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The evolution of myiasis in blowflies (Calliphoridae)

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Abstract

Blowflies (Calliphoridae) are characterised by the ability of their larvae to develop in animal flesh. Where the host is a living vertebrate, such parasitism by dipterous larvae is known as myiasis. However, the evolutionary origins of the myiasis habit in the Calliphoridae, a family which includes the blowflies and screwworm flies, remain unclear. Species associated with an ectoparasitic lifestyle can be divided generally into three groups based on their larval feeding habits: saprophagy, facultative ectoparasitism, and obligate parasitism, and it has been proposed that this functional division may reflect the progressive evolution of parasitism in the Calliphoridae. In order to evaluate this hypothesis, phylogenetic analysis of 32 blowfly species displaying a range of forms of ectoparasitism from key subfamilies, i.e. Calliphorinae, Luciliinae, Chrysomyinae, Auchmeromyiinae and Polleniinae, was undertaken using likelihood and parsimony methods. Phylogenies were constructed from the nuclear 28S large subunit ribosomal RNA gene (28S rRNA), sequenced from each of the 32 calliphorid species, together with suitable outgroup taxa, and mitochondrial cytochrome oxidase subunit I and II (COI + II) sequences, derived primarily from published data. Phylogenies derived from each of the two markers (28S rRNA, COI + II) were largely (though not completely) congruent, as determined by incongruence-length difference and Kishino-Hasegawa tests. However, the phylogenetic relationships of blowfly subfamilies based on molecular data did not concur with the pattern of relationships defined by previous morphological analysis; significantly, molecular analysis supported the monophyly of blowflies (Calliphoridae), distinct from the bot and warble flies (Oestridae). Comparative analysis of the myiasis habit based primarily on the 28S rRNA phylogeny indicated that obligate parasitism, and the ability to initiate myiasis in higher vertebrates, has multiple independent origins across myiasis-causing flies (Calliphoridae and Oestridae) and in at least three subfamilies of blowfly (Calliphoridae). Finally, the general association of various blowfly genera and subfamily clades with particular continental and geographical regions suggests that these groups probably came into existence in the Late Cretaceous period, following the break-up of Gondwana.
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1. Introduction

Blowflies (Calliphoridae) are characterised by the ability of their larvae to develop in the flesh of vertebrates; some species also parasitise invertebrates, e.g. earthworms, while others can complete larval development in animal detritus, e.g. faeces, or by blood-sucking. However, the overall defining characteristic of blowflies (aside from key morphological features) is the requirement for a protein-rich substrate, as provided by animal associated tissues or by-products, in which the larvae can complete their development. When the larvae, indeed any dipterous larvae, develop in a living animal, such parasitism is known as myiasis; blowfly larvae typically cause external myiases and are classified as ectoparasites, as opposed to internal myiases which are often associated with bot and warble flies of the family Oestridae. The evolutionary origins of the myiasis habit in the Calliphoridae, a family which includes the screwworm flies (*Chrysomya* spp., *Cochliomyia* spp.), bluebottles (*Calliphora* spp.) and greenbottles (*Lucilia* spp.), remain unclear (Zumpt, 1965; Erzinclioglu, 1989; Stevens and Wall, 1997a).

Species associated with an ectoparasitic lifestyle can be divided generally into three groups based on their larval feeding habits: (i) saprophages, normally living in decaying matter, which may secondarily invade existing infestations; (ii) facultative ectoparasites, which can live as saprophages or can initiate myiases and live as ectoparasites; and (iii) obligate parasites, which feed only on the tissues of living hosts. It has been proposed (Zumpt, 1965) that this

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functional division may reflect the progressive evolution of parasitism in the Calliphoridae. In support of this proposed evolutionary progression, species displaying a range of dependencies on ectoparasitism can be identified within several calliphorid genera, e.g. *Calliphora* (saprophages), *Lucilia* (facultative ectoparasites) and *Chrysomya* (obligate parasites). However, the evolutionary scenario outlined above is complicated by the existence of other specialised lifestyles, in which calliphorid species have developed novel larval feeding habits, such as various forms of blood-sucking, e.g. *Protocalliphora* spp. and the Congo floor maggot (*Auchmeromyia luteola*), and development in a solitary furuncle (boil) under the skin of the host, e.g. African Tumbu fly (*Cordylobia anthropophaga*). To date, studies of the evolution of parasitism in blowflies (e.g. Zumpt, 1965; Erzinclioglu, 1989) have been limited by the ability to define a consistent phylogeny for the Calliphoridae on the basis of morphological characters alone, while, at the same time, definition of the family and its component subfamilies has undergone many revisions by a variety of taxonomic treatments (e.g. Hall, 1948; Shewell, 1987; Rognes, 1991, 1997).

Accordingly, this paper presents phylogenetic analysis of newly available 28S large subunit ribosomal RNA (28S rRNA) sequences, supplemented by analysis of published mtDNA cytochrome oxidase I and II (COI + II) sequences to build on previous morphology-based studies of Calliphoridae. Species were selected from across all subfamily groups to encompass the full range of forms of the myiasis habit; this also included most major myiasis agents of either economic, medical or veterinary importance. Latterly, interpretation of the myiasis status of taxa in relation to their phylogenetic position was undertaken, allowing the evolutionary origins of ectoparasitism and myiasis in blowflies to be elucidated.

