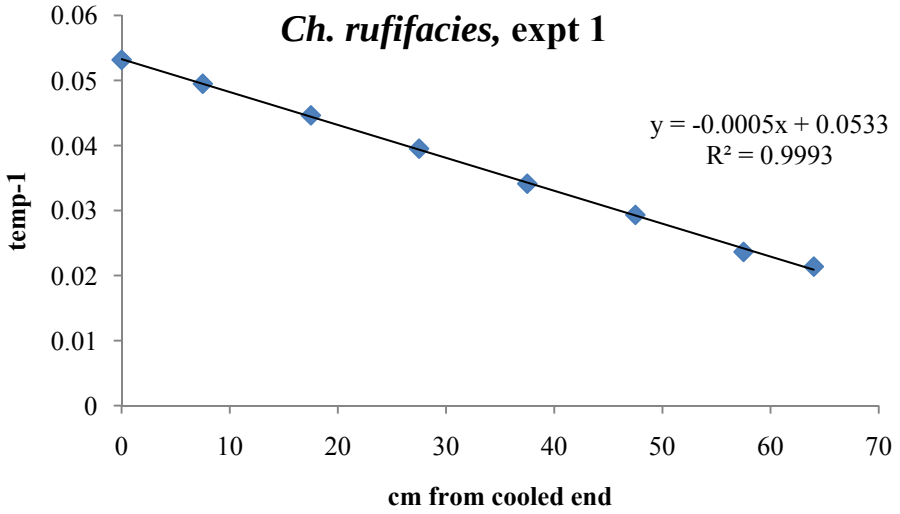

Ch. saffranea, expt 2
y = -0.0006x + 0.0575
R² = 0.9989
temp-1
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end

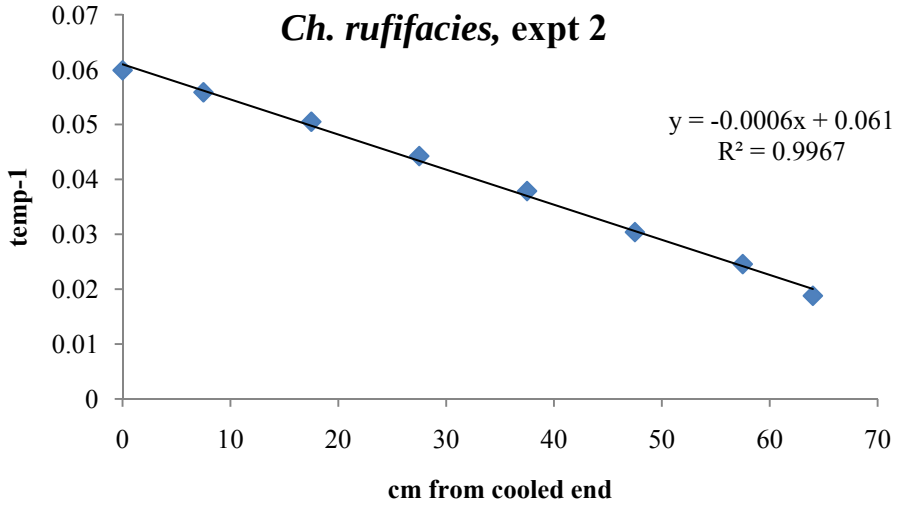

Ch. saffranea, expt 3
y = -0.0006x + 0.0585
R² = 0.9936
temp-1
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end

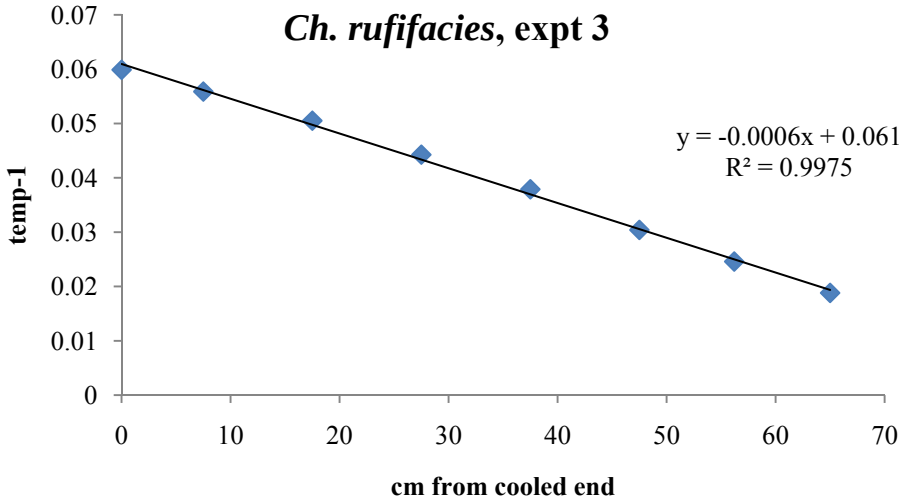

Ch. megacephala, expt 1
y = -0.0006x + 0.0554
R² = 0.999
temp-1
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end


