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Molecular systematics of Australian carrion-breeding blowflies (Diptera : Calliphoridae) based on mitochondrial DNA

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Abstract. Carrion-breeding blowflies have substantial ecological and forensic importance. Because morphological recognition of their immatures is difficult, sequencing of the mtDNA of these flies may assist with their identification. Molecular phylogenetic analysis based on DNA sequences can also clarify evolutionary relationships. In this study, the mitochondrial genes *CO1*, *CO2*, *ND4* and *ND4L* were sequenced for 34 species of blowflies, among which are almost all species known or suspected to breed in carrion in Australia. The resulting sequences were analysed using parsimony and maximum-likelihood Bayesian techniques. The results showed that the combination of these four genes should identify most species reliably, although some very closely related taxa could still be misdiagnosed. The data also helped clarify the life histories of *Calliphora centralis* Malloch, 1927, *C. fuscofemorata* Malloch, 1927 and *C. gilesi* Norris, 1994, which have hitherto only been suspected carrion breeders, and revealed that the current subgeneric assignment of taxa within *Calliphora* Robineau-Desvoidy, based on morphology, requires revision. Unexpectedly, both *Chrysomya rufifacies* (Macquart, 1843) and *Lucilia cuprina* (Wiedemann, 1830) were paraphyletic; each probably comprises two distinct species. The application of a molecular-clock approach to the study of the evolutionary divergence of the carrion-breeding blowflies suggests that the speciation of at least the endemic Australian taxa may have been the result of increasing aridification in Australia during the last five million years.

Additional keywords: *Calliphora, Chrysomya*, evolution, forensic entomology, *Hemipyrellia*, *Lucilia*, mtDNA, *Onesia*.

Introduction

Recent papers featuring the molecular phylogenetic analysis of carrion-breeding blowflies (Calliphoridae) have tended to place greater emphasis on the value of DNA for species identification than for exploring evolutionary ancestry (e.g. Wallman and Donnellan 2001; Wells and Sperling 2001; Chen *et al*. 2004). Reliable identification of these flies is important because they play a vital role in nutrient cycling in ecosystems (Putman 1983) and are useful in forensic science for estimating the time since death of victims of violent crime (Catts and Goff 1992; Greenberg and Kunich 2002). Moreover, a molecular approach to the identification of carrion-breeding blowflies is necessary because the immature stages of many species are difficult or impossible to recognise on the basis of their morphology (Wallman 2001). Deoxyribonucleic acid-based identification combines, in a phylogenetic analysis, sequence data from an unidentified specimen with data from specimens with confirmed identity. The identity of the mystery specimen is inferred from its genetic relatedness to the other specimens.

In addition to inferences about species identification, much other useful information can be gleaned from the evolutionary affinities displayed in a DNA phylogeny. Relationships can sometimes suggest the historical biogeography of species, which in the carrion-breeding blowflies can be especially helpful for understanding species with broad economic and medical significance (e.g. Stevens *et al*. 2002). Furthermore, evolutionary relationships may reveal uncertain or unknown biological attributes. There are several blowfly species that are only suspected to be carrion breeders; many blowflies are, in fact, obligate parasites of other animals. The life histories of these suspect taxa could be indicated by their positions in a phylogenetic tree, relative to species whose biology is known (Stevens 2003). There are also many carrion-breeding blowflies whose specific responses to temperature have not been explored. It would be

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interesting to investigate if the likely development rates of poorly known species could be deduced from their phylogenetic position relative to other species whose behaviour has been better studied. Although of ecological and evolutionary interest, such an approach might also be considered to have applied value in forensics, where the time spent by maggots in a corpse is central to the entomological estimation of time since death. However, given the high level of scientific rigour that forensic conclusions require, it would be dangerous to base these on anything other than actual developmental data.

This paper expands on the earlier work of Wallman and Donnellan (2001) and aims to provide a comprehensive set of DNA sequences, across several mitochondrial genes, for virtually all calliphorid species known or suspected to breed in carrion in Australia. Wallman and Donnellan's analysis concentrated on species of *Calliphora* Robineau-Desvoidy (Calliphorinae). These are among the most common carrion-breeding blowflies in south-eastern Australia, as well as the most speciose genus of these flies across the continent as a whole. This study adds sequence data for 11 *Calliphora* species not included in the previous project. Australian *Calliphora* species have been assigned to four subgenera: *Australocalliphora* Kurahashi, *Calliphora* sensu stricto, *Neocalliphora* Brauer & Bergenstamm and *Paracalliphora* Townsend (Kurahashi 1971, 1989).

Other calliphorid genera containing carrion-breeding species are *Lucilia* Robineau-Desvoidy and *Hemipyrellia* Townsend (Luciliinae), and *Chrysomya* Robineau-Desvoidy (Chrysomyinae). These flies predominate in the tropical north of the continent. Many of the species recorded from northern Australia are found even further north in Asia and other parts of the Old World. Some are also known from the Americas. Overall, 13 species of *Lucilia*, *Hemipyrellia* and *Chrysomya* were added in this new study.

Deoxyribonucleic acid sequences for all species were obtained for the cytochrome oxidase subunit 1 and 2 genes (*CO1* and *CO2*), since these were used in the previous study. Two further, less conserved genes, NADH dehydrogenase subunits 4 and 4L (*ND4* and *ND4L*), were added because very close genetic relationships had earlier been found between certain species pairs, such as *Calliphora augur* (Fabricius, 1775) and *Calliphora dubia* (Macquart, 1855). It was expected that the extra sequence data from *ND4* and *ND4L* would give more certain separation of these taxa. The *ND4* gene has already been applied successfully to the discrimination of various species of flies of other families (Tang *et al*. 1995; Yu *et al*. 1999), as well as beetles (Szalanski and Powers 1996; Huang *et al*. 2001).

To obtain additional insights into the evolution of Australian carrion-breeding blowflies, we employed a relaxed molecular-clock approach to infer the estimates of divergence times of the various taxa outlined above. The method of producing a linearised phylogenetic tree (e.g. Sanderson 1997) avoids the frequently criticised assumption that rates of molecular evolution are constant across all branches in a phylogenetic tree (Sorhannus and Van Bell 1999).

The overall aim of this work was to produce a phylogenetic dataset that should enable the recognition of almost any unidentified calliphorid maggot collected from carrion in Australia, while also clarifying the evolutionary relationships and life histories of Australian blowflies.

Materials and methods

Taxon sampling

Thirty-four species of blowflies (Calliphoridae) belonging to the genera *Calliphora*, *Chrysomya*, *Lucilia*, *Hemipyrellia* and *Onesia* Robineau-Desvoidy were included as ingroup taxa in our analyses. All species are confirmed carrion breeders, other than *Onesia tibialis* (Macquart, 1846), *Calliphora sternalis sternalis* Malloch, 1932, *Calliphora centralis* Malloch, 1927, *Calliphora fuscofemorata* Malloch, 1927 and *Calliphora gilesi* Norris, 1994. These species are frequently found at carrion, but *O. tibialis* and *C. sternalis sternalis* are confirmed parasitoids of earthworms (Norris 1991; K. R. Norris personal communication) and the reproductive habits of the other three are uncertain. Until recently, the breeding habits of two other of the ingroup taxa, *Calliphora fulvicoxa* Hardy, 1930 and *Calliphora macleayi* Malloch, 1927, were also uncertain. However, it is now known that adults of both species have been reared from vertebrate carrion in the Brisbane area (Queensland, Australia) (A. McDowell, personal communication). Hardy (1937) had earlier stated that *C. fulvicoxa* had been shown to oviposit on carrion in the laboratory.

We used two Australian *Sarcophaga* Meigen species (Sarcophagidae: flesh flies) as outgroup taxa. The Sarcophagidae are close relatives of the Calliphoridae, being placed with them in the superfamily Oestroidea (Rognes 1997). McAlpine (1989) considered the Calliphoridae and Sarcophagidae to be the two major components of a subgroup of the Oestroidea that has its origins in coprophagy, but which has become secondarily either sarcophagous or parasitic.

The species used for the analyses in this paper are presented in Table 1, along with locality data, voucher numbers of the Australian Biological Tissue Collection (ABTC) (South Australian Museum) and GenBank accession numbers.

