MOLECULAR SYSTEMATICS AND BIOLOGY OF TWO CLOSELY RELATED BLOWFLIES: *LUCILIA SERICATA* AND *LUCILIA CUPRINA*

A thesis submitted in fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY
of
RHODES UNIVERSITY

by

KIRSTIN ALEXA WILLIAMS

June 2014

ABSTRACT

The greenbottle blowflies, *Lucilia sericata* (Meigen, 1826) and *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) are very difficult to distinguish on the basis of their external morphology. The literature suggests that these two species may be interbreeding. Sequencing two nuclear (*28S rRNA* and *Period*) and one mitochondrial (*COI*) gene indicated that there has been an ancient hybridization event and that mtDNA of *L. sericata* has become fixed in a lineage of *L. cuprina* through mtDNA introgression, possibly involving *Wolbachia* infection. This has implications for identifications of these species based on mtDNA alone.

No study has shown explicitly that hybrids of *L. sericata* and *L. cuprina* can be identified morphologically. Morphological characters used to identify *L. sericata* and *L. cuprina* were scored and tested using specimens of both species and known hybrids. Discriminant function analysis of the characters successfully separated the specimens into three unambiguous groups – *L. sericata*, *L. cuprina* and hybrids. This is the first evidence that hybrids of these two species can be identified from physical characteristics.

Lucilia sericata and *L. cuprina* have medical, veterinary and forensic importance. Knowing their distribution in South Africa would allow more effective management and utilisation of these flies. Their predicted geographic distributions in South Africa were modelled using maximum entropy analysis of selected climatic variables. The most important environmental variables in modelling their distributions were magnitude of monthly rainfall and the magnitude of the monthly maximum temperature for *L. sericata*, and the seasonal variation in monthly mean humidity and

magnitude of monthly rainfall for *L. cuprina*. Both species have a widespread distribution in South Africa and one therefore cannot identify specimens of these flies by locality of capture alone.

Luciliinae is a diverse and geographically widespread subfamily containing four genera - *Hemipyrellia, Lucilia, Dyscritomyia* and *Hypopygiopsis* – that all contain parasitic species ranging from saprophages to obligate parasites. The phylogenetic relationships between these genera are unclear. The *28S rRNA*, *COI* and *Period* genes of 14 species of *Lucilia* and *Hemipyrellia* were partially sequenced and analysed together with 11sequences from GenBank and the Barcode of Life Database (BOLD). *Lucilia sericata* and *L. cuprina* were shown to be sister-species. Three cases of paraphylly were identified within *Lucilia* that affects identification of these species using mtDNA alone. *Hemipyrellia* consistently caused *Lucilia* to be paraphyletic when it was included in analyses, so *Hemipyrellia* should be synonymized with *Lucilia*. The relationships of *Dyscritomyia* and *Hypopygiopsis* to *Lucilia* are unclear and further studies are required. No geographic pattern was found within the different forms of parasitism within this group, but the different degrees of parasitism were phylogenetically clustered.

To Allan, Linda, Liesl and Joanne - the best family in the world

PUBLICACTIONS

Published manuscripts

Williams, K.A. and Villet, M.H. (2013). Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae). *European Journal of Entomology* 110: 187-196.

Williams, K.A., Richards, C.S. and Villet, M.H. (2014). Predicting the geographic distribution of *Lucilia sericata* and *Lucilia cuprina* (Diptera: Calliphoridae) in southern Africa. *African Invertebrates* 55(1): 157 – 170.

Williams, K.A. and Villet, M.H. (2014). Morphological identification of *Lucilia sericata*, *Lucilia cuprina* and their hybrids (Diptera: Calliphoridae). *ZooKeys* 420: 69 -85.

Table of Contents

ABSTRACT ii
PUBLICACTIONS v
Published manuscripts v
LIST OF TABLES ix
LIST OF FIGURES xi
PREFACE xv
CHAPTER 1 1
INTRODUCTION 1
Aims 3
REFERENCES 4
CHAPTER 2 9
ANCIENT AND MODERN HYBRIDIZATION BETWEEN LUCILIA SERICATA AND LUCILIA CUPRINA (DIPTERA: CALLIPHORIDAE) 9
ABSTRACT 9
INTRODUCTION 10
MATERIALS AND METHODS 11
RESULTS 13
DISCUSSION 15
Ancient hybrids and introgression 16
Modern hybrids 17
DNA-based identification 18
ACKNOWLEDGEMENTS 19
REFERENCES 21
CHAPTER 3 40
MORPHOLOGICAL IDENTIFICATION OF LUCILIA SERICATA, LUCILIA CUPRINA AND THEIR HYBRIDS (DIPTERA: CALLIPHORIDAE) 40
ABSTRACT 40
INTRODUCTION 41
MATERIALS AND METHODS 42
RESULTS 43
Univariate assessment of characters 43

Multivariate assessments of characters 46
DISCUSSION 46
Assessment of characters 46
Geographical variation 46
Identifying hybrids 47
CONCLUSION 47
ACKNOWLEDGMENTS 47
REFERENCES 49
CHAPTER 4 67
PREDICTING THE GEOGRAPHIC DISTRIBUTION OF LUCILIA SERICATA AND LUCILIA CUPRINA (DIPTERA: CALLIPHORIDAE) IN SOUTHERN AFRICA 67
ABSTRACT 67
INTRODUCTION 68
MATERIALS AND METHODS 69
Locality records 69
Environmental variables 70
Model building 71
Post hoc comparisons 71
RESULTS 72
DISCUSSION 73
ACKNOWLEDGEMENTS 76
REFERENCES 77
CHAPTER 5 91
BIOGEOGRAPHICAL AND ADAPTIVE RADIATION OF THE GREENBOTTLE FLIES (DIPTERA: CALLIPHORIDAE: LUCILIINAE) 91
ABSTRACT 91
INTRODUCTION 92
MATERIALS AND METHODS 94
DNA data 94
Morphological data 95
Phylogenetic analysis 95
RESULTS 96
Molecular data 96

Morphological data 98
DISCUSSION 98
Relationship of L. sericata *and* L. cuprina 98
Molecular identification of Lucilia *species* 99
Diversification of Luciliinae 101
Taxonomy of Luciliinae 102
CONCLUSION 106
ACKNOWLEDMENTS 107
REFERENCES 108
CHAPTER 6 131
CONCLUSION 131

LIST OF TABLES

Table 2.1: Genes used in studies of *Lucilia sericata* and *Lucilia cuprina*. 28

Table 2.2: Specimen locality data for sequences included from GenBank 29

Table 2.3: Specimen locality data for sequences from this study added to GenBank (* indicate identical sequences that are represented by one sequence in the Bayesian Inference tree, M = Male, F = Female) 30

Table 3.1: Specimens previously identified by molecular markers (Williams & Villet, 2013) used in the morphological analyses. (*hybrids) 51

Table 3.2: Published morphological characters used to distinguish specimens of *Lucilia sericata* and *L. cuprina*. 53

Table 3.3: Eigen vectors and values for the first two roots of the discriminant function analysis 58

Supplementary Table S1: Character-taxon matrix used in the MDS and DFA analyses. 66

Table 4.1: Mean AUC values for whole models... 83

Table 5.1: Specimen locality data for sequences added to GenBank. (Accession numbers starting KF are new sequences from this study). 117

Table 5.2: GenBank sequences included in this study. 120

Table 5.3: Binary coding of 14 morphological characters for the *Lucilia* and *Hemipyrellia* genera. [1 – Colour of the basicostal scale (0 = black/brown, 1 = white/cream); 2 – Number of postsutural acrostichal bristles (0 = two pairs, 1 = three pairs); 3 – Eye separation in the male (0 = distance of greater than the width of the third antennal segment, 1 = less than the width of the third antennal segment); 4 – Number of anterio-dorsal bristles on the mid tibia (0 = one, 1 = two); 5 – Colour of the palpi (0 = yellow/orange, 1 = black/brown); 6 – Subcostal sclerite (0 = bristles absent, 1 = bristles present); 7 – Colour of the squamae (0 = uniform white/cream, 1 = partially or totally brown); 8 – Wings (00 = hyaline, 01 = lightly infuscated, 11 = heavily infuscated); 9 – Eye separation in the female (0 = distance of greater than one quarter of the width of the head, 1 = less than one quarter of the width of the head); 10 – Colour of antennae (0 = uniformly dark, 1 = non-uniform); 11 – Male hypopygium (00 = inconspicuous, 01 = conspicuous, 11 = highly conspicuous); 12 – Colour of abdomen and thorax (0 = predominantly brassy green/green, 1 =

predominantly purple/blue/black); 13 – Colour of the legs (00 = dark brown, 01 = brown/black, 11 = black); 14 – Lower squamal lobe (0 = setae absent, 1 = setae present)] (Stevens & Wall, 1996). 122
Table 5.4. Zoogeographic distribution of Lucilliinae species 124

LIST OF FIGURES

Figure 2.1: World map showing the localities where flies were caught. Insert: Map of South Africa showing the towns where flies were caught……………….…35

Figure 2.2 a and b: Bayesian Inference trees constructed from nuclear genes (*28S* and *Per*) (left) and mitochondrial genes (*COI*) (right) data. Posterior probabilities are indicated on nodes. Blue rectangle = ancient hybrid clade, green rectangle = modern hybrids [C = *cuprina*, S = *sericata*, CV = *Calliphora vicina*, In = *Lucilia infernalis* AUS = Australia, CAN = Canada, FRC = France, GER = Germany, GRC = Greece, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB = Witbank]……………………………………….36

Figure 2.3: NeighborNet network diagram constructed from *28S* & *Per* data. [C = *cuprina*, S = *sericata*, AUS = Australia, CAN = Canada, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB = Witbank]…………………….. 37

Figure 2.4: NeighorNet network diagram constructed from *COI* data. [C = *cuprina*, S = *sericata*, AUS = Australia, CAN = Canada, FRC = France, GER = Germany, JPN =Japan, NAM = Namibia, EGT = Egypt, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB = Witbank]………………………38

Figure 2.5: NeighborNet network diagram constructed from *28S* & *Per* & *COI* concatenated data. [C = *cuprina*, S = *sericata*, AUS = Australia, CAN = Canada, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB =Witbank]………………………………………………………………39

Figure 3.1: Paravertical setulae, distance between the outer and inner vertical setae, the size of the angle at the inner vertical triangle and extent of metallic sheen on parafrontal sclerites. *L. sericata* (A) and *L. cuprina* (B)………………....59
Figure 3.2: Relative width of frontal stripe – *L. sericata* (A) and *L. cuprina* (B)……60
Figure 3.3: Colour of the frontoclypeal membrane. *L. sericata* (A) and *L. cuprina* (B)……………………………………………………………………….61
Figure 3.4: Number of setae on 'quadrat' between the anterior margin and discal setae on the scutellum. *L. sericata* (A) and *L. cuprina* (B)……….……….………62
Figure 3.5: Posterior slope of the humeral callus behind the basal setae and the posterior edge of notopleuron behind the posterior notopleural seta. *L. sericata* (A) and *L. cuprina* (B)……….…………………………………………….…63
Figure 3.6: Non-metric Multi-Dimensional Scaling plot using a Manhattan distance metric using 11 characters. Light blue solid circles = L. sericata, Green open circles = L. cuprina, dark blue squares = introgressed hybrids, purple triangles = modern hybrids……………………………..……………………….……64
Figure 3.7: Ordination plot of the first two roots of the discriminant function analysis using seven characters. Ellipses represent 95% confidence regions. Light blue solid circles = L. sericata, Green open circles = L. cuprina, dark blue squares = introgressed hybrids, purple triangles = modern hybrids ……………………65
Figure 4.1: Map of South Africa showing the collecting trip routes to collect *Lucilia sericata* (A) and *Lucilia cuprina* (B) blowflies. EC – Eastern Cape, FS – Free State, G – Gauteng, KZN – KwaZuluNatal, L – Limpopo, M – Mpumalanga, NC – Northern Cape, NW – North West, WC – Western Cape ….……………84
Figure 4.2: Modified Red-top™ Fly Trap. ……………………......………………85
Figure 4.3: Wool production of magisterial districts, estimated by total grease mass produced in 2011/2012 in South Africa …..…………………………………86
Figure 4.4: Population density in South Africa in 2011. …….………………………87
Figure 4.5: Plots showing the correlation between the predicted distribution of *L.cuprina* (A & C) and *L. sericata* (B & D) and grease mass (kg) and human population density values (log values)……………………………………………88
Figure 4.6: Mean predicted distribution maps for *L. sericata* (A) and *L. cuprina* (B) produced using museum records, survey data and personal contact localities.

The colour range indicates the likelihood of species distribution from dark blue (least likely) to red (most likely)………..………………………………89

Figure 4.7: Jack-knife of climatic variables AUC for *L. sericata* (A) and *L. cuprina* (B)……………………………………………………………………………90

Figure 4.8: Mean distribution maps for *L. sericata* (A &B) and *L. cuprina* (C & D) from museum (A & C) and survey (B & D) data only……….……………………91

Figure 5.1: Bayesian inference tree constructed from concatenated nuclear genes *28S* & *Per*. Posterior probabilities are indicated on nodes. Green box = *Hemipyrellia fernandica* [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica*, IL = *illustris*, In = *infernalis*, Mx = *mexicana*, Pa = *papuensis*, Po = *porphyrina*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town] ……………………….…126

Figure 5.2: Bayesian inference tree constructed from mitochondrial gene *COI*. Posterior probabilities indicated on nodes. Green box = *Hemipyrellia* sp. Blue box = *Dysctritomyia* sp. [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica* IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town] …………………………………………………………………………….127

Figure 5.3: Bayesian inference tree constructed from the concatenated nuclear (*28S* & *Per*) and mitochondrial (*COI*) genes. Posterior probabilities indicated on nodes. Green box = *Hemipyrellia fernandica*. [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica* IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S =

sericata, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town] ……..……………………………………..……128

Figure 5.4: NeighborNet network diagram constructed from *COI* data showing parasitic behaviour and previous sub-generic status of *Lucilia*. Text colours: Red = primary facultative parasite, green = secondary facultative parasite, purple = parasite (unknown if primary or secondary), blue = saprophage, black = unknown parasitic behaviour. [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica*, IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominica, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT=Cape Town] ….…………………………………………………129

Figure 5.5: Bayesian inference tree constructed using *COI* barcode sequences. Posterior probabilities indicated on nodes. Support within the collapsed nodes is variable. Green box = *Hemipyrellia* sp. .………………………………130

Figure 5.6: Majority rule consensus tree for 21 species of *Lucilia* and *Hemipyrellia* constructed from morphological characters listed in Table 5.3. Gren box = *Hemipyrellia* sp……………………………………….………………131

PREFACE

To God be the glory!

I would like to sincerely thank my supervisor and mentor, Prof. Martin Villet, for his continued support and encouragement. His belief that I could achieve this while working full-time has given me the motivation to keep going when at times it seemed never-ending!

To my parents, Allan and Linda, and my sisters, Liesl and Joanne, thank you for allowing me to be me and for your continual support, encouragement and prayers! I couldn't ask for a more supportive or loving family.

Thank you to the management of the Durban Natural Science Museum for financial support and allowing me the opportunity to take a 6 month sabbatical to complete this degree.

Thank you to the National Research Foundation (NRF) of South Africa and Rhodes University for providing funding. Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Research Foundation.

Acknowledgments are listed at the end of each chapter to thank people for specific assistance.

CHAPTER 1

INTRODUCTION

The greenbottle blowflies belong to the genus *Lucilia* and although there are over 200 nominal species, only two occur in South Africa. These two species – *L. sericata* and *L. cuprina* – are cosmopolitan, occurring on six continents (Waterhouse & Paramonov, 1950; Rognes, 1980; Norris, 1990; Bishop, 1991; Holloway, 1991; Rognes, 1994; Bishop, 1995; Fischer, 2000; Harvey *et al*., 2003a, 2003b, 2008; Chen, et al., 2004; Heath & Bishop, 2006; Park *et al*., 2009; Liu *et al*., 2011; Boehme *et al*., 2012; GilArriortua *et al*., 2013).

Lucilia sericata is the most commonly used fly species for maggot debridement therapy (MDT) (Altincicek & Vilcinskas, 2009; Vilcinskas, 2011) and it has recently been shown that *L. cuprina* can also safely be used for MDT (Paul *et al*., 2009; Tantawi *et al*., 2010; Kingu *et al*., 2012). The larvae of both of these species feed on decomposing animal tissue and are thus useful in forensic entomological investigations to determine post mortem intervals (PMI). *Lucilia cuprina* is often referred to as the sheep-strike blowfly (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006) because it is responsible for cutaneous myiasis in sheep which causes millions of dollars' worth of damage in the sheep farming industry each year. *Lucilia sericata* does not appear to strike sheep in the southern hemisphere but has been reported to cause sheep-strike in parts of Europe (Ullyett, 1945; Rose & Wall, 2011). Identification of these flies is therefore important for use in MDT, forensic entomology and controlling sheep-strike.

Ullyett (1945) suggested that *L. sericata* and *L. cuprina* were capable of interbreeding and producing fertile offspring. The discovery of hybrids in a molecular study (Stevens *et al*., 2002) was thought to be a geographically isolated occurrence. The idea of hybrids of these two species has been widely studied with numerous researchers suggesting that two subspecies of *L. cuprina* exist – *L.c. cuprina* and *L.c. dorsalis* or that three species should be recognized (Waterhouse & Paramonov, 1950; Norris, 1990; Stevens & Wall, 1996; Stevens *et al*., 2002; Stevens, 2003; Wallman *et al*., 2005; Wells *et al*., 2007; DeBry *et al*., 2010). Most of these studies were either restricted to specific geographic regions or used very small sample sizes (Waterhouse

& Paramonov, 1950; Norris, 1990; Stevens & Wall, 1996; Stevens *et al*., 2002; Stevens, 2003; Wallman *et al*., 2005; Wells *et al*., 2007; DeBry *et al*., 2010). Specimens from around the world and much larger sample sizes are required to confirm the taxonomic relationship between *L. sericata* and *L. cuprina* and the significance of hybrids.

The morphological identification of *L. sericata* and *L. cuprina* is complicated by the known existence of hybrids of these species. Only one study has attempted to identify the hybrids from their morphology (Tourle *et al*., 2009) using a semi-quantitative morphological index. The results were not conclusive, but did suggest that the hybrids showed more extreme values than the parent species. No studies have shown any statistical support for identifying the hybrids from the morphological characters. Several keys for identifying these species exist (Waterhouse & Paramonov, 1950; Rognes, 1980; Dear, 1986; Holloway, 1991; Rognes, 1994; Wallman, 2001; Whitworth, 2006; 2010). Scoring the characters from these keys and testing them on known hybrid and pure strain specimens, would allow for statistical analysis of the characters. This would determine if the hybrids can be identified from their morphology.

The genus *Lucilia* is part of the subfamily Luciliinae which contains three other genera - *Hemipyrellia*, *Dyscritomyia* and *Hypopygiopsis*. All of these genera are implicated in cutaneous myiasis (Stevens, 2003). There have been several studies on the evolution of parasitism within Calliphoridae (Stevens & Wall, 1997; Otranto & Stevens, 2002; Stevens, 2003) but no research has looked at the geographic distribution of the different forms of parasitism.

The relationship between the genera of Luciliinae is not clearly defined, with some molecular studies suggesting that *Hemipyrellia* and *Dyscritomyia* fall within *Lucilia* (Wells *et al*., 2007; Park *et al*., 2009; Liu *et al*., 2011; McDonagh & Stevens, 2011). Larger sample sizes with specimens from geographically diverse localities and as many species from these genera as possible are required to test this theory.

The existence of paraphyletic species has been shown in molecular studies of *Lucilia*. This has been seen in the species pairs *L. caesar*/*L. illustris*, *L.coeruliviridis*/*L. mexicana* and *L. sericata*/*L. cuprina* (Stevens & Wall, 1996; Stevens *et al*., 2002;

Wallman *et al*., 2005; Tourle *et al*., 2009; DeBry *et al*., 2010, 2012; Sonet *et al*., 2012, 2013; Williams & Villet, 2013). This suggests that hybridization and introgression are more common than was previously thought.

The division of *Lucilia* into (sub)genera such as *Phaenicia* and *Bufolucilia* (Malloch, 1926; Hall, 1948) has not been supported (Aubertin, 1933; Zumpt, 1965) and has thus only persisted in certain parts of the world including North America and some parts of Asia (Stevens & Wall, 1996; Park *et al*., 2009). The use of molecular techniques can assist in clarifying if these divisions have any validity or utility.

The geographic distribution of *L. sericata* and *L. cuprina* in South Africa is important if wild-caught specimens of these two species are to be used in forensic investigations and for augmenting MDT colonies. Knowing their geographic distribution would also assist in developing strategies to control fly strike in sheep farming areas. Limited locality records exist for these two species. The use of distribution modelling techniques would provide predicted distribution maps for these species based on their known locality points. These predicted distribution maps would also show the climatic niche preferences of these species and if they co-exist or have partitioned habitats.

Aims

The aims of this thesis are therefore to:

1. Determine if hybrids of *L. sericata* and *L. cuprina* exist in South Africa or other parts of the world by means of molecular techniques.
2. Determine if the hybrids of *L. sericata* and *L. cuprina* can be determined morphologically.
3. Determine the geographic distributions of *L. sericata* and *L. cuprina* by predictive modelling techniques.
4. Determine if *L. sericata* and *L. cuprina* are sister species, if *Hemipyrellia* and *Dyscritomyia* should be synonymised with *Lucilia* and if any geographic pattern exists within the parasitic behaviour of species of Luciliinae.

REFERENCES

Altincicek B. & Vilcinskas A. (2009) Septic injury-inducible genes in medicinal maggots of the green blow fly *Lucilia sericata*. *Insect Molecular Biology.* 18(1): 119-125.

Aubertin, D. (1933) Revision of the genus *Lucilia* R.-D. (Diptera, Calliphoridae). *Linnean Society Journal of Zoology* 38: 389-463.

Bishop, D.M. (1991) Variations in numbers of occipital setae for two species of *Lucilia* (Diptera: Calliphoridae) in New Zealand. *New Zealand Entomologist* 14: 28-31.

Bishop, D.M. (1995) Subspecies of the Australian green blowfly (*Lucilia cuprina*) recorded in New Zealand. *New Zealand Veterinary Journal* 43: 164-165.

Boehme, P., Amendt, J., and Zehner, R. (2012) The use of *COI* barcodes for molecular identification of forensically important fly species in Germany. *Parasitology Research* 110: 2325-2332.

Chen, W.-Y., Hung, T.-S., and Shiao, S.-F. (2004) Molecular identification of forensically important blow fly species (Diptera: Calliphoridae) in Taiwan. *Journal of Medical Entomology* 41: 47-57.

DeBry R., Timm A.E., Dahlem G.A. & Stamper T. (2010) mtDNA-based identification of Lucilia cuprina (Wiedemann) and Lucilia sericata (Meigen) (Diptera: Calliphoridae) in the continental United States. *Forensic Science International* 202: 102 -109.

DeBry, R., Timm, A.E., Wong, E.S., Stamper, T., Cookman, C., and Dahlem, G. A. (2012) DNA-Based identification of forensically important *Lucilia* (Diptera: Calliphoridae) in the continental United States. *Journal of Forensic Sciences* 58: 73-78.

Dear, J.P. (1986) Calliphoridae (Insecta: Diptera). Fauna of New Zealand no. 8.

Fischer, O.A. (2000) Blowflies of the genera *Calliphora*, *Lucilia* and *Protophormia* (Diptera, Calliphoridae) in South-Moravian urban and rural areas with respect to *Lucilia bufonivora* Moniez, 1876. *Acta Veterinaria Brno* 69: 225-231.

GilArriortua, M., Salona Bordas, M.I., Caine, L.M., Pinheiro, F., and de Pancorbo, M.M. (2013) Cytochrome b as a useful tool for the identification of blowflies of forensic interest (Diptera, Calliphoridae). *Forensic Science International* 228: 132-136.

Hall, D.G. (1948) *The blowflies of North America*. Washington DC: Thomas Say Foundation.

Harvey, M.L., Dadour, I.R., and Gaudieri, S. (2003) Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in western Australia. *Forensic Science International* 131: 134-139.

Harvey, M.L., Mansell, M.W., Villet, M.H., and Dadour, I.R. (2003) Molecular identification of some forensically important blowflies of southern Africa and Australia. *Medical and Veterinary Entomology* 17: 363-369.

Harvey, M.L., Gaudieri, S., Villet, M.H., and Dadour, I.R. (2008) A global study of forensically significant calliphorids: Implications for identification. *Forensic Science International* 177: 66-76.

Heath, A.C.G. and Bishop, D.M. (2006) Flystrike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* 137: 333-344.

Hepburn, G.A. (1943) Sheep blowfly research I - A survey of maggot collections from live sheep and a note on the trapping of blowflies. *Onderstepoort Journal of Veterinary Science and Animal Industry* 18: 13-18.

Holloway, B.A. (1991) Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). *New Zealand Journal of Zoology* 18: 415-420.

Kingu, H.J.C., Kuria, S.K., Villet, M.H., Mkhize, J.N., Dhaffala, A. & Iisa, J.M. (2012) Cutaneous myiasis: is *Lucilia cuprina s*afe and acceptable for maggot debridement therapy? *Journal of Cosmetics, Dermatological Sciences and Applications* 2: 79-82.

Liu, Q.-L., Cai, J.-F., Chang, Y.-F., Gu, Y., Guo, Y.-D., Wang, X.-H., Weng, J.-F., Zhong, M., Wang, X., Yang, L., Wu, K.-L., Lan, L.-M., Wang, J.-F., and Chen, Y.-Q. (2011) Identification of forensically important blow fly species (Diptera: Calliphoridae) in China by mitochondrial cytochrome oxidase I gene differentiation. *Insect Science* 18: 554-564.

Malloch, J.R. (1926) Exotic Muscaridae (Diptera). *Annals and Magazine of Natural History* 17: 489-510.

McDonagh, L.M. and Stevens, J.R. (2011) The molecular systematics of blowflies and screwworm flies (Diptera: Calliphoridae) using *28S* rRNA, COX1 and EF-1α: insights into the evolution of dipteran parasitism. *Parasitology* 138: 1760-177.

Norris, K.R. (1990) Evidence for the multiple exotic origin of Australian populations of the sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Australian Journal of Zoology* 38: 635-648.

Otranto, D. and Stevens, J.R. (2002) Molecular approaches to the study of myiasis-causing larvae. *International Journal for Parasitology* 32: 1345-1360.

Park, S.H., Zhang, Y., Piao, H., Yu, D.H., Jeong, H. J., Yoo, G.Y., Chung, U., Jo, T.-H., and Hwang, J.-J. (2009) Use of cytochrome c oxidase subunit I (*COI*) nucleotide sequences for identification of the Korean Luciliinae fly species (Diptera: Calliphoridae) in forensic investigations. *Journal of Korean Medical Science* 24: 1058-1063.

Paul, A.G., Ahmad, N.W., Lee, H.L., Ariff, A.M., Saranum, M., Naicker, A.S. & Osman, Z. (2009) Maggot debridement therapy with Lucilia cuprina: a comparison with conventional debridement in diabetic foot ulcers. *International Wound Journal* 6(1): 39- 46.

Rognes, K. (1980) The blow-fly genus *Lucilia* Robineau-Desvoidy (Diptera, Calliphoridae) in Norway. *Fauna norvegica Series B* 27: 39-52.

Rognes, K. (1994) First record of the sheep greenbottle fly *Lucilia cuprina* (Wiedemann, 1830) from Europe (Diptera: Calliphoridae) with additional Spanish records of Calliphoridae, Muscidae and Sarcophagidae. *Eos* 69: 41-44.

Rose, H. and Wall, R. (2011) Modelling the impact of climate change on spatial patterns of disease risk: Sheep blowfly strike by *Lucilia sericata* in Great Britain. *International Journal of Parisitology* 41: 739-746.

Sonet, G., Jordaens, K., Braet, Y., and Desmyter, S. (2012) Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic? *Forensic Science International* 223: 153-159.

Sonet, G., Jordaens, K., Braet, Y., Bourguignon, L., Dupont, E., Backeljau, T., De Meyer, M., and Desmyter, S. (2013) Utility of GenBank and the Barcode of

Life Data Systems (BOLD) for the identification of forensically important Diptera from Belgium and France. *ZooKeys* 365: 307-328.

Stevens, J. & Wall, R. (1996) Species, sub-species and hybrid populations of the blowflies Lucilia cuprina and Lucilia sericata (Diptera: Calliphoridae). *Proceedings Biological Sciences* 263: 1335-1341.

Stevens, J. and Wall, R. (1997) The evolution of ectoparasitism in the genus Lucilia (Diptera: Calliphoridae). *International Journal for Parasitology* 27: 51-59.

Stevens, J.R., Wall, R. & Wells, J.D. (2002) Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology*. 11: 141-148.

Stevens, J.R. (2003) The evolution of myiasis in blowflies (Calliphoridae). *International Journal Parasitology* 33: 1105-1113.

Tantawi, T.I., Williams, K.A. & Villet, M.H. (2010) An Accidental but safe and effective use of Lucilia cuprina (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. *Journal of Medical Entomology* 47(3): 491- 494.

Tourle, R., Downie, D. A., and Villet, M. H. (2009) A morphological and molecular comparison of Lucilia cuprina and L. sericata (Diptera: Calliphoridae) in South Africa. *Medical and Veterinary Entomology* 23: 6-14.

Ullyett, G.C. (1945) Species of *Lucilia* attacking sheep in South Africa. *Nature* 155: 636-637.

Vogt, W.G. and Woodburn, T.L. (1979) Ecology, distribution and importance of sheep myiasis flies in Australia. *National Symposium of the sheep blowfly and flystrike in sheep*. N.S.W. Ag. Sydney.

Vilcinskas, A. (2011) From traditional maggot therapy to modern biosurgery. In: Vilcinskas A, ed. *Insect Biotechnology*. Springer, Netherlands, pp. 67-75.

Wallman, J.F. (2001) A key to the adults of species of blowflies in southern Australia known or suspected to breed in carrion. *Medical and Veterinary Entomology* 15: 433-437.

Wallman, J.F., Leys, R. & Hogendoorn, K. (2005) Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. *Invertebrate Systematics* 19: 1-15.

Waterhouse, D.F. and Paramonov, S.J. (1950) The status of the two species of *Lucilia* (Diptera, Calliphoridae) attacking sheep in Australia. *Australian Journal of Scientific Research* 3: 310-336.

Wells, J.D., Wall, R. & Stevens, J.R. (2007) Phylogenetic analysis of forensically important Lucilia flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. *International Journal of Legal Medicine* 121(3): 229-233.

Whitworth, T. (2006) Keys to the genera and species of blow flies (Diptera: Calliphoridae) of America north of Mexico. *Proceedings of the Entomological Society of Washington* 108: 689-725.

Whitworth, T. (2010) Keys to the genera and species of blow flies (Diptera: Calliphoridae) of the West Indies and description of a new species of *Lucilia* Robineau-Desvoidy. *Zootaxa* 2663: 1-35.

Williams, K.A. and Villet, M.H. (2013) Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae). *European Journal of Entomology* 110: 187-196.

Zumpt, F. (1965) *Myiasis in man and animals in the old world: A textbook for physicians, veterinarians and zoologists*. Butterworths, London.

CHAPTER 2

ANCIENT AND MODERN HYBRIDIZATION BETWEEN LUCILIA SERICATA AND LUCILIA CUPRINA (DIPTERA: CALLIPHORIDAE)

ABSTRACT

There are important but inconsistent differences in breeding site preference between the blow flies *Lucilia sericata* (Meigen, 1826) and *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) that have significance for medical and veterinary science. These inconsistencies might arise from hybridisation. The species are difficult to distinguish using external morphology, although the male genitalia are distinctive and there are reliable molecular markers. Molecular evidence of modern hybridisation, derived from a newly developed nuclear marker, the *Period* (*per*) gene, is presented here. This has implications for identifications of these species based on mtDNA, and may lead to an explanation of the medical and veterinary anomalies noted in these species.

INTRODUCTION

The use of *Lucilia* blowflies for maggot debridement therapy (MDT) has become a topic of great interest in South Africa (Williams *et al*., 2008; Cronje & Du Plessis pers. comm). *Lucilia sericata* (Meigen, 1826) is the species of choice for MDT (Altincicek & Vilcinskas, 2009; Vilcinskas, 2011), but the misidentification of *Lucilia cuprina* (Wiedemann, 1830) and *L. sericata* for use in MDT and how best to supplement MDT colonies has raised the issue of species identification (Williams *et al*., 2008; Tantawi *et al*., 2010). *Lucilia cuprina* has recently been used successfully for MDT (Paul *et al*., 2009; Tantawi *et al*., 2010; Kingu *et al*., 2012) although this species is responsible for sheep-strike that causes losses to the wool and meat industries that amount to millions of dollars worldwide each year (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006).

These two species have been suspected of interbreeding and producing fertile hybrids in South Africa (Ullyett, 1945). They have been shown to hybridise under laboratory conditions and to produce fertile hybrids, although there are no reports of this occurring naturally (Ullyett, 1945). *Lucilia cuprina* has consistently been found to be paraphyletic relative to *L. sericata* in studies of several mitochondrial genes (Table 2.1). The biology of these flies is medically and economically important.

Several authors have suggested that these flies should be classified as three species or that *L. cuprina* should be classified as two subspecies – *Lucilia cuprina cuprina* (Wiedemann) and *Lucilia cuprina dorsalis* Robineau-Desvoidy (Waterhouse & Paramonov, 1950; Norris, 1990; Stevens & Wall, 1996; Stevens *et al*., 2002; Stevens, 2003; Wallman *et al*., 2005; Wells *et al*., 2007; DeBry *et al*., 2010). *Lucilia sericata* and *L. cuprina* are morphologically very similar and the adults are difficult to identify using the available keys based on morphological characters without using the male genitalia, which usually requires destructive sampling (Aubertin, 1933; Smith, 1986; Norris, 1990; Holloway, 1991). However, with some experience the females can usually be reliably identified using the characteristics of Holloway (1991).

Molecular methods are useful in confirming the taxonomic status of these two species (Williams *et al*., 2008; Tourle *et al*., 2009; Tantawi *et al*., 2010). The use of more than

one gene for phylogenetic methods is recommended as using only one gene may not give a true picture of phylogenetic relationships (Sperling *et al*., 1994; Nelson *et al*., 2007; Whitworth *et al*., 2007; Tourle *et al*., 2009). Analysing both nuclear and mitochondrial genes simultaneously has highlighted the difference between gene trees and species trees (Nichols, 2001; Stevens *et al*., 2002; Stevens, 2003; Whitworth *et al*., 2007; Tourle *et al*., 2009; DeBry *et al*., 2010). Gene trees model how a gene evolves while a species tree shows the branching of species lineages via the process of speciation.

The purpose of this study was to test if there is evidence of hybridisation between these two species, shown by a difference between the trees produced from sequence data using nuclear as opposed to mitochondrial genes from these flies, from different localities around South Africa and from sites in Africa, Europe, Australia, Asia and North America.

MATERIALS AND METHODS

Adult flies of both *L. sericata* and *L. cuprina* were collected using chicken liver baited fly traps in Britstown, Bloemfontein, Cape Town, Durban, Grahamstown, Nelspruit and Witbank in South Africa (Fig. 2.1 insert). *Lucilia* specimens originating from Welkom and Pretoria were also obtained from a maggot debridement therapy colony at Eugene Marais Hospital in Pretoria. *Lucilia sericata* was also obtained from Australia, Canada, France, Germany, Greece, Japan, Namibia, Switzerland and the United States of America (Fig. 2.1). Additional specimens of *L. cuprina* were obtained from Australia, Egypt, Thailand, the United States of America and Zimbabwe (Fig. 2.1). A total of 84 flies were collected – 11 males and 73 females. They were identified by their morphology using published keys (Aubertin, 1933; Smith, 1986; Holloway, 1991). Due to the biology of these flies, females are attracted to bait traps more than males and therefore characteristics identified by Holloway (1991); specifically the distances and angles between setae on the vertex of females, the extent of metallic sheen on the parafrontal sclerites of females and the number of scutellar setulae were used to identify these flies.

All flies were kept in separate 1.5ml Eppendorf tubes in 96% ethanol and deposited with the Durban Natural Science Museum after analysis. One hind leg of each fly was used for DNA analysis. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions (Qiagen 07/2006).

Three genes were chosen for sequencing – *28S* rRNA (*28S*), a nuclear gene that has been used in previous studies and would allow comparison with other studies (Table 2.1); *Period* (*Per*), a second nuclear gene that is faster-evolving than *28S* to give better resolution; and Cytochrome oxidase I (*COI*), a mitochondrial gene that has been used in previous studies (Table 1). A region of approximately 650bp in Domain 1-2 of the *28S* gene was amplified using the primers 5`-CCCCCTGAATTTAAGCATAT-3` and 5`-GTTAGACTCCTTGGTCCGTG-3` (Stevens *et al*., 2002). A region of approximately 600bp of the *COI* gene was amplified using the primers C1-J1709 (5'-AATTGGGGGTTTGGAAATTG-3`) and C1-N2353 (5'-GCTCGTGTATCAACGTCTATTCC-3`) (Simon *et al*., 2006). This region overlaps the 'barcoding' region for approximately 300 base pairs. A region of approximately 730bp of the *Per* gene, was amplified using the primers *Per*5 (5'-GCCTTCAGATACGGTCAAAC-3') (Warman, pers comm) and *Per* reverse (5`-CCGAGTGTGGTTTGGAGATT-3`) (designed by the author). Polymerase chain reaction (PCR) amplification was performed using 1µL of DNA in a 25µL reaction. Amplification times were 94°C for 5 min denaturation, followed by 36 cycles of 94°C for 30 seconds, 55°C for 1min, 72°C for 30 seconds and a final extension period at 72°C for 7min. PCR products were confirmed by gel electrophoresis stained in ethidium bromide.

PCR products were then sequenced using an ABI 3730l Genetic Analyzer (Applied Biosystems) and the primers used in amplification. Additional DNA sequences for these two species were obtained from GenBank (www.ncbi.nlm.nih.gov) for comparative analysis (Table 2.2). The sequences were aligned and edited using the BioEdit v7.0.9 software (Hall, 1999).

Phylogenetic reconstruction by maximum parsimony analysis was performed using PAUP*4b10 (Swofford, 2003) using the best-fitting model (HKY) from MrModelTest v2.2 (Nylander, 2002) applied in MrMTgui (Nuin, 2005). Statistical support for nodes was assessed by bootstrapping with 100 replicates retaining a maximum of 10 000 trees. Bayesian inference analysis was performed using one cold and three hot chains and the HKY model. Analysis was run for 5 000 000 generations, sampling every 1 000 generations with burn-in of 1000 samples. All phylogenetic analyses used *Calliphora vicina* and *Lucilia infernalis* as outgroups. Incongruence length difference (ILD) tests (Farris *et al*., 1994) were run in PAUP* 4b10 (Swofford, 2003) to quantify the differences in topology between trees for *28S*, *COI* and *Per*. Analysis was then conducted on the partitioned data sets (*28S* and *Per*; *28S*, *Per* and *COI*) with the parameters as above.

When hybridization is involved, a single dichotomising phylogenetic tree will often not be a suitable representation of the phylogenetic history (Huson & Bryant, 2006). This may make it necessary to use a more general graph, such as a network to represent the data. NeighborNet computes a set of splits from the data. If splits are compatible, the resultant graph will be a dichotomous tree, but when the splits are not compatible, it results in a network diagram with multiple parallel branches representing a single split (Huson & Bryant, 2006). Network diagrams were created using NeighborNet in SplitsTree4 (Huson & Bryant, 2008) using the uncorrected P-method for distance.

RESULTS

A total of 654 base pairs for *28S*, 576bp for *COI* and 722bp for *Per* (a total of 1952 bp) were sequenced and aligned. There were no indels in the aligned sequences. A total of 77, 83 and 76 specimens were sequenced respectively for *28S*, *COI* and *Per* (Table 2.3).

The ILD test showed *28S* and *Per* to be congruent (P = 0.99), and the ILD test for *28S* and *COI* was not statistically significant (P = 0.08). *Per* and *COI* were significantly incongruent (P = 0.01) as was the combination of *28S*, *Per* and *COI* (P = 0.01). Due to the high level of congruence between *28S* and *Per*, these two data sets were

concatenated and used for the analyses and network diagrams. Despite the incongruence between the nuclear (*28S* and *Per*) and mitochondrial (*COI*) data, these data sets were also concatenated in order to look at the total evidence and an analysis run on the total evidence.

The Bayesian Inference trees (Fig. 2.2 a) for the nuclear genes (*28S* and *Per*) show both *L. sericata* and *L. cuprina* to be monophyletic clades with strong support (Fig. 2.2 a). The Bayesian Inference tree for *COI* (Fig. 2.2 b) shows *L. sericata* to be monophyletic, but *L. cuprina* is paraphyletic with respect to *L. sericata*, with good posterior probability support. The first *L. cuprina* clade (Fig. 2.2 b) exhibits both nuclear and mitochondrial sequences (and morphology) of "pure *cuprina*", while the second clade (blue rectangle Fig. 2.2 b) exhibits nuclear DNA (and morphology) of *L. cuprina* but mitochondrial DNA of *L. sericata* – a "hybrid" clade. The *L. cuprina* sequences from GenBank from Hawaii, Taiwan and China grouped with the "hybrid" clade (Fig. 2.2 b).

Out of 42 specimens with the morphology of *L. cuprina*, five have mitochondrial genes that are typical of the *L. sericata* clade (green rectangle Fig. 2.2 b), but not of the "ancient hybrid" clade. The maximum parsimony trees were topologically compatible with the Bayesian Inference trees but the trees were less well resolved (trees not shown).

The network diagrams of the nuclear genes (*28S* and *Per*) (Fig. 2.3) indicate a clear and simple split between the *L. sericata* specimens and the *L. cuprina* specimens. The *COI* network diagram (Fig. 2.4) shows two clear splits between a cluster of *L. sericata* specimens, and two clusters of *L. cuprina* specimens. The "hybrid" cluster of *L. cuprina* (blue rectangle Fig 2.2 b) specimens lies closer to the *L. sericata* cluster than to the "pure" *L. cuprina* cluster, but is distinctively monophyletic. The five *L. cuprina* specimens that group within the *L. sericata* clade (green rectangle Fig. 2.2 b) also appear within the *L. sericata* cluster (Fig. 2.4). The network diagram of the total evidence concatenated data sets (Fig. 2.5) shows a clear split between the *L. sericata* and *L. cuprina* clusters, and the *L. cuprina* samples split into two clusters which are linked by more pathways to each other than to the *L. sericata* cluster.

DISCUSSION

A number of studies have been conducted on *L. sericata* and *L. cuprina*, looking at morphological identification, the possibility that they are interbreeding and whether *L. cuprina* should be classified as two subspecies or two independent species (Ullyett, 1945; Waterhouse & Paramonov, 1950; Norris, 1990; Holloway, 1991a,b; Stevens & Wall, 1996; Stevens *et al*., 2002; Stevens, 2003; Wallman *et al*., 2005; Wells *et al*., 2007; Harvey *et al*., 2008; Tourle *et al*., 2009; DeBry *et al*., 2010). This study focuses on these two species in South Africa, but also examines specimens from across the globe to place the South African situation into a global context. This study used two nuclear and one mitochondrial gene where most previous studies have either used only one mitochondrial gene or a combination of mitochondrial genes and one nuclear gene (Table 2.1). Stevens & Wall (1996) used RAPDs which employed a multi-locus nuclear genotype approach.

Individually and together, the nuclear *28S* and *Per* genes show *L. sericata* and *L. cuprina* to be two monophyletic species (Fig. 2.2 a) with very strong posterior probability support (0.99 and 1.00 respectively). However, the mitochondrial *COI* gene suggests that *L. cuprina* is paraphyletic with respect to *L. sericata* (Fig. 2.2 b). There is a monophyletic clade of *L. cuprina* specimens that have *L. sericata*-like mtDNA, which has been seen in previous studies (Table 2.1). The monophyletic clade of *L. cuprina* with *L. sericata*-like mtDNA has been suggested to represent an ancient hybridization event (Stevens & Wall, 1996; Stevens *et al*., 2002; Tourle *et al*., 2009). The *L. sericata* mtDNA appears to have been fixed in this lineage of *L. cuprina* and not lost through lineage sorting.

However, there are also five specimens with the morphology of *L. cuprina* and mtDNA of *L. sericata* that are not representative of the ancient, introgressed clade (Fig. 2.2 b & 2.4), implying novel mismatches of nuclear and mitochondrial genomes. Nuclear genes were not amplified for three of these specimens, but the other two, from Zimbabwe and Thailand, have (different) *28S* and *Per* genotypes typical of *L. cuprina*, which suggests modern hybridization. This has not been seen in any previous studies on *L. sericata* / *L. cuprina* (Table 2.1) and provides the first direct genetic evidence of modern-day natural interbreeding between these species.

Ancient hybrids and introgression

The specimens that form the monophyletic clade of *L. cuprina* with *L. sericata*-like mtDNA originate from Durban, Nelspruit and Cape Town in South Africa, and from Merced in California in the continental USA, Hawaii, China and Taiwan (Tables 2.2 & 2.3). It was once suggested that this lineage was restricted to the Hawaiian Islands (Stevens & Wall, 1996; Stevens *et al*., 2002), but since then the lineage has been found in North America, Africa and Asia. It would be difficult to determine where it originated because it is so widespread. There does not appear to be any geographical coherence within the two *L. cuprina* clades (Fig. 2.2 b). It was suggested that the two named subspecies of *L. cuprina* – *L. c. cuprina* and *L. c. dorsalis* – could be distinguished using *COI* sequences because both subspecies formed monophyletic clades (DeBry *et al*., 2010), with *L. c. cuprina* forming a monophyletic clade that was sister to the *L. sericata* clade, thus suggesting that all *L. cuprina* with *L. sericata*-like mtDNA are *L. c. cuprina*. Sequences from South Africa (Tourle *et al*., 2009) that were included in this analysis (DeBry *et al*., 2010) all grouped with the putative clade of *L. c. cuprina*, although African *L. cuprina* are considered to be *L. cuprina dorsalis* (Waterhouse & Paramonov, 1950). Perhaps *L. c. cuprina* has been introduced into South Africa like some other synanthropic blow flies (Williams & Villet, 2006), but the problem remains of distinguishing them morphologically, an issue that was addressed by Tourle *et al*. (2009), who found the "hybrid" clade to have a morphological index that was more *cuprina*-like than "pure" *cuprina* specimens.

Four cases of mtDNA introgression without detectable nuclear introgression, as seen in this study, were reported for *Protocalliphora* blowflies (Whitworth *et al*., 2007). Interspecific mitochondrial introgression linked to selective sweeps induced by nuclear-cytoplasmic incompatibility due to *Wolbachia* infections has been described in various insects (Ballard, 2000) as an explanation for how mtDNA introgression without nuclear introgression is possible. Cytoplasmic incompatibility is a process where, if uninfected females mate with infected males, some or all of their eggs will die. But if an infected female mates with either an infected or uninfected male, her eggs remain viable but all will be infected with *Wolbachia*. So infected females outcompete uninfected ones and the overall population of *Wolbachia*-infected flies (and therefore *Wolbachia*) increases (Zimmer, 2001). Thus the mitochondria of

infected individuals have a greater chance than uninfected individuals of being passed on because mitochondria are passed down the female line, leading to fixed introgression. *Wolbachia* infection in the blowfly *Protocalliphora sialia* (Baudry *et al*., 2003) and infections of three different strains of *Wolbachia* in *Protocalliphora* in North America (Whitworth *et al*., 2007) have been reported. All of these infections resulted in mtDNA introgression without any detectable nuclear introgression. Further studies are recommended to determine if *Lucilia* blowflies are affected by *Wolbachia* infections as an explanation for the pattern seen in this study. However, such infections can die out over time, so that the only evidence of them may be cytoplasmic introgression (Zimmer, 2001).

The combined *28S* and *Per* data show a very clear split between the *L. sericata* and *L. cuprina* samples (Fig. 2.3). The splits show very little internal incompatibility. The mtDNA (*COI*) shows a much higher degree of incompatibility between the splits (Fig. 2.4) which represents incompatible signals (Huson & Bryant, 2006). There are three important splits that group *L. sericata* together and two *L. cuprina* splits. This grouping is consistent with the Bayesian Inference tree (Fig. 2.2 b). The concatenated total data set (*28S*, *Per* and *COI*) (Fig. 2.5) shows a high level of incompatibility between the *L. cuprina* samples and a high degree of compatibility between the *L. sericata* samples. The *L. cuprina* samples show a number of splits and this incompatibility is probably as a result of the *L. sericata*-like mitochondrial DNA which results in the two clusters of *L. cuprina*.

Modern hybrids

The genetic component of an organism's morphology is determined by its nuclear DNA. One would expect recombination of the nuclear DNA if interbreeding occurs, resulting in morphology that is either intermediate (for multi-locus traits) or a mosaic of the two parental phenotypes (for single-locus traits). However, if one species' alleles are consistently dominant over the other, then despite recombination, the dominant phenotype will prevail (Lewin, 1997). Thus, although the putative modern hybrids had *sericata*-like mtDNA indicating hybridisation, they were still *L. cuprina*-like in morphology, suggesting that *L. cuprina's* alleles for morphology are dominant over those of *L sericata*. In crossing experiments carried out in a laboratory, it was

suggested that the femur colour of *L. cuprina* and the abdomen colour of *L. sericata* were dominant characteristics, giving the hybrids a combination of the two species' morphologies (Ullyett, 1945). However this study used only two characters (femur and abdomen colour) which Ullyett (1945: 636) described as not being "scientific criteria" because there are gradations in both characters depending on both the age and condition of the specimens and the observers' opinion and thus they could not be considered reliable criteria for identification.

Even when hybridization occurred in *Hyalomma* (Acari: Ixodidae), no intermediate morphologies were observed and the morphology of one parent appeared to be inherited over that of the other (Rees *et al*., 2003). Funk & Omland (2003) suggest that most hybrid species originate via asymmetrical hybridization and would be mitochondrially monophyletic. This might explain what we see in this study regarding the ancient hybridization "hybrid" group, but not the modern hybrids (which are derived from several sources). mtDNA may be more susceptible to introgression than nuclear loci (Machado & Hey, 2003). One is therefore less likely to have consistent gene trees for mtDNA and they may even suggest a different phylogeny. This gives support to the well-established idea that more than just one nuclear or mitochondrial gene needs to be used when trying to determine species and gene trees (Funk & Omland, 2003; Machado & Hey, 2003; Hurst & Jiggins, 2005).

