

The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in southeastern Australia

J.F. Wallman^{a,*}, S.C. Donnellan^b

^aDepartment of Environmental Biology, The University of Adelaide, Adelaide, 5005 SA, Australia

^bEvolutionary Biology Unit, South Australian Museum, North Terrace, Adelaide, 5000 SA, Australia

Abstract

The applicability of mitochondrial DNA (mtDNA) sequencing was investigated for the identification of the following forensically important species of blowflies from southeastern Australia: *Calliphora albifrontalis*, *C. augur*, *C. dubia*, *C. hilli hilli*, *C. maritima*, *C. stygia*, *C. vicina*, *Chrysomya rufifacies*, *Ch. varipes* and *Onesia tibialis*. All breed in carrion except *O. tibialis*, which is an earthworm parasitoid. Emphasis was placed on *Calliphora* species because they predominate among the carrion-breeding blowfly fauna of southern Australia and their immatures are difficult to identify morphologically. A partial sequence of the mitochondrial *COII* gene was determined for all species and for *COI* for *C. albifrontalis*, *C. augur*, *C. dubia* and *C. stygia* only. Five other species of blowflies, *Chrysomya albiceps*, *Ch. rufifacies*, *Protophormia terraenovae*, *Lucilia illustris* and *L. sericata*, for which sequence data were already available, were also included. Analysis of the *COI* and *COII* sequences revealed abundant phylogenetically informative nucleotide substitutions that could identify blowfly species to species group. In contrast, because of the low level of sequence divergence of sister species, the data could not distinguish among taxa from the same species group, i.e. the species within the *C. augur* and *C. stygia* groups. The molecular data support the existing species group separation of the taxa within *Calliphora*. Because of the speed and accuracy of current nucleotide sequencing technology and the abundant apomorphic substitutions available from mtDNA sequences, this approach, with the analysis of additional taxa and genes, is likely to enable the reliable identification of carrion-breeding blowflies in Australia. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Blowflies; Calliphora; Calliphoridae; Mitochondrial DNA; Forensic entomology; Australia

1. Introduction

Carrion-breeding blowflies can play a valuable role in the investigation of murders and suspicious deaths in helping to determine time, manner and place of death [1]. They are also crucial for the maintenance of ecological systems [2], and the involvement of some species in myiasis gives them substantial medical and veterinary significance [3,4]. There are, therefore, important reasons for being able to identify adult and immature carrion-breeding blowflies reliably. Correct identification is especially important from a forensic

perspective because flawed forensic evidence may lead to miscarriages of justice [5].

In southern Australia, some of the most forensically important native blowfly species belong to the genus *Calliphora*. Morphological differences between these species are frequently subtle. This is particularly so in the immature stages; third instar larvae of some species are morphologically indistinguishable and the identification of others is difficult even for specialists [6]. Another challenge for correct identification is the possible introduction of carrion-breeding blowflies from overseas or other parts of Australia, some of whose immatures may be undescribed. A diagnostic technique should be capable of distinguishing the immatures of exotic taxa from those of the species already known to be present in the region of interest. Such morphological challenges make a molecular approach to the identification of these species desirable.

* Corresponding author. Tel.: +61-8-8303-4730;
fax: +61-8-8303-6222.
E-mail address: james.wallman@adelaide.edu.au (J.F. Wallman).

As an initial step in the development of a comprehensive molecular diagnostic approach to carrion-breeding blowflies in southern Australia, a study has been undertaken of carrion-breeding *Calliphora* from southeastern Australia. The scope of the study was restricted to this region because of the availability of suitable samples. The first phase of this study employed the technique of allozyme electrophoresis to test existing notions of species boundaries and to detect the presence of cryptic species [7]. This technique also demonstrated the ability of allozyme electrophoresis to identify immatures from southeastern Australia [8].

However, despite being excellent for inferring genetic relatedness, allozymes have limitations as a routine tool for species identification. For successful analysis, specimens must be fresh or frozen. The comparative nature of the technique also means that reference specimens are required. In contrast, analysis using mitochondrial DNA sequencing can be done without the constant need for reference specimens and specimens may be preserved in ethanol or even air dried [9]. Furthermore, facilities for DNA sequencing are now much more widely available than for allozymes and considerably less specialised experience is required to perform the technique.

Recent molecular studies have demonstrated the applicability of mtDNA sequencing to the identification of important Old and New World species of carrion-breeding blowflies [9,10]. The present study explores the utility of DNA sequences from the mitochondrial genes cytochrome oxidase subunits one and two (*COI* and *COII*) to the

identification of some common southeastern Australian carrion-breeding blowflies, with an emphasis on *Calliphora* species. We take a phylogenetic approach to identify diagnostic substitutions, i.e. apomorphic character states, rather than a solely phenetic approach that does not discriminate between plesiomorphic and apomorphic character states.

