DNA Barcoding of Forensically Important Flies in the Western Cape

By

TENIELLE MONIQUE COOKE

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Supervisor: Dr. Marise Heyns

Co-Supervisor: Ms Laura Heathfield

DECLARATION

I Tenielle Cooke, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

One of the central applications of forensic entomology is the determination of the post mortem interval (PMI) from arthropod evidence associated with a corpse. Estimations of the PMI are based on succession and developmental patterns of specific species that visit the body. As first colonisers, Calliphoridae (blow flies) are often used by forensic entomologists to determine the PMI however, developmental rates of visiting fauna differ substantially which makes correct species identification vital. Traditional methods of identification which assign species based on keys that capitalise on morphological differences are insufficient for closely related species, especially during immature stages of the lifecycle or when the specimen is damaged. Molecular identification such as DNA barcoding has therefore become a popular method of identifying species. DNA barcoding characterises species by sequencing and analysing specific regions in the genome. This technique has been used to characterise species in various countries including parts of South Africa. Its application has also been demonstrated in a forensic setting but data for the Western Cape is minimal. This study therefore aimed to assess the utility of DNA barcoding for species level determination of four blow fly species common to the Western Cape of South Africa (*Chrysomya chloropyga*, *Chrysomya albiceps*, *Chrysomya marginalis*, and *Lucilia sericata*) as well as its ability to identify immature specimens. Ten adult specimens from each species were morphologically and molecularly identified using microscopy and DNA barcoding respectively. The standard DNA barcode, *cytochrome c oxidase subunit I* (*COI*) and a secondary marker, the *second internal transcribed spacer* (*ITS2*) were analysed. Phylogenetic analyses for both barcodes showed high interspecific divergence values which are desirable for species level differentiation by DNA barcoding. *COI* sequences from adult flies were also submitted and searched against BOLD for identification and only genus level identity could be achieved, indicating that, COI alone may be insufficient to discriminate between closely related species. DNA sequences from the adult specimens were then used as reference sequences for identification of seven unknown immature specimen using DNA barcoding of both *COI* and *ITS2*. Sequence similarity was assessed and identity was assigned based on >98% similarity scores, and all immatures were successfully identified. The use of more than one DNA marker to complement morphological data ensures higher confidence of species level identification. This method provides a reliable and consistent tool for entomologists to use for species identification which results in higher levels of accuracy in PMI estimations.

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List of Abbreviations

%	Percentage
BOLD	Barcode of life database
Bp	Base pairs
CBOL	Consortium for the barcode of life
COI	Cytochrome c oxidase subunit 1
CYTB	Cytochrome b
DNA	Deoxyribonucleic acid
iBOL	International barcode of life
ITS2	Second internal transcribed spacer
mtDNA	Mitochondrial deoxyribonucleic acid
NADH	Nicotinamide adenine dinucleotide
ND5	Nicotinamide adenine dinucleotide dehydrogenase
N	Number
PCR	Polymerase chain reaction
PMI	Post mortem interval
RFLP	Restriction fragment length polymorphism
UV	UV

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Chapter 1: Research Proposal

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1.1 Introduction

Though not restricted to, forensic entomology, largely involves the use of arthropod evidence associated with a corpse [1] in order to estimate time elapsed since death based on insect colonisation patterns and the developmental stage of a particular species [2], to estimate the post mortem interval (PMI). The first and most crucial step for a forensic entomologist is the correct species identification [3] as this is the basis on which PMI estimations are made. Current methods of identification and classification involve the use of morphological keys and microscopic analysis of the shape, colour and size of different parts or appendages of the adult fly [1]. Identification, however, becomes difficult when collected specimens are at the larval stages of the life cycle [4]. For some species, morphological keys for these stages, are non-existent due to the absence of externally visible distinguishing characteristics [5]. This prompts the rearing of flies until adulthood, which can be time consuming. In addition, further difficulties arise when specimens are damaged due to poor collection or storage techniques which can potentially render these characteristics unrecognizable [6].

Morphological identification of insect species often requires highly skilled entomologists with adequate taxonomic training [1]. This as well the inherent limitations such as rearing of larvae and damaged specimens, has pushed for a DNA-based method that would complement morphological data [7]. DNA barcoding is one such technique. In DNA barcoding, short standardised sequences of DNA are used as an identification tag; these tags exhibit a considerable amount of sequence variation between species and sequence conservation within species [8]. Paul Hebert, [8], proposed the use of a 648 bp fragment of the gene encoding *cytochrome c oxidase* subunit 1 (*COI*) and this fragment has largely become the standard in DNA barcoding of animals. A region of non-coding nuclear ribosomal DNA, the *second internal transcribed spacer* (*ITS2*) was identified as an alternative to *COI* where *COI* did not possess the capability to successfully distinguish between species [9]. The *ITS2* has since been used in conjunction with *COI* for in closely related blow fly species in Australia [9] and China [10].

1.1.1 Research problem

With over 150 genera and 1000 species worldwide [11], the identification of forensically important blow flies is of great importance. Difficulties in identification of larvae is circumvented by rearing to adulthood however, this can be challenging. It is possible that the larvae may die before a definite identification can be made. The time period needed for blow flies to be reared to identifiable adults will also cause delays in an investigation. In cases with damaged specimens, identification by morphological key may be impossible due to the damage of distinguishing characteristics. Due to the challenges presented by morphological identification, a molecular based method incorporating the use of DNA markers called DNA barcoding, was developed [8]. DNA-based identification methods have become a prevalent choice for insect identifications due to their rapidity and reliability in addition to its usefulness for any life stage of an organism [12]. DNA barcoding has the potential to identify organisms down to species level but is yet to be applied to the field of forensic entomology in areas of South Africa.

1.1.2 Rationale and Justification

Evidence garnered though forensic entomology relating to the post mortem interval assists forensic investigations, especially where PMI may be a deciding factor in the determination of guilt of an accused [13] and when used to verify witness statements [14]. As the application of forensic entomology continues to extend to medico-legal cases, it follows that robust methods of species identification should be implemented. The use of DNA barcoding as a means of species identification negates the need to rear larvae to adulthood and identification of damaged specimens will be possible as only a small amount of tissue is needed for DNA extraction. Though successfully used in Germany [15], Portugal [16], North America and West Europe [17] and India [18], there have been few attempts to use this method in the western cape area. However, a study covering other parts of southern Africa has been undertaken [6]. This pilot study will assess the viability of DNA barcoding as a method of species identification for four species of SA blow fly. If successful, the pilot may give way to a larger project that would incorporate the barcoding of all forensically important blow flies in SA.

1.1.3 Aims and Objectives

This pilot study aims to examine the discriminatory value of both *COI* and *ITS2* in four blow flies species common in SA and to assess whether this method could be used as a reliable method of species identification of South African blow flies at both adult and larval stages.

Specific Objectives

- Classify the species of four common blow flies in South Africa by microscopy
- Determine the DNA sequences of the *COI* and *ITS2* regions of these known species
- Initialise the addition of the DNA barcodes of four South African blow flies to the Barcode of Life Database (BOLD).
- Examine the ability of *COI* and *ITS2* to discriminate between the following blow fly species *Chrysomya albiceps, Chrysomya chloropyga, Chrysomya marginalis* and *Lucilia sericata.*
- Assess if blow flies of unknown species to the researcher can be identified using these regions

1.2 Background

Species identification

Taxonomy, the science of naming and classifying biological groups is often referred to as the world's first profession [19]. Modern day forensic entomologists use knowledge garnered from taxonomy to accurately identify species of insects that colonize a dead body to aid criminal investigations. The identification of species is the most important step as this is the basis of any further conclusions. Traditional methods of species identification involve the use of morphological keys to identify certain unifying or distinguishable characteristics [20]. Hebert *et al.,* [8] described four limitations inherent in these methods, firstly phenotypic plasticity – the ability of one genotype to produce more than one phenotype based on the environmental factors, and genetic variability in features used for species recognition could cause misidentification. Secondly, morphologically cryptic taxa are often overlooked. Thirdly, the current morphological keys are only suited for particular life stages and lastly, these keys often require the skill and expertise of highly trained personnel. Therefore different methods allowing for increased accuracy in species identification are required.

Overview of DNA barcoding

Using molecular methods as a tool for species identification can be dated as far back as 1977 when biophysicist and microbiologist, Carl Woese made use of sequence differences in ribosomal RNA in his discovery of Archaea [21]. Molecular advances since then have exponentially increased, and DNA is routinely used in laboratories worldwide. In 2003, Paul Hebert proposed the idea of using short standardized fragments of mitochondrial DNA for species identification. He further proposed creating a library of DNA Barcodes that could have the potential to be used as a new Master Key to accurately identify species within certain taxonomic groups. His research focused on the suitability of the mitochondrial genome and in particular the *cytochrome c oxidase subunit* 1 (*COI*) as the barcode for animal life [8,22]. This *COI* Barcode is now routinely used as the standard for animal barcoding. Subsequently the Consortium for the Barcode of Life (CBOL) was developed and, this international initiative supports the development of DNA Barcoding as a global standard for species identification. Currently the consortium houses 4 013 927 specimens (www.ibol.org – 28/01/16). The Barcode of Life Data systems (BOLD) was also established as a public workbench and database for barcoding projects.

Choice of a Locus

The technique required the use of a universal marker that had sufficient discriminatory value to distinguish across species, ensuring the international exchangeability of the generated data [8]. In order to be standardised, the locus had to be present in all taxa, easily amplified and sequenced and areas flanking the region needed to be highly conserved to allow for the use of standard primers. The locus also needed to provide a large degree of variability between species and a small degree within species [8]. Following these criteria, mitochondrial DNA was used in preference to nuclear DNA. The mitochondrial genome has a relatively fast mutation rate, which translates into a high level of diversity between species [23].

Hebert *et al.,* [22] suggested the use of the gene encoding for subunit one of *cytochrome c oxidase*, an enzyme vital to cellular respiration. The study showed that the mitochondrial genome was further suited for DNA Barcodes due to its lack of recombination and it being void of introns, non-coding regions of a gene present in nuclear DNA. Splicing errors and alternative splicing may occur and would result in ambiguous sequences. Hebert *et al.,* [8] supplied two advantages of using the *COI* gene as a barcode, (1) it had opportunity for robust universal primers enabling recovery of the 5’ end from

representatives from all taxa, and (2) *COI* possessed a greater range of phylogenetic signal when compared to any other mitochondrial gene. They further concluded that the rapidity of the evolution rates of mitochondrial genes were sufficient to allow for the discrimination between closely related as well as phylogeographic groups within species, that is, groups of the same species living in different areas.

Forensically Important Flies and Post Mortem Interval

At the time of death the body starts decomposing and releases apeneumones, a mixture of the liquids and gases produced when the gastrointestinal tract is digested. These apeneumones attract insects to a decomposing body [24] .Flies specifically, are initially attracted to the decomposing body by putrid sulphur-based compounds and the actual oviposition or egg-laying is induced by ammonium-rich samples [25] . Smith [26] showed that four different categories of insect are found on a dead body, (1) Necrophagus, those that feed on carrion, (2) Predators and parasite that feed on the necrophagus insects, (3) Omnivores feeding on the carrion and (4) other species that use the corpse as an extended environment such as spiders. Species of flies from the order Diptera are of most interest to forensic entomology [27], and in particular, those belonging to the families Calliphoridae (blow flies), Sarcophagidae (flesh flies), and Muscidae (house flies).

Blow flies are often the first to colonise a dead body and oviposition occurs at any orifice or open wound on the body. Eggs and larvae of the Diptera need moisture to complete development and it is for this reason that female Diptera do not oviposit in dehydrated or mummified tissue [28]. The presence of insect larvae can therefore give an estimate of PMI up until the dead body has begun the dehydration phase of decomposition – up to 30 days. Routinely, the minimum PMI can be extrapolated by using the age of larvae but this calculation is temperature and species dependant as different species have differing time periods for each life cycle which may or may not be sped up or decreased by changes in temperature [29]. The succession of species is also noted as the insects colonizing the dead body do so in waves according to the state of decomposition the body is in. Both these methods, however require the initial step of correctly identifying the species of larvae and adult flies in which morphological methods are usually used [30]. Identifying forensically important flies to species level generally requires the expertise of specialized taxonomic knowledge. Differentiation at larval stages of certain species is impossible hence rearing the larvae to adulthood is done to circumvent this problem [31]. However, this is time consuming and may delay the progress of an ongoing forensic investigation.

COI Barcoding in Forensically Important Flies

The *COI* gene has been used to successfully identify species of Diptera in Germany [15], Portugal [16], North America and West Europe [17] and India [18]. Meiklejohn *et al.* [32] showed that through *COI* barcoding, flies belonging to the Sarcophagidae family could be identified at all immature stages. However, a few studies have shown that the *COI* barcode does not have the ability to distinguish between closely related species. Contrary to a study done by Boeheme *et al.* [33], Sonet *et al.,* [34] showed that the *COI* barcode was in fact not able to distinguish between *Lucilia caesar and L. illustris.* It was proffered that these two species had a high degree of overlap in their range of intraspecific and interspecific sequence divergences therefore making them indistinguishable. Another study tested the viability of *COI* barcoding on flies with endosymbiotic bacteria Wolbachia, and found that assignment of unknown species was not possible for 60% of the 12 species studied [35]. This prompted the search for other regions that could be amplified and sequenced together with the COI barcode in order to complement the data. Several multigene loci attempts have been made including the use of *cytochrome b* (*CYTB), NADH dehydrogenase 5* (*ND5*), and the first and second nuclear internal transcribed spacers [36].

Second Internal Transcribed Ribosomal Spacer

Recently, the universal DNA Barcode for fungi, the *second internal transcribed ribosomal spacer* (*ITS2*) has shown to be useful in distinguishing between cryptic Calliphoridae species and those that have recently diverged from it. The *ITS2* refers to a non-coding DNA sequence in the nuclear ribosomal cluster situated between the 3' end of the 5.8S ribosomal DNA (rDNA) and the 5' end of the 28S rDNA. Comparisons of the ITS sequences have been used in taxonomy and molecular phylogeny due to the ease of amplification, and high variation between closely related species [37]. Song *et al.,* [10] described the potential of the *ITS2* region in the identification of forensically important flies. They did, however, note that a high level of sequence homology within some species and therefore that the marker could not be used to differentiate between geographical populations. Therefore, analysis of the *ITS2* alone is not sufficient for the identification of cryptic or closely related species of blow fly. However, using both a mitochondrial gene and nuclear gene may provide better resolution for identification.

1.3 Proposed methodology

The study will be conducted in three parts; (i) morphological identification, (ii) molecular identification and (iii) blind molecular species identification.

Specimen Collection

Adult and larvae will be collected from field experiments as well as from established laboratory cultures. Four blow flies common to South Africa will be used for the study, *Chrysomya albiceps, Chrysomya chloropyga, Chrysomya marginalis*, and *Lucilia sericata* [38]. Adult flies will be gassed with ethyl acetate and then collected into 70% ethanol [39]. Larvae will be killed by immersion in near boiling water for no longer than 30 seconds [40] and then stored in 70% ethanol until DNA extraction at room temperature.