DNA-based identification

The use of *COI* sequences to correctly identify the two presumed subspecies seems unlikely to succeed due to the presence of *L. cuprina* flies that group within the *L. sericata* clade (Fig. 2.2 b). The phylogenetic positioning of these flies indicates their relationship relative to other specimens, but does not necessarily give an identification that agrees with the morphology. It also raises the issue of using *COI* as the universal 'barcoding' gene and whether it is suitable, especially for insects (Rubinoff *et al*., 2006; Roe and Sperling, 2007; Whitworth *et al*., 2007; Jordaens *et al*., 2012; Sonet *et al*., 2012). The idea of using part of *COI* as a universal diagnostic gene is to allow the identification of unknown specimens when comparing them to identified species' sequences (Roe and Sperling, 2007). However, using *COI* alone could result in incorrect identifications, as seen in this study, as numerous insect species are infected

with *Wolbachia* or have undergone hybridisation and carry mtDNA of another species (Zimmer, 2001; Baudry *et al*., 2003; Whitworth *et al*., 2007). So the sequences of unknown specimens may align with species that they share mtDNA with, but are in fact a different species based on nuclear DNA or morphology. Although a study on blowflies in Australia suggested that using *COI* for identification is suitable, the authors also raised the issue of misidentifications when hybridisation was involved and suggested the use of a nuclear gene for confirmation (Nelson *et al*., 2007). A study of 1333 mitochondrial sequences (minimum of 300 bp) for 449 species of flies concluded that using *COI* alone for identification had a less than 70% success rate at identifying the species correctly (Meier *et al*., 2006).

The results show that in some cases both nuclear and mitochondrial genes are needed for reliable species identification. It is well known that the use of just one gene can generally be misleading as can be seen in the *L. sericata / L. cuprina* situation, where using only the mitochondrial (*COI*) gene would result in three species (*L. cuprina, L. sericata* and *L. cuprina dorsalis)* being identified, or one species (*L.* sericata) and two subspecies (*L. cuprina cuprina, L. cuprina dorsalis*) (Wallman *et al*., 2005; Harvey *et al*., 2008; Tourle *et al*., 2009; DeBry *et al*., 2010). By using nuclear genes in conjunction with the mitochondrial gene, a potentially misleading situation can be avoided (Rubinoff *et al*., 2006; Nelson *et al*., 2007; Roe & Sperling, 2007; Williams *et al*., 2008; Tantawi *et al*., 2010).

ACKNOWLEDGEMENTS

We thank Frans Cronje, Dave Emery, Georg Goergen, Christine Hänel, Olivia Harrison, Chris Kelly, Ashley Kirk-Spriggs, Nicola Lunt, Mervyn Mansell, Rich Merrit, Nolwazi Mkize, Hideharu Numata, Cameron Richards, Kiyoshi Saigusa, Kerry Sink, Kyle Smith, Kabkaew Sukontason, James Wallman, Gareth Whittington-Jones and Claude Wyss for providing specimens from different localities; Jenny Lamb (University of KwaZulu-Natal) for access to facilities; Jessica Vivian and Theshnie Naidoo for laboratory assistance and advice; Thomas Pape and two anonymous referees for their valuable comments on the draft manuscript; and the Durban Natural Science Museum, the National Research Foundation of South Africa and Rhodes University for financial support. Any opinion, findings and conclusions or

recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Research Foundation.

REFERENCES

Altincicek, B. and Vilcinskas, A. (2009). Septic injury-inducible genes in medicinal maggots of the green blow fly *Lucilia sericata*. *Insect Molecular Biology* 18(1): 119-125.

Anderson, G.S. (2000). Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences* 45: 824-832.

Aubertin, D. (1933). Revision of the genus Lucilia R.-D. (Diptera, Calliphoridae). *Linnean Society Journal of Zoology* 38: 389-463.

Ballard, J.W.O. (2000). When one in not enough: Introgression of mitochondrial DNA in Drosophila. *Molecular Biology and Evolution* 17: 1126-1130.

Ballard, J.W.O. and Whitlock, M.C. (2004). The incomplete natural history of mitochondria. *Molecular Ecology* 13: 729-744.

Baudry, E., Bartos, J., Emerson, K., Whitworth, T. and Werren, H. (2003). Wolbachia and genetic variability in the birdnest blowfly *Protocalliphora sialia*. *Molecular Ecology* 12: 1843-1854.

Clark, K., Evans, L. and Wall, R. (2006). Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. *Forensic Science International* 156: 145-149.

Day, D.M. and Wallman, J.F. (2006). Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae). *Journal of Forensic Science* 51: 657-663.

DeBry, R., Timm, A.E., Dahlem, G.A. and Stamper, T. (2010). mtDNA-based identification of *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) in the continental United States. *Forensic Science International* 202: 102 -109.

Farris, J.S., Källersjö, M., Kluge, A.G. and Bult, C. (1994). Testing significance of congruence. *Cladistics* 10: 315-319.

Funk, D.J. and Omland, K.E. (2003). Species-level paraphyly and polyphyly: Frequency, causes and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology Evolution and Systematics* 34: 397-423.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis programme for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

Harvey, M.L., Gaudieri, S., Villet, M.H. and Dadour, I.R. (2008). A global study of forensically significant calliphorids: Implications for identification. *Forensic Science International* 177: 66-76.

Heath, A.C.G. and Bishop, D.M. (2006). Flystrike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* 137: 333-344.

Hepburn, G.A. (1943). Sheep blowfly research I - A survey of maggot collections from live sheep and a note on the trapping of blowflies. *Onderstepoort Journal of Veterinary Research* 18(1-2): 13-18.

Holloway, B.A. (1991a). Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). *New Zealand Journal of Zoology* 18: 415-420.

Holloway, B.A. (1991b). Identification of third-instar larvae of flystrike and carrion-associated blowflies in New Zealand (Diptera: Calliphoridae). *New Zealand Entomologist* 14: 24-28.

Hurst, G.D.D. and Jiggins, F.M. (2005). Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B* 272: 1525-1534.

Huson, D.H. and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23: 254-267.

Jordaens, K., Sonet, G., Richet, R., Dupont, E., Braet, Y. and Desmyter, S. (2012). Identification of forensically important *Sarcophaga* species (Diptera: Sarcophagidae) using the mitochondrial *COI* gene. *International Journal of Legal Medicine* 127: 491-504.

Kingu, H.J.C., Kuria, S.K., Villet, M.H., Mkhize, J.N., Dhaffala, A. and Iisa, J.M. (2012). Cutaneous myiasis: is *Lucilia cuprina s*afe and acceptable for maggot debridement therapy? *Journal of Cosmetics, Dermatological Sciences and Applications* 2: 79-82.

Lewin, B. (1997). *Genes VI*. Oxford University Press, New York, 1260 pp.

Louw, S.v.d.M and van der Linde, T.C. (1993). Insects frequenting decomposing corpses in central South Africa. *African Entomology* 1(2): 265-269.

Machado, C.A. and Hey, J. (2003). The causes of phylogenetic conflict in a classic Drosophila species group. *Proceedings of the Royal Society B* 270: 1193-1202.

Meier, R., Shiyang, K., Vaidya, G. & Ng, P.K.L. (2006). DNA barcoding and taxonomy in diptera: A tale of high intraspecific variability and low identification success. *Systematic Biology* 55(5): 715-728.

Nakhleh, L., Ruths, D. & Innan, H. Gene trees, species trees and species networks. http://www.phylo.org/pdf_docs/40_(42)Nakhleh_NakhlehRuthsInnan.pdf Accessed 2014-10-21.

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

Nichols, R. (2001). Gene trees and species trees are not the same. *TRENDS in Ecology and Evolution* 16(7): 358-364.

Norris, K.R. (1990). Evidence for the multiple exotic origin of Australian populations of the sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Australian Journal of Zoology* 38: 635-648.

Nylander, J.A.A. (2004). MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Oliva, A. (2001). Insects of forensic significance in Argentina. *Forensic Science International* 120: 145-154.

Page, R.D.M. and Charleston, M.A. (1997). From Gene to Organismal Phylogeny: Reconciled Trees and the Gene Tree/Species Tree Problem. *Molecular Phylogenetics and Evolution* 7(2): 231-240.

Paul, A.G., Ahmad, N.W., Lee, H.L., Ariff, A.M., Saranum, M., Naicker, A.S. and Osman, Z. (2009). Maggot debridement therapy with *Lucilia cuprina*: a comparison with conventional debridement in diabetic foot ulcers. *International Wound Journal* 6(1): 39-46.

Rees, D.J., Dioli, M. and Kirkendall, L.R. (2003). Molecules and morphology: evidence for cryptic hybridization in African *Hyalomma* (Acari: Ixodidae). *Molecular Phylogenetics and Evolution* 27: 131-142.

Rubinoff, D., Cameron, S. and Will, K. (2006). A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. *Journal of Heredity* 97: 581-594.

Sherman, R.A., Hall, M.J.R. and Thomas, S. (2000). Medicinal maggots: an ancient remedy for some contemporary afflictions. *Annual Review of Entomology* 45: 55-81.

Simon, C., Buckley, T.R., Frati, F., Stewart, J.B. and Beckenbach, A.T. (2006). Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. *Annual Review of Ecology,Evolution and Systematics* 37: 545-579.

Smith, K.E. (1986). *A manual of forensic entomology*. British National History Museum, London, 205 pp.

Smith, K.E. and Wall, R. (1997). The use of carrion as breeding site by the blowfly *Lucilia sericata* and other Calliphoridae. *Medical and Veterinary Entomology* 11: 38-44.

Sonet, G., Jordaens, K., Braet, Y., Desmyter, S. (2012). Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic? *Forensic Science International* 223: 153-159.

Sperling, F.A.H., Anderson, G.S. and Hickey, D.A. (1994). A DNA-based approach to the identification of insect species used for postmortem interval estimation. *Journal of Forensic Science* 39: 418-427.

Stevens, J. and Wall, R. (1996). Species, sub-species and hybrid populations of the blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae) *Proceedings Biological Sciences* 263: 1335-1341.

Stevens, J.R., Wall, R. and Wells, J.D. (2002). Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology* 11: 141-148.

Stevens, J.R. (2003). The evolution of myiasis in blowflies (Calliphoridae). *International Journal of Parasitology* 33: 1105-1113.

Swofford, D.L. (2003). PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.

Tantawi, T.I., Williams, K.A. and Villet, M.H. (2010). An Accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. *Journal of Medical Entomology* 47(3): 491- 494.

Tourle, R., Downie, D.A. and Villet, M.H. (2009). A morphological and molecular comparison of *Lucilia cuprina* and *L. sericata* (Diptera: Calliphoridae) in South Africa. *Medical and Veterinary Entomology* 23: 6-14.

Ullyett, G.C. (1945). Species of Lucilia attacking sheep in South Africa. *Nature* 155: 636-637.

Vilcinskas, A. (2011). From traditional maggot therapy to modern biosurgery. In: Vilcinskas A, ed. *Insect Biotechnology*. Springer, Netherlands, pp. 67-75.

Vogt, W.G. and Woodburn, T.L. (1979). Ecology, distribution and importance of sheep myiasis flies in Australia. *National Symposium of the sheep blowfly and flystrike in sheep.* N.S.W. Ag. Sydney, pg23-32.

Wallman, J.F., Leys, R. and Hogendoorn, K. (2005). Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. *Invertebrate Systematics* 19: 1-15.

Waterhouse, D.F. and Paramonov, S.J. (1950). The status of the two species of *Lucilia* (Diptera, Calliphoridae) attacking sheep in Australia. *Australian Journal of Scientific Research* 3: 310-336.

Wells, J.D., Wall, R. and Stevens, J.R. (2007). Phylogenetic analysis of forensically important Lucilia flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. *International Journal of Legal Medicine* 121(3): 229-233.

Whitworth, T.L., Dawson, R.D., Magalon, H. and Baudry, E. (2007). DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proceedings of the Royal Society B* 274: 1731-1739.

Williams, K.A. and Villet, M.H. (2006). A new and earlier record of *Chrysomya megacephala* in South Africa, with notes on another exotic species, *Calliphora vicina* (Diptera: Calliphoridae). *African Invertebrates* 47: 347-350.

Williams, K.A., Cronje, F.J., Avenant, L. and Villet M.H**.** 2008: Identifying flies used for maggot debridement therapy. *South African Medical Journal* 98(3): 196-97.

Wolff, H. and Hansson, C. (2005). Rearing larvae of *Lucilia sericata* for chronic ulcer treatment - an improved method. *Acta Dermato Venereologica* 85: 126-131.
Zimmer, C. (2001). *Wolbachia*: a tale of sex and survival. *Science* 292: 1093-1095.

Table 2.1: Genes used in studies of *Lucilia sericata* and *Lucilia cuprina.*

	Mitochondrial			Nuclear		
Source	CO1	12S rRNA		*28S* rRNA	*Per*	RAPDs
Stevens & Wall. 1996	-	329 bp		-	-	X
Stevens *et al*., 2002	2300 bp (CO1 & 2)	-		2193 bp	-	
Steven, 2003	2300 bp (CO1 & 2)	-		2200 bp	-	
Wallman *et al*., 2005	3008 bp (CO1 & 2 & ND4-ND4L)	-		-	-	
Wells *et al*., 2007	1545 bp	-		-	-	
Harvey *et al*., 2008	1167 bp	-		-	-	
Williams *et al*. 2008	601 bp	-		654 bp	-	
Tourle *et al*., 2009	439 bp	-		678 bp	-	
DeBry *et al*., 2010	1200bp	-		2100 bp	-	
Tantawi *et al*., 2010	576 bp	-		656 bp	746 bp	
This study	576 bp	-		654 bp	722 bp	

Table 2.2. Specimen locality data for sequences included from GenBank

Species	Locality		Accession Number		
			28S	*Per*	*COI*
L. sericata	Langford	UK	AJ300139		
	Hilerod	Denmark	AJ300140		
	Hilerod	Denmark			EF531193
	Kingsbury	UK			AJ417713
	Nerja	Spain			AJ417716
	Harare	Zimbabwe			AJ417717
	-	China			DQ345086
L. cuprina	Townsville	Australia	AJ417709		AJ417710
	Wallaceville	New Zealand		Y19108.1	
	Tororo	Uganda			AJ417711
	-	Taiwan			AY097335
	-	China			DQ345087
	Oahu	Hawaii			DQ453496
	Honolulu	Hawaii			AJ417704
	Waianae				AJ417705

Table 2.3: Specimen locality data for sequences from this study added to GenBank (* indicate identical sequences that are represented by one sequence in the Bayesian Inference tree, M = Male, F = Female)

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
Calliphora vicina	CV_FRC_01	Montferrier-Sur-Lez	France	JN792781		
Lucilia caesar	Ca_FRC_01	Montferrier-Sur-Lez	France	JN792782	JN792858	
Lucilia infernalis	In_RWN_01	Nyungwe Forest Reserve	Rwanda	JN792780	JN792857	JN813094
Lucilia cuprina	C_AUS_01* (M)	Sydney	Australia			JN792622
	C_AUS_02* (F)	Sydney	Australia			JN792623
	C_AUS_03 (F)	Hornsby Heights	Australia	JN792705	JN792783	JN792624
	C_EGT_01 (F)	Alexandria	Egypt	JN792706	JN792784	JN792625
	C_EGT_02 (F)	Alexandria	Egypt	JN792707	JN792785	JN792626
	C_SA_BFN_01(F)	Bloemfontein	South Africa	JN792708	JN792786	JN792627
	C_SA_BFN_02 (F)	Bloemfontein	South Africa	JN792709	JN792787	JN792628
	C_SA_BRT_01 (F)	Britstown	South Africa	JN792710	JN792788	JN792629
	C_SA_BRT_02 (F)	Britstown	South Africa	JN792711	JN792789	JN792630

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
	C_SA_CT_01*(M)	Cape Town	South Africa	JN792712	JN792790	JN792631
	C_SA_CT_02 (F)	Cape Town	South Africa	JN792713	JN792791	JN792632
	C_SA_CT_03*(F)	Cape Town	South Africa	JN792714	JN792792	JN792633
	C_SA_CT_04 (F)	Cape Town	South Africa	JN792715	JN792793	JN792634
	C_SA_CT_05 (F)	Cape Town	South Africa	JN792716	JN792794	JN792635
	C_SA_CT_06 (F)	Cape Town	South Africa	JN792717	JN792795	JN792636
	C_SA_CT_07 (F)	Cape Town	South Africa	JN792718	JN792796	JN792637
	C_SA_CT_08 (F)	Cape Town	South Africa	JN792719	JN792797	JN792638
	C_SA_CT_09*(F)	Cape Town	South Africa	JN792720	JN792798	JN792639
	C_SA_CT_10 (M)	Cape Town	South Africa	JN792721	JN792799	
	C_SA_CT_11*(F)	Cape Town	South Africa	JN792722	JN792800	JN792640
	C_SA_CT_12*(F)	Cape Town	South Africa	JN792723	JN792801	JN792641
	C_SA_DBN_01*(F)	Durban	South Africa	JN792724	JN792802	JN792642
	C_SA_DBN_02 (F)	Durban	South Africa	JN792725	JN792803	JN792643
	C_SA_DBN_03(M)	Durban	South Africa	JN792726	JN792804	JN792644
	C_SA_DBN_04 (F)	Durban	South Africa			JN792645
	C_SA_DBN_05 (F)	Durban	South Africa			JN792646

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
	C_SA_DBN_06 (F)	Durban	South Africa	JN792727	JN792805	JN792647
	C_SA_DBN_07*(F)	Durban	South Africa	JN792728	JN792806	JN792648
	C_SA_DBN_08 (F)	Durban	South Africa	JN792729	JN792807	JN792649
	C_SA_DBN_09 (F)	Durban	South Africa	JN792730	JN792808	JN792650
	C_SA_DBN_10*(F)	Durban	South Africa	JN792731	JN792809	JN792651
	C_SA_DBN_11*(F)	Durban	South Africa	JN792732	JN792810	JN792652
	C_SA_DBN_12 (F)	Durban	South Africa	JN792733	JN792811	JN792653
	C_SA_DBN_13 (F)	Durban	South Africa	JN792734	JN792812	JN792654
	C_SA_DBN_14*(F)	Durban	South Africa	JN792735	JN792813	JN792655
	C_SA_GHT_01(M)	Grahamstown	South Africa	JN792736	JN792814	JN792656
	C_SA_GHT_02 (F)	Grahamstown	South Africa	JN792737	JN792815	JN792657
	C_SA_NEL_01 (F)	Nelspruit	South Africa	JN792738	JN792816	JN792658
	C_SA_NEL_02 (F)	Nelspruit	South Africa	JN792739	JN792817	JN792659
	C_THA_01 (F)	Chiang Mai	Thailand	JN792740	JN792818	JN792660
	C_THA_02 (F)	Chiang Mai	Thailand	JN792741	JN792819	JN792661
	C_THA_03 (F)	Chiang Mai	Thailand	JN792742	JN792820	JN792662
	C_THA_04 (F)	Chiang Mai	Thailand			JN792663

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
	C_USA_01 (F)	Merced	United States of America	JN792743	JN792821	JN792664
	C_USA_02 (F)	Merced	United States of America	JN792744	JN792822	JN792665
	C_ZIM_01 (F)	Matobos	Zimbabwe			JN792666
	C_ZIM_02 (F)	Matobos	Zimbabwe	JN792745	JN792823	JN792667
Lucilia sericata	S_AUS_01 (M)	Seaford	Australia	JN792746	JN792824	JN792668
	S_CAN_01 (F)	Windsor	Canada	JN792747	JN792825	JN792669
	S_CAN_02 (F)	Windsor	Canada	JN792748	JN792826	JN792670
	S_FRC_01 (F)	Montferrier-Sur-Lez	France	JN792749	JN792827	JN792671
	S_FRC_02 (F)	Montferrier-Sur-Lez	France	JN792750	JN792828	JN792672
	S_FRC_03 (F)	Montferrier-Sur-Lez	France	JN792751	JN792829	JN792673
	S_GER_01 (F)	Kempen	Germany	JN792752		JN792674
	S_GER_02 (F)	Kempen	Germany		JN792830	JN792675
	S_GRC_01 (F)	Crete	Greece	JN792753		JN792676
	S_GRC_02 (F)	Crete	Greece			JN792677
	S_JPN_01* (F)	Osaka	Japan	JN792754	JN792831	JN792678
	S_JPN_02* (F)	Osaka	Japan	JN792755	JN792832	JN792679

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
	S_JPN_03* (F)	Iwate	Japan	JN792756	JN792833	JN792680
	S_JPN_04* (F)	Iwate	Japan	JN792757	JN792834	JN792681
	S_NAM_01 (F)	Possession Island	Namibia	JN792758	JN792835	JN792682
	S_NAM_02 (F)	Possession Island	Namibia	JN792759	JN792836	JN792683
	S_SA_CT_01* (F)	Cape Town	South Africa	JN792760	JN792837	JN792684
	S_SA_CT_02 (F)	Cape Town	South Africa	JN792761	JN792838	JN792685
	S_SA_CT_03* (M)	Cape Town	South Africa	JN792762	JN792839	JN792686
	S_SA_CT_04* (F)	Cape Town	South Africa	JN792763	JN792840	JN792687
	S_SA_CT_05 (F)	Cape Town	South Africa	JN792764	JN792841	JN792688
	S_SA_CT_06* (F)	Cape Town	South Africa	JN792765	JN792842	JN792689
	S_SA_CT_07* (F)	Cape Town	South Africa	JN792766	JN792843	JN792690
	S_SA_CT_08* (F)	Cape Town	South Africa	JN792767	JN792844	JN792691
	S_SA_GHT_01 (F)	Grahamstown	South Africa	JN792768	JN792845	JN792692
	S_SA_GHT_02 (F)	Grahamstown	South Africa	JN792769	JN792846	JN792693
	S_SA_PTA_01 (M)	Pretoria	South Africa	JN792770	JN792847	JN792694
	S_SA_PTA_02 (F)	Pretoria	South Africa	JN792771	JN792848	JN792695
	S_SA_PTA_03 (F)	Pretoria	South Africa	JN792772	JN792849	JN792696

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
	S_SA_PTA_04 (M)	Pretoria	South Africa	JN792773	JN792850	JN792697
	S_SA_WLK_01 (F)	Welkom	South Africa	JN792774	JN792851	JN792698
	S_SA_WLK_02 (F)	Welkom	South Africa	JN792775	JN792852	JN792699
	S_SA_WTB_01 (F)	Witbank	South Africa	JN792776	JN792853	JN792700
	S_SA_WTB_02 (F)	Witbank	South Africa	JN792777	JN792854	JN792701
	S_SWZ_01 (M)	Lausanne	Switzerland			JN792702
	S_USA_01 (F)	Michigan	United States of America	JN792778	JN792855	JN792703
	S_USA_02 (M)	Michigan	United States of America	JN792779	JN792856	JN792704

Figure 2.1: World map showing the localities where flies were caught. Insert: Map of South Africa showing the towns where flies were caught

A (28S&Per)

B (COI)

Figure 2.2 a and b: Bayesian Inference trees constructed from nuclear genes (*28S* and *Per*) (left) and mitochondrial genes (*COI*) (right) data. Posterior probabilities are indicated on nodes. Blue rectangle = ancient hybrid clade, green rectangle = modern hybrids [C = *cuprina*, S = *sericata*, CV = *Calliphora vicina*, In = *Lucilia infernalis* AUS = Australia, CAN = Canada, FRC = France, GER = Germany, GRC = Greece, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB = Witbank]

Figure 2.3: NeighborNet network diagram constructed from *28S&Per* data. [C = *cuprina*, S = *sericata*, AUS = Australia, CAN = Canada, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB = Witbank].

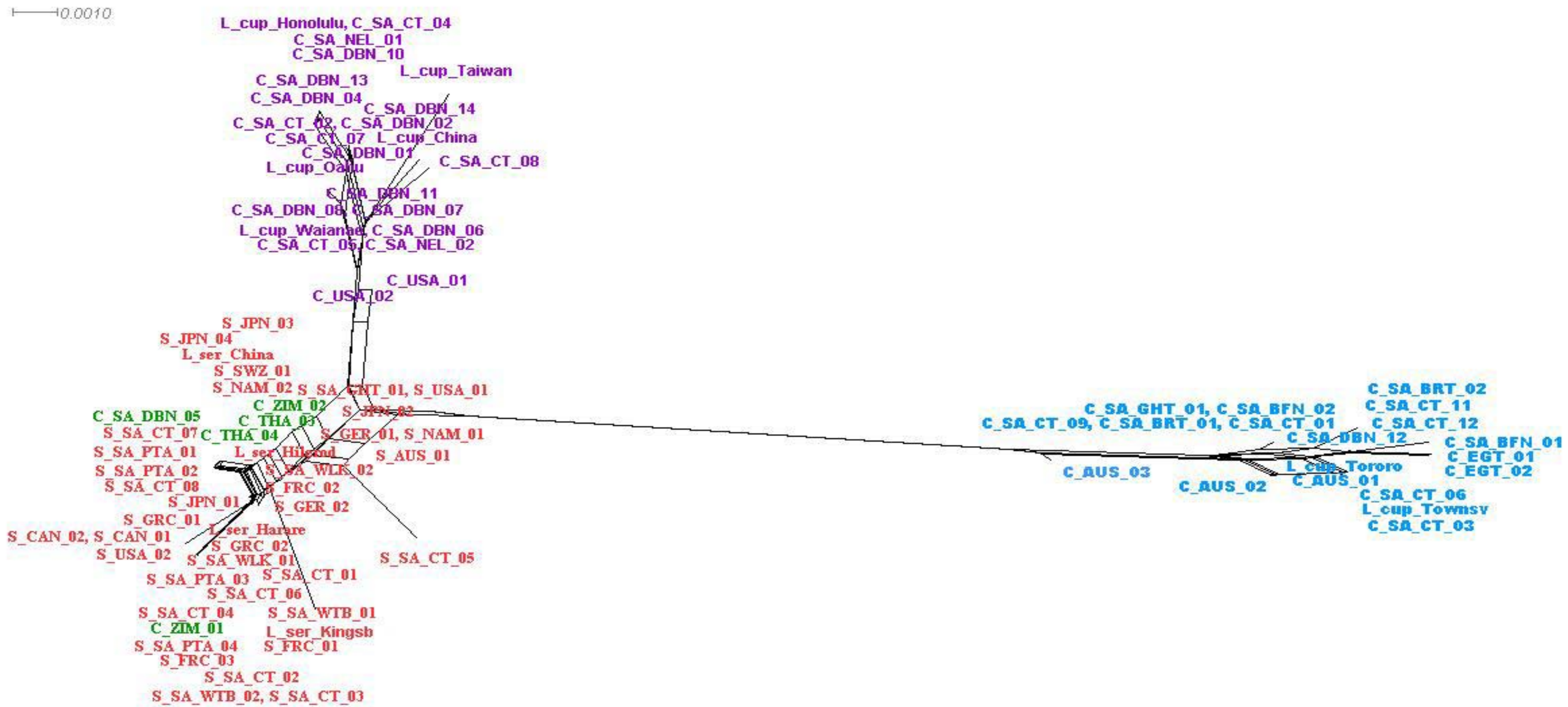

Figure 2.4: NeighorNet network diagram constructed from *COI* data. Purple = *L. cuprina ancient hybrids*, blue = *L. cuprina pure*, red = *L. sericata,* green = *L.cuprina modern hybrids.* [C = *cuprina*, S = *sericata*, AUS = Australia, CAN = Canada, FRC = France, GER = Germany, JPN =Japan, NAM = Namibia, EGT = Egypt, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB = Witbank].

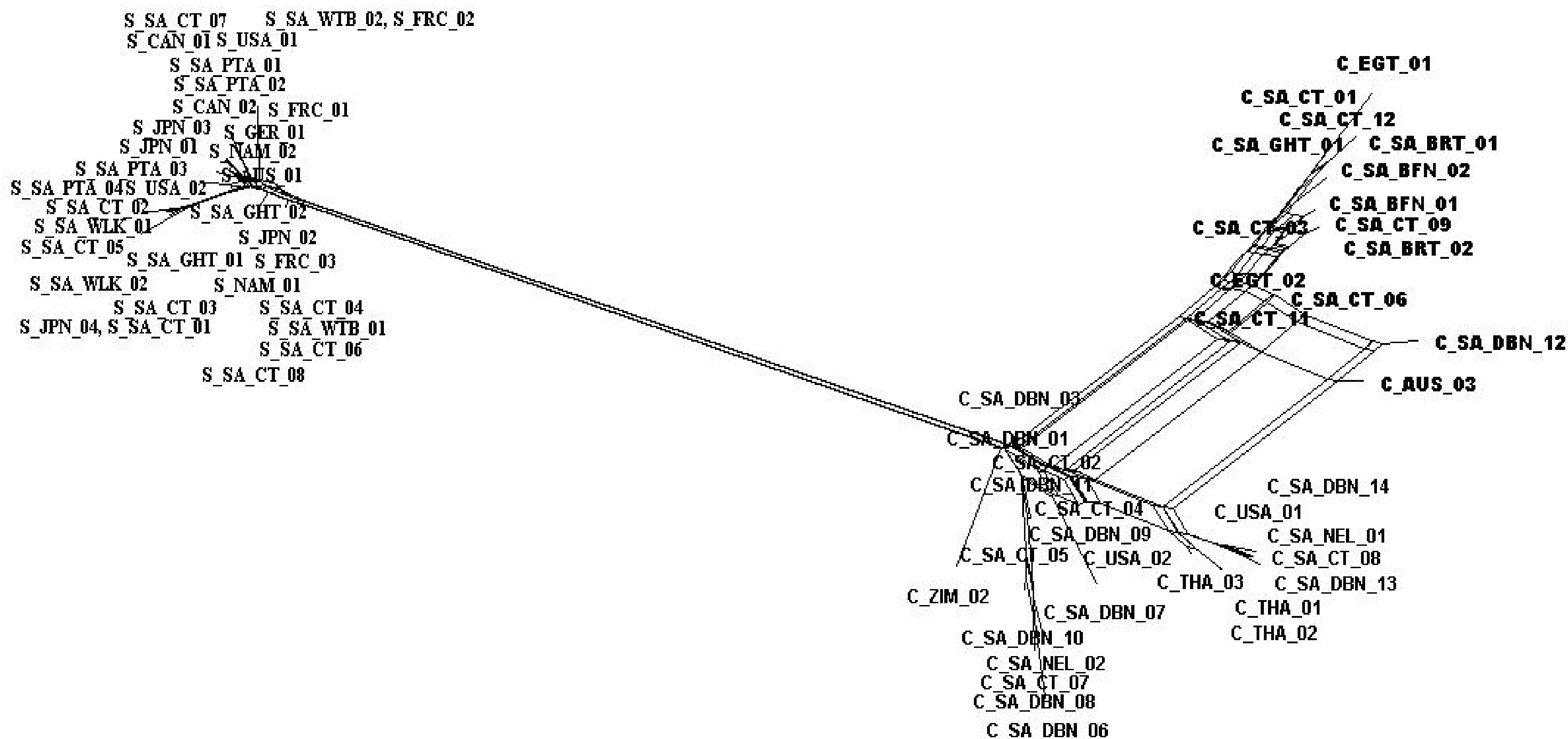

Figure 2.5: NeighborNet network diagram constructed from *28S&Per&COI* concatenated data. [C = cuprina, S = sericata, AUS = Australia, CAN = Canada, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, SA = South Africa, THA = Thailand, ZIM = Zimbabwe, CT = Cape Town, BFN = Bloemfontein, BRT = Britstown, DBN = Durban, GHT = Grahamstown, NEL = Nelspruit, PTA = Pretoria, WLK = Welkom, WTB =Witbank].

CHAPTER 3

MORPHOLOGICAL IDENTIFICATION OF LUCILIA SERICATA, LUCILIA CUPRINA AND THEIR HYBRIDS (DIPTERA: CALLIPHORIDAE)

ABSTRACT

Hybrids of *Lucilia sericata* and *Lucilia cuprina* have been shown to exist in previous studies using molecular methods, but no study has shown explicitly that these hybrids can be identified morphologically. Published morphological characters used to identify *L. sericata* and *L. cuprina* were reviewed, and then scored and tested using specimens of both species and known hybrids. Ordination by multi-dimensional scaling indicated that the species were separable, and that hybrids resembled *L. cuprina*, whatever their origin. Discriminant function analysis of the characters successfully separated the specimens into three unambiguous groups – *L. sericata*, *L. cuprina* and hybrids. The hybrids were morphologically similar irrespective of whether they were from an ancient introgressed lineage or more modern. This is the first evidence that hybrids of these two species can be identified from their morphology. The usefulness of the morphological characters is also discussed and photographs of several characters are included to facilitate their assessment.

INTRODUCTION

The use of maggot debridement therapy (MDT) in South Africa has gained interest in the past decade (Williams *et al*., 2008; Du Plessis & Pretorius, 2011). The identification of the maggots used for this therapy remains an issue, as most medical doctors are not adequately trained in entomology to correctly identify the flies (Williams *et al*., 2008; Tantawi *et al*., 2010). *Lucilia sericata* is the most commonly used species (Sherman *et al*., 2000) but it is often misidentified as *L. cuprina*. These two species are also used in forensic entomology (Louw & van der Linde, 1993; Smith & Wall, 1997; Anderson, 2000; Oliva, 2001; Clark *et al*., 2006; Day & Wallman, 2006) and *L. cuprina* is responsible for sheep strike – myiasis of sheep by the maggots of this fly (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006), but *L. sericata* is responsible for sheep strike in northern Europe where *L. cuprina* is absent (Rose & Wall, 2011). Correct identification of these flies is thus vitally important for these three fields.

Several identification keys have been produced either specifically for *L. sericata* and *L. cuprina*, or for larger suites of Luciliinae or Calliphoridae that included these two species (Waterhouse & Paramonov, 1950; Rognes, 1980; Dear, 1986; Holloway, 1991; Rognes, 1994; Wallman, 2001; Whitworth, 2006, 2010), but several of the diagnostic characters are sometimes omitted while others are included that are less reliable or difficult to observe. Although both of the flies occur worldwide, some of the differences between the character suites in these studies may arise from considering samples from relatively limited geographical regions. The first aim of this study was to consider the value of the published characters based on a sample of specimens from across the world.

A complicating factor is the known existence of natural hybrids of these species (Stevens *et al*., 2002; Wallman *et al*., 2005; Tourle *et al*., 2009; DeBry *et al*., 2010; Williams & Villet, 2013), which has been established by molecular methods. Tourle *et al.* (2009) developed a semi-quantitative morphological index for discriminating *L. sericata* and *L. cuprina*, and it provides some evidence that their hybrids might also be morphologically distinguishable. Specifically, genetically identified hybrid specimens tended to show more extreme index values than either parent species. The index incorporated six characters: femur colour; the numbers of paravertical setulae, scutellar hairs and humeral hairs; the pattern of the postoccular microtrichial pile; the length of the sternal hairs of males; and the position of the inner vertical seta of females. The

second aim of this study was to determine if hybrid specimens can in fact be determined from their morphology.

MATERIALS AND METHODS

Twenty-four specimens of *L. sericata*, *L. cuprina* and their hybrids (Table 3.1) were chosen from specimens that had been sequenced for *28S*, *COI* and *Per* genes (Williams & Villet, 2013). These specimens were chosen to include geographically diverse locations including Egypt, France, Germany, Japan, Namibia, South Africa, Thailand, the United States of America and Zimbabwe.

A total of 18 distinguishing morphological characteristics of adults of *L. sericata* and *L. cuprina* (Table 3.2) were obtained by reviewing several sources (Waterhouse & Paramonov, 1950; Rognes, 1980; Dear, 1986; Holloway, 1991; Rognes, 1994; Wallman, 2001; Tourle *et al*., 2009; Whitworth, 2006, 2010). Three characters referred to the genitalia for males and three characters were specific to females. The male characters could not be viewed without dissecting the specimens and because the majority of the genetically-identified specimens were female (Williams & Villet, 2013), it was decided to include only the 24 females in the analysis. This reduced the number of characters to 15. Photographs of the specimens were taken using a Nikon D800 camera with a 105 mm lens and 124 mm extension to show several of the characters.

Each specimen was scored against the 15 characters (Table 3.2). Each character was then evaluated for its effectiveness in discriminating between the species and its practical value for identification, first univariately and qualitatively, and then multivariately and quantitatively using non-metric multi-dimensional scaling (MDS) in PAST3 (Hammer *et al*., 2001) using a Manhattan distance metric because of the mixed data forms in the character state matrix.

To explore the diagnosibility of the hybrids, a discriminant function analysis (DFA) was performed using PAST3 (Hammer *et al*., 2001) on the scored character matrix to determine which characters were most influential in identifying the taxa. Four of the 15 characters (shape of postocular microtrichial pile, hairiness of metasternal area, contour of the last abdominal tergite, bristles on the scutellum; Table 3.2) were either not easily visible or the hairs were broken or missing in at least half of the specimens and were therefore excluded from the DFA. Another

four of the characters showed no variation within species and therefore had to be excluded from the DFA, which therefore included only seven characters (Table 3.2). The hybrid specimens were treated as a separate group in this analysis, but the introgressed and modern hybrids were not separated.

RESULTS

Univariate assessment of characters

The number of paravertical setulae or occipital bristles (Table 3.2; Figure 3.1). This character was relatively consistent and reliable, but it is not easily viewed and scored if the specimens have been kept in ethanol as the setulae lay flat against the head. The hybrid specimens all keyed out as *L. cuprina*. This character was left out of the DFA analysis due to lack of variation within *L. cuprina*.

The shape of the postocular microtrichial pile on the vertex (Table 3.2) (Holloway, 1991) is a difficult character to see when the specimens have been stored in ethanol because the microtrichia are not visible unless the specimen is dry, and even then the microtrichia sometimes appear to be absent. Due to the difficulty in viewing and scoring this character, it was eventually left out of all further analyses.

The relative positions of the three vertical setae (Table 3.2; Figure 3.1) that form a triangle on either side of the ocellar triangle in females (Holloway, 1991) is a reliable character that consistently separated the two species. This character was excluded from the DFA because it did not show variation within taxa but was included in the MDS analysis. The hybrid specimens consistently keyed out as *L. cuprina*.

The angle formed by the three vertical setae (Table 3.2; Figure 3.1). This character is consistent and easily seen even if the setae have fallen out as they have sockets, which are easily visible. Due to lack of variation within species and the hybrids being identified as *L. cuprina*, this character was also excluded from the discriminant function analysis but it was included in the MDS analysis.

The extent of the metallic sheen on the parafrontal sclerites of females (Table 3.2 & S1; Figure 3.1). This character is easier to observe in dried specimens than ethanol-preserved

specimens and there is some variation. The division between the two species is not absolute - there is some overlap within this character but it was not specific to the hybrids. It was included in both the DFA and MDS analyses.

The relative width of the frontal stripe (frontal vitta) (Table 3.2 & S1; Figure 3.2). Waterhouse & Paramonov (1950) suggested that this character was more reliable in males than females. We found that the width varied from being equal to the parafrontal in *L. cuprina* to being more than twice the width in *L. sericata*. The hybrids were not distinguishable from *L. cuprina*. This character was included in the MDS and the DFA analyses.

The colour of the frontoclypeal membrane (Table 3.2 & S1; Figure 3.3). It was not always easily visible if the proboscis was not extended but it could usually be viewed by either manipulating the proboscis or viewing the specimen from a lateral angle (Waterhouse & Paramonov, 1950). The hybrid specimens were not distinct from *L. sericata* or *L. cuprina*.

The length of the second pair of presutural acrostichals (Table 3.2) is a character that is easier to see in well-preserved specimens (Waterhouse & Paramonov, 1950. This character is not scorable if the bristles are broken or have fallen out. It was left out of the analyses because it does not show any within-species variation.

The number of setae on the scutellum (Table 3.2 & S1; Figure 3.4) in the 'quadrat' between the discal setae and the anterior margin of the scutellum represents the axis in the discriminant analysis that separated *L. sericata* and *L. cuprina* (Holloway, 1991). This character can be used even when the setae have fallen out because they have sockets that are visible and can be counted. There was overlap in the number of setae between the two species, but generally *L. cuprina* had obviously fewer setae. The number of setae in the hybrids was not obviously different from either of the pure species. This overlap may be as a result of the challenge in counting the setae as they are not in straight rows.

The length of the bristles on the scutellum (Table 3.2 & S1) describes the length of the hairs between the two anterior bristles on the lateral margin of the scutellum in relation to the length of the hairs on the dorsal surface of the scutellum (Waterhouse & Paramonov, 1950). This character

was not easy to use as the hairs were broken or had fallen out in half of the specimens and therefore it was left out of the analyses.

The hairiness of the posterior slope of the humeral callus (Table 3.2 & S1; Figure 3.5) behind the basal setae is a reliable character in separating *L. sericata* and *L. cuprina* even though there is variation within species in the number of hairs. The hybrids tended to have more hairs than the pure *L. cuprina* specimens, but there was still overlap in the numbers of hairs between the hybrids and pure *L. cuprina*.

The number of hairs on the edge of the notopleuron (Table 3.2 & S1; Figure 3.5). Both the hairs on the notopleuron and humeral callus are relatively easy to observe although ethanol-preserved specimens need to be dried so that the small hairs are visible. It is another reliable character in separating *L. sericata* from *L. cuprina* despite variation in the number of hairs within species. The hybrids showed no discernable difference in numbers of hairs from *L. cuprina*.

The hairs on the metasternal area (Table 3.2), which is the sclerite mid-ventrally between the middle and hind coxae, are exceedingly difficult to view. If the legs are not set so that one can view between the middle and hind coxae it is very difficult to see the metasternal area. All of the specimens that we examined were preserved in ethanol and it was not easy to view the metasternal area and this character was therefore not analysed.

The colour of the fore femora (Table 3.2 & S1) has long been used as a character to identify *L. sericata* and *L. cuprina* (Ullyet, 1945). It is a controversial character as it varies according to when the flies were killed, if the adults were fully matured and if the specimens were fouled or not during collection and thus is subject to personal interpretation. The hybrids keyed out as *L. cuprina*. Due to the variation in this character it was included in the DFA.

The contour of the last abdominal tergite (Table 3.2) is applicable only to dried specimens (Waterhouse & Paramonov, 1950) as it relies on the hardness of the tergite. It was therefore not a character that could be used in our analyses as all our specimens were ethanol-preserved. It was excluded from the analyses and is probably unreliable even in dried specimens because it relies on the preservation of the specimen and how it is pinned, which affects the contour of the last abdominal tergite.

Multivariate assessments of characters

Superficially, the hybrid specimens were identified as *L. cuprina* when keyed out using any of the published keys. There were no obvious differences in the morphology of the hybrids. When the characters were analysed using MDS, the hybrid specimens were not separated from the *L. cuprina* specimens (Figure 3.6).

However, the ordination plot of the DFA (Figure 3.7) clearly shows three groups – *L. sericata*, *L. cuprina* and hybrids. The most influential characters were the number of setae on the scutellum (Root 1) and the number of hairs on the humeral callus (Root 2) (Table 3.3). It is not obvious in the morphology that there is a difference between the pure and hybrid strains, but statistically one can separate the hybrids from the pure *L. cuprina* specimens.

DISCUSSION

Assessment of characters

Due to the greater number of female flies in the molecular study from which we chose our specimens, we did not include any males. Therefore the male genitalia characters are not discussed in detail. It is not possible to properly view the male genitalia without dissecting them and this is not ideal for non-entomologists such as medical doctors who are using these flies for MDT as one needs experience to dissect out the genitalia (Willaims *et al*., 2008; Tantawi *et al*., 2010). It is possible to correctly identify these flies without using the male genitalia by using the other characters described in Table 3.2.

Geographical variation

Holloway (1991) suggested that the characters that she described were specifically for *L. sericata* and *L. cuprina* from New Zealand and that they might not apply to specimens from other parts of the world. This does not seem to be the case, as the flies examined in this study are from several different countries around the world (Table 3.1) and the characters described (excluding the male genitalia) were useful in identifying these two species and their hybrids.

Identifying hybrids

The DFA unambiguously separated the *L. cuprina* specimens from the hybrids as seen in Fig. 3.7 although it was not statistically significant. This was not noted in previous studies where hybrids were identified only through molecular techniques (Stevens *et al*., 2002; Wallman *et al*., 2005; Tourle *et al*., 2009; DeBry *et al*., 2010; Williams & Villet, 2013). Examination of the number of hairs on the scutellum, humeral callus and notopleuron show a consistent difference that separates these groups. The first two characters were included in the morphological index designed by Tourle *et al*. (2009), which explains the trend found in their results.

The introgressed and modern hybrids were not separated from each other in the DFA ordination plot (Fig. 3.6).

CONCLUSION

Introgressed and modern hybrids of *L. sericata* and *L. cuprina* can be statistically recognized using the characters described in this paper.

Four of the characters used together were consistently successful at separating *L. sericata* and *L. cuprina* (number of paravertical setulae or occipital bristles, distance between the outer and inner vertical setae of females, size of the angle at the inner vertical in triangle joining pre-, outer and inner vertical setae of females, second pair of presutural acrostichals) with little variation within the characters. The number of setae on the scutellum and the number of hairs on the humeral callus and notopleuron are also useful characters although they did show variation within species. It is advisable to use a combination of several characters to identify these two species as no single character was sufficient to separate *L. sericata* and *L. cuprina*.

ACKNOWLEDGMENTS

We thank Georg Goergen, Ashley Kirk-Spriggs, Nicky Lunt, Mervyn Mansell, Hideharu Numata, Cameron Richards, Kiyoshi Saigusa, Kabkaew Sukontason, Tarek Tantawi and Robyn Tourle for providing us with specimens, Emily von Maltitz for taking photographs. Funding was provided by the National Research Foundation (NRF) of South Africa. Any opinion, findings

and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Research Foundation.

REFERENCES

Anderson, G.S. (2000). Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences* 45:824-832.

Clark, K., Evans, L., Wall, R. (2006). Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. *Forensic Science International* 156:145-149.

Day, D.M. and Wallman, J.F. (2006). Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae). *Journal of Forensic Sciences* 51:657-663.

DeBry, R., Timm, A,E., Dahlem, G.A. and Stamper, T. (2010). mtDNA-based identification of *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) in the continental United States. *Forensic Science International* 202: 102-109.

Dear, J.P. (1986). Calliphoridae (Insecta: Diptera). *Fauna of New Zealand* no. 8:1-86.

Du Plessis, H.J.C. and Pretorius, J.P. (2011). The utilisation of maggot debridement therapy in Pretoria, South Africa. *Wound Healing Southern Africa* 4(2): 80-83.

Hammer, Ø,. Harper, D.A.T. and Ryan, P.D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* 4(1): 9pp.

Heath, A.C.G. and Bishop, D.M. (2006). Flystrike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* 137: 333-344.

Hepburn, G.A. (1943). Sheep blowfly research I - A survey of maggot collections from live sheep and a note on the trapping of blowflies. Onderstepoort *Journal of Veterinary Science and Animal Industry* 18(1-2): 13-18.

Holloway, B.A. (1991). Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). *New Zealand Journal of Zoology* 18: 415-420.

Louw, S v.d.M. and van der Linde, T.C. (1993). Insects frequenting decomposing corpses in central South Africa. *African Entomology* 1(2): 265-269.

Oliva, A. (2001). Insects of forensic significance in Argentina. *Forensic Science International* 120: 145-154.

Rognes, K. (1980). The blow-fly genus *Lucilia* Robineau-Desvoidy (Diptera, Calliphoridae) in Norway. *Fauna Norvegica Series B* 27: 39-52.

Rognes, K. (1994). First record of the sheep greenbottle fly *Lucilia cuprina* (Wiedemann, 1830) from Europe (Diptera: Calliphoridae) with additional Spanish records of Calliphoridae, Muscidae and Sarcophagidae. *Eos* 69: 41-44.

Sherman, R.A., Hall, M.J.R. and Thomas, S. (2000). Medicinal maggots: an ancient remedy for some contemporary afflictions. *Annual Review of Entomology* 45: 55-81.

Smith, K.E. and Wall, R. (1997). The use of carrion as breeding site by the blowfly *Lucilia sericata* and other Calliphoridae. *Medical and Veterinary Entomology* 11: 38-44.

Stevens, J.R., Wall, R. and Wells, J.D. (2002). Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology* 11: 141-148.

Tantawi, T.I., Williams, K.A. and Villet, M.H. (2010). An Accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. *Journal of Medical Entomology* 47: 491-494.

Tourle, R., Downie, D.A. and Villet, M.H. (2009). A morphological and molecular comparison of *Lucilia cuprina* and *L. sericata* (Diptera: Calliphoridae) in South Africa. *Medical and Veterinary Entomology* 23: 6-14.

Ullyett, G.C. (1945). Species of *Lucilia* attacking sheep in South Africa. *Nature* 155:636-637.

Vogt, W.G. and Woodburn, T.L. (1979). Ecology, distribution and importance of sheep myiasis flies in Australia. *National Symposium of the sheep blowfly and flystrike in sheep*. New South Wales Department of Agriculture Sydney, Australia p23-32.

Wallman, J.F. (2001). A key to the adults of species of blowflies in southern Australia known or suspected to bred in carrion [corrigendum in Medical and Veterinary Entomology 16, 223]. *Medical and Veterinary Entomology* 15: 433-437.

Wallman, J.F., Leys, R. and Hogendoorn, K. (2005). Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. *Invertebrate Systematics* 19: 1-15.

Waterhouse, D.F. and Paramonov, S.J. (1950). The status of the two species of *Lucilia* (Diptera, Calliphoridae) attacking sheep in Australia. *Australian Journal of Scientific Research* 3: 310-336.

Whitworth, T. (2006). Keys to the genera and species of blow flies (Diptera: Calliphoridae) of America north of Mexico. *Proceedings of the Entomological Society of Washington* 108: 689-725.

Whitworth, T. (2010). Keys to the genera and species of blow flies (Diptera: Calliphoridae) of the West Indies and description of a new species of *Lucilia* Robineau-Desvoidy. *Zootaxa* 2663: 1-35.

Williams, K.A., Cronje, F.J., Avenant, L. and Villet, M.H. (2008). Identifying flies used for maggot debridement therapy. *South African Medical Journal* 98: 196-197.

Williams, K.A. and Villet, M.H. (2013). Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae). *European Journal of Entomology* 110: 187-196.

Table 3.1: Specimens previously identified by molecular markers (Williams & Villet, 2013) used in the morphological analyses. (*hybrids)

Species	Specimen	Country of origin
Lucilia cuprina	C_EGT_01	Egypt - Alexandria
Lucilia cuprina	C_SA_BFN_01	South Africa – Bloemfontein
Lucilia cuprina	C_SA_BFN_02	South Africa – Bloemfontein
Lucilia cuprina	C_SA_BRT_01	South Africa – Britstown
Lucilia cuprina	C_SA_BRT_02	South Africa – Britstown
**Lucilia cuprina*	C_SA_DBN_01	South Africa – Durban
**Lucilia cuprina*	C_SA_DBN_06	South Africa – Durban
Lucilia cuprina	C_SA_DBN_12	South Africa – Durban
**Lucilia cuprina*	C_SA_NEL_01	South Africa – Nelspruit
**Lucilia cuprina*	C_SA_NEL_02	South Africa – Nelspruit
**Lucilia cuprina*	C_THA_03	Thailand – Chiang Mai
**Lucilia cuprina*	C_ZIM_02	Zimbabwe – Matobos
Lucilia sericata	S_FRC_02	France – Montferrier-Sur-Lez
Lucilia sericata	S_GER_01	Germany – Kempen
Lucilia sericata	S_JPN_04	Japan – Iwate
Lucilia sericata	S_NAM_01	Namibia – Possession Island
Lucilia sericata	S_NAM_02	Namibia – Possession Island
Lucilia sericata	S_SA_CT_01	South Africa – Cape Town

Species	Specimen	Country of origin
Lucilia sericata	S_SA_CT_05	South Africa – Cape Town
Lucilia sericata	S_SA_GHT_01	South Africa – Grahamstown
Lucilia sericata	S_SA_GHT_02	South Africa – Grahamstown
Lucilia sericata	S_SA_PTA_02	South Africa – Pretoria
Lucilia sericata	S_SA_WTB_02	South Africa – Witbank
Lucilia sericata	S_USA_01	United States of America – Michigan

Table 3.2. Published morphological characters used to distinguish specimens of *Lucilia sericata* and *L. cuprina*.