2. Materials and methods

2.1. Flies, outgroup and DNA extraction

Blowfly DNA was obtained from the species listed in Table 1. To avoid possible contamination of fly DNA with DNA from ingested protein, gut parasites or eggs, only the thoracic flight muscle of flies was used as a source of DNA; see Stevens and Wall (1995, 2001) and Aljanabi and Martinez (1997) for details. As outlined above, definition of the family Calliphoridae and its component subfamilies has undergone several revisions and, while its exact relationships to the sister families Oestridae, Tachinidae and Sarcophagidae remain in debate, its relationship to family Muscidae within superfamily Muscoidea is generally more unambiguously defined (McAlpine, 1989; Rognes, 1997; Bernasconi et al., 2000; Pape, 2001). Accordingly, a range of closely and more distantly related outgroup taxa were used in the current study; these comprised of a Sarcophagidae species, *Sarcophaga carnaria*, two Oestridae species, *Oestrus ovis* and *Gasterophilus intestinalis*, and a muscid, *Musca domestica*.

2.2. DNA sequences

2.2.1. 28S rRNA sequences

A fragment of 2.2 kb spanning the D1–D7 regions of the 28S rRNA gene was amplified from 46 blowfly taxa (representing 32 species), one flesh fly, two species of bot fly and a house fly (Table 1) by PCR using conserved primers as published; see Stevens and Wall (2001) for details. The 2.2 kb fragment was amplified in two overlapping sections of approximately 0.8 kb (D1–D2) and 1.45 kb (D3–D7), giving an overlap of ~50 bp to facilitate sequence assembly. The inherently diverse rates of genetic evolution of the 28S rRNA gene facilitate its use across a broad phylogenetic range, so that it is suitable for elucidating both higher (older) evolutionary relationships and, by analysis of the divergent 'D' domains, relationships between more recently diverged species (Larson, 1991). Accession numbers for all 50 28S rRNA sequences analysed are given in Table 1.

2.2.2. COI + II sequences

The COI + II sequence of *Lucilia illustris* (Accession number AJ551445) was obtained as follows. A fragment of 2.3 kb spanning the mitochondrial COI and COII subunits, as well as tRNA-leucine, was amplified in two overlapping sections of approximately 1.55 kb (CO1–CO5) and 1.3 kb (CO3–CO8) using published primers (Wells and Sperling, 2001), according to the protocol described by Stevens et al. (2002). All other COI + II sequences, corresponding as far as possible to species included in the 28S rRNA phylogeny, were obtained from the EMBL database.

2.3. Sequence alignment and phylogenetic analysis

Sequences were aligned using the program Clustal X (Thompson et al., 1997) before final adjustments were made by eye. The 28S rRNA alignment included 2,494 nucleotide positions, of which 379 positions could not be unambiguously aligned and were excluded from subsequent phylogenetic analyses. Variation in the 28S rRNA gene was largely confined to four distinct regions within expansion segments: D2, D3, D6 and D7a (Hancock et al., 1988); these were also the regions where most hypervariable non-alignable nucleotides were located. Fortunately, sequences showed sufficient similarity that relatively few nucleotides (379) could not be aligned; of course, such similarity can also result in a reduced number of phylogenetically informative characters which may lead to a loss of resolution later in an analysis. The COI + II alignment included 2,321 nucleotide positions, of which 22 positions were excluded from subsequent analysis of nucleotide data; using the program

Table 1
Specimen details: identity, origin and 28S rRNA sequence accession numbers