Part I – Morphological species identification

Adult fly species will be confirmed using stereomicroscopy and morphological keys for South African blow flies developed by Zumpt [41]

Representative pictures will be taken for each species.

Part II – Molecular Species Identification

DNA Extraction and Quantification

DNA will be extracted from fly legs using the QIAgen DNeasy Tissue DNA Extraction kit or equivalent. Integrity of extracted DNA (i.e whether it is degraded or not) will be determined using gel electrophoresis and its concentration and purity assessed by spectrophotometry. Ten biological repeats will be used to ensure sequence conservation within species.

Amplification of *COI* and *ITS2*

COI will be amplified by polymerase chain reaction (PCR), using the standard primers (table 1.1) which are slightly modified primers designed for *cytochrome C oxidase* by Folmer et al., [42]. The *ITS2* gene will be amplified using the primers in (table 1.1).

Table 1.1 Forward and reverse primers. Primer sequences for amplification of COI and ITS2 regions.

Region to amplify	Primer Name	Sequence (5'-3')	Direction 5'⟶3'	Reference
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Forward	[42]
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Reverse	[42]
ITS2	ITS2_F	TGCTTGGACTACATATGGTTGA	Forward	[10]
	ITS2_R	GTAGTCCCATATGAGTTGAGGTT	Reverse	[10]

Trehalose will be added to the PCR reaction as it lowers the DNA melting temperature and stabilises the *Taq* polymerase therefore acting as a PCR enhancer. It is also useful as a cryoprotectant so samples can be stored frozen, without damage to DNA occurring. PCR will be performed in a thermocycler.

Amplification success will be verified using gel electrophoresis and will be visualised under UV light. *COI* and *ITS2* amplicons are 648 bp and ~310 bp respectively.

PCR product clean up and Sequencing

In order to achieve accurate sequencing, PCR products need to be free from unincorporated nucleotides and residual primers. QIAquick PCR purification kit will be used to purify the PCR products. Sanger sequencing will be used to sequence the COI Barcode and nuclear marker using BigDye Terminator Sequencing kit on an ABI 3100 Genetic Analyser (Applied Biosystems) and will be separated by capillary electrophoresis. The primers used to amplify the DNA will be used for sequencing.

Data Analysis

Electropherograms will be edited (primer sequences removed) and confirmed with ChromasPro software and BioEdit Sequence Alignment Editor. ClustalW will be used for multi alignment of sequences within species.

Sequences will be submitted to Genbank and Barcode of Life Database (BOLD).

Part III – Blind Experiment

The ability of the technique to accurately identify species will be tested through a blind experimental test. Blow fly larvae, with the species only known to the supervisor will be subjected to DNA barcoding

following the methods outlined in Part II. Two biological repeats at each instar stage per species will be used. Sequences of the known blow flies will be used to attempt to identify the unknown species.

1.4 Ethical considerations

The study requires the use of adult and blow fly larvae. The flies and larvae will be euthanised using standardised and accepted methods [40]. Larvae will be euthanised by immersion in near boiling water for 30 seconds and adult flies will first be gassed then collected into 70% ethanol. Due to the ongoing debate about the ability of invertebrates to perceive pain or experience compromised welfare, methods of euthanasia for flies and maggots were of concern. These methods have been accepted by the broader forensic entomology community (both within forensic service delivery and research), as these are deemed to be ethical. It also allows for the preservation of the morphology of the specimen, which is an integral part of the project. These methods have been used extensively in the field – it has been cited by at least 81 research articles. By following these methods, the data collected will be directly comparable to published literature within this field and will ensure no post-mortem damage to the specimen.

1.5 Social value and importance of this research

This pilot study aims to assess the viability of using DNA barcoding as a reliable method for species identification of forensically important blow flies. In forensic entomology, the identity of the species is a vital first step in the estimation of post mortem interval, and if successful, this method could potentially serve to better the field, especially if the research is extended to include all forensically important fly species in SA. Having a database of all forensically important fly species, would mean that any species, at any larval stage can potentially be identified. This translates to investigators receiving information about the deceased in a shorter period of time. Because certain species are known to be indigenous to a particular area, it is possible to deduce whether a body was moved.

1.6 Work plan and budget

The chart that follows (figure 1.1) indicates the general work plan for the period of March – January 2016. Wet laboratory work involves the specimen collection, DNA isolation, PCR amplification and subsequent sequencing. Table 1.2 indicates the proposed budget for the project.

TASK	March	April	May	June	July	August	September	October	November	December	January
Proposal											
Ethics											
Literature Review											
Optimisation											
Wet Lab Experiments											
Data Analysis											
Final Write up											
Submission											

Figure 1.1: Proposed work plan for the intended study

Table 1.2: Proposed budget for the intended work

		Quantity	Cost (Rand)
DNA Extraction	QIAgen DNeasy Extraction Kit	50 reactions	R 3 178.93
Amplification & Electrophoresis	Trehalose	5g	R 253.97
	Tris	300g	R 993.25
	HCl	100mL	R 770.12
	KCl	500g	R 239.23
	dNTPs	.5mL	R 780.50
	Primers (COI)	14675.2 µgrams	R 302.59
	Primers (ITS2)	15557.2 µgrams	R 300.99
	Taq Polymerase		R 1 545.60
	$MgCl_2$	100 g	R 306.40
	Agarose	50 g	R 1 941.00
	Boric acid	500 g	R 824. 18
	EDTA	100 g	R 364.91
	DNA Ladder	0.1mg	R 897.34
	Bromophenol Blue		R 816.63
PCR Clean Up	QIAgen QIAquick PCR Clean-up Kit	50 reactions	R 1 699.51
Sequencing	BigDye Terminator Sequencing Kit	50 reactions	R 3 000
	Sequencing and Capillary Electrophoresis		R 5 000 (at R100 per sample)
	Estimated TOTAL		R 23 215.15

List of Equipment Needed:
Microcentrifuge
Vortex
Pipettes
Agarose Gel Electrophoresis system
UV Doc
Thermal Cycler
Capillary Electrophoresis
Genetic Analyser
Nanodrop/Spectrophotometer

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1.8 Appendices

Appendix A: Amendments to the initial protocol

1. Due to difficulties with rearing, only larvae at the third instar stage were used.
2. In some instances, where stock was finished different but equivalent PCR kits were used.
3. The PCR enhancer Trehalose was not used

Appendix B: Cost incurred

Table A1 Actual expenditure incurred during the course of the project

		Quantity	**Price (R)**
DNA Extraction	ZR Tissue and Insect DNA MiniPrep	1 box (50 reactions)	R 2 759.48
Amplification	KAPA HiFi HotStart ReadyMix PCR Kit	2x 1.25 mL	R 818.37
And	Folmer + ITS2 Primers		R 649.33
	Lep Primers		R 305.00
Electrophoresis	Agarose	1 bottle	R 2 280
	Quick-load 50bp ladder	1.25mL	R 1 108.08
	Loading dye	1.5 mL	R 103.97
	Sequencing	(188 sequences)	R 13 896.00
Sequencing	Pipette tips	3x 96tip box	R 550.00
Consumables	Gloves	2 boxes	R 200.00
	2mL Eppendorf tubes	1 box (1000)	R 300.00
	1.5mL Eppendorf tubes	1 box (1000)	R 300.00
	0.2mL PCR tubes	1 box (1000)	R 500.00
Total			**R 23 770,23**

Chapter 2 Literature Review
Forensic Entomology and DNA Barcoding: A review

2.1 Introduction

Over recent years, forensic entomology has become a common tool in forensic investigations. There are three distinct areas that make up forensic entomology; medicolegal, urban and stored product pests [1]. Medicolegal refers to legal investigations surrounding insects that feed on human corpses. Urban forensic entomology refers to insects that effect man and his immediate environment and lastly, stored product pests deals with contamination of food and drink by insects, answering whether their presence is at allowable levels and whether such levels are accidental or deliberate [1].

Medicolegal forensic entomology makes use of arthropod evidence associated with a corpse [2] . The information garnered from insect analysis gives insight into a forensic investigation on matters such as time elapsed since death and on occasion details about whether a body had been moved [3]. Forensic entomology involves the analysis of insect colonisation patterns and the developmental stage of a particular species at the time of discovery of the body. Using this, forensic entomologists effectively estimate the post mortem interval (PMI) [4].

2.2 Developments in forensic entomology

2.2.1 Brief history

The first recorded use of insects in a forensic setting occurred in 13th century China [3]. In a book titled "*The Washing Away of Wrongs"* by lawyer Sung Tzu, the murder of a man near a rice field is described. The weapon was thought to be a sickle, a tool used for harvesting rice, and hence suspects were asked lay down their working tools. Flies were drawn to what was hypothesised to be traces blood on one of the sickles. When confronted, the suspect subsequently confessed to the murder [5] . In addition to legal and medical experts, artists and sculptors have likewise observed, though perhaps without understanding, the role that insects play in the decomposition of a body [3] .Artwork such as "Danse Macabre" or in English, "Dances of the Death" and carvings like "Skeleton in the Tumba" (16th century) from the Middle Ages illustrate maggots on corpses which represent early skeletonisation and the reduction of body mass which is seen on decaying corpses [6].

2.2.2 Early French cases

From the 18th and 19th centuries, forensic entomology garnered more traction amongst academics as a vital piece of evidentiary information. In 1855 French doctor Bergeret used a rudimentary form of insect succession analysis to determine how long a child had been deceased. The remains had been found behind a chimney [5] and by assessing the fauna associated with the body, Bergeret believed the child had been dead for at least two years. This effectively cleared suspicion that the current occupants of the house committed the murder [2]. It is worthy to mention that Bergeret's ultimate conclusion, a post mortem interval of two years, was not solely based on the entomological findings but on the fact that the body in the final stage of decomposition – skeletionisation. In 1879 French researcher, Paul Brouardel, studied the work Bergeret and applied it to the case of an autopsy of a new born baby.

A few years later, Yavanovich (1888), and Mégnin (1894) raised the profile of forensic entomology, largely through their work on evaluating the succession of insects on corpses [7,8]. Mégnin described eight standard stages of decomposition together with the prediction of the arthropod fauna associated with each stage. [5]. Regardless of on-going research, interest in forensic entomology only

resurfaced in the late 20th century when Doctor Leclercq and Professor Pekka Nuorteva began using forensic entomology as an essential tool for the determination of the PMI [3].

2.3 Insects and the corpse

2.3.1 Post mortem changes

After death, chemical breakdown of cells and the release of enzymes promotes the autolysis of tissue. This is further promoted by bacterial activity in the internal and external environment [9,10]. This results in three specific changes; algor mortis, livor mortis (sometimes referred to as lividity) and rigor mortis. During algor mortis, the body temperature of the deceased decreases. Livor mortis refers to the reddening of skin where blood has settled due to gravitational pull. Lastly, rigor mortis, is the stiffening of muscle fibres [8]. When muscles contract, myosin and actin fuse forming cross bridges between the muscle fibres and during the time of muscle relaxation, the fused myosin and actin separate. This separation requires oxygen, and due to the cessation of respiration after death the muscle cannot relax and hence remains stiff. Eventually enzyme degradation leads to tissue breakdown and the muscles eventually become supple again. [8]. Besides these three changes the body undergoes decomposition which has six distinct stages namely; initial decay, putrefaction, black putrefaction, butyric fermentation, dry decay and skeletonisation [11]. The combined knowledge of the stage of decomposition and the physical changes in the body are used to determine PMI, though occurring in this order, the lengths of each stage may vary considerably according to a variety of factors, both internal and external [8].

2.3.2 Insect colonisation

As decomposition of the body begins, apeneumones are released. These are a mixture of liquids and gases produced during the digestion of the gastrointestinal tract which attracts insects to the body [12]. Blowflies are specifically attracted to putrid sulphur-based compounds which bring them to the body and oviposition or egg-laying is induced by ammonium-rich compounds and hydrogen sulphide [13]. Smith [14], found that four different categories of insect are found on a dead body, (1) Necrophagus, those that feed on carrion, (2) Predators and parasites that feed on the necrophagus insects, (3) Omnivores feeding on the carrion and (4) other species that use the corpse as an extended environment such as spiders. Species of flies from the order Diptera are of most interest to forensic

entomology [15], and in particular, those belonging to the families Calliphoridae (blowflies), Sarcophagidae (flesh flies), and Muscidae (house flies) [16].

2.3.3 Calliphoridae

Blowflies are often the first to colonise a body, arriving within minutes of death and are considered a major vector to tissue degradation due to their great number [17]. Oviposition occurs at orifices or open wounds on the body. Eggs and larvae of insects of the Diptera family need moisture to complete development and it is for this reason that female Diptera do not oviposit in dehydrated or mummified tissue [10,18].

Of the blowflies that visit a body, those in the *Chrysomya* genus constitute a significant proportion [19]. The morphology of flies within the *Chrysomya* genus is quite similar and therefore can make identification challenging [20]. Also of importance are species belonging to the *Lucilia* genus. These flies are commonly known as green bottle flies and extensive research on the life cycle of these flies has been established (Figure 2.1).

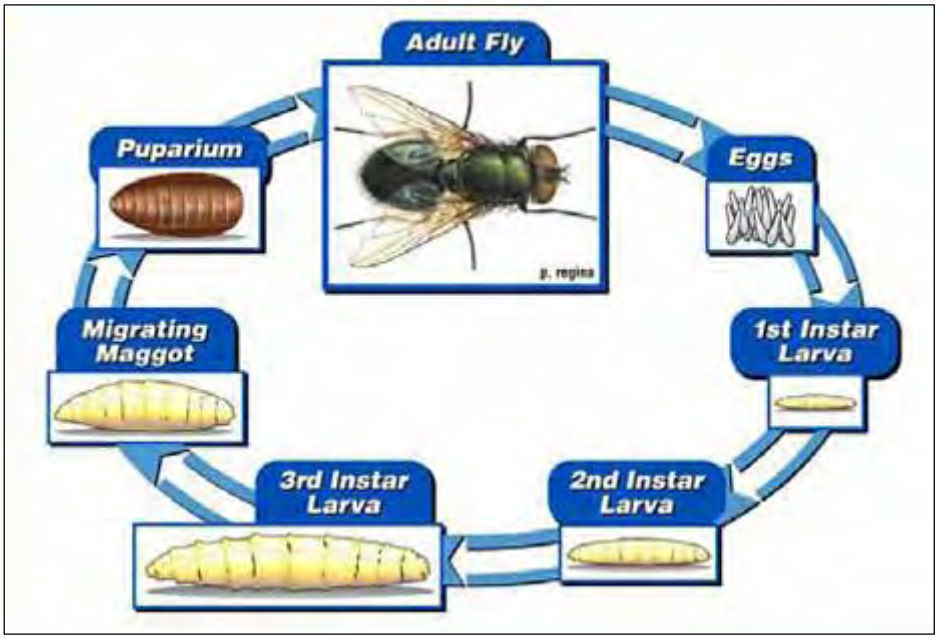

Figure 2.1 Typical life cycle of a blow fly. After eggs are laid, maggots emerge (eclosion). First instar is usually about 5mm in length. Second instar larva doubles in length to about 10mm. Third instar larvae measure at around 18mm in length. Puparium changes colour with age (early and late). Final eclosion; adult fly emerges. After hardening of the cytoskeleton, flies seek mates and copulate. After completion of egg development, eggs are oviposited onto moist carrion. Cleveland Natural Museum.

