

**DNA barcoding of forensically important blow flies (Diptera: Calliphoridae)
within the Western Cape of South Africa**

by

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SUBMITTED TO THE UNIVERSITY OF CAPE TOWN
In partial fulfilment of the requirements for the degree

MPhil (Biomedical Forensic Science)

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

August 2019

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Abstract:

In forensic entomology, determining species identity is a crucial step towards estimating post mortem interval. DNA barcoding can aid in the identification of unknown forensically relevant species, and this requires the comparison of DNA barcodes to reference data from known species. However, there is a lack of DNA barcode reference data of forensically relevant Calliphoridae species in the Western Cape (South Africa). DNA barcodes were generated for the *COI* and *ITS2* markers for 41 forensically relevant Calliphoridae specimens, representing seven species from six localities in the Western Cape: *Chrysomya albiceps* ($n = 3$), *Chrysomya chloropyga* ($n = 8$), *Chrysomya marginalis* ($n = 5$), *Chrysomya megacephala* ($n = 7$), *Hemipyrellia fernandica* ($n = 1$), *Lucilia cuprina* ($n = 8$) and *Lucilia sericata* ($n = 9$). This data was combined with that from Cooke *et al.* (2018) ($n = 40$), and subjected to rigorous statistical and phylogenetic analyses. Phylogenetic analysis which combined data for both *COI* and *ITS2* barcodes returned monophyletic clades for each species with increased support when compared to using each barcode individually. This combined dataset was able to discriminate between *L. cuprina* and *L. sericata* with full support (100% *pP*), which was not achieved previously. DNA barcodes were evaluated for intra- and inter-specific variance as well as haplotype patterning. No haplotype patterning was observed for either barcodes across sampled localities. Lastly, a single-blinded approach was used to assess the dataset, whereby DNA barcodes from 'unknown' specimens were correctly identified using this reference data. These identifications were more accurate than those using GenBank® or BOLD, highlighting the importance of using locally relevant reference data. This study has contributed new data pertaining to DNA barcodes for seven Calliphoridae species, which was previously scarce for the Western Cape, and this has directly contributed to an improvement in the accuracy of local species identification.

Acknowledgements

I would like to acknowledge the National Research Foundation (NRF) for providing me with a scholarship to pursue this research.

I would like to acknowledge the people at CapeNature and the South African National Parks (SANParks) for allowing me to collect samples on their premises.

I would like to acknowledge my supervisor, Laura Heathfield, and co-supervisor, Marise Heyns, for their supervision, support, criticism, patience and guidance.

I would like to acknowledge Gloudi Agenbag for her support with the Sanger Sequencing.

I would like to acknowledge my parents, Sieghard and Julia Kulenkampff, for their love and constant support throughout this endeavour.

I would like to acknowledge my friends and family for putting up with me throughout my research.

Finally, I wish to acknowledge God, who has been with me every step of this journey and without Whom this would have been impossible.

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List of Abbreviations

- AEC: Animal Ethics Committee
- AMOVA: Analysis of molecular variance
- ANP: Agulhas National Park
- BI: Bayesian Inference
- BIC: Bayesian information criterion
- BNP: Bontebok National Park
- BOLD: Barcoding of Life Data Systems
- COI: Cytochrome c oxidase 1
- Cytb: Cytochrome B
- DDBJ: DNA Data Bank of Japan
- DNA: Deoxyribose nucleic acid
- dNTP: Deoxyribonucleotide triphosphate
- EMBL: European Molecular Biology Laboratory
- Exo1: Exonuclease 1
- G: Gamma distribution
- GTR: General time reversible
- HHNR: Hottentots Holland Nature Reserve
- HKY: Hasegawa-Kishino-Yano
- ITS2: Ribosomal second internal transcribed spacer
- MegaBLAST: Nucleotide level Basic Local Alignment Search Tool
- ML: Maximum likelihood
- MRCPU: Medical Research Council Primate Unit
- NADH: Nicotinamide adenine dinucleotide
- NCBI: The National Center for Biotechnology Information
- ND4: Nicotinamide adenine dinucleotide dehydrogenase subunit 4
- PCR: Polymerase chain reaction
- PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism
- PMI: Post mortem interval
- RAPD: Random amplified polymorphic deoxyribose nucleic acid
- rDNA: Ribosomal deoxyribose nucleic acid
- RNA: Ribonucleic acid
- rRNA: Ribosomal Ribonucleic acid
- rSAP: Recombinant Shrimp Alkaline Phosphatase
- SANParks: South African National Parks
- SANS: South African National Standard
- WCNP: West Coast National Park
- WPNR: Wiesenhof Private Nature Reserve
- UCT: University of Cape Town
- UCTMC: University of Cape Town Medical Campus

Chapter 1: Introduction and Literature review

1.1 Introduction

1.1.1 Background

Forensic entomology is a branch of forensic science which uses information about the life stages and behaviour of insect infestations to aid in interpreting evidence within a legal context and can be used in both civil and criminal cases (Lord & Stevenson, 1986). Civil cases include cases where insects or arthropods infest stored produce or in urban contexts, where the insects affect humans or their surroundings. Criminal cases are medico-legal cases where insect infestation occurs on living humans or corpses, where foul play is suspected, or when a transgression of the law has occurred. When aiding in a medico-legal death investigation, the main role of a forensic entomologist is to assist in determining post-mortem interval (PMI) (Kashyap & Pillay, 1989; Benecke, 1998a) with respect to criminal, wildlife and veterinary practices (Anderson, 1999; Dalton & Kotze, 2011). However, forensic entomology is not limited to PMI determination, and can be applied to linking suspects to a crime scene (Webb *et al.*, 1983; Prichard *et al.*, 1986), corpse relocation and the duration of neglect in young (Benecke & Lessig, 2001) or the elderly (Byrd & Castner, 2002).

The determination of PMI has traditionally been done by examining the corpse itself. However, after 72 hours has passed the medical information presented by the corpse, such as livor mortis, rigor mortis and algor mortis from which to correlate PMI, becomes less informative (Henßge & Madea, 2004; Goff, 2009). Forensic entomology can provide a means by which to estimate PMI, especially after 72 hours, by evaluating the life cycle stages of the insects retrieved from the corpse, or from the succession of insects that infest the corpse. This can provide an estimation of PMI for a period of hours, weeks or years since the death of the individual when the general pathological features are no longer of use (Kashyap & Pillay, 1989; Benecke, 1998b; Amendt *et al.*, 2007).

Several methods exist from which forensic entomologists can estimate PMI. Sharma *et al.* (2015) critically reviewed over 20 methods of PMI estimation. When evaluating the limitations of these methods, it was evident that they all rely on accurate species identification and/or reference profiles for comparison purposes. This highlights the value of accurate species identification methods for use in forensic entomological practice. This is especially true in estimating PMI where the results have the potential to add value to a forensic case. For example, the estimation of the PMI results may aid a forensic pathologist to interpret results from ancillary tests (*e.g.* toxicological results) in context of how long the corpse has been decomposing (Amendt *et al.*, 2007; Sharma *et al.*, 2015).

This chapter will focus on explaining these concepts of identification of insects and the limitations with current insect identification techniques. It will also critically discuss the use of 'DNA barcoding' as a means to accurately identify species, with a focus on the family of blow flies Calliphoridae. This will serve as the necessary background and motivation for the current project, with the aims and objectives presented at the end of the chapter.

1.2. Decomposition of a corpse and insect colonisation

Insects are useful in forensic investigations because they infest a corpse in successional stages. As the corpse goes through autolysis, putrefaction and skeletonization, the microclimate that it presents, changes. The decomposition of the corpse is essentially aided by the insect infestations. As these changes in decomposition occur, different species with different ecological needs will make use of the corpse for feeding and breeding purposes. Although the decomposition of a corpse is a continuous process, for ease of convenience it can be divided into five stages: i) fresh, ii) bloated, iii) active decay, iv) post decay and v) skeletonisation as described by Goff (2009).

1.2.1 Insect infestation of a corpse

The insects that infest corpses can also be divided into five categories: i) necrophagous species, which feed on decomposing tissue; ii) necrophiles which are predators and parasites of the necrophagous species; iii) omnivorous species which feed on both the decomposing tissue, as well as, the necrophagous species, iv) adventive or opportunistic species that use the corpse as an extension of their environment (Smith, 1986; Goff, 2009) and v) accidental species that occur on the corpse by chance (Arnaldos *et al.*, 2005). Species that compose the first two categories are typically the most useful in forensic entomology (Joseph *et al.*, 2011), particularly the orders Diptera (flies) and Coleoptera (beetles) (Boehme *et al.*, 2012; Szelecz *et al.*, 2018), and are major contributors to the decomposition of the corpse from the fresh stage to post decay stage. However, this should not devalue the circumstantial evidential value that can be determined from the omnivorous, adventive or accidental species, especially as it is typically overlooked by entomologists (Goff, 2009).

The predominant species of Diptera that are associated with a decomposing corpse are from the families Calliphoridae (blow flies), Sarcophagidae (flesh flies) and Muscidae (house flies) which colonise a corpse in successional stages alongside other insect species (Joseph *et al.*, 2011). Dipteran species are more common in the earlier stages of decomposition, from the fresh to active decay stage, with Coleopterans becoming more predominant in the post decay stages. Insect activity subsides as the corpse skeletonises, though some species such as Coleopterans of the family Nitidulidae, can be present on the skeleton (Goff, 2009; Gennard, 2012).

The succession of species depends on the local fauna which will arrive at the corpse when the microclimate is appropriate for the species' needs. Therefore, it is important that the forensic entomologist knows which species are active in the scene microclimate. Incorrectly identifying a species can result in an incorrect estimation of PMI and in a medico-legal case, this would impair the informative value of other ancillary investigations.

1.2.2 Calliphoridae

As a corpse decomposes and undergoes autolysis, it releases molecules called apneumones which attract a host of insect species (LeBlanc & Logan, 2010). Of these insects, Calliphorid flies are some of the first insects that respond to these molecules and as a result are the first to colonise a corpse. Calliphorid flies use the corpse as a breeding site to oviposit their eggs on. It has been noted that female Calliphorid flies can delay oviposition for up to two weeks until suitable breeding sites are made available (Byrd & Castner, 2002).

Calliphoridae seek out and breed on carcasses of animals and bodies of deceased humans, preferring to oviposit their eggs in areas that are moist and offer protection (Gennard, 2012). Humidity levels play an important role in the dispersal capability of Calliphorid flies because their eggs are vulnerable to desiccation (Williams & Villet, 2006; Richards *et al.*, 2009; Williams *et al.*, 2016). Monthly rainfall is also a limiting factor in the dispersal capabilities of some Calliphorid fly species, with some species limiting flight during rain, while others become more prominent (Mahat *et al.*, 2009). This would temporarily delay, as well as, influence the insect successional infestation of a corpse and is therefore of great forensic importance in medico-legal cases.

Climatic changes can be of vital importance when interpreting information relating to medico-legal investigations as it can influence the PMI determination as well as post mortem corpse relocation. Blow flies are poikilothermic, meaning they are affected by the relative temperature of the surrounding environment (Fraenkel & Herford, 1940; Scholander *et al.*, 1953). Extreme temperature may also limit the dispersal capabilities of the blow flies (Williams & Villet, 2006; Richards *et al.*, 2009; Williams *et al.*, 2016), potentially preventing a species from invading an ecosystem.

1.2.3 Ecology of Calliphoridae

The Calliphoridae species have been documented in several geological regions including the Western Cape of South Africa, with a number of species of Calliphoridae that are of forensic interest (Lutz *et al.*, 2018) (Table 1.1). Species distributions change over time as species adapt, speciate and/or become extinct due to climatic changes affecting the local environment (Thomas *et al.*, 2004; Richards *et al.*, 2009). Due to the Cape Fold mountain ranges, the ecological conditions of the Western Cape are a mosaic of ecosystems with variation in temperature, monthly rainfall and humidity (Cowling *et al.*, 2009). Furthermore, the rainfall patterning of the system shifts from a winter rainfall in the southwest to a year-long or to a summer rainfall towards the Eastern Cape Province of South Africa (Cowling *et al.*, 2009). Local population extinctions due to changes in rainfall patterning could potentially result in the formation of fragmented populations of Calliphorid species within the province. This may lead to a limitation of gene flow between these local populations (Irwin, 2002). Therefore, distinct genetic haplotypes for the fragmented localities could occur.

Table 1.1: A list of Calliphoridae that are known to occur in the Western Cape.

Forensic relevant species are known to colonise corpses. Table is collated from Zumpt, 1956; Williams & Villet, 2006; Richards et al., 2009; Williams et al., 2014 and Lutz et al., 2018.

Genus	Species binomial name	Forensically relevant
<i>Calliphora</i>	<i>C. croceipalpis</i> (Jaenicke, 1867)	Yes
	<i>C. vicina</i> (Robineau-Desvoidy, 1830)	Yes
<i>Chrysomyiini</i>	<i>Ch. albiceps</i> (Wiedemann, 1819)	Yes
	<i>Ch. chloropyga</i> (Wiedemann, 1818)	Yes
	<i>Ch. inclinata</i> (Walker, 1861)	Yes
	<i>Ch. marginalis</i> (Fabricius, 1794)	Yes
	<i>Ch. megacephala</i> (Fabricius, 1794)	Yes
	<i>Ch. laxifrons</i> (Villeneuve, 1914)	Yes
	<i>Ch. putoria</i> (Wiedemann, 1830)	Yes
<i>Hemipyrellia</i>	<i>H. fernandica</i> (Macquart, 1855)	Yes
<i>Lucilia</i>	<i>L. cuprina</i> (Wiedemann, 1830)	Yes
	<i>L. sericata</i> (Meigen, 1826)	Yes
<i>Pachychoeromyia</i>	<i>P. praegrans</i> (Austen, 1911)	No
<i>Phumosi</i>	<i>Paratricylea nigroviolacea</i> (Villeneuve, 1916)	No
<i>Pericallimya</i>	<i>P. perlata</i> (Walker, 1860)	No
	<i>P. westermanni</i> (Wiedemann, 1819)	No
<i>Tricycla</i>	<i>T. bivittata</i> (Curran, 1927)	No

1.3. Species identification

1.3.1 Morphological Identification

Traditionally, identification of insect species is done microscopically, by using morphological characteristics with the aid of identification keys (Boehme *et al.*, 2012; Bunchu *et al.*, 2012). However, when using morphological characteristics to taxonomically identify species, the genetic variability and phenotypic plasticity in the characteristics that are used can lead to inaccuracies with the identification (Herbert *et al.*, 2003a). When non-heritable phenotypic characteristics are present in a taxonomic lineage, the taxonomic lineage would typically be revised (Will & Rubinof, 2004). However, this limits the number of available morphological markers and any identification keys that make use of this morphology would need to be revised and validated before they can be used in forensic investigations.

This limitation is noticeable in many different fly species, where it can be challenging, or near impossible, to distinguish between the juvenile stages (Song *et al.*, 2008; Boehme *et al.*, 2012; Meiklejohn *et al.*, 2012; GilArriortua *et al.*, 2014). To circumvent this, most juvenile specimens have to be incubated to their adult stages before an accurate identification can be made which has the added limitations of being both costly and time consuming (Bunchu *et al.*, 2012). For some congeneric species or recently diverged species, such as members of the genus *Lucilia*, morphological identification even in adult stages can be challenging, especially in species that have hybridised. Furthermore, rearing larvae is not always successful because the larvae can be damaged, perish or escape before reaching maturity, making morphological identification impossible (Amendt *et al.*, 2000; Williams & Villet, 2013, 2014). This is of particular importance in medico-legal investigations where the loss of a specimen can result in loss of evidence that could be informative to the ongoing investigation. Additionally, this method does not account for morphologically cryptic taxa, which may potentially have different developmental rates (Herbert *et al.*, 2003a; Boehme *et al.*, 2012; Meiklejohn *et al.*, 2012).

Morphological keys can only be available for certain life stages or even limited to the sex of the species, impeding their usefulness (Herbert *et al.*, 2003a). Herbert *et al.* (2003a) stated that the

use of morphological keys also requires a high level of expertise to correctly identify species, though this was never substantiated through any form of peer-reviewed research and may be based on the authors' own experiences (Will & Rubinof, 2004). As with all means of visual comparison, comparing various morphological markers are fundamentally subjective, and thus open to scrutiny if used as evidence in a medico-legal investigation. It was suggested that the availability of trained taxonomists willing to do routine identification for medico-legal investigation may be limited, considering that the amount of time that needs to be invested into the identification of a specimen and the stress of being an expert witness (Will & Rubinoff, 2004).

1.3.2 Alternative identification methods

Traditional morphological identification poses a dilemma when it comes to forensic entomology due to the need for accurate species identification when determining PMI. Therefore, new methods have and are still being developed to accurately and efficiently identify these species. Some of these methods of identification are through the use of deoxyribose nucleic acid (DNA). These methods are perhaps a more robust and impartial means of species identification as they are not influenced by phenotypic plasticity (Hebert *et al.*, 2003a). Thus, they may be used to distinguish between cryptic species and can accurately identify the insect species independent of life stages or sex, provided that the reference DNA sequences are available and accurate (Hebert *et al.*, 2003a; Hebert *et al.*, 2004; Hajibabaei *et al.*, 2007). DNA-based identification methods do not require the specimens to be raised to adult stages, to be alive or even whole. This is a notable benefit when one considers the space, time and monetary requirements it takes to routinely rear specimens.

DNA is also one of the few pieces of biological evidence that uses statistical data to support certainty of a conclusion and is thus a highly valuable form of evidence in criminal trials, even more so, if used in conjunction with morphological identification methods (Will & Rubinof, 2004). Finally, molecular methods can be automated, especially for routine identification in medico-legal cases, allowing these identifications to be completed at a quicker pace than through traditional morphological methods.

A limiting factor when using DNA methods for identification is that, in most cases, the voucher specimen will be damaged or destroyed because part of the tissue of the specimen is required for DNA extraction. Retaining vouchers is an important consideration in medico-legal investigations. Vouchers offer the opportunity for the results obtained, for instance DNA reference sequences, to be repeated and reviewed, should the accuracy of the reference be brought into question. The source of the DNA sequence must be known before it can be trusted and if the validity of the sample is brought into question, the voucher can be independently analysed to confirm identity (Wells & Stevens, 2008).

Typical vouchers either include the whole preserved specimen, parts of the specimen or the DNA of the specimen. The retention of a tissue voucher may not pose an issue with some of the larger insect species, especially when a few dissected legs are sufficient for DNA analysis. However, the whole specimen of smaller insects may be required to yield sufficient DNA. Therefore, the only morphological evidence that will be retained will be any photographs taken and a DNA voucher.

Notable genetic techniques that have been previously explored for species identification include polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) (Rugman-Jones *et al.*, 2009; Jenkins *et al.*, 2012), random amplified polymorphic DNA (RAPD) (Benecke, 1998b), as well as analyses of the DNA for specific enzymes such as the reduced form of nicotinamide adenine dinucleotide (NADH), ribonucleic acid (RNA) (Wells & Stevens, 2008) and DNA barcoding. However, each of these methods comes with its own limitations. The DNA of the insect in question needs to be compared to reference DNA from a member of the same species for identification. These require reference 'libraries' to be purpose generated using the molecular technique, which can be costly to maintain. Furthermore, reference sequences may not be available for all species in some localities or may not be open-access.