Deoxyribonucleic acid extraction

Flies were stored at either –80°C or in 100% ethanol. Deoxyribonucleic acid was usually extracted from the legs of the flies, using DNAzol® (Molecular Research Centre Inc., Cincinnati, OH, USA) and following the protocol of Chomczynski *et al*. (1997), with slight modifications. Before centrifugation, the homogenate was incubated at 55°C for two hours with proteinase K (400 μg mL^{-1}), after which DNA was precipitated overnight at –20°C with 100% ethanol. After removal of the legs, the remainder of each fly's body was kept as a voucher specimen. These vouchers are preserved in the Diptera collection in the School of Biological Sciences at the University of Wollongong.

Polymerase chain reaction (PCR) amplification and nucleotide sequencing

Three regions of the mitochondrial genome were amplified and sequenced using PCR methods. An 822-bp region of the 3′ end of the *CO1* gene was amplified using primers M202 (forwards, C1-J-1751, Simon *et al*. 1994) and M70 (reverse, UEA10, Lunt *et al*. 1996). A 638-bp fragment of the *CO2* gene was amplified using the primers M283 (forwards, C2-J-3138, Simon *et al*. 1994) and M284 (reverse, TK-N-3775, Bogdanowicz *et al*. 1993). Comparisons among complete mitochondrial sequences of Diptera (*Cochliomyia*, *Ceratitis*,

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

Species	ABTC no.	GenBank accession numbers			Collection locality
Calliphorinae		*CO1*	*CO2*	*ND4–ND4L*	
Calliphora albifrontalis	3398	AY012546	AY012560	AY842681	Dudley Conservation Pk, SA (35°48′S, 137°51′E)
	3419	AY012547	AY012561	AY842681	Warrenben Conservation Pk, SA (35°09′S, 137°06′E)
C. augur	78509	AY842579	AY842634	AY842682	University of Wollongong, NSW (34°24′S, 150°53′E)
	3187	AY012542	AY012555	AY842683	The University of Adelaide, SA (34°55′S, 138°36′E)
	78472	AY842580	AY842635	AY842684	Coranderrk Bushland Reserve, Vic. (37°40′59″S, 145°31′04″E)
C. centralis	78508	AY842581	AY842636	AY842685	Bawley Point, NSW (35°29′S, 150°23′E)
C. dubia	3205	AY842582	AY012554	AY842688	Cooranga Homestead, SA (36°50′S, 140°17′E)
	3169	AY012540	AY012552	AY842686	Dudley Conservation Pk, SA (35°48′S, 137°51′E)
	3179	AY012541	AY012553	AY842687	The University of Adelaide, SA (34°55′S, 138°36′E)
	78486	AY842583	AY842637	AY842689	Penong, SA (31°59′109″S, 132°57′263″E)
	78485	AY842584	AY842638	AY842690	Cocklebiddy, WA (32°02′S, 126°05′E)
	78488	AY842585	AY842639	AY842691	Mt Gibson, WA (29°31′212″S, 117°19′233″E)
C. fallax	78454	AY842586	AY842640	AY842692	Halfway Creek, NSW (30°14′S, 153°06′E)
C. fulvicoxa	78480	AY842587	AY842641	AY842693	North Brother, NSW (31°39′S, 152°47′E)
C. fuscofemorata	78504	AY842588	AY842642	AY842694	Kuranda, Qld (16°49′S, 145°38′E)
C. gilesi	78482	AY842589	AY842643	AY842695	Penong, SA (31°59′109″S, 132°57′263″E)
C. hilli hillli	3152	AY842590	AY012565	AY842696	Black Mountain, ACT (35°16′S, 149°06′E)
	3135	AY842590	AY012564	AY842696	Cooranga Homestead, SA (36°50′S, 140°17′E)
	78460	AY842591	AY842644	AY842697	Seaford Reserve, Vic. (38°06′S, 145°07′E)
C. macleayi	78453	AY842592	AY842645	AY842698	Halfway Creek, NSW (30°14′S, 153°06′E)
C. maritima	3244	AY842593	AY012562	AY842699	Cape Buffon, SA (37°33′S, 140°06′E)
	78462	AY842594	AY842646	AY842700	Cape Liptrap, Vic. (38°54′S, 145°55′E)
C. nigrithorax	78445	AY842595	AY842647	AY842701	Coranderrk Bushland Reserve, Vic. (37°40′59″S, 145°31′04″E)
C. ochracea	78422	AY842596	AY842648	AY842702	Halfway Creek, NSW (30°14′S, 153°06′E)
C. sp. nov.	78478	AY842597	AY842649	–	The University of Adelaide, SA (34°55′S, 138°36′E)
C. sternalis sternalis	78521	AY842598	AY842650	AY842703	University of Wollongong, NSW (34°24′S, 150°53′E)
C. stygia	78522	AY842599	AY842652	AY842704	University of Wollongong, NSW (34°24′S, 150°53′E)
	78484	AY842600	AY842651	AY842706	Penong, SA (31°59′109″S, 132°57′263″E)
	3371	–	AY012559	AY842705	Spring Gully Conservation Park, SA (33°54′S, 138°36′E)
	78475	AY842601	AY842653	AY842707	Seaford Reserve, Vic. (38°06′S, 145°07′E)
C. varifrons	78476	AY842602	AY842654	–	Perth, WA (31°57′S, 115°51′E)
C. vicina	71190	AY842603	AY012566	AY842708	The University of Adelaide, SA (34°55′S, 138°36′E)
	78464	AY842604	AY842655	AY842709	Seaford Reserve, Vic. (38°06′S, 145°07′E)
Onesia tibialis	71192	AY842605	AY012568	AY842710	The University of Adelaide, SA (34°55′S, 138°36′E)
Chrysomyinae					
Chrysomya flavifrons	78506	AY842615	AY842664	AY842720	Kuranda, Qld (16°49′S, 145°38′E)
Ch. incisuralis	78440	AY842616	AY842665	AY842721	Halfway Creek, NSW (30°14′S, 153°06′E)
	78512	AY842617	AY842666	AY842722	Kuranda, Qld (16°49′S, 145°38′E)
Ch. latifrons	78502	AY842618	AY842667	AY842723	Halfway Creek, NSW (30°14′S, 153°06′E)
Ch. megacephala	78456	AY842619	AY842668	AY842724	Karuah, NSW (32°38′S, 151°57′E)
Ch. nigripes	78496	AY842620	AY842669	AY842725	Kuranda, Qld (16°49′S, 145°38′E)
Ch. rufifacies	78518	AY842621	AY842670	AY842726	North Brother, NSW (31°39′S, 152°47′E)
	78516	AY842622	AY842671	AY842727	Kuranda, Qld (16°49′S, 145°38′E)
	3426	AY842623	AY012548	AY842728	Dudley Conservation Pk, Kangaroo I., SA (35°48′S, 137°51′E)
	78471	AY842624	AY842672	–	Seaford Reserve, Vic. (38°06′S, 145°07′E)
Ch. saffranea	78500	AY842625	AY842673	AY842729	Kuranda, Qld (16°49′S, 145°38′E)
Ch. semimetallica	78458	AY842626	AY842674	AY842730	Halfway Creek, NSW (30°14′S, 153°06′E)
	78519	AY842627	AY842675	AY842731	Kuranda, Qld (16°49′S, 145°38′E)
Ch. varipes	78468	AY842628	AY842676	AY842732	Karuah, NSW (32°38′S, 151°57′E)
	78524	AY842629	AY842677	AY842733	Kuranda, Qld (16°49′S, 145°38′E)
	3602	AY842630	AY012550	AY842734	Dudley Conservation Pk, SA (35°48′S, 137°51′E)
	78466	AY842631	AY842678	AY842735	Coranderrk Bushland Reserve, Vic. (37°40′59″S, 145°31′04″E)
Luciliinae					
Hemipyrellia fergusoni	78450	AY842613	AY842662	AY842718	Halfway Creek, NSW (30°14′S, 153°06′E)
H. ligurriens	78494	AY842614	AY842663	AY842719	Kuranda, Qld (16°49′S, 145°38′E)

(*continued next page*)

Table 1. (continued)

Species	ABTC no.	GenBank accession numbers			Collection locality
L. papuensis	78498	AY842609	AY842659	AY842714	Kuranda, Qld (16°49′S, 145°38′E)
L. porphyrina	78448	AY842610	AY842660	AY842715	Halfway Creek, NSW (30°14′S, 153°06′E)
	78513	AY842611	–	AY842716	Kuranda, Qld (16°49′S, 145°38′E)
L. sericata	78446	AY842612	AY842661	AY842717	Seaford Reserve, Vic. (38°06′S, 145°07′E)
Sarcophagidae					
Sarcophaga impatiens	78526	AY842631	AY842679	AY842736	Coffs Harbour, NSW (30°18′S, 153°07′E)
S. praedatrix	78527	AY842632	AY842680	AY842737	Coffs Harbour, NSW (30°18′S, 153°07′E)

ABTC, Australian Biological Tissue Collection. A dash indicates that the gene region was unable to be sequenced.