Specimen	Subfamily	Collection location	Date	Accession no.	Ref.
Family Muscidae					
Musca domestica Linnaeus	Muscinae	University of Bristol colony, UK	1999	AJ551427	NS
Family Oestridae					
Oestrus ovis L.	Oestrinae	Foggia, Apulia, Italy	1999	AJ551428	NS
Gasterophilus intestinalis (Degeer)	Gasterophilinae	Exeter, Devon, UK	2000	AJ551429	NS
Family Sarcophagidae					
Sarcophaga carnaria (L.)	Sarcophaginae	Exeter, Devon, UK	2001	AJ551430	NS
Family Calliphoridae					
Auchmeromyia luteola (Fabricius)	Auchmeromyiinae	Nguruman, Kenya	1999	AJ551431	NS
Cordylobia anthropophaga (Blanchard)		Yaoundé, Cameroon	1999	AJ551432	NS
Calliphora dubia (Macquart)	Calliphorinae	Adelaide, SA, Australia	1998	AJ558185	NS
Calliphora stygia (Fab.)		Adelaide, SA, Australia	1998	AJ558186	NS
Calliphora quadramaculata (Swederus)		Rangitoto, North Is., New Zealand	1999	AJ558187	NS
Calliphora vicina Robineau-Desvoidy		University of Bristol colony, UK	1995	AJ300131	3
Calliphora vomitoria (L.)		Slapton Ley, Devon, UK	1999	AJ300133	3
Cynomya mortuorum (L.)		Durham, Co. Durham, UK	1999	AJ300135*	3
Cynomyopsis cadaverina (R.-D.)		Ottawa, Canada	1999	AJ300135*	NS
Onesia tibialis (Macq.)		Adelaide, SA, Australia	1998	AJ558188	NS
Chrysomya albiceps (Wiedemann)	Chrysomyinae	Nairobi, Kenya	1999	AJ551433	NS
Chrysomya bezziana Villeneuve		Bogor, Java, Indonesia	2000	AJ551434	NS
Chrysomya chloropyga (Wied.)		Tanzania	1998	AJ558189	NS
Chrysomya megacephala (Fab.)		Fuertaventura, Canary Is., Spain	1997	AJ551435	NS
Chrysomya rufifacies (Macq.)		Adelaide, SA, Australia	1998	AJ551436	NS
Cochliomyia hominivorax (Coquerel)		Jaboticabal, San Paulo, Brazil	2000	AJ551437	NS
Cochliomyia macellaria (Fab.)		Mogi Guaçu, San Paulo, Brazil	2000	AJ551438	NS
Protocalliphora azurea (Fallén)		Antwerp, Belgium	1999	AJ551439	NS
Protocalliphora sialia Shannon & Dobroscky		Kittitas Co., WA, USA	1999	AJ558190*	NS
		Richland Co., OH, USA	1999	AJ558190*	NS
Protophormia terraenovae (R.-D.)		Exeter, Devon, UK	1999	AJ300142	3
Lucilia ampullacea Vill.	Luciliinae	Langford, Somerset, UK	1994	AJ300137	3
Lucilia caesar (L.)		Langford, Somerset, UK	1994	AJ300138	3
Lucilia cluvia Walker		New Orleans, LA, USA	1994	AJ551440	NS
Lucilia cuprina (Wied.)		Townsville, Qld, Australia	1994	AJ417709*	1,2
		Perth, WA, Australia	1995	AJ417709*	1,2
		Dakar, Senegal	1994	AJ417709*	1,2
		Tororo, Uganda	1994	AJ417709*	1,2
		Perth, WA, Australia	1995	AJ417709*	1,2
		Dorie, South Is.,, New Zealand	1994	AJ417709*	1,2
		Hawaii-1: Waianae, Oahu, HI, USA	1994	AJ417709*	1,2
		Hawaii-2: Honolulu, Oahu, HI, USA	1998	AJ417709*	4
Lucilia illustris (Meigen)		Langford, Somerset, UK	1994	AJ300136	3
Lucilia mexicana Macq.		San Francisco, CA, USA	1994	AJ551441	NS
Lucilia richardsi Collin		Usk, Gwent, UK	1995	AJ551442	NS
Lucilia sericata (Mg.)		Hilerod, Sjelland, Denmark	1994	AJ300140*	1,2,3
		Dorie, South Is., New Zealand	1994	AJ300140*	1,2
		Nerja, Andalucia, Spain	1994	AJ300140*	2
		Perth, WA, Australia	1995	AJ300140*	1,2
		Harare, Zimbabwe	1994	AJ300140*	1,2
		Langford, Somerset, UK	1994	AJ300139	3
		Los Angeles, CA, USA	1994	AJ300141	2,3
Lucilia silvarum (Mg.)		Durham, Co. Durham, UK	1999	AJ551443	NS
Lucilia thatuna Shannon		San Francisco, CA, USA	1994	AJ551444	NS
Hemipyrellia fernandica (Macq.)		Tanzania	1994	AJ558191	NS
Pollenia rudis Fab.	Polleniinae	Exeter, Devon, UK	1999	AJ558192	NS

References: 1, Stevens and Wall (1996); 2, Stevens and Wall (1997b); 3, Stevens and Wall (2001); 4, Stevens et al. (2002); NS, new sequence.

*Eight specimens of *L. cuprina* from different regions shared an identical 28S rRNA sequence (AJ417709), as did five specimens of *L. sericata* (AJ300140) and two specimens of *P. sialia* (AJ558190); two species, *C. mortuorum* and *C. cadaverina*, also shared an identical 28S rRNA sequence (AJ300135). In the phylogenies (Figs. 1 and 2) taxa with identical 28S rRNA sequences are represented by a single taxon name; the one exception to this occurs in Fig. 2, where both *L. cuprina* and *L. cuprina* [h. = hawaiiensis] are included by virtue of their different COI + II sequences.

MacClade (see below) nucleotide variation in the COI + II genes was seen to be approximately evenly spread across the length of the sequences. Alignments are available on request from the author. Phylogenetic analysis was performed using the program PAUP (Swofford, 1998) and data were analysed by parsimony and maximum-likelihood methods. Parsimony analysis was implemented using the default options of PAUP, namely: TBR branch-swapping, gaps treated as missing data, zero length branches collapsed. Analyses were performed separately for each set of gene sequences and on a combined data set. For likelihood analysis, the evolutionary model which best fitted the data was derived by a series of hierarchical likelihood ratio tests using the program Modeltest (Posada and Crandall, 1998); resulting parameters were then input to PAUP to implement the model. For all analyses, relative bootstrap support was estimated using 1000 pseudoreplicates for each gene.

The principle phylogenetic analysis of 28S rRNA data included 36 unique sequences (i.e. all identical sequences were excluded); 32 sequences originated from calliphorid taxa, while four were from outgroup taxa, comprising two oestrid taxa, one sarcophagid taxon and one muscid taxon. A complementary analysis of COI + II sequences from 22 corresponding taxa was also performed. Differences between the COI + II and 28S rRNA gene tree topologies were assessed using a parsimony-based Kishino–Hasegawa (K-H) test (Kishino and Hasegawa, 1989). An incongruence-length difference (ILD) test (Farris et al., 1995) was also performed on the combined data to test the phylogenetic homogeneity of the sequences and to assess the combinability of the data from the two genes. While the ILD test is used to assess combinability of data, it also serves as an indicator of phylogenetic congruence (Cunningham, 1997) between genetic markers; it is implemented in PAUP* as a parsimony-based partition-homogeneity test. Two partitions corresponding to 28S rRNA and COI + II were defined within the 4436 characters included in the combined data file.