2. Materials and methods

2.1. DNA sources

DNA was extracted and sequenced from the following species of *Calliphora*: *C. albifrontalis* (Malloch), *C. augur* (Fabricius), *C. dubia* (Macquart), *C. hilli hilli* (Patton), *C. maritima* (Norris), *C. stygia* (Fabricius) and *C. vicina* (Robineau-Desvoidy). Specimens of two common species of *Chrysomya* were also included to use as outgroups: *Ch. rufifacies* (Macquart) and *Ch. varipes* (Macquart). A single representative of the genus *Onesia*, *O. tibialis* (Macquart), an earthworm parasitoid [11] that is frequently found feeding at carrion, was also included because it is difficult to distinguish from species of *Calliphora*. Another reason for its inclusion is that the molecular study of Wallman and Adams [7] placed *Onesia* among the *Calliphora* species examined, thus questioning the monophyly of *Calliphora*.

Collection data for all specimens of the above species included in the study are given in Table 1. These specimens

Table 1
Locality and reference data for specimens from which DNA was extracted

Species	ABTC number	Collection locality
<i>C. stygia</i>	3305	Dudley Conservation Pk, Kangaroo I., SA (35°48'S, 137°51'E)
	3327	Adelaide, SA (34°56'S, 138°36'E)
	3371	Spring Gully Conservation Pk, SA (33°55'S, 138°36'E)
<i>C. albifrontalis</i>	3398	Dudley Conservation Pk, Kangaroo I., SA (35°48'S, 137°51'E)
	3419	Warrenben Conservation Pk, SA (35°09'S, 137°05'E)
<i>C. augur</i>	3187, 3211	Adelaide, SA (34°56'S, 138°36'E)
<i>C. dubia</i>	3169	Dudley Conservation Pk, Kangaroo I., SA (35°48'S, 137°51'E)
	3179	Adelaide, SA (34°56'S, 138°36'E)
	3205	Cooranga Homestead, SA (36°50'S, 140°18'E)
<i>C. hilli hilli</i>	3135	Cooranga Homestead, SA (36°50'S, 140°18'E)
	3152	Canberra, ACT (35°17'S, 149°13'E)
<i>C. maritima</i>	3244	Cape Buffon, Canunda Nat. Pk, SA (37°34'S, 140°07'E)
	3254	Cape Spencer, Innes Nat. Pk, SA (35°18'S, 136°53'E)
<i>C. vicina</i>	71190, 71191	Adelaide, SA (34°56'S, 138°36'E)
<i>O. tibialis</i>	71192, 71193	Adelaide, SA (34°56'S, 138°36'E)
<i>Ch. rufifacies</i>	3426	Dudley Conservation Pk, Kangaroo I., SA (35°48'S, 137°51'E)
	3518	Cooranga Homestead, SA (36°50'S, 140°18'E)
<i>Ch. varipes</i>	3602	Dudley Conservation Pk, Kangaroo I., SA (35°48'S, 137°51'E)
	3603	Meningie, SA (35°42'S, 139°20'E)

are referred to herein by a number assigned to their remnant tissues (see below), which are stored in the Australian Biological Tissue Collection (ABTC) in the South Australian Museum.

The following non-Australian carrion-breeding species, whose nucleotide sequences were available from GenBank, were also included because they have or could potentially become established in Australia: *Chrysomya albiceps* (Wiedemann), *Protophormia terraenovae* (Robineau-Desvoidy),¹ *Lucilia illustris* (Meigen), and *Lucilia sericata* (Meigen). Indeed *L. sericata* is already widespread and *Ch. albiceps* has been found in a corpse accidentally imported into Australia [13].

The above species were assigned to three separate subfamilies by Rognes [14]: Calliphorinae (*Calliphora* and *Onesia*), Luciliinae (*Lucilia*) and Chrysomyinae (*Chrysomya* and *Protophormia*). Species of carrion-breeding *Calliphora* which occur in southeastern Australia comprise six species groups (taxa from the present study in brackets): the *Calliphora augur* group (*C. augur* and *C. dubia*), *C. rufipes* [*hilli*] group (*C. hilli hilli*), *C. maritima* group (*C. maritima*), *C. ochracea* group, *C. stygia* group (*C. albifrontalis* and *C. stygia*) and *C. erythrocephala* [*vicina*] group (*C. vicina*) [7,15]. The selection of species included herein allows a broad assessment of the utility of mitochondrial genes as identification tools for immatures; five of the six species groups of carrion-breeding *Calliphora* that occur in southeastern Australia are represented.