Chrysomya, *Drosophila* and *Anopheles*) from GenBank showed that the *ND4* and *ND4L* genes had the least number of conserved nucleotide sites (63.8%) compared to *CO1* (70.8%), *CO2* (65.5%) or *ND5* (65.7%). Therefore, additional PCR and sequencing primers were developed in conserved sites for almost the complete *ND4* and *ND4L* genes (Table 2). The primer combinations M377–M378 and M379–M380 successfully amplified 884 bp and 968 bp, respectively, in most of the taxa, and have an overlap of ~198 bp in the middle of the *ND4* gene. Primers M430–M432 were developed internally of M377 and M380 in order to amplify gene fragments in some specimens that did not amplify with one or both of the original primer pairs.

Standard PCR amplifications included 1× reaction buffer (Perkin Elmer, Boston, MA, USA), 0.2 mM of each dNTP, 5 pM of each primer, 1 unit of Amplitaq Gold (Perkin Elmer) and 2 mM of $MgCl_2$ in a 50-μL reaction volume. PCR amplifications were carried out on either an OMN-E 500 (Hybaid Ltd, Ashford, UK) thermal cycler or a PC-960G Gradient thermal cycler (Corbett Research, Mortlake, NSW, Australia) for 1 cycle of 92°C for 9 min and 36 cycles (94°C, 45 s; 48–55°C, 45 s; and 72°C, 60 s), followed by a final incubation step at 72°C for 6 min. PCR products were purified using the UltraClean™ PCR DNA purification kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol.

Sequencing was performed using the ABI Prism™ Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in 20-μL or 10-μL reaction volumes according to the manufacturer's instructions. PCR primers were used as sequencing primers and each fragment was sequenced on both strands. Reaction products were purified by isopropanol precipitation (as specified by Applied Biosystems) and sequenced on ABI 3700 (version 3.7) automated DNA sequencers. SeqEd, version 1.0.3 (Applied Biosystems), was used to edit chromotogram files, to determine a consensus of bi-directional sequencing and to manually align sequences across specimens.

Sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/; accession numbers are listed in Table 1).

Sequence analyses

Phylogenetic analyses of aligned sequence data were carried out using the programs PAUP*, version 4.0b8 (Swofford 2001) and MrBayes, version 2.01 (Huelsenbeck and Ronquist 2001). The mitochondrial datasets, *CO1, CO2* and *ND4–ND4L*, are considered to be of one locus and were thus analysed together. Homogeneity of base frequencies among taxa for each gene region was tested using χ^2 analyses in PAUP*. Maximum parsimony (MP) analyses were performed using heuristic searches with 200 random additions of sequences to search for the most parsimonious trees from different islands of trees. Bootstrapping (Felsenstein 1985), with 1000 pseudo-replicates and the heuristic search option, was used to examine the robustness of nodes in the resulting tree. Maximum-likelihood (ML) analyses were carried out using MrBayes.

To find the most appropriate model of molecular evolution for our dataset, a series of nested likelihood-ratio tests were performed on the combined dataset, using MODELTEST 3.06 (Posada and Crandall 1998) and PAUP*. However, rather than a Jukes–Cantor tree, ML scores from different evolutionary models were calculated using the tree resulting from our MP analysis. The significance of the likelihood ratios of different pairs of models was tested against a χ^2 distribution (Posada and Crandall 1998).

High A–T bias in third codon positions is a general phenomenon in insects, especially in Diptera (e.g. Lessinger *et al*. 2000) and Hymenoptera (e.g. Crozier and Crozier 1993). Such bias may be the result of directional mutations causing increased transition rates (Leys *et al*. 2002), which may lead to an increase in homoplasies that act to obscure phylogenetic relationships (Leys *et al*. 2000). The occurrence of directional mutations was investigated by comparing analyses using either the general time reversible model of sequence evolution (GTR, six substitution types) (Rodríguez *et al*. 1990) or a non-reversible model of sequence evolution (12 substitution types). In each case, three site-specific rate parameters were used for the first, second and third codon positions of the three gene regions combined, and nine site-specific rate

Table 2. *ND4–ND4L* primers used in this study

Name	*ND4–ND4L* primers	Position
M377 R	5′-CTTATCATTGACACCACAAAT-′3	8159 in tRNA-^{HIS}
M378 F	5′-CAACCTGAACGATTACAAGC-′3	9148
M379 R	5′-CTAAAATTATCGAACCAGAA-′3	8887
M380 F	5′-GGAGTATTTACTTTTGTGTCTA-′3	9787
M429 F	5′-GAAACTTTATTATCTATATTATTA-′3	Not used
M430 R	5′-GAAACAGGAGCTTCAACAT-′3	8904
M431 F	5′-GCAGGTGTTTATTTATTATT-′3	9120
M432 R	5′-TAAAGGAAATCAATGTAAAA-′3	8249

F, Forwards; R, reverse. 'Position' refers to the 5′ position of the primers in the *Drosophila melanogaster* (NC001709) genome.

parameters for each codon position for each of the three gene regions treated separately.

Bayesian analyses were performed using one cold and three heated chains, which were run for 200 000 generations to explore the effect of parameter use, and for 1 000 000 generations for the final analyses. Trees were sampled every 10 generations. Summarisation of tree topologies and parameter values was done with the first 10 000 trees discarded.

Estimation of divergence times

The phylogram resulting from the Bayesian analysis was used as the preferred tree topology for estimation of divergence times.

The first step in the process of obtaining divergence times for nodes in the tree was to transform the phylogram into a linearised tree, with all tip nodes having the same age. The easiest way to do this was to assume that the molecular rates of evolution in all branches of the topology were behaving as a true molecular clock. However, because molecular datasets are hardly ever truly clock-like (Wu and Li 1985; Muse and Weir 1992; Sorhannus and Van Bell 1999), we first tested whether our dataset would meet the assumption of a molecular clock using a likelihood-ratio test in the program MODELTEST (Posada and Crandall 1998). In such a test, the null hypothesis is the evolutionary model with a global uniform molecular clock, and the alternative hypothesis is the same model with a relaxed molecular clock, ticking at different rates across the phylogeny. On rejection of a global molecular clock, a semi-parametric method (Sanderson 2002*a*) was used to linearise the tree topology. This method allows evolutionary rates to vary between branches, within certain limits, using a penalised-likelihood function. This function includes a roughness penalty (that increases when rate differences in adjacent branches across a tree are large) and a smoothing parameter (which controls the trade off between smoothness and goodness of fit in the model). Cross-validation procedures for finding the optimal smoothing parameters were performed by giving the age of the root of the tree an arbitrary value of 100 million years before present. The program r8s, version 1.05b (Sanderson 2002*a*, 2002*b*), was used to carry out the above-mentioned procedures. The program estimates divergence times and molecular rates when the age of at least one of the nodes is given. Analysis using r8s was performed using the penalised-likelihood method and the quasi-newton algorithm, using 2000 iterations repeated 10 times using different random combinations of initial divergence times.