1.4 DNA Barcoding

DNA barcoding is a taxonomic method that uses short genetic markers within the organism's DNA to differentiate it from other species and to identify it to the species level (Hajibabaei *et al.*, 2007; Jordaens *et al.*, 2013). As such, a DNA barcode must be sufficiently different between species, yet be conserved within species (Hebert *et al.*, 2003a).

Protein coding genes are often considered because they are highly conserved due to their functional requirements for life (Hebert *et al.*, 2003a). If a protein that is vital to cellular function is mutated and cannot fulfil its function, the organism with that mutated protein would not survive to reproductive age and the mutation would not establish in the species population. However, by focusing on the third nucleotide position of a codon it is possible to see variation due to it being less functionally constrained (Hebert *et al.*, 2003a). Therefore, every third nucleotide position offers a point of comparison. As such, a string of 45 base pairs will offer 15 points of comparison, effectively offering over a billion identification labels (Hebert *et al.*, 2003a). However, these protein coding genes must be flanked by conserved DNA sequences in order to amplify the target region across different species with universal polymerase chain reaction (PCR) primers. DNA barcoding, therefore, entails the amplification of target region(s) with universal primers, the sequencing of the region and the subsequent comparison of the resulting DNA sequence(s) to a database containing reference sequences from known species. In such a way, the unknown species can be identified through a matching principle (Ratnasingham & Hebert, 2007; Sonet *et al.*, 2013).

1.4.1 Cytochrome c oxidase 1

Cytochrome c oxidase 1 (COI) is a mitochondrial DNA gene and has been shown to be useful in species diagnosis in many different animal phyla (Hebert *et al.*, 2003a, 2003b). The *COI* marker has three advantages: The first being that the universal primers have shown to be very robust, which allows the 5' end of a gene to be recovered from the majority of phyla. The second advantage is that *COI* shows a high incidence of base substitution when compared with other

protein coding genes which creates a greater range of phylogenetic signal. This allows for phylogenetic discrimination not only between species but potentially also between haplotypes of a single species. It is used in many taxonomic studies, including Calliphoridae, from many locations in the world (Wells & Williams, 2005; Boehme *et al.*, 2012; Jordaens *et al.*, 2013). Finally, *COI* has a high copy number within cells, considering that it is present in the mitochondrial genome, adding an additional benefit when working with low copy DNA, degraded or poor yield DNA samples. However, GilArriortua *et al.* (2015), evaluated the use of several molecular loci including *COI* 5'-end. They determined that the *COI* 5'-end was unable to resolve genetic lineages for some of the more closely related species such as species of the genus *Lucilia*. Sonet *et al.*, (2013) showed that *COI* was unable to distinguish between recently diverged *Lucillia caeser* and *Lucilia illustris*. This may be due to initial morphological misidentification of the specimens within the reference library, which can be problematic on its own, or, due to inconsistencies created by hybridisation and incomplete lineage sorting. In alignment with this Hebert *et al.* (2004) demonstrated that *COI* may potentially be unsuitable for distinguishing between recently diverged species, promoting the necessity of secondary assays, such as the internal transcribed spacer regions.

1.4.2 Ribosomal second internal transcribed spacer

The ribosomal *second internal transcribed spacer* (*ITS2*) is a non-coding region of DNA that is situated in the ribosomal DNA (rDNA) cluster (Song *et al.*, 2008). It has been shown to be beneficial in reconstructing evolutionary relationships at a species level and has also been used in taxonomic studies involving blow fly species (Young & Coleman, 2004; Song *et al.*, 2008). A study by Nelson *et al.* (2008) was successful in distinguishing between *Chrysomya* species using *ITS2*. Song *et al.* (2008), however, noted that *ITS2* was unable to distinguish between some congeneric species, making *ITS2* insufficient for use in analysis relationships between closely related species, cryptic species, as well as, phylogeographically distinct populations. Therefore, it should be used in conjunction with another locus. In contrast to this, GilArriortua *et al.* (2014) noted that *ITS2* potentially had greater discriminatory power than that of *COI*. *ITS2* has a

significant number of informative sites that make it potentially well suited for discriminating between closely related species, and this was corroborated by Cooke *et al.* (2018). Furthermore, *ITS2* is useful for working with potentially degraded samples considering the short length of the amplified sequence.

1.4.3 DNA barcoding in literature

Similar studies to this research study have proved to be successful in other parts of the world including studies done in Germany (Reibe *et al.*, 2009), China (Song *et al.*, 2008), India (Priya Bhaskaran & Sebastian, 2015), Portugal (Oliveira *et al.*, 2011), Taiwan (Chen *et al.*, 2004) as well as in North America and Western Europe (Jordaens *et al.*, 2013). A study by Harvey *et al.* (2003b) successfully evaluated the use of molecular techniques to identify Diptera species in other localities of South Africa, namely Pretoria, KwaZulu-Natal and the Karoo. Furthermore, a study by Cooke *et al.* (2018), demonstrated the potential value of DNA barcoding of forensically relevant blow flies in the Western Cape. In their study, DNA barcodes were successfully generated for four forensically important species. While this was the first contribution of local data, the study was limited to four species and focused on species collected at a single locality. Therefore, the barcodes generated are not representative of the entire blow fly population of the Western Cape of South Africa.

1.5 Genomic databases

As stated previously, in order to utilise DNA barcoding a database of DNA reference sequences is needed. Several genomic databases have been established to date: The National Center for Biotechnology Information's (NCBI) GenBank® ("GenBank® Overview", 2017), the European Molecular Biology Laboratory (EMBL) ("European Molecular Biology Laboratory", 2018), the DNA Data Bank of Japan (DDBJ) ("DNA Data Bank of Japan", 2018) and the Barcoding of Life Database systems (BOLD) ("BOLDSYSTEMS", 2018). These databases function as public libraries of reference sequences. With the availability of these public libraries, methods of molecular

identification such as DNA barcoding become more feasible. Databases such as these rely on submissions from independent researchers.

BOLD is an informatics system which aims to be a single universal system, acquisition, storage, analysis and publication of DNA barcode records with the goal of identifying all eukaryotic organisms at a species level through using DNA barcoding (Kress & Erikson, 2012). The platform also has several requirements for storage of DNA barcodes for species including i) the species name, ii) the voucher data and the associated institution storage information, iii) collection record, iv) the database identifier of the specimen, v) *COI* DNA sequence that is at least 500 bp in length, vi) the PCR primers used and vii) the trace files (Ratnasingham & Hebert, 2007; 2013). As a result BOLD has recently become a prominent database with respect to molecular barcoding (Kress & Erikson, 2012).

A platform such as BOLD is potentially useful to forensic entomologists as it can provide an accurate, efficient and reliable means through which to access reference sequences for comparison in forensic casework. Having voucher specimens and trace files for the DNA barcodes allows for validation of the DNA barcodes and the reference specimen, should the identity of the specimen the barcode was generated from, be brought into question. Furthermore, BOLD collaborates with other databases such GenBank® to further supplement their expanding database.

However, a study by Sonet *et al.* (2013) noted three potential limitations with using databases such as BOLD. The first being that the databases reference sequences may be incomplete, with pertinent species reference sequences not being available. Secondly, the species listed for the reference sequences may be misidentified. Finally, the database may be unable to distinguish between recently diverged or closely related species. This motivates for the need to generate reference samples for all forensically relevant species from different localities. Database platforms like BOLD and GenBank® automatically detected gross errors, such as stop codons in protein coding genes. However, these databases rely on individual researchers to ensure that other sequencing errors or species misidentification are avoided. Errors in reference data inadvertently lead to errors when a questioned sequence is searched against the database.

Hence, submitted reference sequences must be subjected to high levels of quality control and scrutiny. Taxonomic identification by expert taxonomists and the retaining of voucher information or specimens for independent analysis, even standardised photographs, can establish trust in the reference material. However, this information is usually restricted to the information that is published in journal articles and this is often limited (Wells & Stevens, 2008).

1.6. Rationale

DNA barcoding of Calliphoridae flies can be a valuable tool for forensic scientists because it addresses some of the main limitations of traditional morphological methodology. However, DNA barcode reference data of forensically relevant blow fly species is depauperate. The genetic structure of blow flies in the Western Cape has not yet been evaluated in sufficient detail and with large enough sample sizes for use in routine forensic investigation. As such, further research is required to build upon this foundation to add statistical power to the reference library of blow fly sequences. Whilst Cooke *et al.* (2018) did generate DNA for four of the forensically relevant Calliphoridae species within the Western Cape, the study was focused on one locality only. A study design such as this does not account for collection bias. Haplotypes from a single locality is not enough to be an accurate representation of the haplotypes for the province as a whole. Furthermore, not every species will be available at each locality. In order to have a better representation of forensically relevant species of Calliphoridae within the Western Cape, additional localities need to be sampled and reference sequences generated for local species that have yet to have DNA barcodes generated for them.

1.7. Aims

The aim of this research project was to contribute towards a database of reference DNA sequences of blow flies from different localities within the Western Cape to aid the genetic identification of necrophagous blow fly species for medico-legal investigation purposes.

1.7.1 Objectives

The objectives of this study include:

- Collection of blow fly adults from different localities in the Western Cape.
- Taxonomic identification of the blow fly species using established morphological keys.
- Extraction of DNA and sequencing of barcodes from these taxonomically identified blow fly adults.
- Assess the discriminatory value of the reference library by performing phylogenetic analyses, uncorrected pairwise distance analyses, analysis of molecular variance and haplotype patterning.
- Assess the discriminatory value of the reference library by performing DNA barcoding on a blinded selection of specimens and attempting to identify their species.

Chapter 2: Methods

2.1 Specimen collection

Specimens of Calliphoridae were collected using modified Redtop Flycatchers® fly traps (Redtop, Johannesburg) baited with Redtop Flycatchers® fly bait (Redtop, Johannesburg) as described by Williams *et al.* (2014) (Appendix 2.1). These traps were set out at six localities throughout the Western Cape of South Africa: University of Cape Town Health Sciences campus (Observatory), Wiesenhof Private Nature Reserve, Hottentots Holland Nature Reserve, West Coast National Park, Agulhas National Park, and the Bontebok Nature Reserve. This was done to limit the collection bias in the resultant haplotypes for the species as well as to collect different forensically relevant Calliphoridae species that may not be present at a single locality.

A total of 153 flies were collected from the six localities. In order to reduce the number of potentially related individuals, samples were divided into groups based on the species, sample site, day of capture and the location of the trap. A maximum of two flies were selected from each grouping. Appendix 2.2 contains a flow diagram for the selection procedure. Members of *C. croceipalpis* were also captured but were excluded from the barcode generation due to non-specific primer binding for the COI barcode. Additionally, species were not included if they could not be accurately identified using traditional morphological methods. Overall, a total of 41 flies from seven different species were selected for barcode generation. In addition, 40 reference samples from Cooke *et al.* (2018) were included in the analysis section of this minor dissertation, bolstering the analysed samples to 81 samples. Figure 2.1 shows a map of the sampled localities, which species were gathered from for the generated barcodes and the additional reference sequences from Cooke *et al.* (2018) (Sample Localities Map, 2019). Appendix 2.3 contains detailed collection information for each sample.



Figure 2.1: Map of the Western Cape of South Africa showing the sampled localities included in this study.

The coloured gems represent species that were sampled at the respective locality. Localities with a blue center were sampled as part of this study. The locality with the red center was included from Cooke et al. (2018). Localities from left to right are: West Coast National Park in Langebaan; University of Cape Town Medical Campus in Observatory, Cape Town; Medical Research Council Primate Unit premises in Delft, Cape Town; Wiesenhof Private Nature Reserve in Stellenbosch; Hottentots Holland Nature Reserve in Somerset West; Agulhas National Park in Agulhas and Bontebok National Park in Swellendam.

Adult flies were euthanised by gassing with ethyl acetate (Byrd & Castner, 2002; Szelecz *et al.*, 2018). The euthanised specimens were stored in 75 % ethanol at 4 °C to prevent decomposition before analysis (Reibe *et al.*, 2009). This method of euthanasiation and storage is according to previous studies and was commensurate with ethical standards (Byrd and Castner, 2002; Adams and Hall, 200; Reibe *et al.*, 2009; Szelecz *et al.*, 2018) and local forensic entomological practice. This study received ethical approval from the University of Cape Town (UCT) Faculty of Health Sciences Animal Ethics Committee (Reference number: AEC: 015/039) (Appendix 2.4). Collection permits were obtained from CapeNature (Permit number: CN44-59-5015) (Appendix 2.5) and from the South African National Parks (SANParks) (Permit number: CRC/2018-2019/005—2018/v1) (Appendix 2.6).

2.2 Morphological identification

The adult flies were identified under a SteREO Discovery V20.0 stereomicroscope (ZEISS, Oberkochen) with an Axiocam 503 microscope camera (ZEISS, Oberkochen) with the ZEISS ZEN Imaging Software (ZEISS, Oberkochen) using morphological identification keys according to previously published methods (Lutz *et al.*, 2018; Szpila, 2009). Each specimen was photographed from the dorsal, lateral and ventral positions according to the guidelines set out by the BOLD system (Milton *et al.*, 2013).

2.3 Molecular identification

2.3.1 Sample preparation optimisation

Previously, Cooke *et al.* (2018) optimised the sample preparation procedure by grinding the whole specimen using the TissueLyser LT (Qiagen, Hilden). In this study, the TissueLyser II (Qiagen, Hilden) was used which allowed for oscillation at higher speeds; this prompted an assessment whether less input sample could be utilised for DNA extraction so that the majority of the specimen could be retained as a voucher sample.

To this end, six additional specimens of *L. cuprina* were selected. These specimens were not included into the 41 generated barcodes. The specimens were dissected creating batches that used either two, four or six legs or the flight muscle as the tissue used for the DNA extraction. The use of four legs from each sample was the preferable method, with the addition of more legs or the flight muscles not showing a marked improvement (Appendix 2.7). This also allowed the retention of two legs to be retained as part of the voucher. Additionally, the dissection of the flight muscle resulted in damage to the carapace of the voucher, which was seen to be a disadvantage.

For the rest of the specimens, samples were removed from the ethanol, allowed to dry, and four legs for each specimen were dissected from the abdomen. The remainder of the specimen was retained as a voucher specimen and stored in 75 % ethanol at 4 °C.

2.3.2 DNA extraction and quantification

DNA was extracted from the four legs of each of the adult flies using the Quick-DNA™ Tissue/Insect Miniprep kit (Zymo research, California), following the manufacturer's instructions with deviations (Appendix 2.8). The deviations included increasing the incubation time for the Lysis Solution (Zymo research, California) to 10 minutes and the Genomic Lysis Buffer (Zymo

research, California) to 1 hour. An additional wash step, using 98 % ethanol, was included. DNA was eluted into 35 µl of elution buffer and then stored at for 4 °C further analysis.

DNA concentration was quantified using the Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts). The purity ratios determined spectrophotometrically were also recorded.

2.3.3 Amplification

The barcodes within *COI* and *ITS2* were amplified using PCR as described by Cooke *et al.* (2018). However, instead of the KAPA HiFi HotStart ReadyMix, GoTaq 2X ReadyMix (Promega, Wisconsin) were used. Hence, the cycling conditions were adjusted as follows: Initial denaturation was 95 °C for 5 mins; 30 cycles of denaturation at 95 °C for 30 sec, annealing at 45 °C for the Lep and Folmer primers and 50 °C for the *ITS2* primers for 30 sec, elongation at 72 °C for 30 sec; and a final extension time of 72 °C for 5 mins.

The *COI* barcode was amplified initially using Folmer primers - LCO1490 and HCO2198 (Folmer *et al.*, 1994). Previously, Cooke *et al.* (2018) showed that the Folmer primers for *COI* were not optimal for all species encountered in the Western Cape, therefore, a second set of primers was incorporated: Lep primers - LepF1 and LepF2 (Herbert *et al.*, 2004). The *ITS2* barcode was amplified with *ITS2* primers - ITS2_F and ITS2_R (Song *et al.*, 2008) (Appendix 2.9).

In this study, amplification using the Folmer and Lep primers was successful at amplifying some samples from some species: *Ch. marginalis*, *L. cuprina* and *L. sericata* (Folmer primers) and *Ch. albiceps*, *Ch. chloropyga*, *Ch. megacephala* and *Hemphyrellia fernandica* (Lep primers). However, the amplification of some of the samples remained unsuccessful for the species of *Ch. albiceps*, *Ch. marginalis*, *Ch megacephala*, *H. fernandica*, *L. cuprina* and *L. sericata*. Therefore, an additional set of primers, the Marinho primers, TW-J-1289 and C1-N2320 (Marinho *et al.*, 2012) (Appendix 2.9) were included with the addition of 1 µl of 25 mM MgCl₂. For the Marinho primers, initial denaturation was 95 °C for 5 mins. Then the samples were subjected to 35 cycles of

denaturation at 95 °C for 1 min then annealing at 42.7 °C for 1 min followed by elongation at 72 °C for 1 min. Afterwards, the final extension was 72 °C for 10 mins.

Following extensive optimisation (Appendix 2.10), the Marinho primers were sufficient to amplify most problematic samples. Furthermore, if faint bands were present in the electropherograms as a result of low template DNA in the PCR reaction, the sample was reamplified in a secondary PCR, whereby the PCR product from the first PCR substituted the template DNA.

Following thermal cycling, amplification was verified using agarose gel electrophoresis. Post-PCR products underwent electrophoresis in a 1 % (w/v) agarose gel. A Quick-Load® Purple 50 bp DNA Ladder (New England BioLabs, Massachusetts) was used for estimation of amplicon size. SYBR™ safe gel stain (5 µl / 50 ml gel) (Invitrogen, Carlsbad) was used to visualise the DNA. DNA visualisation and image acquisition were accomplished using a Chemi Genius Bio Imaging Transluminator Gel documenter (Syngene, Cambridge) and the GeneSnap Image Acquisition software vs. 7.12.06 (Syngene, Cambridge).

2.3.4 Sanger sequencing

Post-PCR products were then subjected to a clean-up using *recombinant Shrimp Alkaline Phosphatase* (rSAP) (New England Biolabs, Ipswich) and *exonuclease 1* (Exo 1) (New England Biolabs, Ipswich) to remove unincorporated dNTPs and primers. The cleaned products underwent Sanger sequencing using the BigDye Terminator Sequencing kit (Applied Biosystems, California) and separated using capillary electrophoresis on the ABI 3130xl genetic analyser (Applied Biosystems, California) following the internal standard operating procedure. Data were then captured using the GeneMapper v3.1 software (Thermo Fisher Scientific, Massachusetts) and electropherograms were viewed using Chromas (Technelysium Ltd, South Brisbane) to assess the quality of the reads and to identify anomalies or sequencing artefacts.

2.3.5 Identification validation

All sequences were then searched against the BOLD platform using the option "Species Level Barcode Records". GenBank's® Basic Local Alignment Search Tool (MegaBLAST) was then used to confirm the identification as well as to test for the presence of pseudogenes.