2.2. DNA extraction, polymerase chain reaction (PCR) methods and nucleotide sequencing

Total cellular DNA was extracted from the thorax of frozen flies by the protocol of Chomczynski et al. [16] involving DNAzol (Molecular Research Center), except that DNA was dissolved in distilled water rather than in NaOH. The head and abdomen of each specimen was retained to check its identity.

A portion of the *COII* gene was amplified and sequenced using primers C2-J-3138 (sense) [17] and TK-N-3775 (antisense) [18]. Partial *COI* sequences were obtained with UEA7 (sense) [19] and TL2-N-3014 (antisense) [17].

The PCR conditions were: 50–100 ng of DNA, 0.2 μ M of each primer, 0.2 mM of each dNTP, 4 mM of MgCl₂, 5 μ M of GeneAmp 10 \times PCR Buffer II (Perkin-Elmer) and one unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer) in a reaction volume of 50 μ l. PCR cycling conditions on a Omn-E Thermal Cycle (Hybrid) were: cycle 1 (94°C for 3 min, 52°C for 1 min, 75°C for 1 min); cycles 2–35 (93°C

for 45 s, 52°C for 45 s, 72°C for 1 min); cycle 36 (72°C for 6 min, hold at room temperature). PCR products were purified using the Breeze-Clean Nucleic Acid Purification Kit (Geneworks). Sequences of both strands were obtained by direct sequence of the double-stranded PCR product using one of the PCR primers and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). Sequence reactions were electrophoresed on ABI Model 373 and 377 DNA Sequencers.

The sequences have been deposited in GenBank (accession numbers AY012540–69).

2.3. Sequence analyses

The data were analysed in two parts: (1) *COII* data for all *Calliphora* species and *Onesia tibialis*, and (2) *COI* and *COII* data for *Calliphora* species that were poorly differentiated by the *COII* sequences alone: *C. augur* and *C. dubia*, and *C. albifrontalis* and *C. stygia*.

As the sequences were protein encoding and did not contain any insertions or deletions, alignment was done by eye. We tested whether the sequences were of mitochondrial origin or represented paralogous sequences resident in the nucleus [20] in two ways using MEGA [21]. Sequences were translated with the *Drosophila* mitochondrial genetic code and, since nuclear paralogues can lose their coding function, the predicted amino acid sequences were inspected for inappropriate stop codons. We also compared the base composition of the individual sequences since nuclear paralogues can have divergent base compositions relative to mitochondrial genes.

Modeltest2 [22] and PAUP* version 4.0b2 [23] were used to find the optimal model of nucleotide substitution for the data. Iterative rounds of quartet puzzling, a variant of the maximum likelihood method of phylogenetic reconstruction (QP- [24]) were used to find optimal values of parameters specified by the model. Evolutionary trees were found using PAUP* with unweighted heuristic searches under the maximum parsimony criterion of optimality (MP) and heuristic searches under the maximum likelihood criterion of optimality (ML). Trees were rooted by arbitrarily defining the species within the subfamily Chrysomyinae as outgroup taxa. Heuristic searches with multiple taxon addition sequences were used to assess the efficiency of the heuristic algorithm in the MP analysis when the number of taxa was large. Support for relationships was assessed with bootstrapping and also, for the MP analysis, with Bremer decay indices [25], the latter giving an approximation of the number of synapomorphies present for a particular branch. The program Autodecay version 2.9.10 [26] was used with PAUP* to find the Bremer indices. The Incongruence Length Difference test [27] (otherwise called the Partition Homogeneity test) with 1000 permutations was used in PAUP* to assess whether the two gene partitions could be combined for a single analysis.

¹ The sequence of this species included in the study (accession no. L14946) is attributed in Genbank to *Phormia regina* (Meigen), but is actually from a specimen of *Protophormia terraenovae* (Robineau-Desvoidy) [12] (this error is to be amended).

3. Results

3.1. COII analysis

A total of 635 aligned sites for the 27 *COII* sequences were included in the analyses. These included 489 invariant sites and 132 parsimony informative sites. Uncorrected percentage sequence divergence among all taxa ranged from 0.15 to 10%. Third, first and second codon positions had 81, 17 and 2% of the variable sites, respectively. The predicted amino acid sequences were similar, with no premature stop codons predicted and only 12 out of 212 amino acid sites showing variation. Nucleotide base composition was similar for all sequences with a strong AT bias (73.3%), typical of insect mitochondrial DNA [28].