The program MacClade (Maddison and Maddison, 1992) was used to explore the distribution of phylogenetically informative characters (nucleotide changes) within trees. The 'Character Steps' option within the 'Chart' menu was used to evaluate coding position variation and to map the distribution of variable sites along genes.

3. Results

3.1. Phylogenetic analysis

A lack of resolution within the 28S rRNA data did not result in a single most-parsimonious tree (MPT). Instead, parsimony analysis of the 2,115 aligned nucleotides of the 28S rRNA gene yielded 754 MPTs, which are summarised in two forms of consensus tree in Fig. 1. The phylogram (main tree) is a bootstrap majority rule consensus tree derived from 1,000 pseudoreplicates and shows only those branches receiving more than 50% bootstrap support; the corresponding cladogram (inset tree) is the majority-rule consensus tree calculated from the 754 MPTs.

Bootstrap support was also calculated by maximum-likelihood analysis. The best-fit evolutionary model for the likelihood analysis (as determined by Modeltest) was a general time-reversible model (Rodríguez et al., 1990), with the following parameters: A-G, 4.5387; A-T, 3.7549; A-C, 0.5697; G-T, 1.0; G-C, 0.0; T-C, 4.5387; gamma = 0.6386; proportion invariant sites = 0.7560. Levels of support provided by the two methods of analysis were in general agreement; bootstrap values derived by parsimony analysis were in all cases higher than values derived by likelihood, but there was no conflict between clades receiving bootstrap support above 50%.

While bootstrap analysis of these data provided relatively strong support (79%) for the evolutionary separation of Calliphoridae from Oestridae and muscid outgroup taxa, the blowflies were not, on the basis of these gene sequences, well-distinguished from a single representative of family Sarcophagidae (Fig. 1), although in a reduced taxon parsimony analysis (Fig. 2) incorporating a different set of outgroup taxa, support for monophyly of Calliphoridae was observed. Moreover, in relation to the outgroup taxa analysed, the 28S rRNA-based majority-rule consensus tree provided strong support for the monophyly of family Calliphoridae, with more than 95% of most-parsimonious trees grouping Calliphoridae taxa in a single monophyletic clade (Fig. 1, inset). Within family Calliphoridae, the analysis (Fig. 1) identified three main groups, each corresponding to a subfamily identified by classical taxonomic methods, namely, Calliphorinae, Luciliinae and Auchmeromyiinae. Bootstrap support for each of these clades was generally relatively robust (between 63 and 100%) and supported the existence of recognised subfamily groups as monophyletic evolutionary and taxonomic units. However, subfamily Chrysomyinae was not supported by either parsimony or maximum-likelihood bootstrap analyses as being a distinct genetic grouping, although the majority-rule parsimony tree suggested that the subfamily may comprise two distinct groups, one containing New World species (*Cochliomyia* spp.), the other Old World genera (*Chrysomya* spp., *Protophormia terraenovae, Protocalliphora* spp.); this relationship was also supported by a range of distance-based bootstrap analyses (results not shown). As only one species of Polleniinae – *Pollenia rudis* – was included in this study, it is not possible to comment on the phylogenetic robustness of this subfamily.

Phylogenetic analysis of complementary COI + II sequences (Fig. 2) yielded a tree which produced the same arrangement of taxa in subfamilies as that observed for a