In order to constrain the age of nodes in a linearised tree, it is preferable to use data from the fossil record. Unfortunately, the only fossil adult calliphorid to have thus far been reported, *Cretaformia fowleri* McAlpine, 1970, cannot in fact be reliably assigned to the family Calliphoridae (Erzinçlioğlu 1984; Zherikhin 2002). Therefore, in the absence of a fossil record for the blowflies, we used the preliminary molecular clock for arthropod mtDNA, with an evolutionary rate of 2.3% pairwise sequence divergence per million years (Brower 1994) (equivalent to a rate of 0.0115 substitutions per site per million years) for calibrating the divergence times of the nodes. This rate is often used for testing hypotheses involving relatively recent divergences (Knowles 2000; Trewick and Wallis 2001) and it might give reasonable estimates of divergence times of Australian blowflies. The divergence times of the nodes were calibrated using an iterative approach by adjusting the age of the root (Leys *et al*. 2003) until an average rate of substitution was found comparable to the preliminary arthropod mtDNA clock. Ninety-five per cent confidence intervals for divergence times were then calculated for nodes using a cutoff value of four (Cutler 2000), following the methods in r8s (Sanderson 2002*b*).

Results

Sequencing of the *CO1*, *CO2* and *ND4–ND4L* mitochondrial gene fragments resulted in 3008 aligned nucleotide sites. Table 3 shows the number of characters, number of parsimony informative characters, nucleotide frequencies, nucleotide bias and overall χ^2 values of tests for homogeneity of base frequencies across taxa for nine different data partitions. Base composition bias among taxa was found to be significant only for third codon positions of *ND4*. Most of the heterogeneity among taxa was a result of the A–T bias of *ND4* third codon positions, which was lowest in the outgroup species *Sarcophaga impatiens* Walker, 1849 (88.2%) and highest in *O. tibialis* (95.0%). In general, A–T bias was lower in *Calliphora* species and higher in *Chrysomya* and *Lucilia* species, although exceptions were found in all three major clades: *Calliphora ochracea* Schiner, 1868 (94.7%), *Chrysomya incisuralis* (Macquart, 1851) (91.4%) and *Lucilia porphyrina* (Walker, 1856) (91.0%).

Table 3. Relative numbers of parsimony informative characters (Pars. inform.), the probability (*P*) of the χ^2 test of nucleotide bias among taxa, the frequency of nucleotide bases and A-T bias among taxa, and site-specific codon rates (calculated for each gene separately as well as for the three gene regions combined)

Codon position	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Gene	*CO1*	*CO1*	*CO1*	*CO2*	*CO2*	*CO2*	*ND4–ND4L*	*ND4–ND4L*	*ND4–ND4L*
No. characters	274	274	274	213	213	212	516	516	516
Pars. inform.	45	12	200	39	6	152	89	37	346
χ^2	17.668	6.650	128.543	15.972	5.891	153.237	32.928	41.666	236.570
P	1.000	1.000	1.000	1.000	1.000	0.962	1.000	1.000	**0.007**
Frequency A	0.277	0.205	0.458	0.306	0.271	0.438	0.298	0.189	0.429
Frequency C	0.143	0.245	0.084	0.165	0.183	0.084	0.080	0.149	0.009
Frequency G	0.268	0.144	0.017	0.231	0.128	0.015	0.179	0.168	0.676
Frequency T	0.312	0.407	0.441	0.298	0.418	0.463	0.443	0.493	0.494
% A-T bias	58.9	61.2	89.9	60.4	68.9	90.2	74.1	68.3	92.3
Codon rates*	0.166	0.021	2.815						
variance	0.000	0.000	0.000						
Codon rates	0.326	0.025	3.314	0.365	0.041	1.316	0.208	0.057	2.910
variance	0.003	0.000	0.084	0.006	0.000	0.058	0.001	0.000	0.004

*Three gene regions combined.

Apparently, there was no clear phylogenetic structure in the distribution of the bias. The high A–T bias, found for third positions, may have been the result of directional mutations, but the cause of the differences between species is unknown.

Phylogenetic analysis

Maximum-likelihood analyses were performed using Bayesian methods in the program MrBayes. We compared the tree topology and the likelihood value of the resulting tree from several analyses performed using different parameter settings (six or 12 substitution types and three or nine site-specific rate partitions). The four explorative analyses (each with a different combination of site-specific rate partitions and substitutions) resulted in tree topologies that differed only with respect to the placement of *Lucilia papuensis* Macquart, 1843, which was either the sister-group of *L. porphyrina* or the sister-group of all other *Lucilia* species.

The greater the number of parameters possessed by each model, the greater the number of generations it took for the likelihood values to become stable. Although the likelihood values at stability were very similar, average values for the analysis using three site-specific rate partitions were higher than for the analysis using nine partitions, and using the non-reversible model returned a higher average likelihood in comparison with the GTR model. We therefore used three subspecific rate partitions and 12 substitution types for the final analysis. A summary of the site-specific rates is presented in Table 3. The estimated nucleotide substitution rates for the GTR model (six substitution types) and the non-reversible model (12 substitution types) are shown in Table 4. As expected for taxa with considerable A–T bias, transition rates (A↔G, C↔T) are much increased, but substitutions resulting in A (adenine) or T (thymine) appear to have much higher rates than mutations in the opposite direction.

The tree resulting from the phylogenetic analysis using the Bayesian approach is shown in Fig. 1, with posterior probabilities for the nodes given in percentages. The same tree topology was also produced by maximum parsimony analysis. Parsimony analysis with 200 random additions of sequences resulted in a single island with 46 most parsimonious trees (tree length = 4357, *CI* = 0.375, *RI* = 0.679). In general, the MP tree was well supported by bootstrap values: most branches in *Chrysomya* were resolved, as well as several closely related species in *Calliphora* and *Lucilia*. Although parsimony bootstrapping did not support the relative position of the genera *Calliphora* (Calliphorinae) and *Hemipyrellia* and *Lucilia* (Luciliinae) (probably as a result of the above-mentioned A–T bias in *ND4*), the relationships of these groups were resolved using the Bayesian analysis. In almost all cases, nodes that are supported by bootstrap values larger than 50% show posterior probabilities of 100% or very close to 100%. Most of the uncertainty in the topology of the tree is found in the placement of *L. papuensis*. Overall, as there is very little uncertainty in the tree topology, this mitochondrial gene tree might be considered as a reliable framework for systematic and evolutionary research.

Separate analysis of the *CO1* + *CO2* and *ND4–ND4L* datasets largely resulted in the same tree topology, with the only difference being that the deeper nodes in both topologies were not supported by bootstrap values (values <50%).

Table 4. General time-reversible (6 ST) and general non-reversible nucleotide substitution rates (12 ST)

6 ST	A	C	G	T
A	–	1.14	24.73	1.61
C	1.14	–	2.44	37.06
G	24.73	2.44	–	1.00
T	1.61	37.06	1.00	–

12 ST*	A	C	G	T
A	–	0.34	3.75	1.33
C	0.57	–	0.36	31.10
G	18.66	1.16	–	1.00
T	0.31	4.48	0.23	–

*Recalculated by setting G→T rate at 1.0.

Relationships between tree topology and taxonomy and life history

The tree shown in Fig. 1 confirms the monophyly of the three subfamilies included in the analysis: Calliphorinae, Luciliinae and Chrysomyinae. Each of these groups has high posterior probability and/or bootstrap support. Within the Luciliinae, there is also support for the separate monophyletic status of the two main sister-groups, *Lucilia* and *Hemipyrellia*. Our molecular analysis shows a sister-group relationship between the Calliphorinae and Luciliinae. This does not accord with the most comprehensive phylogenetic treatment of the Calliphoridae based on morphology (Rognes 1997), which instead identified the Calliphorinae and Chrysomyinae as sister-groups.

Despite the high overall stability of the phylogenetic tree shown in Fig. 1, there are some individual clades with only a slight degree of genetic distance separating their taxa. This suggests that the divergence within these clades has occurred only very recently (see next section on divergence times). Across the whole tree, minimum pairwise distances between sister clades (at species level) are generally greater than 0.8%. However, in three notable cases the minimum distances are much smaller than this: the *Calliphora hilli*-group (four species), 0.1%; *Calliphora albifrontalis* Malloch, 1932 + *Calliphora stygia* (Fabricius, 1782), 0.1% (Calliphorinae); and *Chrysomya megacephala* (Fabricius, 1794) + *Chrysomya saffranea* (Bigot, 1877), 0.4% (Chrysomyinae) (Table 5). This genetic closeness reflects the morphological similarity between the species in each of these groups. Moreover, Monzu (1977) showed that *C. albifrontalis*/*C. stygia* and *Calliphora hilli hilli* Patton, 1925/*Calliphora*

varifrons Malloch, 1932 produce fertile F1 and F2 hybrids in the laboratory. Nonetheless, the species in each group are regarded as valid (Kurahashi 1982; Wallman and Adams 1997), and reliable morphological characters exist, at least in the adults, enabling their separation.