The model of nucleotide substitution found with a likelihood ratio test implemented with Modest2 was the general time reversible model (GTR- [29]) with a specified proportion of invariant sites (I) and gamma shape parameter of rate variation among sites (Γ). Optimal values for the two specified parameters found with iterative bouts of QP were $I = 0.6358$ and $\Gamma = 1.0845$; base composition: $A = 0.33149$, $C = 0.12171$, $G = 0.12810$, $T = 0.419$; rate matrix: 13,150 ($A \leftrightarrow C$), 108,600 ($A \leftrightarrow G$), 68,700 ($A \leftrightarrow T$), 1.6×10^{-17} ($C \leftrightarrow G$), 691,400 ($C \leftrightarrow T$). The tree found under this model with the maximum likelihood criterion of optimality is presented in Fig. 1A, along with bootstrap proportions (>70%) from 100 pseudo-replicates.

Three equally most parsimonious trees each of length 289 steps were found using a heuristic search with 54 random taxon addition searches. Bootstrap proportions (>70%) from 2000 pseudo-replicates and Bremer decay indices are given on the ML tree in Fig. 1A.

MP and ML analyses are congruent in recognising six lineages within the Calliphorinae all with bootstrap support $\geq 98\%$. Branch support for these lineages from Bremer decay indices was similarly strong (=11–17). The two members of the Luciliinae also comprise a well-supported lineage (bootstrap support: MP = 92%, ML = 91%; Bremer support = 7), as do *Ch. ruffacies* and *Ch. albiceps* (bootstrap support: MP = 96%, ML = 89%; Bremer support = 6). MP and ML analyses are also concordant in including the *L. illustris/L. sericata* lineage in a clade that also includes *Calliphora* and *Onesia*. This clade is well supported by bootstrapping (MP = 89%, ML = 97%) and has a Bremer support value of six. Relationships within two of the lineages that each includes members of two species (*C. augur/C. dubia*; *C. albifrontalis/C. stygia*) are not resolved by our data. It should be noted that, although our data do not demonstrate reciprocal monophyly of the taxa within each of these lineages, the data do not reject reciprocal monophyly. Except for the quite well-supported relationship between *C. hilli hilli* and *C. maritima* (bootstrap support: MP = 93%, ML = 78%; Bremer support = 3), relationships among the *Calliphora*, *Onesia* and *Lucilia* lineages also are not resolved by our data. Among the

nominate outgroup taxa, monophyly of *Ch. ruffacies* and *Ch. albiceps* is well supported (bootstrap support: MP = 96%, ML = 89%; Bremer support = 6).

Table 2 shows that the level of *COII* nucleotide divergence between species groups within *Calliphora* is marked (5.5–10.0%), and is of a comparable magnitude to the level of divergence observed between the two species of *Lucilia* included here (4.9%). In contrast, there is very little divergence based on *COII* between species from the same species group (Table 2). The maximum level of divergence within the *C. stygia* group is 0.5%, while that within the *C. augur* group is 0.8%. These levels contrast with the divergence between congeneric species of *Chrysomya* (*Ch. ruffacies* and *Ch. albiceps* — 3.1%).

3.2. Combined COI and COII analysis

Because of the low level of divergence in *COII* sequences between sister species within the species groups, *C. augur/C. dubia* and *C. albifrontalis/C. stygia*, and the poor resolution of their relationships, we obtained partial sequences of the *COI* gene from a selection of specimens to attempt to resolve relationships within each of these two clusters.

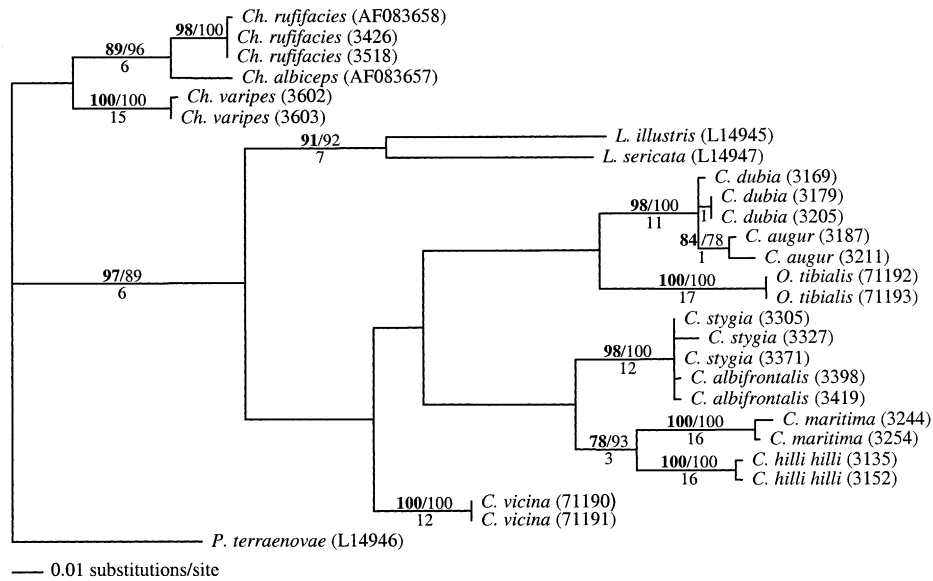
A total of 639 aligned sites for the 11 *COI* sequences were included in the phylogenetic analyses. These included 524 invariant sites and 80 parsimony informative sites. Uncorrected percentage sequence divergence ranged from 0 to 11.1%. Third, first and second codon positions had 77, 19 and 4% of the variable sites, respectively. The predicted amino acid sequences were similar, with no premature stop codons observed and only 10 out of 214 amino acid sites showing variation. Nucleotide base composition was similar for all sequences, with a strong AT bias (69.1%). As the partition homogeneity test did not reject the null hypothesis of data homogeneity ($P = 1.0$), we combined the *COI* and *COII* sequences for analysis.

