The molecular systematics of blowflies and screwworm flies (Diptera: Calliphoridae) using *28S rRNA*, *COX1* and *EF-1α*: insights into the evolution of dipteran parasitism

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(*Received 10 January 2011; revised 28 April and 3 June 2011; accepted 13 June 2011; first published online 26 August 2011*)

SUMMARY

The Calliphoridae include some of the most economically significant myiasis-causing flies in the world – blowflies and screwworm flies – with many being notorious for their parasitism of livestock. However, despite more than 50 years of research, key taxonomic relationships within the family remain unresolved. This study utilizes nucleotide sequence data from the protein-coding genes *COX1* (mitochondrial) and *EF1α* (nuclear), and the *28S rRNA* (nuclear) gene, from 57 blowfly taxa to improve resolution of key evolutionary relationships within the family Calliphoridae. Bayesian phylogenetic inference was carried out for each single-gene data set, demonstrating significant topological difference between the three gene trees. Nevertheless, all gene trees supported a Calliphorinae-Luciliinae subfamily sister-lineage, with respect to Chrysomyinae. In addition, this study also elucidates the taxonomic and evolutionary status of several less well-studied groups, including the genus *Bengalia* (either within Calliphoridae or as a separate sister-family), genus *Onesia* (as a sister-genera to, or sub-genera within, *Calliphora*), genus *Dyscritomyia* and *Lucilia bufonivora*, a specialised parasite of frogs and toads. The occurrence of cross-species hybridisation within Calliphoridae is also further explored, focusing on the two economically significant species *Lucilia cuprina* and *Lucilia sericata*. In summary, this study represents the most comprehensive molecular phylogenetic analysis of family Calliphoridae undertaken to date.

Key words: Calliphoridae, blow-fly, screw-worm, dipteran parasitism, myiasis, hybridization, phylogenetics.

INTRODUCTION

To date, the most comprehensive phylogenetic analyses of Calliphoridae have been based on morphology (Rognes, 1991). Morphological evidence typically supports eight subfamily groupings within Calliphoridae; Calliphorinae, Chrysomyinae, Helicoboscinae, Luciliinae, Melanomyinae, Polliinae, Rhiniinae and Rhinophorinae (Rognes, 1991). Within the key myiasis-causing families, cladistic analysis of morphological characters supports a Calliphorinae-Chrysomyinae grouping (Rognes, 1997), but more recent molecular data instead suggest a Calliphorinae-Luciliinae sister-lineage to Chrysomyinae (Stevens, 2003; Wallman *et al*. 2005). Moreover, while the monophyly of Calliphorinae and Luciliinae are strongly supported (Stevens, 2003; Wallman *et al*. 2005), support for monophyly of subfamily Chrysomyinae is less robust.

One of the defining characteristics of blowflies is the necessity to lay eggs on proteinaceous matter, often the tissue of a living vertebrate host; larvae then develop by feeding on the protein-rich substrate, a form of parasitism known as myiasis (Zumpt, 1965). Within Calliphoridae, a range of larval feeding habits exist, including: coprophagy, saprophagy, sanguinivory and ectoparasitism (both facultative and obligate). Indeed, Calliphoridae represent some of the most economically significant myiasis-causing flies in the world, notorious for their parasitism of livestock. However, despite more than 50 years of research, key taxonomic relationships within Calliphoridae remain ambiguous and understanding the origins of blowfly parasitism is often restricted by the lack of widely agreed theories of evolutionary relationships and taxonomic classification within the family (Stevens, 2003). Consequently, using a multi-gene approach and broad taxon sampling across a range of Calliphoridae subfamilies (Table 1), this study aims to elucidate long-standing ambiguities within the family, including some questions that to date have proved equivocal in single gene studies, together with some issues previously unexplored with molecular methods. Such a robust evolutionary and taxonomic framework is essential to understand fully the evolution of this form of dipteran parasitism.