2.3.6 Data and statistical analysis

The obtained DNA sequences were aligned in Bioedit (Hall, 2011) using ClustalW (Larkin *et al.*, 2007) alongside the 40 reference sequences generated by Cooke *et al.* (2018) and the *COI* and *ITS2* sequences of a *Stomoxys calcitrans* specimen was used as an outgroup (Dsouli *et al.*, 2011). The data were grouped according to species identity. The uncorrected pairwise distance was calculated within groups (intraspecific) and between groups (interspecific) using Mega X (Kumar, 2018). Samples were considered separate species when the uncorrected interspecific distance was ten times that of either intraspecific distance, otherwise referred to as the 10x criterion (Hebert *et al.*, 2003b).

Mega X was used to perform maximum likelihood (ML) analyses for each gene as well as to determine the substitution rate for each analysis. The ML trees were run using the kimura 2 parameter model (Kimura, 1980). The phylogeny established was tested using a bootstrap method with a 1000 bootstrap replicates. Nodes were considered well supported with a bootstrap percentage of $\geq 75\%$ (Hillis & Bull, 1993).

For the Bayesian inference (BI) analyses, separate analyses were run for both markers as well as a combined analysis using MrBayes v3.2.6 (Ronquist *et al.*, 2012; Huelsenbeck *et al.*, 2016). The most optimal substitution models that were recognised by MrBayes were used. DNA substitution models were determined in Mega X using the Bayesian information criterion (BIC). Three hot chains and one cold chain were used. The analysis was run for the number of generations required for the deviation of split frequencies to be below 0.01. Trees were sampled every 100

generations. Burn-in was set to 25 %. Nodes were considered well supported if the posterior probability (pP) was $\geq 95\%$ (Yang & Rannala, 2005).

Haplotype distributions and frequencies were constructed using ARLEQUIN v3.5.2.2 (Excoffier and Lischer, 2010) with a 95 % connection limit. Samples were considered separate groups if there was no shared haplotypes for both markers between groupings of localities.

An analysis of molecular variance (AMOVA) was done using ARLEQUIN and groupings were analysed for nucleotide diversity, mean pairwise difference and number of polymorphic sites within each group.

2.4 Testing the reference library

The generated reference library was tested by using a blinded selection of adults from the collected specimens. Selection and the dissections were done by an independent researcher. The morphological species identity of the blinded selection of flies was recorded. DNA barcodes were generated using the previously established methods. The samples were subjected to BI analysis alongside the reference dataset for *COI*, *ITS2* and the combined *COI* and *ITS2* dataset. Identification was considered successful if the blinded selection of samples clustered within the clades of the species that corresponded with their morphological species identity. Furthermore, the blinded selection of samples was searched against the BOLD platform using the option "Species Level Barcode Records", as well as, GenBank's® MegaBLAST platform. Species identity and supporting values were recorded. The identification success rate of the reference dataset was then compared to the success rate of each platform.

2.5 Uploads

The generated DNA barcodes alongside the sequences trace files, collection information and Z-axial images of the dorsal, ventral and lateral positions of each specimen (Photographic vouchers) were uploaded onto BOLD. Each specimen profile was assigned a unique identifier, called a Process ID. For this study the Process ID's for the generated samples range from BFSAB001-19 to BFSAB041-19. See Appendix 2.3 for which specimen associates with which Process ID.

Chapter 3: Results

3.1 Morphological identification

Each of the included samples were identified using available morphological taxonomic keys. Figure 3.1 include Z axial stacked images of the dorsal, lateral and ventral sections of each of the *Lucilia* species collected in the study, Additional Z axial stacked photos for the remainder of the included species are provided in Appendix 3.1. The morphological features presented by the specimens for *Ch. albiceps*, *Ch. megacephala* and *Ch. chloropyga* were congruent with the morphological features described in the taxonomic keys. Deviations from the taxonomic keys were seen in both species of *Lucilia*, *L. cuprina* and *L. sericata*, when species identity was confirmed using molecular means. Samples of *Ch. marginalis* presented with a darkened fronto-orbital plate indicative of *Ch. laxifrons*. Sampled species showed variation in the colouration of the carapace. Additionally, Appendix 3.2 displays the morphological variation in colouration of the carapace for *L. cuprina*.

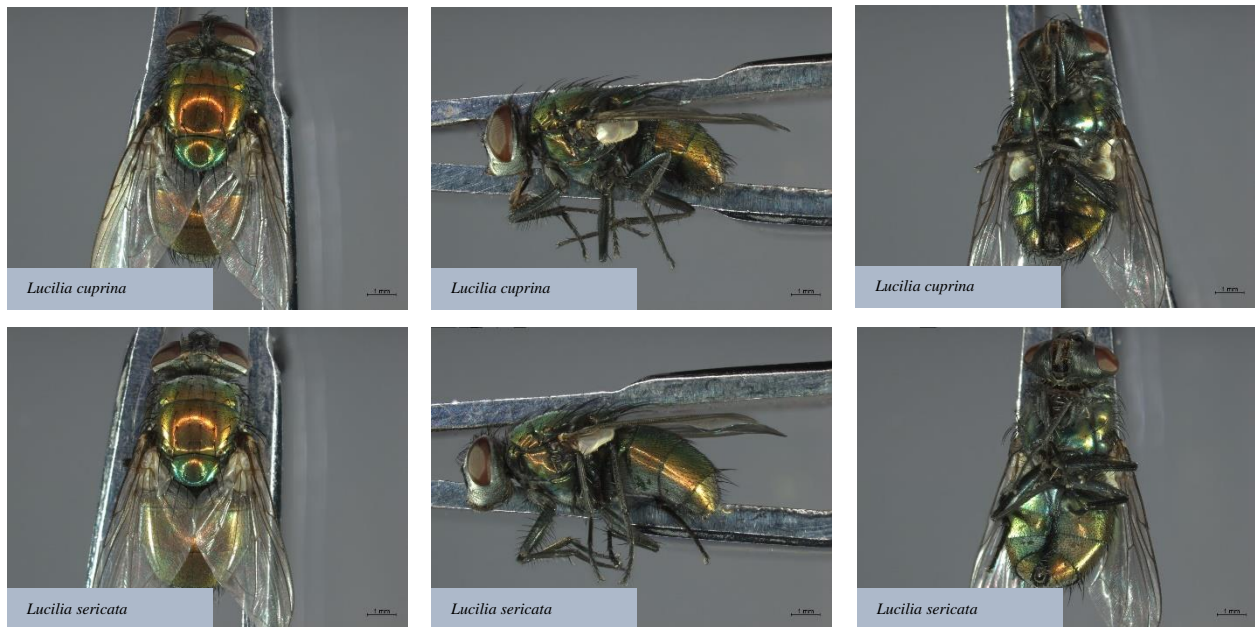


Figure 3.1: Z axial stacked images of the dorsal, lateral and ventral positions of *Lucilia cuprina* and *Lucilia sericata*.

3.2 Sample analysis

DNA was successfully extracted and quantified for all 41 newly collected samples. The mean weight of the tissue used for DNA extraction was $1.0 \text{ mg} \pm 5.8 \text{ mg}$. The mean DNA concentration was $9.6 \pm 3.0 \text{ ng}/\mu\text{L}$. Mean absorbance ratio for 260/280 was 1.63 ± 0.14 and for 260/230 was 0.22 ± 0.04 units (Appendix 3.3).

The 5' *COI* barcode and *ITS2* region was successfully amplified for each sample. Figure 3.2 displays a representative example of successful amplification from a cohort of samples for all the respective primers.

3.3 Phylogenetic trees

3.3.1 Maximum likelihood

For the ML the substitution rates determined were Gamma distributed (G) with the number of discreet Gamma categories being set to 5. For *COI* each species of *Ch. albiceps*, *Ch. chloropyga*, *Ch. marginalis* and *Ch. megacephala* formed distinct monophyletic clades (Figure 3.3). The species *L. sericata* and *L. cuprina* formed a paraphyletic clade, with several samples of *L. cuprina* grouping with the samples of *L. sericata*. The phylogeny developed for the *ITS2* tree (Figure 3.4) showed all species formed distinct monophyletic clades. Bootstrapping support for the *COI* clades was poor (< 75 %) apart from the clade for *Ch. marginalis* (97 %). Support for the *ITS2* clades were poor (< 75 %). The only clade that received sufficient support was that of *Ch. megacephala* (82 %).

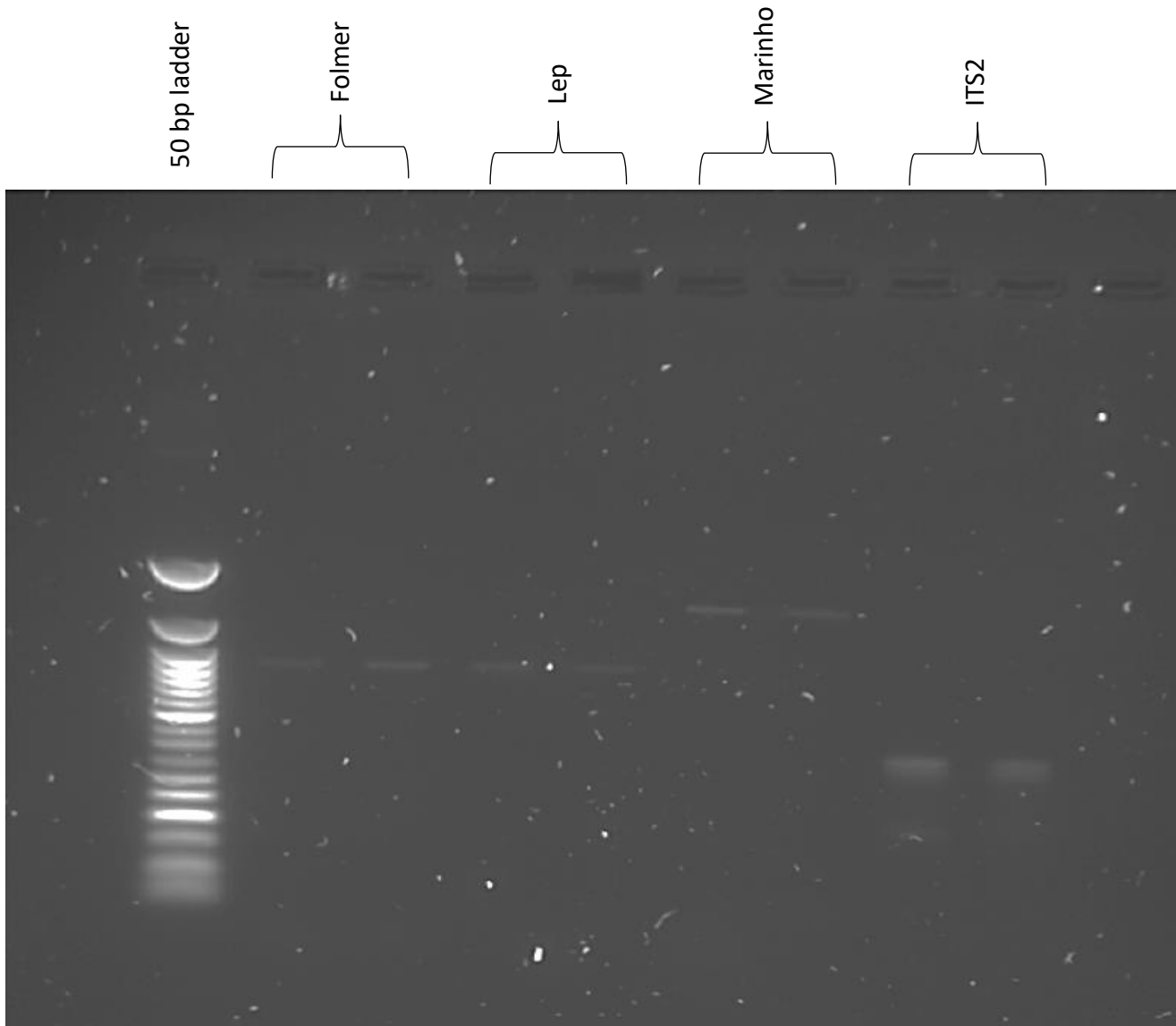


Figure 3.2: Agarose gel showing successful amplifications of DNA using all respective primers included in the study.

From left to right the primers are: the Folmer primers - LCO1490 and HCO2198 (Folmer et al., 1994), the Lep primers - LepF1 and LepF2 (Herbert et al., 2004), the Marinho primers, TW-J-1289 and C1-N2320 (Marinho et al., 2012) and the ITS2 primers - ITS2_F and ITS2_R (Song et al., 2008). A Quick-Load® Purple 50 bp DNA Ladder (New England BioLabs, Massachusetts) was used for estimation of amplicon size. DNA was visualized with a SYBR™ safe gel stain (5 µl / 50 ml gel) (Invitrogen, Carlsbad), using a Chemi Genius Bio Imaging Transluminator Gel documenter (Syngene, Cambridge) using the GeneSnap Image Acquisition software vs. 7.12.06 (Syngene, Cambridge).

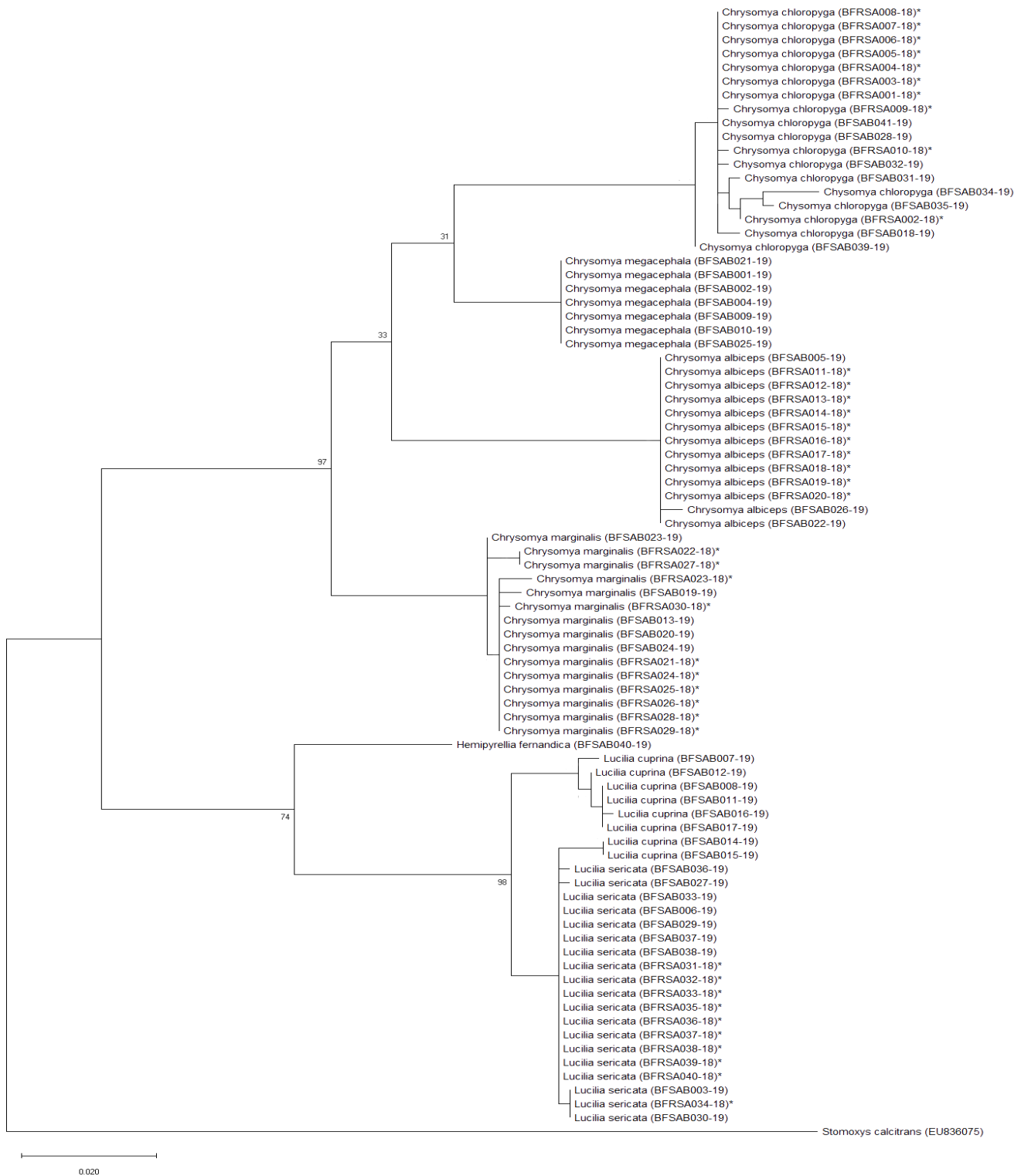


Figure 3.3: Maximum likelihood topology for the 5' section of cytochrome c oxidase 1 gene for seven blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala*, *Hemipyrellia fernandica*, *Lucilia cuprina* and *Lucilia sericata*).

Values on each node refer to bootstrap values shown as a percentage for 1000 replicates. Branches with an * were included from Cooke et al., 2018. The respective BOLD process ID's are listed in parenthesis after each sample. *Stomoxys calcitrans* was included as an outgroup, with the GenBank® accession numbers in parenthesis (Dsouli et al., 2011).

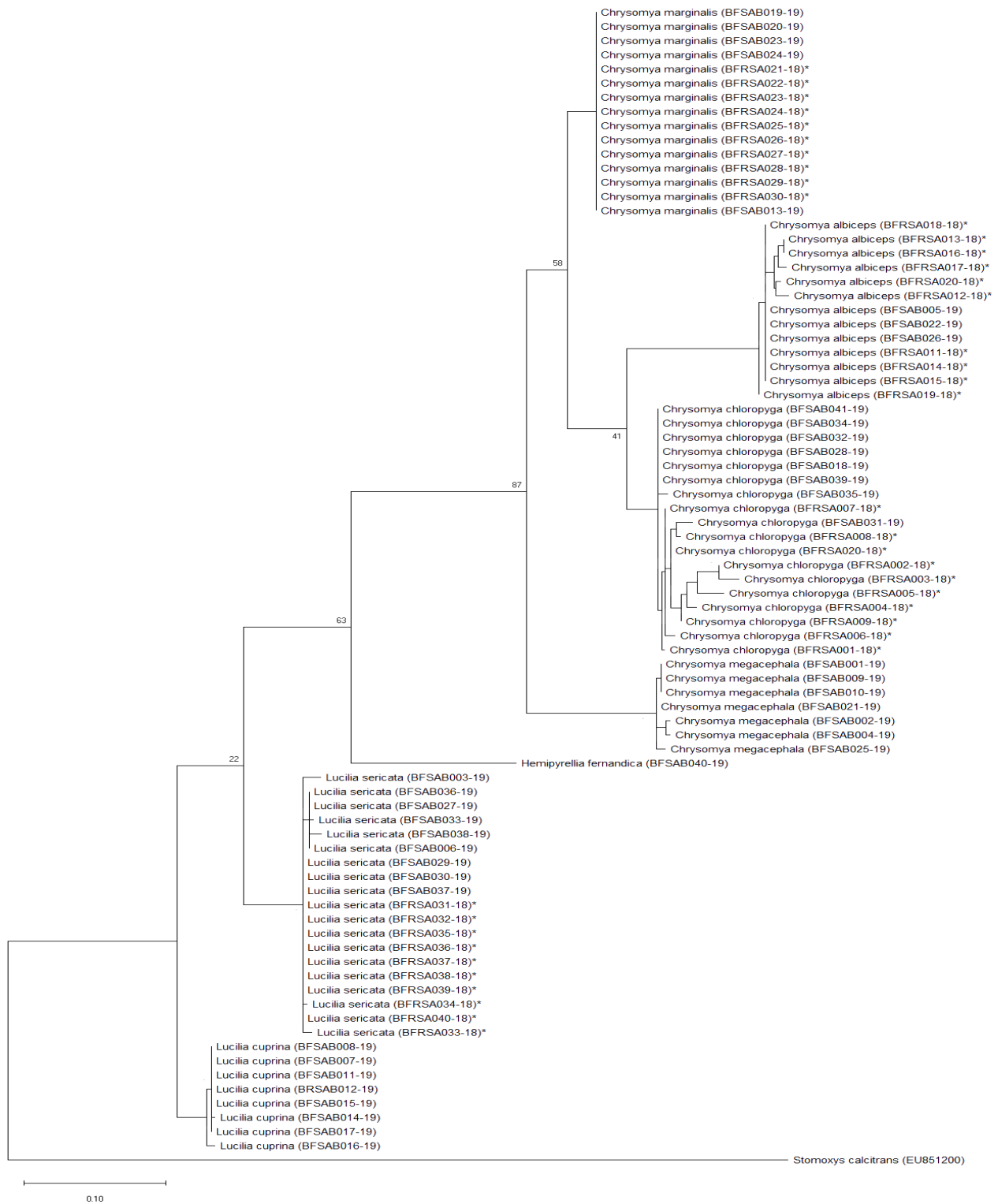


Figure 3.4: Maximum likelihood topology for the ribosomal second internal transcribed spacer gene for seven blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala*, *Hemipyrellia fernandica*, *Lucilia cuprina* and *Lucilia sericata*).