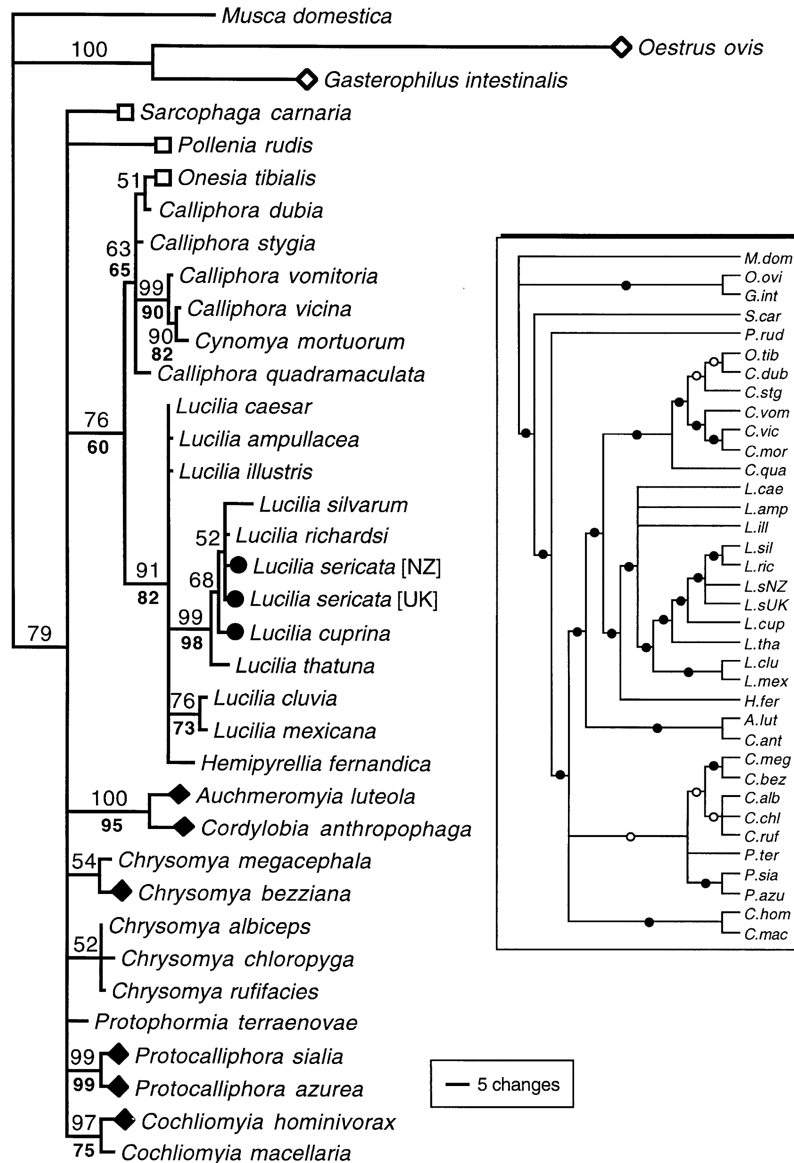

Fig. 1. Phylogeny (main tree) constructed by parsimony analysis of 2115 aligned 28S rRNA nucleotides; percentage bootstrap support values were derived from 1,000 pseudoreplicates by both parsimony (upper values) and maximum-likelihood (lower, bold values) analyses. Thirty-two sequences, representing 46 blowfly taxa from 32 species, in five subfamilies of Calliphoridae are shown, from top to bottom: Polleniinae, Calliphorinae, Luciliinae, Auchmeromyiinae, Chrysomyinae; the four outgroup taxa are *Musca domestica, Oestrus ovis, Gasterophilus intestinalis* and *Sarcophaga carnaria*. Branches of the tree receiving poor (<50%) bootstrap support by both methods of analysis have been collapsed. Symbols ◆, ◇, or □ represent obligate primary myiasis species: ◆ = obligate primary myiasis of warm-blooded vertebrates caused by blowflies (Calliphoridae); □ = obligate primary parasitism of invertebrates (earthworms) caused by blowflies (Calliphoridae) and flesh-flies (Sarcophagidae); ◇ = obligate primary myiasis of warm-blooded vertebrates caused by bot and warble flies (Oestridae). Symbol ● represents facultative primary myiasis species. The majority-rule parsimony tree calculated from the 754 MPTs is shown inset; clades which appear in > 95% of trees are marked ●, clades which appear in > 65% of trees are marked ○.

reduced set of 28S rRNA sequences (Fig. 2). Again, the analysis was supported by both parsimony and maximum-likelihood bootstrap analyses, the best-fit evolutionary model for the likelihood analysis (as determined by Modeltest) being a general time-reversible model with the following parameters: A-G, 79.6283; A-T, 47.5709; A-C, 2.0530; G-T, 1.0; G-C, 3.7515; T-C, 279.7456; gamma = 1.6573; proportion invariant sites = 0.6507. Levels of support provided by the two methods of analysis were in general agreement; bootstrap values derived by parsimony analysis were, in all but one case, higher than values derived by likelihood and there was no conflict between clades receiving bootstrap support above 50%. However, some variation in intra-subfamily relationships and tree topologies (length differences) was apparent between gene phylogenies, as confirmed by K-H tests ($P < 0.0123$, $P < 0.0001$, $n = 22$). Examination of the phylogenies derived from each of the two sets of gene