Within insect systematics, mitochondrial DNA (mtDNA) and nuclear ribosomal DNA molecular markers have traditionally been favoured (Otranto and Stevens, 2002; Shao and Barker, 2007), largely

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doi:10.1017/S0031182011001089

Table 1. Species list for taxa analysed in this study, including subfamily taxonomy, description of larval feeding behaviour, host type and sources used for classifying larval feeding behaviour (myiasis status)

Subfamily	Species	Larval feeding behaviour and typical host	Source reference
Auchmeromyiinae	*Auchmeromyia luteola*	Obligate (sanguinivorous) – vertebrates	1, 2
	Cordylobia anthropophaga	Obligate – vertebrates	1, 2
Bengaliinae	*Bengalia depressa*	Obligate – termites, ant pupae	1, 3
Calliphorinae	*Calliphora dubia*	Secondary facultative – carrion	4, Wallman *pers. comm.*
	Calliphora quadrimaculata	Secondary facultative – carrion, vertebrates	2, 5
	Calliphora stygia	Secondary facultative[1] – carrion, vertebrates	2, 4, Wallman *pers. comm.*
	Calliphora vicina	Secondary facultative – carrion, vertebrates	2, 6
	Calliphora vomitoria	Secondary facultative – carrion, vertebrates	2, 6
	Cynomya cadaverina	Saprophagic – vertebrates	7, 8
	Cynomya mortuorum	Secondary facultative – vertebrates	6, 7
	Onesia tibialis	Primary facultative – earthworms	9, 11
Chrysomyinae	*Chrysomya albiceps*	Secondary facultative – carrion, vertebrates	1, 2
	Chrysomya bezziana	Obligate – vertebrates	1, 2
	Chrysomya chloropyga	Secondary facultative – carrion, vertebrates	1, 2
	Chrysomya megacephala	Secondary facultative – carrion, vertebrates	1, 2
	Chrysomya rufifacies	Secondary facultative – carrion, vertebrates	1, 2
	Cochliomyia hominivorax	Obligate – vertebrates	12, 13
	Cochliomyia macellaria	Secondary facultative – vertebrates	12, 13
	Compsomyiops fulvicrura	Saprophagic – carrion	8, 10
	Phormia regina	Facultative (unknown) – carrion, vertebrates	6, 8
	Protocalliphora azurea	Obligate – birds	2, 6, 14
	Protocalliphora sialia	Primary facultative – carrion, vertebrates	2, 6, 14
	Protophormia terraenovae	Secondary facultative – carrion, vertebrates	2, 6
Helicoboscinae	*Eurychaeta palpalis*	Saprophagic – slugs and snails	6, 7
Luciliinae	*Dyscritomyia lucilioides*	Facultative (unknown) – carrion, vertebrates and/or invertebrates	15, 16
	Dyscritomyia robusta	Facultative (unknown) – carrion, vertebrates and/or invertebrates	15, 16
	Hemipyrellia fergusoni	Saprophagic – carrion	17, 18, 19
	Hemipyrellia fernandica	Saprophagic – carrion	17, 18, 19
	Lucilia ampullacea	Secondary facultative – frogs	2, 6
	Lucilia bufonivora	Obligate – frogs and/or toads	2, 6, 8, 20, *pers. obs.*
	Lucilia caesar	Secondary facultative – carrion, vertebrates	2, 6, 20
	Lucilia cluvia	Saprophagic – carrion	8, 21
	Lucilia cuprina	Primary facultative – carrion, vertebrates	2, 6, 8, 20
	Lucilia illustris	Secondary facultative – carrion, vertebrates	2, 6, 20
	Lucilia mexicana	Saprophagic – carrion	8, 22
	Lucilia papuensis	Saprophagic – carrion	4, 23
	Lucilia porphyrina	Primary facultative – vertebrates	2, 4
	Lucilia richardsi	Facultative (unknown) – vertebrates	2, 6
	Lucilia sericata	Primary facultative – carrion, vertebrates	2, 6, 8, 20
	Lucilia silvarum	Facultative (unknown) – frogs and/or toads	6, 8, 24
	Lucilia thatuna	Facultative (unknown) – vertebrates [To be confirmed]	8, 22
Polleniinae	*Pollenia rudis*	Primary facultative – earthworms	6, 7
Outgroup taxa (Diptera: Muscoidea)			
	Mesembrina meridiana	Facultative (unknown) – carrion, vertebrates	25, 26
	Musca domestica	Secondary facultative – faeces, carrion, vertebrates	2, 27
	Stomoxys calcitrans	Saprophagic – faeces, carrion	2, 28, 29