In some parts of the tree in Fig. 1, the position of taxa and individuals does not always accord with the current taxonomic treatment of the Calliphoridae. In *Chrysomya*, there is a relatively large degree of sequence divergence (more than 4%) between *Chrysomya rufifacies* (Macquart, 1843) specimens from South Australia/Victoria and New South Wales/Queensland (Table 5). This divergence is much greater than that between specimens within either of the two populations (maximum of ~0.1%). This difference suggests that these populations may represent two distinct species.

Within the genus *Lucilia*, the specimens of *Lucilia cuprina* (Wiedemann, 1830) from New South Wales and Queensland show greater affinity with *Lucilia sericata* (Meigen, 1826) than with the *L. cuprina* specimen from Western Australia (minimum divergence: ~0.8% *v.* ~2%) (Table 5). Again, the divergence between eastern and western populations of *L. cuprina* is consistent with them representing separate species.

Within *Calliphora*, none of the three polytypic subgenera (*Australocalliphora*, *Neocalliphora* and *Paracalliphora*) are monophyletic on the basis of these data. This is indicated in Fig. 1 by the subgeneric placement of the *Calliphora* species on the basis of their most recent taxonomic treatment (Kurahashi 1989). Kishino–Hasegawa tests, constraining the subgenera as monophyletic, produced trees with significantly more steps (*Neocalliphora* and *Paracalliphora*: tree length difference = 88, $t = 5.7101$, $P < 0.0001$; *Paracalliphora*: tree length difference = 65, $t = 4.3856$, $P < 0.0001$; *Neocalliphora*: tree length difference = 79,

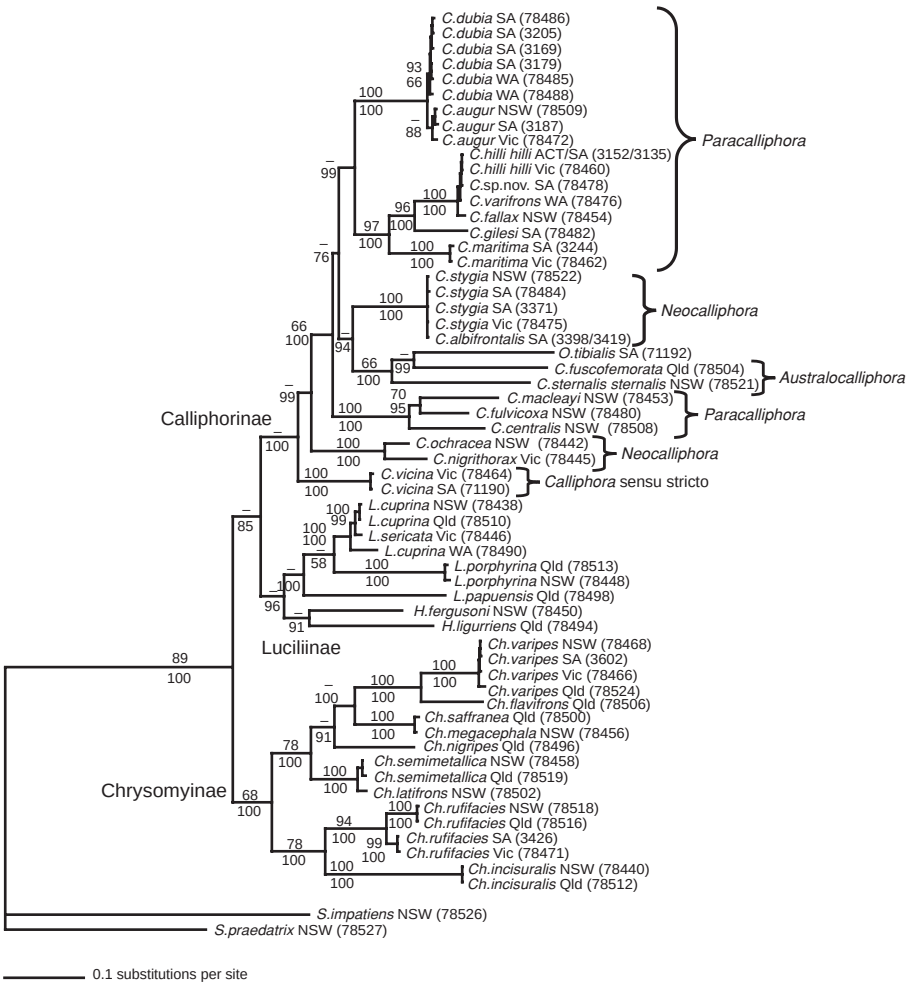

Fig. 1. Maximum-likelihood phylogram showing relationships among mitochondrial *CO1*, *CO2* and *ND4–ND4L* sequences from blowflies (ingroups) and flesh flies (outgroups). Numbers above branches refer to bootstrap proportions among 1000 maximum parsimony pseudo-replicates (>50%) and numbers below the branches to posterior probabilities. Australian Biological Tissue Collection (ABTC) numbers from Table 1 are given in parentheses.

$t = 5.0266$, $P < 0.0001$), indicating that paraphyly of these subgenera is strongly supported. *Australocalliphora*, represented here by *C. fuscofemorata* and *C. sternalis sternalis*, is associated in the tree with *O. tibialis*. There is, therefore, a strong likelihood that *O. tibialis* is a *Calliphora* incorrectly assigned to *Onesia*. The good bootstrap and posterior probability support for the clade containing these three species also makes it very likely that *C. fuscofemorata* (whose biology is unknown) is, in fact, an earthworm parasitoid like *C. sternalis sternalis* and *O. tibialis*.

Within the subgenus *Neocalliphora*, the four species included in the analysis are split into two clades. One, comprising *C. albifrontalis* + *C. stygia* (the *Calliphora stygia*-group of Hardy (1937)), is linked to the clade comprising the *Australocalliphora* species and *O. tibialis*. The other clade comprises *Calliphora nigrithorax* Malloch, 1927 + *C. ochracea* (the *C. ochracea*-group of Hardy (1937)). There is good support for a sister-group relationship between this clade and the other endemic Australian *Calliphora* species.

The subgenus *Paracalliphora* is divided into three distinct clades: (*1*) the *C. augur*-group (*C. augur* and *C. dubia*), recognised by Hardy (1937) and Wallman and Adams (1997); (*2*) the *C. hilli*-group, again recognised by Hardy (1937) (as the *C. rufipes*-group) and Wallman and Adams (1997) (*C. hilli hilli*, *Calliphora* sp. nov. (an undescribed form), *C. varifrons* and *Calliphora fallax* Hardy, 1930) + *Calliphora maritima* Norris, 1994 + *C. gilesi*; and (*3*) *C. macleayi* + *C. fulvicoxa* + *C. centralis*. Since *C. gilesi* is the sister-group of confirmed carrion-breeding blowflies in our phylogram (the *C. hilli*-group), it too is very likely also a carrion breeder. Interestingly, despite *C. macleayi* and *C. centralis* being more similar to each other morphologically than to *C. fulvicoxa*, *C. fulvicoxa* nonetheless forms the sister-group of *C. macleayi*. In light of its placement within this third clade of *Paracalliphora*, *C. centralis* is probably also a carrion breeder.

Calibration and estimation of divergence times

As mentioned in the Introduction, we were also interested in examining whether the phylogenetic data could suggest when the various calliphorid taxa had diverged from each other. The phylogenetic tree in Fig. 1 was transformed into an ultrametric tree using semi-parametric rate smoothing (Sanderson 2002*a*) incorporated in the program r8s. The optimal smoothing parameters, as determined by cross validation, appeared to have a value of 1.0, which indicates that most branches in the trees have their own evolutionary rates. The ultrametric tree was calibrated by substituting different ages for the root node until an average rate for all the branches in the tree was equal to the invertebrate mtDNA clock rate of 0.0115 substitutions per site per million years (Brower 1994).