Character	*Lucilia sericata*	*Lucilia cuprina*	Analysis	
			MDS	DFA
General				
Number of paravertical setulae or occipital bristles (Waterhouse & Paramonov, 1950; Dear, 1986; Holloway, 1991; Rognes, 1994; Whitworth, 2006, 2010)	Usually 2+2 but up to 8+8 (not always equal numbers i.e. can be 1+2 etc.)	1+1	yes	no
Shape of postocular microtrichial pile on vertex (viewed obliquely from behind) (Holloway, 1991)	Boundary between pale and dark areas not straight or sharply defined	Boundary straight and sharply defined	no	no
Width of the frontal stripe (frontal vitta) (Waterhouse & Paramonov, 1950; Rognes 1980, 1994)	Twice as wide as a parafrontal (fronto-orbital) plate	As wide as a parafrontal (fronto-orbital) plate	yes	yes
Colour of the frontoclypeal membrane (Waterhouse & Paramonov, 1950; Wallman, 2001)	Light brown	Dark brown to black	yes	yes
Second pair of presutural acrostichals (Waterhouse & Paramonov, 1950)	Extend at least as far as insertions of the first pair of postsutural acrostichals	Do not extend to first pair of postsutural acrostichals	yes	no
Number of setulae on 'quadrat' between discal setae and anterior margin of scutellum (Holloway, 1991)	35 - 55	15 - 25	yes	yes
Bristles on the scutellum (Waterhouse & Paramonov, 1950)	Dorsal bristles distinctly smaller than lateral hairs	Dorsal bristles slightly smaller than or equal to lateral hairs	no	no

Character	*Lucilia sericata*	*Lucilia cuprina*	Analysis	
			MDS	DFA
Number of hairs on the posterior slope of the humeral callus behind the basal setae (Waterhouse & Paramonov, 1950; Rognes, 1994; Whitworth, 2006)	6 – 8	0 - 4	yes	yes
Number of hairs on the edge of the notopleuron behind the posterior notopleural seta (Waterhouse & Paramonov, 1950; Rognes, 1994; Whitworth, 2006)	8 - 16	2 – 5	yes	yes
Metasternal area – sclerite midventrally between middle and hind coxae (Rognes, 1994, Wallman, 2001, Whitworth, 2006)	Hairy	Bare	no	no
Colour of the fore femora (Waterhouse & Paramonov, 1950; Dear, 1986; Wallman, 2001)	Dark metallic blue to black or dark brown	Metallic green	yes	yes
Contour of the last abdominal tergite (Waterhouse & Paramonov, 1950)	Irregular depressions	Generally smooth	no	no
Females				
Distance between the outer and inner vertical setae of females (Holloway, 1991)	Equal to 0.5 - 0.7 distance between prevertical and inner vertical setae	Equal to the distance between prevertical and inner vertical setae	yes	no
Size of the angle formed by the inner vertical seta relative to the prevertical and outer vertical setae of females (Holloway, 1991)	Obtuse	Right angle	yes	no

Character	*Lucilia sericata*	*Lucilia cuprina*	Analysis	
			MDS	DFA
Extent of metallic sheen on parafrontal sclerites of females (Holloway, 1991)	From vertex barely to base of upper orbital seta and not enclosing bases of any frontal setae	From vertex almost to base of lower orbital seta and enclosing bases of 1 or 2 frontal setae	yes	yes
Males				
Shape of apical halves of cerci (Waterhouse & Paramonov 1950; Holloway, 1991)	Broad and tapering	Slender and parallel	no	no
Shape of apical halves of surstyli (Waterhouse & Paramonov; 1950, Rognes, 1980; Holloway, 1991)	Curved and broad	Straight and slender	no	no
Form of apical setae of cerci (Holloway, 1991)	Long and wavy	Minute and straight	no	no

Table 3.3: Eigen vectors and values for the first two roots of the discriminant function analysis

Character	Root 1	Root 2
Number of setulae on 'quadrat' between discal setae and anterior margin of scutellum	**1.5822**	0.0324
Number of hairs on edge of notopleuron behind posterior notopleural seta	0.5576	**0.3300**
Number of hairs on posterior slope of humeral callus behind basal setae	0.4216	**0.9066**
Colour of fore femora	0.2591	**-0.2023**
Relative width of frontal stripe (frontal vitta)	0.1551	0.0104
Extent of metallic sheen on parafrontal sclerites of females	0.0519	-0.0697
Colour of frontoclypeal membrane	-0.1551	-0.0104
Eigenvalue	18.5560	0.7406

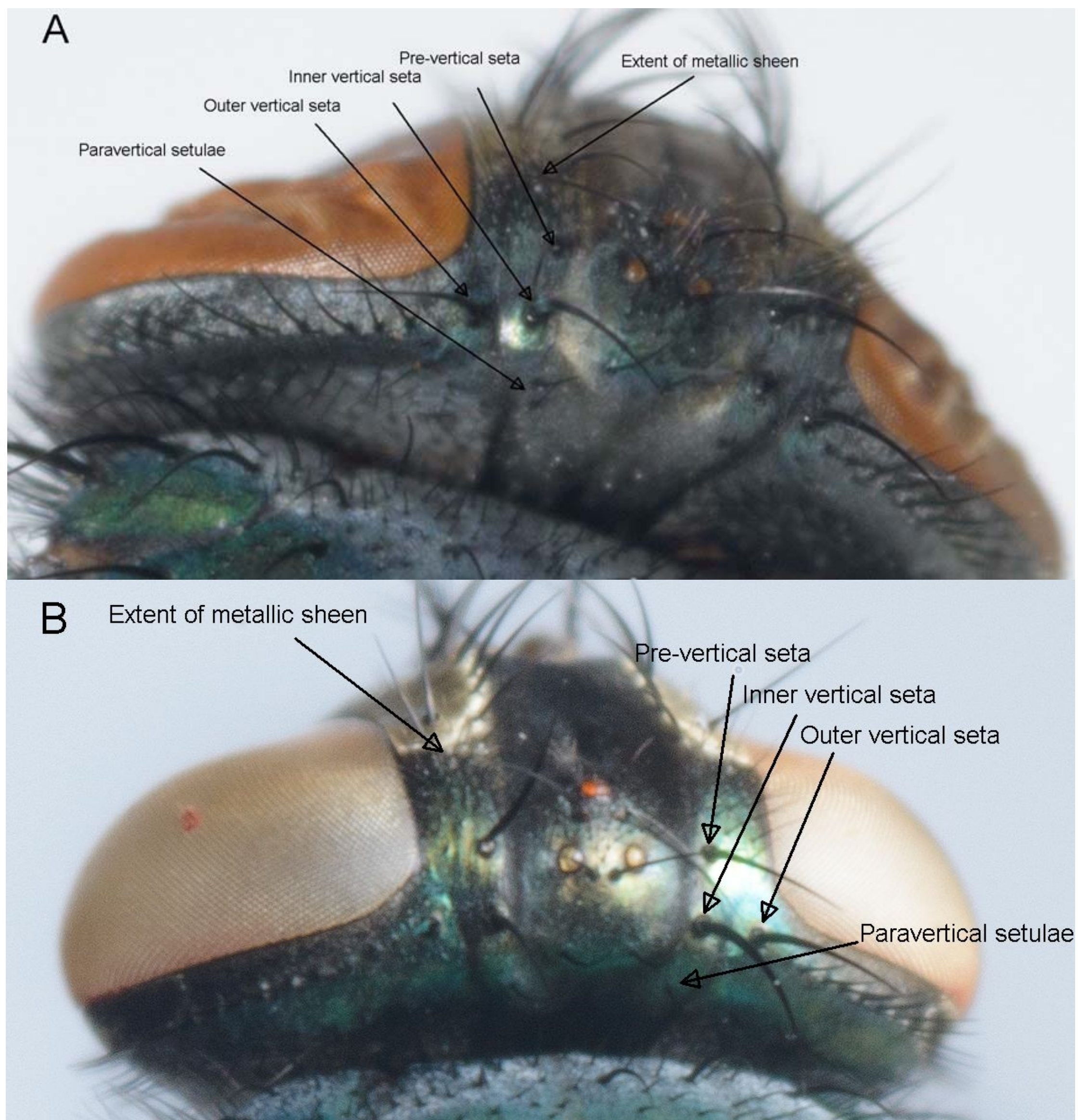

Figure 3.1: Paravertical setulae, distance between the outer and inner vertical setae, the size of the angle at the inner vertical triangle and extent of metallic sheen on parafrontal sclerites. *L. sericata* (A) and *L. cuprina* (B).

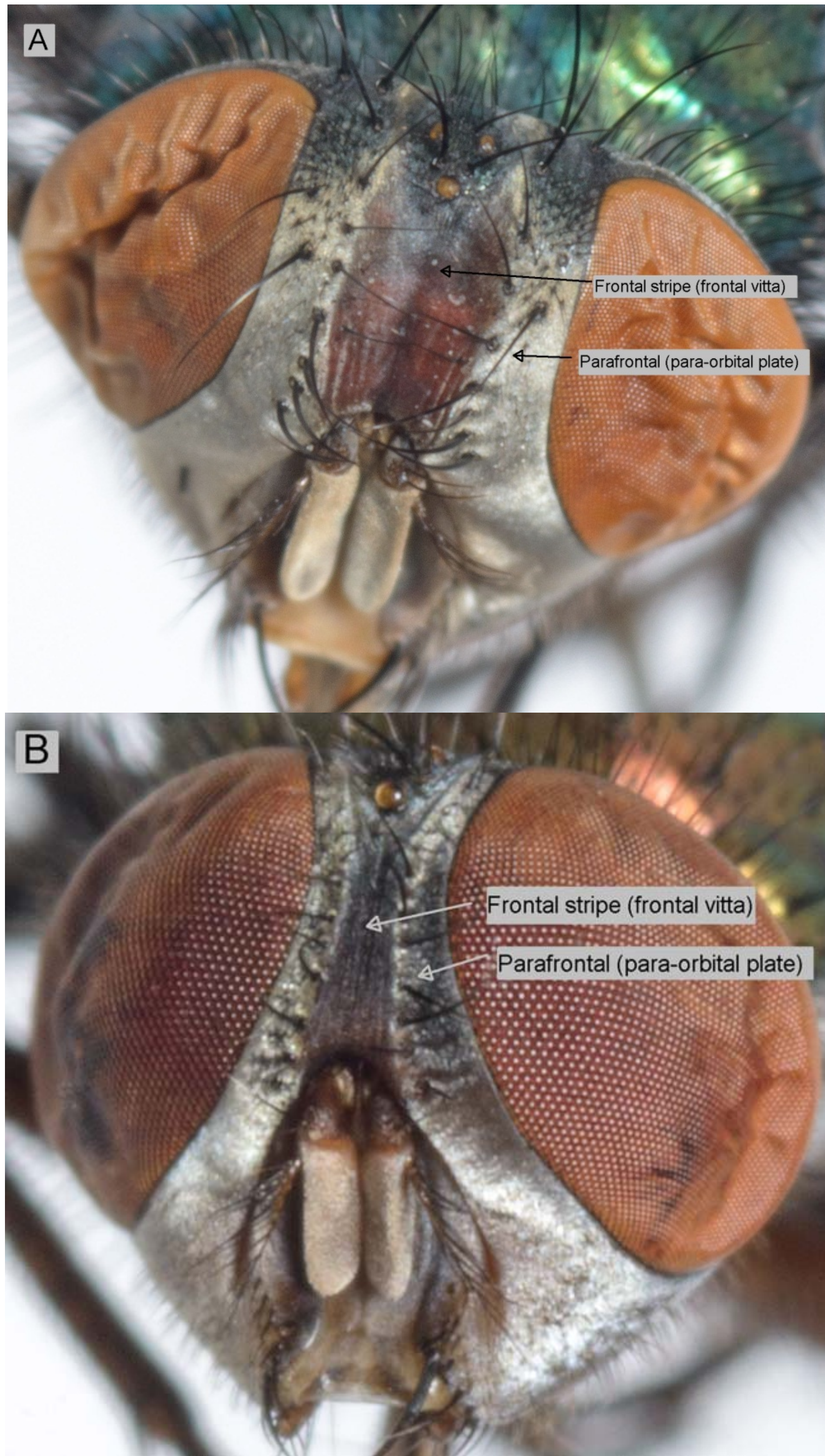

Figure 3.2: Relative width of frontal stripe – *L. sericata* (A) and *L. cuprina* (B).

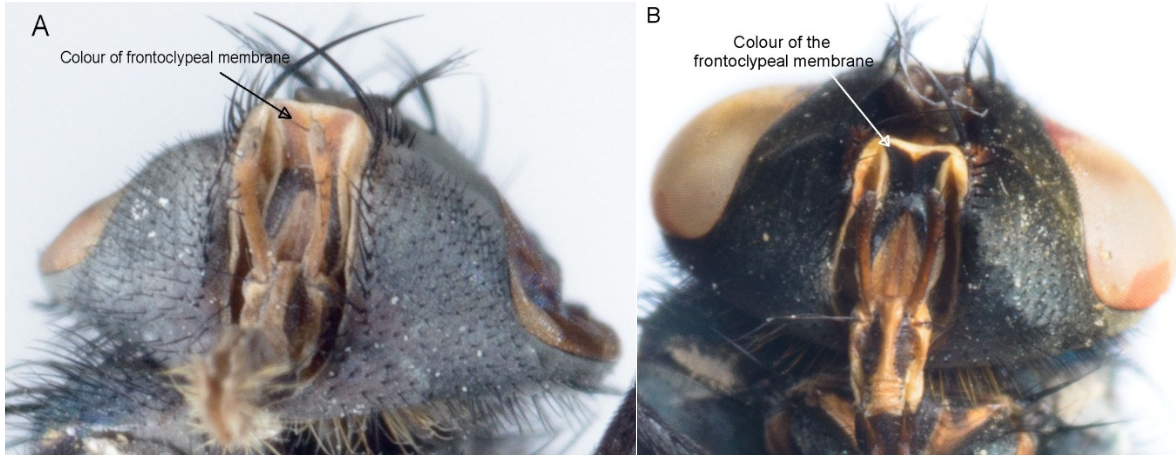

Figure 3.3: Colour of the frontoclypeal membrane. *L. sericata* (A) and *L. cuprina* (B).

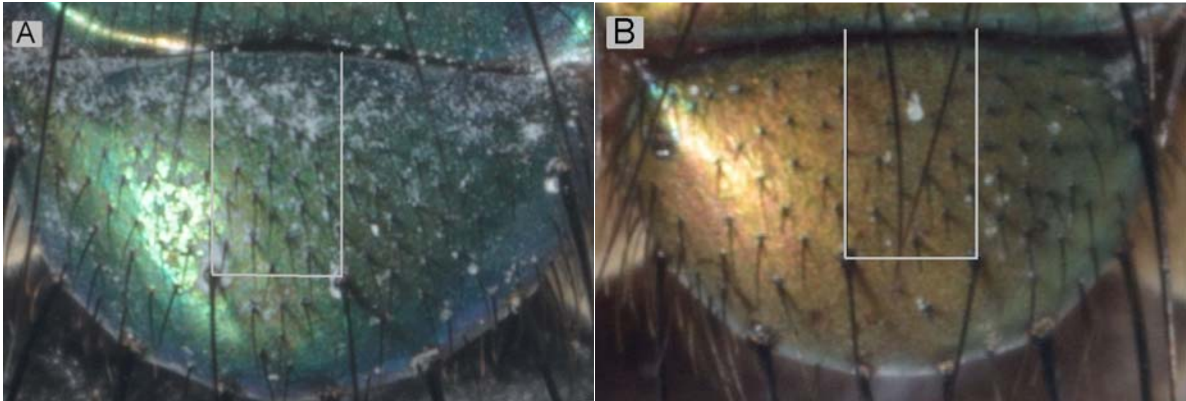

Figure 3.4: Number of setae on ‘quadrat’ between the anterior margin and discal setae on the scutellum. *L. sericata* (A) and *L. cuprina* (B).

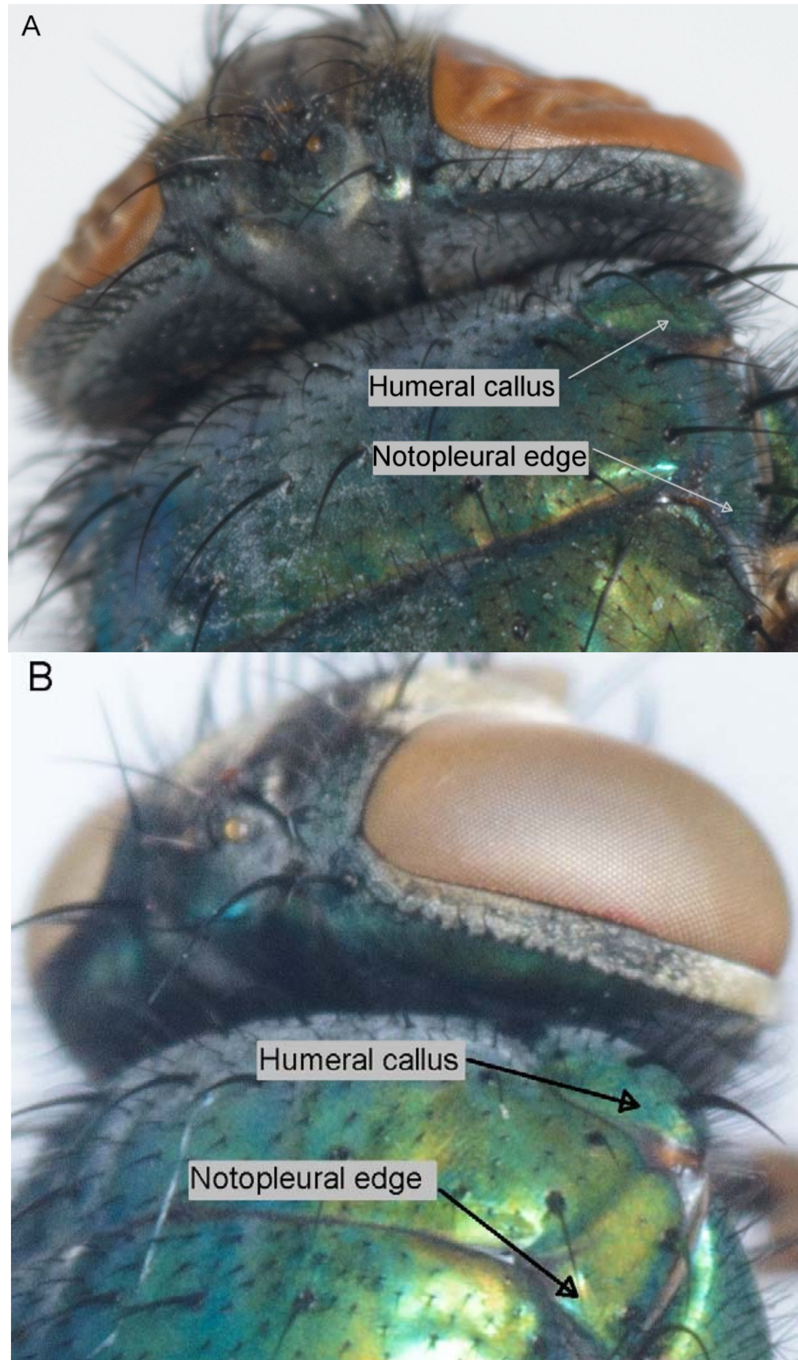

Figure 3.5: Posterior slope of the humeral callus behind the basal setae and the posterior edge of notopleuron behind the posterior notopleural seta. *L. sericata* (A) and *L. cuprina* (B).

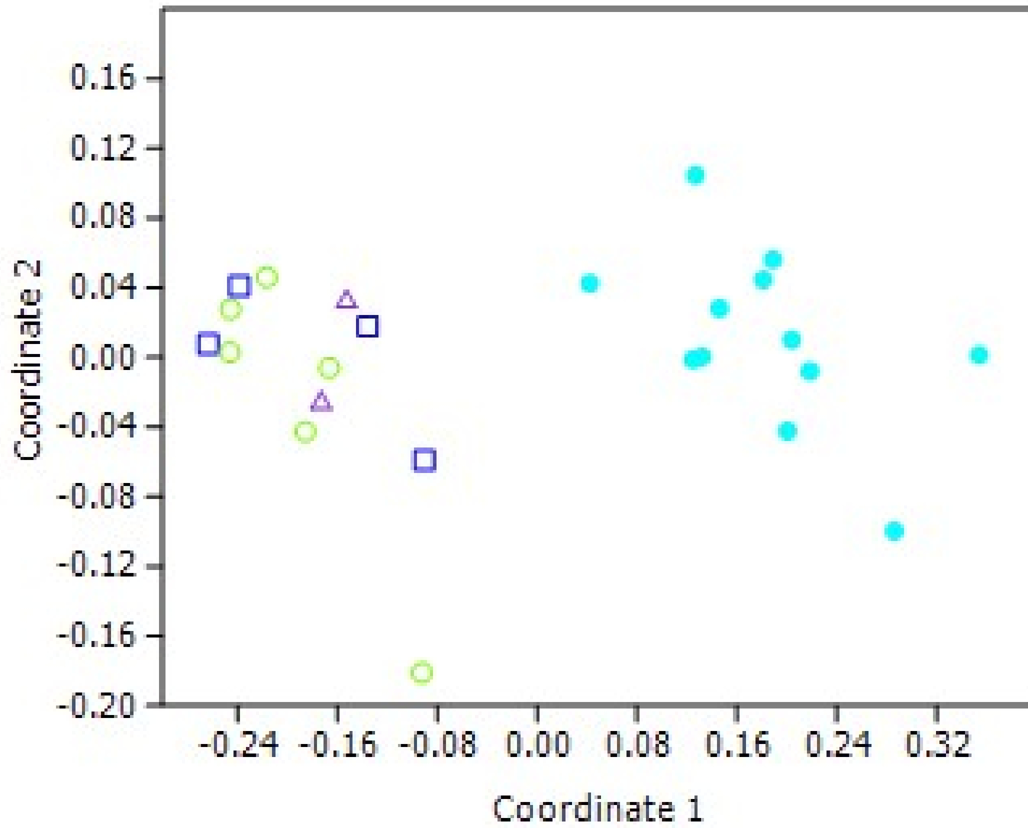

Figure 3.6: Non-metric Multi-Dimensional Scaling plot using a Manhattan distance metric using 11 described characters. Light blue solid circles = *L. sericata*, Green open circles = *L. cuprina*, dark blue squares = introgressed hybrids, purple triangles = modern hybrids.

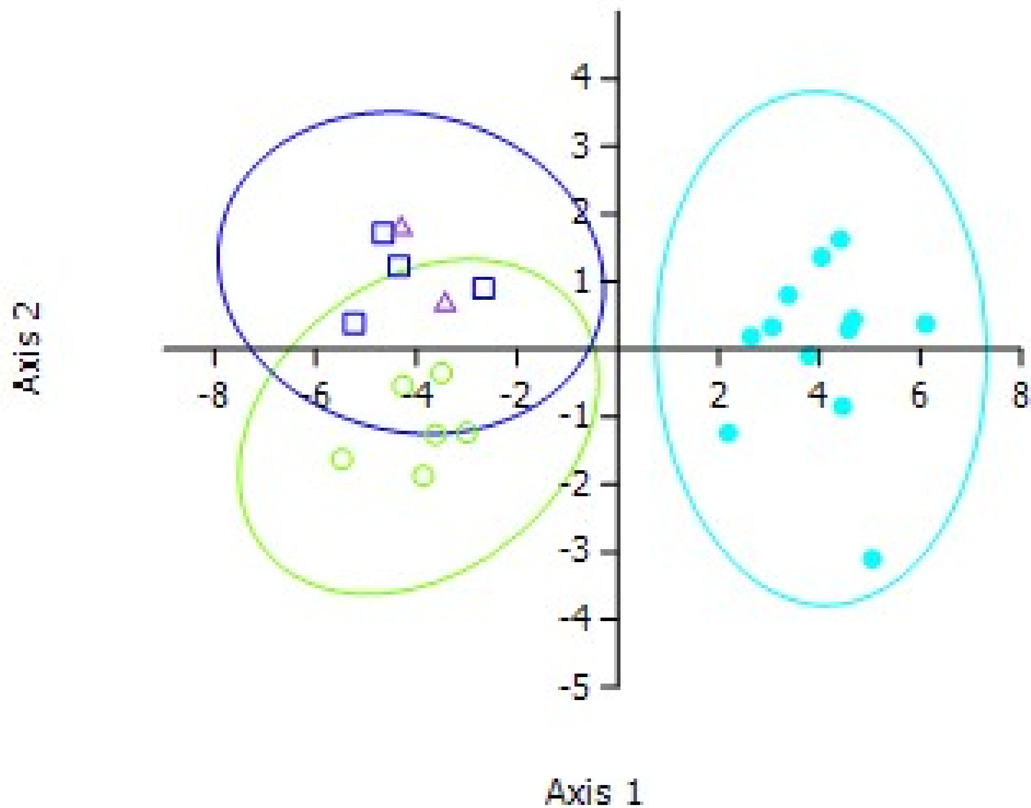

Figure 3.7: Ordination plot of the first two roots of the discriminant function analysis using seven characters. Ellipses represent 95% confidence regions. Light blue solid circles = *L. sericata*, Green open circles = *L. cuprina*, dark blue squares = introgressed hybrids, purple triangles = modern hybrids.

Supplementary Table S1. Character-taxon matrix used in the MDS and DFA analyses.

	L. cuprina						hybrids						*L. sericata*											
	C_EGT_01	C_SA_BFN_01	C_SA_BFN_02	C_SA_BRT_01	C_SA_BRT_02	C_SA_DBN_12	C_SA_DBN_01	C_SA_DBN_06	C_SA_NEL_01	C_SA_NEL_02	C_THA_03	C_ZIM_02	S_FRC_02	S_GER_01	S_JPN_04	S_NAM_01	S_NAM_02	S_SA_CT_01	S_SA_CT_05	S_SA_GHT_01	S_SA_GHT_02	S_SA_PTA_02	S_SA_WTB_02	S_USA_01
Extent of metallic sheen on parafrontal sclerites of females (0 = enclosing; 1 = not enclosing)	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	1	1
Frontal stripe (0 = equal, 1 = one and a half times; 2 = twice as wide, 3 = more than twice as wide)	2	0	2	0	1	2	2	1	0	0	2	2	2	2	3	2	2	2	2	3	3	3	2	3
Clypeus colour (0 = light brown; 1 = dark brown; 2 = black)	1	1	2	2	1	1	1	2	1	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0
Number of setulae on quadrat between anterior margin & discal setae (0 = 1 hair; 1 = 2; 2 = 3 etc.)	21	16	20	31	16	14	26	22	14	15	21	19	34	37	33	31	26	34	43	32	29	32	31	26
Humeral callus - posterior slope of humeral callus behind the basal setae (0 = 0; 1 = 1; 2 =2 etc.)	0	1	1	0	3	2	4	3	2	4	2	4	6	8	5	7	6	2	7	5	5	5	7	4
Notopleura - posterior edge of notopleuron behind the posterior notopleural setae (0 = 0,	2	3	4	0	3	2	1	5	2	3	2	5	8	6	8	8	9	8	8	6	7	5	9	4

1 = 1, 2 =2 etc.)																								
Colour of fore femora (0 = metallic coppery green; 1 = metallic coppery brown; 2 = brown; 3 = black)	0	0	1	0	1	1	0	0	0	0	0	0	2	3	2	2	3	3	3	2	2	2	2	2
Number of occipital bristles (0 = 1+1, 1 = 2+2 or more)	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
Second pair of presutural acrostichals (0 = do not extend, 1 = extend beyond point on insertion)	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
Distance between outer and inner vertical setae (0 = equal, 1 = 0.1, 2 = 0.2 etc)	0	0	0	0	0	0	0	0	0	0	0	0	7	9	7	5	5	6	5	3	7	7	5	3
Size of angle formed by inner vertical seta relative other vertical setae (0 = right, 1 = obtuse)	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1

CHAPTER 4

PREDICTING THE GEOGRAPHIC DISTRIBUTION OF LUCILIA SERICATA AND LUCILIA CUPRINA (DIPTERA: CALLIPHORIDAE) IN SOUTHERN AFRICA

ABSTRACT

Lucilia sericata (Meigen, 1826) and *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae: Luciliinae) have medical, veterinary and forensic importance. Knowing their distribution in South Africa would allow more effective management and utilisation of these flies. Their predicted geographic distributions in South Africa were modelled using maximum entropy analysis of selected climatic variables. The most important environmental variables in modelling the distributions were the magnitude of monthly rainfall and the magnitude of the monthly maximum temperature for *L. sericata* and the seasonal variation in monthly mean humidity and magnitude of monthly rainfall for *L. cuprina*. A clear geographical bias was shown in museum records and supports the need for focused surveys. There was no correlation between the predicted distribution of *L. cuprina* and sheep farming in South Africa, nor between the predicted distribution of *L. sericata* and human population density. Although their distributions differed, both species have a widespread distribution in South Africa and one cannot therefore identify these flies by locality alone – morphological or molecular identification is necessary.

INTRODUCTION

Lucilia sericata is a cosmopolitan greenbottle blowfly originating from Europe that is used in maggot debridement therapy (MDT) – the use of maggots to clean necrotic wounds on living human beings (Sherman *et al*., 2000; Wolff & Hansson, 2005; Williams *et al*., 2008; Altincicek & Vilcinskas, 2009; Paul *et al*., 2009; Tantawi *et al*., 2010); forensic entomology (Louw & van der Linde, 1993; Smith & Wall, 1997; Anderson, 2000; Oliva, 2001; Clark *et al*., 2006; Day & Wallman, 2006) and, to a lesser extent in South Africa, involved in sheep strike – the process where these flies lay their eggs on living sheep and the maggots damage the wool and skins by feeding on the sheep (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006). It has been suggested that in South Africa this species occurs in urban areas and is not found in rural settings (Meskin, 1986; Braack & deVos, 1987). *Lucilia cuprina*, its sister species, is indigenous to Africa and Asia. It is a huge problem in sheep strike (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006), has been successfully used in MDT (Paul *et al*., 2009; Tantawi *et al*., 2010) and is useful in forensic investigations (Louw & van der Linde, 1993; Day & Wallman, 2006). It is thought that in South Africa this species occurs primarily in rural environments and seldom near human habitation (Meskin, 1986; Braack & de Vos, 1987). Both of these species have the potential to spread disease because they breed in decaying and rotting organic matter (Zumpt, 1952).

To understand these flies for forensic investigations and veterinary research, one needs to know where they occur both locally and country-wide. Knowing their geographic distribution would also assist in developing strategies to control fly strike in sheep farming areas. Mapping the distribution of *L. sericata* is complicated by the species being introduced to the country, so that it may still be spreading to the limits of its potential distribution. In this situation, old maps need to be updated with new distribution records. Climate change creates a greater challenge for understanding both species because changing conditions at a locality may alter its suitability for either species, so that old historical locality records eventually need to be revised.

Species distribution modelling techniques produce maps of the potential distribution of species (Elith & Leathwick, 2009). MaxEnt (Phillips *et al*., 2006; Phillips & Dudík,

2008) is a predictive biogeography programme that uses a maximum entropy algorithm to match known locality points of a species to potential localities based on their environmental characteristics. It is a useful technique because it does not require absence records to build a predictive model. This allows one to use museum and other occurrence records without having to do field work to provide absence records, which is costly, time-consuming and often ambiguous in outcome (Phillips & Dudík, 2008).

In this paper we present models of predicted geographic distributions for *L. sericata* and *L. cuprina* in South Africa and discuss the environmental variables highlighted by these models. The correlations between where these species are predicted to occur and the distribution of sheep farming and human settlements are also examined.

MATERIALS AND METHODS

Locality records

Historical occurrence records for *L. sericata* and *L. cuprina* were obtained from the following museums: KwaZulu-Natal Museum (formerly the Natal Museum; Pietermaritzburg); Albany Museum (Grahamstown); Iziko Museum (formerly the South African Museum; Cape Town); Durban Natural Science Museum (Durban) and the National Museum (Bloemfontein). The identifications of the specimens were confirmed by the authors. Indeterminable specimens were excluded. Ten additional records with co-ordinates were obtained from literature surveys (Braack, 1986; Braack & de Vos, 1987; Louw & van der Linde, 1993).

Current occurrence records were obtained from personal contacts (see acknowledgements) and from five collecting surveys, undertaken after the literature survey and museum records were obtained, to collect data from poorly-sampled and under-represented areas of the country. Traps were hung a metre above the ground from available vegetation at ~50 km intervals along the chosen route (Fig. 4.1 A & B) and left for four days. They were placed in rural areas along the roadside, at least 2 km away from towns and out of sight of human settlements. Field trips were conducted year-round except for winter (May – August), when blowfly numbers are known to be low (Williams, 2002). Redtop™ fly traps (Miller Methods, Ltd.) were modified by removing the base of the traps and attaching screw-top jars containing

fresh chicken liver to them (Fig. 4.2). The centres of the jars' lids were cut out and the mouth of the jars covered in netting. The jars were then screwed onto the plastic base of the traps with the lids. This allowed flies to detect the odour of the bait and enter the trap, but prevented them from getting into the bait and thus becoming too fouled to identify. The flies were therefore confined to the bag of the trap and after being placed in a freezer, were easily removed.

A total of 132 records (60 collection and 72 survey records) were obtained for *L. cuprina* and 120 (39 collection and 81 survey records) records for *L. sericata.* There were several survey sites that did not record both or either species (Fig. 4.1 A & B). No museum or literature records more than 50 years old were used for the analysis as these are not within the temporal span of the climatic variables used in this study (Schulze *et al*., 1997). Duplicated site records were removed to prevent pseudoreplication.

Environmental variables

Eleven climatic predictor variables were selected for building the models. These represented variables that are regarded as appropriate to ectotherms at global and regional scales (Mackey & Lindenmayer, 2001; Phillips, 2008; Richards *et al*., 2009). No vegetation variables were included because these flies are habitat generalists and do not require a particular type of vegetation to breed. It was anticipated that climatic variables would have the greatest abiotic influence on their distribution (Meskin, 1986; Braack & de Vos, 1987; Williams, 2002). Digital maps of the variables for South Africa were developed by Schulze *et al*. (1997) to produce continuous digital maps at a resolution of 60 pixels per degree (i.e. about 1.6 x 1.6 km) by interpolating from point data obtained from a network of weather stations throughout South Africa and averaged over 10 years (Schulze *et al*., 1997). Principle component analyses (PCA) were performed on the climatic variable maps as per Richards *et al*. (2009), which resulted in two summary layers for each variable (Table 4.1). The first variable generally represents the magnitude of the climatic variable while the second generally represents the seasonal variation of the variable (Richards *et al*., 2009).

Model building

MaxEnt 3.3.3k software was used as it requires only presence records and its efficacy has been well documented (Elith *et al*., 2006; Phillips *et al*., 2006; Phillips & Dudík, 2008). The default parameters of MaxEnt were used (Phillips & Dudík, 2008). These included the regularization multiplier (1), maximum number of iterations (500), maximum number of background points (10 000) and convergence threshold (0.00001). Only hinge features were used as this avoids the overfitting of MaxEnt models when dealing with alien species (Elith *et al*., 2010) and 25% of the data were reserved to test the model. The outputs of ten replicates were combined to give a mean output. A logistic output for constructing the predictive maps was selected as it is the easiest to comprehend, giving a value between 0 and 1 as a probability of an organism occurring (Phillips & Dudík, 2008). Jackknife analyses and mean area-under-curve (AUC) plots were created using MaxEnt. AUC is commonly used as a test of the overall performance of the model (Elith *et al*., 2006) and although reservations have been expressed about its utility (Lobo *et al*., 2008), it continues to be used as a handy indication of the usefulness of a model (Elith *et al*., 2006, 2011). A value of 1.00 is perfect agreement of the model while a value of 0.50 represents a random fit. Jackknife analysis indicates which variable has the greatest influence on the model and the overall success of the model.

The data were then divided into survey records and museum records and each partition was modelled separately to assess the importance of doing a focused survey.

Post hoc comparisons

To examine the putative relationship of each species to sheep strike, data for wool production in South Africa for 2011/2012 were obtained from Cape Wools SA and mapped to the magisterial level as kg/km^2 (Fig. 4.3) to show the areas of highest density of sheep farming in South Africa. The average predicted likelihood values from the MaxEnt models for both *L. sericata* and *L. cuprina* at a magisterial level were plotted against the values for wool production and the correlation coefficient was calculated using PAST3 (Hammer *et al*., 2001) (Fig. 4.5 A & B).

To evaluate the putative synanthropy of *L. sericata*, human population density figures for South Africa were obtained from the Census 2011 national census. These were mapped as people/km² at the municipal level (Fig. 4.4). The average predicted likelihood values from the MaxEnt models for both *L. sericata* and *L. cuprina* were plotted against the values for the population density figures at a municipal level and the correlation coefficient was calculated using PAST3 (Hammer *et al*., 2001) (Fig. 4.5 C & D). This allowed quantitative comparison between the areas of highest human habitation and the predicted distribution of the flies.

RESULTS

Both species are predicted to occur in large areas of South Africa (Fig. 4.6 A & B). *Lucilia sericata* has a predicted central and western distribution with a very low likelihood of occurring in the northern parts of South Africa. *Lucilia cuprina* has a central and eastern predicted distribution including the northern parts of South Africa. The mean area-under-curve (AUC) for *L. sericata* and *L. cuprina* was 0.78 and 0.77 respectively, for the training model and 0.69 for both test models (Table 4.1), indicating moderately good fits of the models to the data.

Jackknife analysis showed that the magnitude of monthly rainfall and the magnitude of the maximum monthly temperature were the most important climatic variables predicting the distribution of *L. sericata* (Figure 4.7A). The seasonal variation in humidity and magnitude of monthly rainfall were the most important predictors for *L. cuprina* (Figure 4.7B).

The museum data models (Fig. 4.8 A & C) show distinctly different areas of suitability for both *L. sericata* and *L. cuprina* when compared to the survey data models for the same species (Fig. 4.8 B & D).

The comparison between the predicted distribution of *L. sericata* and wool production in South Africa showed no correlation ($r = 0.067$; $p = 0.190$) between where this species is predicted to occur and where large numbers of sheep occur due to wool farming (Figs 4.3, 4.5B & 4.6A). The comparison between *L. cuprina* and wool production also showed no correlation ($r = 0.017$; $p = 0.735$) between sheep farming

areas and the predicted areas that this species is likely to occur (Figs 4.3, 4.5A & 4.6B).

The comparison between the predicted distribution of *L. sericata* and human population density distribution showed no correlation (r = 0.102; p = 0.121). Large areas of low population density in the Northern Cape were predicted to be areas suitable for this species, while areas of high population density in Limpopo and eastern Mpumalanga were areas of low suitability for this species (Figs 4.4, 4.5D & 4.6A).

The predicted distribution of *L. cuprina* showed no statistically significant correlation (r = 0.019; p = 0.769) with human settlement despite areas of high population density particularly in the Western Cape and eastern parts of South Africa (Figs 4.4, 4.5C & 4.6B) being areas of higher suitability for this species.

DISCUSSION

The maximum entropy modelling technique has been used to model plant and insect distributions for purposes such as monitoring invasive species and disease vectors and their potential spread due to climate change (Chamaillé *et al*., 2010; Kulkarni *et al*., 2010; Fisher *et al*., 2011; Gormley *et al*., 2011; Gurgel-Concalves *et al*., 2012; Petersen, 2012). It performs well on small sample sizes (Pearson *et al*., 2007), which indicates that the generative methods used in MaxEnt give better predictions than the discriminative methods employed by other techniques (Elith *et al*., 2006; Phillip & Dudík, 2008).

The mean AUC for both species models is on the low side (0.69 for both species); models with values above 0.75 are considered potentially useful (Elith, 2002). This could be explained by the fact that models show greater uncertainty for species that show temporal or spatial variation in their habitats; that tolerate a large variety of habitats or have large ranges; or that are migrants or nomadic (McPherson & Jetz, 2007). Blowflies are typically r-selected (Elzinga, 1997), mobile opportunists (Smith & Wall, 1998) that make use of most available carrion resources to breed (Richards *et al*., 2009). This means they may occur in an area as a result of factors other than the

local environmental variables used in this study e.g. transient food and breeding resources.

By using climatic variables for predicting species distributions, the assumption is made that those variables actually define the limits of the species' distribution. Other factors, like geographic barriers and biotic interactions, may limit the species so that it does not or cannot occupy all of the climatically suitable areas (Meskin, 1986; Soberо̉n & Peterson, 2005; Pearson *et al*., 2007). Certain ecological traits such as physiological tolerance and home range size exert real effects on the accuracy of distribution models that are not explained by methodology (McPherson & Jetz, 2007). MaxEnt predicts potential distributions, not realised ones (Phillips & Dudík, 2008), which means that some areas may be predicted to be suitable for these blowflies based on the environmental variables used, but the flies are not found in those areas because there are other factors such as competition with other blowflies for resources that may affect their ability to survive in those areas.

The model for *L. sericata* is most influenced by the magnitude of monthly rainfall and the magnitude of the maximum monthly temperatures (Fig. 4.7A). This species originated in Europe and has been present in South Africa for over 100 years (museum records). Braack & Retief (1986) showed that the blowflies, *Chrysomya albiceps* and *C. marginalis,* were able to travel up to 2.25 km/day. Flies dusted with radioactive Phosphorous-powder were recovered as far as 63.5 km from the release site. Studies on *Lucilia cuprina* in Australia recovered fluorescent dusted flies 17 km from the release point (Gilmour *et al.,* 1946). Assuming *L. sericata* is equally dispersive, this supports the idea that *L. sericata* has spread throughout South Africa to all the niches it will inhabit. The east coast and northern parts of South Africa are generally hotter (Schulze *et al*., 1997), which appears to limit the likelihood of this species occurring in these regions. Although *Lucilia sericata* is an introduced species, we do not believe that there are any barriers to its dispersal in South Africa. The rate at which *Chrysomya megacephala* was recorded to spread in South Africa after being introduced in 1971, suggests that blowflies are capable of spreading very rapidly in the country, likely due to their r-selected reproductive biology (Williams & Villet, 2006).

The model for *L. cuprina* is most influenced by the seasonal variation of humidity and the magnitude of monthly rainfall (Fig. 4.7B). The species is predicted to occur along the east coast, and into the northern parts of South Africa which are all areas that are known for their humidity. Adult blowflies are very dependent on moisture and humidity is very important for egg development as blowfly eggs desiccate easily (Richards *et al*., 2009).

The models for both *L. sericata* and *L. cuprina* (Fig 8) using the museum data and survey data separately, support the recommendations for focused surveys of areas with very little data (Newbold, 2010; Elith *et al*., 2011). Museum records are known to be biased in sampling effort and location and can influence the accuracy of predictive distribution models if no surveys are done to minimise the bias (Newbold, 2010). The areas shown by the survey data to be suitable for these flies strongly reflect the areas that were surveyed (Figs. 4.1 & 4.8 B & D). These data are biased due to the surveys, but when combined with the museum records, give a more complete depiction of where these flies are likely to occur in the country as most of the climatically extreme areas of South Africa have been included. This must be considered when using modelling programmes to predict occurrences of species that may inhabit diverse climatic zones (Newbold, 2010).

Lucilia sericata is reported to be associated with areas inhabited by humans (Meskin, 1986; Braack & de Vos, 1987). However, the predicted occurrence of *L. sericata* shows no correlation with human population density, suggesting that this species is not an "urban" fly and occurs in both urban and rural environments. The potential distribution of *L. sericata* is also not correlated with wool production in South Africa, which is expected because this fly is not considered a pest in sheep farming in South Africa (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979).

Lucilia cuprina was thought to occur only in rural settings and not in areas populated by humans (Meskin, 1986; Braack & de Vos, 1987). The correlation between human population density and the predicted distribution of *L. cuprina* was not statistically significant, which supports this idea (Figures 4.4 and 4.6). *Lucilia cuprina* has been a

pest in sheep farming (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006). The correlation between the areas in South Africa that have higher wool production (and therefore more sheep) and the predicted distribution of *L. cuprina* is not statistically significant (Figures 4.3 and 4.6). This is unexpected but may be explained by the fact that sheep farmers in South Africa are selecting breeds of sheep that do not have the skin fold around the anal area that promotes sheep strike, thereby eliminating the most common site of egg laying of this fly (A.R. Palmer, pers. comm.).

These two species appear to be largely generalists in that they are predicted to occur in most parts of South Africa except for a small region of the south western and north eastern parts. This suggests that it is not possible to tell which species of *Lucilia* one is dealing with based only on geographic location. Morphological and molecular methods are therefore advocated for identifying these two species, especially if they are being used in forensic investigations.

ACKNOWLEDGEMENTS

We thank Bruce Davidson, Peter Illgner, James Kay, John Midgley, Kyla Orr, Warren Potts, Mark Robertson and Garreth Whittington-Jones for locality records; Cameron Richards for the extensive trapping excursions; Ashley Kirk-Sprigs (Albany Museum and National Museum), Simon van Noort (Iziko Museum), B. Muller (KwaZulu-Natal Museum) for museum records; John Midgley for field assistance; Olivia Harrison for maps; Mark Robertson for comments on this manuscript; and Rhodes University and the Durban Natural Science Museum for funding this research. Funding was provided by the National Research Foundation (NRF) of South Africa. Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Research Foundation.

REFERENCES

Anderson, G.S. (2000). Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences* 45: 824-832.

Altincicek, B. and Vilcinskas, A. (2009). Septic injury-inducible genes in medicinal maggots of the green blow fly *Lucilia sericata*. *Insect Molecular Biology* 18(1): 119-125.

Braack, L.E.O. (1986). Arthropods associated with carcasses in the northern Kruger National Park. *South African Journal of Wildlife Research* 16: 91-98.

Braack, L.E.O. and Retief, P.F. (1986). Dispersal, density and habitat preference of the blowflies *Chrysomya albiceps* and *Chrysomya marignalis* (Wd.) (Diptera: Calliphoridae). *Onderstepoort Journal of Veterinary Research*. 53: 13-18.

Braack, L.E.O. and de Vos, V. (1987). Seasonal abundance of carrion-frequenting blow-flies (Diptera: Calliphoridae) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research* 54: 591-597.

Chamaille, L., Tran, A., Meunier, A., Bourdoiseau, G., Ready, P. and Dedet, J-P. (2010). Environmental risk mapping of canine leishmaniasis in France. *Parasites and Vectors* 3:31. doi: 10.1186/1756-3305-3-31.

Clark, K., Evans, L. and Wall, R. (2006). Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. *Forensic Science International* 156: 145-149.

Day, D.M. and Wallman, J.F. (2006). Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae). *Journal of Forensic Sciences* 51: 657-663.

Elith, J. (2002). Quantitative methods for modelling species habitat: comparative performance and an application to Australian plants. *In*: Ferson, S. and Burgman, M. (eds) *Quantitative methods for conservation biology*. Springer, pp. 38-39.

Elith, J., Graham, C.H. and the NCEAS Species Distribution Modelling Group. (2006). Novel methods improved prediction of species' distributions from occurrence data. *Ecography* 30: 609-628.

Elith, J. and Leathwick, J.R. (2009). Species distribution models: ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution and Systematics* 40: 677-697.

Elith, J., Kearney, M. and Philliphs, S. (2010). The art of modelling range-shifiting species. *Methods in Ecology and Evolution*. 1: 330-342.

Elith, J., Phillips, S.J., Hastie, T., Dudík, M., Chee, Y.E. and Yates, C.J. (2011). A statistical explanation of MaxEnt for ecologists. *Diversity and Distributions* 17: 43-57.

Elzinga, R.J. (1997). *Fundamentals of Entomology*. New Jersey: Prentice Hall.

Fisher, D., Moeller, P., Thomas, S.M., Naucke, J. and Beierkuhnlein, C. (2011). Combining climatic projections and dispersal ability: a method for estimating the responses of sandfly vector species to climate change. *PLoS Neglected Tropical Diseases* 5(11): e1407. doi:10.1371/journal.pntd.0001407.

Gilmour, D., Waterhouse, D.F. and McIntyre, G.A. (1946). An account of experiments undertaken to determine the natural population density of the sheep blowfly, *Lucilia cuprina* Wied. *Bulletin of the Council for Scientific and Industrial Research Australia* 195: 1-39.

Gormley, A. M., Forsyth, D.M., Griffioen, P., Lindeman, M., Ramsey, D.S.L., Scroggie, M.P. and Woodford, L. (2011). Using presence-only and presence-absence data to estimate the current and potential distributions of established invasive species. *Journal of Applied Ecology* 48: 25-34.

Gurgel-Goncalves, R., Galvao, C., Costa, J. and Townsend Peterson, A. (2012). Geographic distribution of Chagas disease vectors in Brazil based on ecological niche modelling. *Journal of Tropical Medicine*. doi: 10.1155/2012/705326.

Heath, A.C.G. and Bishop, D.M. (2006). Flystrike in New Zealand: an overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* 137: 333-344.

Hepburn, G.A. (1943). Sheep blowfly research I – a survey of maggot collections from live sheep and a note on the trapping of blowflies. *Onderstepoort Journal of Veterinary Science and Animal Industry* 18: 13-18.

Kulkarni, M.A., Desrochers, R.E. and Kerr, J.T. (2010). High resolution niche models of malaria vectors in northern Tanzania: a new capacity to predict malaria risk? *PLoS ONE* 5(2): e9396. doi: 10.1371/journal.pone.0009396

Lobo, M., Jimenez-Valverde, A. and Real, R. (2007). AUC: a misleading measure of the performance of predictive distribution models. *Global Ecology and Biogeography* 17: 145-151.

Louw, M. and van der Linde, T.C. (1993). Insects frequenting decomposing corpses in central South Africa. *African Entomology* 1(2): 265-269.

Mackey, B.G. and Lindenmayer, D.B. (2001). Towards a hierarchical framework for modelling the spatial distribution of animals. *Journal of Biogeography* 28: 1147-1166.

McPherson, J.M. and Jetz, W. (2007). Effects of species' ecology on the accuracy of distribution models. *Ecography* 30: 135-151.

Meskin, I. (1986). Factors affecting the coexistence of blowflies (Diptera: Calliphoridae) on the Transvaal Highveld, South Africa. *South African Journal of Science* 82: 244-250.

Newbold, .T. (2010). Applications and limitations of museum data for conservation and ecology, with particular attention to species distribution models. *Progress in Physical Geography* 34(1): 3-22.

Oliva, A. (2001). Insects of forensic significance in Argentina. *Forensic Science International* 120: 145-154.

Paul, A.G., Ahmad, N.W., Lee, H.L., Ariff, A.M., Saranum, M., Naicker, A.S. and Osman, Z. (2009). Maggot debridement therapy with *Lucilia cuprina*: a comparison with conventional debridement in diabetic foot ulcers. *International Wound Journal* 6(1): 39-46.

Pearson, R.G., Raxworthy, C.J., Nakamura, M. and Townsend Peterson, A. (2007). Predicting species distributions from small numbers of occurrence records: a test case using cryptic geckos in Madagascar. *Journal of Biogeography* 34(1): 102-117.