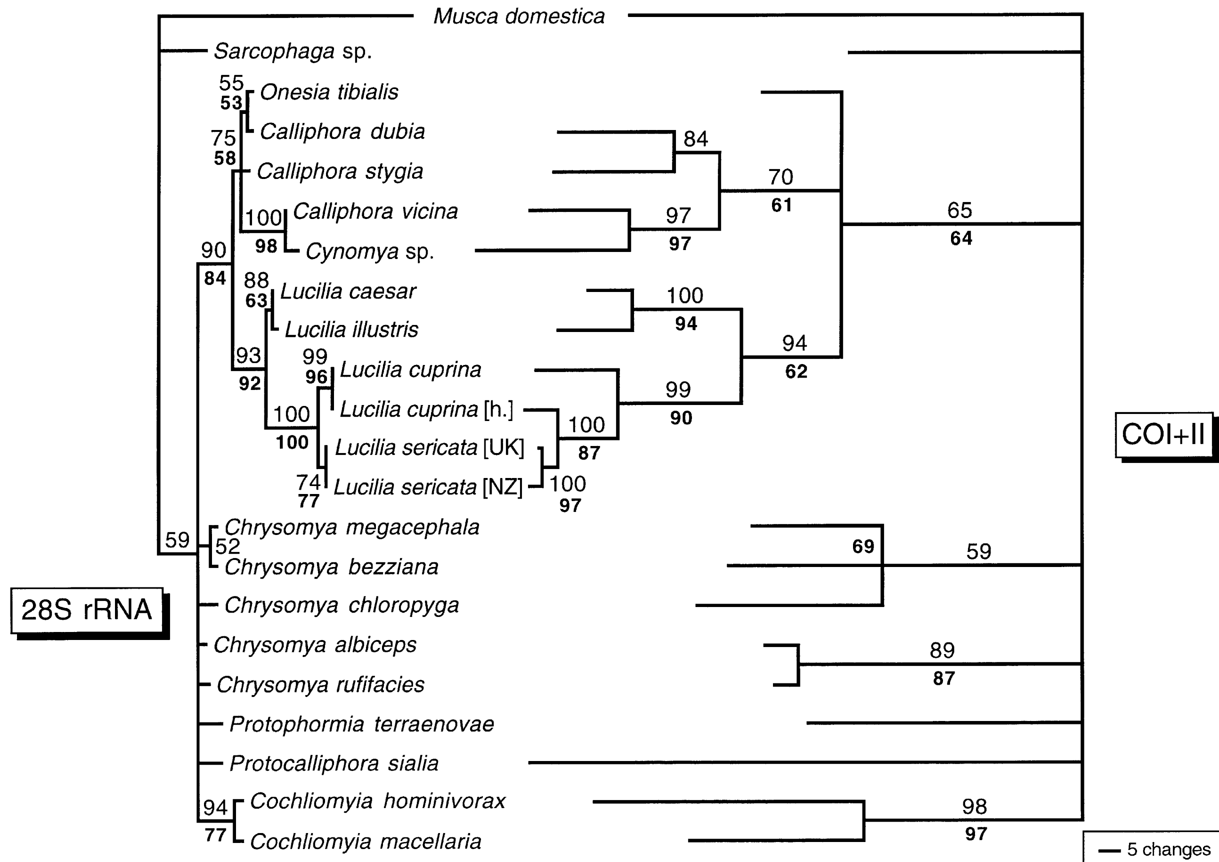

Fig. 2. Corresponding 28S rRNA and COI + II phylogenies constructed by parsimony analysis of 2137 (28S rRNA) and 2299 (COI + II) aligned nucleotides; percentage bootstrap support values were derived from 1000 pseudoreplicates by both parsimony (upper values) and maximum-likelihood (lower, bold values) analyses. Twenty-two COI + II sequences were analysed: *Musca domestica*, AF104622; (Bernasconi et al., 2000); *Sarcophaga* sp., AF259509 (Wells et al., 2001a); *Cochliomyia hominivorax*, AF260826 (Lessinger et al., 2000); *Calliphora vicina*, AJ417702; *Lucilia caesar*, AJ417703; *Lucilia illustris*, AJ551445; *Lucilia cuprina* [h. = *hawaiiensis*], AJ417704; *Lucilia cuprina*, AJ417707; *Lucilia sericata* [NZ], AJ417713; *Lucilia sericata* [UK], AJ417714 (Stevens et al., 2002); *Calliphora dubia*, AY012540; *Calliphora stygia*, AY012544; *Chrysomya rufifacies,* AY012548; *Onesia tibialis*, AY012568; (Wallman and Donnellan, 2001); *Chrysomya albiceps*, AF083657 (Wells and Sperling, 1999); *Chrysomya bezziana*, AF295548; *Chrysomya megacephala*, AF295551; *Protophormia terraenovae*, AF295553; *Chrysomya chloropyga*, AF295554; *Cochliomyia macellaria*, AF295555; *Protocalliphora sialia*, AF295559 (Wells and Sperling, 2001); *Cynomya* sp., AF259505 (Wells et al., 2001b). Details of the corresponding 28S rRNA sequences are provided in Table 1; *L. cuprina* and *Lucilia cuprina* [h. = *hawaiiensis*] share an identical rRNA sequence (AJ417709).

sequences (Fig. 2) suggested that the difference in topologies was due to incongruent characterisation of several key taxa (*Calliphora stygia, Chrysomya chloropyga*) and, indeed, successive removal of these taxa resulted in less extreme significance values in subsequent K-H tests. Finally, removal of either *Lucilia cuprina* taxon resulted in phylogenies that showed no significant difference ($P > 0.05$, $n = 19$) in topology between the two genes.

Additionally, two partitions corresponding to the two genes were defined within the 4436 characters included in the combined data file using an incongruence-length difference (ILD) test. The parsimony-based ILD test showed that sequence data for the two genes were not homogeneous across the 22 taxa analysed (see above) and that the two data sets did not describe exactly the same phylogeny. Of the 4436 characters, 483 were parsimony-informative and analysis yielded a tree of length 1677 for the original partition, which was shorter than 248 trees derived from 1,000 pseudoreplicate partitions ($P = 0.752$). Following the same strategy of data exploration as used for the comparison of tree topologies, i.e. removing candidate incongruent taxa (see above), a P value > 95% was reached upon removal of either *L. cuprina* taxon or *C. stygia* from the analysis ($n = 21$), confirming the different evolutionary histories of the 28S rRNA and COI + II genes in these taxa.

3.2. Substitution distribution analysis

Analysis of the distribution of phylogenetically informative characters (nucleotide changes) within trees showed that variation in 28S rRNA gene sequences was largely confined to four main regions within D domains: D2, D3, D6 and D7a (Hancock et al., 1988). However, nucleotide variation in the COI + II sequences appeared evenly spread across the length of the genes, but with an obvious bias to non-coding sites (1st position: 253/1500 = 17%; 2nd

position: 35/1500 = 2%; 3rd position: 1212/1500 = 81%), suggesting that substitutions are predominantly neutral and that variation is non-adaptive. Such a result indicates that the evolution of these COI + II data follows a molecular clock, a finding supported for COI by the recently published work of Gaunt and Miles (2002).