The age of the root node, the node separating the Chrysomyinae clade from the Luciliinae and Calliphorinae clades, was estimated at 21.7 million years ago (mya) (Fig. 2). Based on this estimate, divergence of the Calliphorinae and Luciliinae occurred about two million years later, whereas divergences within genera occurred mostly between about one and 15 mya. Within-species divergences occurred mostly less than 0.5 mya. However, some speciation events also occurred that recently, these being in the above-mentioned *C. hilli*-group, and in the clades *C. albifrontalis* + *C. stygia*, and *Ch. megacephala* + *Ch. saffranea*.

Table 5. Percentage uncorrected sequence divergence between selected sister clades for the combined *CO1*, *CO2*, *ND4* and *ND4L* genes

	Minimum	Maximum
C. augur v. C. dubia	0.857	1.374
C. fallax v. rest of *C. hilli*-group (three spp.)	0.141	1.098
C. albifrontalis v. C. stygia	0.134	0.572
L. cuprina NSW/Qld *v. L. sericata*	0.805	0.932
L. cuprina NSW/Qld *v.* WA	2.082	2.263
Ch. megacephala v. Ch. saffranea	0.403	0.403
Ch. latifrons v. Ch. semimetallica	1.103	1.365
Ch. rufifacies NSW/Qld *v.* SA/Vic	2.170	4.102

Discussion

Species identification

A key aim of this project was to determine if the *ND4–ND4L* gene segment could improve the genetic diagnosis of *Calliphora* species that were previously shown to be inseparable based only on the *CO2* gene sequence or the combined gene sequences of *CO1* and *CO2* (Wallman and Donnellan 2001). The inclusion of *ND4–ND4L* in the new phylogenetic analysis has assisted somewhat in improving the delineation of *C. augur* and *C. dubia*. However, although the overall statistical support for the phylogenetic tree in Fig. 1 is very good, some clades still have very little sequence variation. This could doubtless make some DNA-based identifications unreliable. An unknown blowfly larva diagnosed as belonging to either *C. albifrontalis* + *C. stygia* or the *C. hilli*-group could certainly still be misidentified, especially since we do not know how much genetic variation exists within the taxa comprising these clades. Overall, therefore, *ND4–ND4L* has not appreciably improved the resolution of these very closely related Australian blowflies.

Nonetheless, most of the other species included in the analysis would be unambiguously identified if an unknown specimen were compared against the total dataset. Exceptions are *Ch. saffranea* and *Ch. megacephala*, which are also very closely related, and *L. cuprina*, whose paraphyletic status is discussed below. As noted by Wallman and Donnellan (2001), there may be no imperative from a forensic perspective to separate genetically very similar species if their biologies are not shown to be significantly different. In

any case, molecular differentiation of such species might still be possible by targeting other variable gene regions, such as *ITS1* and *ITS2* (Walton *et al*. 1999), and by using allozymes, as already done for *Calliphora* species (Wallman and Adams 1997, 2001).

Phylogenetic and biological affinities

The much greater body of sequence data and the broader taxonomic scope of this study, compared with previous work, have helped clarify the relationships among the four genera and three subfamilies included herein. A sister-group relationship between the Calliphorinae and Luciliinae, despite not agreeing with the morphological results of Rognes (1997), does accord with the work of Stevens (2003) in analyses based on *CO1* + *CO2* and *28S* rRNA genes. Rognes (1997) based the morphological association of the Calliphorinae with the Chrysomyinae, to the exclusion of the Calliphorinae, on details of the female terminalia. As Stevens (2003) implies, the molecular data indicate the need to review the morphological characters used to support this relationship.

The results in this paper also provide important new information about the evolution of the individual species and species-groups, which is a significant advance. Based on their phylogenetic affinities, the breeding habits of *C. centralis*, *C. gilesi* and *C. fuscofemorata* are now much more certain. Hardy (1937) placed both *C. centralis* and *C. macleayi* (based predominantly on the similarity of their

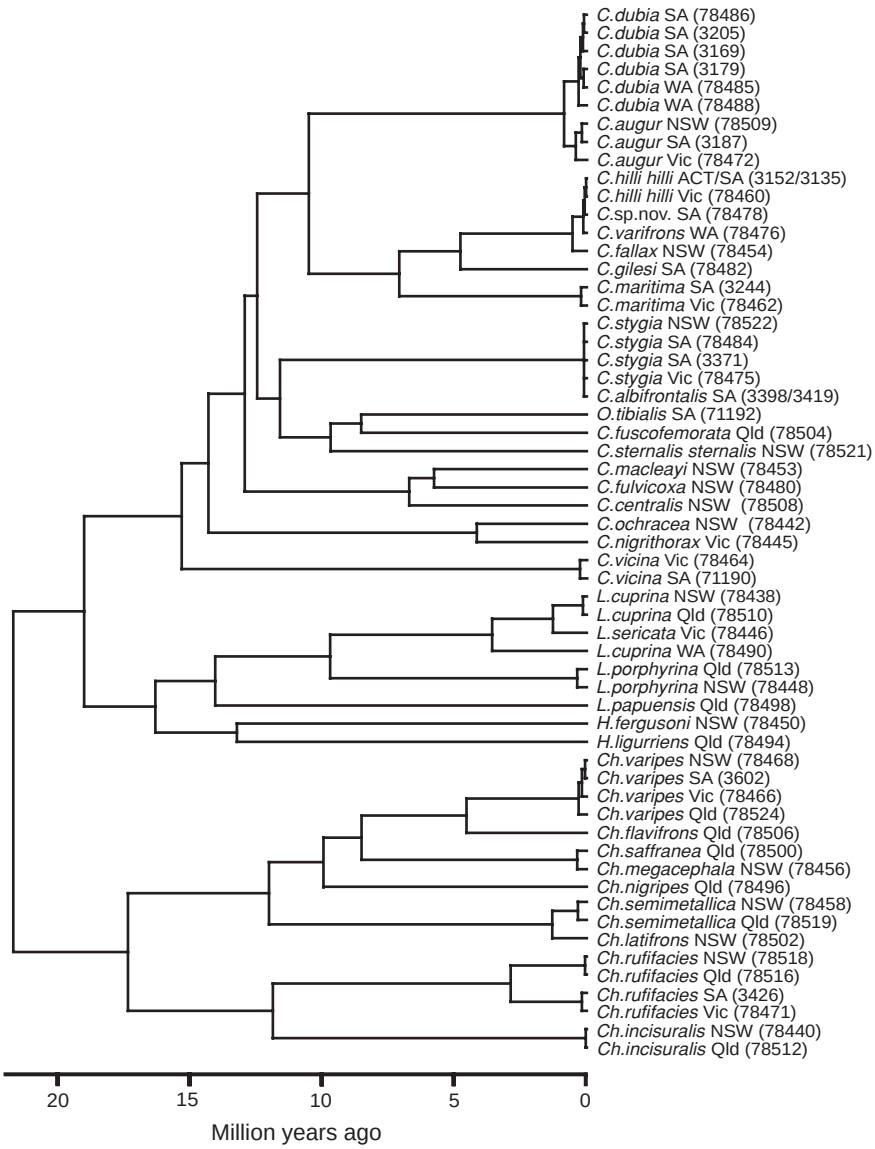

Fig. 2. Linearised phylogenetic tree calibrated with the preliminary mtDNA clock of Brower (1994).

genitalia) in the *C. centralis*-group. However, he placed *C. fulvicoxa* in a separate group, believing that this species had little in common with those of the *C. centralis*-group (he placed the two groups in separate subgenera). Kurahashi (1971) rejected this interpretation, reuniting the three species within the subgenus *Paracalliphora*. Neither he, nor any workers since, suggested the relationship between *C. centralis*, *C. fulvicoxa* and *C. macleayi* revealed by our data.

Calliphora fuscofemorata is also attracted to carrion, at which the senior author has trapped this species, particularly females, in abundance. However, based on the current results, this behaviour is probably associated only with feeding rather than reproduction, as also observed in *O. tibialis* and *C. sternalis sternalis* (Norris 1991; K. R. Norris personal communication). Workers have varied in their views of the evolutionary affinities of *C. fuscofemorata*. Hardy (1937) believed that the species was related to the members of his *C. centralis*- and *C. augur*-groups. By contrast, Patton (1935) considered it a member of his '*canimicans*-group', noting that its genital structure was close to that of *C. sternalis sternalis* (this group corresponds to Kurahashi's (1971) subgenus *Australocalliphora*).