For the combined data, the model of nucleotide substitution found with a Likelihood Ratio test implemented with Modest2 was the GTR + Γ [29]. Optimal values for the specified parameters found with iterative bouts of QP were $\Gamma = 0.1221$; base composition: $A = 0.31903$, $C = 0.15189$, $G = 0.131031$, $T = 0.399$; rate matrix: 7.54 ($A \leftrightarrow C$), 75.12 ($A \leftrightarrow G$), 46.09 ($A \leftrightarrow T$), 6.422 ($C \leftrightarrow G$), 227 ($C \leftrightarrow T$). The tree found under this model with ML is presented in Fig. 1B, along with bootstrap proportions (>70%) from 100 pseudo-replicates.

One tree of length 278 steps was found with a branch and bound search under MP. Bootstrap proportions (>70%; heuristic searches) from 2000 pseudo-replicates and Bremer decay indices for this tree are included on the ML tree in Fig. 1B.

Despite the addition of 116 more variable sites from the *COI* data, relationships between the sister species within the *C. augur/C. dubia* and *C. albifrontalis/C. stygia* lineages are no better resolved; indeed, both bootstrap and Bremer support indicators in both MP and ML analyses are similar to the analyses based on the *COII* data alone.

A



B

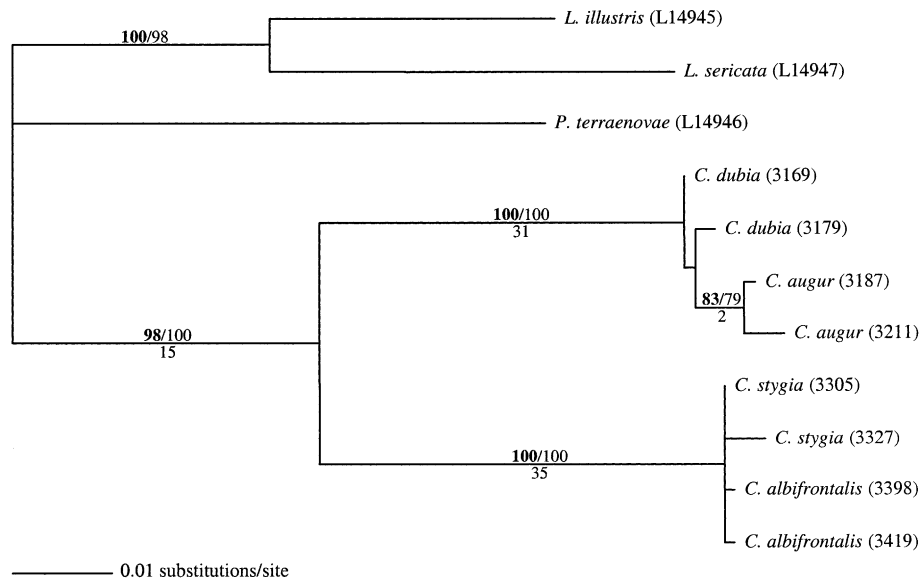


Fig. 1. Maximum likelihood tree of relationships among mitochondrial *COII* sequences (A) and combined mitochondrial *COI* and *COII* sequences (B) from blowflies. Numbers above branches refer to bootstrap proportions (>70%) among 100 ML pseudo-replicates (in bold) and 2000 MP pseudo-replicates and numbers below branches to Bremer decay indices. Numbers relate to the node to the immediate left. ABTC or accession numbers from Table 1 or GenBank are given in brackets.