3.3. Comparative analysis of blowfly myiasis status

Comparative analysis indicates that primary obligate parasitism appears to have arisen on at least five independent occasions in the taxa included in this study (symbols ◆, ◇ or □, Fig. 1). Within the blowflies (Calliphoridae) all primary obligate parasites of higher vertebrates (◆, Fig. 1) are found in the Auchmeromyiinae clade, or amongst the Chrysomyinae taxa. *Onesia tibialis* and *P. rudis* are both obligate parasites of earthworms (□, Fig. 1); *O. tibialis* is classified in the Calliphorinae clade, while *P. rudis*, the single representative of subfamily Polleniinae, is classified separately from all other calliphorid subfamiles. Both primary non-obligate agents of myiasis, *L. cuprina* and *Lucilia sericata*, are placed in a single clade within the subfamily Luciliinae (●, Fig. 1). The status of other analysed taxa as agents of myiasis is less well defined, but includes many facultative secondary myiasis agents, e.g. *Lucilia caesar, Chrysomya albiceps, Chrysomya rufifacies*, together with a variety of saprophagous species, e.g. *Calliphora vicina* and *Lucilia richardsi*.

4. Discussion

The taxon complement of the subfamily clades defined by phylogenetic analysis of 28S rRNA sequence data is in general overall agreement with the taxonomic status of the taxa as defined by classical morphology-based analysis, although monophyly of subfamily Chrysomyinae is not supported by these rRNA data; taxa from this subfamily (as classically defined) form an unresolved polytomy in the analyses presented here. These findings are supported by analysis of COI + II gene sequences from a reduced taxon set.

Inter-subfamily phylogenetic relationships derived from the molecular data also do not concur fully with the pattern of relationships defined by morphological analysis (Rognes, 1997). Molecular analysis (Fig. 1) provides some support for the monophyly of Calliphoridae (based on the taxa analysed) with calliphorid subfamilies forming a well-supported grouping, distinct from family Oestridae (the bot and warble flies); previous morphology-based analysis grouped family Oestridae with Chrysomyinae, Calliphorinae and Luciliinae (Rognes, 1997). However, the relationship of Calliphoridae with Sarcophagidae is less well resolved, though at least some parsimony analyses identify Sarcophagidae as a sister group to a monophyletic Calliphoridae clade; perhaps significantly, no analyses –either parsimony or maximum-likelihood – group the sarcophagid taxon, *S. carnaria*, within Calliphoridae. Molecular analysis also classifies Calliphorinae and Luciliinae as sister groups on the basis of both nuclear (28S rRNA) and mitochondrial (COI + II) sequences (Fig. 2); previous morphology-based analysis identified Calliphorinae and Chrysomyinae as sister groups (Rognes, 1997). While analysis of additional gene markers may help to resolve this issue, the data presented here suggest that the morphology-based sister group status of subfamilies Calliphorinae and Chrysomyinae may require revision.

Relative levels of molecular evolution within some groups, notably Chrysomyinae, e.g. *Protocalliphora sialia*, differed substantially between the two molecular phylogenies (Fig. 2), with particularly rapid evolution (inferred from disproportionately long branch lengths) being observed in some lineages within the COI + II phylogeny compared with those in the rRNA-based phylogeny. Overall, subfamily Chrysomyinae was undefined in both the 28S rRNA and COI + II phylogenies. However, while resolution of relationships between Chrysomyinae taxa (as classically defined) was poor, no Chrysomyinae taxa were grouped with or within any other subfamilies; clearly, sequence data from another genetic marker will be required to reliably resolve the phylogeny of subfamily Chrysomyinae. In the case of *P. sialia*, the original analysis (Wells and Sperling, 2001) of this COI + II sequence also classified this unusual bird parasite as notably distinct from all other Chrysomyinae taxa. As shown by the tests of phylogenetic congruence and topology, however, the exact evolutionary relationships of several species varied according to the genetic marker used. Similarly, a recent study by Stevens et al. (2002) demonstrated divergent nuclear and mitochondrial phylogenies in hybrid *Lucilia* spp. Given the apparently great age (see below) of these subfamilies (Chrysomyinae and Luciliinae), and by definition of the lineages within them, it is perhaps not surprising that some minor variation in the intra-subfamily relationships defined by such diverse genes (nuclear/non-protein coding versus mitochondrial/protein coding) should occur. Acceptance of such variation, however, does not help in deciding which (if any) of these two gene phylogenies best represents the true evolutionary history of the species under study; on one hand we have the general (but by no means complete) agreement of the 28S rRNA phylogeny with trends in the morphological evidence, on the other is the reported clock-like nature of at least the COI gene (Gaunt and Miles, 2002).