Our conclusion that *O. tibialis* is most likely a *Calliphora* was previously canvassed by Wallman and Adams (1997) in an analysis based on allozyme data. Their assessment of the genetic affinities of *O. tibialis* and certain *Calliphora* species found that *tibialis* fell within *Calliphora* rather than outside it. Although Australian *Onesia* (as interpreted by Kurahashi (1989)) contains numerous species other than *O. tibialis*, several authors have believed that this genus, at least in relation to its Australian representatives, does not warrant generic status at all (e.g. Bezzi 1927; Malloch 1932; Hardy 1937; Norris 1994). Of course, to test this hypothesis, an analysis including a wider selection of *Onesia* species, other than simply *O. tibialis*, would be required. The likely placement of *O. tibialis* within *Calliphora* implies that it is also a member of the subgenus *Australocalliphora*. Of course, if *O. tibialis* really is an *Onesia*, then *Australocalliphora*, being paraphyletic in the current analysis, requires revision. Either way, the clade comprising *O. tibialis*, *C. fuscofemorata* and *C. sternalis sternalis* must have evolved a parasitic life history by independent evolution from a saprophagous ancestor.

There are grounds for a review of Kurahashi's (1971) subgenera of *Calliphora* as a result of the evolutionary relationships suggested by our results. These relationships suggest that, from a strict cladistic perspective, only *Calliphora* sensu stricto is a valid subgenus. *Australocalliphora* has already been mentioned above. Of the other subgenera, *Neocalliphora* is polyphyletic and *Paracalliphora* is paraphyletic. Since the type species of *Neocalliphora* is *C. ochracea*, the clade *C. ochracea* + *C. nigrithorax* should remain within *Neocalliphora*, but the clade *C. stygia* + *C. albifrontalis* must either form part of *Australocalliphora* or *Paracalliphora*, or comprise a new subgenus. The placement in our analysis of *C. ochracea* + *C. nigrithorax* as the most basal clade of the Australian *Calliphora* supports the view of Dear (1986) that these two species, along with the similar New Zealand species *Calliphora quadrimaculata* (Swederus, 1787), constitute 'something near to the ancestral stock of present-day *Calliphora* species'.

The type species of the fourth subgenus included in the analysis, *Paracalliphora*, is *Calliphora oceaniae* Robineau-Desvoidy, 1830 [= *Calliphora augur*]. Thus, the *C. augur*-group clade certainly should remain within *Paracalliphora*, as should possibly also the clade *C. hilli*-group + *C. gilesi* + *C. maritima*. However, the clade *C. macleayi* + *C. fulvicoxa* + *C. centralis* has no place there and ought to be renamed. We do not propose to name this new subgenus here, but suggest that this be deferred until completion of a broader analysis of *Paracalliphora* species, several of which inhabit non-Australian parts of the Australasian and Oceanian region.

Wallman and Adams (1997) suggested that, on the basis of female reproductive anatomy, a new genus of Calliphorinae might be needed to contain *Australocalliphora*, *Paracalliphora* and two other subgenera of *Calliphora* erected by Kurahashi (1971), *Oceanocalliphora* and *Papuocalliphora*. These groups are all ovoviviparous or viviparous, whereas *Neocalliphora*, *Calliphora* sensu stricto and other *Calliphora* species are oviparous. This study does not lend support to the view of Wallman and Adams (1997). Instead, assuming that ovipary in blowflies, at least in the Calliphorinae, is the plesiomorphic condition, associated with a saprophagous origin (Zumpt 1965; Erzinçlioğlu 1989), it seems likely from our results that ovovivipary/vivipary has either evolved independently among Australian members of *Calliphora* at least three times, or evolved once and been subsequently lost in the *C. stygia*-group. Furthermore, as noted above, this evolutionary transition from ovipary to ovovivipary/vivipary has also involved the evolution of parasitism.

The Australian Calliphoridae currently contain five subfamilies in addition to the three included here: Ameniinae, Aphyssurinae, Phumosiinae, Polleniinae and Rhiniinae. As far as is known, all of these are exclusively parasitic. The inclusion in future studies of a broad range of genera from these groups might further elucidate the evolution of parasitism among the Australian blowfly fauna and indicate how frequently it has evolved. However, it must be emphasised that the evolutionary integrity of the family as a whole is still very much in question. The morphological phylogenetic analysis of Rognes (1997), in fact, placed the Ameniinae, Phumosiinae, Polleniinae and Rhiniinae outside a clade containing the remainder of the calliphorid subfamilies, making the Calliphoridae polyphyletic. Even excluding these additional subfamilies, the evolution of parasitism within the Calliphorinae needs further investigation.

Unexpectedly high intraspecific sequence divergences

The phylogenetic data in this paper reveal some very interesting results for the common species *Chrysomya rufifacies* and *Lucilia cuprina*. Both are widespread throughout Australia, but are also found in many other parts of the world. Their geographic origins remain largely unclear.

Chrysomya rufifacies

Chrysomya rufifacies reaches great abundance in the summer months in southern Australia, when it is among the most dominant blowflies in carrion. Its larvae ('hairy maggots') are predatory on the larvae of other species (Baumgartner 1993). Since *Ch. rufifacies* is probably native to Australia (Holdaway 1933), the genetic separation shown by the current results suggest that, since the original spread of its ancestral form, a barrier to gene flow has developed in eastern Australia that has created two separate populations, one in the south-east and the other in the north-east. The calibration of divergence times from our molecular data suggests that the divergence of the two different populations occurred around three mya. These populations now appear, on the basis of more than 4% sequence divergence, to be different species. To test this conclusion, we compared our *Ch. rufifacies* data for the *CO1* + *CO2* gene regions with data for the same regions from a *Ch. rufifacies* specimen from Florida, as well as from a specimen of *Chrysomya albiceps* (Wiedemann, 1819) from Egypt. These additional *Chrysomya* gene sequences are from the work of Wells and Sperling (1999). The relationship of *Ch. albiceps* to populations of *Ch. rufifacies* is of interest because the two species are, on morphological and ecological grounds, extremely similar (Tantawi and Greenberg 1993). Our comparison of the two datasets revealed that *Ch. rufifacies* from Florida is conspecific with *Ch. rufifacies* from South Australia and Victoria (0.07% sequence divergence). However, Australian *Ch. rufifacies* is paraphyletic with reference to *Ch. albiceps*, *Ch. albiceps* being the sister-group of the south-eastern Australian population of *Ch. rufifacies*. *Chrysomya albiceps* shows ~3.4% sequence divergence from the south-eastern Australian population, and 6.4% divergence from the north-eastern population.

This persuasive evidence that *Ch. rufifacies* constitutes two species may be associated with the variation in its reproductive behaviour; it is a primary invader of carrion in some places, e.g. Queensland (O'Flynn and Moorhouse 1979), but a secondary invader in others, e.g. Guam (Bohart and Gressitt 1951). Preliminary inspection of the morphology of specimens from each of the Australian populations did not reveal any notable differences.

Harvey *et al*. (2003), comparing *Ch. rufifacies* populations from Western Australia and Queensland using *CO1* alone, found sequence divergence of <1%. This is consistent with these specimens being of the same species. Since these sequences were very similar to the *CO1* sequence of the American *Ch. rufifacies* specimen mentioned above (comparison done by Harvey *et al*. 2003), and would therefore by default be closest to our South Australian and Victorian specimens, both *Ch. rufifacies* sibling species might occur in Queensland.