2.3.4 Post mortem interval

The PMI is defined as the period between death and the time of discovery of the death. The PMI could contribute vital information during a homicide investigation such as adding to the reconstruction of the crime by providing a timeline history of the corpse, establishing credibility of witness statements as well as narrowing the suspect pool [21,22]. During the first 72 hours after the death, the PMI can be estimated by a forensic pathologist using the physical changes of algor mortis, lividity and rigor mortis, as these changes occur within specific timeframes [23,24].

The general opinion is that the body loses heat at a rate of 1 °C per hour and that it takes the body 6-8 hours to reach ambient temperature. This however, is all dependent on the temperature of the body prior to death, the surrounding temperature and the body mass of the deceased. Factors such as whether the body was clothed or naked can also affect the rate at which it loses heat [8]. Livor mortis begins at least 15-20 minutes after death but is most visible after approximately 2 hours. The pattern of the lividity can give information about the position of the deceased around the time of death. Lividity is not as apparent in darker skinned people as it is in lighter skinned people which limits its utility. Rigor mortis starts 2-4 hours after death and usually in small muscles first and the maximum stiffness is often reached 24 hours after death. Stiffness lasts for 24-48 hours and the muscles then begin to relax as the fibres start to decay [7].

A forensic pathologist uses these indicators to give an estimation of a possible PMI but these changes can only provide information regarding the deceased for up to 72 hours. Once the body has passed the initial putrefaction stage of decomposition and equilibration of temperature between the environment and the body has been reached, no reliable PMI estimations can be made from these specific factor and therefore PMI estimations rely largely on the insect flora found on the corpse [11].

2.3.5 Estimating the post mortem interval using forensic entomology

PMI estimations essentially aim to provide a maximum and minimum period of time between time of probable death and time of discovery of the body. Though the exact time of death is difficult to estimate, insect activity aids in refining the estimation. Estimations of PMI using forensic entomology can be done in one of two ways; a time period can be determined based on the life cycle of insects associated with the corpse, and proposed as the minimum PMI [12]. This time period would essentially

correspond not to the time of death but to the time of colonisation instead [22]. If it is suspected that a longer period of time has passed since death, the PMI can be estimated by the observation and analysis of the succession of arthropods present [12,25]. The succession often follows the stages of tissue decomposition as each stage attracts a different group of insects. The two methods however, are generally complementary techniques.

Due to the need of moist flesh for the larvae to feed on, the analysis of blowfly larvae can be used up until the dehydration phase of decomposition – up to a month, as oviposition would occur up until the body dehydrated. When, the minimum PMI is estimated using the age of larvae there are a few things to consider; the growth of the larvae is species and temperature dependant as different species have differing time periods for each life cycle which may or may not be sped up or decreased by changes in temperature [26].

2.4 Species identification

2.4.1 Importance and rationale

Both insect succession observation and the use of life cycle analysis require an initial step of correctly identifying the species of larvae and adult flies. Identification of species is usually done via the observation of morphological features of the fly in question [2]. Identifying forensically important flies to species level generally requires specialised taxonomic knowledge and differentiation at larval stages of certain species is impossible due to the absence of distinguishing external features. To circumvent this, larvae can be reared to adulthood [8]. However, this is a time-consuming process and may delay the progress of an ongoing forensic investigation. Larval rearing may also fail, and any information that would have been gathered would be lost [27].

The identification of species is a vital first step for forensic entomologists as different species have differing lengths of life cycles and are affected differently by external factors such as temperature and humidity [28]. An incorrect assignment of species may lead to incorrect deductions of the PMI, which could have negative effects especially when the deductions are to be presented as official evidence in court proceedings [29].

2.4.2 Morphological species identification

Modern day forensic entomologists use knowledge garnered from taxonomy to accurately identify species of insects that colonise a dead body. The identification of species is the most important step as this is the basis on any further conclusions. Traditional methods of species identification involve the use of morphological keys to identify certain unifying or distinguishable characteristics between flies [30]. Hebert *et al.,* [31] described four limitations inherent in these methods:

(1) Phenotypic plasticity – the ability of one genotype to produce more than one phenotype based on environmental factors, and genetic variability in features used for species recognition could cause misidentification;
(2) Morphologically cryptic taxa are often overlooked;
(3) Current morphological keys are only suited for particular life stages, and
(4) These keys often require the skill and expertise of highly trained personnel.

2.4.3 Molecular species identification

With the difficulties facing traditional morphological species identification, a more robust and impartial method was found in genomic approaches. Differences in DNA sequences could be exploited as a method of species identification. Several molecular methods have been tested such as polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) in which the discrimination of species is based on restriction profile of amplicons [32,33] and multiplex PCR which combines primer sets with different specificities in a single assay essentially detecting multiple species in a single assay [34,35]. More recently, DNA barcoding which involves PCR amplification and sequencing of a genetic marker [36–38].

2.5. DNA barcoding

2.5.1 Principle of DNA barcoding

In 2003, Paul Hebert *et al.,* proposed the idea of using short standardised fragments of mitochondrial DNA for species identification. They further proposed creating a library of the standard DNA sequences that could have the potential to be used as a new "Master Key" to accurately identify species within certain taxonomic groups. Their research focused on the suitability of the mitochondrial genome and in particular, the *cytochrome c oxidase subunit 1* (*COI*), as the barcode for animal life [31]. The technique was named "DNA barcoding" and the *COI* barcode is now routinely used as the standard for animal barcoding.

DNA barcoding involves the isolation of DNA from source material and the amplification of standardised regions using universal or custom primers. The amplicons are then sequenced and subsequently compared to reference data (Figure 2.2). If sequences match with a high degree of similarity, they are placed in that particular reference taxon. When no matches occur, the new data can be used to describe geographical variants or in some cases new species [8,30].

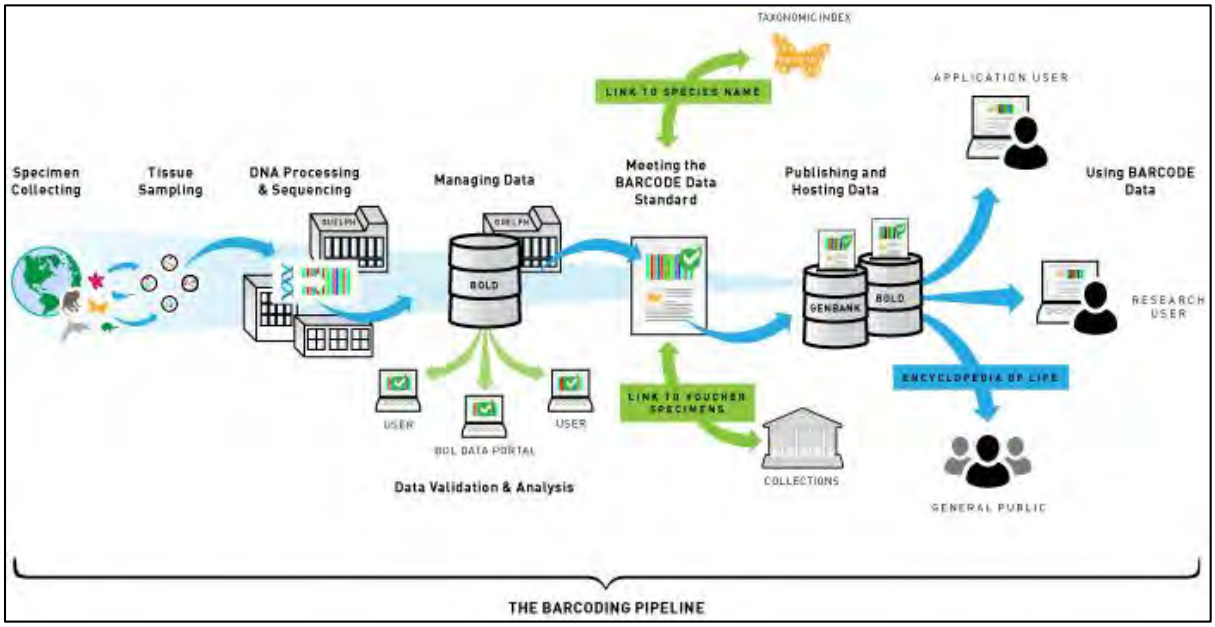

Figure 2.2 The workflow of DNA barcoding from specimen collection to the data outputs. www.ibol.org. DNA barcoding begins with sample collection and continues to tissue sampling, DNA processing, data management and the respective applications.

After the initiation of the Barcode of Life project, interest in this technique increased and DNA barcoding is now a funded global effort with three dedicated websites and partner organisations the

world over [39]. Barcoding projects began in 2004 and the number of projects has increased. The technique's ability to identify species has since been assessed for many taxa [40–43] and it has been used to resolve taxonomic ambiguity [44,45].

In 2004, the Consortium for the Barcode of Life (CBOL) was developed. CBOL was tasked with the development of standard protocols for the extraction and amplification of DNA and subsequent sequencing. The sequence data is loaded and freely available to researchers on the database – Barcode of Life Database (BOLD), which also serves as a public workbench for identification, visualisation, interpretation and the sharing and publishing of sequence data [46]. More recently, the international Barcode of Life (iBOL) project was initiated in 2010. This international collaboration with a host of 26 countries aim to establish an identification system based on a DNA library inclusive of all eukaryotes that can be fully automated. Currently the database contains 4 013 927 specimens [47].

In their article, Hebert *et al.,* [31] suggested a single gene to identify all animal life; they proposed that not only would the use of DNA provide better taxonomic resolution to morphological methods of taxonomy, but it would address the observed decline in traditional taxonomic knowledge prevalent. In addition to this, it would provide a means to identify as well as define boundaries and aid in species delimitation [48]. These statements were met with mixed reception [39]. This was due to wide perception that this method would diminish and replace traditional taxonomy and identification methods [49–51]. In addition, it was believed that if species determination were to be based solely on the genetic divergence, errors would be inevitable [52]. According to Meyer and Paulay [53], the success of DNA barcoding is largely dependent on the 'barcoding gap' which is established when the claim of interspecific divergence is greater than intraspecific variation and amongst some species this does not exist. Furthermore, the use of DNA barcoding for not only species identification but for species discovery was highly discouraged [54]. According to DeSalle [55] DNA barcoding should not be looked to as a method of species discovery (the finding and naming of new species) as this is a primary domain of taxonomy. DeSalle explains that the use of a signal locus to describe new species or infer phylogeny may be premature without additional evidence. In response to claims of DNA barcoding being used for species discovery and delimitation, DeSalle [56] proposed DNA barcoding should only be used to corroborate taxonomic data.

2.5.2 The locus

DNA barcoding required a universal marker that had sufficient discriminatory value to distinguish between species, ensuring the international exchangeability of the generated data [31]. In order to be standardised, the locus had to be present in all taxa, easily amplified and sequenced, and the areas flanking the region needed to be highly conserved to allow for the use of standard primers. The locus also needed to provide a large degree of variability between species but a small degree within species [31].

Mitochondrial Genome

The mitochondrial genome (Figure 2.3) typically consists of a circular genome, that comprises 13 protein coding genes, 22 transfer RNAs and 2 ribosomal RNAs [57]. Mitochondrial DNA (mtDNA) has previously been used to study evolutionary relationships within and among species [58] as well as identification of species [59] and based on the requirements of a suitable barcode sequence, mtDNA was used in preference to nuclear DNA. The main advantage being that the mitochondrial genome has a relatively fast mutation rate, which translates into a high level of diversity between species [60]. Hundreds to 1000s of mitochondria are in any given cell, with each containing 5-15 copies of mtDNA, making it more accessible. Inn addition, mtDNA has no recombination, making it a good source of markers for the study of closely related taxa.

Hebert *et al.,* [31] suggested the use of the gene that encodes for subunit one of cytochrome C oxidase (Figure 2.3), an enzyme vital to cellular respiration. The study showed that the mitochondrial genome was further suited for DNA barcodes due to its low level of recombination and it being void of introns..

Hebert *et al.,* [48] supplied two advantages of using the *COI* gene as a barcode; (i) it had the opportunity for robust universal primers enabling recovery of the 5' end from representatives from all taxa, and (ii) *COI* possessed a greater range of phylogenetic signal than any other mitochondrial gene. They further concluded that the rapid evolutionary rates were enough to allow for the discrimination between closely related as well as phylogeographic (organisms of the same species but differing geographical locations) groups within species.

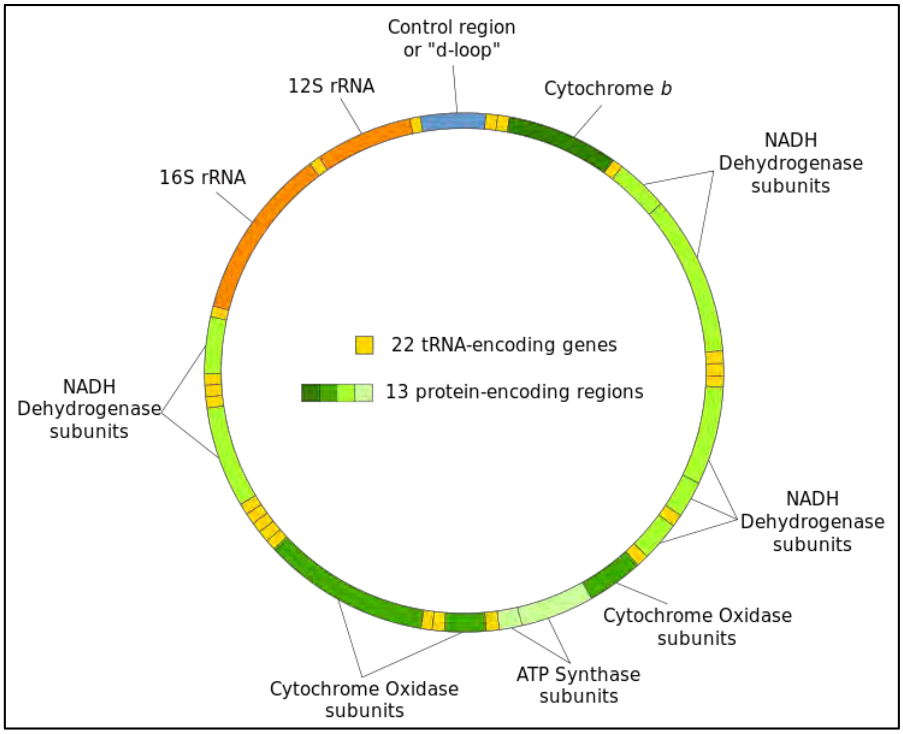

Figure 2.3 Circular mitochondrial genome. The genome consists of 22 transfer RNA genes and 13 genes encoding for proteins adapted from [62]

Nuclear ribosomal DNA

Genes within nuclear ribosomal DNA (rDNA) are another source of molecular markers useful for comparisons of closely related taxa due to their rapidly evolving nature [61]. rDNA genes comprise of tandemly repeated transcription units with intergenic spacers between them. The units contain conserved regions for ribosomal subunits 18S, 5.8S and 28S. These are further separated by two non-conserved areas known as internal transcribed spacer (ITS) regions [61] (Figure 2.4). Comparisons of the ITS sequences have been used in taxonomy and molecular phylogeny due to the ease of amplification, and high variation between closely related species, [62]. This high variation is a direct effect of their rapid rates of evolutionary divergence. Amplification of ITS2 has also been suggested as a universal barcode for fungi [62].