Petersen, M.J. (2012). Evidence of a climatic niche shift following North American introductions of two crane flies (Diptera: genus Tipula). *Biological Invasions*. doi: 10.1007/s10530-012-0337-3.

Phillips, S.J. (2008). Transferability, sample selection bias and background data in presence-only modelling: a response to Peterson *et al*. (2007). *Ecography* 31: 272-278

Phillips, S.J., Anderson, R.P. and Schapire, R.E. (2006). Maximum entropy modelling of species geographic distributions. *Ecological Modelling* 190: 231- 259.

Phillips, S.J. and Dudík, M. (2008). Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecography* 31: 161-175.

Richards, C.S., Williams, K.A. and Villet, M.H. (2009). Predicting geographic distribution of seven forensically significant blowfly species (Diptera: Calliphoridae) in South Africa. *African Entomology* 17(2): 170-182.

Schulze, R.E., Maharaj, M., Lynch, S.D., Howe, B.J. and Melvil-Thomson, B. (1997). *South African atlas of agrohydrology and climatology*. 1st edn. Water Research Commission, Pretoria.

Sherman, R.A., Hall, M.J.R. and Thomas, S. (2000). Medicinal maggots: an ancient remedy for some contemporary afflictions. *Annual Review of Entomology* 45: 55-81.

Smith, K.E. and Wall, R. (1997). The use of carrion as breeding site by the blowfly *Lucilia sericata* and other Calliphoridae. *Medical and Veterinary Entomology* 11: 38-44.

Smith, K.E. and Wall, R. (1998). Estimates of population density and dispersal in the blowfly *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of Entomological Research* 88: 65-73.

Soberȯn, J. and Townsend Peterson, A. (2005). Interpretation of models of fundamental ecological niches and species' distributional areas. *Biodiversity Informatics* 2: 1-10.

Tantawi, T.I., Williams, K.A. and Villet, M.H. (2010). An accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. *Journal of Medical Entomology* 47(3): 491- 494.

Ullyett, G.C. (1945). Species of *Lucilia* attacking sheep in South Africa. *Nature* 155: 636-637.

Vogt, W.G. and Woodburn, T.L. (1979). Ecology distribution and importance of sheep myiasis flies in Australia. *National Symposium of the sheep blowfly and flystrike in sheep*. N.S.W. Ag. Sydney.

Williams, K.A. (2002). *Spatial and temporal occurrence of forensically important South African blowflies (Diptera: Calliphoridae)*. MSc Thesis, Rhodes University.

Williams, K.A. and Villet, M.H. (2006). A new and earlier record of *Chrysomya megacephala* in South Africa, with notes on another exotic species, *Calliphora vicina* (Diptera: Calliphoridae). *African Invertebrates* 47: 347-350

Williams, K.A., Cronje, F.J., Avenant, L. and Villet, M.H. 2008. Identifying flies used for maggot debridement therapy. *South African Medical Journal* 98(3): 196 - 197.

Wolff, H. and Hansson, C. (2005). Rearing larvae of *Lucilia sericata* for chronic ulcer treatment - an improved method. *Acta Dermato Venereologica* 85: 126-131.

Zumpt, F. and Patterson, P.M. (1952). Flies visiting human faeces and carcasses. *South African Journal of Clinical Science* 3: 92-106.

Table 4.1. Mean AUC values for whole models.

	AUC	
	L. sericata	*L. cuprina*
Training	0.78	0.77
Test data	0.69	0.69
Standard deviation	0.0489	0.0471

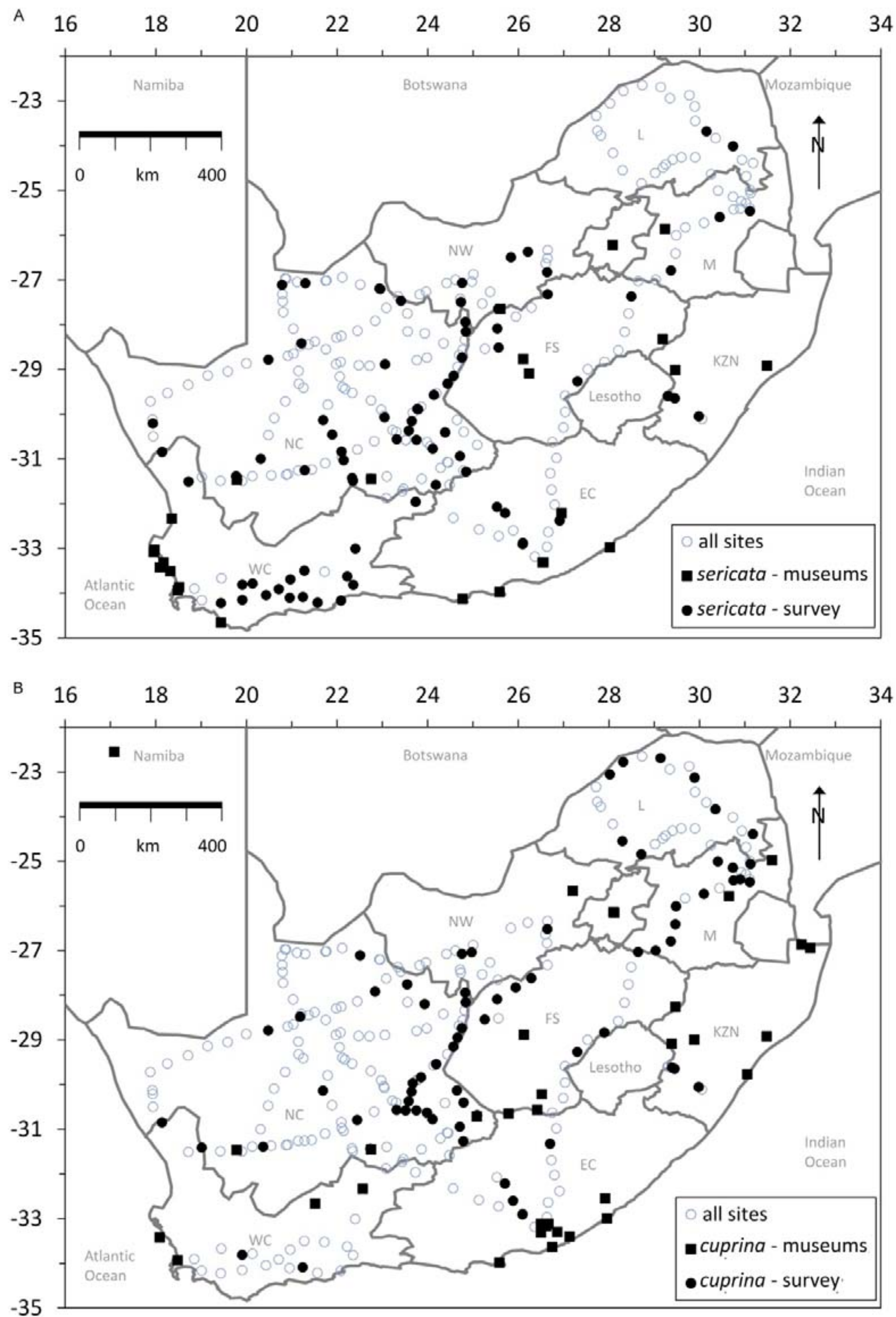

Figure 4.1: Map of South Africa showing the collecting trip routes to collect *Lucilia sericata* (A) and *Lucilia cuprina* (B) blowflies. EC – Eastern Cape, FS – Free State, G – Gauteng, KZN – KwaZuluNatal, L – Limpopo, M – Mpumalanga, NC – Northern Cape, NW – North West, WC – Western Cape

Figure 4.2: Modified Red-top™ Fly Trap.

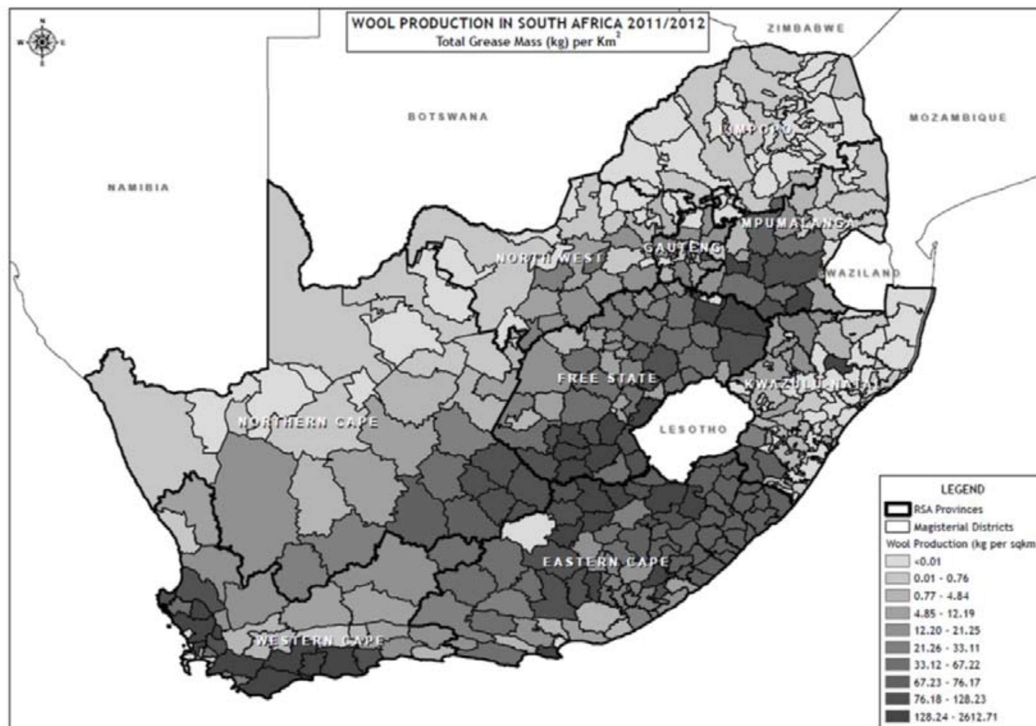

Figure 4.3: Wool production of magisterial districts, estimated by total grease mass produced in 2011/2012 in South Africa

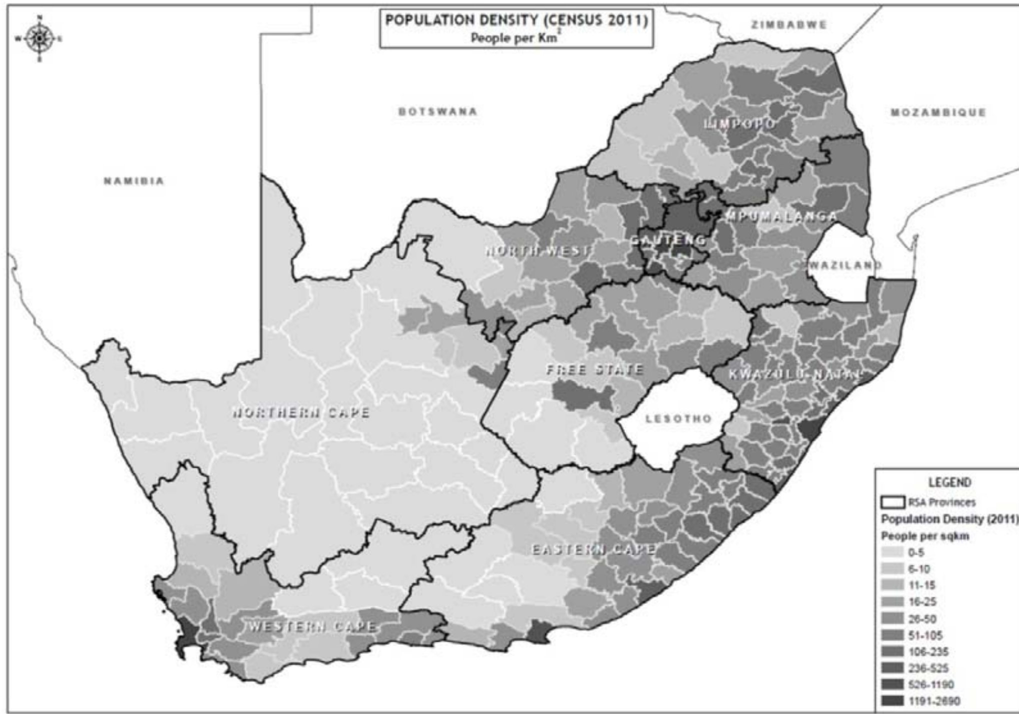

Figure 4.4: Population density in South Africa in 2011.

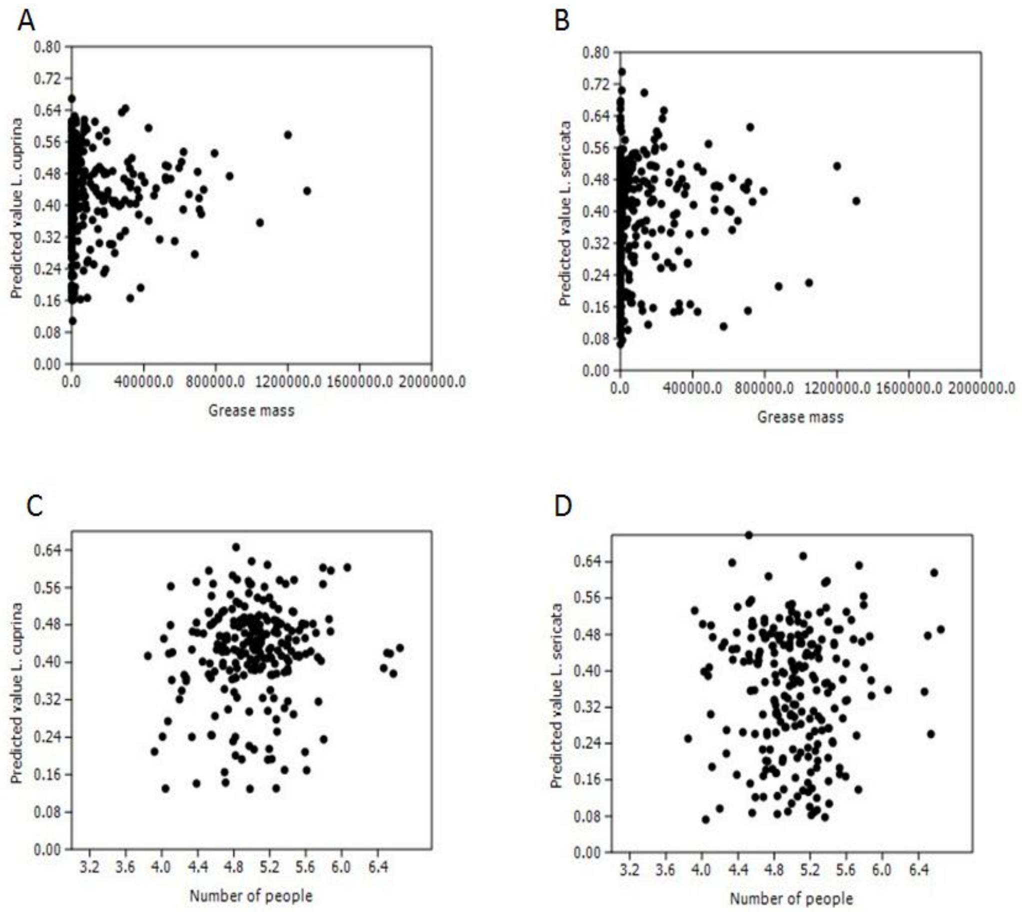

Figure 4.5: Plots showing the correlation between the predicted distribution of *L.cuprina* (A & C) and *L. sericata* (B & D) and grease mass (kg) and human population density values (log values).

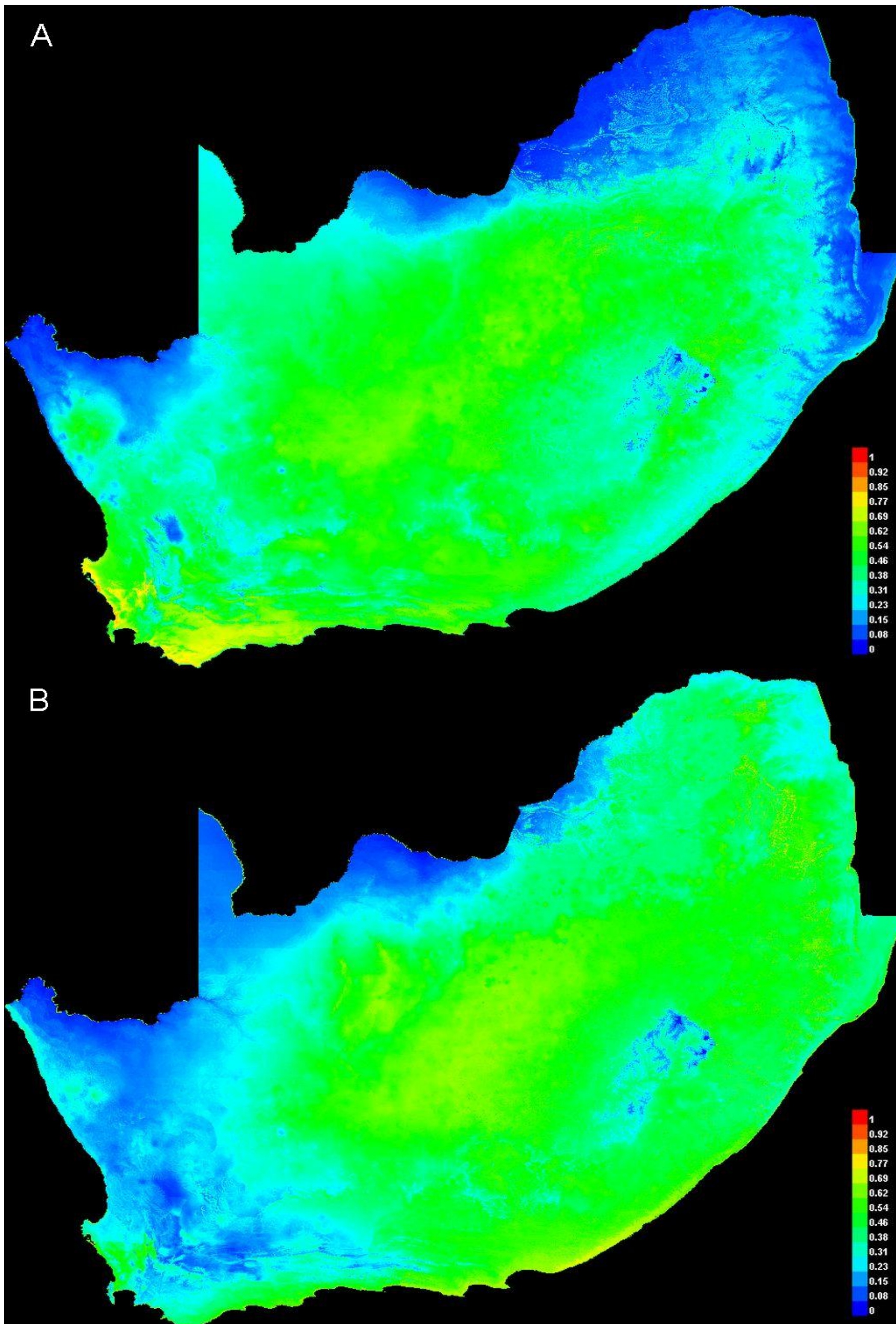

Figure 4.6: Mean predicted distribution maps for *L. sericata* (A) and *L. cuprina* (B) produced using museum records, survey data and personal contact localities. The colour range indicates the likelihood of species distribution from dark blue (least likely) to red (most likely).

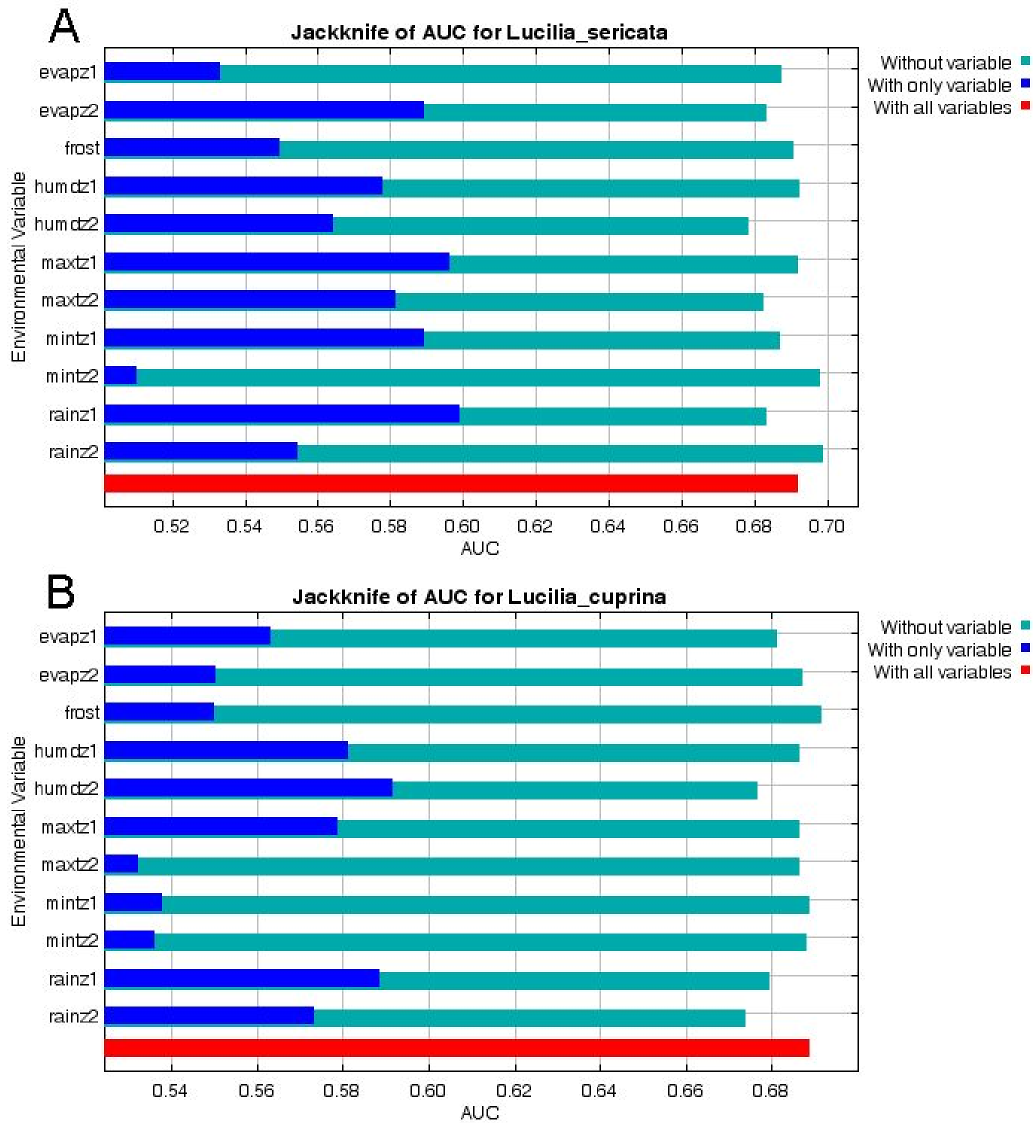

Figure 4.7: Jack-knife of climatic variables AUC for *L. sericata* (A) and *L. cuprina* (B).

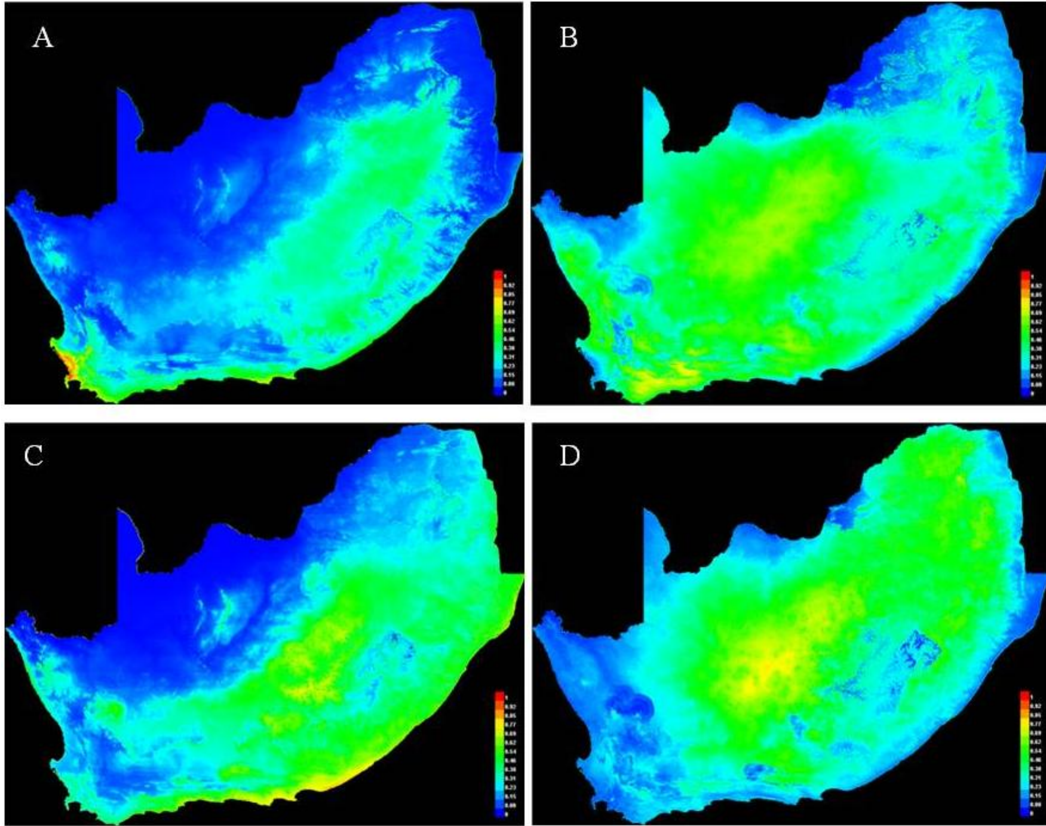

Figure 4.8: Mean distribution maps for *L. sericata* (A & B) and *L. cuprina* (C & D) from museum (A & C) and survey (B & D) data only.

CHAPTER 5

BIOGEOGRAPHICAL AND ADAPTIVE RADIATION OF THE GREENBOTTLE FLIES (DIPTERA: CALLIPHORIDAE: LUCILIINAE)

ABSTRACT

The subfamily Luciliinae is diverse and geographically widespread. Its four currently recognised genera – *Hemipyrellia, Lucilia, Dyscritomyia* and *Hypopygiopsis* – contain species that are parasitic, ranging from saprophages to obligate parasites, but their phylogenetic pattern of diversification is unclear. The *28S rRNA*, *COI* and *Period* genes of 14 species of *Lucilia* and *Hemipyrellia* were partially sequenced and analysed together with sequences of 11 further species from public databases. The molecular data yielded three cases of species-level paraphyly within *Lucilia* that hamper barcode identifications of those six species. The placement of *Dyscritomyia* and *Hypopygiopsis* was ambiguous, since both made *Lucilia* paraphyletic in some analyses. Recognising *Hemipyrellia* as a genus consistently left *Lucilia* s.l. paraphyletic, and the occasionally recognised (sub)genus *Phaenicia* was consistently paraphyletic. *Hemipyrellia* and *Phaenicia* should therefore be synonymised with *Lucilia*. Analysis of a matrix of 14 morphological characters scored for adults of all genera and most of the species included in the molecular analysis confirmed several of these findings. The different degrees of parasitism were phylogenetically clustered within this genus but did not form a graded series of evolutionary stages, and there was no particular relationship between feeding habits and biogeography.

INTRODUCTION

All of the genera of the subfamily Luciliinae are reported to exhibit parasitism in the form of myiasis – the infestation of living animals with dipteran larvae (Stevens, 2003). This is expressed in different forms from facultative secondary necrophagous myiasis to obligate primary carnivorous myiasis. Evidence of an evolutionary pattern underlying this feeding behaviour has not been found (Stevens & Wall, 1997; Otranto & Stevens, 2002; Stevens, 2003), and a pattern in the geographic distribution of the different forms of myiasis has not been sought.

There are few quantitative studies of relationships within the genus *Lucilia* Robineau-Desvoidy, 1830 (Aubertin, 1933; Stevens & Wall, 1996, 1997; Wells *et al*., 2007; Park *et al*., 2009; DeBry *et al*., 2012; Sonet *et al*., 2012), with research generally focusing on species of medical, veterinary or forensic interest in specific geographic regions (Stevens & Wall, 2001; Chen *et al*., 2004; Wallman *et al*., 2005; Harvey, *et al*., 2008; Reibe *et al*., 2009; Liu *et al*., 2011; Boehme *et al*., 2012; DeBry *et al*., 2012; Nelson *et al*., 2012; Sonet *et al*., 2013). The most comprehensive revision of the genus was published by Aubertin (1933), who recognised 37 species. Since then revisions of the genus and keys for identification have been produced, but only for specific geographic regions (Hall, 1948; James, 1971; Rognes, 1980; Smith, 1986; Rognes, 1991; Whitworth, 2006, 2010). Most species of *Lucilia* are limited to particular continents or islands and very few, such as *Lucilia sericata* (Meigen, 1826), are cosmopolitan. It is difficult to assess relationships when studies are taxonomically fragmented and parochial.

At the species level, *Lucilia sericata* and *L. cuprina* (Wiedemann, 1830) have been referred to as sister-species (Ash & Greenberg, 1974) because they are very similar morphologically and each is often misidentified as the other. They are now both found in Australia, New Zealand, South Africa, large parts of Asia, Europe and North America (Waterhouse & Paramonov, 1950; Rognes, 1980; Norris, 1990; Bishop, 1991, 1995; Holloway, 1991; Rognes, 1994; Fischer, 2000; Harvey *et al*., 2003a, 2003b, 2008; Chen, *et al*., 2004; Heath & Bishop, 2006; Park *et al*., 2009; Liu *et al*., 2011; Boehme *et al*., 2012; GilArriortua *et al*., 2013). They have each received

intensive biological investigation, and it would benefit comparative studies if it could be confirmend that they are actually sister species.

Several studies have established that natural hybrids of *L. sericata* and *L. cuprina* exist (Stevens & Wall, 1996; Stevens *et al*., 2002; Wallman *et al*., 2005; Tourle *et al*., 2009; DeBry *et al*., 2010; Williams & Villet, 2013). Two other species pairs, *Lucilia coeruleiviridis* Macquart, 1855 and *L. mexicana* Macquart, 1843, and *L. caesar* (Linnaeus, 1758) and *L. illustris* (Meigen, 1826), have also been shown to be paraphyletic, possibly due to introgressive hybridisation (DeBry *et al*., 2012; Sonet *et al*., 2012, 2013). The frequency and phylogenetic distribution of this phenomenon in the genus is of general interest because of its implications for understanding speciation and diversification in the group.

At a slightly higher taxonomic level, *Lucilia* has been variously divided into subgenera or genera by Malloch (1926) and Hall (1948), respectively. *Phaenicia* Robineau-Desvoidy, 1863 has been the most used of these names and its use persists in North America and sometimes in Korea (Stevens & Wall, 1996; Park *et al*., 2009). Its validity has been rejected by some researchers (Aubertin, 1933; Zumpt, 1965) who argue that there is no taxonomic value to the subdivision and who have therefore retained *Phaenicia* as a junior synonym of *Lucilia*. A phylogenetic study of *Lucilia* presents an opportunity to assess this matter.

At a yet higher taxonomic level, Rognes (1991) suggested that the genera *Dyscritomyia* Grimshaw, 1901, *Hemipyrellia* Townsend, 1918, *Hypopygiopsis* Townsend, 1916, and *Lucilia* should be united in the subfamily Luciliinae. Several phylogenetic studies have placed species of *Hemipyrellia* within *Lucilia* (Wells *et al*., 2007; Park *et al*., 2009; Liu *et al*., 2011; McDonagh & Stevens, 2011). Whether *Dyscritomyia* is related to *Lucilia* or nested within it has depended on which gene was analysed (Wells *et al*., 2007; McDonagh & Stevens, 2011). Several other genera have been included in the Luciliinae, such as *Bufolucilia* Townsend, 1919, *Francilia* Shannon, 1924, *Acrophagella* Ringdahl, 1942, *Phumonesia* Villeneuve, 1914 and *Viridinsula* Shannon, 1926 but most of these are now treated as synonyms of *Lucilia*.

The aims of this study are therefore to confirm if *L. sericata* and *L. cuprina* are sister-species; to explore if *L. coeruleiviridis/L. mexicana* and *L. caesar/L. illustris* are paraphyletic species; to examine the relationships between the species of *Lucilia* and clarify the taxonomic status of *Phaenicia*; to estimate the relationships of *Hemipyrellia*, *Dyscritomyia*, *Hypopygiopsis* and *Lucilia;* and to assess the geographical and phylogenetic patterns of myiasis-causing behaviour in these flies.

MATERIALS AND METHODS

DNA data

Adult *Lucilia* flies were obtained from countries around the world (Table 5.1). *Hemipyrellia fernandica* (Macquart, 1855) were obtained from Benin, South Africa and Tanzania, and *Calliphora vicina* Robineau-Desvoidy, 1830 were obtained from France and used as an outgroup (Table 5.1). Idientifications were made morphologically by the donors and verified using published keys (Aubertin, 1931, 1933; Smith, 1986; Holloway, 1991; Whitworth, 2006, 2010). All flies were kept in separate 1.5 ml Eppendorf tubes in 96% ethanol or as dried pinned specimens and deposited with the Durban Natural Science Museum after analysis.

One hind leg of each fly was used for DNA analysis. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Three genes were chosen for sequencing – *28S* rRNA (*28S*), a nuclear gene that has been used in previous studies and would allow comparison with other studies (Stevens *et al*., 2002; Stevens, 2003; Tourle *et al*., 2009; DeBry *et al*., 2010; Sonet *et al*., 2012); *Period* (*Per*), a second nuclear gene that is faster-evolving than *28S* to give better resolution and *Cytochrome oxidase I* (*COI*), the DNA barcoding gene of choice that has been used in previous studies (Stevens *et al*., 2002; Stevens, 2003; Wallman *et al*., 2005; Wells *et al*., 2007; Harvey *et al*., 2008; Liu *et al*., 2009; Park *et al*., 2009; Tourle *et al*., 2009; DeBry *et al*,. 2010; DeBry *et al*., 2012; Sonet *et al*., 2012). A region of approximately 650bp in the Domain 1-2 of the *28S* gene was amplified using the primers 5`-CCCCCTGAATTTAAGCATAT-3` and 5`-TTAGACTCCTTGGTCCGTG-3` (Stevens *et al*., 2002). A region of approximately 600bp of the *COI* gene was amplified using the primers C1-J1709 (5'-ATTGGGGGGTTTGGAAATTG-3`) and

C1-N2353 (5'-GCTCGTGTATCAACGTCTATTCC-3`) (Simon *et al*., 2006). A region of approximately 730bp of the *Per* gene, was amplified using the primers Per5 (5'-GCCTTCAGATACGGTCAAAC-3') (Warman, pers comm) and Per reverse (5`-CCGAGTGTGGTTTGGAGATT-3`) (designed by the author). Polymerase chain reaction (PCR) amplification was performed using 1µL of DNA in a 25µL reaction. Amplification times were 94°C for 5 min denaturation, followed by 36 cycles of 94°C for 30 seconds, 55°C for 1min, 72°C for 30 seconds and a final extension period at 72°C for 7min. PCR products were confirmed by gel electrophoresis stained in ethidium bromide. PCR products were then sequenced using an ABI 3730l Genetic Analyzer (Applied Biosystems) and the primers used in amplification.

Additional DNA sequences of *28S*, *Per* and *COI* were obtained from GenBank (www.ncbi.nlm.nih.gov) (Table 5.2). Additional *COI* barcode sequences were downloaded from the Barcode of Life Database (BOLD) website for all available *Lucilia*, *Hemipyrellia* and *Hypopygiopsis* species and for *Paralucilia* Bauer & Bergenstamm, 1891 and *Chrysomya chloropyga* (Wiedemann, 1818) which were included as additional outgroups. Duplicate sequences from the same studies were removed and a total of 207 sequences were included in the analysis. The sequences were aligned and edited using the BioEdit v7.0.9 software (Hall, 1999).

Morphological data

The states of the 14 morphological characters defined by Stevens & Wall (1996) were obtained from Aubertin (1931, 1933), Stevens & Wall (1996) and Whitworth (2010) for all of the *Lucilia* and *Hemipyrellia* species for which sequences were available (Table 5.3). Museum specimens were inspected where possible to complete the character state matrix. *Calliphora vicina* was included as an outgroup.

Phylogenetic analysis

The DNA data for each gene was first used in phylogenetic reconstruction by maximum parsimony analysis using PAUP*4b10 (Swofford, 2003) and the best-fitting model (GTR+G) from jModelTest (Posada, 2008). Statistical support for nodes was assessed by bootstrapping with 100 replicates, retaining a maximum of 10 000 trees.

Separate Bayesian inference analyses were performed on each gene in MrBayes (Huelsenbeck & Ronquist, 2001). One cold and three hot chains were run for 5 000 000 generations, sampling every 1 000 generations with burn-in of 1 000 samples. Incongruence length difference (ILD) tests (Farris *et al*., 1994) were run in PAUP*4b10 (Swofford, 2003) to quantify the differences in topology between trees for *28S*, *COI* and *Per*. An analysis was then conducted on the total data set partitioned by gene (*28S* and *Per*; *28S*, *Per* and *COI*) with the parameters as above.

A network analysis for the *COI* data was created using the NeighborNet algorithm in SplitsTree4 (Huson & Bryant, 2008) and the uncorrected P-distance method.

The *COI* barcode sequences were aligned for a region of approximately 800 bps. Bayesian inference analysis was performed in MrBayes (Huelsenbeck & Ronquist, 2001) with the parameters as described above.

Maximum parsimony analysis of the morphological data (Table 5.3) was performed in Paup*4b10 (Swofford, 2003). Statistical support for nodes was assessed by bootstrapping with 100 replicates retaining a maximum of 10 000 trees. Strict consensus and 50% majority rule trees were produced from the analysis.

The zoogeographic distributions of species in the Lucilliinae are shown in Table 5.4.

RESULTS

Molecular data

Sequencing of the *28S*, *Per* and *COI* genes resulted in 1932 bp being aligned – 656 bp for *28S*, 700 bp for *Per* and 576 bp for *COI*. A total of 46 specimens were sequenced for *28S*, 41 specimens for *Per* and 39 specimens for *COI*. These sequences were submitted to GenBank (Table 5.1).

The ILD test for *28S* and *Per* showed these two genes to be highly congruent ($p = 1.00$) and the datasets were therefore concatenated for the analyses. The ILD test for *28S*, *Per* and *COI* showed the combination of these genes to be incongruent ($p =$

0.03). Despite the incongruence between the nuclear (*28S* and *Per*) and mitochondrial (*COI*) data, these data sets were also concatenated and an analysis run on the total molecular evidence.

The Bayesian inference tree (Fig. 5.1) for the nuclear genes (*28S* and *Per*) clearly shows that *L. sericata* and *L. cuprina* are sister clades with 100% support. *Lucilia thatuna* Shannon, 1926 and *L. silvarum* Meigen, 1826 form a sister clade to the *L. sericata* + *L. cuprina* clade. The specimens of *H. fernandica* all group together and are sister to *L. papuensis* Macquart, 1843. The *Hemipyrellia* clade sits within the *Lucilia* clade (Fig. 5.1).

In the Bayesian inference tree for the mitochondrial gene (*COI*) (Fig. 5.2), *L. cuprina* is paraphyletic with respect to *L. sericata*. The *L. cuprina* + *L. sericata* clade is poorly resolved with respect to the *L. silvarum* + *L. taiyuanensis* Chu, 1975 clade. The *H. fernandica* sequences group with those of *H. ligurriens* and *H. pulchra* from GenBank and this clade is sister to *Lucilia infernalis* Villeneuve, 1914. This *Hemipyrellia* + *L. infernalis* clade sits within the *Lucilia* clade on the tree. Two specimens of *H. ligurriens* from Taiwan group with the *L. cuprina* specimens. The three *Dyscritomyia* sequences included in the analysis, group together monophyletically outside *Lucilia*.

The Bayesian inference tree for the incongruent concatenated total evidence molecular dataset (*28S*, *Per* and *COI*) (Fig. 5.3) shows *L. sericata* and *L. cuprina* to be sister clades with strong support. The *H. fernandica* sequences sit within *Lucilia*, and the rest of the tree was topologically similar to the individual gene trees.

The NeighborNet analysis (Fig. 5.4) clearly shows seven distinct major splits. The New World species (*L. coeruleiviridis*, *L. cluvia* Walker, 1849, *L. eximia* Wiedemann, 1819, *L. mexicana* and *L. fayeae* Whitworth, 2010) group together; *L. caesar/*, *L. illustris, L. porphyrina* Walker, 1856, *L. ampullacea* Villeneuve, 1922, *L. adiosoemartoi*, *L. papuensis* Macquart, 1843, *L. bazini* Séguy, 1934 and *L. hainanensis* Fan, 1965 form a group; *L. infernalis* is isolated, as is *H. fernandica*; the bulk of the *Lucilia* species that are primary facultative parasites (*L. sericata, L. cuprina*, *L. silvarum* and *L. thatuna*) group together; and *Calliphora vicina* and the

Dyscritomyia species as the outgroups form separate but neighbouring splits. The parasitic behaviour of the species is indicated in coloured text and the ellipses on the diagram show the (sub)generic classification according to Hall (1948).

Bayesian inference analysis of the *COI* barcode data set generated a tree (Fig. 5.5) with very strong posterior probabilities for most clades except for the *L. sericata* + *L. cuprina* + *L. taiyuanensis* (0.61) and *L. caesar* + *L. illustris* (0.58) clades. The *Hemipyrellia* species all form a distinct clade within *Lucilia* with 100% support. One of the *Hypopygiopsis infumata* (Bigot, 1877) sequences forms a clade with *L. hainanensis* + *L. papuensis* + *L. bazini* and the other sequence groups with the *Hemipyrellia* sequences. *Paralucilia paraensis* (Mello, 1972) sits outside *Lucilia* with *Chrysomya chloropyga*, confirming its classification as a chrysomyine.

Morphological data

The strict consensus parsimony tree for the morphological characters was largely uninformative, forming only two clades, with the majority of the species being unresolved (tree not shown). The majority rule consensus tree (Fig. 5.6) grouped *L. sericata, L. cuprina, L. silvarum, L. bufonivora* Moniez, 1876 and *L. thatuna* together. *Lucilia coeruleiviridis* and *L. cluvia* group together in all of the trees. The *Hemipyrellia* species form a clade within *Lucilia*, and *L. caesar* and *L. illustris* group together.

DISCUSSION

Relationship of **L. sericata** *and* **L. cuprina**

Although only about half of the *Lucilia* species listed as valid by Aubertin (1933) were included, these results strongly suggest that *L. sericata* and *L. cuprina* are indeed sister species. All of the Bayesian inference analyses (Figs 5.1–5.3) indicate that *L. sericata* and *L. cuprina* are sister taxa with strong support from the nuclear gene (*28S* & *Per*) and total data (*28S*, *Per* & *COI*) trees and weaker support from the *COI* gene alone. *Lucilia cuprina* is paraphyletic (Fig. 5.2) with respect to *L. sericata* in the mitochondrial gene (*COI*) tree, as has been shown previously (using the same sequences but weaker auxiliary taxon sampling) to be the result of introgressive hybridisation between these two species (Williams & Villet, 2013). In another study,

the nuclear gene elongation factor-1 alpha (*EF-1α*) did not recover *L. sericata* and *L. cuprina* as sister-species (McDonagh & Stevens, 2011), but the clade containing *L. sericata* was poorly resolved and thus the conclusion was not well supported. In the same study, the *28S* and *COI* gene trees both recovered *L. sericata* and *L. cuprina* as sister species with strong support (McDonagh & Stevens, 2011).

The Bayesian inference analysis of the *COI* barcode sequences included 45 *L. sericata* sequences and 42 *L. cuprina* sequences. Despite the number of sequences, *L. sericata* was poorly resolved, which explains the poor node support (0.61) of the *L. sericata* + *L. cuprina* + *L. taiyuanensis* clade. *Lucilia taiyuanensis* is represented by only one sequence and it forms a sister clade with *L. silvarum* in the Bayesian inference tree of the *COI* sequences (Fig. 5.2). *Lucilia silvarum* forms a clade with *L. richardsi* in the barcode tree that is sister to the *L. elongata* Shannon, 1924 + *L. bufonivora* clade, and these four species form a sister clade to a *L. sericata* + *L. cuprina* + *L. taiyuanensis* clade (Fig. 5.5). In the parsimony analysis of the barcode data, *L. taiyuanensis* was recovered as a sister clade to *L. sericata* + *L. cuprina* but with poor support (tree not shown), but despite that, shows *L. sericata* and *L. cuprina* to be sister species.

Molecular identification of* Lucilia *species

It has already been established that *L. sericata* and *L. cuprina* show a case of ancient introgression, and that they still interbreed (Williams & Villet, 2013). This is a widely acknowledged problem for identification by *COI* sequences alone (Rubinoff *et al*., 2006; Nelson *et al*., 2007; Roe & Sperling, 2007; Williams *et al*., 2008; Tantawi *et al*., 2010; Williams & Villet, 2013). Other problematic species pairs occur in the genus (DeBry *et al*., 2012; Sonet *et al*., 2012), and it is important to recognise the cause(s) and to document genes that are more useful for identification in these contexts.

In the Bayesian inference trees based on mitochondrial (*COI*) (Fig. 5.2) and total evidence (*28S*, *Per* and *COI*) (Fig. 5.3), *L. mexicana* is paraphyletic with respect to *L. coeruleiviridis*. This has been observed in the continental United States of America (DeBry *et al*., 2012), where these two species were found to share a mitochondrial

haplotype. The *L. mexicana* specimens with this *L. coeruleiviridis* haplotype appear to be limited to a geographic area including Texas and New Mexico (DeBry *et al*., 2012). This study confirms this pattern, since the new sequences of *L. mexicana* from New Mexico grouped with *L. coeruleiviridis*, and the GenBank specimens of *L. mexicana* from California formed a distinct clade (Fig 5.2 & 5.3). This suggests that there has been introgression between these two species. The nuclear genes separate *L. coeruleiviridis* and *L. mexicana*, although *L. mexicana* is not resolved in this tree (Fig 5.1.). In the Bayesian inference tree based on the *Period* gene alone (tree not shown), these two species are recovered as sister clades with 100% support, which suggests that nuclear genes will separate these two species as they do for *L. sericata* and *L. cuprina* (Williams & Villet, 2013).

A similar problem of shared haplotypes occurs in *L. caesar* and *L. illustris* (Sonet *et al*., 2012). In the *COI* tree (Fig. 5.2), *L. caesar* specimens from France and Korea and one specimen of *L. illustris* from the UK are not resolved, but the remainder of the *L. caesar* and *L. illustris* specimens form a mixed clade with 100% support. These two species can therefore not be unambiguously identified using only *COI*. The nuclear genes in this study (Fig. 5.1) separated these two species but used only two specimens of *L. caesar* from France and seven specimens of *L. illustris* from Japan, Switzerland, Canada and the United States of America. More specimens from other countries may give a different result as was seen in a previous study (Sonet *et al*., 2012) where *L. caesar* and *L. illustris* could not be reliably identified using either mitochondrial or nuclear genes as the intraspecific and interspecific differences were very low. This might be as a result of hybridisation or incomplete lineage sorting (Sonet *et al*., 2012).

These three species pairs highlight the need for using more than one gene to identify species, as has been suggested in previous studies (Rubinoff *et al*., 2006; Nelson *et al*., 2007; Roe & Sperling, 2007; Williams *et al*., 2008; Tantawi *et al*., 2010; Williams & Villet, 2013). It also highlights a problem in using *COI* as a universal 'barcoding' gene (Rubinoff *et al*., 2006; Roe and Sperling, 2007; Whitworth *et al*., 2007; Sonet *et al*., 2012; van Nieukerken *et al*., 2012; Jordaens *et al*., 2013), especially in a forensic context. While cases of ancient introgression remain identifiable (DeBry *et al*., 2012; Williams &Villet, 2013), cases of incomplete lineage sorting may be intractable, and

morphological ideintification may be the best solution, especially if the identifications need to go to court (cf. Hebert *et al*., 2003).

Diversification of Luciliinae

The Luciliinae shows a strong pattern of diversification (Fig. 5.2). Two underlying patterns are discernible: biogeographical radiation (Fig. 5.4) and the diversification of parasitism (Fig. 5.4).

The NeighborNet analysis (Fig. 5.4) showed geographically distinct clusters of species from the New World (*L. eximia* + *L. mexicana* + *L. coeruleiviridis* + *L. cluvia* + *L. fayeae*) the Oriental region (*L. hainanensis* + *L. bazini* + *L. papuensis* + *L. adiosoemartoi* Kurahashi, 1988*)* and Eurasia (*L. porphyrina* + *L. ampullacea*). *Hemipyrellia* formed a monophyletic Old World lineage (Aubertin, 1931). *Lucilia infernalis* is found only in Africa (Aubertin, 1933) and the sequences from Rwanda and Burundi form a separate group.

The *L. sericata* + *L. cuprina* + *L. thatuna* + *L. silvarum* split represents a group of species that are facultative parasites, with *L. sericata* and *L. cuprina* being primary facultative parasites. This group is geographically diverse, with only *L. thatuna* being restricted to one region, the United States of America. Likewise, *L. caesar* and *L. illustris* form a split that represents secondary facultative parasites. *Lucilia illustris* is Holarctic, while *L. caesar* is restricted to the Palaearctic (DeBry *et al*., 2012). *Dyscritomyia* is endemic to the Hawaiian Islands (Wells *et al*., 2002) and phylogenetically coherent.

Many *Lucilia* species are myiasis-causing (Zumpt, 1965), with *L. cuprina* being the most recognised and often referred to as the sheep-strike blowfly (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006). Other species of *Lucilia* known to be facultative parasites include *L. sericata*, *L. silvarum*, *L. thatuna*, *L. richardsi*, *L. porphyrina*, *L. illustris*, *L. caesar*, *L. ampullacea* and *L. bufonivora*, which is the only known obligate parasitic species in the genus (Aubertin, 1933; Hall, 1948; Zumpt, 1965; Rognes, 1991; McDonagh & Stevens, 2011). There are also saprophagous species within *Lucilia*, including *L. mexicana, L. cluvia, L. papuensis*

and *L. infernalis* (Hall, 1948; Zumpt, 1965). None of these different parasitic behaviours are limited to any particular geographical area (Fig. 5.4).