[1] Identified by Zumpt (1965) as originally a primary myiasis fly, a role it is not now generally recognised as fulfilling (Stevens and Wallman, 2006).

[1] Zumpt, 1956; [2] Zumpt, 1965; [3] Rognes, 1998; [4] Fuller, 1934; [5] Dear, 1985*a*; [6] Rognes, 1991; [7] van Emden, 1954; [8] Hall, 1948; [9] Norris, 1991; [10] Dear, 1985*b*; [11] Hardy, 1937; [12] James, 1947; [13] Shewell, 1987; [14] Sabrosky *et al*. 1989; [15] Pollock, 1974; [16] James, 1981; [17] So and Dudgeon, 1989; [18] Chen *et al*. 2004; [19] Sukontason *et al*. 2008; [20] Aubertin, 1933; [21] Nelder *et al*., 2009; [22] James, 1955; [23] Shah and Sakhawat, 2004; [24] Eaton *et al*. 2008; [25] Meier *et al*. 1999; [26] Karpa *et al*. 2007; [27] Dogra and Mahajan, 2010; [28] Bishop, 1913; [29] Parr, 1962.

due to the relative ease with which these markers can be amplified (Baker *et al*. 2001). The vast majority of molecular phylogenetic studies of parasitic arthropods to date have used single protein-coding genes (Shao and Barker, 2007), however, analyses are increasingly combining mitochondrial and nuclear genes, in an attempt to utilize unlinked sets of data that have evolved under essentially different constraints (Stevens, 2003; Lin and Danforth, 2004). Focusing on molecular markers with different rates of substitution in this way not only improves phylogenetic resolution at different levels of divergence, but also allows comparisons between evolutionary histories of gene trees and species trees to be explored. Accordingly, this study uses nucleotide sequence data from two protein coding genes (mitochondrial *COX1* and nuclear *EF-1α*) and one nuclear rRNA gene (*28S*). These three genes have been used extensively in insect systematics, including blowfly studies (e.g. Gleeson and Sarre, 1997; Stevens and Wall, 2001; Stevens *et al*. 2002; Stireman, 2002; Wells *et al*. 2002, 2004, 2007; Stevens, 2003; Petersen *et al*. 2007; Harvey *et al*. 2008; Kutty *et al*. 2008; Tourle *et al*. 2009), and as such provide sequences from a broad range of species across multiple genes for inclusion in this study. As a mitochondrial gene, *COX1* has several advantages including a lack of recombination during cell division, high copy number, relative ease of isolation, availability of universal primers and the presence of both conserved and variable regions. Mitochondrial genes are also expected to reach reciprocal monophyly before nuclear genes, due to generally higher rates of sequence change (Avise *et al*. 1979; Lunt *et al*. 1996; Monteiro and Pierce, 2001; Funk and Omland, 2003; Dowton, 2004; Lin and Danforth, 2004), making *COX1* particularly useful for inferring relationships between recently diverged species and in population genetics (Stevens and Wall, 1997*b*; Shao and Barker, 2007). Nuclear genes such as *EF-1α* also may offer several advantages over mitochondrial genes, for example, by having a generally low level of biased base composition (Friedlander *et al*. 1992, 1994; Brower and DeSalle, 1994; Lin and Danforth, 2004). However, paralogous copies of *EF-1α* have been identified in some insect species (e.g. Danforth and Ji, 1998), which may differ by up to 25% of the nucleotide sites in the coding regions. Consequently, multiple sequence alignments and BLAST searching were used across all three gene data sets to ensure that only the correct copies of the target genes were included in this study. The 28S subunit is a popular rRNA gene within insect systematics, as it displays both conserved and highly variable regions (D expansions) suitable for resolving relationships at a range of different hierarchical levels, and even having the ability to distinguish between closely related species (Otranto *et al*. 2005). This study focuses on these D expansions in an attempt to resolve relationships between closely related species, including apparent hybrid specimens.