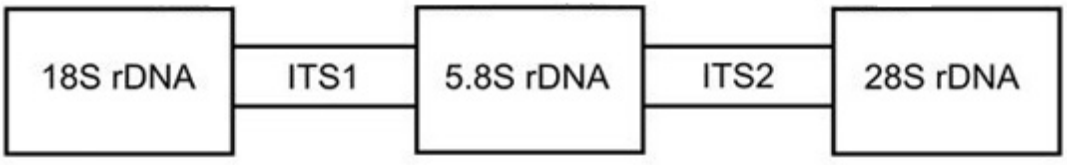

Figure 2.4 Schematic diagram of the repeat units of rDNA which comprises the 18S, 5.8S and 28S conserved rDNA genes and the internal spacers, internal transcribed spacer 1 (ITS2) and internal transcribed spacer 2 (ITS2) adapted from[63]

2.5.3 Barcoding of forensically important flies

The *COI* has been used to successfully identify species of Diptera in Germany [64], Portugal [65], North America and West Europe [66] and India. Flies are collected from death scenes or at the mortuary on the deceased directly into ethanol [67]. DNA is usually extracted from the legs or the thorax of the fly followed by the amplification of the *COI* region. Amplicons are sequenced and compared to known sequences housed in a database. High percentages of similarity indicate identity of unknown samples.

Meiklejohn [37] showed that through *COI* barcoding, flies belonging to the Sarcophagidae family could be identified at all immature stages. However, a few studies [68,69] have shown that the *COI* barcode does not have the ability to distinguish between closely related species. Contrary to a study done by Boeheme *et al.,* [70], Sonet [68] showed that the *COI* barcode was in fact not able to distinguish between *Lucilia caesar and L. illustris.* It was proffered that these two species had a high degree of overlap in their range of intraspecific and interspecific sequence divergences therefore making them indistinguishable.

Recently, the universal DNA barcode for fungi, the *second internal transcribed ribosomal spacer* (*ITS2*) has shown to be useful in distinguishing between cryptic Calliphoridae species and those that have recently diverged from it. The *ITS2* refers to a non-coding DNA sequence in the nuclear ribosomal cluster situated between the 3' end of the 5.8S ribosomal DNA (rDNA) and the 5' end of the 28S rDNA. Song *et al.,* [71] described the potential of the *ITS2* region in the identification of forensically important flies. They did however, note that there is a high level of sequence homology between some species and that the marker could not be used to differentiate between geographical populations. Therefore, analysis of the *ITS2* alone is not sufficient for the identification of cryptic or closely related species of blow fly, however, using both a mitochondrial gene and nuclear gene may provide better resolution for identification.

2.6. Forensic entomology in South Africa

Forensic entomology has been used in South Africa since the early 20th century [72]. André Prins directed the first attempts at forensic research in southern Africa at the South African Museum. He published details regarding the life cycle of several South African blowflies, keys to their third instar larvae, illustrated morphology and indicated developmental rates [73]. In addition, Prins made a series of notes on the arthropods associated with decaying matter [74].

The year 1992 saw the founding of the Forensic Entomology Investigation Team of the then Universiteit van die Oranje Vry Staat (now called the University of the Free State) which was situated at the Department of Forensic Medicine under the leadership of medical entomologist Theunis C. van der Linde [72]. Van der Linde and his students went onto to report largely on maggots, their development rates [75], anatomy and use as toxicological indicators [76]. They performed several experiments on pig carcasses to monitor insect succession under differing conditions such as wrapped versus clothed [77], burned versus frozen [78], in the shade versus exposed [79] to the sun as well as suspended [80] or stabbed [81].

Research regarding the identification of adult flies in South Africa was explored by Martin H. Villet (1993) at Rhodes University in Grahamstown. An electronic key has since been developed (IdentiFly) for southern African blowflies and fleshflies. This centre is now known as the Southern African Forensic Entomology Research (SAFER) laboratory.

From the late 1950s, the South African Police Service (SAPS) occasionally consulted with forensic entomologists [72]. In the year 2000 entomological evidence was used in court to support the conviction Albert du Preez in a case of indecent assault and murder in the Johannesburg Supreme High Court [72]. The evidence was provided by Mervyn W. Mansell who had been working with SAPS five years prior to this case and continues to provide vital entomological evidence in a wide range of cases including high profile cases [72]. In 2003, Harvey *et al*., [82] collaborating with Australian forensic entomologists used molecular methods as a method for accurate species identification. They commented that though forensic entomology in South Africa has come a long way, DNA based identification is more a curiosity than a reality [82].

2.7. Conclusion

Forensic entomology has developed from humble beginnings to a vital part in a forensic investigation, providing investigators with tools that can assist in court proceedings. Morphological identification, though very useful, has limitations in that cryptic species, larvae and damaged specimens may be indistinguishable. Morphological identification also requires skilled taxonomists for accurate identification, a profession where numbers are slowly decreasing. DNA barcoding offers a reliable method to complement morphological data. The dependence of accurate species identification for PMI estimations signifies the importance of a reliable and reproducible method. The efficacy of DNA barcoding however, is only as reliable as the database is representative. With geographical influences playing a part in evolution of species, the database should have representative species from multiple locations to ensure correct identification. As only a few studies have been conducted for South Africa and less for the Western Cape, studies are needed to increase representation in the database and could have applications in the forensic cases where PMI needs to be determined.

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Chapter 3 : Minor-dissertation in the format of a manuscript

DNA Barcoding of Forensically Important Flies in the Western Cape of South Africa

Keywords: COI, ITS2, forensic entomology, post mortem interval, species identification

Abstract

Forensic entomology provides a method to determine post mortem interval based on the age of and stage of life cycle of arthropods associated with a dead body. This requires knowledge of the life cycle of insects that visit the body, especially first colonisers such as Calliphoridae (Diptera). Traditional species identification has been hampered by morphological indistinguishability especially between immature specimens or when the specimen is damaged. Implementation of molecular methods such as DNA barcoding has introduced methods to complement morphological findings. However, in order to provide effective and correct identifications, databases need to be well represented. Four species of blow fly common to the Western Cape of South Africa, (*Chrysomya chloropyga*, *Chrysomya albiceps*, *Chrysomya marginalis*, and *Lucilia sericata*) were used to assess the utility of DNA barcoding for species level determination in a local context, as well as its ability to identify immature specimens. The standard *COI* barcode as well as a secondary barcode *ITS2* were amplified and sequenced. Sequence divergences within and between species were analysed. Intraspecific divergence showed a maximum of 0.003% and 0.043% for *COI* and *ITS2* respectively. Higher interspecific divergence values were found in *COI* sequences compared to *ITS2*. DNA sequences from the adult specimens were then used as reference sequences for identification of seven unknown immature specimen using DNA barcoding of both *COI* and *ITS2*. Sequence similarity was assessed and identity was assigned based on >98% similarity scores, and all immatures were successfully identified. According to these results, *COI* and *ITS2* have sufficient discriminatory power for species level identification for the four species studied. Additionally, this technique of DNA barcoding is suitable for the identification of immature specimens.

3.1 Introduction

The use of insect evidence to estimate the post mortem interval (PMI) has proven to be valuable in forensic investigations [1,2]. Traditional methods of PMI determination by assessing livor mortis, algor mortis and rigor mortis are commonly unreliable after 72 hours hereafter forensic entomology becomes a vital role in PMI determination [3]. Insects within the taxon family Calliphoridae (blow flies) are first colonisers of the deceased and as such offer the most information regarding time since death. PMI can be calculated based on succession patterns of insects and the life cycle stage of blow flies collected at the scene.

[4,5].

Calliphoridae comprise over one thousand species, each with differing life cycle patterns. Due to the life cycle being central to this method of PMI determination, correct species identification is critical [2]. Traditionally, species identification involves the use of a morphological key that assigned species based on distinguishing characteristics [6]. This method requires personnel with specialised taxonomic knowledge especially in closely related or recently divergent species, where changes appear subtle [7].

Due to the lack of distinguishable external features in blow flies during immature stages (especially first and second instar stages), morphological keys do not exist for many of these initial developmental stages and species identification is not possible. Therefore, larvae need to be reared to adulthood for definite identification, however, these efforts are time consuming [8] and often fail [1]. In addition, specimen that are damaged, brought about by incorrect storage or handling methods, may not be identifiable due to the lack of distinctive characteristics [9,10].

For the past decade, attempts to circumvent the shortfalls of morphological identification have been focused on using molecular techniques which take advantage of diversity in different species' DNA [11]. Methods such as PCR-RFLP [12,13] and multiplex PCR [14,15] have been assessed and validated for use in species identification. More recently, DNA barcoding has become a common means of species identification for forensically important flies whereby specifically chosen target regions (barcodes) in the DNA are amplified and sequenced, followed by comparison to reference sequences of known species in a database. [16–18].

Mitochondrial DNA has been the preference for molecular analysis due to its comparably higher mutation rate than nuclear DNA [19]. Focus has been centred on a 658 bp region of a mitochondrial

gene that encodes the cytochrome oxidase subunit I (COI). Hebert *et al.* [11], suggested this region as a universal genetic marker to classify and identify all of animal life. DNA barcoding projects have since escalated and *COI* is currently the standard marker for animal identification [20]. According to Hebert *et al.* [21], barcoding has the potential for species level identification in at least 95% of cases, as well as the identification of phylogeographic subspecies. Since blow fly species often exist in distinct localised populations, a technique that allows for the identification of subspecies would be very useful to forensic entomologists. The tendency of particular species and sub-species to remain in localised populations has the potential to offer additional investigative information such as whether the body had been moved or interfered with, provided the species and sub-species preferred habitats are known[20].

Several studies however have found limitations in the discriminating power of the *COI* barcode in closely related species of blow fly, another region, the *second internal transcribed spacer* (*ITS2*), has been assessed as an alternative or supplementary barcode [25,26].

Since the initiation of DNA barcoding, a substantial amount of barcode sequences have accumulated in databases such as BOLD and Genbank. However the information on these databases can be incorrect. For instance, Park *et al.*, [27] found that a fly first identified by Chinese researchers as *Aldrichina graham* through DNA barcoding had considerable distance (6.5-6.9%) to their own specimens and Chinese specimens of the same species. In addition Park *et al*., [27] found the specimen had remarkable sequence similarity (0.7-1.7% sequence distance) to that of a different species (*Calliphora vicinia*). Park *et al.,* [27] concluded that this sequence was most likely a misidentification. The reliance of DNA barcoding on a database stresses the need for correct sequences to be uploaded. Forensically, searching against incorrect sequences can lead to misidentification which may influence PMI determinations [28].

The most valuable information garnered from insect evidence is its ability to provide a method of PMI estimation, especially in cases where time since death is estimated to be longer than 72 hours [29]. The dependence of PMI calculations on the life cycle patterns makes correct species identification crucial. The presence of geographical differences within and between species requires a database that is both comprehensive and representative of species from various regions [20]. This pilot study aimed to assess the intraspecific and interspecific similarity within, at any developmental stage, within and among four species of blowfly common to the Western Cape area of South Africa.

3.2 Materials and Methods

Specimens

Ten adult specimens from *Lucilla sericata*, *Chrysomya albiceps*, *Chrysomya marginalis* and *Chrysomya chloropyga* were collected directly into 70% ethanol in accordance with previously published methods [30] from field experiments in the Western Cape area. Each specimen was identified using a stereomicroscope (Carl Zeiss, Oberkochen, Germany) and morphological keys adapted from Zumpt [31]. Images of all flies were captured. Unrelated larvae of unknown species to the researcher but known to an independent researcher, were sampled at the third instar stage. This was carried out to test the reliability of barcoding on immature specimen. This study received ethics approval from the Animal Ethics Committee, Faculty of Health Sciences at the University of Cape Town (FHS-AEC REF: 015/039)

DNA extraction, amplification and sequencing

Genomic DNA was extracted from whole flies and immature specimens using the ZR Insect extraction kit (Zymo Research, CA, USA) according to the manufacturer's instructions. The *COI* barcode was first amplified using Folmer primers LCO1490 and HCO2198 [32]. Upon analysis, two species failed to amplify and an additional set of primers was used, LepF1 and LepR1 [33]. In addition, the *ITS2* was amplified using previously published primers ITS2_F and ITS2_R [26]. Each 25µL reaction contained 0.3µM of each primer, 1X HiFi Hotstart Ready Mix (KAPA Biosystems, South Africa) All reaction mixtures contained 10ng. PCR was carried out on T100 thermal cycler (BioRad, Berkeley, CA, USA) at the following cycling conditions; Initial denaturation, 95°C for 3 minutes, followed by 25 cycles of denaturation at 98°C for 20 seconds, annealing at 55°C for 15 seconds, elongation at 72°C for 30 seconds. Final extension was carried out at 72°C for 5 minutes. PCR amplicons were then purified with *Exonuclease-1* and shrimp alkaline phosphatase. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA,) according to manufacturer's instructions. Labelled products were cleaned using ZR-96 DNA Sequencing Clean-up Kit (Zymo Research, California) and separated using an ABI 3500*xl* analyser (Applied Biosystems, CA, USA).

Analysis

Reverse and forward sequences were visualised and analysed using ChromasLite [34] and BioEdit 7.2.5 [35] to remove reading errors and remove primers. Multiple sequence alignments were performed for each marker using MUSCLE as given in MEGA version 6 [36]. Phylogenetic analyses were carried out in MEGA 6 using Maximum Likelihood, and to assess the reliability of phylogenetic tree

construction bootstrapping was performed with 1000 replicates. Intraspecific and interspecific differences and pairwise distances were calculated using Arlequin version 3.5. Consensus sequences were compiled from adult flies using Seaview, these were used as reference sequences for species identification of immatures. BOLD [37] was used a secondary identification for COI sequences.

3.3 Results

The molecular phylogeny of *Ch. chloropyga, Ch. albiceps, Ch. marginalis* and *L. sericata* was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [38] based on *COI* (Figure 3.1A) and *ITS2* (Figure 3.1B) in MEGA 6 [36]. For each species node, phylogenetic support was high (>94%), though phylogenetic support for *ITS2* was higher than *COI* (>99%). *Stomoyxs calcitrans* (Diptera, Muscidae) was used as an out group.