Ch. megacephala, expt 2
y = -0.0005x + 0.0494
R² = 0.9985
temp-1
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cold end


Ch. megacephala, expt 3
y = -0.0005x + 0.0523
R² = 0.9957
temp-1
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cold end

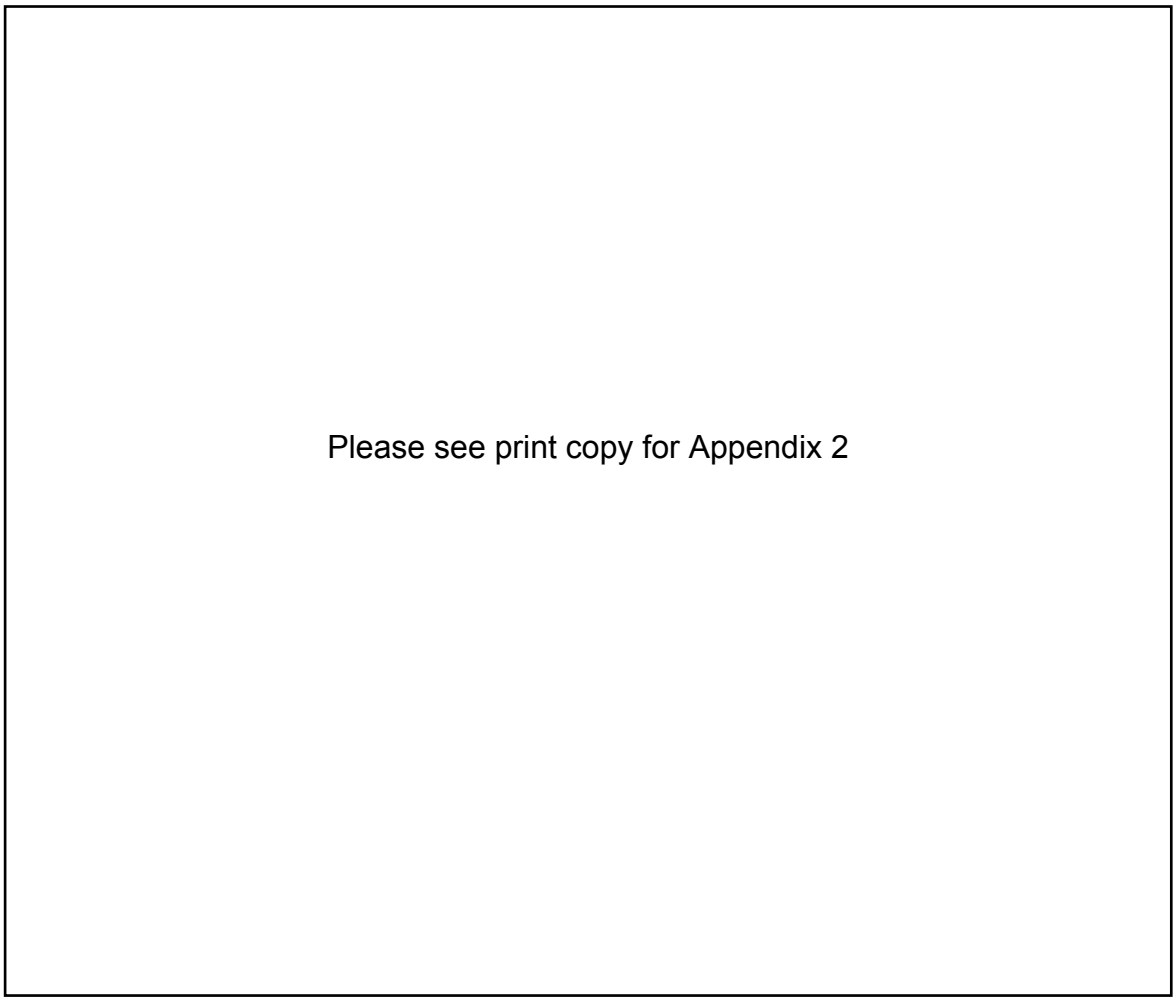

Ch. rufifacies, expt 1
y = -0.0005x + 0.0533
R² = 0.9993
temp-1
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end


Ch. rufifacies, expt 2
y = -0.0006x + 0.061
R² = 0.9967
temp-1
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end


Ch. rufifacies, expt 3
y = -0.0006x + 0.061
R² = 0.9975
temp-1
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
cm from cooled end

Appendix 2. Comparison of *Chrysomya* and *Calliphora* larval lengths at three temperatures

Chrysomya larval lengths (± SE) plotted with corresponding data for *Calliphora augur* (Day, 2006) at 25, 30 and 35ºC. *Calliphora augur* lengths differed significantly from *Chysomya* lengths at each time investigated ($P<0.05$).



Please see print copy for Appendix 2

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