Values on each node refer to bootstrap values shown as a percentage for 1000 replicates. Branches with an * were included from Cooke et al., 2018. The respective BOLD process ID's are listed in parenthesis after each sample. *Stomoxys calcitrans* was included as an outgroup, with the GenBank® accession numbers in parenthesis (Dsouli et al., 2011).

3.3.2 Bayesian Inference

For the BI analysis, the substitution model for *COI* was determined to be GTR + G. For *ITS2* the substitution model was HKY + G. Five million generations were sufficient for the deviation of split frequencies to be below 0.01. Trees were sampled every 100 generations. Burn-in was set to 12500.

For *COI*, species of *Ch. albiceps*, *Ch. chloropyga*, *Ch. marginalis* and *Ch. megacephala* formed monophyletic clades, with the *Lucilia* species forming paraphyletic clades (Figure 3.5). The clades were well supported (> 95 % pP) for the *Lucilia* species as well as *Ch. albiceps*. The clades for *Ch. chloropyga*, *Ch. marginalis* and *Ch. megacephala* were poorly supported (< 95 % pP) with the clades for *Ch. marginalis* and *Ch. chloropyga* having the least support (66 % pP). The topology for the *ITS2* tree showed each species forming distinct clades, however, support for the clades was low with the only well supported clades being for *Ch. marginalis* and *Ch. megacephala* (100 % pP) (Figure 3.6). The combined *COI* and *ITS2* phylogenetic tree returned a tree topology with each species presenting with distinct phylogenetic clades (Figure 3.7). The clades for *Ch. marginalis*, *L. cuprina* and *L. sericata* were well supported (≥ 99 % pP) whilst the clades for *Ch. albiceps*, *Ch. chloropyga* and *Ch. megacephala* showed moderate support (≈ 80 % pP).

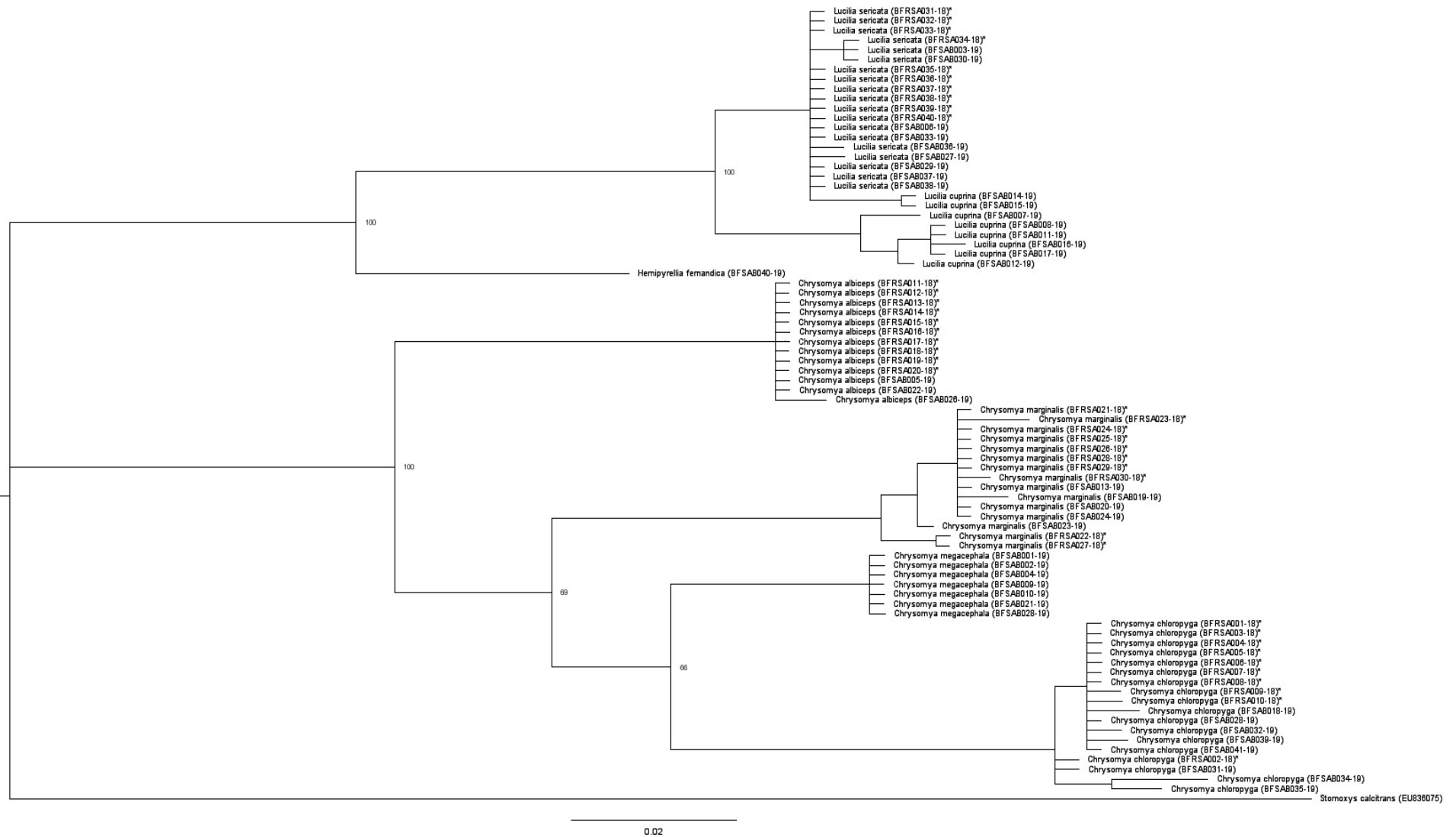


Figure 3.5: Bayesian inference topology for the 5' section of cytochrome c oxidase 1 gene for seven blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala*, *Hemipyrellia fernandica*, *Lucilia cuprina* and *Lucilia sericata*). Values on each node represent the posterior probability value (pP) derived from the Bayesian inference analysis. Branches with an * were included from Cooke *et al.*, 2018. The respective BOLD process ID's are listed in parenthesis after each sample. *Stomoxys calcitrans* was included as an outgroup, with the GenBank® accession numbers in parenthesis (Dsouli *et al.*, 2011).

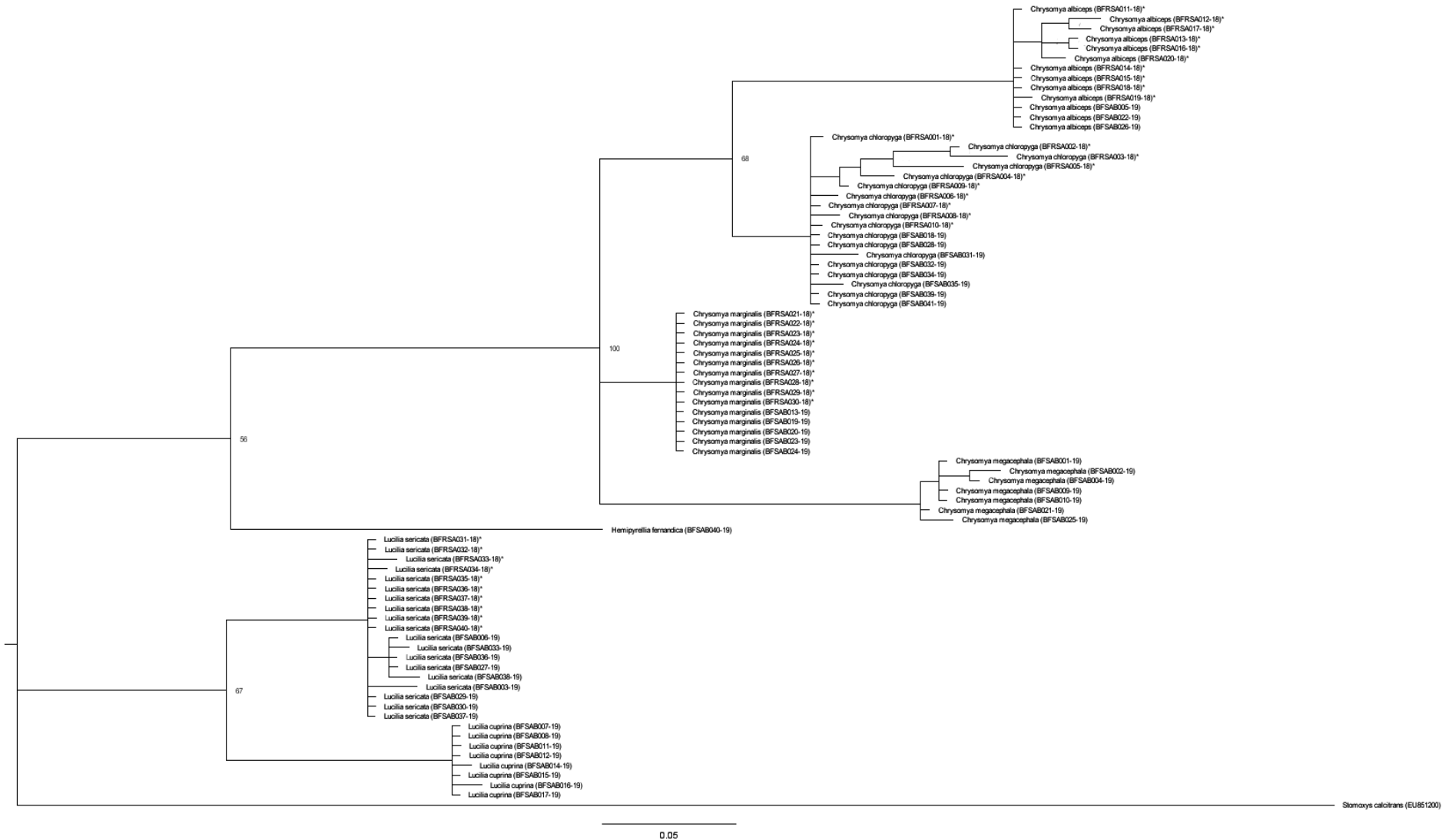


Figure 3.6: Bayesian inference topology for the second internal transcribed spacer for seven blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala*, *Hemipyrellia fernandica*, *Lucilia cuprina* and *Lucilia sericata*). Values on each node represent the posterior probability value (pP) derived from the Bayesian inference analysis. Branches with an * were included from Cooke et al., 2018. The respective BOLD process ID's are listed in parenthesis after each sample. *Stomoxys calcitrans* was included as an outgroup, with the GenBank® accession numbers in parenthesis (Dsouli et al., 2011).

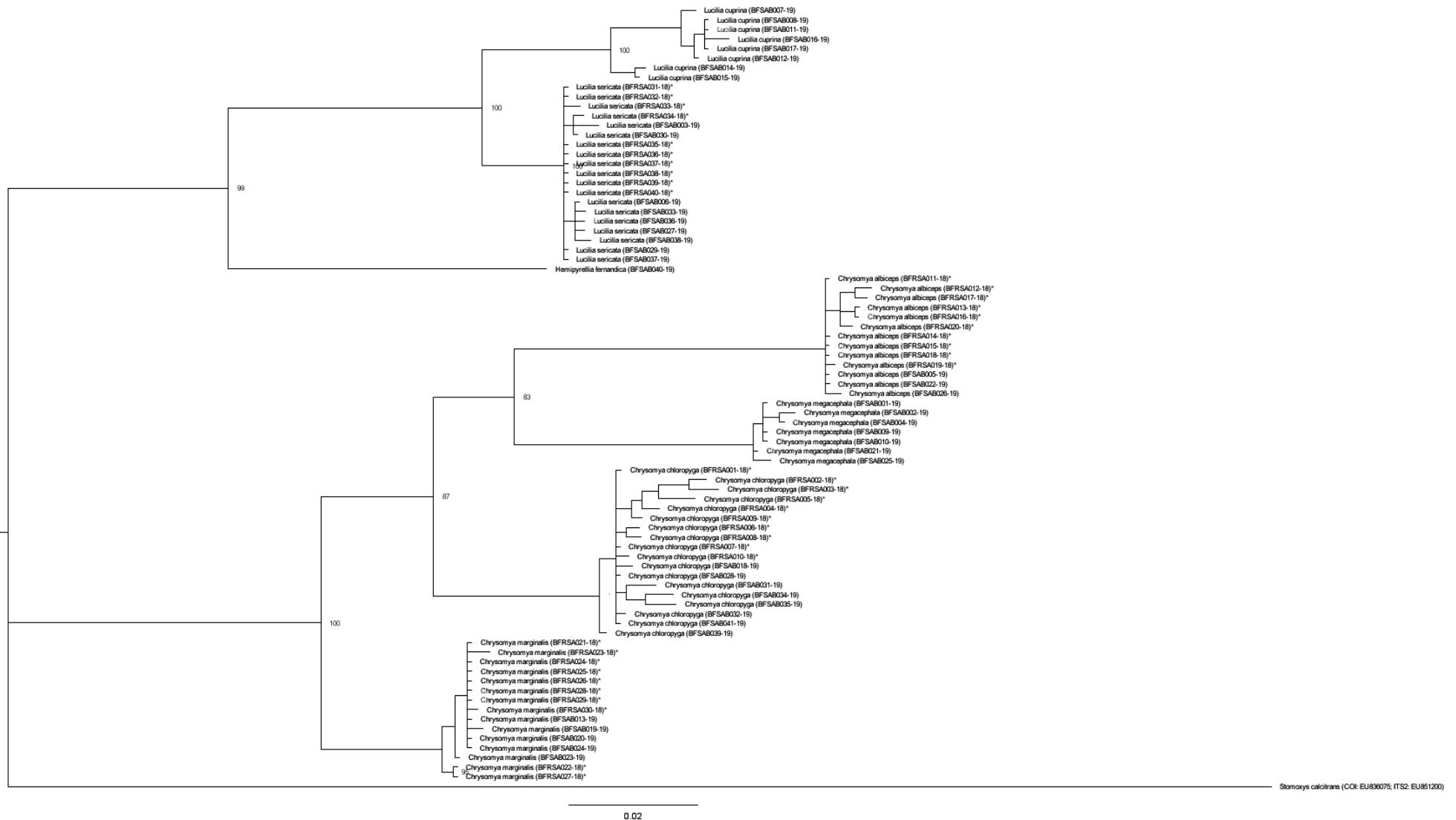


Figure 3.7: Bayesian inference topology for the combined 5' section of cytochrome c oxidase 1 and second internal transcribed spacer for seven blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala*, *Hemipyrellia fernandica*, *Lucilia cuprina* and *Lucilia sericata*).

Values on each node represent the posterior probability value (pP) derived from the Bayesian inference analysis. Branches with an * were included from Cooke *et al.*, 2018. The respective BOLD process ID's are listed in parenthesis after each sample. *Stomoxys calcitrans* was included as an outgroup, with the GenBank® accession numbers in parenthesis (Dsouli *et al.*, 2011).

3.4 Population statistics

3.4.1 Haplotype distribution and frequencies

The haplotype distribution and frequencies across the various sampled localities for each sampled species are displayed in Table 3.1 for *COI* and Table 3.2 for *ITS2*. Each species showed no distinctive haplotype patterning, with haplotypes being shared across the localities where they occurred when both markers were viewed together. When each marker was viewed independently, some haplotype patterning was observed. For the *COI* marker for *Ch. chloropyga* sampled from the Agulhas National Park and Wiesenhof Private Nature Reserve had haplotypes that were only seen from these sampled areas. Samples of *Ch. chloropyga* from the Agulhas National Park each presented with a unique haplotype. The haplotype distribution for *L. sericata* for *COI* showed a unique haplotype for the sample from the West Coast National Park and a unique haplotype for *ITS2* for the sample from the Bontebok National Park. For *Ch. marginalis* for *ITS2* each of the sampled localities presented with a unique haplotype. This was similarly seen with *Ch. megacephala* for *ITS2* across its sampled haplotypes.

Table 3.1: Haplotype frequency and distribution across sampled localities for the cytochrome c oxidase 1 (COI).

Localities sampled were the Agulhas National Park (ANP), Bontebok National Park (BNP), Hottentots Holland Nature Reserve (HHNR), West Coast National Park (WCNP), Wiesenhof Private Nature Reserve (WPNR), the University of Cape Town Medical Campus (UCTMC), Medical Research Council Primate Unit premises (MRCPU).

Locality → Haplotype number ↓	MRCPU	WCNP	UCTMC	WPNR	HHNR	ANP	BNP	Total number of observations
<i>Ch. albiceps</i>								
1	10			2				12
2				1				1
<i>Ch. chloropyga</i>								
1	7	1					1	9
2	1							1
3	1							1
4	1							1
5				1				1
6						1		1
7						1		1
8						1		1
9						1		1
10							1	1
<i>Ch. marginalis</i>								
1	6			3				9
2	2							2
3	1							1
4	1							1
5				1				1
6				1				1
<i>Ch. megacephala</i>								
1			2	5				7
<i>L. cuprina</i>								
1			2	1				3
2				1				1
3			1					1
4			2					2
5			1					1
<i>L. sericata</i>								
1	9			1	1	2	1	14
2	1			1	1			3
3		1						1
4							1	1

Table 3.2: Haplotype frequency and distribution across sampled localities for the second internal transcribed spacer (ITS2).

Localities sampled were the Agulhas National Park (ANP), Bontebok National Park (BNP), Hottentots Holland Nature Reserve (HHNR), West Coast National Park (WCNP), Wiesenhof Private Nature Reserve (WPNR), the University of Cape Town Medical Campus (UCTMC), Medical Research Council Primate Unit premises (MRCPU).