The majority rule consensus tree of the morphological characters (Fig. 5.6) is largely incongruent with the molecular phylogenetic trees (Figs. 5.1-5.3, 5.5). The only clade that is congruent contains *L. sericata* + *L. cuprina* + *L. richardsi* + *L. silvarum* + *L. bufonivora* + *L. thatuna*. In the *COI* Bayesian inference tree this clade includes *L. elongata* too (Fig. 5.5). All of these species are known to be facultative parasitic agents of myiasis, with *L. bufonivora* and possibly *L. elongata* being obligate parasites of toads (Aubertin, 1933; Zumpt, 1965). The same pattern is seen in the network analysis (Fig. 5.4), where these facultatively parasitic species group together. The majority rule consensus tree is comparable with that of Stevens & Wells (1996), with three groups in common: *L. caesar* + *L. illustris*; *L. coeruleiviridis* + *L. cluvia*; and *L. sericata* + *L. cuprina* + *L. richardsi* + *L. silvarum* + *L. bufonivora* + *L. thatuna.* Due to disparities in taxon sampling, the remainder of the tree is dissimilar. The incongruence with the phylogenetic trees is possibly as a result of the limited character set available for the morphological parsimony analysis. It is ideal to have more characters than species in this type of analysis (Stevens & Wall, 1996), whereas we have 21 species and 17 character states. This limits the conclusions about general trends that can be drawn from these morphological data.

Taxonomy of Luciliinae

Lucilia Robineau-Desvoidy, 1830 (type species: *Lucilia caesar*) has a complex nomenclatural history that is integrally related to its biogeographical and dietary radiation. Several authors including Bigot, van der Wulp, Brauer and Bergenstamm, Girschner, Hough, Kramer, Shannon and Malloch (Aubertin, 1933) contributed to the ultimate development of this genus. Early studies of the European Lucilia were conducted by Stein (1924), Richards (1926), Collin (1926) and Séguy (1928) and Shannon published on the North and South Americican *Lucilia* (1926) (Aubertin, 1933). Aubertin (1933) published the most comprehensive review of the genus and recognised 27 species. This genus is widely spread across with world. The adults of this genus feed on nectar, carrion and decomposing material and the females are oviparous (Aubertin, 1933). The larvae of this genus develop on decomposing animal

material. Several species have developed specialised parasitic behaviour such as *L. cuprina* which lays its eggs on living sheep and the larvae feed on the live animals causing myiasis. *Lucilia bufonivora* is a parasite of toads.

Phaenicia Robineau-Desvoidy, 1863 (type species: *Phaenicia concinna* = *Musca sericata*) has a history of varied usage. Hall (1948) divided *Lucilia* into several separate genera including *Bufolucilia*, *Phaenicia* and *Lucilia sensus stricto*. Hall's (1948) separation of species into the genera *Phaenicia* and *Lucilia* was primarily based on the presence or absence of bristles on the subcostal sclerite and the character of the ocellar triangle. In contrast, Malloch (1926) used the yellow colour of the basicostal scale and the presence of three postsutural acrostichal bristles to define his concept of *Phaenicia*. The use of *Phaenicia* has persisted in North American literature (Stevens & Wall, 1996; Byrd & Castner, 2010), but is not generally used in other parts of the world as it is seen as a junior synonym of *Lucilia* (Zumpt, 1965).

In the network analysis (Fig. 5.4) the species that would be assigned to *Phaenicia* based on Hall's (1948) criteria can clearly be seen to be part of two distant splits. These species occur in both the Old and New Worlds, showing huge geographic diversity. The group includes species that are primary facultative parasites and species that are saprophages. Hall's (1948) usage of *Lucilia s.str.* refers only to *Lucilia illustris* (and *L. caesar* for clarity between the two) as he focused only on the blowflies of North America. The remaining species that would fall into this clade based on his diagnostic criteria grouped with *L. caesar* and *L. illustris* (Fig. 5.4), and includes species that are primary and secondary facultative parasites as well as species that are saprophagous.

Bufolucilia Townsend, 1919 (type species: *Lucilia bufonivora*) includes the species *bufonivora*, *silvarum* and *elongata*, which are found in Europe and North America. *Bufolucilia* forms a part of the split that includes most of the facultatively parasitic *Lucilia* species (Fig. 5.4). There is no obvious reason to separate *Lucilia* into (sub)genera based on the parasitic behaviour of the species because primary and secondary facultatively parasitic and saprophagous species are spread throughout the

genus (Fig. 5.4). Recognising *Bufolucilia* also makes *Phaenicia* paraphyletic (Fig. 5.4).

Phumonesia Villeneuve, 1914 and ***Roubaudiella*** Séguy, 1925 (type species: *Phumonesia infernalis* = *Roubaudiella caerulea*) are monotypic genera founded on the same species, and therefore objective synonyms. Similarly, *Francilia* Shannon, 1924, and *Acrophagella* Ringdahl, 1942, are objective synonyms based on the same species. Several other genus-group taxa have been placed within the Luciliinae, including *Caesariceps* Rodendorf, 1926*, Dasylucilia* Rodendorf, 1926*, Luciliella* Malloch, 1926 and *Viridinsula* Shannon, 1926. Their status needs assessment, and the results presented here suggest that morphological analyses alone will not be sufficient. Phylogenetic studies including a selection of both nuclear and mitochondrial genes are recommended.

Hemipyrellia Townsend, 1918 (type species: *Lucilia fernandica*) was erected as a genus by Townsend (1918) and revised by Aubertin (1931). It had previously been suggested that *Hemipyrellia* was a synonym of *Lucilia* (Shannon, 1926). *Hemipyrellia* is restricted to the Old World and the species are saprophagous. The results of this study place *Hemipyrellia* within *Lucilia* for both nuclear and mitochondrial analyses with 100% support (Figs 5.1 and 5.2), the *COI* barcode Bayesian tree (Fig. 5.5) with very strong support, and the morphological majority rule consensus tree (Fig. 5.6) with weaker (56%) support.

Two specimens of *H. ligurriens* from Taiwan (Fig. 5.2) group within the *L. cuprina* clade. This is probably a misidentification as the specimens of *H. ligurriens* and *H. pulchra*, both from China, group with *H. fernandica* sequenced in this study. In two studies of Australian blowflies, *Hemipyrellia* was found to be a sister-group to *Lucilia* (Wallman *et al*., 2005; Nelson, *et al*., 2012) but these studies included only species of *Lucilia* that occur in Australia, thus *Hemipyrellia* may be a sister-clade to Australian *Lucilia* as an artefact of taxon sampling. Similarly in another study (Singh & Wells, 2013) *Hemipyrellia* was found to be a sister-group to *Lucilia* but this was based on one specimen of *Lucilia sericata* and one specimen of *Hemipyrellia fernandica.* Several other studies have sequenced *Hemipyrellia* specimens and found them to lie

within *Lucilia* (Wells *et al*., 2007; Park *et al*., 2009; Liu *et al*., 2011; McDonagh & Stevens, 2011). These studies together with the results of this study provide strong support the synonymy of *Hemipyrellia* and *Lucilia*.

Dyscritomyia Grimshaw, 1901 (type species: *Prosthetochaeta robusta*) contains 35 nominal species that are all found exclusively on the Hawaiian Islands (James, 1981). The biology of *Dyscritomyia* differs from the other Luciliinae in that they are viviparous and produce only one larva at a time that is retained in the uterus for the first two instar stages. Little is known about their parasitic behaviour but it is assumed that *Dyscritomyia* species are facultatively parasitic saprophages (Hardy, 1981). *Dyscritomyia* was included in the *COI* Bayesian inference analysis and was recovered as a separate clade to *Lucilia* (Fig. 5.2). In previous studies, *Dyscritomyia* was recovered within *Lucilia* when analysing the *COI* and *EF-1α* genes (Wells *et al*., 2007; McDonagh & Stevens, 2001) but it was recovered as a sister clade to *Lucilia* when analysing the *28S* gene (McDonagh & Stevens, 2011). *Dyscritomyia* was also recovered as a sister group to *Lucilia* in a study of the *COI* and *COII* genes (Wells, *et al*., 2002). The current study used only a 576 bp region of the total *COI* gene from the sequences available on GenBank that were used in the Wells et al. (2002) study, but still recovered *Dyscritomyia* as a sister clade to *Lucilia*. It therefore does not appear that the length of the *COI* sequence affects the analysis.

This study used 20 species of *Lucilia* in the *COI* analysis while the previous studies used six and 13 species respectively (Wells *et al*., 2002; McDonagh & Stevens, 2011). The position of *Dyscritomyia* relative to *Lucilia* may be determined by the taxon sampling of *Lucilia*, as mentioned regarding *Hemipyrellia*. This highlights the need for a more comprehensive study of this genus and inclusion of as many *Dyscritomyia* and *Lucilia* species as possible to confirm the taxonomic relationship between *Dyscritomyia* and *Lucilia.*

Hypopygiopsis Townsend, 1916 (type species: *Hypopygiopsis splendens* = *Hypopygiopsis fumipennis*) is restricted to the Asian and Australasian regions of the world (Kurahashi, 1977). This genus exhibits both oviparous and larviparous behaviour. The larval behaviour includes both facultative parasitism and saprophagy.

Hypopygiopsis was included in the Bayesian inference analysis of the *COI* barcode dataset. One *Hypopygiopsis infumata* sequence grouped within *Lucilia* (Fig. 5.5) as part of a clade including *L. hainanensis*+ *L. papuensis* + *L. bazini*. On closer examination of the sequences, *Hypopygiopsis infumata* was identical to the *Lucilia bazini* sequence from China. The *L. hainanensis* sequence from China that groups with these two sequences only differs by one base pair. This places doubt on the identification of these sequences and prevents any meaningful inferences being drawn. The second *Hypopygiopsis infumata* sequence groups with *Hemipyrellia*. There are only five sequences of *Hypopygiopsis* publically available and therefore the limited number of sequences constrains the credibility of this result and it is recommended that more sequences of this genus are examined to clarify if this genus should also be synonymised with *Lucilia*.

CONCLUSION

Lucilia sericata and *L. cuprina* are inded sister-species. *Lucilia mexicana* is confirmed to be paraphyletic with respect to *L. coeruleiviridis* possibly as a result of hybridisation and introgression. *Lucilia caesar* and *L. illustris* are both paraphyletic and further studies with different genes are needed to determine if these two species can be identified using molecular methods. *Hemipyrellia* should be synonimised with *Lucilia* as this genus sits within *Lucilia* in all of the analyses conducted in this study. *Dyscritomyia* requires further studies to confirm its phylogenetic positioning with regard to *Lucilia* as taxon sampling appears to have an impact on the analysis. The limited number of sequences available for *Hypopygiopsis* and the apparent misidentification of sequences prevent any conclusions being drawn about its relationship to *Lucilia*. In this study we have identified at least three cases of misidentified sequences from GenBank, which is a well-known problem (Bridge *et al*., 2003; Harris 2003; Nilsson *et al*., 2006; Valkiūnas *et al*., 2008). There is no geographic pattern to the distribution of the different parasitic behaviours within the Luciliinae and no reason to sub-divide *Lucilia* into other genera or sub-genera based on either geographic location or parasitic behaviour.

ACKNOWLEDMENTS

We thank Dave Emery, Georg Goergen, Chris Kelly, Ashley Kirk-Spriggs, Nicky Lunt, Rich Merritt, Hideharu Numata, Cameron Richards, Kiyoshi Saigusa, Kabkaew Sukontason, Robyn Tourle, James Wallman, Terry Whitworth and Claude Wyss for providing us with specimens. Funding was provided by the National Research Foundation (NRF) of South Africa. Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Research Foundation.

REFERENCES

Ash, N. and Greenberg, B. (1975). Developmental temperature responses of the sibling species *Phaenicia sericata* and *Phaenicia pallescens*. *Annals of the Entomological Society of America* 68: 197-200.

Aubertin, D. (1931). Revision of the genus *Hemipyrellia* Tns. (Diptera, Calliphoridae). *Proceedings of the Zoological Society of London*, 497-509.

Aubertin, D. (1933). Revision of the genus *Lucilia* R.-D. (Diptera, Calliphoridae). *Linnean Society Journal of Zoology* 38: 389-463.

Bishop, D.M. (1991). Variations in numbers of occipital setae for two species of *Lucilia* (Diptera: Calliphoridae) in New Zealand. *New Zealand Entomologist* 14: 28-31.

Bishop, D.M. (1995). Subspecies of the Australian green blowfly (*Lucilia cuprina*) recorded in New Zealand. *New Zealand Veterinary Journal* 43: 164-165.

Boehme, P., Amendt, J., and Zehner, R. (2012). The use of *COI* barcodes for molecular identification of forensically important fly species in Germany. *Parasitology Research* 110: 2325-2332

Bridge, P.D., Spooner, B.M., Roberts, P.J., and Panchal, G. (2003). On the unreliability of published DNA sequences. *New Phytologist* 160: 43-48.

Byrd, J.H & Castner, J.L. 2010. *Forensic Entomology: The utility of arthropods in legal investigations.* CRC Press, Taylor & Francis Group, Boca Raton. 681pp.

Chen, W.-Y., Hung, T.-S., and Shiao, S.-F. (2004). Molecular identification of forensically important blow fly species (Diptera: Calliphoridae) in Taiwan. *Journal of Medical Entomology* 41: 47-57.

DeBry, R., Timm, A.E., Dahlem, G.A., and Stamper, T. (2010.) mtDNA-based identification of *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) in the continental United States. *Forensic Science International* 202: 102-109.

DeBry, R., Timm, A.E., Wong, E.S., Stamper, T., Cookman, C., and Dahlem, G. A. (2012). DNA-Based identification of forensically important *Lucilia* (Diptera: Calliphoridae) in the continental United States. *Journal of Forensic Sciences* 58: 73-78.

Farris, J.S., Kallerjo, M., Kluge, A.G. and Bult, C. (1994). Testing significance of congruence. *Cladistics* 10: 315-319.

Fischer, O.A. (2000). Blowflies of the genera *Calliphora*, *Lucilia* and *Protophormia* (Diptera, Calliphoridae) in South-Moravian urban and rural areas with respect to *Lucilia bufonivora* Moniez, 1876. Acta Veterinaria Brno 69: 225-231.

GilArriortua, M., Salona Bordas, M.I., Caine, L.M., Pinheiro, F., and de Pancorbo, M.M. (2013). Cytochrome b as a useful tool for the identification of blowflies of forensic interest (Diptera, Calliphoridae). *Forensic Science International* 228: 132-136.

Hall, D.G. (1948). *The blowflies of North America*. Washington DC: Thomas Say Foundation.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

Hardy, D.E. (1981). *Insects of Hawaii. Vol 14. Diptera: Cyclorrhapha IV. Series Schizophora Section Calypteratae*. University Press of Hawaii, Honolulu.

Harris, D.J. (2003). Can you bank on GenBank? *Trends in Ecology and Evolution* 18: 317-319.

Harvey, M.L., Dadour, I.R., and Gaudieri, S. (2003). Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in Western Australia. *Forensic Science International* 131: 134-139.

Harvey, M.L., Mansell, M.W., Villet, M.H., and Dadour, I.R. (2003). Molecular identification of some forensically important blowflies of southern Africa and Australia. *Medical and Veterinary Entomology* 17: 363-369.

Harvey, M.L., Gaudieri, S., Villet, M.H., and Dadour, I.R. (2008). A global study of forensically significant calliphorids: Implications for identification. *Forensic Science International* 177: 66-76.

Heath, A.C.G. and Bishop, D.M. (2006). Flystrike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* 137: 333-344.

Hebert, P.D.N., Cywinska, A., Ball, S.L., and deWaard, J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B* 270: 313-321.

Hepburn, G.A. (1943). Sheep blowfly research I - A survey of maggot collections from live sheep and a note on the trapping of blowflies. *Onderstepoort Journal of Veterinary Science and Animal Industry* 18: 13-18.

Holloway, B.A. (1991). Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). *New Zealand Journal of Zoology* 18: 415-420.

Huelsenbeck, J.P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

Huson, D.H., and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23: 254-267.

James, M.T. (1971). New species and records of Australasian Calliphorinae, with special reference to the fauna of New Guinea (Diptera: Calliphoridae). *Pacific Insects* 13: 1-12.

James, M.T. (1981). Genus *Dyscritomyia* Grimshaw. In *A Manual of the Insects of the Hawaiian Islands, including an Ennumeration of the Species and Notes on the Origin, Distribution, Hosts, Parasites, etc.* (D.E. Hardy) Vol. 14: 294-349. The University Press of Hawaii, Honolulu.

Jordaens, K., Sonet, G., Richet, R., Dupont, E., Braet, Y., and Desmyeter, S. (2013). Identification of forensically important *Sacrcophaga* species (Diptera: Sarcophagidae) using the mitochondrial *COI* gene. *International Journal of Legla Medicine* 127: 491-504.

Kurahashi, H. (1977). The tribe Luciliini from Australian and Oriental regions I. Genus *Hypopygiopsis* Townsend (Diptera, Calliphoridae). *The Entomological Society of Japan* 45: 553-562.

Liu, Q.-L., Cai, J.-F., Chang, Y.-F., Gu, Y., Guo, Y.-D., Wang, X.-H., Weng, J.-F., Zhong, M., Wang, X., Yang, L., Wu, K.-L., Lan, L.-M., Wang, J.-F., and Chen, Y.-Q. (2011). Identification of forensically important blow fly species (Diptera: Calliphoridae) in China by mitochondrial cytochrome oxidase I gene differentiation. *Insect Science* 18: 554-564.

Malloch, J.R. (1926). Exotic Muscaridae (Diptera). *Annals and Magazine of Natural History* 17: 489-510.

Marinho, M.A.T., Junqueira, A.C.M., Paulo, D.F., Esposito, M.C., Villet, M.H., and Azeredo-Espin, A.M.L. (2012). Molecular phylogenetics of Oestroidea

(Diptera: Calyptratae) with emphasis on Calliphoridae: insights into the inter-familial relationships and additional evidence for paraphyly among blowflies. *Molecular Phylogenetics and Evolution* 65: 840-854.

McDonagh, L.M. and Stevens, J.R. (2011). The molecular systematics of blowflies and screwworm flies (Diptera: Calliphoridae) using *28S* rRNA, COX1 and EF-1α: insights into the evolution of dipteran parasitism. *Parasitology* 138: 1760-177.

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

Nelson, L.A., Lambkin, C.L., Batterham, P., Wallman, J.F., Dowton, M., Whiting, M.F., Yeates, D.K., and Cameron, S.L. (2012). Beyond barcoding: A mitochondrial genomics approach to molecular phylogenetics and diagnostics of blowflies (Diptera: Calliphoridae). *Gene* 511: 131-142.

Nilsson RH, Ryberg M, Kristiansson E, Abarenkov K, Larsson K-H, et al. (2006). Taxonomic Reliability of DNA Sequences in public sequence databases: a fungal perspective. *PLoS ONE* 1(1): e59. doi:10.1371/journal.pone.0000059

Norris, K.R. (1990). Evidence for the multiple exotic origin of Australian populations of the sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Australian Journal of Zoology* 38: 635-648.

Otranto, D. and Stevens, J.R. (2002). Molecular approaches to the study of myiasis-causing larvae. *International Journal for Parasitology* 32: 1345-1360.

Park, S.H., Zhang, Y., Piao, H., Yu, D.H., Jeong, H. J., Yoo, G.Y., Chung, U., Jo, T.-H., and Hwang, J.-J. (2009). Use of cytochrome c oxidase subunit I (*COI*) nucleotide sequences for identification of the Korean Luciliinae fly species (Diptera: Calliphoridae) in forensic investigations. *Journal of Korean Medical Science* 24: 1058-1063.

Posada D. (2008). jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253-1256.

Reibe, S., Schmitz, J., and Madea, B. (2009). Molecular identification of forensically important blowfly species (Diptera: Calliphoridae) from Germany. *Parasitology Research* 106: 257-261.

Roe, A.D., and Sperling, F.A.H. (2007). Patterns of evolution of mitochondrial cytochrome c oxidase I and II DNA and implications for DNA barcoding. *Molecular Phylogenetics and Evolution* 44: 325-345.

Rognes, K. (1980). The blow-fly genus *Lucilia* Robineau-Desvoidy (Diptera, Calliphoridae) in Norway. *Fauna Norvegica Series B* 27: 39-52.

Rognes, K. (1991). Blowflies (Diptera, Calliphoridae) of Fennoscandia and Denmark, *Fauna Enomogolica Scandanavia* 245: 1-277.

Rognes, K. (1994). First record of the sheep greenbottle fly *Lucilia cuprina* (Wiedemann, 1830) from Europe (Diptera: Calliphoridae) with additional Spanish records of Calliphoridae, Muscidae and Sarcophagidae. *Eos* 69: 41-44

Rubinoff, D., Cameron, S., and Will, K. (2006). A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. *Journal of Heredity* 97: 581-594.

Shannon, R.C. (1926). Synopsis of the American Calliphoridae (Diptera). *Proceedings of the Entomological Society of America* 28: 115-133.

Simon, C., Buckley, R.T., Frati, F., Stewart, J.B., and Beckenbach, A.T. (2006). Incorporating Molecular Evolution into Phylogenetic Analysis, and a New Compilation of Conserved Polymerase Chain Reaction Primers for Animal Mitochondrial DNA. *Annual Review of Ecological Evolutionary Systematics* 37: 545-579.

Singh, B. and Wells, J.D. (2013). Molecular systematics of the Calliphoridae (Diptera: Oestroidea): Evidence from one mitochondrial and three nuclear genes. *Journal of Medical Entomology* 50(1): 15-23.

Smith, K.E. (1986). *A manual of forensic entomology*. British National History Museum, London. pp205.

Sonet, G., Jordaens, K., Braet, Y., and Desmyter, S. (2012). Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic? *Forensic Science International* 223: 153-159.

Sonet, G., Jordaens, K., Braet, Y., Bourguignon, L., Dupont, E., Backeljau, T., De Meyer, M., and Desmyter, S. (2013). Utility of GenBank and the Barcode of Life Data Systems (BOLD) for the identification of forensically important Diptera from Belgium and France. *ZooKeys* 365: 307-328.

Stevens, J. and Wall, R. (1996). Classification of the genus *Lucilia* (Diptera: Calliphoridae): a preliminary parsimony analysis. *Journal of Natural History* 30: 1087-1094.

Stevens, J. and Wall, R. (1997). The evolution of ectoparasitism in the genus *Lucilia* (Diptera: Calliphoridae). *International Journal for Parasitology* 27: 51-59.

Stevens, J. and Wall, R. (2001). Genetic relationships between blowflies (Calliphoridae) of forensic importance. *Forensic Science International* 120: 116-123.

Stevens, J.R., Wall, R., and Wells, J.D. (2002). Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology* 11: 141-148.

Stevens, J.R. (2003). The evolution of myiasis in blowflies (Calliphoridae). *International Journal for Parasitology* 33: 1105-1113.

Stevens, J.R. and Wallman, J.F. (2006). The evolution of myiasis in humans and other animals in the Old and New Worlds (part I): phylogenetic analyses. *TRENDS in Parisitology* 22: 129-136.

Stevens, J.R., Wallman, J.F., Otranto, D., Wall, R., and Pape, T. (2006). The evolution of myiasis in humans and other animals in the Old and New Worlds (part II): biological and life-history studies. *TRENDS in Parisitology* 22: 181-188.

Swofford, D.L. (2003). *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, MA.

Tantawi, T.I., Williams, K.A. and Villet, M.H. (2010). An Accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. *Journal of Medical Entomology* 47: 491- 494.

Tourle, R., Downie, D. A., and Villet, M. H. (2009). A morphological and molecular comparison of *Lucilia cuprina* and *L. sericata* (Diptera: Calliphoridae) in South Africa. *Medical and Veterinary Entomology* 23: 6-14.

Ullyett, G.C. (1945). Species of *Lucilia* attacking sheep in South Africa. *Nature* 155: 636-637.

Van Nieukerken, E.J., Doorenweerd, C., Stokvis, F.R., and Groenenberg, D.S.J. (2012). DNA barcoding of the leaf-mining moth subgenus *Ectoedemia* s. str.

(Lepidoptera: Nepticulidae) with *COI* and EF1-α: two are better than one in recognizing cryptic species. *Contributions to Zoology* 81: 1-24.

Valkiūnas, G., Atkinson, C.T., Bensch, S., Sehgal, R.N.M., and Ricklefs, R. (2008). Parasite misidentifications in GenBank: how to minimize their number? *Trends in Parasitology* 24: 247–248.

Vogt, W.G. and Woodburn, T.L. (1979). Ecology, distribution and importance of sheep myiasis flies in Australia. *National Symposium of the sheep blowfly and flystrike in sheep*. N.S.W. Ag. Sydney.

Wallman, J.F., Leys, R., and Hogendoorn, K. (2005). Molecular systematics of Australian carrion-breeding blowflies (Diptera: Callihporidae) based on mitochondrial DNA. *Invertebrate Systematics* 19: 1-15.

Waterhouse, D.F. and Paramonov, S.J. (1950). The status of the two species of *Lucilia* (Diptera, Calliphoridae) attacking sheep in Australia. *Australian Journal of Scientific Research* 3: 310-336.

Wells, J.D., Goff, M.L., and Tomberlin, K. (2002). Molecular systematics of the endemic Hawaiian blowfly genus *Dyscritomyia* Grimshaw (Diptera: Calliphoridae). *Medical Entomology and Zoology* 53: 231-238.

Wells, J.D., Wall, R., and Stevens, J.R. (2007). Phylogenetic analysis of forensically important *Lucilia* flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. *International Journal of Legal Medicine* 121: 229-233.

Whitworth, T. (2006). Keys to the genera and species of blow flies (Diptera: Calliphoridae) of America north of Mexico. *Proceedings of the Entomological Society of Washington* 108: 689-725.

Whitworth, T. (2010). Keys to the genera and species of blow flies (Diptera: Calliphoridae) of the West Indies and description of a new species of Lucilia Robineau-Desvoidy. *Zootaxa* 2663: 1-35.

Whitworth, T.L., Dawson, R.D., Magalon, H., and Baudry, E. (2007). DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proceedings of the Royal Society B* 274: 1731-1739.

Williams, K.A., Cronje, F. J., Avenant, L., and Villet, M.H. (2008). Identifying flies used for maggot debridement therapy. *South African Medical Journal* 98: 196-197.

Williams, K.A. and Villet, M.H. (2013). Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae). *European Journal of Entomology* 110: 187-196.

Zumpt, F.K.E. (1965). *Myiasis in man and animals in the old world: a textbook for physicians, veterinarians and zoologists*. Butterworths, London.

Table 5.1. Specimen locality data for sequences added to GenBank. (Accession numbers starting KF are new sequences from this study).

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
Calliphora vicina	CV_FRC_01(F)	Montferrier-Sur-Lez	France	JN792781	KF839531	KF839562
	CV_FRC_02(M)	Montferrier-Sur-Lez	France	KF839506		
Lucilia caesar	Ca_FRC_01(M)	Montferrier-Sur-Lez	France	JN792782	JN792858	KF839556
	Ca_FRC_02(F)	Montferrier-Surz-Lez	France	KF839501	KF839532	KF839557
Lucilia coeruleiviridis	Co_CAN_01(M)	Windsor	Canada	KF839502	KF839533	KF839558
	Co_CAN_02(M)	Windsor	Canada	KF839503		KF839559
	Co_USA_03(F)	Putnam Co. Missouri	United States of America	KF839504	KF839534	KF839560
	Co_USA_04(F)	Martinstown, Missouri	United States of America	KF839505		KF839561
Lucilia cuprina	C_AUS_01 (M)	Sydney	Australia	KF856254		JN792622
	C_EGT_01 (F)	Alexandria	Egypt	JN792706	JN792784	JN792625
	C_SA_CT_02 (F)	Cape Town	South Africa	JN792713	JN792791	JN792632
	C_SA_DBN_01(F)	Durban	South Africa	JN792724	JN792802	JN792642
	C_THA_02 (F)	Chiang Mai	Thailand	JN792741	JN792819	JN792661
	C_THA_03 (F)	Chiang Mai	Thailand	JN792742	JN792820	JN792662
	C_ZIM_02 (F)	Matobos	Zimbabwe	JN792745	JN792823	JN792667
Lucilia eximia	Ex_CSR_01(F)	Santo Domingo	Costa Rica	KF839507	KF839535	KF839563

	Ex_CSR_02(F)	Santo Domingo	Costa Rica	KF839508	KF839536	KF839564
Lucilia fayeae	Fa_DOM_01(F)	Calibishie	Dominica	KF839509	KF839537	KF839565
	Fa_DOM_02(F)	Calibishie	Dominica	KF839510	KF839538	KF839566
Lucilia illustris	IL_CAN_01(F)	Windsor	Canada	KF839516	KF839544	KF839572
	IL_CAN_02(F)	Windsor	Canada	KF839517	KF839545	KF839573
	IL_JPN_01(F)	Iwate Medical University	Japan	KF839518	KF839546	KF839574
	IL_JPN_02(F)	Iwate Medical University	Japan	KF839519	KF839547	KF839575
	IL_SWZ_01(F)	Lausanne-Suisse	Switzerland	KF839520	KF839548	
	IL_USA_01(F)	Michigan	United States of America	KF839521	KF839549	
	IL_USA_02(F)	Michigan	United States of America	KF839522	KF839550	KF839576
Lucilia infernalis	In_BRN_01(F)	Parc National de la Kibira	Burundi	KF839523	KF839551	KF839577
	In_RWN_01(F)	Nyungwe Forest Reserve	Rwanda	JN792780	JN792857	JN813094
Lucilia mexicana	Mx_USA_01(F)	New Mexico	United States of America	KF839524	KF839552	KF839578
	Mx_USA_02(F)	New Mexico	United States of America	KF839525		KF839579
Lucilia papuensis	Pa_AUS_01	-	Australia	KF839526		
Lucilia porphyrina	Po_AUS_01	-	Australia	KF839527	KF839553	
Lucilia sericata	S_AUS_01 (M)	Seaford	Australia	JN792746	JN792824	JN792668
	S_FRC_01 (F)	Montferrier-Sur-Lez	France	JN792749	JN792827	JN792671

	S_JPN_01 (F)	Osaka	Japan	JN792754	JN792831	JN792678
	S_NAM_01 (F)	Possession Island	Namibia	JN792758	JN792835	JN792682
	S_SA_CT_07 (F)	Cape Town	South Africa	JN792766	JN792843	JN792690
	S_USA_01 (F)	Michigan	United States of America	JN792778	JN792855	JN792703
Lucilia silvarum	Si_GER_01(F)	Kempen	Germany	KF839528		KF839580
Lucilia thatuna	Th_USA_01(F)	Del Norte Co. California	United States of America	KF839529	KF839554	KF839581
	Th_USA_02(F)	Del Norte Co. California	United States of America	KF839530	KF839555	KF839582
Hemipyrellia fernandica	H_BEN_01(M)	Contonou	Benin	KF839511	KF839539	KF839567
	H_BEN_02(M)	Contonou	Benin	KF839512	KF839540	KF839568
	H_SA_DBN_01(F)	Durban	South Africa	KF839513	KF839541	KF839569
	H_TAN_01(M)	Mkuraja	Tanzania	KF839514	KF839542	KF839570
	H_TAN_02(M)	Mkuraja	Tanzania	KF839515	KF839543	KF839571

Table 5.2: GenBank sequences included in this study.

Species	Locality		Accession Number		
			28S	*Per*	*COI*
L. adiosoemartoi	-	Indonesia			AY074901
L. ampullacea	Langford	UK	AJ300137		
L. ampullacea	Bristol	UK			DQ453487
L. ampullacea	-	Korea			EU925394
L. bazini	-	Taiwan			AY346450
L. bazini	-	China			DQ345082
L. caesar	Langford	UK	AJ300138		AY417703
L. caesar	Bristol	UK			DQ453488
L. caesar	-	Korea			EU880196
L. cluvia	New Orleans	USA	AJ551440		DQ453490
L. cluvia	Volusia Co. Florida	USA			JQ942371
L. coeruleiviridis	New York	USA			FJ650558
L. cuprina	-	China			DQ345087
L. cuprina	Honolulu	Hawaii			AJ417704
L. cuprina	Oahu	Hawaii			DQ453496
L. cuprina	-	Taiwan			AY097335
L. cuprina	-	Thailand			EU418577
L. cuprina	Tororo	Uganda			AJ417711
L. cuprina	Townsville	Australia	AJ417709		AJ417710
L. cuprina	Waianae	Hawaii			AJ417705
L. cuprina	Wallaceville	New Zealand		Y19108.1	
L. cuprina	Noordhoek	South Africa	EU626549		
L. cuprina	Cincinnati	USA	FJ650542		
L. eximia	-	Brazil			DQ453491
L. hainanensis	-	Taiwan			AY346451
L. hainanensis	-	China			DQ345084
L. illustris	Langford	UK	AJ300136		AJ551445
L. illustris	-	Korea			EU880204
L. illustris	-	China			DQ345090
L. illustris	-	India			DQ200168
L. mexicana	San Francisco	USA	AJ551441		DQ453492

Species	Locality		Accession Number		
			28S	*Per*	*COI*
L. mexicana	California	USA			FJ650563
L. mexicana	California	USA			FJ650562
L. papuensis	-	China			DQ345085
L. porphyrina	-	Taiwan			AY097336
L. porphyrina	-	Japan			AY074900
L. porphyrina	-	China			DQ345089
L. richardsi	Usk	-	AJ551142		
L. sericata	Perth	Australia			AB112833
L. sericata	Nerja	Spain			AJ417716
L. sericata	Kingsbury	UK			AJ417713
L. sericata	Hilerod	Denmark	AJ300140		EF531193
L. sericata	Harare	Zimbabwe			AJ417717
L. sericata	-	China			DQ345086
L. sericata	Langford	UK	AJ300139		
L. sericata	Los Angeles	USA	AJ300141		
L. silvarum	Durham	UK	AJ551443		
L. silvarum	-	USA			FJ650564
L. silvarum	Linn Co., OR	USA			JQ942455
L. taiyuanensis	-	China			DQ345088
L. thatuna	San Francisco	USA	AJ551444		DQ453489
L. thatuna	Del Norte Co., California	USA			JQ942464
H. ligurriens	-	China			DQ345092
H. ligurriens	-	Taiwan			AY097334
H. ligurriens	-	Taiwan			DQ453493
H. pulchra	-	China			DQ345091
D. fasciata	-	Hawaii			AY074902
D. lucilioides	-	Hawaii			AY074903
D. robusta	-	Hawaii			AY074898
C. vicina	Bristol	UK	AJ300131		AJ417702

Table 5.3: Binary coding of 14 morphological characters for the *Lucilia* and *Hemipyrellia* genera. [1 – Colour of the basicostal scale (0 = black/brown, 1 = white/cream); 2 – Number of postsutural acrostichal bristles (0 = two pairs, 1 = three pairs); 3 – Eye separation in the male (0 = distance of greater than the width of the third antennal segment, 1 = less than the width of the third antennal segment); 4 – Number of anterio-dorsal bristles on the mid tibia (0 = one, 1 = two); 5 – Colour of the palpi (0 = yellow/orange, 1 = black/brown); 6 – Subcostal sclerite (0 = bristles absent, 1 = bristles present); 7 – Colour of the squamae (0 = uniform white/cream, 1 = partially or totally brown); 8 – Wings (00 = hyaline, 01 = lightly infuscated, 11 = heavily infuscated); 9 – Eye separation in the female (0 = distance of greater than one quarter of the width of the head, 1 = less than one quarter of the width of the head); 10 – Colour of antennae (0 = uniformly dark, 1 = non-uniform); 11 – Male hypopygium (00 = inconspicuous, 01 = conspicuous, 11 = highly conspicuous); 12 – Colour of abdomen and thorax (0 = predominantly brassy green/green, 1 = predominantly purple/blue/black); 13 – Colour of the legs (00 = dark brown, 01 = brown/black, 11 = black); 14 – Lower squamal lobe (0 = setae absent, 1 = setae present)] (Stevens & Wall, 1996).

Species	Character number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lucilia ampullacea	0	0	1	0	0	1	0	00	0	0	00	0	01	0
Lucilia bufonivora	0	0	0	0	1	0	0	00	0	0	01	0	11	0
Lucilia coeruleiviridis	1	0	1	0	0	0	0	00	1	1	00	0	00	0
Lucilia caesar	0	0	1	0	0	1	0	00	0	0	11	0	01	0
Lucilia cluvia	1	0	0	0	0	0	0	00	1	0	00	0	00	0
Lucilia cuprina	1	1	0	0	0	0	0	00	0	0	01	0	11	0
Lucilia eximia	0	0	1	0	0	0	1	00	0	1	00	0	00	0
Lucilia fayeae	0	0	1	0	0	0	1	01	0	0	00	1	00	0
Lucilia illustris	0	0	1	0	0	1	0	00	0	0	01	0	11	0
Lucilia infernalis	0	1	1	0	0	1	1	11	0	1	00	1	01	0
Lucilia mexicana	0	0	1	0	0	0	1	01	0	1	00	0	11	0
Lucilia papuensis	0	0	1	1	0	1	1	01	0	1	00	0	11	0
Lucilia porphyrina	0	0	1	0	0	1	1	01	1	0	00	1	00	0
Lucilia richardsi	1	1	0	1	1	0	0	00	0	0	00	0	11	0

Lucilia sericata	1	1	0	0	0	0	0	00	0	0	00	0	11	0
Lucilia silvarum	0	1	0	0	1	0	0	00	0	0	01	0	11	0
Lucilia thatuna	1	1	1	0	0	0	0	00	1	0	00	0	11	0
Hemipyrellia fernandica	0	0	0	0	1	1	0	00	1	0	00	0	11	0
Hemipyrellia ligurriens	0	0	0	0	0	1	0	00	1	1	01	0	11	0
Hemipyrellia pulchra	0	0	0	1	0	?	0	00	0	1	00	0	11	0
Calliphora vicina	1	1	1	1	0	0	1	00	0	0	11	1	01	1

Table 5.4. Zoogeographic distribution of Lucilliinae species.

Species	Region						
	Afrotropical	Australasian	Neararctic	Neotropical	Oriental	Pacific	Palaearctic
L. adiosoemartoi					X		
L. ampullacea							X
L. bazini					X		
L. caesar							X
L. cluvia			X				
L. coeruleiviridis			X				
L. cuprina	X	X	X		X		
Dysctritomyia sp.						X	
L. eximia				X			
L. fayeae				X			
L. hainanensis					X		
H. fernandica	X						
Hemipyrellia sp.		X			X		
Hypopygiopsis sp.		X					
L. illustris			X				X
L. infernalis	X						
L. mexicana			X				
L. papuensis		X			X		

Species	Region						
	Afrotropical	Australasian	Neararctic	Neotropical	Oriental	Pacific	Palaearctic
L. porphyrina		X					X
L. sericata	X	X	X	X	X		X
L. silvarum			X				X
L. taiyuanensis					X		
L. thatuna			X				

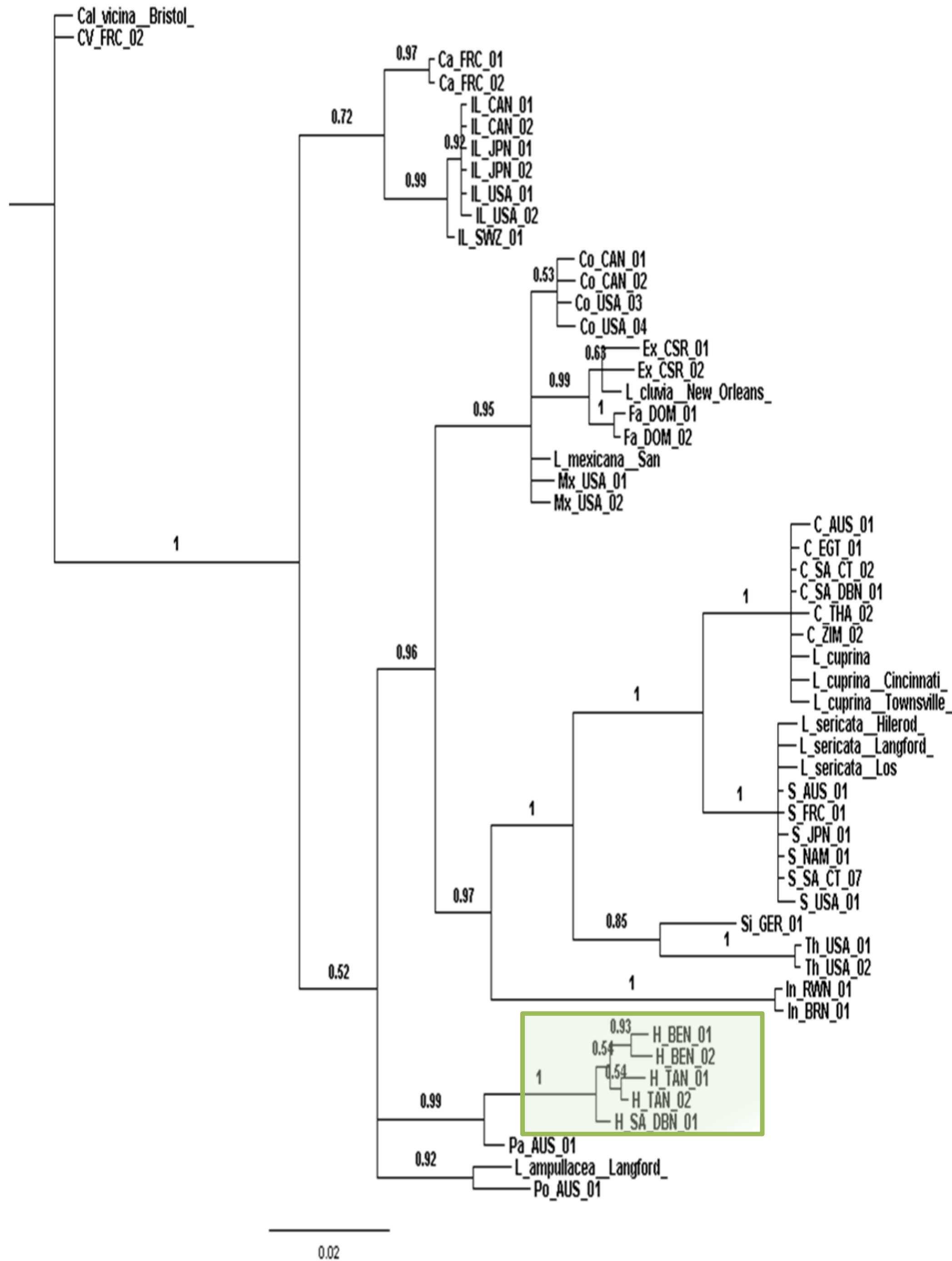

Figure 5.1. Bayesian inference tree constructed from concatenated nuclear genes *28S&Per*. Posterior probabilities are indicated on nodes. Green box = *Hemipyrellia fernandica* [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica*, IL = *illustris*, In = *infernalis*, Mx = *mexicana*, Pa = *papuensis*, Po = *porphyrina*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town].

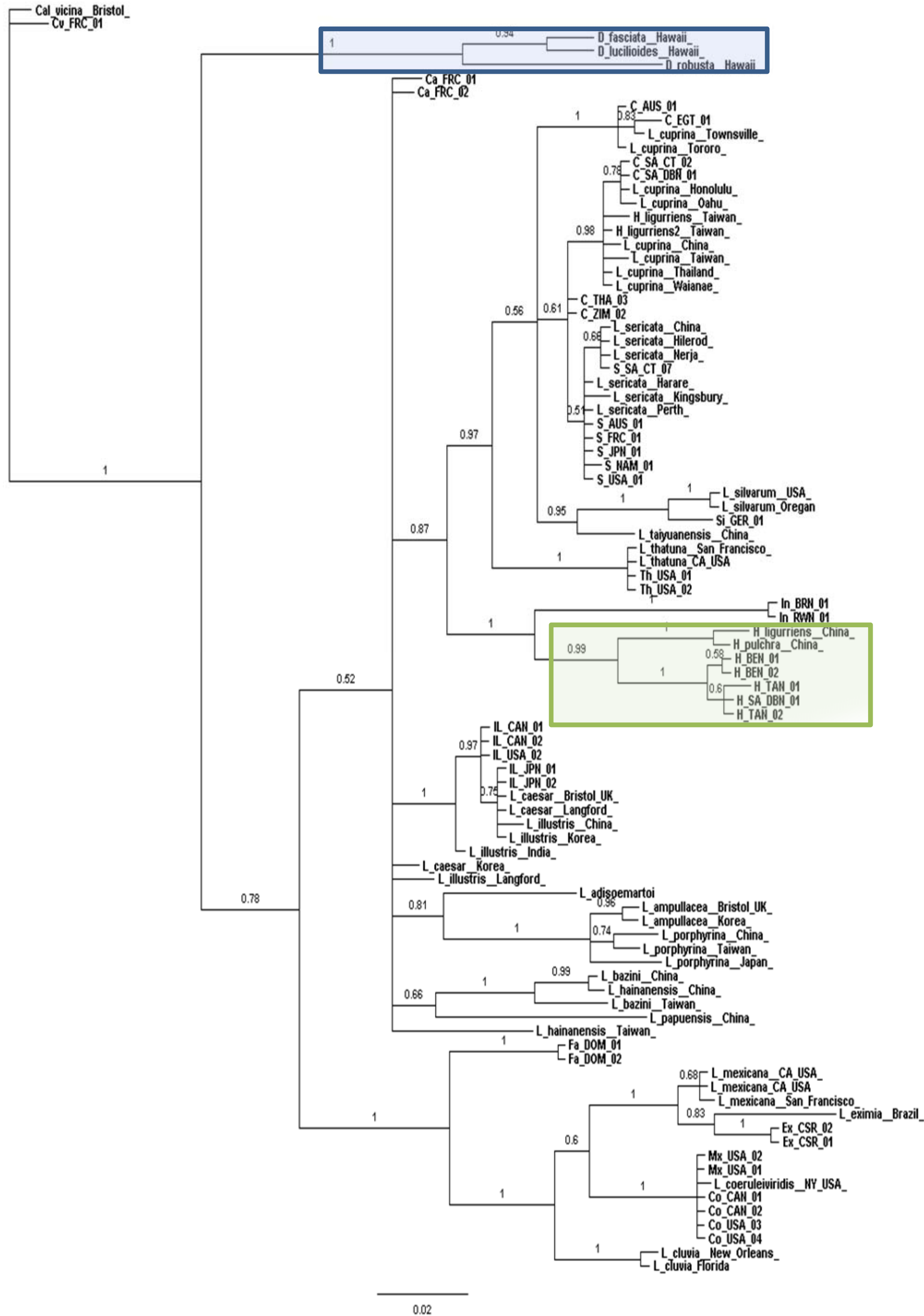

Figure 5.2. Bayesian inference tree constructed from mitochondrial gene *COI*. Posterior probabilities indicated on nodes. Green box = *Hemipyrellia* sp. Blue box = *Dysctritomyia* sp. [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica* IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town].

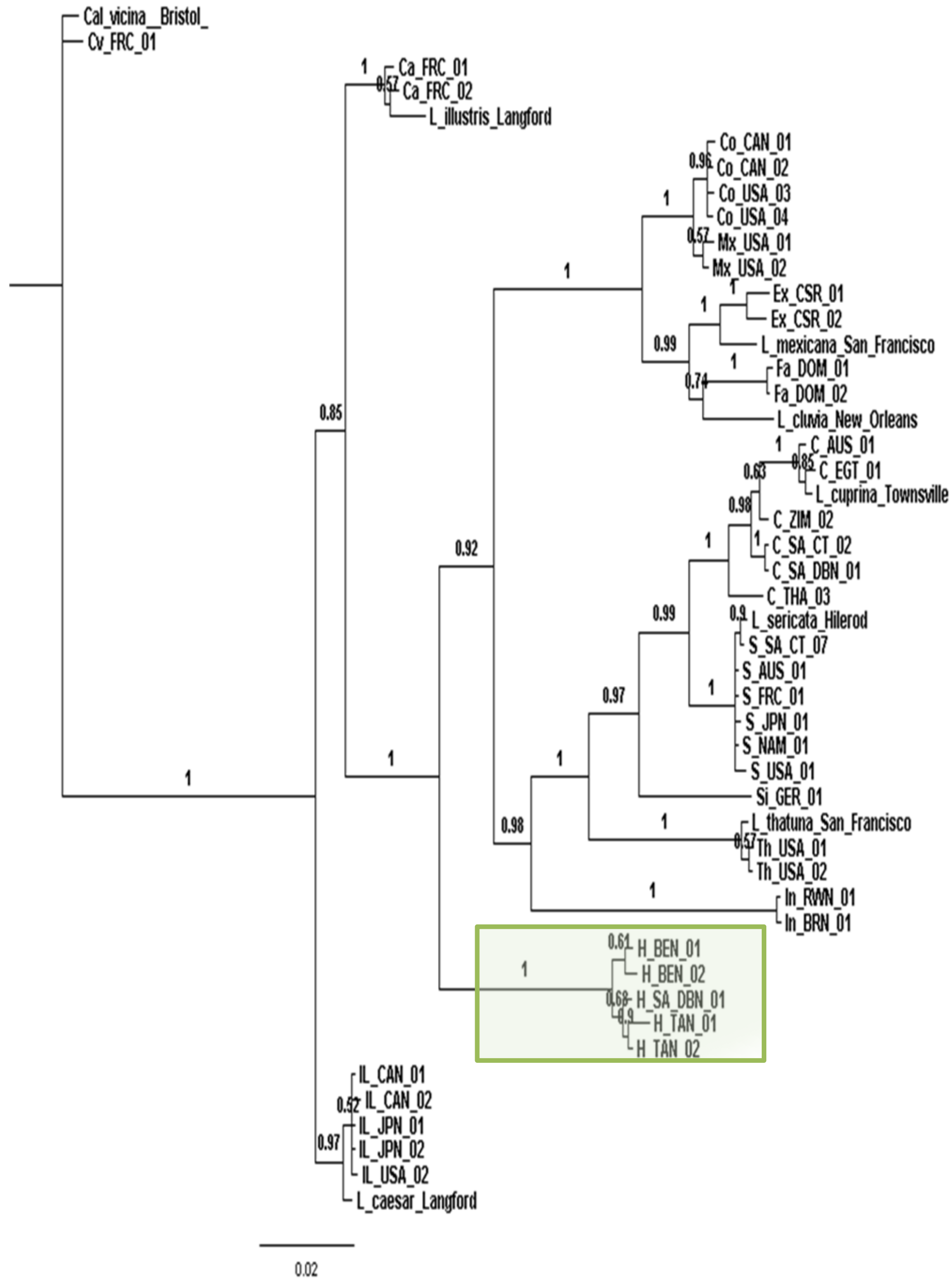

Figure 5.3. Bayesian inference tree constructed from the concatenated nuclear (*28S* & *Per*) and mitochondrial (*COI*) genes. Posterior probabilities indicated on nodes. Green box = *Hemipyrellia fernandica*. [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica* IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town].