The sequence data collected from these three genes were then analysed using Bayesian phylogenetic inference, an increasingly popular choice over Maximum Parsimony and Maximum Likelihood methods due to an apparent increased sensitivity to phylogenetic signal (Alfaro *et al*. 2003) and better estimates of phylogenetic accuracy in terms of nodal support (Hillis and Bull, 1993; Wilcox *et al*. 2002). In addition to the status of subfamily Chrysomyinae outlined above, a number of, to date, unresolved taxonomic relationships are also addressed. The identity of the genus *Bengalia* as true blowflies remains ambiguous, with two main hypotheses having been proposed, namely that *Bengalia* represent either a subfamily, Bengaliinae (Rognes, 2005), or a completely separate sister-family to Calliphoridae (Lehrer, 2003). The endemic Hawaiian genus *Dyscritomyia,* on the other hand, while differing from the majority of other blowflies in that it is viviparous (Pollock, 1974), are currently placed within the subfamily Luciliinae. To date, very little molecular research has focused on *Dyscritomyia* (see Wells *et al*. 2002). Within the Calliphorinae the group *Onesia* has traditionally been granted genus status but several phylogenetic studies have reported evidence that *Onesia* is in fact a sub-group within the genus Calliphora (Wallman and Adams, 1997; Wallman and Donnellan, 2001).

Within the genus *Lucilia*, the species *L. bufonivora* represents a specialist parasite which has evolved to feed exclusively on members of *Anura* (frogs and toads), with a high host specificity for the common toad, *Bufo bufo* (Brumpt, 1934; Strijbosch, 1980). *L. bufonivora* is thought to have diverged from its sister taxon, *L. silvarum*, relatively recently (Stevens and Wall, 1996*a*), with some authors, e.g. Townsend (1935), grouping both species as a separate subgenus '*Bufolucilia*'. Recent field studies in the German region of North Rhine-Westphalia revealed myiasis infestation rates in frogs and toads of up to 70%, causing significant mortality (Weddeling and Kordges, 2008). Nevertheless, *L. bufonivora* remains poorly studied (Neumann and Meyer, 2008) and the work presented here represents the first molecular phylogenetic study to include *L. bufonivora*, and includes analysis of larvae collected from a case of nasal myiasis in a toad collected in Suffolk, UK (Fig. 1).

Finally, this study provides new insights into the status of cross-species hybridisation between, *L. sericata* and *L. cuprina*, which together are the primary causal agents of 'sheep strike' in cool temperate (Europe and New Zealand) and sub-tropical/warm temperate regions (Australia and South Africa), respectively (Stevens and Wall, 1997*a*), and which represent some of the most well studied of all blowflies. Numerous morphological (Holloway 1991*a*,*b*; Stevens and Wall, 1996*a*) and genetic

Fig. 1. Toad with nasal myiasis, Suffolk, UK; posterior ends of live larvae are visible within the enlarged wounds at the site of the original nostrils (left nostril shown arrowed). Larvae were collected and preserved in alcohol prior to DNA extraction (photographs courtesy of Mr M. Porter).

(Stevens and Wall, 1996*b*, 1997*b*, Stevens *et al*. 2002; Stevens, 2003; Wallman *et al*. 2005; Harvey *et al*. 2008) studies have focused on trying to separate these ambiguous species, although few have employed a multi-gene approach to exploring the status of hybrid populations (Stevens and Wall, 1996*b*, 1997*b*; Nelson *et al*. 2007; Tourle *et al*. 2009). The work presented here aims to explore the hybridisation between these two economically significant blowflies within a multi-gene framework, and utilises specimens from a variety of geographical locations, including a hybrid from Hawaii (Stevens and Wall, 1996*b*), and a recently confirmed *L. cuprina* × *L. sericata* hybrid from South Africa (Tourle *et al*. 2009).