Locality → Haplotype number ↓	MRCPU	WCNP	UCTMC	WPNR	HHNR	ANP	BNP	Total number of observations
<i>Ch. albiceps</i>								
1	4			3				7
2	1							1
3	2							2
4	1							1
5	1							1
6	1							1
<i>Ch. chloropyga</i>								
1	2	1		1		2	1	7
2	1							1
3	1							1
4	1							1
5	1							1
6	1							1
7	1							1
8	1							1
9	1							1
10						1		1
11						1		1
12							1	1
<i>Ch. marginalis</i>								
1	10							10
2				5				5
<i>Ch. megacephala</i>								
1				1				1
2				1				1
3				1				1
4			1					1
5			1					1
6				1				1
7				1				1

Table 3.2: Haplotype frequency and distribution across sampled localities for the second internal transcribed spacer (ITS2) (continued).

Localities sampled were the Agulhas National Park (ANP), Bontebok National Park (BNP), Hottentots Holland Nature Reserve (HHNR), West Coast National Park (WCNP), Wiesenhof Private Nature Reserve (WPNR), the University of Cape Town Medical Campus (UCTMC), Medical Research Council Primate Unit premises (MRCPU).

Locality → Haplotype number ↓	MRCPU	WCNP	UCTMC	WPNR	HHNR	ANP	BNP	Total number of observations
<i>L. cuprina</i>								
1			6	1				7
2			1					1
<i>L. sericata</i>								
1	5				2	1		8
2	1							1
3	1							1
4	3							3
5		1		1		1		3
6						1		1
7				1				1
8							1	1

3.4.2 Intra and Inter specific analysis

Table 3.3 shows the intraspecific analysis for *COI* and *ITS2* sequences for each species. Across all the species and for both markers the nucleotide diversity was low ($\leq 0.01 \pm 0.01$). For *COI*, *L. cuprina* presented with the most variability amongst the samples with the highest mean pairwise difference between samples (7.79 ± 4.05) and the most polymorphic sites (number of polymorphic sites = 18). *Chrysomya chloropyga* also presented with a high amount of variability in the *COI* marker (mean pairwise difference = 2.42 ± 1.37 ; number of polymorphic sites = 16), as well as, having the most variability for *ITS2* (mean pairwise difference = 3.75 ± 1.98 ; number of polymorphic sites = 24). The species *Ch. chloropyga* also presented with the highest number of observed haplotypes (*COI* = 10; *ITS2* = 12). *Ch. megacephala* presented with homogeneity for the *COI* marker.

Table 3.3: Intraspecific analysis for *COI* and *ITS2* sequences for the included species. Parenthesis indicate number of the sample sequences of the whole taken from Cooke et al. (2018).

Species	Number of specimens	Molecular Marker	Number of haplotypes observed	Nucleotide diversity	Mean pairwise difference	Number of polymorphic sites
<i>Ch. albiceps</i>	13(10)	COI	2	0.00 ± 0.00	0.31 ± 0.34	2
	13(10)	ITS2	6	0.01 ± 0.00	2.17 ± 1.28	5
<i>Ch. chloropyga</i>	18(10)	COI	10	0.00 ± 0.00	2.42 ± 1.37	16
	18(10)	ITS2	12	0.01 ± 0.01	3.75 ± 1.98	24
<i>Ch. marginalis</i>	15(10)	COI	6	0.00 ± 0.00	1.24 ± 0.82	6
	15(10)	ITS2	2	0.00 ± 0.00	0.48 ± 0.44	1
<i>Ch. megacephala</i>	7	COI	1	0.00 ± 0.00	0.00 ± 0.00	0
	7	ITS2	7	0.01 ± 0.01	3.62 ± 2.08	8
<i>L. cuprina</i>	8	COI	5	0.01 ± 0.01	7.79 ± 4.05	18
	8	ITS2	2	0.00 ± 0.00	0.25 ± 0.31	1
<i>L. sericata</i>	19(10)	COI	4	0.00 ± 0.00	0.49 ± 0.44	3
	19(10)	ITS2	8	0.00 ± 0.00	0.73 ± 0.57	4

Table 3.4 displays the intraspecific and interspecific uncorrected pairwise distance values for both *COI* and *ITS2*. The intraspecific uncorrected pairwise distance was low (< 3 %) across all the species with the highest divergence for the *COI* marker being *L. cuprina* (1.27 %) and for the *ITS2* marker being *Ch. chloropyga* (1.63 %). Interspecific uncorrected pairwise distance was high (> 3 %) for all species for both markers, apart from the distance values for *L. cuprina* and *L. sericata* (1.65 %). For *COI*, the distance values ranged between 4.97 % to 11.60 %, again besides the *Lucilia* species. For *ITS2* the interspecific uncorrected pairwise distance ranged between 5.36 % and 21.71 %.

Table 3.4: Uncorrected pairwise distance values between included species for *COI* (below the diagonal and *ITS2* (above the diagonal) markers.

*Bolded values represent uncorrected pairwise distances for *COI/ITS2*.*

	<i>Ch. albiceps</i>	<i>Ch. chloropyga</i>	<i>Ch. marginalis</i>	<i>Ch. megacephala</i>	<i>L. cuprina</i>	<i>L. sericata</i>
<i>Ch. albiceps</i>	0.05/0.68	6.61	9.25	11.74	20.06	21.71
<i>Ch. chloropyga</i>	6.80	0.39/1.63	5.36	10.94	19.43	20.76
<i>Ch. marginalis</i>	6.24	6.16	0.30/0.00	11.31	20.23	20.97
<i>Ch. megacephala</i>	5.17	4.97	5.05	0.00/0.74	19.74	21.51
<i>L. cuprina</i>	10.41	11.60	9.98	9.26	1.27/0.26	6.88
<i>L. sericata</i>	10.52	10.79	9.62	8.59	1.65	0.08/0.43

3.5 Testing the reference library

For the BI analysis, the substitution models did not change with the addition of the blinded selection of samples. The substitution model was determined for *COI* to be GTR + G and for *ITS2* it was HKY + G. Five million generations were again sufficient for the deviation of split frequencies to be below 0.01. Trees were sampled every 100 generations. Burn-in was set to 12500.

Table 3.5 details the results of the identification for the phylogenetic analysis as well as the output from BOLD and GenBank®. When assessing *COI*, seven out of the eight samples were grouped in clusters that corresponded with their morphological identification (Appendix 3.4). For *ITS2* all of the blinded selection grouped within a clade that corresponded with their morphological identification (Appendix 3.5). For the combined dataset all the blinded selection of samples grouped into the respective phylogenetic clades that corresponded to the morphological identification (Appendix 3.6).

The sequences for the 5' *COI* marker were compared to BOLD's search platform under "Species Level Barcode Records". Out of the eight samples, BOLD was only able to identify three of the specimens. When the sequences were searched against using GenBank's® MegaBLAST feature, only six out of the eight samples were identified correctly for the *COI* marker, and four for the *ITS2* marker. Two of the samples (samples 2 and 8) were identified by both platforms as *Chrysomya vicina*. Morphological analysis of these two samples showed the anterior part of the genal dilation as being black in colour with the basicosta being brownish-black, which is not indicative of *Ch. vicina* but rather *C. croceipalpis*.

Table 3.5: The identification of eight blinded samples of Calliphoridae species using the barcoding of life data systems (BOLD) platform using the "Species Level Barcode Records", as well as, GenBank's® Basic Local Alignment Search Tool (MegaBLAST).

Morphological identification was done using identification keys by Lutz et al. (2017) and Szpila (2009).

Unknown sample	Morphological Identification	Identification using COI dataset from this study	Identification using ITS2 dataset from this study	Identification using the combined COI and ITS2 dataset from this study	BOLD identification	BOLD reported similarity		GenBank® MegaBLAST identification	GenBank® reported similarity	
						Top%	Low%		COI	ITS2
1	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	100	100	<i>Ch. megacephala</i>	100	100
								<i>Ch. saffranaea</i>	N/A	100
2	<i>C. croceipalpis</i>	<i>C. croceipalpis</i>	<i>C. croceipalpis</i>	<i>C. croceipalpis</i>	<i>C. vicina</i>	98.4	97.22	<i>C. vicina</i>	97.4	92.03
3	<i>Ch. marginalis</i>	<i>Ch. marginalis</i>	<i>Ch. marginalis</i>	<i>C. marginalis</i>	<i>Ch. marginalis</i>	99.65	94.89	<i>Ch. marginalis</i>	99.66	100
4	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	<i>Ch. megacephala</i>	100	100	<i>Ch. megacephala</i>	100	99.68
								<i>Ch. saffranaea</i>	N/A	99.68
5	<i>L. cuprina</i>	<i>L. cuprina</i>	<i>L. cuprina</i>	<i>L. cuprina</i>	<i>L. cuprina</i>	100	94.84	<i>L. cuprina</i>	100	99.42
								<i>L. cuprina X sericata</i>		
								<i>L. sericata</i>		
6	<i>Ch. chloropyga</i>	<i>Ch. chloropyga</i>	<i>Ch. chloropyga</i>	<i>Ch. chloropyga</i>	<i>Ch. chloropyga</i>	98.81	95.43	<i>Ch. chloropyga</i>	99.81	100
								<i>Ch. putoria</i>		
7	<i>L. sericata</i>	<i>L. sericata</i>	<i>L. sericata</i>	<i>L. sericata</i>	<i>L. sericata</i>	100	99.84	<i>L. sericata</i>	100	100
								<i>L. cuprina</i>		
8	<i>C. croceipalpis</i>	<i>C. croceipalpis</i>	<i>C. croceipalpis</i>	<i>C. croceipalpis</i>	<i>C. vicina</i>	98.4	97.06	<i>C. vicina</i>	97.42	92.41

Chapter 4: Discussion and Conclusion

4.1 Introduction

This study aimed to contribute towards the database of reference barcodes for the Calliphorid species that are present in different localities in the Western Cape of South Africa. To this end, 153 flies were sampled from six localities in the Western Cape, of which 41 unrelated specimens, representing seven species, were included in the study. Reference barcodes were successfully generated for both *COI* and *ITS2* for the 41 specimens of Calliphorid flies. Furthermore, they were compliant with the requirements for BOLD and together with the photographs representing morphology, were published on both BOLD and GenBank®. These barcodes were assessed with and compared to previously generated reference material from the Western Cape taken from a pilot study done by Cooke *et al.* (2018).

4.2 Assessing haplotype distribution by sampled localities

Part of the aim of this project was to sample flies from different localities, since previous research in the Western Cape assessed flies from a single locality only. As such, it was important to assess the haplotype patterning before assessing the discriminatory capacity of the barcodes, in order to determine if there were any distinct subpopulations. This would inform the AMOVA test as well as the interpretation of the phylogenetic trees.

When assessing the haplotype patterning using both markers, no specific haplotype patterning was seen (Table 3.3 and Table 3.4). This would suggest that there is sufficient gene flow between the sampled localities, which is likely due to the capacity of the Calliphorid species to travel long distances in search of suitable breeding environments. Blow flies have been known to travel distances of up to 40 km in a single lifetime and up to 2 km per day (Braack & Retief, 1986; Braack & de Vos, 1987; Smith & Wall, 1998).

However, it should be taken into consideration that the absence of haplotype patterning may be due to the sampled subpopulations not being representative of the genetic subpopulations that are available in the Western Cape. Thus, there is a possibility that an unsampled locality, perhaps also in a different season, may return a distinct subpopulation within a species. A more comprehensive sampling regime would need to be implemented in order to accurately assess the haplotype patterning of Calliphoridae species within the Western Cape, which was beyond the scope of this study. However, given the data generated by this study, it was deemed unnecessary to evaluate any of the species as separate subspecies in downstream analyses, since the haplotype distributions showed no distinct genetic patterning between the sampled localities.

4.3 Evaluation of the barcodes for species discrimination

Hebert *et al.* (2003a) stated that the criterion for assessing a taxonomic system is the ability for the system to deliver accurate species identification. In the case of a phylogenetic analysis this would require that there is a monophyletic association for each species group that has sufficient phylogenetic support.

4.3.1 *COI* marker

COI is traditionally considered the 'barcode of life' and is one of the original markers for species identification (Hebert *et al.*, 2003a; Ratnasingham & Hebert, 2007). *COI* presented with clusters for the respective species showing distinct monophyletic clades for both analyses performed (Figure 3.4 and 3.6). However, the phylogenetic analysis for *COI* did not show monophyletic association for the *Lucilia* species. Furthermore, the nodal phylogenetic support for many of the clades were well below the cut-off values (75 % bootstrapping and 95 % *pP* for the ML and BI trees respectively). This, as well as the inability to discriminate between the *Lucilia* species, results in a failure in the criterion of monophyletic association.

As sample numbers increase, so should the phylogenetic support (Pollock *et al.*, 2002; Zwickl & Hillis, 2002; Hillis *et al.*, 2003). This was also not observed in this study, where clade support decreased compared to the previous cohort of samples in the pilot study by Cooke *et al.* (2018). This may be indicative of variation in the barcodes being introduced due to an increase in sampled localities. However, the intraspecific nucleotide diversity for *COI* was low for all the included species (Table 3.2) indicating that the degree of polymorphism within each species sample set was low (Nei & Li, 1979). This was reinforced by low intraspecific mean pairwise difference values for each species sample set (Table 3.1). Additionally, the interspecific uncorrected pairwise distance for the *COI* marker satisfied the 10x criterion (Hebert *et al.*, 2003a) as well as being above 3 % for the majority of the samples, with an exception for the *Lucilia* species (Table 3.2). Furthermore, the intraspecific uncorrected pairwise distance values for *COI* were all greater than 3 % with the exception of *L. cuprina* (Table 3.2). This demonstrates the capacity of the *COI* marker to discriminate between the included species, apart from the *Lucilia* species.

4.3.2 *Lucilia* spp. and *COI*

The *COI* marker was unable to distinguish between the *Lucilia* species. In the Western Cape members of the *Lucilia* species can be difficult to accurately identify using morphology, with some of the only morphological markers being the position, number and length of setulae and hairs (Tourle *et al.*, 2008; Lutz *et al.*, 2018). These morphological traits are not reliable for identification purposes because the hairs or the setulae can be detached or damaged (Harvey *et al.*, 2003a; Williams & Villet, 2014).

Many studies have raised the concern that *COI* does not have the capacity to distinguish between recently diverged species (Wallman & Donnellan, 2001; Hebert *et al.*, 2004; Wells *et al.*, 2007; GilArriortua *et al.*, 2014, 2015). This included the members of the *Lucilia* genus, *L. cuprina* and *L. sericata*. In this study paraphyletic association is observed with both *Lucilia* species. What is of interest is that when evaluating the uncorrected pairwise difference, the intraspecific values for these *Lucilia* species were considerably lower than the other species pairs and failed not only the

10x criterion but also did not surpass the general 3 % threshold. Furthermore, the intraspecific value for the *L. cuprina* grouping was high (1.27 %). This would indicate that the cohort of samples that formed the *L. cuprina* group in this study may not be a homogenous species.

There are two possible reason for this. The first is that this group may represent two subspecies of *L. cuprina*. Whilst this would account for the high intraspecific uncorrected pairwise difference, it would not account for the relatively low interspecific distance between the *L. cuprina* and the *L. sericata* groups. Considering that *L. sericata* had a relatively low intraspecific uncorrected pairwise distance, a more plausible reason would be due to hybridisation events between *L. cuprina* and *L. sericata*. A study by Nelson *et al.* (2008) demonstrated that ancient hybridisation events occurred in the Australian Calliphoridae species. A hybridisation event like this could result in a hybridised subspecies of *L. cuprina* that is localised within the Western Cape of South Africa. This would pose a challenge when determining PMI in medico-legal death investigations involving *L. cuprina* when using the *COI* marker. Accidentally using the hybrid to estimate PMI in place of *L. cuprina* could result in misleading estimates, due to potential variations within the lifecycles of the hybrid (Tourle *et al.*, 2008).

4.3.3 ITS2 marker

For the *ITS2* marker, the results of the phylogenetic analysis presented with monophyletic clades for the all species, including the *Lucilia* species. However, like *COI*, not all of the clades were well supported, with the nodal support being relatively poor, returning less than or equal to 68 % *pP*. Therefore, the *ITS2* marker also failed to meet the criterion for monophyletic association, even though all species presented with distinct monophyletic clades. This result was also observed in the pilot dataset (Cooke *et al.*, 2018), where the authors demonstrated that *ITS2* was not suitable for use as a standalone marker for species identification.

The *ITS2* marker did pass the 10x criterion for the interspecific uncorrected pairwise distance for the majority of species, apart from two, which is the interspecific distance between *Ch. albiceps* and *Ch. chloropyga*, as well as between *Ch. chloropyga* and *Ch. marginalis*. This may be a result

of the higher molecular variation seen in *Ch. albiceps*, *Ch. chloropyga* and *Ch. marginalis*. Furthermore, when compared to the pilot study dataset, there was a significant decrease in the interspecific uncorrected pairwise distance values, with some species greater than 40 %. This is potentially due to the introduced variation from the bolstered sample sizes, additional sample localities as well as the additional species; which would allow for a more accurate representation of the discriminatory capacity for *ITS2*.

4.3.4 Evaluation of the combined dataset

The combined dataset for the *COI* and *ITS2* markers proved to be more effective at species delineation than either marker when used individually (Figures 3.5, 3.6 and 3.7). The combined dataset returned distinct monophyletic clades with improved phylogenetic support for every species included and it provided a more robust taxonomic assessment for the included samples. Furthermore, the combined dataset was able to distinguish between the *Lucilia* species, with full phylogenetic support. This was accomplished despite the potential hybridisation between the *L. cuprina* and *L. sericata* species.

Overall, the combined dataset did not meet the criterion of well-supported monophyletic association for all the included species and thus did not satisfy the requirements of an independent taxonomic system. However, this may not be a result of the capacity of the barcodes to discriminate between Calliphoridae species but rather due to a limitation of the current reference material available for the Western Cape. Several studies have assessed the effect of taxonomic sampling with the capacity of phylogenetic inference, and suggest that increasing sample number increases the accuracy of correct taxonomic association (Poe, 1998; Zwickl & Hillis, 2002; Pollock *et al.*, 2002). Whilst dated, the study by Poe (1998) concluded that a minimum of 20 samples are needed in order to properly assess the phylogenetic inference.

This study contributed to increasing reference sample numbers for the Western Cape of South Africa and bolstered the number of available reference sequences for *Ch. albiceps*, *Ch. chloropyga*, *Ch. marginalis* and for *L. sericata*. Furthermore, the reference samples that were

generated for *Ch. megacephala*, *H. fernandica* and *L. cuprina* are the first in the area. Therefore, this study substantially increased the reference material available for the Province. Despite this, none of the seven included species in this study reached 20 specimens each. Therefore, the lower than optimally supported clades may be a result of insufficient reference materials, and there is thus a strong motivation to expand the sample size for each species so as to increase the value of this data in forensic casework.