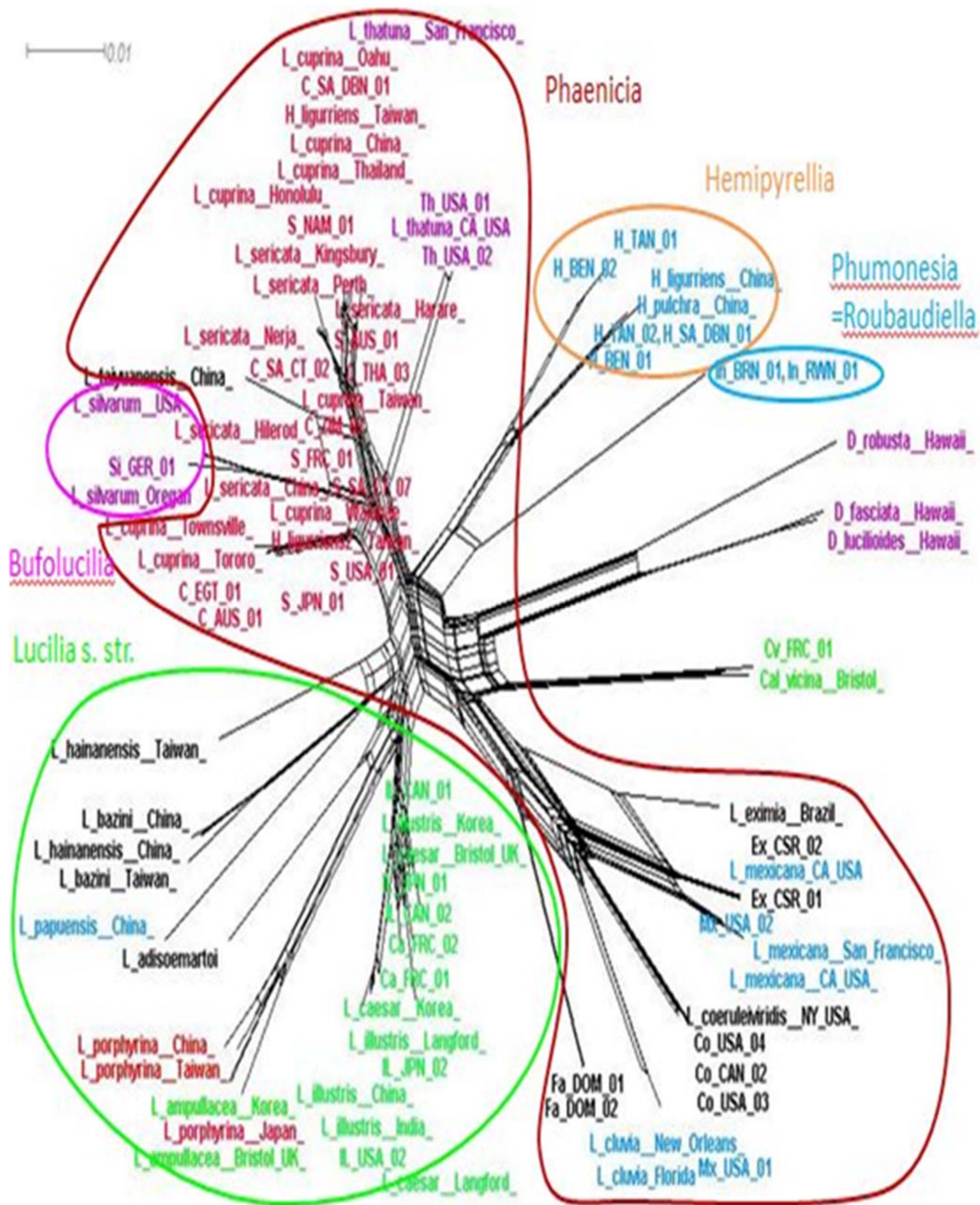

Figure 5.4. NeighborNet network diagram constructed from *COI* data showing parasitic behaviour and previous sub-generic status of *Lucilia*. Text colours: Red = primary facultative parasite, green = secondary facultative parasite, purple = parasite (unknown if primary or secondary), blue = saprophage, black = unknown parasitic behaviour. [C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica*, IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominica, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT=Cape Town].

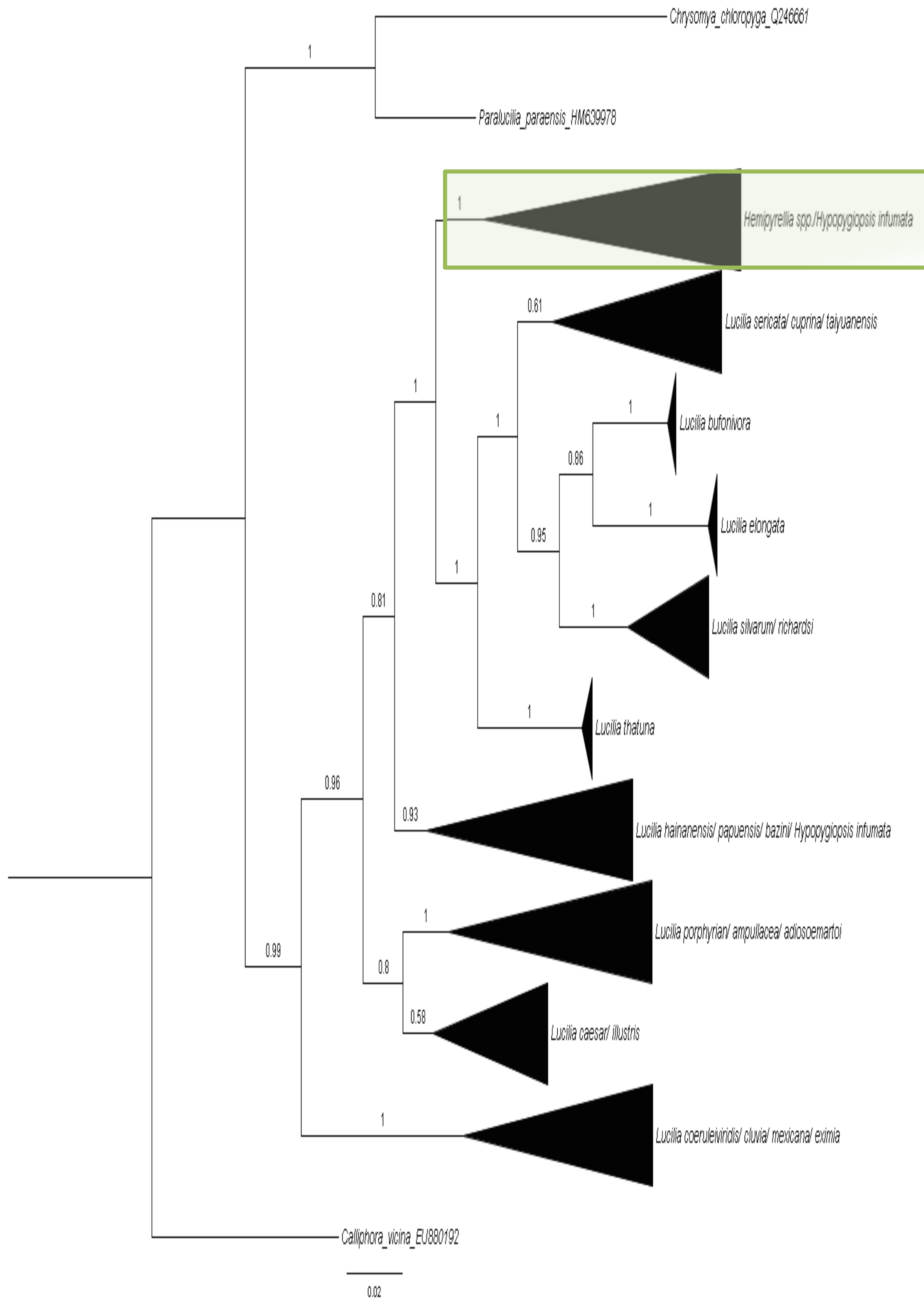

Figure 5.5. Bayesian inference tree constructed using *COI* barcode sequences. Posterior probabilities indicated on nodes. Support within the collapsed nodes is variable. Green box = *Hemipyrellia* sp.

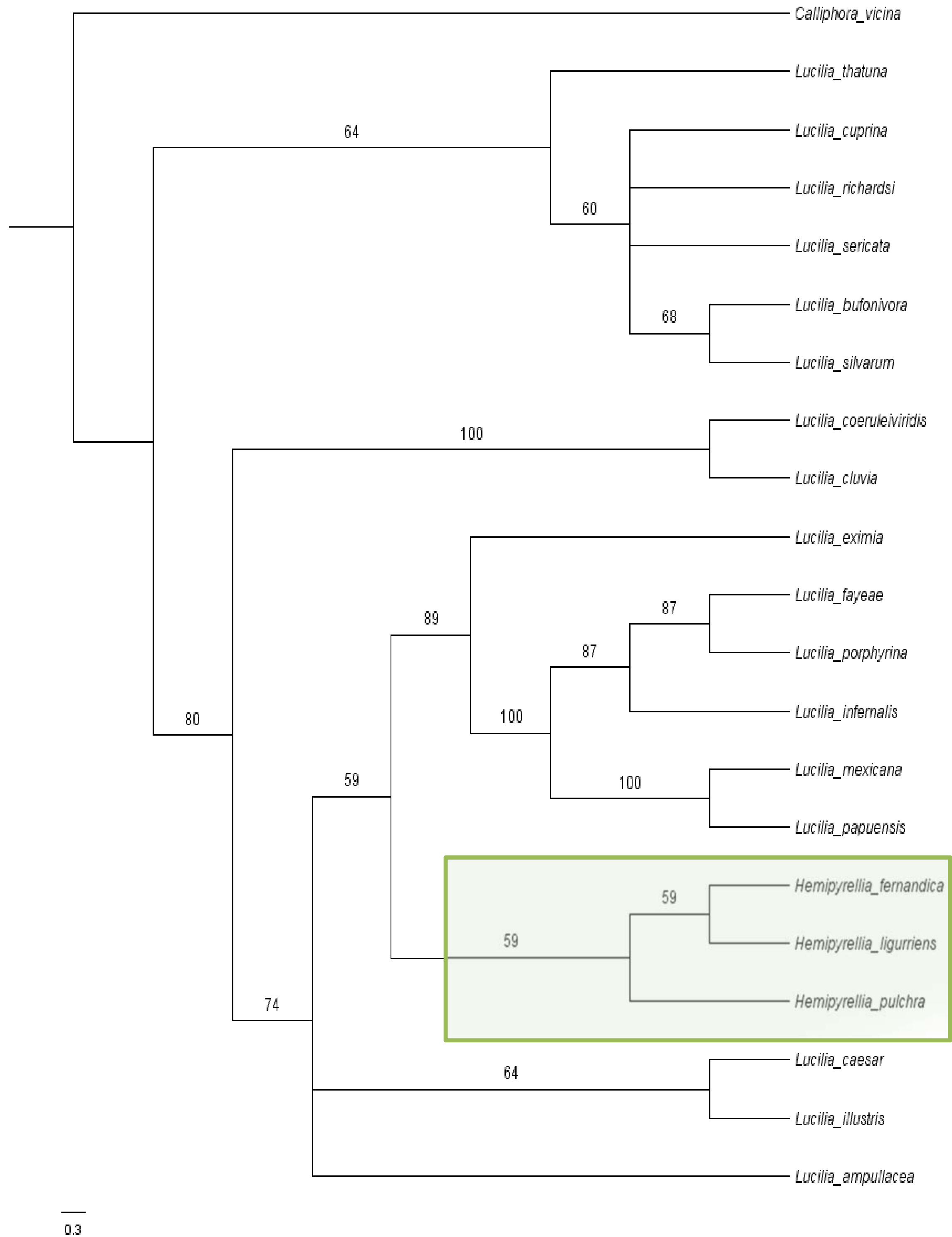

Figure 5.6. Majority rule consensus tree for 21 species of *Lucilia* and *Hemipyrellia* constructed from morphological characters listed in Table 5.3. Green box = *Hemipyrellia* sp.

CHAPTER 6

CONCLUSION

Lucilia sericata and *L. cuprina* are the only species of the genus *Lucilia* that occur in South Africa. The forensic, veterinary and medical importance of these flies underlies the need to examine the relationship between these species, their relationship within *Lucilia*, their geographic distribution in South Africa and the use of morphological identification.

Sequencing the mitochondrial and nuclear DNA of *Lucilia sericata* and *L. cuprina* showed the existence of both ancient and modern hybrid specimens of these two species. The existence of modern hybrids has not been shown before. This is the possible result of introgression and incomplete lineage sorting. The presence of *Wolbachia* bacteria was not investigated, but is suspected of being originally responsible for the introgression of *L. sericata* mitochondrial DNA into *L. cuprina* without any nuclear DNA transfer; it may since have bred out of the host population. The existence of hybrids of these species has now been shown to occur in several continents and is not limited to Hawaii. Knowledge of the existence of these hybrids is important in forensic entomology when molecular techniques are used for identification of flies found on a body. The biology of the hybrids has not been studied and may differ from the pure strains which could affect their growth rates and therefore affect the post mortem interval (PMI) determination. Similarly, no studies have looked at whether these hybrids are involved in sheep strike or have been used in MDT. The biology and lifecycle of the hybrids therefore needs to be determined by laboratory experiments and applied to these fields.

Morphological identification of *L. sericata* and *L. cuprina* is possible using the published keys but this is complicated by the existence of hybrids of these species. By scoring selected morphological characters and using these to identify specimens, the hybrids can be identified statistically. This is important for forensic entomology when determining PMIs and when identifying flies responsible for sheep strike, as the biology of the hybrids has not yet been studied and may differ from the parental strains. Morphological identification in conjunction with molecular methods will ensure correct species identifications. MDT is becoming popular as an alternative to traditional medical treatments because of the increase in antibiotic resistant bacteria and the correct identification of the flies used for this treatment is therefore important.

Both *Lucilia sericata* and *L. cuprina* are predicted to occur in most parts of South Africa. Neither species shows specialized habitat requirements and their predicted ranges overlap to a large extent. No correlation was found between the geographical distributions of sheep farming and where *L. cuprina* was predicted to occur. Similarly, no correlation between human populations and *L. sericata* occurrence was found. If either of these species is present in forensic investigations, sheep strike or obtained for MDT colonies, they cannot be identified from the associated locality data, and morphological or molecular methods will have to be used.

Lucilia sericata and *L. cuprina* were consistently represented as sister-species in several different analyses. No geographic patterns were evident in *Lucilia* with regard to the parasitic behavior of its species. Two additional cases of introgression within *Lucilia* were confirmed in this study. This is important for forensic entomology as it affects the correct identification of species by molecular methods and may have implications for determining PMIs if it affects the hybrids lifecycles. The other genera of the subfamily Luciliinae are not clearly separated from *Lucilia*. *Hemipyrellia* should be synonymized with *Lucilia* as it consistently renders *Lucilia* paraphyletic in all of the analyses conducted (Chapter 5). *Dyscritomyia* and *Hypopygiopsis* require further study to confirm their taxonomic relationship to *Lucilia*.

The cosmopolitan distribution of *L. sericata* and *L. cuprina* makes the results of this study relevant, not only to South Africa, but to large parts of the world. The identification of species that are sequenced and submitted to GenBank affirms concerns because at least three different species' sequences from GenBank that were used in this research were identical. Further taxonomic research on the Luciliinae is required to clarify the taxonomic status of the genera in this subfamily.

Due to *L. sericata* and *L. cuprina* being used for MDT, being responsible for sheep strike, and both species being used in forensic entomology investigations because of their habit of breeding in dead bodies, the existence of hybrids of these two species is very important. It has been shown in this study that they can be identified both by molecular and morphological methods. The biology of the hybrids however, has not been studied and future research to determine if their biology differs from that of the parental strains is required to make their use in forensics, veterinary and medical entomology more reliable.

Eur. J. Entomol. **110**(2): 187–196, 2013
http://www.eje.cz/pdfs/110/2/187
ISSN 1210-5759 (print), 1802-8829 (online)

Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae)

KIRSTIN WILLIAMS[1, 2] and MARTIN H. VILLET[2]

[1]Durban Natural Science Museum, PO Box 4085, Durban, 4000, South Africa; e-mail: WilliamsK@durban.gov.za
[2]Southern African Forensic Entomology Research Laboratory, Department of Zoology and Entomology, Rhodes University, PO Box 94, Grahamstown, 6140, South Africa; e-mail: M.Villet@ru.ac.za

Key words. Diptera, Calliphoridae, *Lucilia sericata*, *L. cuprina*, hybrids, mDNA, phylogenetic, taxonomy, introgression

Abstract. There are important but inconsistent differences in breeding site preference between the blow flies *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) that have significance for medical and veterinary science. These inconsistencies might arise from hybridisation. The species are difficult to distinguish using external morphology, although the male genitalia are distinctive and there are reliable molecular markers. Molecular evidence of modern hybridisation, derived from a newly developed nuclear marker, the *period* (*per*) gene, is presented here. This has implications for identifications of these species based on mtDNA, and may lead to an explanation of the medical and veterinary anomalies noted in these species.

INTRODUCTION

The use of *Lucilia* blowflies for maggot debridement therapy (MDT) has become a topic of great interest in South Africa (Williams et al., 2008; F. Cronje & Du Plessis H.J.C, pers. comm). *Lucilia sericata* (Meigen, 1826) is the species of choice for MDT (Altincicek & Vilcinskas, 2009; Vilcinskas, 2011), but the misidentification of *Lucilia cuprina* (Wiedemann, 1830) and *L. sericata* for use in MDT and how best to supplement MDT colonies has raised the issue of species identification (Williams et al., 2008; Tantawi et al., 2010). *Lucilia cuprina* has recently been used successfully for MDT (Paul et al., 2009; Tantawi et al., 2010; Kingu et al., 2012) although this species is responsible for sheep-strike that causes losses to the wool and meat industries that amount to millions of dollars worldwide each year (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006). Different populations of *L. sericata* show different degrees of *cuprina*-like attraction to sheep (Crombe, 1944; Cragg, 1956), but no clear pattern in this myiasis has been noted.

These two species have been suspected of interbreeding and producing fertile hybrids in South Africa (Ullyett, 1945). They have been shown to hybridise under laboratory conditions and to produce fertile hybrids, although there are no reports of this occurring naturally (Ullyett, 1945). *Lucilia cuprina* has consistently been found to be paraphyletic relative to *L. sericata* in studies of several mitochondrial genes (Table 1). If they are interbreeding, this leads to an explanation of the medical and veterinary anomalies noted in the biology of these species.

Several authors have suggested that these flies should be classified as three species or that *L. cuprina* should be classified as two subspecies – *Lucilia c. cuprina* (Wiedemann, 1830) and *L. c. dorsalis* Robineau-Desvoidy, 1830 (Waterhouse & Paramonov, 1950; Norris, 1990; Stevens & Wall, 1996; Stevens et al., 2002; Stevens, 2003; Wallman et al., 2005; Wells et al., 2007; DeBry et al., 2010). *Lucilia sericata* and *L. cuprina* are morphologically very similar and the adults are difficult to identify using the available keys based on morphological characters without using the male genitalia, which usually requires destructive sampling (Aubertin, 1933; Smith, 1986; Norris, 1990; Holloway, 1991). However, with some experience, the females can usually be reliably identified using the characteristics described by Holloway (1991a).

Molecular methods are useful in confirming the taxonomic status of these two species (Williams et al., 2008; Tourle et al., 2009; Tantawi et al., 2010). The use of more than one gene for taxonomic and phylogenetic studies is recommended as using only one gene may not give a true picture of relationships or patterns of gene flow (Sperling et al., 1994; Nelson et al., 2007; Whitworth et al., 2007; Tourle et al., 2009). Analysing both nuclear and mitochondrial genes simultaneously has highlighted introgression and the difference between gene trees and species trees (Page & Charleston, 1997; Nichols, 2001; Stevens et al., 2002; Stevens, 2003; Whitworth et al., 2007; Tourle et al., 2009; DeBry et al., 2010).

The purpose of this study was to test for evidence of hybridisation between these two species, shown by a difference between the gene trees produced from sequence data using nuclear, as opposed to mitochondrial, genes from these flies from different localities around South Africa and from sites in Africa, Europe, Australia, Asia, and North America.

MATERIAL AND METHODS

Adult flies of both *L. sericata* and *L. cuprina* were collected in Britstown, Bloemfontein, Cape Town, Durban, Grahamstown, Nelspruit, and Witbank in South Africa (Fig. 1 insert). *Lucilia* specimens originating from Welkom and Pretoria were

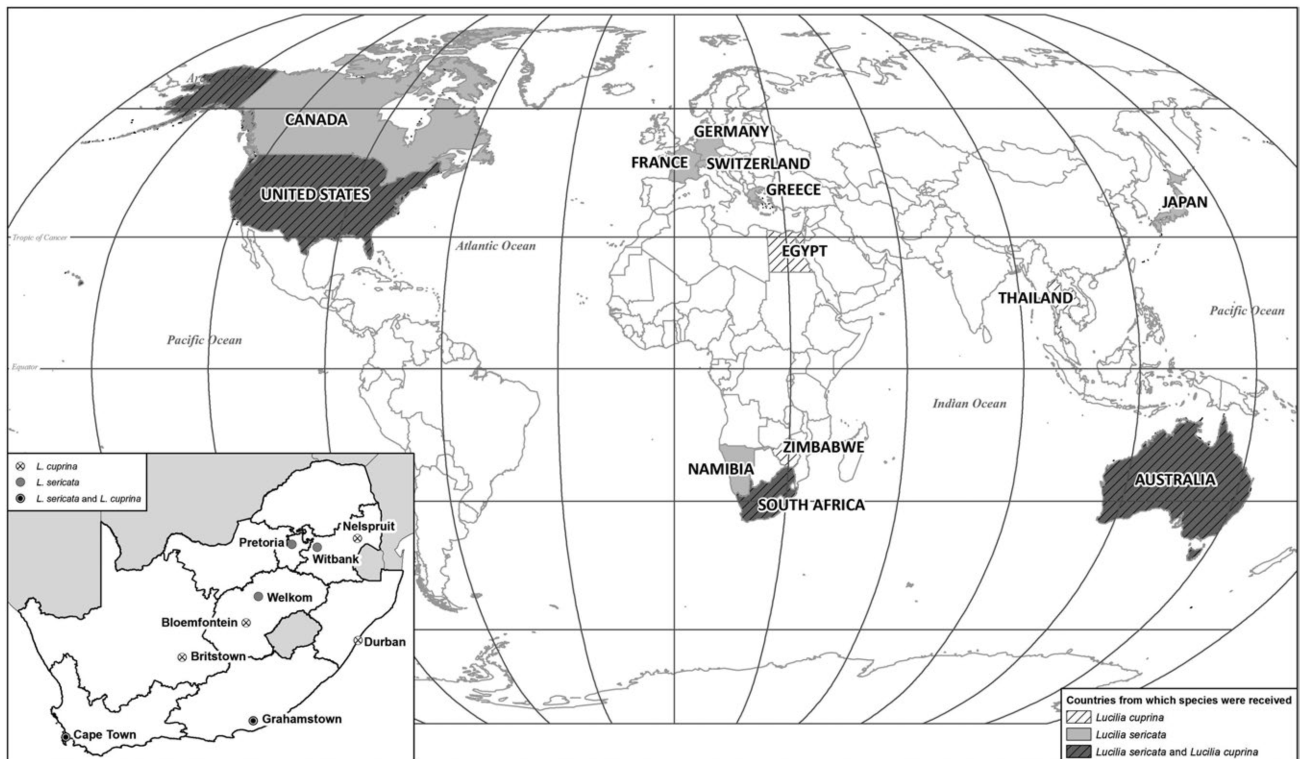

Fig. 1. World map showing the localities where flies were caught. Insert: map of South Africa showing the towns where flies were caught.

also obtained from a maggot debridement therapy colony at Eugene Marais Hospital in Pretoria. *Lucilia sericata* was also obtained from Australia, Canada, France, Germany, Greece, Japan, Namibia, Switzerland, and the United States of America (Fig. 1). Additional specimens of *L. cuprina* were obtained from Australia, Egypt, Thailand, the United States of America, and Zimbabwe (Fig. 1). A total of 84 flies were collected – 11 males and 73 females. They were identified by their morphology using published keys (Aubertin, 1933; Smith, 1986; Holloway, 1991a). Due to the biology of these flies, females are attracted to bait traps more than males and therefore characteristics identified by Holloway (1991a); specifically the distances and angles between setae on the vertex of females, the extent of metallic sheen on the parafrontal sclerites of females and the number of scutellar setulae were used to identify these flies.

All flies were kept in separate 1.5 ml Eppendorf tubes in 96% ethanol and deposited with the Durban Natural Science Museum after analysis. One hind leg of each fly was used for DNA analysis. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions (Qiagen 07/2006).

Three genes were chosen for sequencing – *28S* rRNA (*28S*), a nuclear gene that has been used in previous studies and would allow comparison with other studies (Table 1); *period* (*per*), a second nuclear gene that is faster-evolving than *28S* to give better resolution; and cytochrome oxidase I (*COI*), that has been used in previous studies (Table 1). A region of approximately 650 bp in domain 1–2 of the *28S* gene was amplified using the primers 5'-CCCCCTGAATTTAAGCATAT-3' and 5'-GTTAGACTCCTTGGTCCGTG-3' (Stevens et al., 2002). A region of approximately 600 bp of the *COI* gene was amplified using the primers C1-J1709 (5'-AATTGGGGGGTTTGGAA ATTG-3') and C1-N2353 (5'-GCTCGTGTATCAACGTCTA TTCC-3') (Simon et al., 2006). This region overlaps the "barcoding" region for approximately 300 base pairs. A region of approximately 730 bp of the *per* gene, was amplified using the primers *per*5 (5'-GCCTTCAGATACGGTCAAAC-3') (G. Warman, pers. comm.) and *per* reverse (5'-CCGAGTGTGGTTTG

TABLE 1. Genes used in studies of *Lucilia sericata* and *Lucilia cuprina*.

Source	Mitochondrial		Nuclear		
	CO1	12S rRNA	*28S* rRNA	*per*	RAPDs
Stevens & Wall, 1996	–	329 bp	–	–	X
Stevens et al., 2002	2300 bp (*CO1* & *2*)	–	2193 bp	–	
Stevens, 2003	2300 bp (*CO1* & *2*)	–	2200 bp	–	
Wallman et al., 2005	3008 bp (*CO1* & *2* & *ND4-ND4L*)	–	–	–	
Wells et al., 2007	1545 bp	–	–	–	
Harvey et al., 2008	1167 bp	–	–	–	
Williams et al., 2008	601 bp	–	654 bp	–	
Tourle et al., 2009	439 bp	–	678 bp	–	
DeBry et al., 2010	1200bp	–	2100 bp	–	
Tantawi et al., 2010	576 bp	–	656 bp	746 bp	
This study	576 bp	–	654 bp	722 bp	

Table 2. Specimen locality data for sequences included from GenBank.

Species	Locality	Country	Accession Number		
			28S	*per*	*COI*
L. sericata	Langford	UK	AJ300139		
	Hilerod	Denmark	AJ300140		
	Hilerod	Denmark			EF531193
	Kingsbury	UK			AJ417713
	Nerja	Spain			AJ417716
	Harare	Zimbabwe			AJ417717
	–	China			DQ345086
L. cuprina	Townsville	Australia	AJ417709		AJ417710
	Wallaceville	New Zealand		Y19108.1	
	Tororo	Uganda			AJ417711
	–	Taiwan			AY097335
	–	China			DQ345087
	Oahu	Hawaii			DQ453496
	Honolulu	Hawaii			AJ417704
	Waianae				AJ417705

GAGATT-3') (designed by the authors). Polymerase chain reaction (PCR) amplification was performed using 1 µL of DNA in a 25 µL reaction. Amplification times were 94°C for 5 min denaturation, followed by 36 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 30 s and a final extension period at 72°C for 7 min. PCR products were confirmed by gel electrophoresis stained in ethidium bromide.

PCR products were then sequenced using an ABI 3730l Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the primers used in amplification. Additional DNA sequences for these two species were obtained from GenBank (www.ncbi.nlm.nih.gov) for comparative analysis (Table 2). The sequences were aligned and edited using the BioEdit v7.0.9 software (Hall, 1999).

Phylogenetic reconstruction by maximum parsimony analysis was performed using PAUP*4b10 (Swofford, 2003) using the best-fitting model (HKY) from MrModelTest v2.2 (Nylander, 2004) applied in MrMTgui (Nuin, 2005). Statistical support for nodes was assessed by bootstrapping with 100 replicates retaining a maximum of 10,000 trees. Bayesian inference analysis was performed using one cold and three hot chains and the HKY model. Analysis was run for 5,000,000 generations, sampling every 1,000 generations with burn-in of 1,000 samples. All phylogenetic analyses used *Calliphora vicina* and *Lucilia infernalis* as outgroups. Incongruence length difference (ILD) tests (Farris et al., 1994) were run in PAUP* 4b10 (Swofford, 2003) to quantify the differences in topology between trees for *28S*, *COI* and *per*. Analysis was then conducted on the partitioned data sets (*28S* and *per*; *28S*, *per* and *COI*) with the parameters as above.

When hybridization is involved, a single dichotomising phylogenetic tree will often not be a suitable representation of the phylogenetic history (Huson & Bryant, 2006). This may make it necessary to use a more general graph, such as a network to represent the data. NeighborNet computes a set of splits from the data. If splits are compatible, the resultant graph will be a dichotomous tree, but when the splits are not compatible, it results in a network diagram with multiple parallel branches representing a single split (Huson & Bryant, 2006). Network diagrams were created using NeighborNet in SplitsTree4 (Huson & Bryant, 2006) using the uncorrected P-method for distance.

RESULTS

A total of 654 base pairs for *28S*, 576 bp for *COI* and 722 bp for *per* (a total of 1952 bp) were sequenced and aligned. There were no indels in the aligned sequences. A total of 77, 83 and 76 specimens were sequenced respectively for *28S*, *COI* and *per* (Table 3).

The ILD test showed *28S* and *per* to be congruent (P = 0.99), and the ILD test for *28S* and *COI* was not statistically significant (P = 0.08). *per* and *COI* were significantly incongruent (P = 0.01) as was the combination of *28S*, *per* and *COI* (P = 0.01). Due to the high level of congruence between *28S* and *per*, these two data sets were concatenated and used for the analyses and network diagrams. Despite the incongruence between the nuclear (*28S* and *per*) and mitochondrial (*COI*) data, these data sets were also concatenated and an analysis run on the total evidence.

The Bayesian Inference trees (Fig. 2A) for the nuclear genes (*28S* and *per*) show both *L. sericata* and *L. cuprina* to be monophyletic clades with strong support (Fig. 2A). The Bayesian Inference tree for *COI* (Fig. 2B) shows that *L. cuprina* is paraphyletic with respect to *L. sericata*, with good posterior probability support. The first *L. cuprina* clade (Fig. 2B) exhibits both nuclear and mitochondrial sequences (and morphology) of "pure *cuprina*", while the second clade exhibits nuclear DNA (and morphology) of *L. cuprina* but mitochondrial DNA of *L. sericata* – a "hybrid" clade. The *L. cuprina* sequences from GenBank from Hawaii, Taiwan and China grouped with the "hybrid" clade (Fig. 2B).

Out of 42 specimens with the morphology of *L. cuprina*, five have mitochondrial genes that are typical of the *L. sericata* clade (Fig. 2B), but not of the "ancient hybrid" clade. The maximum parsimony trees were topologically compatible with the Bayesian Inference trees but the trees were less well resolved (trees not shown).

The network diagrams of the nuclear genes (*28S* and *per*) (Fig. 3) indicate a clear and simple split between the *L. sericata* specimens and the *L. cuprina* specimens. The *COI* network diagram (Fig. 4) shows two clear splits between a cluster of *L. sericata* specimens, and two clusters of *L. cuprina* specimens. The "hybrid" cluster of *L. cuprina* specimens lies closer to the *L. sericata* cluster than to the "pure" *L. cuprina* cluster, but is distinctively monophyletic. The five *L. cuprina* specimens that group

TABLE 3. Specimen locality data for sequences from this study added to GenBank (* indicate identical sequences that are represented by one sequence in the Bayesian Inference tree, M – Male, F – Female).

Species	Specimen	Locality		Accession Number		
				28S	*per*	*COI*
Calliphora vicina	CV_FRC_01	Montferrier-Sur-Lez	France	JN792781		
Lucilia caesar	Ca_FRC_01	Montferrier-Sur-Lez	France	JN792782	JN792858	
Lucilia infernalis	In_RWN_01	Nyungwe Forest Reserve	Rwanda	JN792780	JN792857	JN813094
	C_AUS_01* (M)	Sydney	Australia			JN792622
	C_AUS_02* (F)	Sydney	Australia			JN792623
	C_AUS_03 (F)	Hornsby Heights	Australia	JN792705	JN792783	JN792624
	C_EGT_01 (F)	Alexandria	Egypt	JN792706	JN792784	JN792625
	C_EGT_02 (F)	Alexandria	Egypt	JN792707	JN792785	JN792626
	C_SA_BFN_01(F)	Bloemfontein	South Africa	JN792708	JN792786	JN792627
	C_SA_BFN_02 (F)	Bloemfontein	South Africa	JN792709	JN792787	JN792628
	C_SA_BRT_01 (F)	Britstown	South Africa	JN792710	JN792788	JN792629
	C_SA_BRT_02 (F)	Britstown	South Africa	JN792711	JN792789	JN792630
	C_SA_CT_01*(M)	Cape Town	South Africa	JN792712	JN792790	JN792631
	C_SA_CT_02 (F)	Cape Town	South Africa	JN792713	JN792791	JN792632
	C_SA_CT_03*(F)	Cape Town	South Africa	JN792714	JN792792	JN792633
	C_SA_CT_04 (F)	Cape Town	South Africa	JN792715	JN792793	JN792634
	C_SA_CT_05 (F)	Cape Town	South Africa	JN792716	JN792794	JN792635
	C_SA_CT_06 (F)	Cape Town	South Africa	JN792717	JN792795	JN792636
	C_SA_CT_07 (F)	Cape Town	South Africa	JN792718	JN792796	JN792637
	C_SA_CT_08 (F)	Cape Town	South Africa	JN792719	JN792797	JN792638
	C_SA_CT_09*(F)	Cape Town	South Africa	JN792720	JN792798	JN792639
	C_SA_CT_10 (M)	Cape Town	South Africa	JN792721	JN792799	
	C_SA_CT_11*(F)	Cape Town	South Africa	JN792722	JN792800	JN792640
	C_SA_CT_12*(F)	Cape Town	South Africa	JN792723	JN792801	JN792641
	C_SA_DBN_01*(F)	Durban	South Africa	JN792724	JN792802	JN792642
	C_SA_DBN_02 (F)	Durban	South Africa	JN792725	JN792803	JN792643
Lucilia cuprina	C_SA_DBN_03(M)	Durban	South Africa	JN792726	JN792804	JN792644
	C_SA_DBN_04 (F)	Durban	South Africa			JN792645
	C_SA_DBN_05 (F)	Durban	South Africa			JN792646
	C_SA_DBN_06 (F)	Durban	South Africa	JN792727	JN792805	JN792647
	C_SA_DBN_07*(F)	Durban	South Africa	JN792728	JN792806	JN792648
	C_SA_DBN_08 (F)	Durban	South Africa	JN792729	JN792807	JN792649
	C_SA_DBN_09 (F)	Durban	South Africa	JN792730	JN792808	JN792650
	C_SA_DBN_10*(F)	Durban	South Africa	JN792731	JN792809	JN792651
	C_SA_DBN_11*(F)	Durban	South Africa	JN792732	JN792810	JN792652
	C_SA_DBN_12 (F)	Durban	South Africa	JN792733	JN792811	JN792653
	C_SA_DBN_13 (F)	Durban	South Africa	JN792734	JN792812	JN792654
	C_SA_DBN_14*(F)	Durban	South Africa	JN792735	JN792813	JN792655
	C_SA_GHT_01(M)	Grahamstown	South Africa	JN792736	JN792814	JN792656
	C_SA_GHT_02 (F)	Grahamstown	South Africa	JN792737	JN792815	JN792657
	C_SA_NEL_01 (F)	Nelspruit	South Africa	JN792738	JN792816	JN792658
	C_SA_NEL_02 (F)	Nelspruit	South Africa	JN792739	JN792817	JN792659
	C_THA_01 (F)	Chiang Mai	Thailand	JN792740	JN792818	JN792660
	C_THA_02 (F)	Chiang Mai	Thailand	JN792741	JN792819	JN792661
	C_THA_03 (F)	Chiang Mai	Thailand	JN792742	JN792820	JN792662
	C_THA_04 (F)	Chiang Mai	Thailand			JN792663
	C_USA_01 (F)	Merced	USA	JN792743	JN792821	JN792664
	C_USA_02 (F)	Merced	USA	JN792744	JN792822	JN792665
	C_ZIM_01 (F)	Matobos	Zimbabwe			JN792666
	C_ZIM_02 (F)	Matobos	Zimbabwe	JN792745	JN792823	JN792667
	S_AUS_01 (M)	Seaford	Australia	JN792746	JN792824	JN792668
	S_CAN_01 (F)	Windsor	Canada	JN792747	JN792825	JN792669
	S_CAN_02 (F)	Windsor	Canada	JN792748	JN792826	JN792670
	S_FRC_01 (F)	Montferrier-Sur-Lez	France	JN792749	JN792827	JN792671
	S_FRC_02 (F)	Montferrier-Sur-Lez	France	JN792750	JN792828	JN792672
	S_FRC_03 (F)	Montferrier-Sur-Lez	France	JN792751	JN792829	JN792673
	S_GER_01 (F)	Kempen	Germany	JN792752		JN792674
	S_GER_02 (F)	Kempen	Germany		JN792830	JN792675
	S_GRC_01 (F)	Crete	Greece	JN792753		JN792676
	S_GRC_02 (F)	Crete	Greece			JN792677
	S_JPN_01* (F)	Osaka	Japan	JN792754	JN792831	JN792678
	S_JPN_02* (F)	Osaka	Japan	JN792755	JN792832	JN792679
	S_JPN_03* (F)	Iwate	Japan	JN792756	JN792833	JN792680
	S_JPN_04* (F)	Iwate	Japan	JN792757	JN792834	JN792681
	S_NAM_01 (F)	Possession Island	Namibia	JN792758	JN792835	JN792682
	S_NAM_02 (F)	Possession Island	Namibia	JN792759	JN792836	JN792683
	S_SA_CT_01* (F)	Cape Town	South Africa	JN792760	JN792837	JN792684
	S_SA_CT_02 (F)	Cape Town	South Africa	JN792761	JN792838	JN792685
Lucilia sericata	S_SA_CT_03* (M)	Cape Town	South Africa	JN792762	JN792839	JN792686
	S_SA_CT_04* (F)	Cape Town	South Africa	JN792763	JN792840	JN792687
	S_SA_CT_05 (F)	Cape Town	South Africa	JN792764	JN792841	JN792688
	S_SA_CT_06* (F)	Cape Town	South Africa	JN792765	JN792842	JN792689
	S_SA_CT_07* (F)	Cape Town	South Africa	JN792766	JN792843	JN792690
	S_SA_CT_08* (F)	Cape Town	South Africa	JN792767	JN792844	JN792691
	S_SA_GHT_01 (F)	Grahamstown	South Africa	JN792768	JN792845	JN792692
	S_SA_GHT_02 (F)	Grahamstown	South Africa	JN792769	JN792846	JN792693
	S_SA_PTA_01 (M)	Pretoria	South Africa	JN792770	JN792847	JN792694
	S_SA_PTA_02 (F)	Pretoria	South Africa	JN792771	JN792848	JN792695
	S_SA_PTA_03 (F)	Pretoria	South Africa	JN792772	JN792849	JN792696
	S_SA_PTA_04 (M)	Pretoria	South Africa	JN792773	JN792850	JN792697
	S_SA_WLK_01 (F)	Welkom	South Africa	JN792774	JN792851	JN792698
	S_SA_WLK_02 (F)	Welkom	South Africa	JN792775	JN792852	JN792699
	S_SA_WTB_01 (F)	Witbank	South Africa	JN792776	JN792853	JN792700
	S_SA_WTB_02 (F)	Witbank	South Africa	JN792777	JN792854	JN792701
	S_SWZ_01 (M)	Lausanne	Switzerland			JN792702
	S_USA_01 (F)	Michigan	USA	JN792778	JN792855	JN792703
	S_USA_02 (M)	Michigan	USA	JN792779	JN792856	JN792704

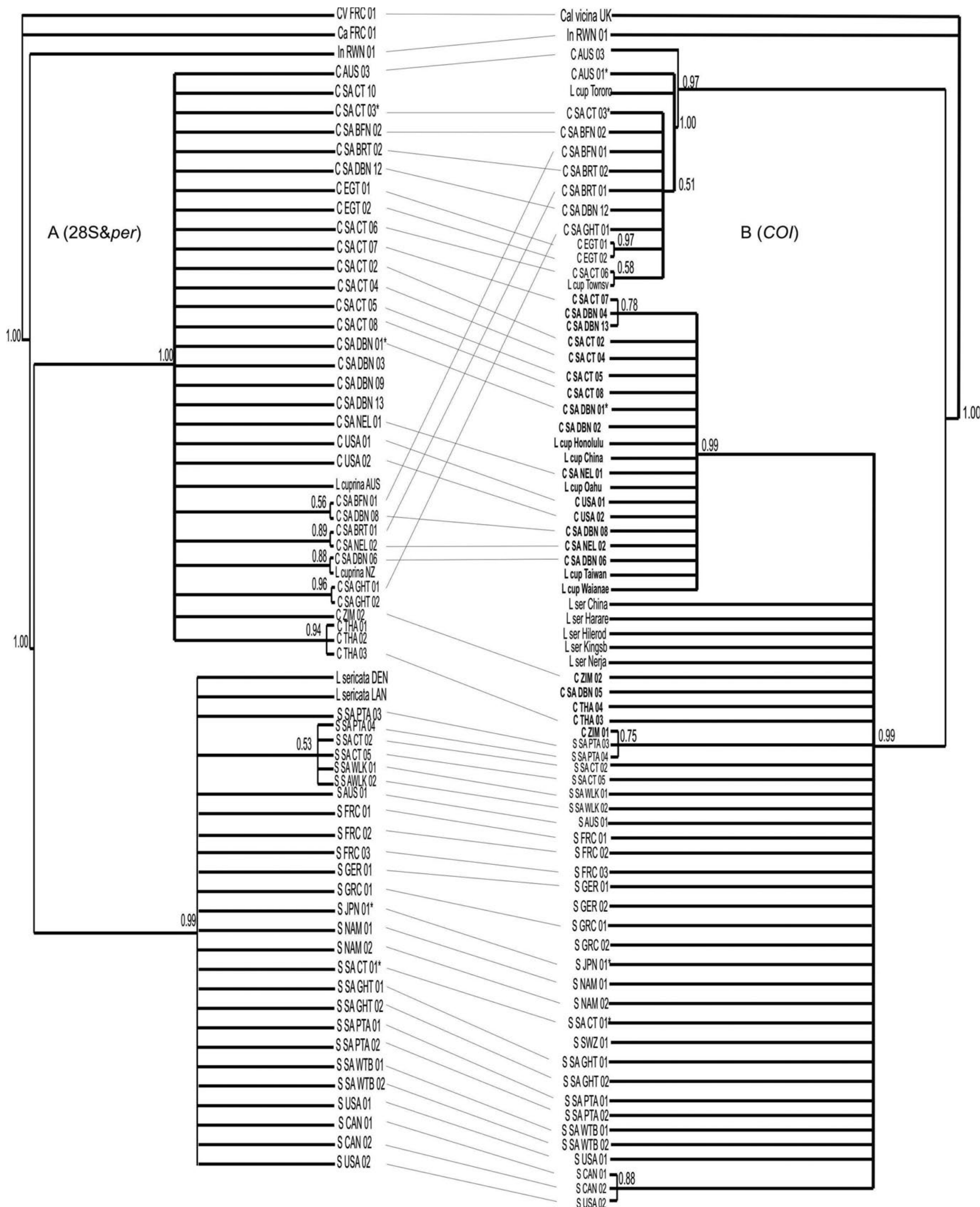

Fig. 2. Bayesian Inference trees constructed from nuclear genes (*28S* and *per*) (A) and mitochondrial genes (*COI*) (B) data. Posterior probabilities are indicated on nodes. C – *cuprina*, S – *sericata*, CV – *Calliphora vicina*, In – *Lucilia infernalis*, CA - *Lucilia caesar*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, GRC – Greece, JPN – Japan, NAM – Namibia, EGT – Egypt, RWN – Rwanda, SWZ – Switzerland, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

Fig. 3. NeighborNet network diagram constructed from *28S* & *per* data. C – *cuprina*, S – *sericata*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, JPN – Japan, NAM – Namibia, EGT – Egypt, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

within the *L. sericata* clade (Fig. 2B) also appear within the *L. sericata* cluster (Fig. 4). The network diagram of the total evidence concatenated data sets (Fig. 5) shows a clear split between the *L. sericata* and *L. cuprina* clusters, and the *L. cuprina* samples split into two clusters which are linked by more pathways to each other than to the *L. sericata* cluster.

DISCUSSION

A number of studies have been conducted on *L. sericata* and *L. cuprina*, looking at morphological identification, the possibility that they are interbreeding and whether *L. cuprina* should be classified as two subspecies or two independent species (Ullyett, 1945; Waterhouse & Paramonov, 1950; Norris, 1990; Holloway, 1991a, b; Stevens & Wall, 1996; Stevens et al., 2002; Stevens, 2003; Wallman et al., 2005; Wells et al., 2007; Harvey et al., 2008; Tourle et al., 2009; DeBry et al., 2010). This study focuses on these two species in South Africa, but also examines specimens from across the globe to place the South African situation into a global context. This study used two nuclear and one mitochondrial gene where most previous studies have either used only one mitochondrial gene or a combination of mitochondrial genes and one nuclear gene (Table 1). Stevens & Wall (1996) used RAPDs, which encompasses multi-locus nuclear genotype data, but without targeting explicit genes (Table 1).

Individually and together, the nuclear *28S* and *per* genes show *L. sericata* and *L. cuprina* to be two monophyletic clades (Fig. 2A) with very strong posterior probability support (0.99 and 1.00 respectively). However, the mitochondrial *COI* gene suggests that *L. cuprina* is paraphyletic with respect to *L. sericata* (Fig. 2B). There is a monophyletic clade of *L. cuprina* specimens that have *L. sericata*-like mtDNA, which has been seen in previous studies (Table 1). This monophyletic clade of *L. cuprina* with *L. sericata*-like mtDNA has been suggested to represent an ancient hybridization event (Stevens & Wall, 1996; Stevens et al., 2002; Tourle et al., 2009). The *L. sericata* mtDNA appears to have been fixed in this lineage of *L. cuprina* and not lost through lineage sorting.

However, there are also five specimens with the morphology of *L. cuprina* and mtDNA of *L. sericata* that are not representative of the ancient, introgressed clade (Figs

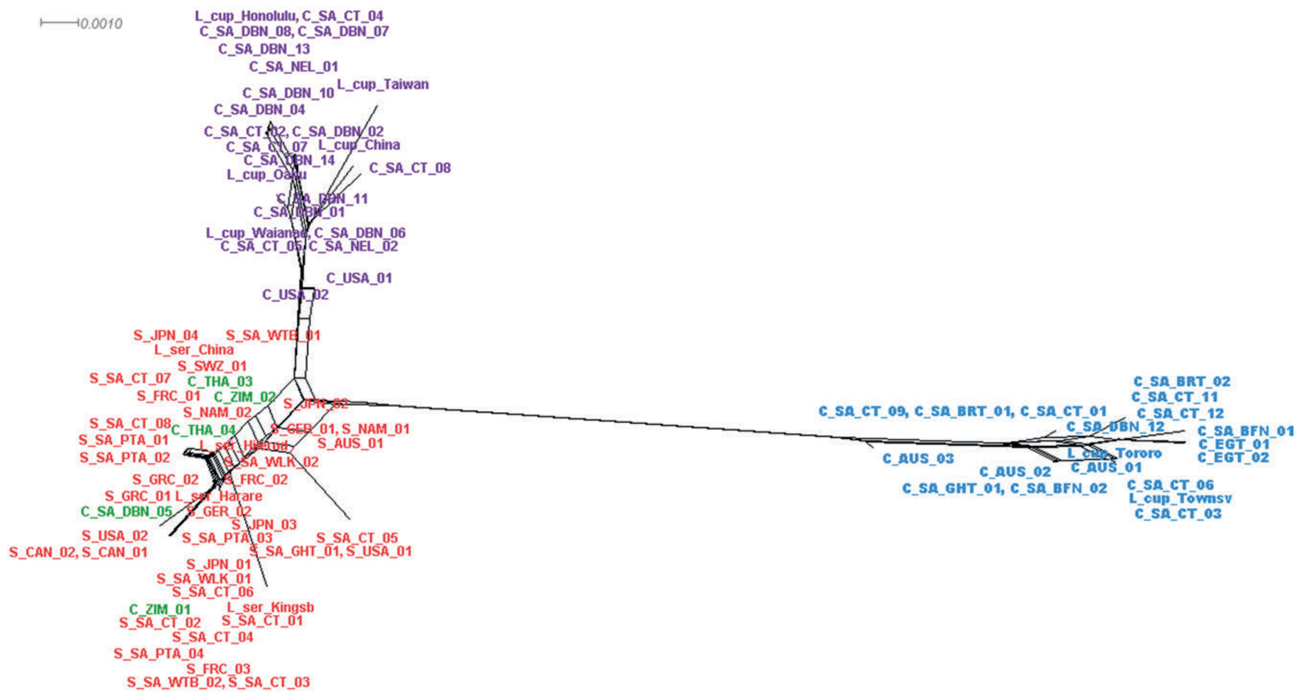

Fig. 4. NeighborNet network diagram constructed from *COI* data. C – *cuprina*, S – *sericata*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, JPN – Japan, NAM – Namibia, EGT – Egypt, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

2B and 4), implying novel mismatches of nuclear and mitochondrial genomes. Nuclear genes were not amplified for three of these specimens, but the other two, from Zimbabwe and Thailand, have (different) *28S* and *per* genotypes typical of *L. cuprina*, which suggests modern hybridization. This has not been seen in any previous studies on *L. sericata* / *L. cuprina* (Table 1) and provides the first direct genetic evidence of modern-day natural interbreeding between these species.

Ancient hybrids and introgression

The specimens that form the monophyletic clade of *L. cuprina* with *L. sericata*-like mtDNA originate from Durban, Nelspruit and Cape Town in South Africa, and from Merced in California in the continental USA, Hawaii, China, and Taiwan (Tables 2 and 3). It was once suggested that this lineage was restricted to the Hawaiian Islands (Stevens & Wall, 1996; Stevens et al., 2002), but since then the lineage has been found in North America, Africa, and Asia. It would be difficult to determine where it originated because it is so widespread. There does not appear to be any geographical coherence within the two *L. cuprina* clades (Fig. 2B). It was suggested that the two named subspecies of *L. cuprina* – *L. c. cuprina* and *L. c. dorsalis* – could be distinguished using *COI* sequences because both subspecies formed monophyletic clades (DeBry et al., 2010), with *L. c. cuprina* forming a monophyletic clade that was sister to the *L. sericata* clade, thus suggesting that all *L. cuprina* with *L. sericata*-like mtDNA are *L. c. cuprina*. Sequences from South Africa (Tourle et al., 2009) that were included in this analysis (DeBry et al., 2010) all grouped with the putative clade of *L. c. cuprina*, although African *L. cuprina* are considered to be *L. cuprina dorsalis* (Waterhouse & Paramonov, 1950). Perhaps *L. c. cuprina* has been introduced into South Africa like some other synanthropic blow flies (Williams & Villet, 2006), but the problem remains of distinguishing them morphologically, an issue that was addressed by Tourle et al. (2009), who found the "hybrid" clade to have a morphological index that was more *cuprina*-like than "pure" *cuprina* specimens.