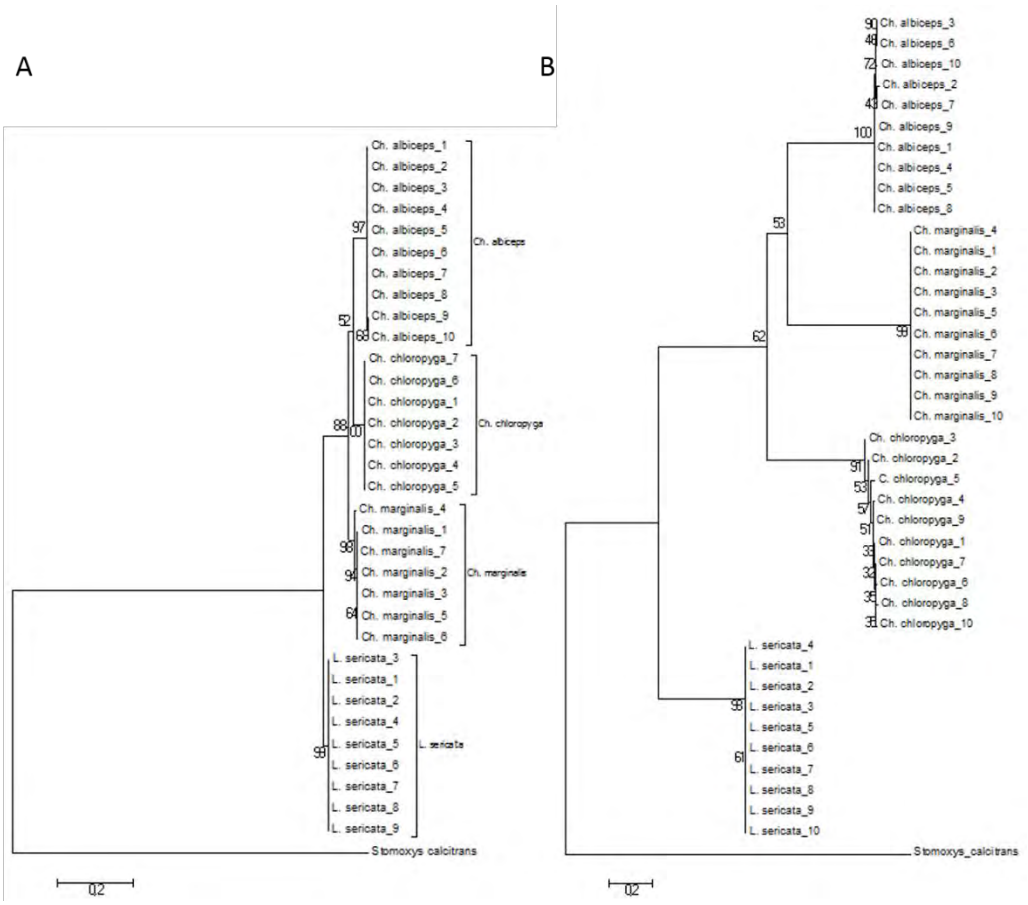

Figure 3.1 Maximum likelihood tree for 33 COI sequences (a) and 40 ITS2 sequences (b) from four blow fly species and one out group Stomoxys calcitrans. Values on tree branches refer to bootstrap values shown as a percentage of 1000 replicates and indicate support for nodes.

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Intra and interspecific analysis of four species studied

Intraspecific analysis was performed using Arlequin [39] for each species based on *COI* and *ITS2* sequences. *ITS2* in *Ch. chloropyga* appeared to be the most variable, with nine different haplotypes and a total of 30 polymorphic sites. In *Ch. albiceps, ITS2* displayed homogeneity across all ten specimens (table 3.2).

Table 3.1 The intraspecific analyses performed on each species for COI and ITS2 sequences. Table displays the species, number of specimen per species (N), molecular marker, the number of haplotypes observed, the nucleotide diversity, the mean number of pairwise differences and the number of polymorphic sites.

Species	N	Molecular marker	Number of haplotypes observed	Nucleotide diversity	Mean number of pairwise differences	Number of polymorphic sites
Ch. chloropyga	8	COI barcode	3	0.002±0.001	1.410±0.921	5
	10	ITS2	9	0.022±0.012	6.768±3.329	10
Ch. albiceps	10	COI barcode	10	0.002±0.001	1.415±0.903	6
	10	ITS2	6	0.003±	1.516±0.950	6
Ch. marginalis	7	COI barcode	10	0.003±0.002	2.500±1.484	6
	10	ITS2	1	0.000	0.000	0
L. sericata	9	COI barcode	2	0.000±	0.233±0.285	1
	10	ITS2	5	0.003±0.002	1.205±0.802	6

To demonstrate intra- and interspecific divergence, distance matrices (Table 3.2) showed nucleotide divergence values within and between species for *COI*. Minimum intraspecific was 0% across all four species with maximums reaching 0.003 for *Ch. chloropyga, Ch. albiceps*, *Ch. marginalis* and 0.002 for *L. sericata* (Table 4.3, Appendix 4.8). Interspecific divergence for species used in this study varied from 3.3-9.96%. The smallest divergence values corresponded to congeneric species *Ch. albiceps* and *Ch. chloropyga.* The highest divergence value was seen between *L. sericata* and *Ch. marginalis*. Table 3.2 also shows nucleotide divergence values within and between species for *ITS2*. Minimum intraspecific variation from all 10 specimen in each species had a minimum of 0% with the exception of *Ch. chloropyga* which had 0.007%. Maximum percentage variation was 0.043, 0.015 and 0.003 for *Ch. chloropyga, Ch. albiceps*, *L. sericata* respectively. *Ch. marginalis* displayed no variation (Table 4.4, Appendix 4.8). Interspecific divergence of *ITS2* for species used in this study varied from 2.1-4.2%. The smallest divergence values corresponded congeneric species *Ch. albiceps* and *Ch. marginalis.* The highest divergence value was seen between *L. sericata* and *Ch. marginalis.*

Table 3.2 Percentage of divergence values between L. sericata, Ch. albiceps, Ch. marginalis and Ch. chloropyga at the COI *(below the diagonal) and* ITS2 *(above the diagonal) regions. Bolded values indicate intraspecific distances for* COI/ITS2

	Ch. chloropyga	***Ch. albiceps***	***Ch. marginalis***	***L. sericata***
Ch. chloropyga	**0.001/0,024**	2.349	3.385	3.956
Ch. albiceps	3.275	**0.001/0.009**	2.112	4.067
Ch. marginalis	5.941	3.299	**0.004/0.001**	4.211
L. sericata	8.481	8.820	9.937	**0.001/0.004**

Identification of immature specimen

Adult specimens were used as references for identification of immature specimens. Table 3.3 reports the similarity scores reported after alignment using Basic Local Alignment Search Tool (BLAST) The majority of sequences matched with 100% similarity. Furthermore pairwise difference between unknown and reference sequences were computed and are also shown in Table. 3.3. COI sequences from immature specimen were also submitted to BOLD to assess its ability to identify sequences. Table 3.3 shows the BOLD identification and the top result and the reported percentage similarity.

Table 3.3 Identification of immature specimen, percentage of sequence similarity to specimens used in this study. Also indicated are identifications reported by BOLD and corresponding similarity

Unknown specimen	**Most similar to**	**% Similarity COI**	**% similarity ITS2**	**BOLD identification**	**BOLD reported % similarity**
1	*Ch. albiceps*	100%	100%	*Ch. albiceps*	100%
2	*L. sericata*	100%	99%	*L. sericata* *L. cuprina*	100% 100%
3	*Ch. chloropyga*	99%	99%	*Ch. chloropyga*	99.5%
4	*L. sericata*	100%	99%	*L. sericata* *L. cuprina* *L. cuprina x sericata*	100% 100% 98.8%
5	*Ch. albiceps*	98%	100%	*Ch. albiceps*	99.83%
6	*Ch. albiceps*	99%	100%	*Ch. albiceps*	100%
7	*Ch. albiceps*	99%	100%	*Ch. albiceps*	100%

3.4 Discussion

The determination of the PMI is often an integral part forensic investigations. After 72 hours, conventional methods of PMI determination become less reliable and forensic entomology assumes a more prominent role. Due to the shortcomings of morphological identification, molecular methods such as DNA barcoding have become increasingly popular for the forensic identification of insects. The aim of this study was to assess whether the standard DNA barcode *COI* and a supplementary region, *ITS2* could provide enough resolution to sufficiently identify species of blow flies common to the Western Cape of South Africa. In addition, the ability of DNA barcoding to identify larval samples was assessed.

Criteria for species identification, according to the DNA barcode Consortium, requires that the species in a phylogeny share monophyletic association [40]. Phylogenetic analysis of both *COI* and *ITS2* by means of Maximum likelihood trees show each species as a monophyletic group (Figure 3.1),

indicating that both barcodes share the ability to distinguish the four species in this study. High support values at species nodes of both trees, demonstrate the suitability of *COI* and *ITS2* for species determination, which is the foundation of DNA barcoding. Node support values for *ITS2* were higher (>99%) than *COI* (>94%) which suggests that *ITS2* may have better discrimination power than *COI* for the studied species. This is in line with other studies that have found success in using *ITS2* [25,26].

Threshold values within the context of percentage diversity between sequences constitute another criterion for DNA barcoding. These are based on the observation that nucleotide divergence between insect species, in most cases, exceed 3% or that there is a 10x or greater difference in nucleotide distance among species than within [11] . Also referred to as the barcoding gap, this was calculated using mean interspecific distances however, Meier *et al.* showed that this exaggerated the barcoding gap and proposed the use of the smallest interspecific distance value instead [41]. In this study, the smallest interspecific divergence for *COI* was 3.2% and 2.1% for *ITS2.* Intraspecific variation within each species for *COI* is 0.001, for both *Ch. chloropyga* and *Ch. albiceps,* 0.004 for *Ch. marginalis* and 0.002 for *L. sericata*. According to 10x criterion, this would correspond to maximum sequence divergences of 0.01, 0.01, 0.4 and 0.02% respectively. Interspecific distances for *COI* exceed both thresholds whereas *ITS2* only maintains the 10x threshold. The low sequence divergence in *ITS2* demonstrates what even though the phylogenetics produced a well-defined tree, there may be overlap between species and its use in species level identification should be carried out with caution.

According Hebert *et al.* [11], a criterion for the selection of a universal barcode is ease of amplification. In this study, the *ITS2* sequence was easier to amplify than *COI* due to the failure of universal primers to amplify *COI* in two of four species. A similar study performed on mites, found amplification of *COI* problematic and suggested the utilisation of *ITS2* over *COI* for convenience [42]. Though the use of universal primers was a key aim for DNA barcoding, the use of primers that only amplify certain species may add another layer of discrimination. *COI* displayed higher interspecific differences across all four species when compared to those of *ITS2* however, *ITS2* showed higher rates of intraspecific variability, suggesting that *COI* may be superior to *ITS2* in these four species. One exception was *Ch. marginalis* which displayed a very low degree of intraspecific variability in *ITS2*, while demonstrating highest genetic variation for *COI* compared to other species. In Table 3.1, *ITS2* presents with what appears to be complete homogeneity for *Ch. marginalis* which is indicative of minimum variability in this region.

Based on the low intraspecific variation and high interspecific variation for most species studied, *COI* appears to be more suited to species identification than *ITS2*, which is also seen by Yao *et al*., [43] who suggested that *ITS2* be used as complementary to COI for animal identifications. In addition, for *COI* all species had divergence values higher than 3% (Table 3.2) the threshold indicated by Hebert [11].

Conversely, taking into account the phylogenetic trees, though both reported each species as monophyletic groups, phylogenetic support was higher in *ITS2* than in *COI* suggesting *ITS2* as a better option for these four species. According to a similar study on these and two additional markers by Lv *et al.* [44], *COI* was not found to be significantly superior to the other markers in terms of correct species identification.

Sequences from adult specimens were used as reference material for immature specimens. Table 3.3 shows the similarity scores, for each marker. Species were assigned to immature specimen based on high (>98%) sequence similarity. All specimens were correctly identified. Sample 6 was identified as *Ch. albiceps*, however initial morphological identification by an independent researcher reported it to be *Ch. rufifacies*, sister species of *Ch. albiceps*. Genbank and BOLD were used to confirm the identity of this sample, which both reported it to be *Ch. albiceps*. Due to the likeness of these and other sister species, errors like this can be become common practice. Since the development stages within the life cycle of blow flies differ, PMI estimations inferred by forensic entomology may be incorrect if based on incorrect data.

Immature specimens were also identified using BOLD, which displays the 99 best matches and details species-level identification for sequences that show less than 1% sequence divergence [37]. Most of the larval specimens matched with 100% percent similarity to their own species from other parts of South Africa as well as other countries. This indicated that *COI,* in the studied species did not have the capacity to distinguish between phylogeographic groups within a species. The database was able to confirm identity to species level for *Ch. chloropyga, Ch. albiceps* and *Ch. marginalis*. Interestingly, *L. sericata* only be identified to genus level and was reported as being 100% to both *L. sericata* and *L. cuprina* as well as having a 99.5% similarity score to the hybrid species *L. cuprina x sericata.* These two species have been reported as being almost morphologically indistinguishable [45]. According to Wells *et al.* [23], *COI* alone is insufficient to distinguish between these two species due to some cases showing *L. cuprina* haplotypes being more similar to those of *L. sericata* than other *L. cuprina.* This means that within phylogenetic trees, these two would not exhibit a pattern of monophyly. However, when using nuclear data, such as the *ITS2,* monophyly of the species can be seen [46], demonstrating that the incorporation of a secondary marker would be useful for closely related species. Recently Willlams and Villet [47], developed a key to distinguish not only *L. sericata* and *L. cuprina* and their naturally occurring hybrids. In this circumstance, morphological data appears to be superior to molecular therefore combining both disciplines could strengthen the final conclusions as suggested by Chan *et al*., [48].

The findings in this pilot study demonstrate the suitability of both *COI* and *ITS2* for species level identification based on DNA barcoding methods. However, this is only for the studied species and as such further studies that include more species can provide a more in holistic overview of these methods.

3.5 Conclusion

The ability of the *COI* and *ITS2* regions was assessed for se as a marker for species identification for flies common to the Western Cape of South Africa. The results showed that *COI* and *ITS2* had sufficient discriminatory value to allow for species level identification for the studied species. The maintenance of threshold values and monophyletic grouping of the specimen based on the regions allow for correct species identification. *ITS2* had higher levels of interspecific diversity than *COI* and thus supplements *COI* data especially in closely related species. Immature specimens were successfully identified. Additionally, ambiguous results reported on *COI* sequences by databases can be refined by the analysis of supplementary regions. To the author's knowledge, this is the first time the *ITS2* sequence for *Ch. marginalis* has been sequenced and analysed.