The introduction of new species into reference database can also result in lowered phylogenetic support for the respective clades. A study by Will & Rubinoff (2004) showed that the addition of taxa in the analysis was enough to decrease the phylogenetic support. Therefore, the assessment of the phylogeny is dependent on the choice of taxa that are included (Will *et al.*, 2005). From a medico-legal perspective it would then be prudent to establish a robust means of identifying unknown specimens; therefore, the analysis would need to be able to withstand the addition of new taxa or even samples of previously existing taxa to the reference database. A means to accomplish this would be to increase the number of markers, such as Cytochrome b (Cytb), NADH dehydrogenase subunit 4 (ND4), 18S ribosomal RNA (rRNA) or 28S rRNA that are recognised in phylogenetic inferences (Marinho *et al.*, 2012; GilArriortua *et al.*, 2013, 2014).

4.4 Assessment of DNA barcodes

Whilst phylogenetic support offers a means of assessing the capacity for discrimination between species, it should be assessed with caution. A well-supported statistical probability linking an unknown specimen with a monophyletic clade of reference samples may seem like a reliable test, however, it is uncertain how a future unknown sample would fare, considering that there may still be a possibility of incorrect assortment (Wells & Stevens, 2008). The nodal phylogenetic support is an assessment of probability and is subject to failure, despite the low possibility. Additionally, the phylogenetic association relies heavily on the quality of the reference materials that are available. This does not only apply to the molecular references but also to the taxonomic keys that are available.

Another means of assessing the discriminatory capacity of the barcodes would be to test the barcodes using a known specimen and to calculate the percentage of incorrect identifications. When attempting to identify the blinded cohort of unknown samples in this study, the use of the generated reference data set proved to be more accurate than the search functions of BOLD and GenBank's® megaBLAST, with all of the unknown species being accurately clustered within their respective clades. Both online platforms suffer from incomplete reference material, as noted with the *C. croceipalpis* sequences being closely related to *C. vicina*. The capacity of an assessment to exclude unknown samples, for which there is not any reference material available, is important to prevent the unknown sample from being misidentified.

Whilst GenBank's® megaBLAST may offer a convenient way to compare a sample to a multitude of reference sequences, it is more suitable to enable one to gain a general impression of the identity of the sample. This is due to the quantity of gaps or errors in the reference material that has been deposited on the database (Wells *et al.*, 2007). BOLD may not contain as many gaps or errors as GenBank®, due to the stricter criteria BOLD utilises for obtaining barcode status. However, the BOLD platform is predominantly 5'COI based for animal identification, which may not prove to be an optimal means of identifying Calliphoridae without a combined approach with more selective markers, whereas, COI may be sufficient for the majority of animal identifications.

4.5 Technical considerations

When assessing the use of the markers for DNA barcoding it is important to take into consideration some of the technical aspects involved in processing the samples to generate DNA barcodes.

4.5.1 DNA yields

When assessing the DNA yield from the samples included in this study it was noted that the samples with low DNA yield, negatively impacted the ease at which DNA barcodes were generated. Therefore, to improve DNA barcode generation workflow, the DNA yield needs to be optimal. The first means to accomplish this would be to use more tissue from the samples for the extraction. In this study only four legs were used during the extraction in order to retain the morphological features of the vouchers, however, this resulted in relatively low DNA concentrations. Consequentially, increased incubation times and an extra ethanol wash step were included to increase the purity of the extracted DNA. This increased the overall processing time for the extraction. Whilst suitable for research purposes, the consequences of this need to be considered for inclusion in routine medico-legal investigations.

While it is recommended to dissect tissue from the head and thorax of adult specimens and from the mid-section of maggots, in order to prevent contamination from endo-parasites or digested biological material (Genard, 2012), this will be time consuming and will damage the voucher specimen. Furthermore, in the DNA extraction optimisation, the extra tissue gained from dissecting the thorax did not grant any noticeable benefit to DNA yield. Utilising more of the voucher would render it unsuitable for morphology. Therefore, other avenues need to be explored in order to improve the yield from these extractions, without compromising what can be retained as a voucher. Possible avenues include how samples are euthanised, dissected and stored.

When doing molecular analysis on specimens, all the specimens should be killed and stored appropriately. This has more value in medico-legal investigations where the specimens used in the investigation, may need to be called upon again, should further ancillary investigation(s) be required. The preparation of the specimens can influence the outcome of the analysis. The initial preservation of samples is important, and ideally, samples should be stored in 95 % ethanol at the crime scene; with the euthanisation method being to flash freeze them at -70 °C. However, this may be impractical to implement at a crime scene. Furthermore, the length of time the specimens remain frozen may influence the rate of DNA degradation (Lonsdale *et al.*, 2004).

Storing samples, without refrigeration in 99 % ethanol provided DNA fragments of 1400 bp in length and preserving the samples in 75 % ethanol returned fragments of up to 350 bp (Sperling *et al.*, 1994). Due to the rapid degradation of DNA it would be beneficial to keep the samples cold, around 4 ° C, if the samples cannot be frozen.

Additionally, attention needs to be directed to the euthanasia methods that are in practice by forensic entomologists and the impact that these have on DNA extraction. For instance, using ethyl acetate can possibly reduce the amount of DNA extracted (Dillon *et al.*, 1996) even though it is considered a humane means to euthanise insects. Furthermore, the life stage of the insect, such as its larval stage, may require a different euthanasia method which may influence the downstream applications (Adams & Hall, 2003).

Ethical considerations are important when using animals in research. The *South African National Standard for the care and use of animals for scientific purposes* (SANS 10386:2008) is a set of accepted guidelines that provides general principles for the use and care of animals in research. These guidelines specify the responsibilities of researchers and associated institutions. Furthermore, it details the terms of operation, reference, as well as, membership of institutional animal ethics committees (AECs). It also provides guidelines for the humane conduct of scientific studies and teaching activities, and for the acquisition of animals and the caring for said animals. In these guidelines Calliphoridae are considered lower invertebrates and do not require ethical approval to conduct scientific processes. However, it is advised that the invertebrates still be treated with the same consideration with respect to replacement, reduction and refinement, and are given the benefit of the doubt as to whether they can suffer or not. Therefore, Calliphoridae used in forensic practices should be euthanised humanely.

Sample euthanasia, preservation, storage and DNA extraction methods still have scope to be optimised further for efficient DNA barcode generation in South Africa. Whilst this study can offer insight into the challenges that are associated with some of these aspects, a thorough evaluation and optimisation study for use in a routine medico-legal practice is required, before DNA barcoding can be properly implemented as a tool for forensic investigations.

4.5.2 Amplification

Another consideration that needs to be taken into account is the capacity of the respective primer set to amplify the target region from preferably all species. In the pilot study it was noted that not all *COI* primers were able to amplify the whole cohort of species included in the study (Cooke *et al.*, 2018). Cooke *et al.* (2018) suggested that the *COI* primer specificity could act as an additional layer of discrimination between the Calliphoridae species. This is because some primer sets were able to amplify all members of one species and not amplify all members of other species. However, in this study it was noted that the two sets of *COI* primers (Lep and Folmer (Folmer *et al.*, 1994; Hebert *et al.*, 2004)) were unable to amplify *all* members of a single species but were able to amplify *some* members from a species. When the primers were aligned to a template sequence for cytochrome oxidase complex along with the Marinho primers (Marinho *et al.*, 2012) the Lep and Folmer primers bound to a similar region of *COI*, which was indeed a variable region for *COI*. The Marinho primers proved to be successful with problematic samples. However, due to the increased size of the amplicon, there was minimal sequencing data overlap between the forward and reverse strand. This can make clean-up and alignment of the sequences challenging. Therefore, a more reliable and universal primer set is recommended for use in routine medico-legal investigations.

4.5.3 Taxonomic keys

In this study it was noted that members of the *Ch. marginalis* species that were captured had darkened frontal occipital lobes. This would suggest that the identification be that of *Ch. laxifrons*. However, the morphological identification was confirmed molecularly, with both BOLD and GenBank® resulted in a *Ch. marginalis* identification. Furthermore, the samples grouped into a well-supported clade with the samples from the study done by Cooke *et al.* (2018) in the combined BI analysis (Figure 3.6). Finally, the entire cohort of *Ch. marginalis* showed minimal genetic variation for both markers (Table 3.3) and satisfied the 10x criterion for the uncorrected interspecific pairwise difference (Table 3.4). This indicates that the identification is indeed *Ch.*

marginalis and not *Ch. laxifrons*. Additionally, members of *L. cuprina* demonstrated greater amounts of variation in the colourations of the exoskeleton (Appendix 3.2). Although subjective, this morphological trait can be used in discriminating between *Lucilia* species and other genera of Calliphoridae. These deviations from the available morphological taxonomic keys for South Africa indicate that they need to be revised for some of the local variations in Calliphorid species.

4.6 Study design limitations

When assessing the overall design and execution of this study several limitations were noted during the course of the research. The primary challenge that this study faced was collecting an adequate number of samples within the study timeframe and sampling sites. As discussed previously, whilst this study doubled the pre-existing DNA barcodes that were available for the local Calliphoridae species, it did not establish enough unrelated specimens to accurately assess the discriminatory capacity of the barcodes. This was inherently due to the conservative sampling protocol that was conducted during the research period, resulting in a small, unrelated but trusted selection of flies.

Several factors influenced this, with the first being the number of sampled localities. In this study sampling was limited to six sampled localities. Increasing the number of localities would be the most effective way of collecting unrelated samples for all species that are available at the sampling site, provided that an adequate distance separates it from other sampled localities. Furthermore, increasing the number of sampled localities would offer the chance of collecting other forensically relevant Calliphoridae species that were not collected or available at the other localities. This would also allow for better assessment of the haplotype distributions for Calliphoridae species and add valuable collection data for use in forensic entomological practices.

Apart from increasing the number of sampled localities one can also address the way in which sampling was conducted at each locality. For this study, each locality was sampled for only a single season for the majority of the sampled localities. This introduced a few factors that were unavoidable. The first was that not all species may have been active and attracted to the bait, simply due to the environmental conditions that were present when sampling. Different

Calliphoridae species may seek out a corpse during different seasons at any given sampled locality. This behavioural component would make capturing the flies with a bait trap challenging during non-breeding seasons. Furthermore, certain seasons have different weather effects such as increases or decreases in the average rainfall, humidity, windspeed and temperature (Cowling *et al.*, 2009). Different species become more prominent during different periods of weather conditions. Simply revisiting sampled localities during different seasons would not only offer the possibility to increase the number of unrelated specimens that have been captured but also offer the chance to capture species that may not have been active during previous sampling trips. Furthermore, this would add to the availability of seasonal collection information, which can be of aid in forensic entomological practises. Additionally, it would also be beneficial to sample the same season multiple times, over different years, in order to account for annual changes in the abundance of Calliphorid species and also to alleviate any temporal factors that may affect a specific breeding year, such as drought.

Finally, increasing the number of traps employed at each sampling locality would increase the chances of successfully capturing flies during each sampling trip. This would, however, require that more traps are constructed, set up and maintained during the sampling period, and in turn potentially require more than one researcher to be active on a sampling site. While the solutions to the limitations that were experienced in this study design seem easy to execute, employing these solutions subsequently require additional time and resources, that were beyond the scope of what was available for this minor research dissertation.

4.7 Conclusion

This study has expanded the available local DNA barcode references for the Western Cape of South Africa and is one step closer to establishing a comprehensive reference library for use in forensic entomological practices. The combined dataset proved to be a capable tool for delineating Calliphoridae, as well as being able to discriminate between *L. cuprina* and *L. sericata*. Furthermore, the dataset was able to successfully identify the blinded selection of Calliphoridae, surpassing both search platforms. This study also highlighted the limitations of DNA barcoding within the Western Cape of South Africa, demonstrating the need for sufficient sample numbers and the inclusion of all local species within the reference library. This study has doubled the DNA barcode data available for Western Cape of South Africa, and has meaningfully contributed towards a valuable tool for species identification of blow flies and by implications PMI determination in forensic investigations.

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Appendix 2.1



Figure 2.2: Photograph of set up collection trap used in the study. Taken at the University of Cape Town, Faculty of Health Sciences.

Appendix 2.2

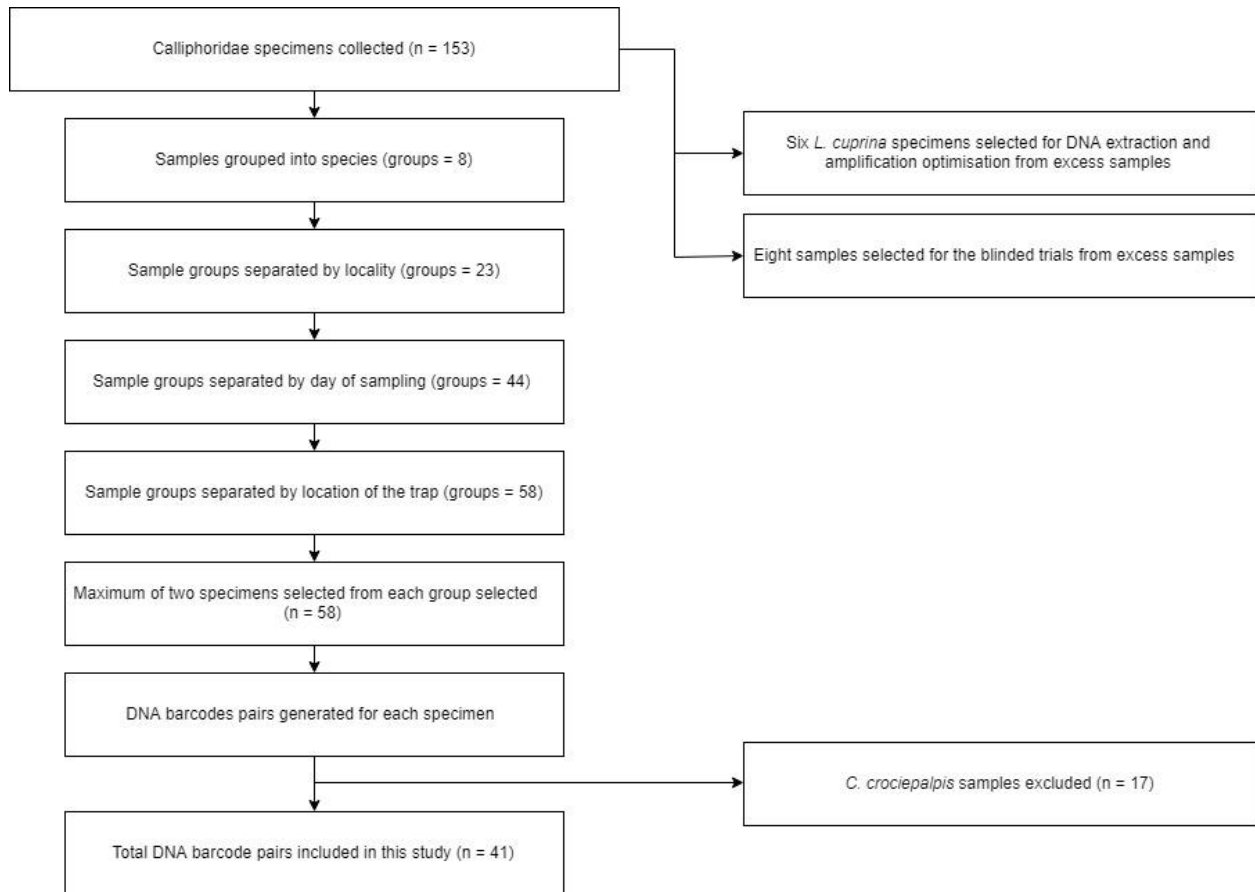


Figure 2.3: Diagram depicting the sample selection procedure used in this study.

Appendix 2.3

Table 2.1: Collection information for the Calliphoridae specimens included in this study for six localities in the Western Cape of South Africa, as well as, the corresponding barcoding of life data system (BOLD) process ID.

Morphological identification was done using taxonomic keys from Lutz et al. (2018) and Szpila (2009). Molecular identification was accomplished by using GenBank® MegaBLAST and BOLD platform species level barcode records search option. Localities sampled were the Agulhas National Park (ANP), Bontebok National Park (BNP), Hottentots Holland Nature Reserve (HHNR), West Coast National Park (WCNP), Wiesenhof Private Nature Reserve (WPNR) and the University of Cape Town Medical Campus (UCTMC). Species included are Chrysomya albiceps, Chrysomya chloropyga, Chrysomya marginalis, Chrysomya megacephala, Hemipyrellia fernandica, Lucilia cuprina and Lucilia sericata.

Process ID	Morphological ID	Molecular ID	Locality	Date	Season	GPS
BFSAB001-19	Ch. megacephala	Ch. megacephala	WPNR	30/04/2018	Autumn	S 33° 50' 19.5" E 18° 52' 84.0"
BFSAB002-19	Ch. megacephala	Ch. megacephala	WPNR	30/04/2018	Autumn	S 33° 50' 19.5" E 18° 52' 84.0"
BFSAB003-19	L. sericata	L. sericata	WPNR	30/04/2018	Autumn	S 33° 50' 19.5" E 18° 52' 84.0"
BFSAB004-19	Ch. megacephala	Ch. megacephala	WPNR	01/05/2018	Autumn	S 33° 50' 19.5" E 18° 52' 84.0"
BFSAB005-19	Ch. albiceps	Ch. albiceps	WPNR	01/05/2018	Autumn	S 33° 50' 19.5" E 18° 52' 84.0"
BFSAB006-19	L. cuprina	L. sericata	WPNR	01/05/2018	Autumn	S 33° 50' 16.7" E 18° 52' 82.4"
BFSAB007-19	L. cuprina	L. cuprina	WPNR	01/05/2018	Autumn	S 33° 50' 16.7" E 18° 52' 82.4"
BFSAB008-19	L. cuprina	L. cuprina	UCT	06/05/2018	Winter	S 33° 56' 32.4" E 18° 28' 05.3"
BFSAB009-19	Ch. megacephala	Ch. megacephala	UCT	14/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB010-19	Ch. megacephala	Ch. megacephala	UCT	14/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB011-19	L. cuprina	L. cuprina	UCT	14/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB012-19	L. cuprina	L. cuprina	UCT	16/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB013-19	Ch. marginalis	Ch. marginalis	WPNR	17/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB014-19	L. cuprina	L. cuprina	UCT	16/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB015-19	L. cuprina	L. cuprina	UCT	16/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB016-19	L. sericata	L. cuprina	UCT	16/05/2018	Autumn	S 33° 56' 59.1" E 18° 27' 94.7"
BFSAB017-19	L. cuprina	L. cuprina	WPNR	26/05/2018	Autumn	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB018-19	Ch. chloropyga	Ch. chloropyga	WPNR	24/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"

Table 2.1: Collection information for the Calliphoridae specimens included in this study for six localities in the Western Cape of South Africa, as well as, the corresponding barcoding of life data system (BOLD) process ID continued.

Morphological identification was done using taxonomic keys from Lutz et al. (2018) and Szpila (2009). Molecular identification was accomplished by using GenBank® MegaBLAST and BOLD platform species level barcode records search option. Localities sampled were the Agulhas National Park (ANP), Bontebok National Park (BNP), Hottentots Holland Nature Reserve (HHNR), West Coast National Park (WCNP), Wiesenhof Private Nature Reserve (WPNR) and the University of Cape Town Medical Campus (UCTMC). Species included are Chrysomya albiceps, Chrysomya chloropyga, Chrysomya marginalis, Chrysomya megacephala, Hemipyrellia fernandica, Lucilia cuprina and Lucilia sericata.