Four cases of mtDNA introgression without detectable nuclear introgression, as seen in this study, were reported for *Protocalliphora* blowflies (Whitworth et al., 2007). Interspecific mitochondrial introgression linked to selective sweeps induced by nuclear-cytoplasmic incompatibility due to *Wolbachia* infections has been described in various insects (Ballard, 2000) as an explanation for how mtDNA introgression without nuclear introgression is possible. Cytoplasmic incompatibility is a process where, if uninfected females mate with infected males, some or all of their eggs will die. But if an infected female mates with either an infected or uninfected male, her eggs remain viable but all will be infected with *Wolbachia*. So infected females outcompete uninfected ones and the overall population of *Wolbachia*-infected flies (and therefore *Wolbachia*) increases (Zimmer, 2001). Thus the mitochondria of infected individuals have a greater chance than uninfected individuals of being passed on because mitochondria are passed down the female line, leading to fixed introgression. *Wolbachia* infection in the blowfly *Protocalliphora sialia* (Baudry et al., 2003) and infections of three different strains of *Wolbachia* in *Protocalliphora* in North America (Whitworth et al., 2007)

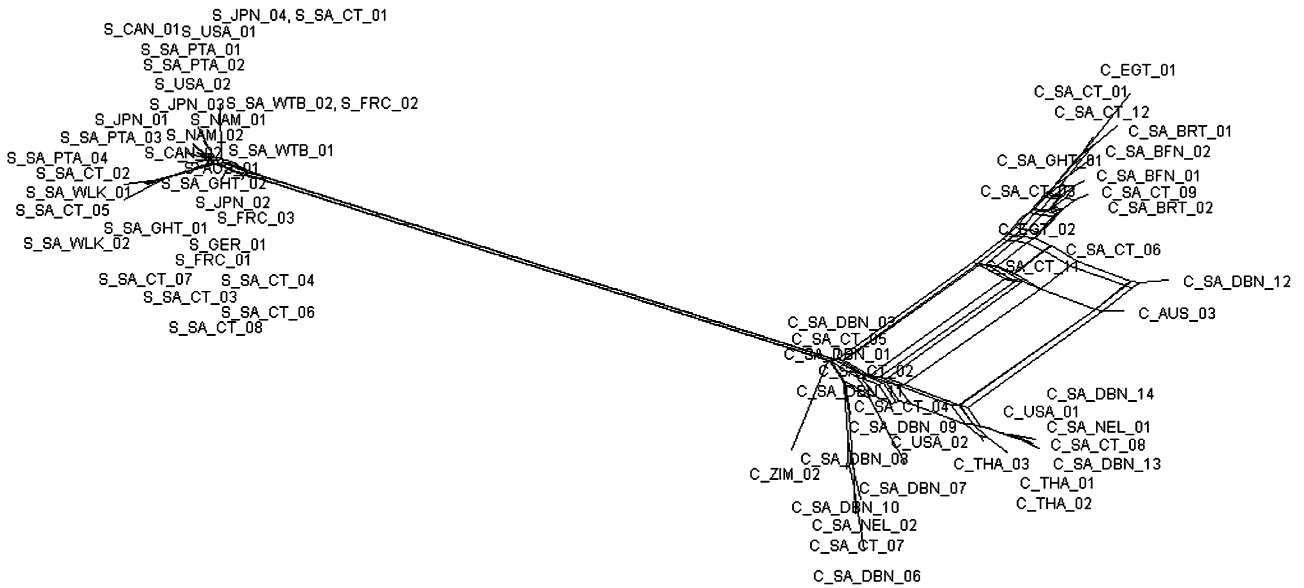

Fig. 5. NeighborNet network diagram constructed from *28S* & *per* & *COI* concatenated data. C – *cuprina*, S – *sericata*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, JPN – Japan, NAM – Namibia, EGT – Egypt, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

have been reported. All of these infections resulted in mtDNA introgression without any detectable nuclear introgression. Further studies are recommended to determine if *Lucilia* blowflies are affected by *Wolbachia* infections as an explanation for the pattern seen in this study. However, such infections can die out over time, so that the only evidence of them may be cytoplasmic introgression (Zimmer, 2001).

The combined *28S* and *per* data show a very clear split between the *L. sericata* and *L. cuprina* samples (Fig. 3). The splits show very little internal incompatibility. The mtDNA (*COI*) shows a much higher degree of incompatibility between the splits (Fig. 4) which represents incompatible signals (Huson & Bryant, 2006). There are three important splits that group *L. sericata* together and two *L. cuprina* splits. This grouping is consistent with the Bayesian Inference tree (Fig. 2B). The concatenated total data set (*28S*, *per* and *COI*) (Fig. 5) shows a high level of incompatibility between the *L. cuprina* samples and a high degree of compatibility between the *L. sericata* samples. The *L. cuprina* samples show a number of splits and this incompatibility is probably as a result of the *L. sericata*-like mitochondrial DNA which results in the two clusters of *L. cuprina*.

Modern hybrids

The genetic component of an organism's morphology is determined by its nuclear DNA. One would expect recombination of the nuclear DNA if interbreeding occurs, resulting in morphology that is either intermediate (for multi-locus traits) or a mosaic of the two parental phenotypes (for single-locus traits). However, if one species' alleles are consistently dominant over the other, then despite recombination, the dominant phenotype will prevail (Lewin, 1997). Thus, although the putative modern hybrids had *sericata*-like mtDNA indicating hybridisation, they were still *L. cuprina*-like in morphology, suggesting that *L. cuprina*'s alleles for morphology are dominant over those of *L sericata*. In crossing experiments carried out in a laboratory, it was suggested that the femur colour of *L. cuprina* and the abdomen colour of *L. sericata* were dominant characteristics, giving the hybrids a combination of the two species' morphologies (Ullyett, 1945). However, this study used only two characters (femur and abdomen colour) which Ullyett (1945) described as not being "scientific criteria" because there are gradations in both characters depending on both the age and condition of the specimens and the observers' opinion and thus they could not be considered reliable criteria for identification.

Even when hybridization occurred in *Hyalomma* (Acari: Ixodidae), no intermediate morphologies were observed and the morphology of one parent appeared to be inherited over that of the other (Rees et al., 2003). Funk & Omland (2003) suggest that most hybrid species originate via asymmetrical hybridization and would be mitochondrially monophyletic. This might explain what we see in this study regarding the ancient hybridization "hybrid" group, but not the modern hybrids (which are derived from several sources). mtDNA may be more susceptible to introgression than nuclear loci (Machado & Hey, 2003). One is therefore less likely to have consistent gene trees for mtDNA and they may even suggest a different phylogeny. This gives support to the well-established idea that more than just one nuclear or

mitochondrial gene needs to be used when trying to determine species and gene trees (Funk & Omland, 2003; Machado & Hey, 2003; Hurst & Jiggins, 2005).

DNA-based identification

The use of *COI* sequences to correctly identify the two presumed subspecies of *L. cuprina* seems unlikely to succeed due to the presence of *L. cuprina* flies that group within the *L. sericata* clade (Fig. 2B). The phylogenetic positioning of these flies indicates their relationship relative to other specimens, but does not necessarily give an identification that agrees with their morphology. This problem is even more acute for modern hybrids. It also raises the issue of using *COI* as the universal "barcoding" gene and whether it is suitable, especially for insects (Rubinoff et al., 2006; Roe & Sperling, 2007; Whitworth et al., 2007; Jordaens et al., 2012; Sonet et al., 2012). The idea of using part of *COI* as a universal diagnostic gene is to allow the identification of unknown specimens when comparing them to identified species' sequences (Roe & Sperling, 2007). However, using *COI* alone could result in incorrect identifications, as seen in this study, as numerous insect species have undergone hybridisation and may carry mtDNA of another species (Zimmer, 2001; Baudry et al., 2003; Whitworth et al., 2007). The sequences of unidentified specimens may align with species with which they share mtDNA, but which are in fact a different species based on nuclear DNA or morphology. Although a study on blowflies in Australia suggested that using *COI* for identification is tenable, the authors also raised the issue of misidentifications when hybridisation was involved and suggested the use of a nuclear gene for confirmation (Nelson et al., 2007). A study of 1333 mitochondrial sequences (minimum of 300 bp) for 449 species of flies concluded that using *COI* alone for identification had a less than 70% success rate at identifying the species correctly (Meier et al., 2006).

The results show that in some cases both nuclear and mitochondrial genes are needed for reliable species identification and hybrid detection. It is well known that the use of just one gene can generally be taxonomically misleading as can be seen in the *L. sericata / L. cuprina* situation (Wallman et al., 2005; Harvey et al., 2008; Tourle et al., 2009; DeBry et al., 2010), especially if modern hybridisation is occurring at any appreciable rate. By using nuclear genes in conjunction with mitochondrial genes, a potentially misleading situation can be avoided (Rubinoff et al., 2006; Nelson et al., 2007; Roe & Sperling, 2007; Williams et al., 2008; Tantawi et al., 2010).

ACKNOWLEDGEMENTS. We thank F. Cronje, D. Emery, G. Goergen, C. Hänel, O. Harrison, C. Kelly, A. Kirk-Spriggs, N. Lunt, M. Mansell, R. Merrit, N. Mkize, H. Numata, C. Richards, K. Saigusa, K. Sink, K. Smith, K. Sukontason, J. Wallman, G. Whittington-Jones, and C. Wyss for providing specimens from different localities; O. Harrison for generating the locality map; J. Lamb (University of KwaZulu-Natal) for access to facilities; J. Vivian and T. Naidoo for laboratory assistance and advice; T. Pape and two anonymous referees for their valuable comments on the draft manuscript; and the Durban Natural Science Museum, the National Research Foundation of South Africa and Rhodes University for financial support. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Research Foundation.

REFERENCES

Altincicek B. & Vilcinskas A. 2009: Septic injury-inducible genes in medicinal maggots of the green blow fly *Lucilia sericata*. — *Insect Mol. Biol.* **18**: 119–125.

Aubertin D. 1933: Revision of the genus *Lucilia* R.-D. (Diptera, Calliphoridae). — *J. Linn. Soc. Lond. (Zool.)* **38**: 389–463.

Ballard J.W.O. 2000: When one in not enough: Introgression of mitochondrial DNA in *Drosophila*. — *Mol. Biol. Evol.* **17**: 1126–1130.

Baudry E., Bartos J., Emerson K., Whitworth T. & Werren H. 2003: *Wolbachia* and genetic variability in the birdnest blowfly *Protocalliphora sialia*. — *Mol. Ecol.* **12**: 1843–1854.

Cragg J.B. 1956: The olfactory behaviour of *Lucilia* species (Diptera) under natural conditions. — *Ann. Appl. Biol.* **44**: 467–477.

Crombe A.C. 1944: On the measurement and modification of the olfactory responses of blowflies. — *J. Exp. Biol.* **120**: 159–166.

DeBry R., Timm A.E., Dahlem G.A. & Stamper T. 2010: mtDNA-based identification of *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) in the continental United States. — *Forensic Sci. Int.* **202**: 102–109.

Farris J.S., Källersjö M., Kluge A.G. & Bult C. 1994: Testing significance of congruence. — *Cladistics* **10**: 315–319.

Funk D.J. & Omland K.E. 2003: Species-level paraphyly and polyphyly: Frequency, causes and consequences, with insights from animal mitochondrial DNA. — *Annu. Rev. Ecol. Evol. Syst.* **34**: 397–423.

Hall T.A. 1999: BioEdit: a user-friendly biological sequence alignment editor and analysis programme for Windows 95/98/NT. — *Nucl. Acid Symp. Ser.* **41**: 95–98.

Harvey M.L., Gaudieri S., Villet M.H. & Dadour I.R. 2008: A global study of forensically significant calliphorids: Implications for identification. — *Forensic Sci. Int.* **177**: 66–76.

Heath A.C.G. & Bishop D.M. 2006: Flystrike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). — *Vet. Parisitol.* **137**: 333–344.

Hepburn G.A. 1943: Sheep blowfly research I – A survey of maggot collections from live sheep and a note on the trapping of blowflies. — *Onderstepoort J. Vet.* **18**: 13–18.

Holloway B.A. 1991a: Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). — *N. Z. J. Zool.* **18**: 415–420.

Holloway B.A. 1991b: Identification of third-instar larvae of flystrike and carrion-associated blowflies in New Zealand (Diptera: Calliphoridae). — *N. Z. Entomol.* **14**: 24–28.

Hurst G.D.D. & Jiggins F.M. 2005: Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. — *Proc. R. Soc. (B)* **272**: 1525–1534.

Huson D.H. & Bryant D. 2006: Application of phylogenetic networks in evolutionary studies. — *Mol. Biol. Evol.* **23**: 254–267.

Jordaens K., Sonet G., Richet R., Dupont E., Braet Y. & Desmyter S. 2012: Identification of forensically important *Sarcophaga* species (Diptera: Sarcophagidae) using the mito-

chondrial *COI* gene. — *Int. J. Legal Med.* (in press) DOI: 10.1007/s00414-012-0767-6.

Kingu H.J.C., Kuria S.K., Villet M.H., Mkhize J.N., Dhaffala A. & Iisa J.M. 2012: Cutaneous myiasis: is *Lucilia cuprina* safe and acceptable for maggot debridement therapy? — *J. Cosmet. Dermatol. Sci. Appl.* **2**: 79–82.

Lewin B. 1997: *Genes VI*. Oxford University Press, New York, 1260 pp.

Louw S.v.d.M. & van der Linde T.C. 1993: Insects frequenting decomposing corpses in central South Africa. — *Afr. Entomol.* **1**: 265–269.

Machado C.A. & Hey J. 2003: The causes of phylogenetic conflict in a classic *Drosophila* species group. — *Proc. R. Soc. (B)* **270**: 1193–1202.

Meier R., Shiyang K., Vaidya G. & Ng P.K.L. 2006: DNA barcoding and taxonomy in Diptera: A tale of high intraspecific variability and low identification success. — *Syst. Biol.* **55**: 715–728.

Nelson L.A., Wallman J.F. & Dowton M. 2007: Using *COI* barcodes to identify forensically and medically important blowflies. — *Med. Vet. Entomol.* **21**: 44–52.

Nichols R. 2001: Gene trees and species trees are not the same. — *Trends Ecol. Evol.* **16**: 358–364.

Norris K.R. 1990: Evidence for the multiple exotic origin of Australian populations of the sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). — *Aust. J. Zool.* **38**: 635–648.

Nuin P. 2005: *MrMTgui 1.0 (Version 1.6)*. Program distributed by the author at http://www.genedrift.org/mtgui.php

Nylander J.A.A. 2004: *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Page R.D.M. & Charleston M.A. 1997: From gene to organismal phylogeny: reconciled trees and the gene tree/species tree problem. — *Mol. Phylogenet. Evol.* **7**: 231–240.

Paul A.G., Ahmad N.W., Lee H.L., Ariff A.M., Saranum M., Naicker A.S. & Osman Z. 2009: Maggot debridement therapy with *Lucilia cuprina*: a comparison with conventional debridement in diabetic foot ulcers. — *Int. Wound J.* **6**: 39–46.

Rees D.J., Dioli M. & Kirkendall L.R. 2003: Molecules and morphology: evidence for cryptic hybridization in African *Hyalomma* (Acari: Ixodidae). — *Mol. Phylogenet. Evol.* **27**: 131–142.

Rubinoff D., Cameron S. & Will K. 2006: A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. — *J. Hered.* **97**: 581–594.

Simon C., Buckley T.R., Frati F., Stewart J.B. & Beckenbach A.T. 2006: Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. — *Annu. Rev. Ecol. Evol. Syst.* **37**: 545–579.

Smith K.E. 1986: *A Manual of Forensic Entomology*. British National History Museum, London, 205 pp.

Sonet G., Jordaens K., Braet Y. & Desmyter S. 2012: Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic? — *Forensic Sci. Int.* **223**: 153–159.

Sperling F.A.H., Anderson G.S. & Hickey D.A. 1994: A DNA-based approach to the identification of insect species used for postmortem interval estimation. — *J. Forensic Sci.* **39**: 418–427.

Stevens J.R. 2003: The evolution of myiasis in blowflies (Calliphoridae). — *Int. J. Parasitol.* **33**: 1105–1113.

Stevens J. & Wall R. 1996: Species, sub-species and hybrid populations of the blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae) — *Proc. Biol. Sci.* **263**: 1335–1341.

Stevens J.R., Wall R. & Wells J.D. 2002: Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. — *Insect Mol. Biol.* **11**: 141–148.

Swofford D.L. 2003: *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, MA.

Tantawi T.I., Williams K.A. & Villet M.H. 2010: An accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. — *J. Med. Entomol.* **47**: 491–494.

Tourle R., Downie D.A. & Villet M.H. 2009: A morphological and molecular comparison of *Lucilia cuprina* and *L. sericata* (Diptera: Calliphoridae) in South Africa. — *Med. Vet. Entomol.* **23**: 6–14.

Ullyett G.C. 1945: Species of *Lucilia* attacking sheep in South Africa. — *Nature* **155**: 636–637.

Vilcinskas A. 2011: From traditional maggot therapy to modern biosurgery. In Vilcinskas A. (ed.): *Insect Biotechnology*. Springer, Dordrecht, pp. 67–75.

Vogt W.G. & Woodburn T.L. 1979: Ecology, distribution and importance of sheep myiasis flies in Australia. In: *National Symposium of the Sheep Blowfly and Flystrike in Sheep.* N.S.W. Dep. Agric., Sydney, pp. 23–32.

Wallman J.F., Leys R. & Hogendoorn K. 2005: Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. — *Invertebr. Syst.* **19**: 1–15.

Waterhouse D.F. & Paramonov S.J. 1950: The status of the two species of *Lucilia* (Diptera, Calliphoridae) attacking sheep in Australia. — *Aust. J. Sci. Res.* **3**: 310–336.

Wells J.D., Wall R. & Stevens J.R. 2007: Phylogenetic analysis of forensically important *Lucilia* flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. — *Int. J. Legal Med.* **121**: 229–233.

Whitworth T.L., Dawson R.D., Magalon H. & Baudry E. 2007: DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). — *Proc. R. Soc. (B)* **274**: 1731–1739.

Williams K.A. & Villet M.H. 2006: A new and earlier record of *Chrysomya megacephala* in South Africa, with notes on another exotic species, *Calliphora vicina* (Diptera: Calliphoridae). — *Afr. Invertebr.* **47**: 347–350.

Williams K.A., Cronje F.J., Avenant L. & Villet M.H. 2008: Identifying flies used for maggot debridement therapy. — *S. Afr. Med. J.* **98**: 196–197.

Zimmer C. 2001: *Wolbachia*: A tale of sex and survival. — *Science* **292**: 1093–1095.

Received May 31, 2012; revised and accepted November 19, 2012

ZooKeys 420: 69–85 (2014)
doi: 10.3897/zookeys.420.7645
www.zookeys.org

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

Morphological identification of *Lucilia sericata*, *Lucilia cuprina* and their hybrids (Diptera, Calliphoridae)

Kirstin A. Williams[1], Martin H. Villet[2]

1 *Entomology Department, Durban Natural Science Museum, Durban, South Africa* **2** *Southern African Forensic Entomology Research Laboratory, Department of Zoology and Entomology, Rhodes University, Grahamstown, 6140 South Africa*

Corresponding author: *Kirstin A. Williams* (Kirstin.Williams@durban.gov.za)

Academic editor: *P. Cerretti* | Received 8 April 2014 | Accepted 9 June 2014 | Published 25 June 2014

http://zoobank.org/A13A1EBC-6A83-4001-BC62-387C978E86A1

Citation: Williams KA, Villet MH (2014) Morphological identification of *Lucilia sericata*, *Lucilia cuprina* and their hybrids (Diptera, Calliphoridae). ZooKeys 420: 69–85. doi: 10.3897/zookeys.420.7645

Abstract

Hybrids of *Lucilia sericata* and *Lucilia cuprina* have been shown to exist in previous studies using molecular methods, but no study has shown explicitly that these hybrids can be identified morphologically. Published morphological characters used to identify *L. sericata* and *L. cuprina* were reviewed, and then scored and tested using specimens of both species and known hybrids. Ordination by multi-dimensional scaling indicated that the species were separable, and that hybrids resembled *L. cuprina*, whatever their origin. Discriminant function analysis of the characters successfully separated the specimens into three unambiguous groups – *L. sericata*, *L. cuprina* and hybrids. The hybrids were morphologically similar irrespective of whether they were from an ancient introgressed lineage or more modern. This is the first evidence that hybrids of these two species can be identified from their morphology. The usefulness of the morphological characters is also discussed and photographs of several characters are included to facilitate their assessment.

Keywords

Greenbottle blowflies, keys, morphology, discriminant analysis

Copyright *K.A.Williams, M.H.Villet.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Introduction

The use of maggot debridement therapy (MDT) in South Africa has gained interest in the past decade (Williams et al. 2008, Du Plessis and Pretorius 2011). The identification of the maggots used for this therapy remains an issue, as most medical doctors are not adequately trained in entomology to correctly identify the flies (Williams et al. 2008, Tantawi et al. 2010). *Lucilia sericata* is the most commonly used species (Sherman et al. 2000) but it is often misidentified as *L. cuprina*. These two species are also used in forensic entomology (Louw and van der Linde 1993, Smith and Wall 1997, Anderson 2000, Oliva 2001, Clark et al. 2006, Day and Wallman 2006) and *L. cuprina* is the species most often responsible for sheep strike – myiasis of sheep by the maggots of this fly (Hepburn 1943, Ullyett 1945, Vogt and Woodburn 1979, Heath and Bishop 2006), but *L. sericata* is responsible for sheep strike in northern Europe where *L. cuprina* is absent (Rose and Wall 2011). Correct identification of these flies is thus vitally important for these three fields.

Several identification keys have been produced either specifically for *L. sericata* and *L. cuprina*, or for larger suites of Luciliinae or Calliphoridae that included these two species (Waterhouse and Paramonov 1950, Rognes 1980, 1994, Dear 1986, Holloway 1991, Wallman 2001, Whitworth 2006, 2010), but several of the diagnostic characters are sometimes omitted while others are included that are less reliable or difficult to observe. Although both species occur worldwide, some of the differences between the character suites in these studies may arise from considering samples from relatively limited geographical regions. The first aim of this study was to consider the value of the published characters based on a sample of specimens from across the world.

A complicating factor is the known and widespread existence of natural hybrids of these species (Stevens et al. 2002, Wallman et al. 2005, Tourle et al. 2009, DeBry et al. 2010, Williams and Villet 2013), which has been established by molecular methods. Tourle et al. (2009) developed a semi-quantitative morphological index for discriminating *L. sericata* and *L. cuprina*, and it provides some evidence that their hybrids might also be morphologically distinguishable. Specifically, genetically identified hybrid specimens tended to show more extreme index values than either parent species. The index incorporated six characters: femur colour; the numbers of paravertical setulae, scutellar hairs and humeral hairs; the pattern of the postoccular microtrichial pile; the length of the sternal hairs of males; and the position of the inner vertical seta of females. The second aim of this study was to determine if hybrid specimens can in fact be determined from their morphology.

Materials and methods

Twenty-four specimens of *L. sericata*, *L. cuprina* and their hybrids (Table 1) were chosen from specimens that had been sequenced for 28S, COI and Per genes (Williams and Villet 2013). These specimens were chosen to include geographically diverse locations

Table 1. Specimens previously identified by molecular markers (Williams and Villet 2013) used in the morphological analyses. (*hybrids).

Species	Specimen	Country of origin
Lucilia cuprina	C_EGT_01	Egypt - Alexandria
Lucilia cuprina	C_SA_BFN_01	South Africa – Bloemfontein
Lucilia cuprina	C_SA_BFN_02	South Africa – Bloemfontein
Lucilia cuprina	C_SA_BRT_01	South Africa – Britstown
Lucilia cuprina	C_SA_BRT_02	South Africa – Britstown
Lucilia cuprina	C_SA_DBN_12	South Africa – Durban
**Lucilia cuprina*	C_SA_DBN_01	South Africa – Durban
**Lucilia cuprina*	C_SA_DBN_06	South Africa – Durban
**Lucilia cuprina*	C_SA_NEL_01	South Africa – Nelspruit
**Lucilia cuprina*	C_SA_NEL_02	South Africa – Nelspruit
**Lucilia cuprina*	C_THA_03	Thailand – Chiang Mai
**Lucilia cuprina*	C_ZIM_02	Zimbabwe – Matobos
Lucilia sericata	S_FRC_02	France – Montferrier-Sur-Lez
Lucilia sericata	S_GER_01	Germany – Kempen
Lucilia sericata	S_JPN_04	Japan – Iwate
Lucilia sericata	S_NAM_01	Namibia – Possession Island
Lucilia sericata	S_NAM_02	Namibia – Possession Island
Lucilia sericata	S_SA_CT_01	South Africa – Cape Town
Lucilia sericata	S_SA_CT_05	South Africa – Cape Town
Lucilia sericata	S_SA_GHT_01	South Africa – Grahamstown
Lucilia sericata	S_SA_GHT_02	South Africa – Grahamstown
Lucilia sericata	S_SA_PTA_02	South Africa – Pretoria
Lucilia sericata	S_SA_WTB_02	South Africa – Witbank
Lucilia sericata	S_USA_01	United States of America – Michigan

including Egypt, France, Germany, Japan, Namibia, South Africa, Thailand, the United States of America and Zimbabwe.

A total of 18 distinguishing morphological characteristics of adults of *L. sericata* and *L. cuprina* (Table 2) were obtained by reviewing several published sources (Waterhouse and Paramonov 1950, Rognes 1980, 1994, Dear 1986, Holloway 1991, Wallman 2001, Tourle et al. 2009, Whitworth 2006, 2010). Three characters referred to the male genitalia and three characters were specific to females. The males' characters could not be viewed without dissecting the specimens and because the majority of the genetically-identified specimens were female (Williams and Villet 2013), it was decided to include only females in the analysis. This reduced the number of characters to 15. Photographs of the specimens were taken using a Nikon D800 camera with a 105 mm lens and 124 mm extension to show several of the characters.

Each specimen was scored against the 15 characters (Table 2). Each character was then evaluated for its effectiveness in discriminating between the species and its practical value for identification, first univariately and qualitatively, and then multivariately and quantitatively using non-metric multi-dimensional scaling (MDS) in PAST3

Table 2. Published morphological characters used to distinguish specimens of *Lucilia sericata* and *L. cuprina*.

Character	Lucilia sericata	Lucilia cuprina	Analysis	
			MDS	DFA
General				
Number of paravertical setulae or occipital bristles (Waterhouse and Paramonov 1950, Dear 1986, Holloway 1991, Rognes 1994, Whitworth 2006, 2010)	Usually 2+2 but up to 8+8 (not always equal numbers i.e. can be 1+2 etc.)	1+1	yes	no
Shape of postocular microtrichial pile on vertex (viewed obliquely from behind) (Holloway 1991)	Boundary between pale and dark areas not straight or sharply defined	Boundary straight and sharply defined	no	no
Width of the frontal stripe (frontal vitta) (Waterhouse and Paramonov 1950, Rognes 1980, 1994)	Twice as wide as a parafrontal (fronto-orbital) plate	As wide as a parafrontal (fronto-orbital) plate	yes	yes
Colour of the frontoclypeal membrane (Waterhouse and Paramonov 1950, Wallman 2001)	Light brown	Dark brown to black	yes	yes
Second pair of presutural acrostichals (Waterhouse and Paramonov 1950)	Extend at least as far as insertions of the first pair of postsutural acrostichals	Do not extend to first pair of postsutural acrostichals	yes	no
Number of setulae on 'quadrat' between discal setae and anterior margin of scutellum (Holloway 1991)	35–55	15–25	yes	yes
Bristles on the scutellum (Waterhouse and Paramonov 1950)	Dorsal bristles distinctly smaller than lateral hairs	Dorsal bristles slightly smaller than or equal to lateral hairs	no	no
Number of hairs on the posterior slope of the humeral callus behind the basal setae (Waterhouse and Paramonov 1950, Rognes 1994, Whitworth 2006)	6 – 8	0–4	yes	yes
Number of hairs on the edge of the notopleuron behind the posterior notopleural seta (Waterhouse and Paramonov 1950, Rognes 1994, Whitworth 2006)	8–16	2 – 5	yes	yes
Metasternal area – sclerite midventrally between middle and hind coxae (Rognes 1994, Wallman 2001, Whitworth 2006)	Hairy	Bare	no	no
Colour of the fore femora (Waterhouse and Paramonov 1950, Dear 1986, Wallman 2001)	Dark metallic blue to black or dark brown	Metallic green	yes	yes
Contour of the last abdominal tergite (Waterhouse and Paramonov 1950)	Irregular depressions	Generally smooth	no	no
Females				
Distance between the outer and inner vertical setae of females (Holloway 1991)	Equal to 0.5–0.7 distance between prevertical and inner vertical setae	Equal to the distance between prevertical and inner vertical setae	yes	no

Character	Lucilia sericata	Lucilia cuprina	Analysis	
			MDS	DFA
Size of the angle formed by the inner vertical seta relative to the prevertical and outer vertical setae of females (Holloway 1991)	Obtuse	Right angle	yes	no
Extent of metallic sheen on parafrontal sclerites of females (Holloway 1991)	From vertex barely to base of upper orbital seta and not enclosing bases of any frontal setae	From vertex almost to base of lower orbital seta and enclosing bases of 1 or 2 frontal setae	yes	yes
Males				
Shape of apical halves of cerci (Waterhouse and Paramonov 1950, Holloway 1991)	Broad and tapering	Slender and parallel	no	no
Shape of apical halves of surstyli (Waterhouse and Paramonov 1950, Rognes 1980, Holloway 1991)	Curved and broad	Straight and slender	no	no
Form of apical setae of cerci (Holloway 1991)	Long and wavy	Minute and straight	no	no

(Hammer et al. 2001) using a Manhattan distance metric because of the mixed data forms in the character state matrix.

To explore the diagnosibility of the hybrids, a discriminant function analysis (DFA) was performed using PAST3 (Hammer et al. 2001) on the scored character matrix to determine which characters were most influential in identifying the species. Four of the 15 characters (shape of postocular microtrichial pile, hairiness of metasternal area, contour of the last abdominal tergite, bristles on the scutellum; Table 2) were either not easily visible or the hairs were broken or missing in at least half of the specimens and were therefore excluded from the DFA. Another four of the characters showed no variation within species and therefore had to be excluded from the DFA, which therefore included only seven characters (Table 2). The hybrid specimens were treated as a separate group in this analysis, but the introgressed and modern hybrids were not separated.

Results

Univariate assessment of characters

The number of paravertical setulae or occipital bristles (Table 2; Figure 1). This character was relatively consistent and reliable, but it is not easily viewed and scored if the specimens have been kept in ethanol. The hybrid specimens all keyed out as *L. cuprina*. This character was left out of the DFA analysis due to lack of variation within *L. cuprina*.

The shape of the postocular microtrichial pile on the vertex (Table 2) (Holloway 1991) is a difficult character to see when the specimens have been stored in ethanol because the microtrichia are not visible unless the specimen is dry, and even then the microtrichia sometimes appear to be absent. Due to the difficulty in viewing and scoring this character, it was eventually left out of all further analyses.

The relative positions of the three vertical setae (Table 2; Figure 1) that form a triangle on either side of the ocellar triangle in females (Holloway 1991) is a reliable character that consistently separated the two species. This character was excluded from the DFA because it did not show variation within taxa but was included in the MDS analysis. The hybrid specimens consistently keyed out as *L. cuprina*.

The angle formed by the three vertical setae (Table 2; Figure 1). This character is consistent and easily seen even if the setae have fallen out as they have sockets, which are easily visible. Due to lack of variation within species and the hybrids being identified as *L. cuprina*, this character was also excluded from the discriminant function analysis but it was included in the MDS analysis.

The extent of the metallic sheen on the parafrontal sclerites of females (Table 2 and Suppl. material 1; Figure 1). This character is easier to observe in dried specimens than ethanol-preserved specimens and there is some variation. The division between the two species is not absolute – there is some overlap within this character but it was not specific to the hybrids. It was included in both the DFA and MDS analyses.

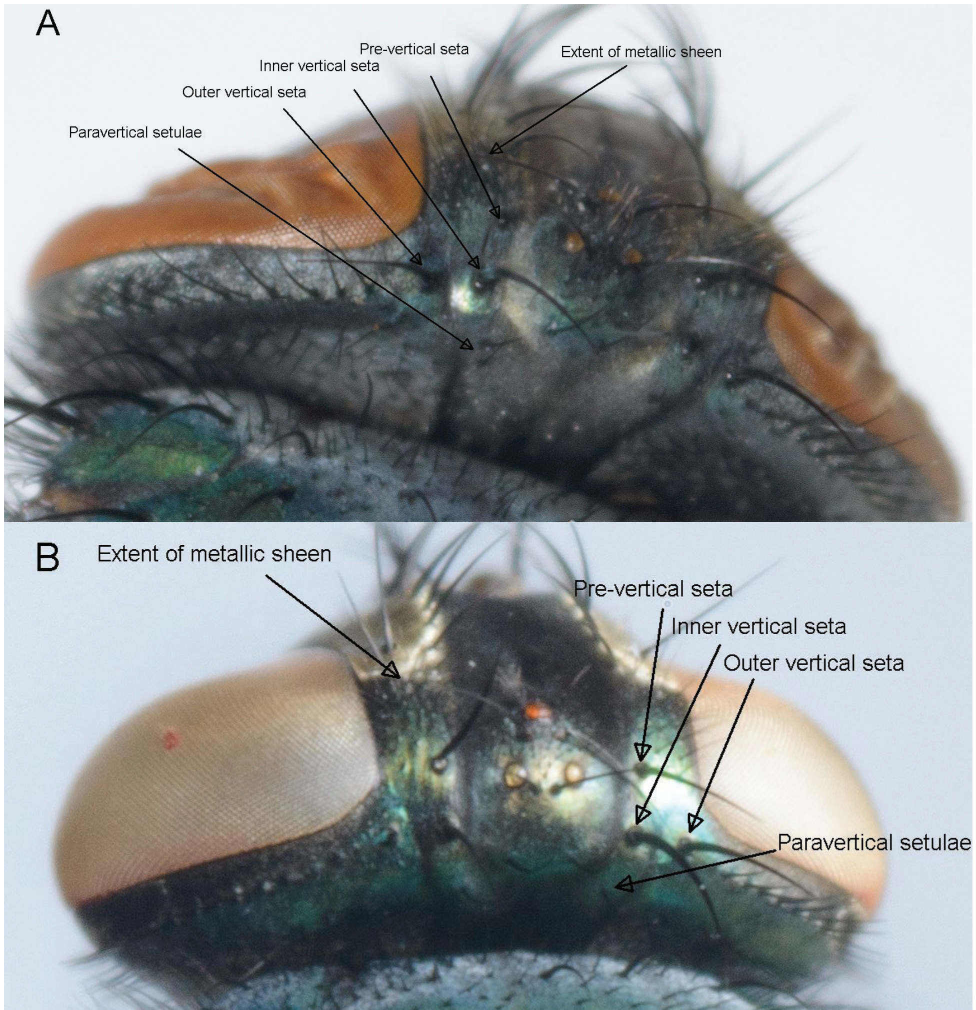

Figure 1. Paravertical setulae, distance between the outer and inner vertical setae, the size of the angle at the inner vertical triangle and extent of metallic sheen on parafrontal sclerites. *L. sericata* (**A**) and *L. cuprina* (**B**).

The relative width of the frontal stripe (frontal vitta) (Table 2 and Suppl. material 1; Figure 2). Waterhouse and Paramonov (1950) suggested that this character was more reliable in males than females. We found that the width varied from being equal to the parafrontal to being more than twice the width in both species. The hybrids were not distinguishable from *L. cuprina*. This character was included in the MDS and the DFA analyses.

The colour of the frontoclypeal membrane (Table 2 and Suppl. material 1; Figure 3). It was not always easily visible if the proboscis was not extended but it could usually be viewed by either manipulating the proboscis or viewing the specimen from

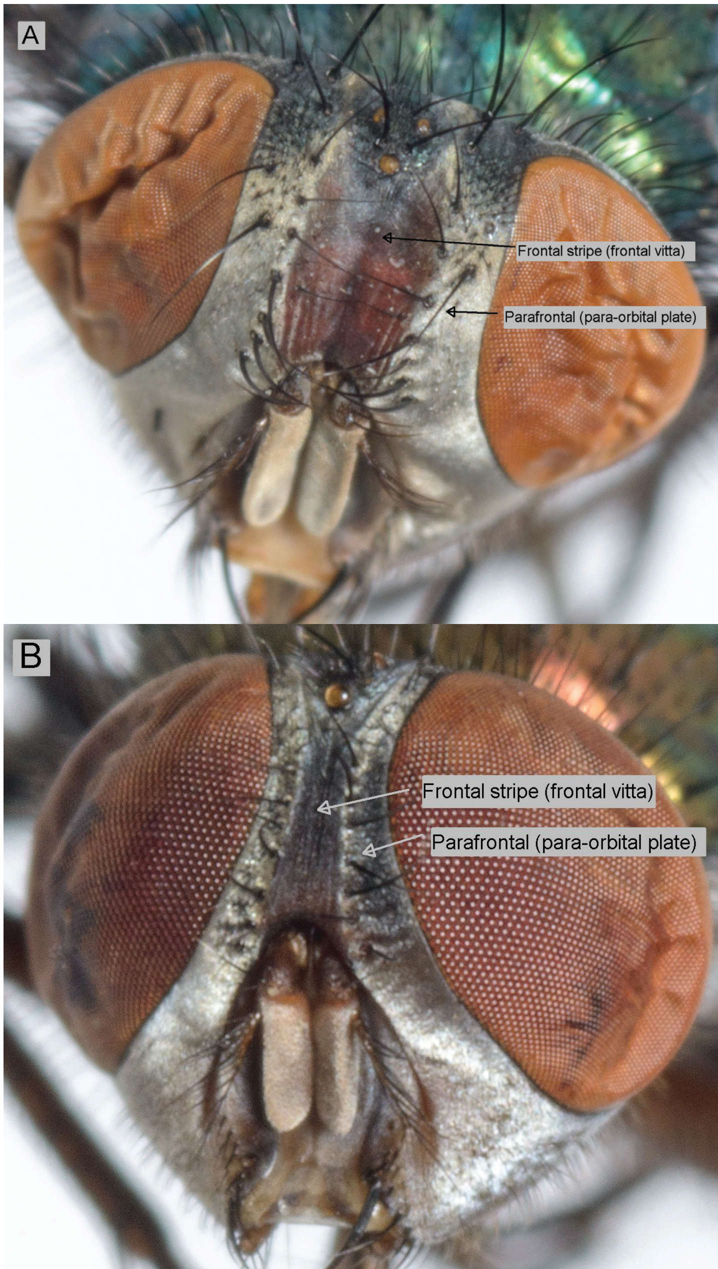

Figure 2. Frontal stripe – *L. sericata* (**A**) and *L. cuprina* (**B**).

a lateral angle (Waterhouse and Paramonov 1950). The hybrid specimens were not distinct from *L. sericata* or *L. cuprina*.

The length of the second pair of presutural acrostichals (Table 2) is a character that is easier to see in well-preserved specimens (Waterhouse and Paramonov 1950).

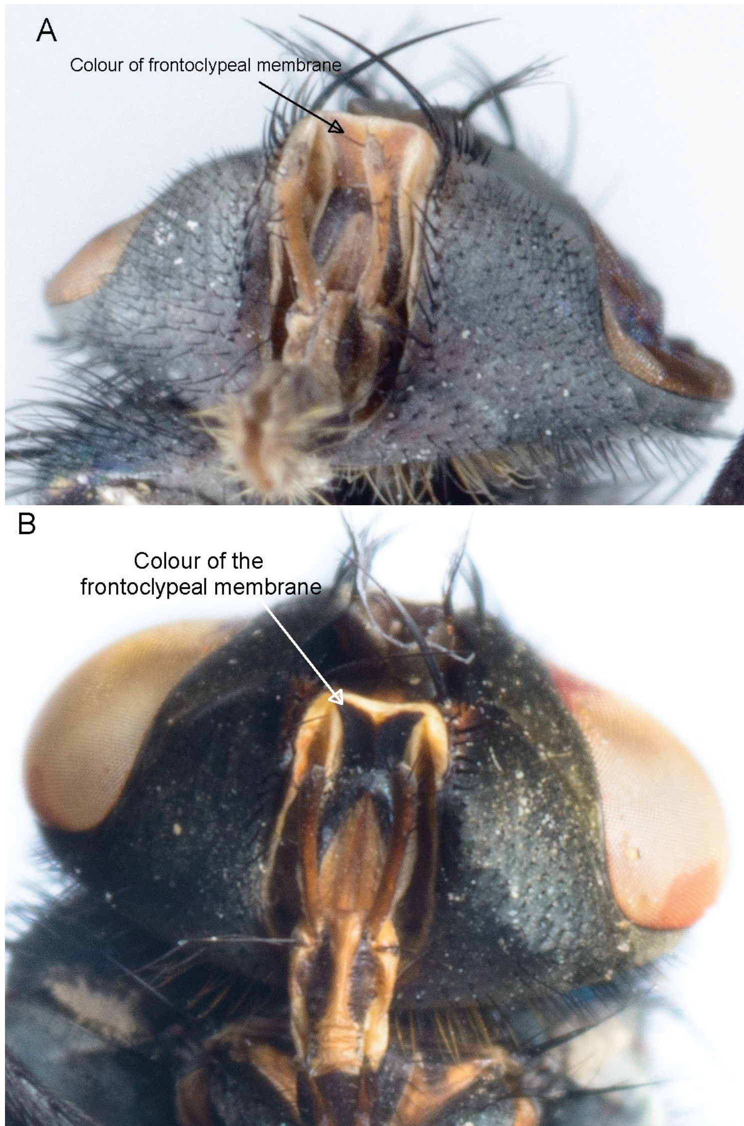

Figure 3. Colour of the frontoclypeal membrane. *L. sericata* (**A**) and *L. cuprina* (**B**).

This character is not scorable if the bristles are broken or have fallen out. It was left out of the analyses because it does not show any intraspecies variation.

The number of setae on the scutellum (Table 2 and Suppl. material 1; Figure 4) in the 'quadrat' demarcated by the discal setae and the anterior margin of the scutellum represents the axis in the discriminant analysis that separated *L. sericata* and *L. cuprina* (Holloway 1991). This character can be used even when the setae have fallen out because they have sockets that are visible and can be counted. There was overlap in the number of setae between the two species, but generally *L. cuprina* had obviously fewer

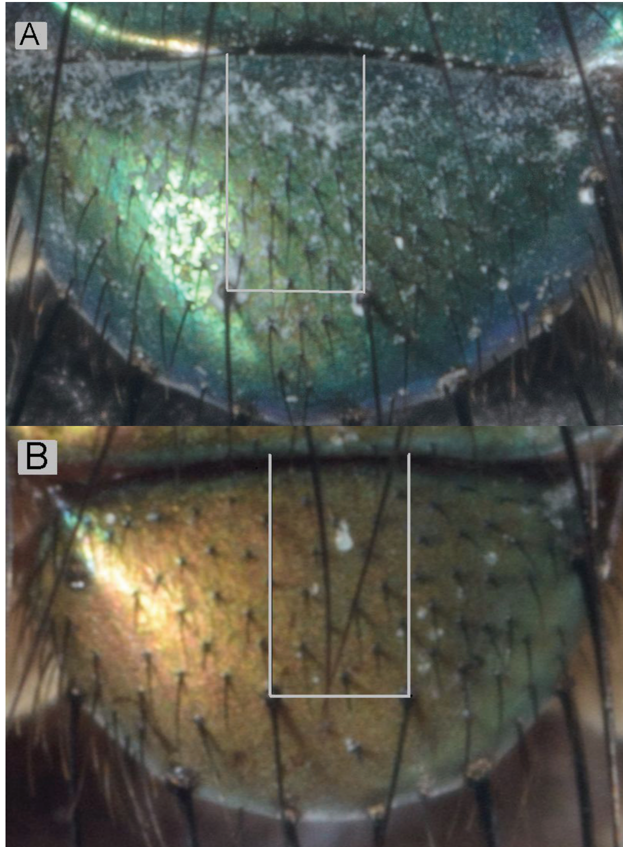

Figure 4. Number of setae on 'quadrat' between the anterior margin and discal setae on the scutellum. *L. sericata* (**A**) and *L. cuprina* (**B**).

setae. The number of setae in the hybrids was not obviously different from either of the pure species. This overlap may be as a result of the challenge of counting the setae as they are not in straight rows.

The length of the bristles on the scutellum (Table 2 and Suppl. material 1) describes the length of the hairs between the two anterior bristles on the lateral margin of the scutellum in relation to the length of the hairs on the dorsal surface of the scutellum (Waterhouse and Paramonov 1950). This character was not easy to use as the hairs were broken or had fallen out in half of the specimens and therefore it was left out of the analyses.

The hairiness of the posterior slope of the humeral callus (Table 2 and Suppl. material 1; Figure 5) behind the basal setae is a reliable character in separating *L. sericata* and *L. cuprina* even though there is variation within species in the number of hairs. The hybrids tended to have more hairs than the pure *L. cuprina* specimens, but there was still overlap in the numbers of hairs between the hybrids and pure *L. cuprina*.

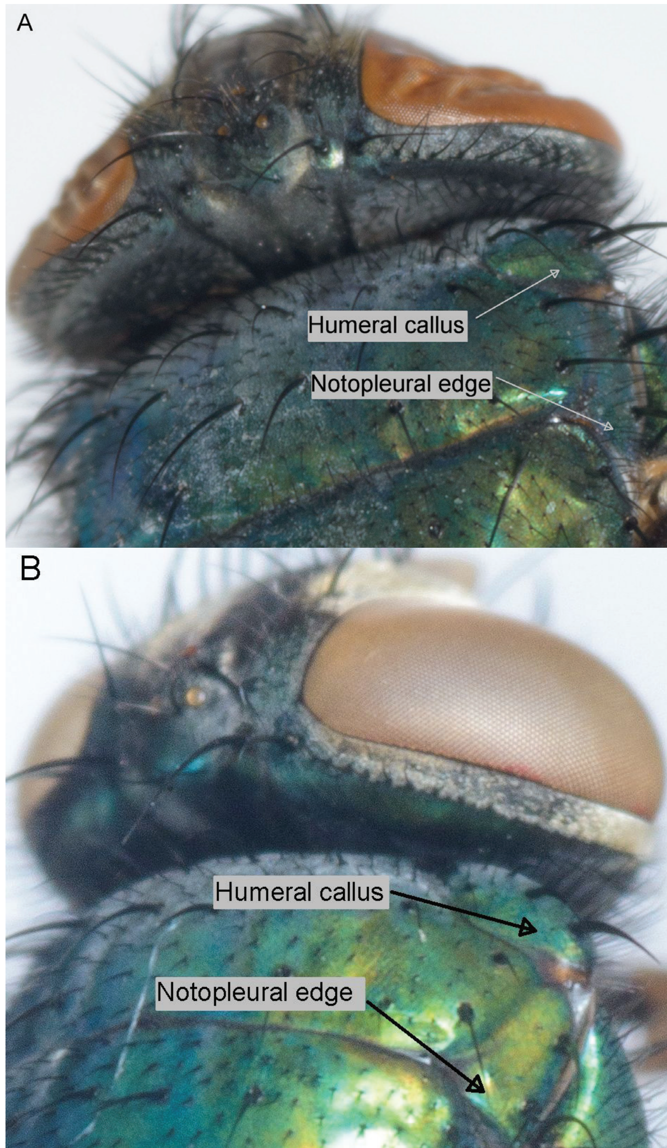

Figure 5. Posterior slope of the humeral callus behind the basal setae and the posterior edge of notopleuron behind the posterior notopleural seta. *L. sericata* (**A**) and *L. cuprina* (**B**).

The number of hairs on the edge of the notopleuron (Table 2 and Suppl. material 1; Figure 5). Both the hairs on the notopleuron and the humeral callus are relatively easy to observe although ethanol-preserved specimens need to be dried so that the small hairs are visible. It is another reliable character in separating *L. sericata* from *L. cuprina* despite variation in the number of hairs within species. The hybrids showed no discernable difference in numbers of hairs from *L. cuprina*.

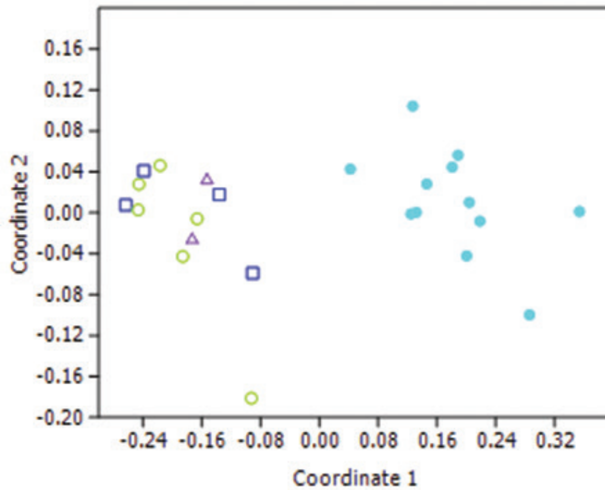

Figure 6. Non-metric Multi-Dimensional Scaling plot using a Manhattan distance metric using 11 characters. Light blue solid circles = *L. sericata*, Green open circles = *L. cuprina*, dark blue squares = introgressed hybrids, purple triangles = modern hybrids.

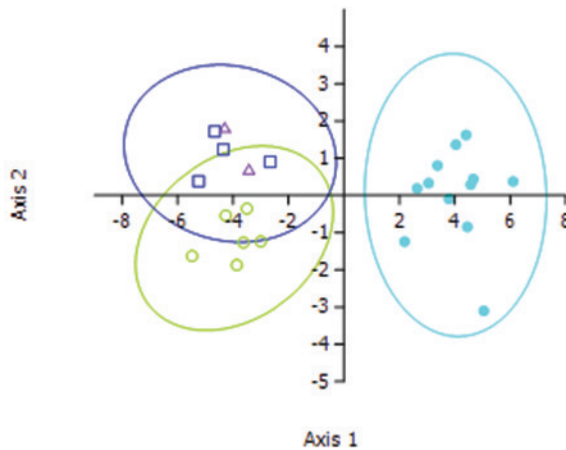

Figure 7. Ordination plot of the first two roots of the discriminant function analysis using seven characters. Ellipses represent 95% confidence regions. Light blue solid circles = *L. sericata*, Green open circles = *L. cuprina*, dark blue squares = introgressed hybrids, purple triangles = modern hybrids.

The hairs on the metasternal area (Table 2), which is the sclerite mid-ventrally between the middle and hind coxae, are exceedingly difficult to view if the legs are not set appropriately to facilitate this. . All of the specimens that we examined were preserved in ethanol and it was not easy to view the metasternal area and this character was therefore not analysed.

The colour of the fore femora (Table 2 and Suppl. material 1) has long been used as a character to identify *L. sericata* and *L. cuprina* (Ullyett 1945). It is a controversial

Table 3. Eigen vectors and values for the first two roots of the discriminant function analysis.