3.6 Acknowledgements

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3.7 References

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Chapter 4 : Appendices

Appendix 4.1 Adult specimen

Table 4.1 Sample ID, species name and origin of specimen used in this study

Sample ID	Species	Origin
BFSA01*	*C. chloropyga*	Western Cape, SA
BFSA02*	*C. chloropyga*	Western Cape, SA
BFSA03	*C. chloropyga*	Western Cape, SA
BFSA04	*C. chloropyga*	Western Cape, SA
BFSA05*	*C. chloropyga*	Western Cape, SA
BFSA06	*C. chloropyga*	Western Cape, SA
BFSA07	*C. chloropyga*	Western Cape, SA
BFSA08	*C. chloropyga*	Western Cape, SA
BFSA09	*C. chloropyga*	Western Cape, SA
BFSA10	*C. chloropyga*	Western Cape, SA
BFSA11	*C. albiceps*	Western Cape, SA
BFSA12	*C. albiceps*	Western Cape, SA
BFSA13	*C. albiceps*	Western Cape, SA
BFSA14	*C. albiceps*	Western Cape, SA
BFSA15	*C. albiceps*	Western Cape, SA
BFSA16	*C. albiceps*	Western Cape, SA
BFSA17	*C. albiceps*	Western Cape, SA
BFSA18	*C. albiceps*	Western Cape, SA
BFSA19	*C. albiceps*	Western Cape, SA
BFSA20	*C. albiceps*	Western Cape, SA
BFSA21	*C. marginalis*	Western Cape, SA
BFSA22*	*C. marginalis*	Western Cape, SA
BFSA23*	*C. marginalis*	Western Cape, SA

BFSA24*	*C. marginalis*	Western Cape, SA
BFSA25	*C. marginalis*	Western Cape, SA
BFSA26	*C. marginalis*	Western Cape, SA
BFSA27	*C. marginalis*	Western Cape, SA
BFSA28	*C. marginalis*	Western Cape, SA
BFSA29	*C. marginalis*	Western Cape, SA
BFSA30	*C. marginalis*	Western Cape, SA
BFSA31*	*L. sericata*	Western Cape, SA
BFSA32	*L. sericata*	Western Cape, SA
BFSA33	*L. sericata*	Western Cape, SA
BFSA34	*L. sericata*	Western Cape, SA
BFSA35	*L. sericata*	Western Cape, SA
BFSA36	*L. sericata*	Western Cape, SA
BFSA37	*L. sericata*	Western Cape, SA
BFSA38	*L. sericata*	Western Cape, SA
BFSA39	*L. sericata*	Western Cape, SA
BFSA40	*L. sericata*	Western Cape, SA

*denotes samples that were omitted from the *COI* analysis due to failed sequencing reactions.

Appendix 4.2 Immature specimen

Table 4.2 Sample ID, larval stage and species of immature specimen used in this study

Specimen name	Instar stage	Species	Origin
MG1	Third	*Ch. albiceps*	Western Cape, SA
MG2	Third	*L. sericata*	Western Cape, SA
MG3	Third	*Ch. chloropyga*	Western Cape, SA
MG4	Third	*L. sericata*	Western Cape, SA
MG5	Third	*Ch. albiceps*	Western Cape, SA
MG6	Third	*Ch. albiceps*	Western Cape, SA
MG7	Third	*Ch. albiceps*	Western Cape, SA

Appendix 4.3 Representative gel of PCR amplification

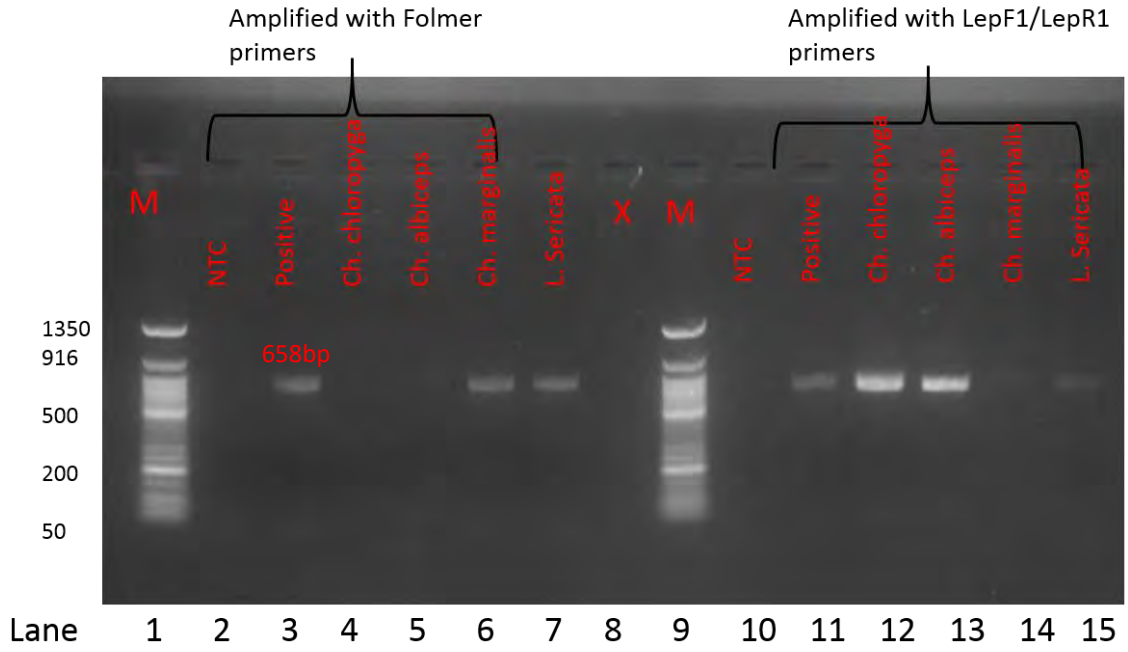

Figure 4.1 1.5% Agarose gel showing amplification of COI with Folmer and Lep primers. Electrophoresis was performed at 100V for one hour. L= ladder, NTC = no template control, +ve= positive control. 1-4 corresponds to Ch. chloropyga, Ch. albiceps, Ch. marginalis *and* L. sericata *respectively.*

Appendix 4.4 Molecular weight marker

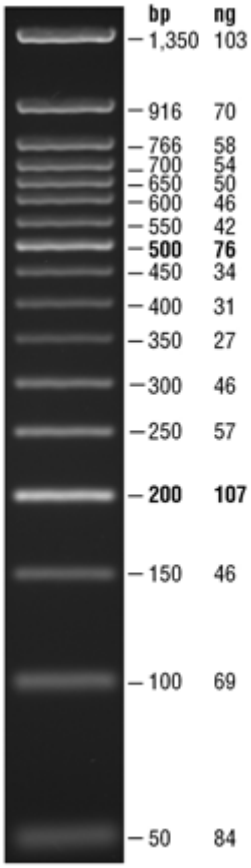

Figure 4.2Quick-Load 50 bp DNA Ladder visualized by ethidium bromide staining on a 3% TBE agarose gel. Mass values are for 1 µg/lane.

Appendix 4.5 Consensus sequences in FASTA format

COI Barcode

>Consensus_chloropyga_COI

TCATAAAGATATTGGTACTTTATATTTCATTTTCGGAGCTTGATCCGGAATAGTAGGAAC

TTCATTAAGTATTTTAATTCGAGCCGAATTAGGACACCCTGGGGCACTAATTGGAGATGA

CCAAATTTATAATGTAATTGTAACAGCTCACGCTTTTATTATAATTTTCTTTATAGTAAT

GCCAATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATACTAGGAGCTCCAGA

TATAGCTTTCCCACGAATAAATAATATAAGTTTCTGACTTTTACCTCCTGCATTAACTTT

ACTATTAGTAAGTAGTATAGTAGAAAATGGGGCTGGAACAGGATGAACTGTTTATCCACC

TTTGTCATCTAATATTGCCCATGGTGGTGCATCAGTTGATTTAGCTATTTTCTCTTTACA

TCTAGCCGGGATTTCTTCAATTTTAGGAGCTGTAAATTTTATTACAACTGTAATTAATAT

ACGGTCTACAGGAATTACATTTGACCGAATACCACTATTCGTTTGATCTGTAGTTATTAC

TGCTCTATTATTATTATTATCTTTACCAGTATTAGCAGGAGCTATTACTATATTATTAAC

CGACCGAAATTTAAATACTTCATTCTTTGACCCAGCAGGAGGGGGAGACCCTATTTTATA

CCAACACTTATTTTGATTTTTTGGACATCCAGAAANTTAT

>Consensus_albiceps_COI

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AATTGTAACAGCTCATGCCTTTATTATAATTTTCTTTATAGTAATACCAATTATAATTGG

AGGATTTGGAAATTGACTAGTTCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCACG

AATAAATAATATAAGTTTCTGACTTTTACCTCCTGCATTAACTTTACTATTAGTAAGTAG

TATAGTAGAAAATGGAGCTGGAACAGGATGAACTGTTTATCCACCTTTATCATCTAATAT

TGCTCATGGTGGAGCATCAGTTGATTTAGCTATTTTTTCTTTACACTTAGCTGGAATTTC

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CACATTTGATCGAATACCTTTATTCGTATGATCTGTAGTTATTACTGCTCTTCTTTTATT

ATTATCATTACCAGTATTAGCCGGTGCAATTACTATATTATTAACTGATCGAAATTTAAA

TACTTCATTCTTTGATCCAGCAGGAGGAGGAGATCCTATTTTATATCAACATTTATTT

>Consensus_marginalis_COI

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TTCCCTAAGTATCTTAATTCGAGCTGAATTAGGACATCCTGGAGCACTAATTGGAGATGA

CCAAATTTATAATGTAATTGTAACAGCTCACGCTTTTATTATAATTTTCTTTATAGTAAT

ACCAATTATAATTGGAGGATTTGGAAATTGACTAGTTCCTTTAATATTAGGAGCTCCAGA

TATAGCATTCCCACGAATAAACAATATAAGTTTCTGACTTTTACCTCCTGCTTTAACTCT

ACTATTAGTAAGTAGTATAGTAGAAAATGGAGCTGGAACAGGATGAACTGTTTACCCACC

TTTATCATCTAATATTGCCCATGGAGGTGCATCAGTTGATTTAGCTATTTTCTCACTACA

TTTAGCTGGAATTTCCTCAATTTTAGGAGCTGTAAATTTTATTACAACTGTAATTAATAT

ACGATCTACAGGAATTACATTTGATCGAATACCTTTATTTGTTTGATCTGTAGTAATTAC

TGCTTTATTATTATTGTTATCTTTACCAGTATTAGCAGGAGCTATTACTATACTATTAAC

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>Consensus_sericata_COI

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AATTCGAGCTGAATTAGGACATCCTGGAGCTTTAATTGGAGATGATCAAATTTATAATGT

AATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTATAGTAATGCCAATTATAATTGG

AGGATTTGGAAATTGATTAGTTCCATTAATACTAGGAGCTCCAGATATAGCATTCCCTCG

AATAAATAATATAAGTTTTTGACTTTTACCTCCTGCATTAACTTTATTATTAGTTAGTAG

TATAGTAGAAAACGGAGCTGGAACAGGATGAACAGTTTACCCTCCTCTATCTTCTAATAT

TGCTCATGGAGGAGCTTCTGTTGATTTAGCTATTTTCTCTCTTCATTTAGCAGGAATTTC

TTCAATTTTAGGAGCTGTAAATTTTATTACTACAGTTATTAATATACGATCAACAGGAAT

TACTTTTGATCGAATACCTTTATTTGTTTGATCAGTAGTAATTACAGCTTTATTACTTTT

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Consensus sequences – ITS2

>Consensus_chloropyga_ITS2

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TAAAATCAGAGTATTTTAATAATATGTTTAAATATTCTTTTTTTATTGAGGAAGGTCTAG
CATAAAAATTTATGAAACTAGAATTGCCTCTTTAATATAAAGAATCTCATTTATGTGGAT
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>Consensus_albiceps_ITS2

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TCTAAATGAATAAAATCAGAGTATTTTAATAAAATTTTAAAATATTCTTTTTTTATTGAG
GAAAGTCTAGCATAAAAATTTATGAAACTAGAATTGCCTCTTTAAAATAAAGAATTTCAT
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TCAAATGAATAAAATCAGAGTATTTTAATAATATATTATTAAAATGTTCTTTTTTTATTG
AGGAAGGTCTAGCATAAAAATTTATGAAACTAGAATTGCCTCTTTAATATAAAGAATTAA
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>Consensus_sericata_ITS2

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AAACCTCTTATTGAATAAAATCAGAGTATTTTTAAAATTACATTTTATTATATTCTTTTT
TTATTGAGGAAAGTCTAGCATAAAATATTTATGAAACTAGAATTGCCTCTCTAAAAGAAG
AAAAAGAAAAATACAGAAAAAAAAAGAAATGATTCTTATTCATGGTTTTGATATTTAAAT
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Appendix 4.6 Alignments of *COI* sequences

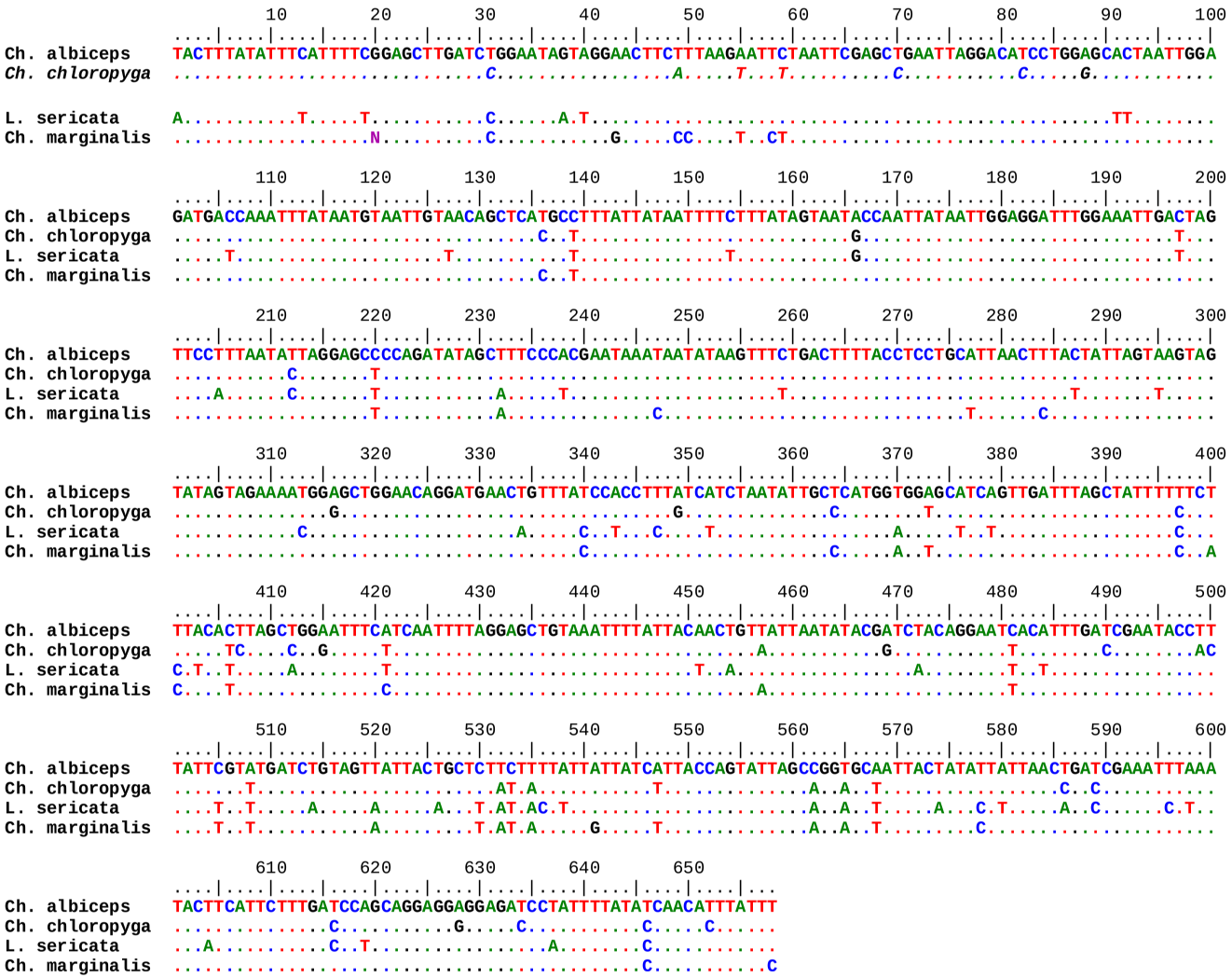

Figure 4.3 COI *sequence alignment from consensus sequences from* Ch. chloropyga, Ch. albiceps, Ch. marginalis *and* L. sericata. Identity is indicated with a full stop (.).