MATERIALS AND METHODS

Specimens

A total of 57 Calliphoridae taxa were used in this study, along with 3 outgroup taxa, comprising 90 previously published sequences and 90 new sequences (Table 2). These taxa represent a range of subfamilies, genera, parasitic larval feeding behaviours and, where possible, different geographic populations (Tables 1 and 2).

Specimens used to produce new sequence data came from in-house collections at the University of Exeter, freshly collected samples provided by colleagues, and specimens on loan from external collections. All samples were stored at 4 °C, either in 100% ethanol, or as dried pinned specimens.

Molecular analysis

DNA extractions were carried out using a salt extraction method (Aljanabi and Martinez, 1997), except in instances where only a limited amount of sample was available, for which DNA extraction was carried out using a Qiagen DNeasy® Blood & Tissue Kit (Qiagen GmbH, Germany).

DNA extractions were subject to PCR procedures to amplify regions of the nuclear protein-coding gene *elongation factor-1 alpha* (*EF-1α*), the ribosomal DNA *28S* (*28S rRNA*) and the mitochondrial protein-coding gene *cytochrome oxidase I* (*COX1*). Published universal insect primers (Table 3) and their corresponding PCR protocols were used to amplify *COX1* (Bogdanowicz *et al*. 1993; Simon *et al*. 1994; Sperling and Hickey, 1994; Sperling *et al*. 1995; Lunt *et al*. 1996; Wells and Sperling, 1999), *EF-1α* (McDonagh *et al*. 2009) and the D1–D7 expansion regions and related core elements of the large subunit *28S rRNA* (Hoelzel and Green, 1992; Friedrich and Tautz, 1997*a*, *b*); overlapping amplifications were performed according to the primer map shown in Fig. 2. These primers have been shown to work well in a broad range of insect species, including blowflies (e.g. Stevens and Wall, 2001; Stevens, 2003). Control samples (i.e. no DNA) were included in each set of PCR reactions to ensure no PCR carry-over or reagent contamination had occurred. PCR products were separated by gel electrophoresis and bands of appropriate sizes were cut out and purified. A fragment of ∼2·2 kb spanning the D1–D7 regions of the *28S rRNA* gene 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. For most taxa, amplification of the 1·6 kb *COX1* gene proceeded via a single fragment, though for a number of taxa amplification of a second shorter fragment of 0·68 kb (between primers COI F1 – COI R1) improved sequence read quality. The *EF-1α* fragment was ∼1·35 kb and was amplified in a single fragment. *COX1* fragment sizes were checked against published blowfly mtDNA genomes (Stevens *et al*. 2008), while *28S rRNA* and *EF-1α* fragments were checked against previously published sequences (Hovemann *et al*. 1988; Moulton, 2000; Friedrich and Tautz, 1997*a*, *b*; Stevens, 2003). Purified PCR products were then sequenced using a commercial sequencing facility (COGENICS, formally Lark Technologies Inc.).

Sequence alignment and verification of sequence identity

Sequence fragments were checked for quality and edited manually before being assembled into a single consensus sequence, using AutoAssembler 2.0 (Applied Biosystems, Inc.). Any ambiguities in the consensus sequence were resolved or standard IUPAC/IUB codes (Leonard, 2003) used.