Process ID	Process ID	Process ID	Process ID	Process ID	Process ID	Process ID
BFSAB019-19	Ch. marginalis	Ch. marginalis	WPNR	16/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB020-19	Ch. marginalis	Ch. marginalis	WPNR	16/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB021-19	Ch. megacephala	Ch. megacephala	WPNR	16/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB022-19	Ch. albiceps	Ch. albiceps	WPNR	16/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB023-19	Ch. marginalis	Ch. marginalis	WPNR	17/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB024-19	Ch. marginalis	Ch. marginalis	WPNR	18/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB025-19	Ch. megacephala	Ch. megacephala	WPNR	18/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB026-19	Ch. albiceps	Ch. albiceps	WPNR	18/07/2018	Winter	S 33° 50' 16.4" E 18° 52' 77.4"
BFSAB027-19	L. sericata	L. sericata	WCNP	06/09/2018	Spring	S 33° 07' 06.5" E 18° 03' 30.1"
BFSAB028-19	Ch. chloropyga	Ch. chloropyga	WCNP	06/09/2018	Spring	S 33° 07' 06.5" E 18° 03' 30.1"
BFSAB029-19	L. sericata	L. sericata	HHNR	11/09/2018	Spring	S 34° 00' 96.6" E 19° 07' 30.2"
BFSAB030-19	L. sericata	L. sericata	HHNR	11/09/2018	Spring	S 34° 01' 01.3" E 19° 07' 26.2"
BFSAB031-19	Ch. chloropyga	Ch. chloropyga	ANP	26/10/2018	Spring	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB032-19	Ch. chloropyga	Ch. chloropyga	ANP	27/10/2018	Spring	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB033-19	L. cuprina	L. sericata	ANP	27/10/2018	Spring	S 34° 49' 62.6" E 19° 57' 26.7"
BFSAB034-19	Ch. chloropyga	Ch. chloropyga	ANP	28/10/2018	Spring	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB035-19	Ch. chloropyga	Ch. chloropyga	ANP	29/10/2018	Spring	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB036-19	L. cuprina	L. sericata	ANP	29/10/2018	Spring	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB037-19	L. sericata	L. sericata	ANP	29/10/2018	Spring	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB038-19	L. sericata	L. sericata	BNP	31/10/2018	Summer	S 34° 02' 59.1" E 20° 26' 36.7"
BFSAB039-19	Ch. chloropyga	Ch. chloropyga	BNP	01/11/2018	Summer	S 34° 02' 59.1" E 20° 26' 36.7"
BFSAB040-19	H. fernandica	H. fernandica	ANP	27/10/2018	Summer	S 34° 49' 68.8" E 20° 00' 54.9"
BFSAB041-19	Ch. chloropyga	Ch. chloropyga	BNP	03/11/2018	Summer	S 34° 02' 59.1" E 20° 26' 36.7"



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee



Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

30 April 2018

FHS AEC REF NO: 015/039

Dr M Heyns
Pathology
Forensic Medicine & Toxicology
Falmouth Building

Dear Dr Heyns

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

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

I am pleased to inform you that the FHS AEC has **approved** the following amendments to the above mentioned study:

- Additional research participant: Kyle Kulenkampff
- Extension of the project until March 2019

A Form for amendment (version October 2014) is also available at <http://www.health.uct.ac.za/fhs/research/animalethics/forms>

Yearly progress report submitted to the ethics office is a requirement for on-going approval of studies. Notification of study closure is a requirement.

Ethics approval letter and copy of the application form to be submitted to the Animal Unit when commencing the study for release of animals.

The principal investigator has to:

Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as approved, or as amended.

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).

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.

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.

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.

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.

All animals found dead must be reported to the RAF on the appropriate form:

<http://www.health.uct.ac.za/fhs/research/animalethics/forms>

All animals found in distress must be reported to the RAF on the appropriate form.

Please quote the REC. REF in all your correspondence

Yours sincerely

Signature Removed

PROF PJ COMMERFORD
CHAIR, FHS AEC

Appendix 2.5

Western Cape Province

Telephone No: (027) 021 483 0000
 E-mail: permits.fax@capenature.co.za
 PGWC Shared Services Centre
 cnr Bosduif and Volstruis Streets
 Bridgetown
 7764



Facsimile No: (027) 0865567734
 Internet: www.capenature.co.za
 Private Bag X29
 Gatesville
 7766

PERMIT TO HUNT WITH PROHIBITED HUNTING METHOD OF WILD ANIMALS – RESEARCH PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance 1974, (Ord 19 of 1974)(Section 29 & 33)
 Not Transferable

HOLDER			
Full Name:	Mr. Kyle Kulenkampff	Identity No:	9206115314084
Trade Name:	University of Cape Town	Registration No:	
Postal Address	PO Box 13914	Physical Address:	Division of Forensic Medicine and
City / Town:	Mowbray	City / Town:	Observatory
Province / State:	Western Cape	Province / State:	Western Cape
Country:	South Africa	Country:	South Africa
Postal / Zip Code:	7705	Longitude:	
		Latitude:	

In terms of and to the provisions of the abovementioned Ordinance and the Regulations framed thereunder, the holder of this permit is hereby authorised to Hunt (capture/disturb/stampede/kill) the protected wild animal(s) specified below on the property mentioned on this permit. See conditions on last page.

DETAILS		
Permit / License No:	CN44-59-5015	Stamp:
Expiry Date:	31/12/2018	
Date Issued:	20/07/2018	
Amount Paid:	R 0.00	
Reference:		
File Code:	1/2/1/6/5/K	

DESCRIPTION	PROPERTY
Organization	University of Cape Town
Full Name:	Mr. Kyle Kulenkampff
Identity Number:	9206115314084
Postal Address	See special conditions for details
City / Town:	NA
Province / State:	Western Cape
Country:	South Africa
Postal / Zip Code:	0000
Longitude:	
Latitude:	

SPECIES (SCIENTIFIC NAME)	QTY	NOTE
Blow Flies (Calliphoridae)	30	Maximum at each nature reserve. For collection purposes.
Note. (Note.)	0	Method: Fly traps
Note. (Note.)	0	Conditions apply. Note special conditions.

F. Arnold

20/07/2018

Issued By:	Approved on Behalf CEO	Effective Date:	Signature of Holder:
Fadwa Arnold	Western CapeNature Conservation Board		I acknowledge, accept and understand fully the permit conditions as described.

STANDARD CONDITIONS

1. When the holder of this permit *kills/captures/collect any wild animal in terms thereof, he shall, before leaving the abovementioned property, or if he does not leave it, after each day's hunt/capture/collection, record the particulars regarding the date, species and number of each sex of each species, or if it is impossible to distinguish the sex, the total number of each species of such wild animals which he had *killed/capture/collected.
2. The holder of this permit shall return it to the Chief Executive Officer: Western Cape Nature Conservation Board, Private Bag X29, Gatesville, 7766, within 14 days of the date of expiry thereof.
3. THIS PERMIT IS SUBJECT TO THE SPECIAL CONDITIONS AS SET OUT IN THE ADDENDUM HERETO.

SPECIAL CONDITIONS

PROJECT TITLE: DNA barcoding of forensically important blowflies (Diptera: Calliphoridae) within the Western Cape of South Africa.

COLLECTION AREAS: Hottentots Holland Nature Reserve; Wesehof Private Nature Reserve; University of Cape Town Medical Campus; West Coast National Park; Bontebok National Park; Agulhas National Park.

METHOD: Fly traps.

NUMBER OF PERSONS ENGAGED IN THIS PROJECT:

1. Kyle Kulenkampff, 9206115314084
2. Dr Marise Heyns, 6701150061080

THIS PERMIT IS ISSUED SUBJECT TO THE FOLLOWING SPECIAL CONDITIONS:

1. The West Coast, Bontebok and Agulhas National Parks are managed by the South African National Parks Board and this institution must be approached for permits to collect specimens in these parks.
2. The data of all species collected must be captured on the SOB datasheet and submitted electronically to CapeNature (avlok@capenature.co.za) for inclusion in the SOB database.
3. Copies of all reports or publications emanating from this research must be forwarded to CapeNature (avlok@capenature.co.za) for dissemination to relevant staff.
4. The Conservation Manager of Hottentots Holland Nature Reserve (Monique Ruthenberg: mruthenberg@capenature.co.za), must be contacted at least a week in advance to discuss logistical arrangements for the field work.
5. The Conservation Manager has the right to refuse access to sensitive areas.
6. The permit holder must give a presentation about the research findings at one of CapeNature's Quarterly Ecological Meetings of the Region East. Kindly contact Dr AnneLise Vlok (avlok@capenature.co.za) or Ms Natalie Baker (nbaker@capenature.co.za) to arrange for a suitable date.

CONDITIONS APPLICABLE TO RESEARCHERS UNDERTAKING RESEARCH OR OTHER COLLECTING WORKS ON PROVINCIAL CONSERVATION AREAS AND / OR PRIVATELY OWNED LAND IN THE PROVINCE OF WESTERN CAPE:

1. THE MANAGER OF THE RELEVANT CONSERVATION AREA(S) (IF ANY) MUST BE INFORMED TIMEOUSLY BEFORE ANY CONSERVATION AREA IS ENTERED FOR COLLECTING OR RESEARCH PURPOSES AND THE MANAGER'S WRITTEN PERMISSION TO ENTER SUCH RESERVE MUST BE ACQUIRED BEFOREHAND. THIS PERMIT DOES NOT GRANT THE PERMIT HOLDER AUTOMATIC ACCESS TO ANY NATURE RESERVE, CONSERVATION AREA, WILDERNESS AREA AND / OR STATE FOREST. ANY OTHER / FURTHER CONDITIONS OR RESTRICTIONS THAT THE MANAGER MAY STIPULATE AT HIS / HER DISCRETION MUST ALSO BE ADHERED TO. THIS PERMIT MUST BE AVAILABLE TO BE SHOWN ON DEMAND.
2. The owner of any other land concerned (be it privately or publicly owned land) must give WRITTEN consent allowing the permit holder to enter said property to collect flora / fauna. This written permission must reflect the full name and address of the property owner (or of the person authorised to grant such permission), the full name and address of the person to whom the permission is granted and the number and species of the flora / fauna, the date or dates on which such flora / fauna may be picked / collected and the land in respect of which permission is granted. Copies of this written permission must be made available to The Western Cape Nature Conservation Board upon request.
3. Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognised South African scientific institution / museum / herbarium (preferably within the Province of Western Cape) where such material will be available to other researchers. For every flora specimen collected on a Western Cape Nature Conservation Board nature reserve, one additional (extra) herbarium specimen must be forwarded to the Western Cape Nature Conservation Board Herbarium at Jonkershoek (c/o MJ Simpson, Private Bag X5014, Stellenbosch 7599).
4. A list of all collected specimens / material including the; species name, the number collected, the collection date and the precise locality of the collection must be submitted within 14 days from the date of expiry of your permit to The Chief Executive Officer: CapeNature, Private Bag X29, Gatesvilles, 7766
5. The maximum number of specimens per species specified in the permit (if at all) may not be exceeded without the prior permission of The Chief Executive Officer: Western Cape Nature Conservation Board.
6. For projects of more than one year's duration a progress report must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board before 31 December of each year.
7. One copy of all completed reports, publications, or articles (including books, videos, CDs, DVDs etc.) resulting from the project/collection must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board free of charge.
8. Should a report, publication, article or thesis arise from this project/collection, an acknowledgement to Western Cape Nature Conservation Board must be included.
9. The Forest Act 1984 (Act 122 of 1984) and regulations, the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) and all regulations in terms of the Ordinance must be adhered to.
10. Should it be envisaged to export any material / specimens across the boundaries of the Western Cape Province, an export permit will be required in respect of certain species and a further application form will have to be completed. The permit holder must confirm with the Western Cape Nature Conservation Board whether an export permit is required BEFORE exporting any material / specimens from the Western Cape Province.
11. No species that appear on the Red Data List or species listed as endangered in terms of the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) may be collected, except for those mentioned on the permit.

12. Unless otherwise specifically indicated in writing, no material or specimens collected with this permit or material or specimens bred or propagated, from material or specimens collected with this permit, may be donated, sold or used for any commercial purpose by any party.

13. IF APPLICABLE, ETHICS CLEARANCE MUST BE ACQUIRED FROM YOUR RESEARCH INSTITUTE PRIOR TO COLLECTION.

CHIEF EXECUTIVE OFFICER
WCNCB

Appendix 2.6

To develop, expand, manage and promote a system of sustainable national parks that represent biodiversity and heritage assets, through innovation and best practice for the just and equitable benefit of current and future generations.



Permit number: CRC/2018-2019/005--2018/V1

CAPE RESEARCH CENTRE

P.O. Box 216, STEENBERG, 7947

Tel: +27 (0)21 713 7511; Fax: +27 (0)21 712 0131

19 June 2018

Research Permit: BONTEBOK, WEST COAST & AGULHAS NATIONAL PARKS

Kyle Kulenkampff - "DNA barcoding of forensically important blowflies (Diptera: Calliphoridae) within the Western Cape of South Africa"

University of Cape Town, Department of Pathology

Herewith the permit for your research project from **19 June 2018** until **30 June 2019**. The approval is subject to the following conditions. The Park Management staff must be to **all sections** of the Park.

Standard Conditions:

- The use of non-demarcated areas will lead to the disturbance of animals and eco-systems, trampling of vegetation and soil erosion and only the use of accepted pathways and areas is therefore permitted, **UNLESS BY SPECIAL ARRANGEMENTS. PLEASE CONTACT THE PARK MANAGEMENT STAFF IF RESTRICTED AREAS NEED TO BE ACCESSED.**
- No damage shall be permitted to any natural vegetation, environment or property.
- Animals may not be disturbed in any way.
- Uncontrolled vehicle access and parking could cause damage to vegetation and soil erosion and therefore only the use of approved vehicles routes and parking areas is allowed.
- Fires can cause loss of vegetation, soil erosion and life and therefore fires, and braai's are not permitted unless in dedicated braai areas.
- Remove all rubbish and waste as it has an effect on the health of visitors, animals and plants.
- Other visitors to the area and or neighbours may not be hindered in any way.
- Pollution affects the health and safety of animals, plants, visitors and neighbours and is not permitted.
- Excessive noise affects animals (e.g. birds nesting in the areas), visitors and or neighbours and is not permitted.
- Your permit and identification must be retained and kept on your person at all times, and produced on request.
- The areas under the control of SANParks are used entirely at your own risk. South African National Parks, its Board, directors, employees and agents are not liable for any loss or damage to the property or possession of any guest or participant (or accompanying minor) whether such damage is caused by the negligent act or omission of South African National Parks; arising from death or any bodily injuries of whatsoever nature sustained by a guest or participant (or accompanying minor) whether such injuries are caused by the negligent act or omission by South African National Parks, and/or by the defective functioning of any apparatus. The guest or participant and/or his/her/their estate hereby indemnifies South African National Parks against any claim, action, judgment, costs and/or expenses which may

addo elephant
agulhas
augrabies
bontebok
camdeboo
golden gate highlands
karoo
kglalagadi transfrontier
knysna lake area
kruger
mapungubwe
marakele
mokala
mountain zebra
namaqua
table mountain
tankwa karoo
tsitsikamma
richtersveld
west coast
wilderness

be made against South African National Parks and as may in any way be related to the above.

The onus lies with the company or applicant to ensure that they are adequately insured

- Please note that you (your staff etc) are subject to the conditions of Section 86 of the National Environmental Management Act (107 of 1998) and the National Environmental Act: Protected Areas Act (Act 57 of 2003) and any other relevant legislation for the duration of your stay in the National Park (e.g. adherence to the Registrar's requirements for guides).
- SANParks staff's instructions, notices, regulations and signs must be complied with.
- The activity shall be restricted to the area applied for.
- Gate and operating times to be complied with.
- NO PETS ALLOWED.

Special Conditions:

- The researcher may collect blowflies using a baited fly trap.
- 30 specimens from each of the common blow fly species may be collected

Area	Park Management Staff	Telephone Number:
ANP	Johan Taljaard (Park Manager) Johan.Taljaard@sanparks.org	028 435 6078
BNP	Bradley Koopman Bradley.Koopman@sanparks.org	028 514 3206
WCNP	Carmen Gagiano carmen.gagliano@sanparks.org	022 772 2144

Yours faithfully,

Signature Removed

Debbi Winterton- Science Liaison Officer; E-mail: Deborah.winterton@sanparks.org

- addo elephant
- agulhas
- augrabies
- bontebok
- camdeboo
- golden gate highlands
- karoo
- kgalagadi transfrontier
- knysna lake area
- kruger
- mapungubwe
- marakele
- mokala
- mountain zebra
- namaqua
- table mountain
- tankwa karoo
- tsitsikamma
- richtersveld
- west coast
- wilderness

Appendix 2.7

DNA extraction optimisation

Six specimens of *L. cuprina* were selected. The specimens were dissected creating batches that used either two, four or six legs or the flight muscle as the tissue used for the DNA extraction (Table 2.2). DNA extraction was done following the previously established methodology.

Table 2.2: DNA quantification results of extracted DNA of six *L. cuprina* specimens.

Tissue weight (g) refers to the weight of four legs from each specimen that went through the DNA extraction process. The DNA concentration, and absorbance values were determined using Nanodrop™ spectrophotometry using a Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts).

Sample	Tissue used	Tissue weight (mg)	mean DNA concentration (ng/μl)	mean A260/208	mean A260/230
OE1	2 legs	0.1	8.7	1.47	0.15
OE1	4 legs	0.3	10.3	1.52	0.18
OE2	2 legs	0.2	6.8	1.63	0.20
OE2	4 legs	0.7	8.1	1.53	0.56
OE3	2 legs	0.4	7.8	1.69	0.20
OE3	4 legs	0.6	10.2	1.63	0.18
OE4	2 legs	0.2	11.1	1.67	0.49
OE4	4 legs	0.3	9.0	1.54	0.32
OE5	6 legs	1.8	7.5	1.69	0.46
OE5	Flight muscle	1.8	7.7	1.67	0.35
OE6	6 legs	1.4	8.6	1.62	0.25
OE6	Flight muscle	1.7	7.3	1.73	0.14

Appendix 2.8

Protocol for DNA extraction:

Note: A precipitate may have formed in the DNA Pre-Wash Buffer during shipping. To completely resuspend the buffer, incubate the bottle at 30 – 37 °C for 30 minutes and mix by inversion. **DO NOT MICROWAVE.**

(For optimal performance, add beta-mercaptoethanol (user supplied) to the **Genomic Lysis Buffer** to a final dilution of 0.5%(v/v) i.e., 500 µl per 100 ml.)

1. Add specimen(s) to a **ZR BashingBead™ Lysis Tube (2.0 mm)**. Add 750 µl **Lysis Solution** to the tube and cap tightly to prevent leakage.

Note: *Generally, no more than 50 mg tissue should be sampled, for larger samples will exceed the DNA binding capacity of the spin column (See **Specifications** on page 1 of the manual provided with the extraction kit).*

Note: *4 legs were used in this experiment, to retain two legs on the voucher specimen in case further morphological analysis is required*

2. Place the tubes in the TissueLyser Adapter Set 2 x 24.

Note: *The adapter set contains 2 adapters, with each adapter comprised of a top plate and a bottom plate for holding a rack of capped collection microtubes (Figure 5). Sample disruption can be carried out at room temperature or after storing the adapter set at – 80°C for at least 2 hours. The adapter set can be cleaned with detergent, microbicides, or up to 96% ethanol.*

(For this experiment the adapter set was placed in a -20°C freezer overnight.)