Table 2 shows that the level of combined *COI* and *COII* nucleotide divergence between species groups within *Caliphora* is marked (6.1–6.8%), and is again of a comparable magnitude to the level of divergence observed between the two species of *Lucilia* included here (5.4%).

As with the *COII* data alone, there is very little divergence based on combined *COI* and *COII* data between species from the same species group (Table 2). The maximum level of divergence within both *C. augur* and *stygia* groups is 0.8%.

Table 2

Percent uncorrected sequence divergence between calliphorid taxa for the *COII* gene (above diagonal) and the combined *COI* and *COII* genes (below diagonal)^a

Species/specimens	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	–	4.9	7.2	7.8	8.6	6.9	7.1	7.4	7.4	7.2	8.2	8.7	8.3	9.1	9.0	6.9	7.4
2	5.4	–	8.8	8.2	8.5	8.3	7.1	7.4	7.7	7.5	7.5	8.1	7.7	9.1	7.9	6.9	7.4
3			–	3.1	6.8	6.1	7.4	7.4	7.5	7.4	6.8	7.1	6.9	9.6	9.0	8.5	8.5
4				–	6.8	5.8	7.9	8.0	8.0	7.9	7.1	7.4	7.2	10.0	9.6	8.5	9.0
5					–	5.5	9.2	9.1	9.1	8.9	8.8	9.2	8.6	10.0	9.8	8.4	9.0
6	7.8	8.5				–	7.7	7.7	8.0	7.9	6.8	7.1	6.6	9.9	9.3	6.3	8.4
7	8.1	8.3				9.0	–	0.2	0.6	0.5	5.6	6.1	5.8	7.7	7.2	6.5	6.8
8	8.3	8.6				9.2	0.2	–	0.8	0.6	5.5	6.0	5.7	7.7	7.2	6.5	6.9
9	8.4	8.6				9.4	0.6	0.6	–	0.1	5.8	6.2	6.0	7.8	7.4	6.6	6.8
10	8.5	8.6				9.4	0.8	0.8	0.4	–	5.7	6.1	5.8	7.7	7.2	6.5	6.6
11	8.6	8.6				8.9	6.1	6.2	6.4	6.4	–	0.3	0.1	7.4	6.8	6.3	7.6
12	9.0	9.0				9.1	6.5	6.6	6.8	6.8	0.3	–	0.5	7.7	7.1	6.5	7.9
13	8.6	8.7				8.8	6.2	6.3	6.5	6.4	0.1	0.4	–	7.5	6.9	6.2	7.7
14														–	6.1	8.3	8.0
15															–	7.9	8.0
16																–	7.3
17																	–

^a For species that diverged from all others by a relatively large percentage and where within-species variation was small, i.e. less than 0.8%, the minimum sequence divergence percentages are given that distinguish any individual within that species from any other species. Species/specimen codes: 1: *L. illustris*; 2: *L. sericata*; 3: *Ch. rufifacies*; 4: *Ch. albiceps*; 5: *Ch. varipes*; 6: *P. terraenovae*; 7: *C. dubia* (3169); 8: *C. dubia* (3179/3205, 3179 only for combined *COI* and *COII* data); 9: *C. augur* (3187); 10: *C. augur* (3211); 11: *C. stygia* (3305); 12: *C. stygia* (3327/3371 for *COII* data, 3327 only for combined *COI* and *COII* data); 13: *C. albifrontalis*; 14: *C. maritima*; 15: *C. hilli hilli*; 16: *C. vicina*; 17: *O. tibialis*.

4. Discussion

Despite the limited phylogenetic resolution of these gene sequences, they nonetheless have potential for identification of these calliphorid flies. The four species groups of *Calliphora* recognised in earlier molecular work on these species [7], along with *C. vicina*, could all be diagnosed using these DNA sequences. This is reflected in the strong bootstrap and Bremer support for each group (apomorphic character states) and the level of nucleotide divergence between groups (overall differences). At present the nucleotide data can diagnose flies to species group, which may be an adequate level of identification based on what is known of the variation in the forensically important thermobiological characteristics of these flies [30–32]. Since adults and immatures have identical genotypes, these sequences would have applicability to the identification of all life stages.

However, within the *C. augur*- and *C. stygia* groups, there was insufficient sequence variation to show monophyly of haplotypes from each species. The low within species group discrimination could be due either to sampling of too few informative characters or to these species yet to become reciprocally monophyletic for their respective mitochondrial lineages, i.e. the taxa have diverged only very recently. It is in any case still unclear whether species from the same species group differ sufficiently in their thermobiology to warrant their discrimination for forensic purposes.