All gene sequence identities were checked against GenBank using BLAST. While only a single copy of *EF-1α* has so far been found in members of Oestroidea, two copies of *EF-1α* have been identified

Table 2. Taxon list, including subfamily taxonomy, country of origin of specimen, collector, sources used for specimen identification and accession numbers for new DNA sequences

Subfamily	Species	Location (new sequences)	ID	*EF-1α*[a]	*COX1*[b]	*28S rRNA*[c]
Auchmeromyiinae	*Auchmeromyia luteola*	Nguruman, Kenya	JRS [1]	FR719213	FR719153	AJ551431 [30]
	Cordylobia anthropophaga	Yaoundé, Cameroon	JRS [1]	FR719229	FR719158	AJ551432 [30]
Bengaliinae	*Bengalia depressa*	Nairobi, Kenya	JRS/NW [1]	FR719214	FR719154	FR719270
Calliphorinae	*Calliphora dubia*	Adelaide, Australia	JFW [2]	FR719215	EU418556 [15]	AJ558185 [30]
	Calliphora quadrimaculata	Rangitoto, N.I., New Zealand	RDN/JRS [3]	FR719216	FR719155	AJ558187 [30]
	Calliphora stygia	Adelaide, Australia	JFW [2]	FR719217	AY842601 [16]	AJ558186 [30]
	Calliphora vicina	Adelaide, Australia	JFW [2]	FR719218	EU418571 [15]	AJ300132 [17]
	Calliphora vicina	Bristol, UK	JRS [4]	FR719219	AJ417702 [17]	AJ300131 [17]
	Calliphora vomitoria	Devon, UK	JRS [4]	FR719220	FR719156	AJ300133 [17]
	Calliphora vomitoria	Sonoma, USA	JRS [4]	FR719221	FR719157	AJ300134 [17]
	Cynomya cadaverina	Ottawa, Canada	LD [5]	FR719230	AF259505 [18]	AJ300135 [30]
	Cynomya mortuorum	Durham, UK	LD [4]	FR719231	FR719159	AJ300135 [17]
	Onesia tibialis	Adelaide, Australia	JFW [6]	FR719263	AY842605 [16]	AJ558188 [30]
Chrysomyinae	*Chrysomya albiceps*	Nairobi, Kenya	JRS [1,7]	FR719222	AF083657 [19]	AJ551433 [30]
	Chrysomya bezziana	Bogor, Java, Indonesia	RT/MJRH [1,7]	FR719223	AF295548 [20]	AJ551434 [30]
	Chrysomya chloropyga	Tanzania	JRS/RLW [1,8]	FR719223	AF295554 [20]	AJ558189 [30]
	Chrysomya megacephala	Calicut, Kerala, India	RLW [7]	FR719225	AF295551 [20]	FR719281
	Chrysomya rufifacies	Adelaide, Australia	JRS [7]	FR719226	AB112845 [21]	AJ551436 [30]
	Cochliomyia hominivorax	San Paulo, Brazil	AMLAE [5,9]	FR719227	EU418550 [15]	AJ551437 [30]
	Cochliomyia macellaria	San Paulo, Brazil	AMLAE [5,9]	FR719228	AF295555 [20]	AJ551438 [30]
	Compsomyiops fulvicrura	As published	As published	FJ025667 [22]	FJ025607 [22]	FJ025504 [22]
	Phormia regina	Brno, Czech Republic	OAF/JRS [4]	FR719264	AF295550 [20]	AF366685 [23]
	Protocalliphora azurea	Antwerp, Belgium	SH-B [4,10]	FR719266	FR719180	AJ551439 [30]
	Protocalliphora sialia	Kittitas Co., WA, USA	TLW [10]	FR719267	AF295559 [20]	AJ558190 [30]
	Protophormia terraenovae	Czech Republic	JRS/OAF [4]	FR719268	AF295553 [20]	AJ300142 [17]
Luciliinae	*Dyscritomyia lucilioides*	Hawaii, USA	JDW [11]	FR719232	AY074903 [24]	FR719288
	Dyscritomyia robusta	Hawaii, USA	JDW [11]	FR719233	AY074899 [24]	FR719289
	Hemipyrellia fergusoni	Tanzania	RLW/JFW [1,2]	FR719234	AY842613 [16]	FR719290
	Hemipyrellia fernandica	Tanzania	RLW [1]	FR719235	FR719160	AJ558191 [30]
	Lucilia ampullacea	Somerset, UK	RLW [4,12]	FR719236	EU925394 [25]	AJ300137 [17]
	Lucilia bufonivora[I]	Suffolk, UK	JRS[1] [4,12]	FR719237	FR719161	FR719293
	Lucilia bufonivora	Suffolk, UK	RLW [4,12]	FR719238	FR719162	FR719294
	Lucilia caesar	Somerset, UK	JRS [4,12]	FR719239	AJ417703 [27]	AJ300138 [17]
	Lucilia cluvia	New Orleans, USA	RLW [12]	FR719240	DQ453490 [26]	AJ551440 [30]
	Lucilia cuprina × *Lucilia sericata hybrid*[II]	Hawaii, USA	RLW/JDW [12,13]	FR719241	AJ417704 [27]	AJ417709 [30]
	Lucilia cuprina × *Lucilia sericata hybrid*[III]	Cape Town, South Africa	MHV [12,13]	FR719242	FR719164	FR719298
	Lucilia cuprina	Perth, Australia	RLW [12,13]	FR719245	AJ417707 [27]	AJ417709 [30]
	Lucilia cuprina	Townsville, Australia	RLW [12,13]	FR719247	AJ417710 [27]	AJ417709 [30]
	Lucilia cuprina	Nairobi, Kenya	JRS [12,13]	FR719243	FR719165	FR719299