By virtue then of the greater species coverage achieved with 28S rRNA sequences in this study and in the light of recent reports questioning the resolution of COI variation in Calliphoridae taxa (e.g. Stevens and Otranto, in press), the 28S rRNA tree (Fig. 1) was used as the basis for comparative analysis. Using such an approach, it was apparent that primary obligate parasitism has arisen on numerous (probably at least five) independent occasions in

the taxa included in this study (symbols ◆, ◇ or □, Fig. 1). The most parsimonious interpretation of the phylogeny (Fig. 1) also suggests that parasitism arose independently in Calliphoridae/Sarcophagidae and Oestridae, a result which is perhaps not surprising when the ectoparasitic form of myiasis exhibited by many Calliphoridae species is contrasted with the obligate, effectively endoparasitic lifestyles of the Oestridae. Focusing on the blowflies (family Calliphoridae), it is apparent that all primary obligate parasites of warm-blooded vertebrates (◆, Fig. 1) are Auchmeromyiinae or Chrysomyinae taxa, suggesting (based on the taxa analysed) that obligate parasitism of vertebrates arose independently on at least three separate occasions within the Calliphoridae. Alternatively, it may be hypothesised that obligate parasitism was the ancestral state and has subsequently been lost in those blowflies which are not obligate parasites, e.g. *C. albiceps* and *Cochliomyia macellaria*. Such a hypothesis, however, does not take into account the large number of non-obligate parasites and saprophagous species which are present in subfamilies Auchmeromyiinae and Chrysomyinae, but which, due to limitations of the specimen collection, were not included in the current study. The existence of ectoparasitic species, albeit facultative, in subfamily Luciliinae indicates a fourth apparently independent evolution of the myiasis habit within the Calliphoridae. Moreover, placement of the facultative primary myiasis species, *L. cuprina* and *L. sericata* (●, Fig. 1), in a clade with other secondary and saprophagous blowfly species, i.e. *L. richardsi* and *L. silvarum*, also suggests possible multiple independent evolution of the myiasis habit within this subfamily. This scenario is further supported by the existence of the reportedly obligate toad parasite, *Lucilia bufonivora* (not included in this study), which is morphologically all but identical to *L. silvarum* (Aubertin, 1933).

As noted above, the exact status of other blowfly taxa as agents of myiasis is less well defined, but includes many facultative secondary myiasis agents and saprophagous species, the status of which may change according to local factors, particularly climate. For example, *P. terraenovae* and *L. caesar* both become more common, and in the case of *P. terraenovae* sometimes predominant, in sheep strike in more northerly regions of their range, e.g. Finland (Wall and Shearer, 1997). Such apparent changes in myiasis status serve to underline that the distinction between primary and secondary myiasis agents is not always well defined. Nevertheless, the results of this study indicate that obligate parasitism and the ability to initiate myiasis in higher vertebrates has multiple independent origins across myiasis-causing flies (Calliphoridae and Oestridae) and in at least three subfamilies of blowfly (Calliphoridae). Such multiple origins of parasitism and a general propensity across the family to cause both primary and secondary facultative myiasis, and/or for larvae to develop in carrion, indicate that the ancestral state of larval development in Calliphoridae may have been carrion feeding and opportunistic myiasis; such a conclusion is in general agreement with the saprophagous origins proposed by Zumpt (1965) and Erzinclioglu (1989). Such a life-history strategy would have left ancestral blowflies well-placed to exploit new niches, such as obligate parasitism of mammals, as opportunities arose over evolutionary time.

Finally, evaluation of the distribution of taxa across the phylogeny (Fig. 1) may provide further clues to the timing and pattern of blowfly evolution. In particular, the association of various blowfly genera and subfamilies with particular geographical regions (e.g. *Cochliomyia* in the New World, *Chrysomya* in the Old World, Auchmeromyiinae in the Afrotropical and Oriental regions) suggests that perhaps many blowfly groups came into existence at a time of significant geographical isolation and subsequently underwent localised divergence and speciation. In geological time, such a situation was present at the beginning of the Late Cretaceous period, following the final break-up of the southern super-continent of Gondwana, around 105 Myr ago (Smith et al., 1994). This was accompanied by (i) the rise and spread of flowering plants in the Cretaceous period (146–65 Myr ago), which, given the reliance of many adult flies – including blowflies – on flower nectar, may have at least helped to facilitate the continued evolution of the various calliphorid groups, and (ii) the rapid spread and diversification of mammals (= potential hosts; based on the requirements of extant species) in the Tertiary, 65–1.8 Myr ago. Of course, as demonstrated by the existence of the specialised toad parasite, *L. bufonivora*, care should be exercised when relating blowfly evolution to any one specific host type (obligate toad parasitism is almost certainly a derived state and thus relatively recent in relation to the age of Calliphoridae and genus *Lucilia*). Nevertheless, the idea of distinct ancestral forms of contemporary Calliphoridae subfamilies having existed since the Late Cretaceous is not at variance with available fossil evidence, which reports an extinct calliphorid[1] species, *Cretaformia fowleri*, from the Upper Cretaceous (105–65 Myr ago) of Canada (McAlpine, 1970). Unfortunately, we can only speculate as to what the larvae of this species were developing on or in.

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[1] Zherikhin (2002) has recently re-evaluated the antiquity of Calliphoridae and, accordingly, suggests that this fossil specimen, whilst being a schizophoran type, may predate the origin of family Calliphoridae.

specimens and material: D. Otranto, L. Davies, J. Wallman, P. Grébaut, R. Tellman, R. Bisset, N. Springate, R. Newcomb, D. Gleeson, J. Shears, S. Hurtrez, T. Whitworth, R. Wootton, A. Lessinger and A. Azeredo-Espin.

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