Note: The Following protocol was used following the guidelines and safety protocols outlined in the TissueLyser II user manual. **(Make sure that the adapter sets are balanced.)**

3. Operate the TissueLyser for 10 minutes at 15 Hz.
4. Allow to incubate for **10 minutes**.
5. Centrifuge the **ZR BashingBead™ Lysis Tube (2.0 mm)** in a microcentrifuge at $\geq 10,000 \times g$ for 1 minute.
6. Transfer up to 400 μl supernatant to a **Zymo-Spin™ IV Filter** in a **Collection Tube** and centrifuge at 7000 rpm (approximately 7,000 $\times g$) for 1 minute.

Note: *Snap off the base of the Zymo-Spin™ IV Filter prior to use.*

7. Transfer the supernatant in a **Standard 2ml tube**.
8. Add 1,200 μl of **Genomic Lysis Buffer** to the filtrate in the **Standard 2ml tube** from Step 7. Mix using an agitator.
9. Allow to incubate for **1 hour**.
10. Transfer 800 μl of the mixture from Step 9 to a **Zymo-Spin™ IIC Column** in a **Collection Tube** centrifuge at 10,000 $\times g$ for 1 minute.

Note: *the Zymo-Spin™ IIC Column has a capacity of about 800 μl .*

11. Discard the flow through from the **Collection Tube** and **repeat Step 10**.
12. Add 200 μl **DNA Pre-Wash Buffer** to the **Zymo-Spin™ IIC Column** in a new **Collection Tube** and centrifuge at 10,000 $\times g$ for 1 minute.

13. Add 500 μ l **g-DNA Wash Buffer** to the **Zymo-Spin™ IIC Column** and centrifuge at 10,000 $\times g$ for 1 minute.
14. Discard the flow through from the **Collection Tube**.
15. Add 500 μ l **ethanol (96-100%)** to the **Zymo-Spin™ IIC Column** and centrifuge at 10,000 $\times g$ for 1 minute.
16. Discard the flow through from the **Collection Tube**.
17. **Centrifuge at full speed (20,000 $\times g$; 14,000 rpm) for 3 min to dry the membrane completely.**

***Note:** This step is necessary, since ethanol carryover into the elute may interfere with some downstream applications.*

18. Transfer the **Zymo-Spin™ IIC Column** to a clean 1.5 ml microcentrifuge tube and add 100 μ l (35 μ l minimum) **DNA Elution Buffer** directly to the column matrix. Centrifuge at 10,000 $\times g$ for 30 seconds to elute the DNA.

***Note:** Elute w/ 35 μ l for highly concentrated DNA.*

(To obtain maximal yields of DNA it will be necessary to elute with 100-150 μ l DNA elution buffer).

Appendix 2.9

Table 2.3: A Table showing a list of primers that were used in this study.

Region to amplify	Primer Name	Sequence (5'-3')	Direction	Reference
<i>COI</i>	LCO1490	GGTCAACAAATCATAAAGATATTGG	Forward	Folmer <i>et al.</i> , 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Reverse	Folmer <i>et al.</i> , 1994
	LepF1	ATTCAACCAATCATAAAGATAT	Forward	Herbert <i>et al.</i> , 2004
	LepR1	TAAACTTCTGGATGTCCAAAAA	Reverse	Herbert <i>et al.</i> , 2004
	TW-J-1289	ACTAATAGCCTTCAAAGC	Forward	Marinho <i>et al.</i> , 2012
	C1-N2320	AATCCTAATAATCCAATAGC	Reverse	Marinho <i>et al.</i> , 2012
<i>ITS2</i>	ITS2_F	TGCTTGACTACATATGGTTGA	Forward	Song <i>et al.</i> , 2008
	ITS2_R	GTAGTCCCATATGAGTTGAGGTT	Reverse	Song <i>et al.</i> , 2008

Appendix 2.10

DNA amplification optimisation:

The amplification of DNA was optimised in multiple stages. For these optimisation stages a single specimens DNA was used. Gel electrophoresis was run according to the previously established methods. Band intensity between the sample run at different conditions was used to determine the most optimal method, Figure 2. gives an example of different band intensity.

The first stage was to perform a temperature gradient for each primer. Temperature ranges were determined by selecting a range of 5 °C above and below the melting temperatures of the respective primers. Following this the number of cycles that the PCR was run for was increased from 30 to 35 cycles along with doubling the time for each phase of the PCR. The last stage of the optimisation was to include a cofactor. MgCl₂ was introduced in concentrations of 2 mM, 2.5mM and 3 mM. 1 µl of 5x AmpSolution (Promega, Madison) was also tested.

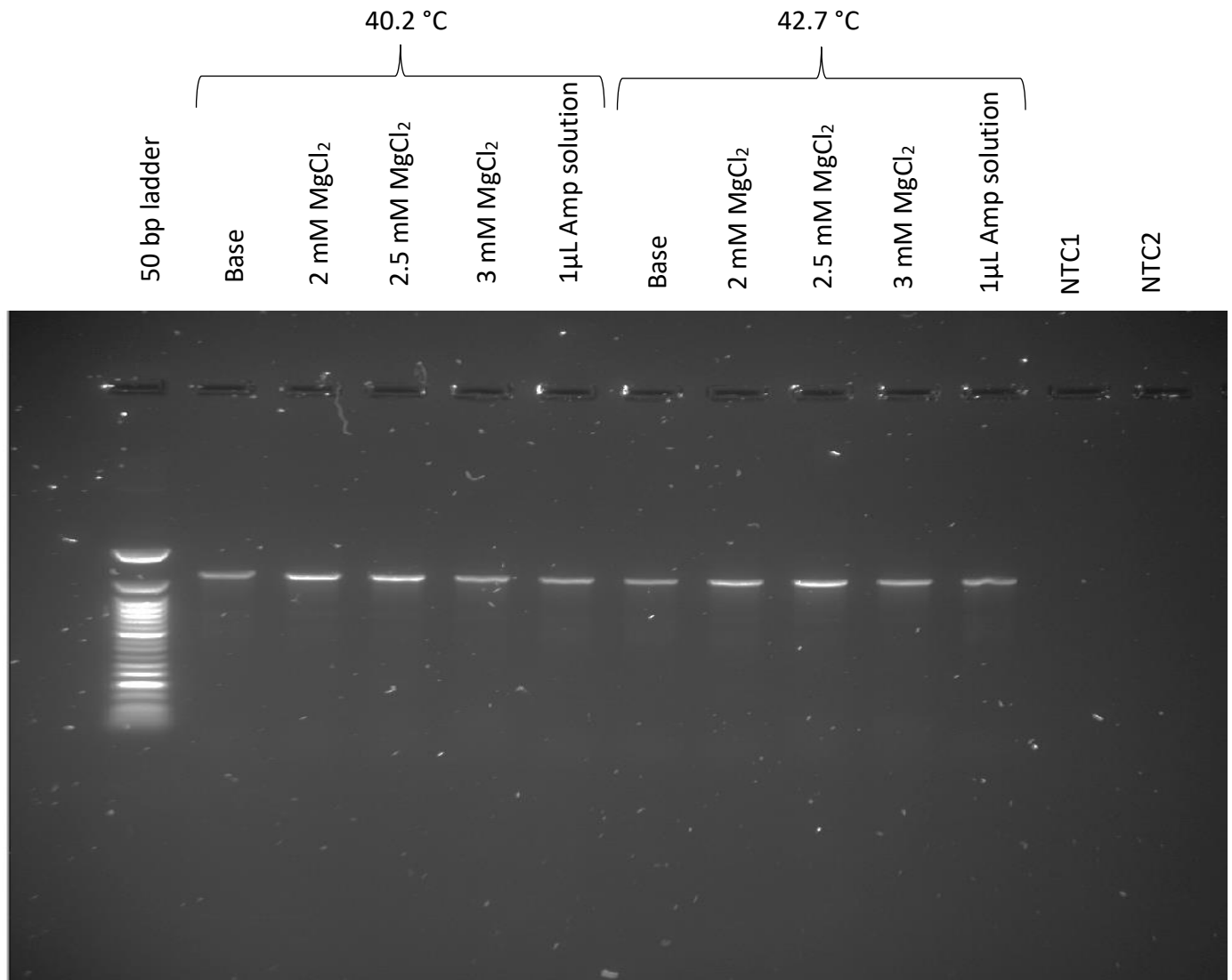


Figure 2.4: Agarose gel showing chemical gradient.

MgCl₂ was introduced in concentrations of 2 mM, 2.5mM and 3 mM, as well as, 1 μl of 5x AmpSolution (Promega, Madison). The samples were tested at different annealing temperatures in the PCR cycle conditions. Base refers to a baseline sample without the addition of cofactors. NTC refers to a no template control. A Quick-Load® Purple 50 bp DNA Ladder (New England BioLabs, Massachusetts) for confirmation of amplification of the loci and estimation of amplicon size. DNA was visualized with a SYBR™ safe gel stain (5 μl / 50 ml gel) (Invitrogen, Carlsbad), using a Chemi Genius Bio Imaging Transluminator Gel docuenter (Syngene, Cambridge) using the GeneSnap Image Acquisition software vs. 7.12.06 (Syngene, Cambridge).

Appendix 3.1



Figure 3.8: Z axial stacked images of the dorsal, lateral and ventral positions of *Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala* and *Hemipyrella fernandica*.

Appendix 3.2



Figure 3.9: Z axial stacked images of the dorsal positions of *Lucilia cuprina* showing the morphological variation in colour of the exoskeleton.

Appendix 3.3

Table 3.6: DNA quantification results of extracted DNA of 41 blow fly specimens for the included Calliphoridae species.

Tissue weight (g) refers to the weight of four legs from each specimen that went through the DNA extraction process. The DNA concentration, and absorbance values were determined using Nanodrop™ spectrophotometry using a Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific™, Massachusetts).

Process ID	Species	Tissue weight (mg)	Mean DNA concentration (ng/ul)	Mean A260/280	Mean A260/230
BFSAB001-19	<i>Ch. megacephala</i>	1.1	10.7	1.76	0.24
BFSAB002-19	<i>Ch. megacephala</i>	0.9	10.6	1.68	0.23
BFSAB003-19	<i>L. sericata</i>	0.6	14.9	1.55	0.23
BFSAB004-19	<i>Ch. megacephala</i>	1.2	12.3	1.53	0.23
BFSAB005-19	<i>Ch. albiceps</i>	1.7	8.3	1.77	0.23
BFSAB006-19	<i>L. cuprina</i>	0.5	5.4	2.03	0.23
BFSAB007-19	<i>L. cuprina</i>	0.9	11.4	1.60	0.23
BFSAB008-19	<i>L. cuprina</i>	0.1	13.5	1.59	0.23
BFSAB009-19	<i>L. cuprina</i>	0.8	7.9	1.75	0.23
BFSAB010-19	<i>Ch. megacephala</i>	1.6	7.6	1.67	0.23
BFSAB011-19	<i>Ch. megacephala</i>	1.6	4.0	1.70	0.23
BFSAB012-19	<i>L. cuprina</i>	1.3	11.1	1.58	0.23
BFSAB013-19	<i>L. cuprina</i>	1.0	14.2	1.55	0.23
BFSAB014-19	<i>Ch. marginalis</i>	1.7	10.6	1.46	0.22
BFSAB015-19	<i>L. cuprina</i>	0.4	12.6	1.66	0.23
BFSAB016-19	<i>L. cuprina</i>	0.4	14.4	1.61	0.23
BFSAB017-19	<i>L. sericata</i>	1.0	8.9	1.66	0.23
BFSAB018-19	<i>L. cuprina</i>	0.7	11.4	1.53	0.23
BFSAB019-19	<i>Ch. chloropyga</i>	1.4	10.3	1.62	0.23
BFSAB020-19	<i>Ch. marginalis</i>	1.7	4.3	1.70	0.24
BFSAB021-19	<i>Ch. marginalis</i>	1.1	7.5	1.41	0.24
BFSAB022-19	<i>Ch. megacephala</i>	1.7	6.3	1.79	0.24
BFSAB023-19	<i>Ch. albiceps</i>	1.2	6.0	1.77	0.25
BFSAB024-19	<i>Ch. marginalis</i>	2.4	4.8	1.73	0.24
BFSAB025-19	<i>Ch. megacephala</i>	0.8	6.5	1.49	0.25
BFSAB026-19	<i>Ch. marginalis</i>	2.2	7.0	1.85	0.25
BFSAB027-19	<i>Ch. megacephala</i>	1.8	8.9	1.58	0.25

Table 3.6: DNA quantification results of extracted DNA of 41 blow fly specimens for the included Calliphoridae species continued.

Tissue weight (g) refers to the weight of four legs from each specimen that went through the DNA extraction process. The DNA concentration, and absorbance values were determined using Nanodrop™ spectrophotometry using a Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific™, Massachusetts).

Process ID	Species	Tissue weight (mg)	Mean DNA concentration (ng/ul)	Mean A260/280	Mean A260/230
BFSAB028-19	<i>Ch. albiceps</i>	1.2	5.0	1.58	0.26
BFSAB029-19	<i>L. sericata</i>	1.2	7.3	1.66	0.23
BFSAB030-19	<i>Ch. chloropyga</i>	0.9	9.3	1.66	0.22
BFSAB031-19	<i>L. sericata</i>	0.8	11.1	1.66	0.23
BFSAB032-19	<i>L. sericata</i>	0.5	9.5	1.42	0.22
BFSAB033-19	<i>Ch. chloropyga</i>	0.7	11.9	1.40	0.19
BFSAB034-19	<i>Ch. chloropyga</i>	1.5	6.6	1.43	0.19
BFSAB035-19	<i>L. cuprina</i>	0.7	12.3	1.47	0.18
BFSAB036-19	<i>Ch. chloropyga</i>	0.1	10.1	1.49	0.19
BFSAB037-19	<i>L. cuprina</i>	0.5	10.6	1.42	0.20
BFSAB038-19	<i>Ch. chloropyga</i>	0.2	10.5	1.43	0.17
BFSAB039-19	<i>L. cuprina</i>	1.3	9.6	1.72	0.19
BFSAB040-19	<i>L. sericata</i>	0.7	15.2	1.59	0.21
BFSAB041-19	<i>L. sericata</i>	0.3	11.2	1.68	0.21
BFSAB001-19	<i>Ch. chloropyga</i>	0.4	10.9	1.58	0.25
BFSAB002-19	<i>H. fernandica</i>	0.3	14.0	1.56	0.24
BFSAB003-19	<i>Ch. chloropyga</i>	0.2	9.4	1.83	0.03
	Mean value:	1.0 ± 5.8	9.6 ± 3.0	1.63 ± 0.14	0.22 ± 0.04

Appendix 3.6

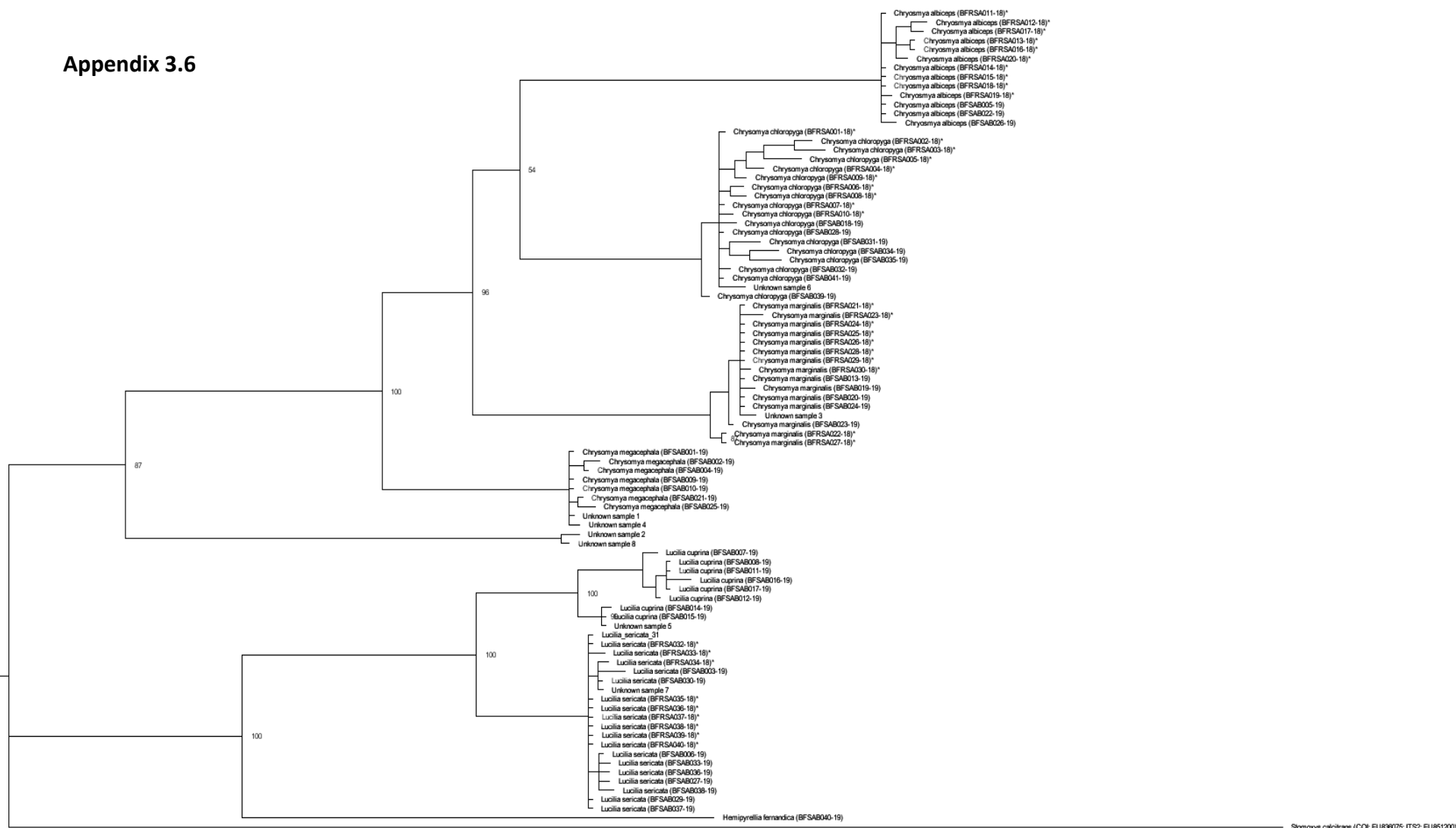


Figure 3.12: Bayesian inference topology for the combined 5' section of cytochrome c oxidase 1 and second internal transcribed spacer for seven blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga*, *Chrysomya marginalis*, *Chrysomya megacephala*, *Hemipyrellia fernandica*, *Lucilia cuprina* and *Lucilia sericata*) and eight unknown samples.

Values on each node represent the posterior probability value (pP) derived from the Bayesian inference analysis. Branches with an * were included from Cooke et al., 2018. The respective BOLD process ID's are listed in parenthesis after each sample. *Stomoxys calcitrans* was included as an outgroup, with the GenBank® accession numbers in parenthesis (Dsouli et al., 2011).