Future work could develop a PCR-based restriction endonuclease assay (PCR-RFLP) to assist with identification of all five groups of *Calliphora* [9]. However, with the speed and efficiency of current nucleotide sequencing technology this would scarcely improve the time for identification beyond that of the techniques described herein (<48 h). Furthermore, because PCR-RFLP would utilise only a small fraction of the apomorphic sites, the technique has the potential to misallocate the identity of unexpected exotic taxa that have recently entered a region, especially if few restriction enzymes are used. With the identification of additional taxa, the PCR-RFLP test would likely need to be developed further and require extensive revalidation.

While phylogenetic analysis was not employed specifically to help to resolve the evolutionary relationships of this group of flies, it nonetheless gives an indication of the utility of the *COI* and *COII* genes for this purpose. The present molecular data support the separate phylogenetic status of all of the species groups within *Calliphora* that were analysed here, as well as the separate status of the additional member of the Calliphorinae analysed, *O. tibialis*. However, there is no resolution of the relationships among groups as assessed by bootstrap support and Bremer decay indices. We do not comment on the evolutionary relationships among the three subfamilies of Calliphoridae, since a more distant outgroup and more taxon representation would be required to test this. Overall, the minimal level of information available about the relationships between and within groups

again suggests that the taxa examined here diverged too recently for the *COI* and *COII* genes to be informative phylogenetically for deeper relationships within the group. Since most of the variation between sequences was accounted for by third codon positions, further phylogenetic information should be derived from loci with a higher contribution toward variation from the first and second codon positions, such as the six mitochondrial NADH dehydrogenase subunit genes. Future DNA analysis of relationships within *Calliphora* might therefore usefully include such genes. Such analysis should also be expanded to include species with life histories other than carrion-breeding and a range of outgroups from other genera within the Calliphoridae.

This report shows that, with further development, mitochondrial nucleotide sequences can provide a means of identifying forensically important Australian blowflies. Future work could include the examination of more variable mitochondrial genes to, if necessary, distinguish species within species groups, and the analysis of additional species of blowflies from other parts of Australia and overseas that are likely to occur in Australia in the future.

Acknowledgements

We thank Jan Armstrong for valuable assistance with technical aspects of the study and Steve Cooper, Mark Adams and Remko Leijts for providing helpful comments on the manuscript. This work was partially supported by a grant from the Sir Mark Mitchell Research Foundation to J.F. Wallman.