Lucilia cuprina

Within *Lucilia*, our data suggest that *L. cuprina* likely also constitutes two species. This is suggested by the genetic distinctiveness of the Western Australian *L. cuprina* specimen, compared with specimens from the eastern states and with *L. sericata*. For many years *L. cuprina* has been known to display morphological variation in different parts of its very wide distribution (Aubertin 1933), but the taxonomic significance of this has never been satisfactorily resolved. Waterhouse and Paramonov (1950) interpreted the variation as evidence for two subspecies, *L. cuprina cuprina* (Wiedemann) and *L. cuprina dorsalis* Robineau-Desvoidy, 1830, but most calliphorid taxonomists have not acknowledged these forms. *Lucilia cuprina cuprina* is coppery red or brassy, with greenish tinges, and occurs in the New World, Asia, Indonesia and Oceania. By contrast, *L. cuprina dorsalis* is mostly bright green and found in Australia, Africa and India (Waterhouse and Paramonov 1950; Norris 1990). Furthermore, the two subspecies are believed to behave quite differently; *L. cuprina cuprina* is synanthropic and therefore concentrated around urban areas, whereas *L. cuprina dorsalis* is a serious agricultural pest, initiating cutaneous myiasis in sheep (Norris 1990). *Lucilia cuprina dorsalis* was probably introduced into Australia from South Africa in the 1860s, with later, separate invasions of the east coast of Australia, again by flies of African provenance (Waterhouse and Paramonov 1950; Norris 1990). Norris (1990) presented evidence for occasional introductions of *L. cuprina cuprina* into eastern Australia from areas of the Pacific. Norris (1990) also showed that *L. cuprina cuprina* and *L. cuprina dorsalis* probably hybridise in eastern Australia, but they have not been known to interbreed elsewhere.

Morphological examination of the *L. cuprina* specimens included in our study revealed characteristics typical of *L. cuprina dorsalis* in the Western Australian specimen, whereas the flies from New South Wales and Queenland had some features indicative of *L. cuprina cuprina* ancestry (making them possible hybrids). Thus the divergent populations of *L. cuprina* in our data might represent these two subspecies. But since *L. cuprina* is paraphyletic in our analysis with reference to *L. sericata*, *L. cuprina cuprina* and *L. cuprina dorsalis* could not be subspecies, but must instead constitute distinct species, *Lucilia cuprina* sensu stricto and *Lucilia dorsalis*. The degree of divergence between the *L. cuprina* sensu stricto and *L. dorsalis* populations suggests that these species have been evolving independently for at least 3.5 million years. The two populations sampled by us

would presumably have had separate origins in Africa (*L. dorsalis*) and one or more regions of the Pacific, such as Papua New Guinea (*L. cuprina* sensu stricto).

Further evidence for the genetic distinctiveness of *L. dorsalis* and *L. sericata* comes from comparison of the present results with those of Stevens *et al*. (2002). Stevens *et al*. compared specimens of *L. cuprina* sensu lato from Western Australia (Perth) and Queensland (Townsville) using mitochondrial DNA (*CO1* and *CO2*), and found only a 0.1% sequence divergence between populations. We compared the *CO1* + *CO2* sequences of our Western Australian specimen of *L. dorsalis* with those of the Perth specimen of Stevens *et al*. (2002) and, similarly, found only 0.07% sequence divergence. Stevens *et al*. (2002) also included a specimen of *L. sericata* from Perth in their study. There was no difference between the *CO1* + *CO2* sequences of this fly and those of our *L. sericata* specimen from Melbourne. These comparisons of our data with those of Stevens *et al*. (2002) help confirm that *L. dorsalis* and *L. sericata* are valid species, widespread across the Australian continent.

However, uncertainty remains regarding the taxonomic status of our putative *L. cuprina* sensu stricto specimens from News South Wales and Queensland, especially as they may in fact be crosses between *L. cuprina* sensu stricto and *L. dorsalis*. As part of their aforementioned study, Stevens *et al*. (2002) recognised possible hybrids between *L. sericata* and (probably) *L. cuprina* sensu stricto from Hawaii. These flies possessed *L. sericata*-type mitochondrial DNA and *L. cuprina*-type nuclear DNA. Our results could also be explained if the New South Wales and Queensland specimens were such hybrids. This issue could be resolved either with the joint application of mitochondrial and nuclear DNA (as employed by Stevens *et al*. (2002)) or with allozymes (e.g. Wallman and Adams 1997), along with greater sampling of flies resembling either *L. cuprina* sensu stricto or *L. dorsalis* throughout eastern Australia. Such additional sampling is, of course, also needed for a more thorough assessment of the taxonomic status of *Ch. rufifacies*.

Estimated divergence times and blowfly evolution

As a last step, it is worthwhile examining how the divergence times estimated for the evolution of the blowflies (Fig. 2) relate to the historical biogeography of the Australian region. The latter might clarify the mode of speciation of the Australian blowflies over time.

Based on Fig. 2, the divergences of the major clades (subfamily level) of blowflies all occurred before 15 mya. Most divergences coincide with the Miocene epoch (which ended five mya), straddling a major transition in the Australian climate from wet and warm in the early Miocene to much dryer and cooler in the present day (Markgraf *et al*. 1995; Martin 1998).

Among the subfamilies Luciliinae and Chrysomyinae, most of the species included here are also found in other parts of the world, especially in the south-east Asian and Oriental regions. However, three are endemic to Australia: *Hemipyrellia fergusoni* Patton, 1925, *Chrysomya latifrons* (Malloch, 1927) and *Ch. incisuralis*. All are paired in the phylogenetic results with non-endemic species, to which the endemics are most closely related on morphological grounds. All of these species-pairs, except the *Hemipyrellia* species, diverged from each other more recently than five mya. Since we have not included representatives of the non-endemics from other parts of their distributions and non-Australian species from these genera, it is not possible to speculate on whether these species originated in Australia and subsequently invaded other areas, or *vice versa*. Dispersals from or to south-east Asia could have been facilitated by the increased proximity of Australia to south-east Asia ~10 mya and by low sea levels as a result of the subsequent periodic glaciations during the Pleistocene (the last of which was 18 000 years ago) (Markgraf *et al*. 1995). Because of their reproductive dependency on the carcasses of vertebrate animals, these flies would be expected to be able to disperse wherever the vertebrate fauna was also found.

In contrast to the Luciliinae and Chrysomyinae, the species within the Calliphorinae included here are almost all Australian endemics (the exception is *Calliphora vicina* Robineau-Desvoidy, 1830). Indeed, because of the abundance of *Calliphora* species in Australia, it has been suggested that the genus evolved there (Erzinçlioğlu 1984). That cannot be confirmed from the current results, especially as the genus has additional members, not included here, that are endemic to other regions. It appears from the estimation of divergence times that, like the endemic Luciliinae and Chrysomyinae species, most *Calliphora* species diverged since the late Miocene (around five mya). The notable exception to this is the clade containing the earthworm parasites. Why might so much blowfly speciation have occurred only in the last five million years? The answer to this might lie in the dramatic changes in Australia's climate that occurred in the Pliocene and Pleistocene. These changes had their origins in the middle Miocene, but later became more pronounced and spread to the south of the continent. There was a steady loss of wet sclerophyll forest across Australia, giving way to open vegetation, which was associated with greatly reduced rainfall. This was most pronounced in the centre of the continent. Around 500 000 years ago the increasing aridity reached its current level (Martin 1998). Since the distribution and abundance of blowflies is heavily influenced by moisture and temperature (Norris 1959), this aridity may have been instrumental in directing their evolution. If so, it is particularly likely to have played a role in the very recent speciations seen in the *C. hilli*-group, *C. augur*-group and *C. stygia*-group, given the south-western *v*. south-eastern concentrations of the distributions of species in each group, i.e. *C. dubia* (south-western and central-southern) and *C. augur* (south-eastern), and *C. varifrons* (south-western)

and *C. hilli hilli*/ *C.* sp. nov. (south-eastern). The vicariance barrier formed by the Nullarbor Plain presumably induced these speciations. Paramonov (1959) noted the often-close relationship between fly species from the south-west and south-east of the continent.

In conclusion, this paper gives the first comprehensive indication of molecular relationships among Australian carrion-breeding blowflies and allied species and shows that mitochondrial DNA continues to have scope for their molecular identification. Our results for *Ch. rufifacies* and *L. cuprina* indicate the likely presence of additional species among the Australian blowfly fauna, and their taxonomy therefore requires much closer scrutiny. There is also clear evidence for the independent evolution, from carrion-breeding ancestors, of parasitism among Australian blowflies. Finally, estimation of the divergence times of the Australian carrion-breeding blowflies using the molecular-clock approach suggests that the aridification of Australia during the late Neogene and Quarternary periods might have contributed to their speciation. It would be well worthwhile directing future work to the addition of representative species from other clades within the Australian Calliphoridae to examine whether the aridification hypothesis agrees more broadly with the evolution of the family in Australia.

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