Appendix 4.7 Alignments of ITS2 sequences

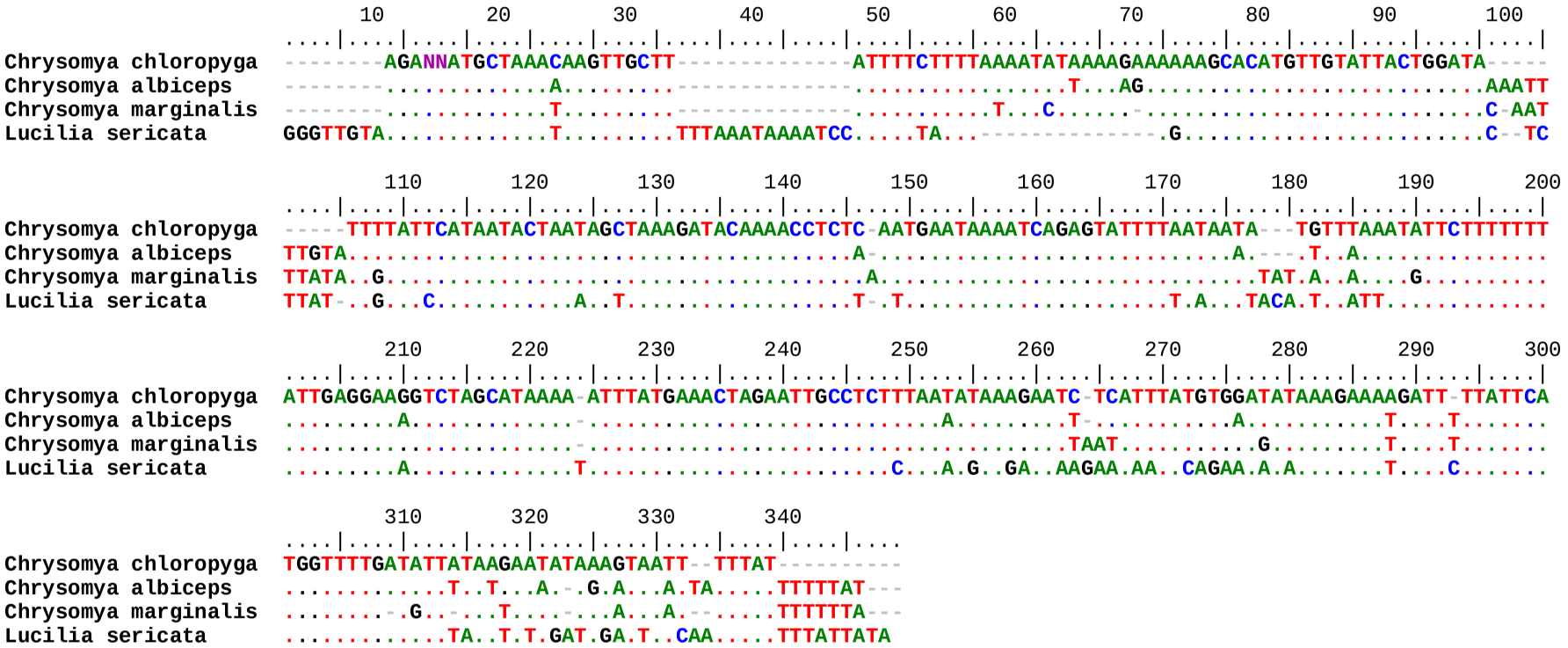

Figure 4.4 ITS2 sequence alignment from consensus sequences from Ch. chloropyga, Ch. albiceps, Ch. marginalis and L. sericata. Identity is indicated with a full stop (.)

Appendix 4.8 Distance matrices for all specimen for *COI* and *ITS2*

Table 4.3 Pairwise sequence divergence between the studied species (Ch. chloropyga, Ch, marginlais, Ch, albiceps and L. sericata) for COI. Nucleotide divergence in percentage is shown below the diagonal and standard error estimates are shown above.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Chrysomya_chloropyga_BFSA10		0,002	0,001	0,001	0,001	0,001	0,001	11,719	11,719	11,706	11,706	11,706	11,706	11,706	11,706	11,706	11,706
2 Chrysomya_chloropyga_BFSA09	0,003		0,001	0,001	0,001	0,001	0,001	11,714	11,714	11,703	11,703	11,703	11,703	11,703	11,703	11,703	11,703
3 Chrysomya_chloropyga_BFSA08	0,002	0,002		0,000	0,000	0,000	0,000	11,727	11,727	11,713	11,713	11,713	11,713	11,713	11,713	11,713	11,713
4 Chrysomya_chloropyga_BFSA07	0,002	0,002	0,000		0,000	0,000	0,000	11,727	11,727	11,713	11,713	11,713	11,713	11,713	11,713	11,713	11,713
5 Chrysomya_chloropyga_BFSA06	0,002	0,002	0,000	0,000		0,000	0,000	11,727	11,727	11,713	11,713	11,713	11,713	11,713	11,713	11,713	11,713
6 Chrysomya_chloropyga_BFSA04	0,002	0,002	0,000	0,000	0,000		0,000	11,727	11,727	11,713	11,713	11,713	11,713	11,713	11,713	11,713	11,713
7 Chrysomya_chloropyga_BFSA03	0,002	0,002	0,000	0,000	0,000	0,000		11,727	11,727	11,713	11,713	11,713	11,713	11,713	11,713	11,713	11,713
8 Chrysomya_albiceps_BFSA20	32,439	32,427	32,468	32,468	32,468	32,468	32,468		0,001	0,002	0,002	0,002	0,002	0,002	0,001	0,002	0,002
9 Chrysomya_albiceps_BFSA19	32,439	32,427	32,468	32,468	32,468	32,468	32,468	0,002		0,001	0,001	0,001	0,001	0,001	0,002	0,001	0,001
10 Chrysomya_albiceps_BFSA18	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002		0,000	0,000	0,000	0,000	0,001	0,000	0,000
11 Chrysomya_albiceps_BFSA17	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002	0,000		0,000	0,000	0,000	0,001	0,000	0,000
12 Chrysomya_albiceps_BFSA16	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002	0,000	0,000		0,000	0,000	0,001	0,000	0,000
13 Chrysomya_albiceps_BFSA15	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002	0,000	0,000	0,000		0,000	0,001	0,000	0,000
14 Chrysomya_albiceps_BFSA14	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002	0,000	0,000	0,000	0,000		0,001	0,000	0,000
15 Chrysomya_albiceps_BFSA13	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,002	0,003	0,002	0,002	0,002	0,002	0,002		0,001	0,001
16 Chrysomya_albiceps_BFSA12	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002	0,000	0,000	0,000	0,000	0,000	0,002		0,000
17 Chrysomya_albiceps_BFSA11	32,398	32,386	32,427	32,427	32,427	32,427	32,427	0,003	0,002	0,000	0,000	0,000	0,000	0,000	0,002	0,000	
18 Chrysomya_marginalis_BFSA21	0,065	0,065	0,063	0,063	0,063	0,063	0,063	33,019	33,019	32,975	32,975	32,975	32,975	32,975	32,975	32,975	32,975
19 Chrysomya_marginalis_BFSA25	0,065	0,065	0,063	0,063	0,063	0,063	0,063	33,062	33,062	33,019	33,019	33,019	33,019	33,019	33,019	33,019	33,019
20 Chrysomya_marginalis_BFSA26	0,065	0,065	0,063	0,063	0,063	0,063	0,063	33,062	33,062	33,019	33,019	33,019	33,019	33,019	33,019	33,019	33,019
21 Chrysomya_marginalis_BFSA27	0,061	0,061	0,060	0,060	0,060	0,060	0,060	33,496	33,496	33,450	33,450	33,450	33,450	33,450	33,450	33,450	33,450
22 Chrysomya_marginalis_BFSA28	0,065	0,065	0,063	0,063	0,063	0,063	0,063	33,062	33,062	33,019	33,019	33,019	33,019	33,019	33,019	33,019	33,019
23 Chrysomya_marginalis_BFSA29	0,065	0,065	0,063	0,063	0,063	0,063	0,063	33,062	33,062	33,019	33,019	33,019	33,019	33,019	33,019	33,019	33,019
24 Chrysomya_marginalis_BFSA30	0,063	0,063	0,061	0,061	0,061	0,061	0,061	33,019	33,019	32,975	32,975	32,975	32,975	32,975	32,975	32,975	32,975
25 Lucilia_sericata_BFSA32	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
26 Lucilia_sericata_BFSA33	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
27 Lucilia_sericata_BFSA34	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,129	0,131	0,129	0,129	0,129	0,129	0,129	0,127	0,129	0,129
28 Lucilia_sericata_BFSA35	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
29 Lucilia_sericata_BFSA36	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
30 Lucilia_sericata_BFSA37	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
31 Lucilia_sericata_BFSA38	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
32 Lucilia_sericata_BFSA39	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127
33 Lucilia_sericata_BFSA40	31,897	31,858	31,897	31,897	31,897	31,897	31,897	0,127	0,129	0,127	0,127	0,127	0,127	0,127	0,125	0,127	0,127

Table 4.3 cont. Pairwise sequence divergence between the studied species (Ch. chloropyga, Ch. marginalis, Ch. albiceps and L. sericata) for COI. Nucleotide divergence in percentage is shown below the diagonal and standard error estimates are shown above

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Chrysomya_chloropyga_BFSA10	0,012	0,012	0,012	0,012	0,012	0,012	0,012	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544
Chrysomya_chloropyga_BFSA09	0,012	0,012	0,012	0,011	0,012	0,012	0,012	11,531	11,531	11,531	11,531	11,531	11,531	11,531	11,531	11,531
Chrysomya_chloropyga_BFSA08	0,012	0,012	0,012	0,011	0,012	0,012	0,011	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544
Chrysomya_chloropyga_BFSA07	0,012	0,012	0,012	0,011	0,012	0,012	0,011	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544
Chrysomya_chloropyga_BFSA06	0,012	0,012	0,012	0,011	0,012	0,012	0,011	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544
Chrysomya_chloropyga_BFSA04	0,012	0,012	0,012	0,011	0,012	0,012	0,011	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544
Chrysomya_chloropyga_BFSA03	0,012	0,012	0,012	0,011	0,012	0,012	0,011	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544	11,544
Chrysomya_albiceps_BFSA20	11,896	11,906	11,906	11,934	11,906	11,906	11,896	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA19	11,896	11,906	11,906	11,934	11,906	11,906	11,896	0,022	0,022	0,023	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA18	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA17	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA16	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA15	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA14	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA13	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA12	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_albiceps_BFSA11	11,882	11,896	11,896	11,921	11,896	11,896	11,882	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022	0,022
Chrysomya_marginalis_BFSA21		0,002	0,002	0,004	0,002	0,002	0,001	11,671	11,671	11,671	11,671	11,671	11,671	11,671	11,671	11,671
Chrysomya_marginalis_BFSA25	0,003		0,000	0,004	0,000	0,000	0,001	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683
Chrysomya_marginalis_BFSA26	0,003	0,000		0,004	0,000	0,000	0,001	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683
Chrysomya_marginalis_BFSA27	0,009	0,009	0,009		0,004	0,004	0,004	11,558	11,558	11,558	11,558	11,558	11,558	11,558	11,558	11,558
Chrysomya_marginalis_BFSA28	0,003	0,000	0,000	0,009		0,000	0,001	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683
Chrysomya_marginalis_BFSA29	0,003	0,000	0,000	0,009	0,000		0,001	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683	11,683
Chrysomya_marginalis_BFSA30	0,002	0,002	0,002	0,008	0,002	0,002		11,671	11,671	11,671	11,671	11,671	11,671	11,671	11,671	11,671
Lucilia_sericata_BFSA32	32,364	32,405	32,405	31,771	32,405	32,405	32,364		0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000
Lucilia_sericata_BFSA33	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000		0,002	0,000	0,000	0,000	0,000	0,000	0,000
Lucilia_sericata_BFSA34	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,002	0,002		0,002	0,002	0,002	0,002	0,002	0,002
Lucilia_sericata_BFSA35	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000	0,000	0,002		0,000	0,000	0,000	0,000	0,000
Lucilia_sericata_BFSA36	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000	0,000	0,002	0,000		0,000	0,000	0,000	0,000
Lucilia_sericata_BFSA37	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000	0,000	0,002	0,000	0,000		0,000	0,000	0,000
Lucilia_sericata_BFSA38	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000	0,000	0,002	0,000	0,000	0,000		0,000	0,000
Lucilia_sericata_BFSA39	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000	0,000	0,002	0,000	0,000	0,000	0,000		0,000
Lucilia_sericata_BFSA40	32,364	32,405	32,405	31,771	32,405	32,405	32,364	0,000	0,000	0,002	0,000	0,000	0,000	0,000	0,000	

Table 4.4 Pairwise sequence divergence between the studied species (Ch. chloropyga, Ch. marginalis, Ch. albiceps and L. sericata) for ITS2. Nucleotide divergence in percentage is shown below the diagonal and standard error estimates are shown above