Character	Root 1	Root 2
Number of setulae on 'quadrat' demarcated by discal setae and anterior margin of scutellum	**1.5822**	0.0324
Number of hairs on edge of notopleuron behind posterior notopleural seta	0.5576	0.3300
Number of hairs on posterior slope of humeral callus behind basal setae	0.4216	**0.9066**
Colour of fore femora	0.2591	-0.2023
Relative width of frontal stripe (frontal vitta)	0.1551	0.0104
Extent of metallic sheen on parafrontal sclerites of females	0.0519	-0.0697
Colour of frontoclypeal membrane	-0.1551	-0.0104
Eigenvalue	18.5560	0.7406

character as it varies according to when the flies were killed, if the adults were fully matured and if the specimens were fouled or not during collection and thus is subject to personal interpretation. The hybrids keyed out as *L. cuprina*. Due to the variation in this character it was included in the DFA.

The contour of the last abdominal tergite (Table 2) is applicable only to dried specimens (Waterhouse and Paramonov 1950) as it relies on the hardness of the tergite. It was therefore not a character that could be used in our analyses as all our specimens were ethanol-preserved. It was excluded from the analyses and is probably unreliable even in dried specimens because it relies on the preservation of the specimen and how it is pinned, which affects the contour of the last abdominal tergite.

Multivariate assessments of characters

Superficially, the hybrid specimens were identified as *L. cuprina* when keyed out using any of the published keys. There were no obvious differences in the morphology of the hybrids. When the characters were analysed using MDS, the hybrid specimens were not separated from the *L. cuprina* specimens (Figure 6).

However, the ordination plot of the DFA (Figure 7) clearly shows three groups – *L. sericata*, *L. cuprina* and hybrids. The most influential characters were the number of setae on the scutellum (Root 1) and the number of hairs on the humeral callus (Root 2) (Table 3). It is not obvious in the morphology that there is a difference between the pure and hybrid strains, but statistically one can separate the hybrids from the pure *L. cuprina* specimens.

Discussion

Assessment of characters

Due to the greater number of female flies in the molecular study from which we chose our specimens, we did not include any males. Therefore the male genitalia characters

are not discussed in detail. It is not possible to properly view the male genitalia without dissecting them and this is not ideal for non-entomologists such as medical doctors who are using these flies for MDT as one needs experience to dissect out the genitalia. It is possible to correctly identify these flies without using the male genitalia by using the other characters described in Table 2.

Geographical variation

Holloway (1991) suggested that the characters that she described were specifically for *L. sericata* and *L. cuprina* from New Zealand and that they might not apply to specimens from other parts of the world. This does not seem to be the case, as the flies examined in this study are from several different countries around the world (Table 1) and the characters described (excluding the male genitalia) were useful in identifying these two species and their hybrids.

Identifying hybrids

The DFA unambiguously separated the *L. cuprina* specimens from the hybrids and it was statistically significant. This was not noted in previous studies where hybrids were identified only through molecular techniques (Stevens et al. 2002, Wallman et al. 2005, Tourle et al. 2009, DeBry et al. 2010, Williams and Villet 2013). Examination of the number of hairs on the scutellum, humeral callus and notopleuron show a consistent difference that separates these groups. The first two characters were included in the morphological index designed by Tourle et al (2009), which explains the trend found in their results.

The introgressed and modern hybrids were not separated in the DFA ordination plot (Fig. 6).

Conclusion

Introgressed and modern hybrids of *L. sericata* and *L. cuprina* can be statistically recognized using the characters described in this paper.

Four of the characters were consistently successful at separating *L. sericata* and *L. cuprina* (number of paravertical setulae or occipital bristles, distance between the outer and inner vertical setae of females, size of the angle at the inner vertical in triangle joining pre-, outer and inner vertical setae of females, second pair of presutural acrostichals) with little variation within the characters. The number of setae on the scutellum and the number of hairs on the humeral callus and notopleuron are also useful characters although they did show variation within species. It is advisable to use a combination of several characters to identify these two species as no single character was sufficient to separate *L. sericata* and *L. cuprina*.

Acknowledgments

We thank Georg Goergen, Ashley Kirk-Spriggs, Nicky Lunt, Mervyn Mansell, Hideharu Numata, Cameron Richards, Kiyoshi Saigusa, Kabkaew Sukontason, Tarek Tantawi and Robyn Tourle for providing us with specimens, Emil von Maltitz for taking photographs. Funding was provided by the National Research Foundation (NRF) of South Africa. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Research Foundation.

References

Anderson GS (2000) Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). Journal of Forensic Sciences 45: 824–832.

Clark K, Evans L, Wall R (2006) Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. Forensic Science International 156: 145–149. doi: 10.1016/j.forsciint.2004.12.025

Day DM, Wallman JF (2006) Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae). Journal of Forensic Sciences 51: 657–663. doi: 10.1111/j.1556-4029.2006.00127.x

DeBry R, Timm AE, Dahlem GA, Stamper T (2010) mtDNA-based identification of *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) in the continental United States. Forensic Science International 202: 102–109. doi: 10.1016/j.forsciint.2010.04.038

Dear JP (1986) Calliphoridae (Insecta: Diptera). Fauna of New Zealand 8: 1–86.

Du Plessis HJC, Pretorius JP (2011) The utilisation of maggot debridement therapy in Pretoria, South Africa. Wound Healing Southern Africa 4: 80–83.

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4: 9 pp.

Heath ACG, Bishop DM (2006) Flystrike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). Veterinary Parasitology 137: 333–344. doi: 10.1016/j.vetpar.2006.01.006

Hepburn GA (1943) Sheep blowfly research I - A survey of maggot collections from live sheep and a note on the trapping of blowflies. Onderstepoort Journal of Veterinary Science and Animal Industry 18: 13–18.

Holloway BA (1991) Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). New Zealand Journal of Zoology 18: 415–420. doi: 10.1080/03014223.1991.10422847

Louw S v.d.M, van der Linde TC (1993) Insects frequenting decomposing corpses in central South Africa. African Entomology 1: 265–269.

Oliva A (2001) Insects of forensic significance in Argentina. Forensic Science International 120: 145–154. doi: 10.1016/S0379-0738(01)00423-6

Rognes K (1980) The blow-fly genus *Lucilia* Robineau-Desvoidy (Diptera, Calliphoridae) in Norway. Fauna Norvegica Series B 27: 39–52.

Rognes K (1994) First record of the sheep greenbottle fly *Lucilia cuprina* (Wiedemann, 1830) from Europe (Diptera: Calliphoridae) with additional Spanish records of Calliphoridae, Muscidae and Sarcophagidae. Eos 69: 41–44.

Rose H, Wall R (2011) Modelling the impact of climate change on spatial patterns of disease risk: Sheep blowfly strike by *Lucilia sericata* in Great Britain. International Journal of Parasitology 41: 739–746. doi: 10.1016/j.ijpara.2011.01.012

Sherman RA, Hall MJR, Thomas S (2000) Medicinal maggots: an ancient remedy for some contemporary afflictions. Annual Review of Entomology 45: 55–81. doi: 10.1146/annurev.ento.45.1.55

Smith KE, Wall R (1997) The use of carrion as breeding site by the blowfly *Lucilia sericata* and other Calliphoridae. Medical and Veterinary Entomology 11: 38–44. doi: 10.1111/j.1365-2915.1997.tb00287.x

Stevens JR, Wall R, Wells JD (2002) Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. Insect Molecular Biology 11: 141–148. doi: 10.1046/j.1365-2583.2002.00318.x

Tantawi TI, Williams KA, Villet MH (2010) An Accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. Journal of Medical Entomology 47: 491–494. doi: 10.1603/ME09183

Tourle RA, Downie DA, Villet MH (2009) A morphological and molecular comparison of *Lucilia cuprina* and *L. sericata* (Diptera: Calliphoridae) in South Africa. Medical and Veterinary Entomology 23: 6–14. doi: 10.1111/j.1365-2915.2008.00765.x

Ullyett GC (1945) Species of *Lucilia* attacking sheep in South Africa. Nature 155: 636–637. doi: 10.1038/155636b0

Vogt WG, Woodburn TL (1979) Ecology, distribution and importance of sheep myiasis flies in Australia. National Symposium of the Sheep Blowfly and Flystrike in Sheep. New South Wales Department of Agriculture Sydney, Australia, 23–32.

Wallman JF (2001) A key to the adults of species of blowflies in southern Australia known or suspected to bred in carrion [corrigendum in Medical and Veterinary Entomology 16, 223]. Medical and Veterinary Entomology 15: 433–437. doi: 10.1046/j.0269-283x.2001.00331.x

Wallman JF, Leys R, Hogendoorn K (2005) Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. Invertebrate Systematics 19: 1–15. doi: 10.1071/IS04023

Waterhouse DF, Paramonov SJ (1950) The status of the two species of *Lucilia* (Diptera, Calliphoridae) attacking sheep in Australia. Australian Journal of Scientific Research 3: 310–336.

Whitworth T (2006) Keys to the genera and species of blow flies (Diptera: Calliphoridae) of America north of Mexico. Proceedings of the Entomological Society of Washington 108: 689–725.

Whitworth T (2010) Keys to the genera and species of blow flies (Diptera: Calliphoridae) of the West Indies and description of a new species of *Lucilia* Robineau-Desvoidy. Zootaxa 2663: 1–35.

Williams KA, Cronje FJ, Avenant L, Villet MH (2008) Identifying flies used for maggot debridement therapy. South African Medical Journal 98: 196–197.

Williams KA, Villet MH (2013) Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae). European Journal of Entomology 110: 187–196. doi: 10.14411/eje.2013.029

Supplementary material 1

Character-taxon matrix

Kirstin A. Williams, Martin H. Villet

Data type: Species data

Explanation note: Character-taxon matrix used in the MDS and DFA analyses

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: doi: 10.3897/zookeys.420.7645.app1

African Invertebrates Vol. 55 (1): 157–170 Pietermaritzburg 30 June 2014

Predicting the geographic distribution of *Lucilia sericata* and *Lucilia cuprina* (Diptera: Calliphoridae) in South Africa

K.A. Williams[1,2*], **C.S. Richards**[3] and **M.H. Villet**[2]

[1] Department of Entomology, Durban Natural Science Museum, P.O. Box 4085, Durban, 4000 South Africa; Kirstin.Williams@durban.gov.za
[2] Southern African Forensic Entomology Research Laboratory, Department of Zoology and Entomology, Rhodes University, P.O. Box 94, Grahamstown, 6140 South Africa; M.Villet@ru.ac.za
[3] Agriprotein Technologies, Poultry, Mariendahl Research Farm, 7607 South Africa; Cameron@agriprotein.com
*Corresponding author

ABSTRACT

Lucilia sericata (Meigen, 1826) and *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae: Luciliinae) have medical, veterinary and forensic importance. Knowing their distribution in South Africa would allow more effective management and utilisation of these flies. Their predicted geographic distributions in South Africa were modelled using maximum entropy analysis of selected climatic variables. The most important environmental variables in modelling the distributions were the magnitude of monthly rainfall and the magnitude of the monthly maximum temperature for *L. sericata* and the seasonal variation in monthly mean humidity and magnitude of monthly rainfall for *L. cuprina*. A clear geographical bias was shown in museum records and supports the need for focused surveys. There was no correlation between the predicted distribution of *L. cuprina* and sheep farming in South Africa, nor between the predicted distribution of *L. sericata* and human population density. Although their patterns of occurrence differed, both species are widely distributed in South Africa and therefore one cannot identify these flies by locality alone – morphological or molecular identification is necessary.

KEY WORDS: Calliphoridae, *Lucilia cuprina*, *Lucilia sericata*, blowflies, environmental variables, MaxEnt, modelling.

INTRODUCTION

Lucilia sericata (Meigen, 1826) is a cosmopolitan greenbottle blowfly that originates from Europe and which is used in maggot debridement therapy (MDT) – the use of maggots to clean necrotic wounds on living human beings (Sherman *et al*. 2000; Wolff & Hansson 2005; Williams *et al*. 2008; Altincicek & Vilcinskas 2009; Paul *et al*. 2009; Tantawi *et al*. 2010). The maggots are also important in forensic entomology (Louw & van der Linde 1993; Smith & Wall 1997; Anderson 2000; Oliva 2001; Clark *et al*. 2006; Day & Wallman 2006) and, to a lesser extent, in sheep strike – the process whereby these flies lay their eggs on living sheep and the maggots damage the wool and skin by feeding on the sheep (Hepburn 1943; Ullyett 1945; Vogt & Woodburn 1979; Heath & Bishop 2006). It has been suggested that in South Africa, *L. sericata* occurs in urban areas and is not found in rural settings (Meskin 1986; Braack & de Vos 1987). *Lucilia cuprina* (Wiedemann, 1830), its sister species, is indigenous to Africa and Asia. It is a huge problem in sheep strike (Hepburn 1943; Ullyett, 1945; Vogt & Woodburn 1979; Heath & Bishop 2006), has been successfully used in MDT (Paul *et al*. 2009; Tantawi *et al*. 2010), and is useful in forensic investigations (Louw & van der Linde 1993; Day & Wallman 2006). It is thought that in South Africa, this species occurs primarily in rural environments and seldom near human habitation (Meskin 1986; Braack & de Vos 1987). Both species have the potential to spread disease because they breed in decaying and rotting organic matter (Zumpt & Patterson 1952).

http://africaninvertebrates.org
urn:lsid:zoobank.org:pub:F8A75272-90DD-4963-9EC2-BF0040012C2F

To understand these flies for forensic investigations and veterinary research, one needs to know where they occur, both locally and country-wide. Knowing their geographic distribution would also assist in developing strategies to control fly strike in sheep-farming areas. Mapping the distribution of *L. sericata* is complicated by it being an introduced species to the country, and that it may still be in the process of spreading to the limits of its potential distribution. In this situation, old maps need to be updated with new distribution records. Climate change creates a greater challenge for understanding both species because changing conditions at a locality may alter its suitability for either species, so that old historical locality records eventually need to be revised.

Species distribution modelling techniques produce maps of the potential distribution of species (Elith & Leathwick 2009). MaxEnt (Phillips *et al*. 2006; Phillips & Dudík 2008) is a predictive biogeography programme that uses a maximum entropy algorithm to match known locality points for a species to potential localities, based on their environmental characteristics. It is a useful technique because it does not require absence records to build a predictive model. This allows one to use museum and other occurrence records without having to do fieldwork to provide absence records, which is costly, time-consuming and often ambiguous in outcome (Phillips & Dudík 2008).

In this paper, we present models of predicted geographic distributions for *L. sericata* and *L. cuprina* in South Africa and discuss the environmental variables highlighted by these models. The correlations between where these species are predicted to occur and the distribution of sheep farming and human settlements are also examined.

MATERIAL AND METHODS

Locality records

Historical occurrence records for *Lucilia sericata* and *Lucilia cuprina* were obtained from the following museums: KwaZulu-Natal Museum (formerly the Natal Museum, Pietermaritzburg); Albany Museum (Grahamstown); Iziko Museum (formerly the South African Museum, Cape Town); Durban Natural Science Museum (Durban) and the National Museum (Bloemfontein). The identifications of the specimens were confirmed by the authors. Indeterminable specimens were excluded. Ten additional records with co-ordinates were obtained from literature surveys (Braack 1986; Braack & de Vos 1987; Louw & van der Linde 1993).

Current occurrence records were obtained from personal contacts (see acknowledgements) and from five collecting surveys, undertaken following the literature survey and after museum records had been obtained, to collect data from poorly-sampled and under-represented areas of the country. Traps were set at ~50 km intervals along the chosen route (Fig. 1A & B) and left for four days. They were placed in rural areas along the roadside, at least 2 km away from towns and out of sight of human settlements. Field trips were conducted year-round except during winter (May to August), when blowfly numbers are known to be low (Williams 2002). Redtop™ fly traps (Miller Methods, Ltd) were modified by removing the base of the traps and attaching screw-top jars containing fresh chicken liver to them (Fig. 2). The centres of the jars' lids were cut out and the mouth of the jars covered by netting. The jars were then screwed on to the plastic base of the traps with the lids. This allowed flies to detect the odour of the bait and enter the trap, but prevented them from getting to the bait and thus becoming too fouled to identify. The flies were therefore confined to the bag of the trap and were easily removed.

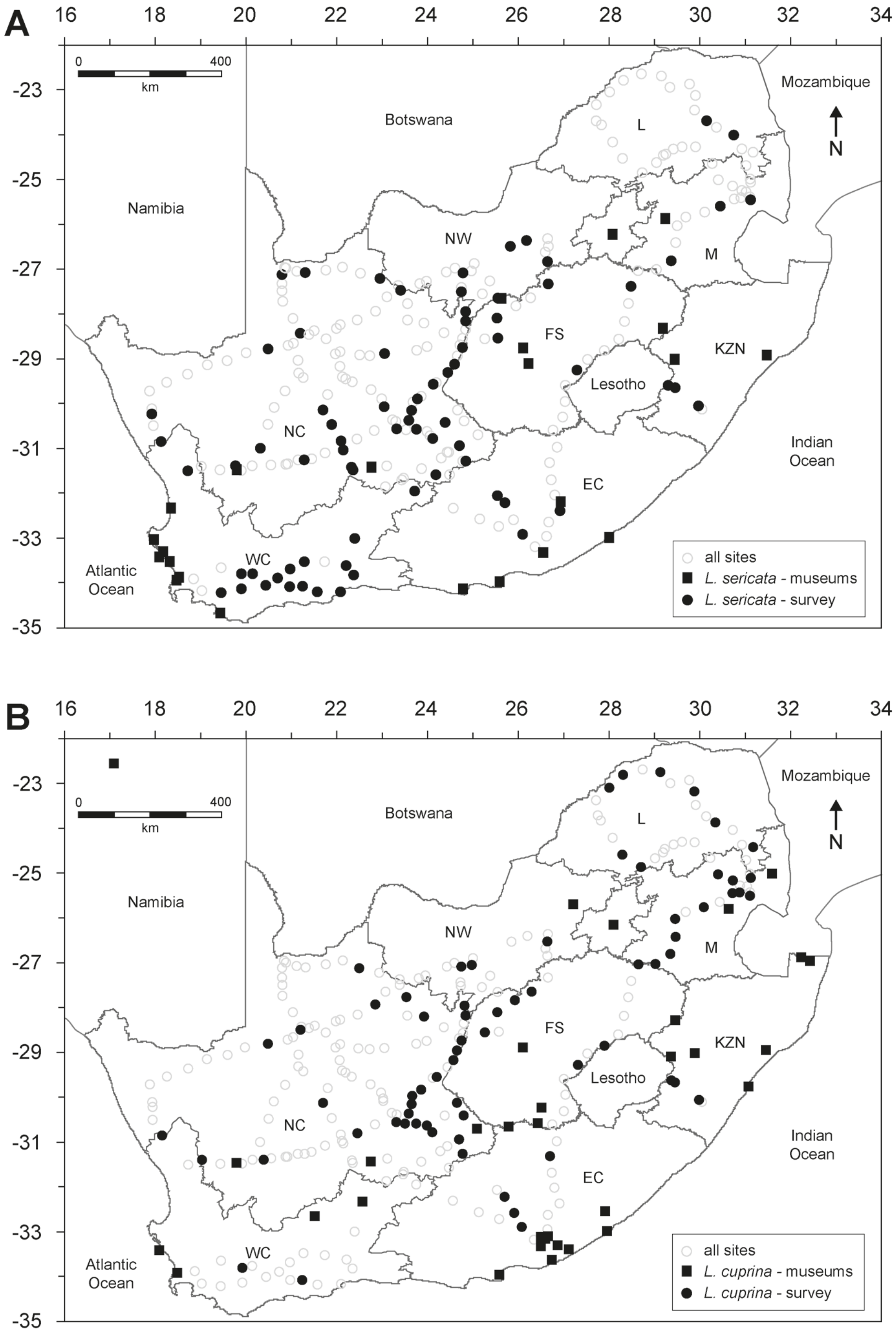

Fig. 1. Map of South Africa, showing the occurrence records and collection trip routes for (A) *Lucilia sericata* and (B) *Lucilia cuprina*.

Fig. 2. Modified Red-top™ fly trap used in surveys.

A total of 132 records (60 collection and 72 survey records) were obtained for *L. cuprina* and 120 (39 collection and 81 survey records) for *L. sericata.* There were several survey sites where both or either species were not recorded (Fig. 1A & B). No museum or literature records more than 50 years old were used for the analysis, as these are not within the temporal span of the climatic variables used in this study (Schulze *et al*. 1997). Duplicated site records were not taken into account so as to prevent pseudoreplication.

Environmental variables

Eleven climatic predictor variables were selected for building the models. These represented variables that are regarded as appropriate to ectotherms at global and regional scales (Mackey & Lindenmayer 2001; Phillips 2008; Richards *et al*. 2009). No vegetation variables were included because these flies are habitat generalists and do not require a particular type of vegetation to breed, although they probably do not occur in forests. It was anticipated that climatic variables would have the greatest abiotic influence on their distribution (Meskin 1986; Braack & de Vos 1987; Williams 2002). Digital maps

TABLE 1
Mean area-under-curve (AUC) values for whole models.

	AUC	
	Lucilia sericata	*Lucilia cuprina*
Training data	0.78	0.77
Test data	0.69	0.69
Standard deviation	0.0489	0.0471

of the variables for South Africa were developed by Schulze *et al*. (1997) to produce continuous digital maps at a resolution of 60 pixels per degree (i.e. about 1.6 x 1.6 km) by interpolating from point data obtained from a network of weather stations throughout South Africa and averaged over 10 years (Schulze *et al*. 1997). Principal component analyses (PCA) were performed on the climatic variable maps as per Richards *et al*. (2009), which resulted in two summary layers for each variable (Table 1). The first variable generally represents the magnitude of the climatic variable while the second generally represents the seasonal variation of the variable (Richards *et al*. 2009).

Model building

MaxEnt 3.3.3k software was used as it requires only presence records and its efficacy has been well documented (Elith *et al*. 2006; Phillips *et al*. 2006; Phillips & Dudík 2008). The default parameters of MaxEnt were used (Phillips & Dudík 2008). These included the regularization multiplier (1), maximum number of iterations (500), maximum number of background points (10 000) and convergence threshold (0.00001). Only hinge features were used as this avoids the overfitting of MaxEnt models when dealing with alien species (Elith *et al*. 2010); and 25 % of the data were reserved to test the model. The outputs of ten replicates were combined to give a mean output. A logistic output for constructing the predictive maps was selected as it is the easiest to comprehend, giving a value between 0 and 1 as the probability of occurrence of an organism (Phillips & Dudík 2008). Jackknife analyses and mean area-under-curve (AUC) plots were created using MaxEnt. AUC is commonly used as a test of the overall performance of the model (Elith *et al*. 2006) and although reservations have been expressed about its utility (Lobo *et al*. 2008), it remains a handy indication of the usefulness of a model (Elith *et al*. 2006, 2011). A value of 1.00 is perfect agreement with the model, while a value of 0.50 represents a random fit. Jackknife analysis indicates which variable has the greatest influence on the model and the overall success of the model.

The data were then divided into survey records and museum records and each partition was modelled separately to assess the importance of doing a focused survey.

Post hoc comparisons

To examine the putative relationship of each species to sheep strike, data for wool production in South Africa for 2011/2012 were obtained from Cape Wools SA and mapped to the magisterial district level as kg/km² (Fig. 3) to show the areas of highest density of sheep farming in South Africa. The average predicted likelihood values from the MaxEnt models for both *L. sericata* and *L. cuprina* at a magisterial district level were plotted against the values for wool production and the correlation coefficient was calculated using PAST3 (Hammer *et al*. 2001) (Fig. 5A & B).

Fig. 3. Wool production of magisterial districts, estimated by total grease mass produced in 2011/2012 in South Africa.

Fig. 4. Distribution of human population in South Africa in 2011 in terms of density.

To evaluate the putative synanthropy of *L. sericata*, human population density figures for South Africa were obtained from the 2011 national census results. These data were mapped as people/km² at the municipal level (Fig. 4). The average predicted likelihood values from the MaxEnt models for both *L. sericata* and *L. cuprina* were plotted against the values for the population density figures at a municipal level and the correlation coefficient was calculated using PAST3 (Hammer *et al*. 2001) (Fig. 5C & D). This allowed quantitative comparison between the areas of highest human habitation and the predicted distribution of the flies.

RESULTS

It is predicted that both species occur in large areas of South Africa (Fig. 6A & B). *Lucilia sericata* has a predicted central and western distribution, with a very low likelihood of occurring in the northern parts of South Africa. *Lucilia cuprina* has mainly a central and eastern predicted distribution, but it also includes the northern parts of South Africa. The mean area-under-curve (AUC) for *L. sericata* and *L. cuprina* was 0.78 and 0.77, respectively, for the training model and 0.69 for both test models (Table 1), indicating moderately good fits of the models to the data.

Jackknife analysis showed that the magnitude of monthly rainfall and the magnitude of the maximum monthly temperature were the most important climatic variables predicting the distribution of *L. sericata* (Fig. 7A). The seasonal variation in humidity and magnitude of monthly rainfall were the most important predictors for *L. cuprina* (Fig. 7B).

The museum data models (Fig. 8A & C) show distinctly different areas of suitability for both *L. sericata* and *L. cuprina* when compared to the survey data models for the same species (Fig. 8B & D).

The comparison between the predicted distribution of *L. sericata* and wool production in South Africa showed no correlation ($r = 0.067$; $p = 0.190$) between where this species is predicted to occur and where there are large numbers of sheep because of wool farming (Figs 3, 5B & 6A). The comparison between *L. cuprina* and wool production also revealed no correlation ($r = 0.017$; $p = 0.735$) between sheep-farming areas and the predicted areas in which this species is likely to occur (Figs 3, 5A & 6B).

The comparison between the predicted distribution of *L. sericata* and human population density distribution showed no correlation ($r = 0.102$; $p = 0.121$). Large areas of low population density in the Northern Cape were predicted to be areas suitable for this species, whereas areas of high population density in Limpopo and eastern Mpumalanga were areas of low suitability for this species (Figs 4, 5D & 6A).

The predicted distribution of *L. cuprina* showed no statistically significant correlation ($r = 0.019$; $p = 0.769$) with human settlement despite areas of high population density, particularly in the Western Cape and eastern parts of South Africa (Figs 4, 5C & 6B), being areas of higher suitability for this species.

DISCUSSION

The maximum entropy modelling technique has been used to model plant and insect distributions for purposes such as monitoring invasive species and disease vectors and their potential spread due to climate change (Chamaillé *et al*. 2010; Kulkarni *et al*.

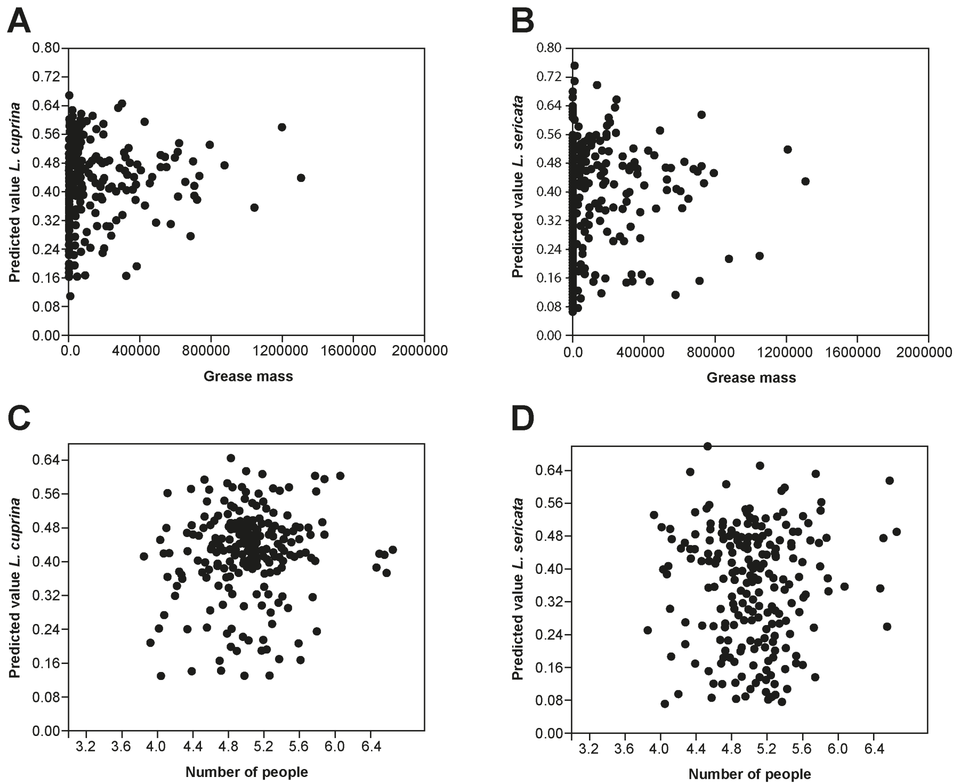

Fig. 5. Plots showing the correlation between the predicted distribution of *Lucilia cuprina* (A & C) and *Lucilia sericata* (B & D) to grease mass (kg) and human population density values (log values).

2010; Fischer *et al*. 2011; Gormley *et al*. 2011; Gurgel-Gonçalves *et al*. 2012; Petersen 2013). It performs well on small sample sizes (Pearson *et al*. 2007), which indicates that the generative methods used in MaxEnt give better predictions than the discriminative methods employed by other techniques (Elith *et al*. 2006; Phillip & Dudík 2008).

The mean AUC for the species models is on the low side (0.69 for both species); models with values above 0.75 are considered potentially useful (Elith 2002). This could be explained by the fact that the validity of models is more uncertain when species show temporal or spatial variation in their habitats; or are tolerant of a wide variety of habitats or have large distributional ranges; or are are migrants or nomadic (McPherson & Jetz 2007). Blowflies are typically r-selected (Elzinga 1997), mobile opportunists (Smith & Wall 1998) that make use of most available carrion resources to breed (Richards *et al*. 2009). This means that they may occur in an area as a result of factors other than the local environmental variables used in this study, e.g. transient food and breeding resources.

By using climatic variables for predicting species distributions, the assumption is made that those variables actually define the limits of the distribution of the species. Other factors, like geographic barriers and biotic interactions, may limit the species so that it does not or cannot occupy all of the climatically suitable areas (Meskin 1986; Soberón

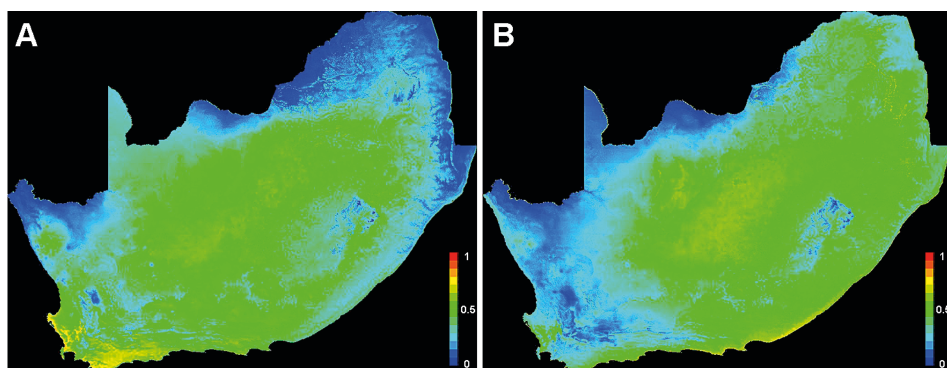

Fig. 6. Mean predicted distribution maps for *Lucilia sericata* (A) and *Lucilia cuprina* (B), produced using museum records, survey data and personal contact localities. The colour range indicates the likelihood of species distribution from dark blue (least likely) to red (most likely).

& Peterson 2005; Pearson *et al*. 2007). Certain ecological traits such as physiological tolerance and home range size exert real effects on the accuracy of distribution models that are not explained by methodology (McPherson & Jetz 2007). MaxEnt predicts potential distributions, not realised ones (Phillips & Dudík 2008), which means that some areas may be predicted to be suitable for these blowflies based on the environmental variables used, yet the flies are not found in those areas because there are other factors, such as competition with other blowflies, for resources that may affect their ability to survive in those areas.

The model for *L. sericata* is most influenced by the magnitude of monthly rainfall and the magnitude of the maximum monthly temperatures (Fig. 7A). This species originated in Europe and has been present in South Africa for over 100 years (museum records). Braack & Retief (1986) showed that the blowflies *Chrysomya albiceps* (Wiedemann, 1819) and *C. marginalis* (Wiedemann, 1830) were able to travel up to 2.25 km/day. Some of these flies dusted with radioactive P-powder were recovered as far as 63.5 km from the release site. In Australia, fluorescent dusted *L. cuprina* were found 17 km from the release point (Gilmour *et al.* 1946). This supports the idea that *L. sericata* has spread throughout South Africa to all the niches it can inhabit. The east coast and northern parts of South Africa are generally hotter (Schulze *et al*. 1997), which appears to limit the likelihood of this species occurring in these regions. Although *L. sericata* is an introduced species, we do not believe that there are any major barriers to its dispersal in South Africa. The rate at which *Chrysomya megacephala* (Fabricius, 1794) was recorded as spreading in South Africa after being introduced in 1971, suggests that blowflies are capable of doing so very rapidly in the country, likely because of their r-selected reproductive biology (Williams & Villet, 2006).

The model for *L. cuprina* is most influenced by the seasonal variation in humidity and the magnitude of monthly rainfall (Fig. 7B). The species is predicted to occur along the east coast, and into the northern parts of South Africa, which are all regions that are known for their humidity. Adult blowflies are very dependent on moisture; and humidity is very important for egg development as blowfly eggs desiccate easily (Richards *et al*. 2009).

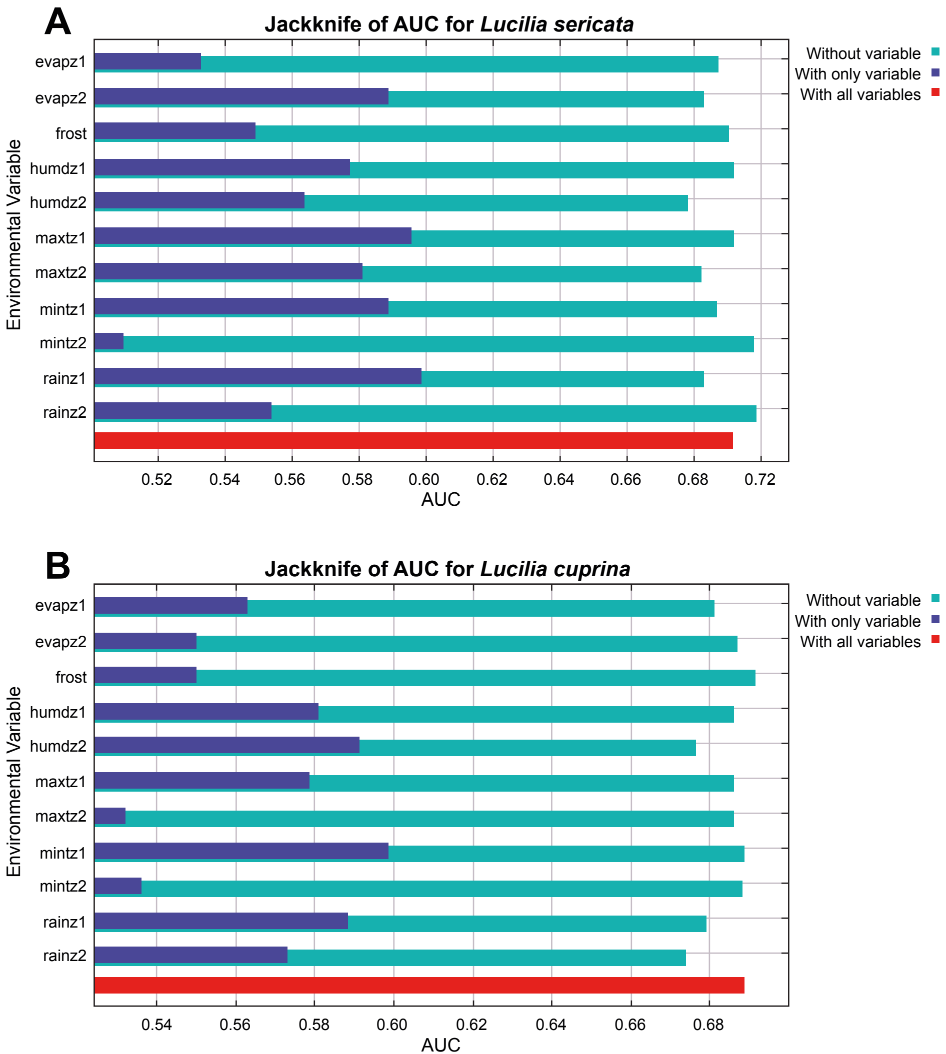

Fig. 7. Jackknife analysis of climatic variables area-under-curve (AUC) for *Lucilia sericata* (A) and *Lucilia cuprina* (B).

The models for both *L. sericata* and *L. cuprina* (Fig. 8) using the museum data and survey data separately, support the recommendations for focused surveys of areas for which there are very few records (Newbold 2010; Elith *et al*. 2011). Museum specimens are known to reflect bias in sampling effort and location and their use can influence the accuracy of predictive distribution models if no fieldwork is carried out to minimise the bias (Newbold 2010). The areas shown by the survey data to be suitable for these flies strongly correspond to the areas that were visited (Figs 1 & 8B & D). These data are biased due to the surveys, but when combined with the museum records, give a more complete depiction of whereabouts in the country these flies are likely to occur, as most of the climatically extreme areas of South Africa have been included. This must

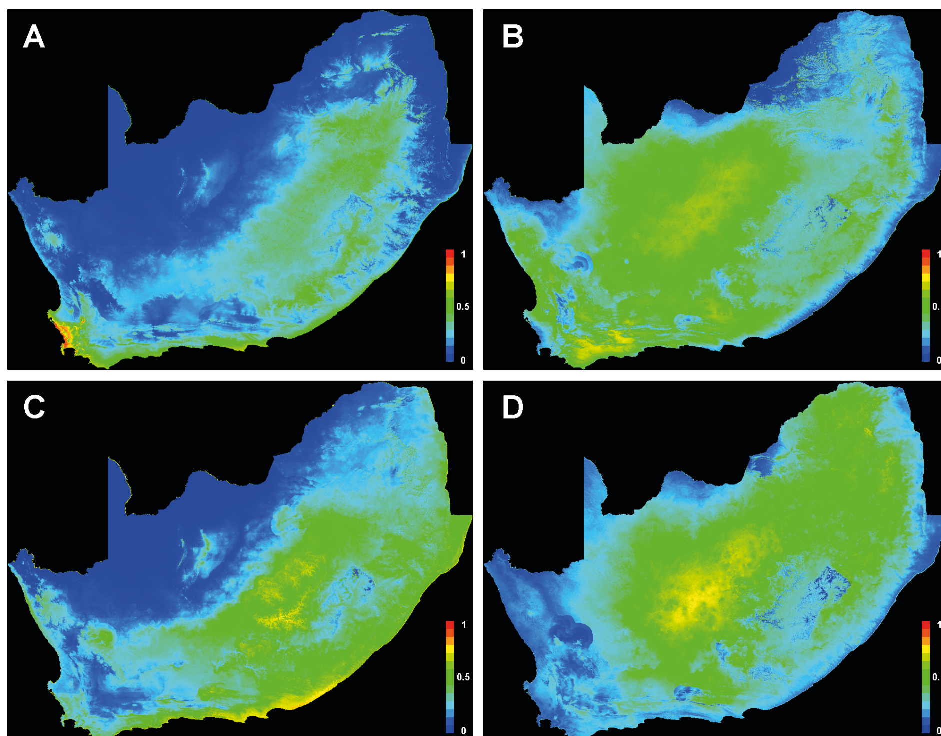

Fig. 8. Mean distribution maps for *Lucilia sericata* (A & B) and *Lucilia cuprina* (C & D) from museum (A & C) and survey (B & D) data only.

be considered when using modelling programmes to predict occurrences of species that may inhabit diverse climatic zones (Newbold 2010).

It has been reported that *L. sericata* is associated with areas inhabited by humans (Meskin 1986; Braack & de Vos 1987). However, the predicted occurrence of *L. sericata* shows no correlation with human population density, suggesting that this species is not an "urban" fly and occurs in both urban and rural environments. The potential distribution of *L. sericata* is also not correlated with wool production in South Africa, which is to be expected because this fly is not considered a pest in sheep-farming areas in South Africa (Hepburn 1943; Ullyett 1945; Vogt & Woodburn 1979).

L. cuprina has been thought to occur only in rural settings and not in areas populated by humans (Meskin 1986; Braack & de Vos 1987). The correlation between human population density and the predicted distribution of *L. cuprina* was not statistically significant, which supports this idea (Figs 4 and 6). *L. cuprina* has been a pest in sheep farming (Hepburn 1943; Ullyett 1945; Vogt & Woodburn 1979; Heath & Bishop 2006). The correlation between the areas in South Africa that have higher wool production (and therefore more sheep) and the predicted distribution of *L. cuprina* is not statistically significant (Figs 3 and 6). This is unexpected but may be explained by the fact that sheep farmers in South Africa are selecting breeds of sheep that do not have the skin fold around the anal area that promotes sheep strike, thereby eliminating the most common site of egg laying by this fly (A.R. Palmer pers. comm.).

L. sericata and *L. cuprina* appear to be largely generalists in that they are predicted to occur in most parts of South Africa, except in small areas of the south western and north eastern regions. This suggests that it is not possible to tell which species of *Lucilia* one is dealing with based only on geographic location. Morphological and molecular techniques are therefore advocated for identifying these two species, especially if they are being used in forensic investigations.

ACKNOWLEDGEMENTS

We thank Bruce Davidson, Peter Illgner, James Kay, John Midgley, Kyla Orr, Warren Potts, Mark Robertson and Garreth Whittington-Jones for locality records; Ashley Kirk-Sprigs (Albany Museum and National Museum), Simon van Noort (Iziko Museum) and Burgert Muller (KwaZulu-Natal Museum) for museum records; John Midgley for assistance in the field; Olivia Harrison for maps; Mark Robertson for comments on the manuscript; and Rhodes University and the Durban Natural Science Museum for funding this research. Financial support was also provided by the National Research Foundation (NRF) of South Africa. Any opinions, findings, conclusions and recommendations in this paper are those of the authors and do not necessarily reflect the views of the NRF.

REFERENCES

Altincicek, B. & Vilcinskas, A. 2009. Septic injury-inducible genes in medicinal maggots of the green blow fly *Lucilia sericata*. *Insect Molecular Biology* **18** (1): 119–125.

Anderson, G.S. 2000. Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences* **45**: 824–832.

Braack, L.E.O. 1986. Arthropods associated with carcasses in the northern Kruger National Park. *South African Journal of Wildlife Research* **16**: 91–98.

Braack, L.E.O. & Retief, P.F. 1986. Dispersal, density and habitat preference of the blow-flies *Chrysomyia albiceps* (Wd.) and *Chrysomyia marignalis* (Wd.) (Diptera: Calliphoridae). *Onderstepoort Journal of Veterinary Research* **53**: 13–18.

Braack, L.E.O. & de Vos, V. 1987. Seasonal abundance of carrion-frequenting blow-flies (Diptera: Calliphoridae) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research* **54**: 591–597.

Chamaillé, L., Tran, A., Meunier, A., Bourdoiseau, G., Ready, P. & Dedet, J.-P. 2010. Environmental risk mapping of canine leishmaniasis in France. *Parasites and Vectors* **3**: 31. Doi: 10.1186/1756-3305-3-31.

Clark, K., Evans, L. & Wall, R. 2006. Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. *Forensic Science International* **156**: 145–149.

Day, D.M. & Wallman, J.F. 2006. Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae). *Journal of Forensic Sciences* **51**: 657–663.

Elith, J. 2002. Quantitative methods for modeling species habitat: comparative performance and an application to Australian plants. *In*: Ferson, S. and Burgman, M., eds, *Quantitative methods for conservation biology*. New York: Springer, pp. 38–58.

Elith, J., Graham, C.H. & the NCEAS Species Distribution Modelling Group. 2006. Novel methods improve prediction of species' distributions from occurrence data. *Ecography* **29**: 129–151.

Elith, J. & Leathwick, J.R. 2009. Species distribution models: ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution, and Systematics* **40**: 677–697.

Elith, J., Kearney, M. & Phillips, S. 2010. The art of modelling range-shifting species. *Methods in Ecology and Evolution* **1**: 330–342.

Elith, J., Phillips, S.J., Hastie, T., Dudík, M., Chee, Y.E. & Yates, C.J. 2011. A statistical explanation of MaxEnt for ecologists. *Diversity and Distributions* **17**: 43–57.

Elzinga, R.J. 1997. *Fundamentals of entomology*. 4th edn. New Jersey: Prentice Hall.

Fischer, D., Moeller, P., Thomas, S.M., Naucke, J.T. & Beierkuhnlein, C. 2011. Combining climatic projections and dispersal ability: a method for estimating the responses of sandfly vector species to climate change. *PLoS Neglected Tropical Diseases* **5** (11): e1407. Doi: 10.1371/journal.pntd.0001407.

Gilmour, D., Waterhouse, D.F. & McIntyre, G.A. 1946. An account of experiments undertaken to determine the natural population density of the sheep blowfly, *Lucilia cuprina* Wied. *Bulletin of the Council for Scientific and Industrial Research, Australia* **195**: 1–39.

Gormley, A.M., Forsyth, D.M., Griffioen, P., Lindeman, M., Ramsey, D.S.L., Scroggie, M.P. & Woodford, L. 2011. Using presence-only and presence-absence data to estimate the current and potential distributions of established invasive species. *Journal of Applied Ecology* **48**: 25–34.

Gurgel-Gonçalves, R., Galvão, C., Costa, J. & Peterson, A.T. 2012. Geographic distribution of Chagas disease vectors in Brazil based on ecological niche modelling. *Journal of Tropical Medicine*. Doi: 10.1155/2012/705326.

Hammer, Ø., Harper D.A.T. & Ryan, P.D. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* **4** (1): 4.

Heath, A.C.G. & Bishop, D.M. 2006. Flystrike in New Zealand: an overview based on a 16-year study, following the introduction and dispersal of the Australian sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* **137**: 333–344.

Hepburn, G.A. 1943. Sheep blowfly research I – a survey of maggot collections from live sheep and a note on the trapping of blowflies. *Onderstepoort Journal of Veterinary Science and Animal Industry* **18**: 13–18.

Kulkarni, M.A., Desrochers, R.E. & Kerr, J.T. 2010. High resolution niche models of malaria vectors in northern Tanzania: a new capacity to predict malaria risk? *PLoS ONE* **5** (2): e9396. Doi: 10.1371/journal.pone.0009396.

Lobo, J.M., Jiménez-Valverde, A. & Real, R. 2008. AUC: a misleading measure of the performance of predictive distribution models. *Global Ecology and Biogeography* **17**: 145–151.

Louw, S.v.d.M. & van der Linde, T.C. 1993. Insects frequenting decomposing corpses in central South Africa. *African Entomology* **1** (2): 265–269.

Mackey, B.G. & Lindenmayer, D.B. 2001. Towards a hierarchical framework for modelling the spatial distribution of animals. *Journal of Biogeography* **28**: 1147–1166.

McPherson, J.M. & Jetz, W. 2007. Effects of species' ecology on the accuracy of distribution models. *Ecography* **30**: 135–151.

Meskin, I. 1986. Factors affecting the coexistence of blowflies (Diptera: Calliphoridae) on the Transvaal Highveld, South Africa. *South African Journal of Science* **82**: 244–250.

Newbold, T. 2010. Applications and limitations of museum data for conservation and ecology, with particular attention to species distribution models. *Progress in Physical Geography* **34** (1): 3–22.

Oliva, A. 2001. Insects of forensic significance in Argentina. *Forensic Science International* **120**: 145–154.

Paul, A.G., Ahmad, N.W., Lee, H.L., Ariff, A.M., Saranum, M., Naicker, A.S. & Osman, Z. 2009. Maggot debridement therapy with *Lucilia cuprina*: a comparison with conventional debridement in diabetic foot ulcers. *International Wound Journal* **6** (1): 39–46.

Pearson, R.G., Raxworthy, C.J., Nakamura, M. & Peterson, A.T. 2007. Predicting species distributions from small numbers of occurrence records: a test case using cryptic geckos in Madagascar. *Journal of Biogeography* **34** (1): 102–117.

Petersen, M.J. 2013. Evidence of a climatic niche shift following North American introductions of two crane flies (Diptera; genus *Tipula*). *Biological Invasions* **15** (4): 885–897. Doi: 10.1007/s10530-012-0337-3.

Phillips, S.J. 2008. Transferability, sample selection bias and background data in presence-only modelling: a response to Peterson *et al*. (2007). *Ecography* **31**: 272–278.

Phillips, S.J., Anderson, R.P. & Schapire, R.E. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* **190**: 231–259.

Phillips, S.J. & Dudík, M. 2008. Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecography* **31**: 161–175.

Richards, C.S., Williams, K.A. & Villet, M.H. 2009. Predicting geographic distribution of seven forensically significant blowfly species (Diptera: Calliphoridae) in South Africa. *African Entomology* **17** (2): 170–182.

Schulze, R.E., Maharaj, M., Lynch, S.D., Howe, B.J. & Melvil-Thomson, B. 1997. *South African atlas of agrohydrology and climatology*. 1st edn. Pretoria: Water Research Commission.

Sherman, R.A., Hall, M.J.R. & Thomas, S. 2000. Medicinal maggots: an ancient remedy for some contemporary afflictions. *Annual Review of Entomology* **45**: 55–81.

Smith, K.E. & Wall, R. 1997. The use of carrion as breeding sites by the blowfly *Lucilia sericata* and other Calliphoridae. *Medical and Veterinary Entomology* **11**: 38–44.

———1998. Estimates of population density and dispersal in the blowfly *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of Entomological Research* **88**: 65–73.

Soberón, J. & Peterson, A.T. 2005. Interpretation of models of fundamental ecological niches and species' distributional areas. *Biodiversity Informatics* **2**: 1–10.

Tantawi, T.I., Williams, K.A. & Villet, M.H. 2010. An accidental but safe and effective use of *Lucilia cuprina* (Diptera: Calliphoridae) in maggot debridement therapy in Alexandria, Egypt. *Journal of Medical Entomology* **47** (3): 491–494.

Ullyett, G.C. 1945. Species of *Lucilia* attacking sheep in South Africa. *Nature* **155**: 636–637.
Vogt, W.G. & Woodburn, T.L. 1979. Ecology, distribution and importance of sheep myiasis flies in Australia. *National symposium on the sheep blowfly and flystrike in sheep*. Sydney: New South Wales Department of Agriculture, pp. 23–32.
Williams, K.A. 2002. *Spatial and temporal occurrence of forensically important South African blowflies (Diptera: Calliphoridae)*. Grahamstown: MSc Thesis, Rhodes University.
Williams, K.A. & Villet, M.H. 2006. A new and earlier record of *Chrysomya megacephala* in South Africa, with notes on another exotic species, *Calliphora vicina* (Diptera: Calliphoridae). *African Invertebrates* **47**: 347–350.
Williams, K.A., Cronje, F.J., Avenant, L. & Villet, M.H. 2008. Identifying flies used for maggot debridement therapy. *South African Medical Journal* **98** (3): 196–197.
Wolff, H. & Hansson, C. 2005. Rearing larvae of *Lucilia sericata* for chronic ulcer treatment – an improved method. *Acta Dermato-Venereologica* **85**: 126–131.
Zumpt, F. & Patterson, P.M. 1952. Flies visiting human faeces and carcasses in Johannesburg, Transvaal. *South African Journal of Clinical Science* **3**: 92–106.