References

- [1] E.P. Catts, M.L. Goff, Forensic entomology in criminal investigations, *Ann. Rev. Entomol.* 37 (1992) 253–272.
- [2] R.J. Putman, Carrion and Dung: Decomposition of Animal Wastes, Edward Arnold, London, 1983.
- [3] R.W. Crosskey, R.P. Lane, House-flies, blow-flies and their allies (calyptrate Diptera), in: R.P. Lane, R.W. Crosskey (Eds.), *Medical Insects and Arachnids*, Chapman & Hall, London, 1993, pp. 403–428.
- [4] M.J.R. Hall, R. Wall, Myiasis of humans and domestic animals, *Adv. Parasitol.* 35 (1995) 257–334.
- [5] P. Gerber, Playing dice with expert evidence: the lessons to emerge from *Regina v. Chamberlain*, *Med. J. Aust.* 147 (1987) 243–247.
- [6] J.F. Wallman, Third-instar larvae of common carrion-breeding blowflies of the genus *Calliphora* in South Australia, *Invertebr. Taxon.* 15 (2001) 37–51.
- [7] J.F. Wallman, M. Adams, Molecular systematics of carrion-breeding blowflies of the genus *Calliphora* (Diptera: Calliphoridae), *Aust. J. Zool.* 45 (1997) 337–356.
- [8] J.F. Wallman, M. Adams, The forensic application of allozyme electrophoresis to the identification of blowfly larvae (Diptera: Calliphoridae) in southern Australia, *J. Forensic Sci.* 46 (2001) 681–684.
- [9] F.A.H. Sperling, G.S. Anderson, D.A. Hickey, A DNA-based approach to the identification of insect specimens used for postmortem interval estimation [published erratum appears in letter from Wells and Sperling, *J. Forensic Sci.* 45 (2000) 1358–1359], *J. Forensic Sci.* 39 (1994) 418–427.
- [10] J.D. Wells, F.A.H. Sperling, Molecular phylogeny of *Chrysomya albiceps* and *C. rufifacies* (Diptera: Calliphoridae), *J. Med. Entomol.* 36 (1999) 222–226.
- [11] K.R. Norris, General biology, in: I.D. Naumann, P.B. Carne, J.F. Lawrence, E.S. Nielsen, J.P. Spradbery, R.W. Taylor, M.J. Whitten, M.J. Littlejohn (Eds.), *The Insects of Australia*, Melbourne University Press, Carlton, 1991, pp. 68–108.
- [12] J.D. Wells, F.A.H. Sperling, Commentary on: F.A.H. Sperling, G.S. Anderson, D.A. Hickey, A DNA-based approach to the identification of insect species used for postmortem interval estimation, *J. Forensic Sci.* 39 (1994) 418–427 and on S. Vincent, J.M. Vian, M.P. Carlotti, Partial sequencing of the cytochrome oxidase *b* subunit gene I: a tool for the identification of European species of blow flies for post-mortem interval estimation, *J. Forensic Sci.* 45 (2000) 820–823, *J. Forensic Sci.* 45 (2000) 1358–1359.
- [13] R.A. James, D.A. Eitzen, R. Hall, J.F. Wallman, C.J. McKenna, R.W. Byard, Autopsy and quarantine considerations in a case of illegal entry of a corpse into Australia, *J. Law Med.* 8 (2000) 89–91.
- [14] K. Rognes, Blowflies (Diptera: Calliphoridae) of Fennoscandia and Denmark, *Fauna Entomol. Scand.* 24 (1991) 1–272.
- [15] G.H. Hardy, Notes on the genus *Calliphora* (Diptera). Classification, synonymy, distribution and phylogeny, *Proc. Lin. Soc. N.S.W.* 62 (1937) 17–26.
- [16] P. Chomczynski, K. Mackey, R. Drews, W. Wilfinger, DNAzol[®]: a reagent for the rapid isolation of genomic DNA, *BioTechniques* 22 (1997) 550–553.
- [17] C. Simon, F. Frati, A. Beckenbach, B. Crespi, H. Liu, P. Flook, Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers, *Ann. Entomol. Soc. Am.* 87 (1994) 651–701.
- [18] S.M. Bogdanowicz, W.E. Wallner, T.M. Bell, R.G. Harrison, Asian gypsy moths (Lepidoptera: Lymantriidae) in North America: evidence from molecular data, *Ann. Entomol. Soc. Am.* 86 (1993) 710–715.
- [19] D.H. Lunt, D.-X. Zhang, J.M. Szymura, G.M. Hewitt, The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic study, *Insect Mol. Biol.* 5 (1996) 153–165.
- [20] D.-X. Zhang, G.M. Hewitt, Nuclear integrations: challenges for mitochondrial DNA markers, *Tr. Ecol. Evo.* 11 (1996) 247–251.
- [21] S. Kumar, K. Tamura, M. Nei, MEGA: Molecular Evolutionary Genetics Analysis, Version 1.0, The Pennsylvania State University, University Park, Pennsylvania, 1993.
- [22] D. Posada, K.A. Crandall, MODELTEST: testing the model of DNA substitution, *Bioinformatics* 14 (1998) 817–818.
- [23] D.L. Swofford, PAUP: Phylogenetic Analysis Using Parsimony, Version 4.0b2, Sinauer Associates, Sunderland, MA, 1999.
- [24] K. Strimmer, A. Von Haeseler, Likelihood-mapping: a simple method to visualise phylogenetic content of a sequence

- alignment, *Proc. Natl. Acad. Sci. U.S.A.* 94 (1997) 6815–6819.
- [25] K. Bremer, The limits of amino acid sequence data in angiosperm phylogenetic reconstruction, *Evolution* 42 (1988) 795–803.
- [26] T. Eriksson, Autodecay, Version 2.9.10 (Hypercard stack distributed by the author), Botaniska Institutionen, Stockholm University, Stockholm, 1997.
- [27] J.S. Farris, M. Källersjö, A.G. Kluge, C. Bult, Testing significance of incongruence, *CLADES* 10 (1994) 315–319.
- [28] R.H. Crozier, Y.C. Crozier, The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organisation, *Genetics* 133 (1993) 97–117.
- [29] F.J. Rodríguez, J.L. Oliver, A. Marín, J.R. Medina, The general stochastic model of nucleotide substitution, *J. Theor. Biol.* 142 (1990) 485–501.
- [30] M.A. O'Flynn, The succession and rate of development of blowflies in southern Queensland and the application of these data to forensic entomology, *J. Aust. Entomol. Soc.* 22 (1983) 137–148.
- [31] B. Morris, Physiology and taxonomy of blowflies, MAgSc thesis, University of Adelaide, Adelaide, 1993.
- [32] N. Monzu, Coexistence of carrion-breeding Calliphoridae (Diptera) in Western Australia, Ph.D. thesis, University of Western Australia, Perth, 1977.