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 Chrysomya_chloropyga_BFSA09		0,011	0,011	0,008	0,012	0,006	0,003	0,007	0,005	0,005	1,324	1,485	1,451	1,324	1,324	1,451	1,324	1,324	1,376	1,413
2 Chrysomya_chloropyga_BFSA10	0,032		0,007	0,010	0,011	0,013	0,012	0,012	0,010	0,011	1,440	1,611	1,572	1,440	1,440	1,572	1,440	1,440	1,493	1,532
3 Chrysomya_chloropyga_BFSA01	0,032	0,014		0,013	0,014	0,012	0,011	0,013	0,012	0,012	1,284	1,436	1,401	1,284	1,284	1,401	1,284	1,284	1,324	1,368
4 Chrysomya_chloropyga_BFSA02	0,017	0,028	0,043		0,011	0,010	0,008	0,009	0,006	0,008	1,434	1,607	1,564	1,434	1,434	1,564	1,434	1,434	1,482	1,519
5 Chrysomya_chloropyga_BFSA03	0,035	0,032	0,043	0,032		0,011	0,011	0,012	0,010	0,010	1,602	1,801	1,725	1,602	1,602	1,725	1,602	1,630	1,793	1,674
6 Chrysomya_chloropyga_BFSA04	0,010	0,043	0,039	0,028	0,032		0,005	0,006	0,008	0,006	1,393	1,547	1,516	1,393	1,393	1,516	1,393	1,393	1,443	1,466
7 Chrysomya_chloropyga_BFSA05	0,003	0,035	0,032	0,021	0,032	0,007		0,006	0,006	0,003	1,324	1,485	1,451	1,324	1,324	1,451	1,324	1,324	1,376	1,413
8 Chrysomya_chloropyga_BFSA06	0,014	0,039	0,043	0,024	0,035	0,010	0,010		0,007	0,005	1,445	1,621	1,568	1,445	1,445	1,568	1,445	1,445	1,492	1,530
9 Chrysomya_chloropyga_BFSA07	0,007	0,024	0,039	0,010	0,028	0,017	0,010	0,014		0,005	1,421	1,601	1,546	1,421	1,421	1,546	1,421	1,421	1,472	1,508
10 Chrysomya_chloropyga_BFSA08	0,007	0,032	0,035	0,017	0,028	0,010	0,003	0,007	0,007		1,367	1,531	1,492	1,367	1,367	1,492	1,367	1,367	1,418	1,455
11 Chrysomya_albiceps_BFSA11	2,982	3,181	2,905	3,169	3,487	3,157	2,982	3,257	3,169	3,073		0,007	0,005	0,000	0,000	0,005	0,000	0,003	0,006	0,006
12 Chrysomya_albiceps_BFSA12	3,327	3,564	3,236	3,550	3,983	3,536	3,327	3,657	3,550	3,436	0,014		0,007	0,007	0,007	0,007	0,007	0,007	0,006	0,007
13 Chrysomya_albiceps_BFSA13	3,257	3,487	3,169	3,473	3,845	3,459	3,257	3,576	3,473	3,362	0,010	0,015		0,005	0,005	0,000	0,005	0,007	0,006	0,006
14 Chrysomya_albiceps_BFSA14	2,982	3,181	2,905	3,169	3,487	3,157	2,982	3,257	3,169	3,073	0,000	0,014	0,010		0,000	0,005	0,000	0,003	0,006	0,006
15 Chrysomya_albiceps_BFSA15	2,982	3,181	2,905	3,169	3,487	3,157	2,982	3,257	3,169	3,073	0,000	0,014	0,010	0,000		0,005	0,000	0,003	0,006	0,006
16 Chrysomya_albiceps_BFSA16	3,257	3,487	3,169	3,473	3,845	3,459	3,257	3,576	3,473	3,362	0,010	0,015	0,000	0,010	0,010		0,005	0,007	0,006	0,006
17 Chrysomya_albiceps_BFSA17	2,982	3,181	2,905	3,169	3,487	3,157	2,982	3,257	3,169	3,073	0,000	0,014	0,010	0,000	0,000	0,010		0,003	0,006	0,006
18 Chrysomya_albiceps_BFSA18	2,982	3,181	2,905	3,169	3,590	3,157	2,982	3,257	3,169	3,073	0,003	0,014	0,014	0,003	0,003	0,014	0,003		0,006	0,007
19 Chrysomya_albiceps_BFSA19	3,073	3,282	2,993	3,269	3,861	3,257	3,073	3,362	3,269	3,169	0,010	0,011	0,010	0,010	0,010	0,010	0,010	0,010		0,007
20 Chrysomya_albiceps_BFSA20	3,245	3,473	3,157	3,459	3,784	3,409	3,245	3,562	3,459	3,349	0,010	0,014	0,010	0,010	0,010	0,010	0,010	0,014	0,014	
21 Chrysomya_marginalis_BFSA21	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
22 Chrysomya_marginalis_BFSA22	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
23 Chrysomya_marginalis_BFSA23	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
24 Chrysomya_marginalis_BFSA24	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
25 Chrysomya_marginalis_BFSA25	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
26 Chrysomya_marginalis_BFSA26	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
27 Chrysomya_marginalis_BFSA27	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
28 Chrysomya_marginalis_BFSA28	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
29 Chrysomya_marginalis_BFSA29	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
30 Chrysomya_marginalis_BFSA30	5,679	5,901	5,276	6,409	6,009	6,092	5,679	6,328	6,131	5,901	3,163	3,355	3,464	3,163	3,163	3,464	3,163	3,163	3,441	3,451
31 Lucilia_sericata_BFSA31	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
32 Lucilia_sericata_BFSA32	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
33 Lucilia_sericata_BFSA33	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
34 Lucilia_sericata_BFSA34	7,986	8,466	8,061	8,302	8,859	7,900	7,986	8,087	8,518	8,251	8,576	8,852	8,576	8,576	8,576	8,576	8,576	8,799	8,302	8,302
35 Lucilia_sericata_BFSA35	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
36 Lucilia_sericata_BFSA36	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
37 Lucilia_sericata_BFSA37	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
38 Lucilia_sericata_BFSA38	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
39 Lucilia_sericata_BFSA39	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576
40 Lucilia_sericata_BFSA40	8,251	8,734	8,325	8,570	9,128	8,163	8,251	8,354	8,787	8,518	8,852	9,129	8,852	8,852	8,852	8,852	8,852	9,076	8,576	8,576

Table 4.4 Cont. Pairwise sequence divergence between the studied species (Ch. chloropyga, Ch. marginalis, Ch. albiceps and L. sericata) for ITS2. Nucleotide divergence in percentage is shown below the diagonal and standard error estimates are shown above

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1 Chrysomya_chloropyga_BFSA09	2,757	2,757	2,757	2,757	2,757	2,757	2,757	2,757	2,757	2,757	4,310	4,310	4,310	4,073	4,310	4,310	4,310	4,310	4,310	4,310
2 Chrysomya_chloropyga_BFSA10	2,982	2,982	2,982	2,982	2,982	2,982	2,982	2,982	2,982	2,982	4,836	4,836	4,836	4,636	4,836	4,836	4,836	4,836	4,836	4,836
3 Chrysomya_chloropyga_BFSA01	2,511	2,511	2,511	2,511	2,511	2,511	2,511	2,511	2,511	2,511	4,461	4,461	4,461	4,187	4,461	4,461	4,461	4,461	4,461	4,461
4 Chrysomya_chloropyga_BFSA02	3,282	3,282	3,282	3,282	3,282	3,282	3,282	3,282	3,282	3,282	4,617	4,617	4,617	4,422	4,617	4,617	4,617	4,617	4,617	4,617
5 Chrysomya_chloropyga_BFSA03	3,057	3,057	3,057	3,057	3,057	3,057	3,057	3,057	3,057	3,057	5,295	5,295	5,295	4,909	5,295	5,295	5,295	5,295	5,295	5,295
6 Chrysomya_chloropyga_BFSA04	3,037	3,037	3,037	3,037	3,037	3,037	3,037	3,037	3,037	3,037	4,249	4,249	4,249	4,058	4,249	4,249	4,249	4,249	4,249	4,249
7 Chrysomya_chloropyga_BFSA05	2,757	2,757	2,757	2,757	2,757	2,757	2,757	2,757	2,757	2,757	4,310	4,310	4,310	4,073	4,310	4,310	4,310	4,310	4,310	4,310
8 Chrysomya_chloropyga_BFSA06	3,250	3,250	3,250	3,250	3,250	3,250	3,250	3,250	3,250	3,250	4,516	4,516	4,516	4,329	4,516	4,516	4,516	4,516	4,516	4,516
9 Chrysomya_chloropyga_BFSA07	3,097	3,097	3,097	3,097	3,097	3,097	3,097	3,097	3,097	3,097	4,801	4,801	4,801	4,586	4,801	4,801	4,801	4,801	4,801	4,801
10 Chrysomya_chloropyga_BFSA08	2,937	2,937	2,937	2,937	2,937	2,937	2,937	2,937	2,937	2,937	4,584	4,584	4,584	4,336	4,584	4,584	4,584	4,584	4,584	4,584
11 Chrysomya_albiceps_BFSA11	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	5,111	5,111	5,111	4,842	5,111	5,111	5,111	5,111	5,111	5,111
12 Chrysomya_albiceps_BFSA12	1,216	1,216	1,216	1,216	1,216	1,216	1,216	1,216	1,216	1,216	5,321	5,321	5,321	5,106	5,321	5,321	5,321	5,321	5,321	5,321
13 Chrysomya_albiceps_BFSA13	1,277	1,277	1,277	1,277	1,277	1,277	1,277	1,277	1,277	1,277	5,072	5,072	5,072	4,839	5,072	5,072	5,072	5,072	5,072	5,072
14 Chrysomya_albiceps_BFSA14	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	5,111	5,111	5,111	4,842	5,111	5,111	5,111	5,111	5,111	5,111
15 Chrysomya_albiceps_BFSA15	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	5,111	5,111	5,111	4,842	5,111	5,111	5,111	5,111	5,111	5,111
16 Chrysomya_albiceps_BFSA16	1,277	1,277	1,277	1,277	1,277	1,277	1,277	1,277	1,277	1,277	5,072	5,072	5,072	4,839	5,072	5,072	5,072	5,072	5,072	5,072
17 Chrysomya_albiceps_BFSA17	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	5,111	5,111	5,111	4,842	5,111	5,111	5,111	5,111	5,111	5,111
18 Chrysomya_albiceps_BFSA18	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	1,140	5,251	5,251	5,251	4,979	5,251	5,251	5,251	5,251	5,251	5,251
19 Chrysomya_albiceps_BFSA19	1,276	1,276	1,276	1,276	1,276	1,276	1,276	1,276	1,276	1,276	4,802	4,802	4,802	4,542	4,802	4,802	4,802	4,802	4,802	4,802
20 Chrysomya_albiceps_BFSA20	1,241	1,241	1,241	1,241	1,241	1,241	1,241	1,241	1,241	1,241	4,931	4,931	4,931	4,606	4,931	4,931	4,931	4,931	4,931	4,931
21 Chrysomya_marginalis_BFSA21		0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
22 Chrysomya_marginalis_BFSA22	0,000		0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
23 Chrysomya_marginalis_BFSA23	0,000	0,000		0,000	0,000	0,000	0,000	0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
24 Chrysomya_marginalis_BFSA24	0,000	0,000	0,000		0,000	0,000	0,000	0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
25 Chrysomya_marginalis_BFSA25	0,000	0,000	0,000	0,000		0,000	0,000	0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
26 Chrysomya_marginalis_BFSA26	0,000	0,000	0,000	0,000	0,000		0,000	0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
27 Chrysomya_marginalis_BFSA27	0,000	0,000	0,000	0,000	0,000	0,000		0,000	0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
28 Chrysomya_marginalis_BFSA28	0,000	0,000	0,000	0,000	0,000	0,000	0,000		0,000	0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
29 Chrysomya_marginalis_BFSA29	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000		0,000	6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
30 Chrysomya_marginalis_BFSA30	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000		6,150	6,150	6,150	5,887	6,150	6,150	6,150	6,150	6,150	6,150
31 Lucilia_sericata_BFSA31	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964		0,000	0,000	0,003	0,000	0,000	0,000	0,000	0,000	0,000
32 Lucilia_sericata_BFSA32	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000		0,000	0,003	0,000	0,000	0,000	0,000	0,000	0,000
33 Lucilia_sericata_BFSA33	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000		0,003	0,000	0,000	0,000	0,000	0,000	0,000

Table 4.4 Cont. Pairwise sequence divergence between the studied species (Ch. chloropyga, Ch. marginalis, Ch. albiceps and L. sericata) for ITS2. Nucleotide divergence in percentage is shown below the diagonal and standard error estimates are shown above

34	Lucilia_sericata_BFSA34	9,693	9,693	9,693	9,693	9,693	9,693	9,693	9,693	9,693	9,693	0,003	0,003	0,003		0,003	0,003	0,003	0,003	0,003	0,003
35	Lucilia_sericata_BFSA35	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000	0,000	0,003		0,000	0,000	0,000	0,000	0,000
36	Lucilia_sericata_BFSA36	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000	0,000	0,003	0,000		0,000	0,000	0,000	0,000
37	Lucilia_sericata_BFSA37	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000	0,000	0,003	0,000	0,000		0,000	0,000	0,000
38	Lucilia_sericata_BFSA38	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000	0,000	0,003	0,000	0,000	0,000		0,000	0,000
39	Lucilia_sericata_BFSA39	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000	0,000	0,003	0,000	0,000	0,000	0,000		0,000
40	Lucilia_sericata_BFSA40	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	9,964	0,000	0,000	0,000	0,003	0,000	0,000	0,000	0,000	0,000	

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

14 October 2015

Ms TM Cooke
C/o Dr M Heyns
Division of Forensic Medicine
Level 5
Falmouth Building

Dear Ms Cooke

PROTOCOL TITLE: DNA BARCODING OF FORENSICALLY IMPORTANT FLIES IN THE WESTERN CAPE

FHS AEC REF NO: 015/039

Thank you for submitting your protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **authorised** your protocol, which will terminate on **30 October 2018**

Number of animals & species: 72 Blowflies

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the authorisation of this protocol imposes the following obligations on the (PI) principal investigator:

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **29 February 2016**. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **29 February 2016**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as authorised, or as amended.

4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).
5. Ensuring that you as the PI (principal investigator) immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
6. Ensuring that you as the PI (principal investigator) alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
7. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
8. If the principal investigator or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
9. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
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My best wishes for a successful research and /or teaching endeavour.

Yours sincerely

Signed

PROF PJ COMMERFORD
CHAIRPERSON, FHS ANIMAL ETHICS COMMITTEE

Appendix 4.10 – Guidelines to author

FORENSIC SCIENCE INTERNATIONAL

An international journal dedicated to the applications of medicine and science in the administration of justice.

AUTHOR

INFORMATION PACK

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ISSN: 0379-0738

DESCRIPTION

Forensic Science International publishes original contributions in the many different scientific disciplines pertaining to the forensic sciences. Fields include forensic pathology and histochemistry, chemistry, biochemistry and toxicology (including drugs, alcohol, etc.), biology (including the identification of hairs and fibres), serology, odontology, psychiatry, anthropology, the physical sciences, firearms, and document examination, as well as investigations of value to public health in its broadest sense, and the important marginal area where science and medicine interact with the law.

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Types of paper

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Forensic Anthropology Population Data

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Sexing

Aging sub adult skeletal remains

Aging adult skeletal remains

Aging living sub adults and adults

Determining ancestry

Stature estimation

Facial reconstruction

Non metric trait distribution, pathology and trauma

Positive identification of human skeletal remains

Positive identification of the living

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C. Cattaneo: Osteology, Anthropology and Odontology

Tel: +39 2 5031 5678

Fax: +39 2 5031 5724

E-mail: cristina.cattaneo@unimi.it

P. Margot: Questioned Documents and Physical Science: ballistics, tool marks, contact traces, drugs analysis, fingerprints and identification, etc.

Tel: +41 21 692 4605

Fax: +41 21 692 4605

E-mail: pierre.margot@unil.ch

O.H. Drummer: Toxicology

Tel: +61 3 9684 4334

Fax: +61 3 9682 7353

E-mail: olaf.drummer@vifm.org

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