	Lucilia cuprina	Dorie, S.I., New Zealand	AH/DMB [12,13]	FR719244	AJ417706 [27]	AJ417709 [30]
	Lucilia cuprina	Grahamstown, South Africa	MHV [12,13]	FR719246	FR719167	FR719302
	Lucilia cuprina	Tororo, Uganda	JRS [12,13]	FR719248	AJ417711 [27]	AJ417709 [30]
	Lucilia illustris	Somerset, UK	RLW [4,12]	FR719249	AJ551445 [30]	AJ300136 [17]
	Lucilia mexicana	San Francisco, USA	RLW [12]	FR719250	DQ453492 [26]	AJ551441 [30]
	Lucilia papuensis	Kuranda, QLD, Australia	JFW [2,12]	FR719251	AY842609 [16]	FR719307
	Lucilia porphyrina	Kuranda, QLD, Australia	JFW [2,12]	FR719252	AY842610 [16]	FR719308
	Lucilia richardsi	Usk, Gwent, UK	RLW [4,12]	FR719253	FR872384	AJ551442 [30]
	Lucilia sericata	Dorie, S.I., New Zealand	AH/DMB [12,13]	FR719254	AJ417713 [27]	AJ300140 [30]
	Lucilia sericata	Perth, Australia	JRS [12,13]	FR719255	AJ417715 [27]	AJ300140 [30]
	Lucilia sericata	Somerset, UK	JRS [12,13]	FR719256	AJ417714 [27]	AJ300139 [17]
	Lucilia sericata	Los Angeles, USA	JRS [12,13]	FR719257	AJ417715 [27]	AJ300141 [17]
	Lucilia sericata	Harare, Zimbabwe	RLW [12,13]	FR719258	AJ417717 [27]	AJ300140 [30]
	Lucilia silvarum	Durham, UK	LD/JRS [4,12]	FR719260	FR719176	AJ551443 [30]
	Lucilia silvarum	San Francisco, USA	RLW [4,12]	FR719259	FR719175	FR719316
	Lucilia thatuna	San Francisco, USA	RLW [12]	FR719261	FR719177	AJ551444 [30]
Polleniinae	*Pollenia rudis*	Devon, UK	JRS [4]	FR719265	FR719179	AJ558192 [30]
Helicoboscinae	*Eurychaeta palpalis*	As published	As published	FJ025672 [22]	FJ025612 [22]	FJ025512 [22]
Muscoidea	*Mesembrina meridiana*	Devon, UK	JRS [14]	FR719262	FR719178	FR719318
	Musca domestica	As published	As published	AF503149 [28]	AB479528 [29]	AJ551427 [30]
	Stomoxys calcitrans	As published	As published	FJ025698 [22]	AB479521 [29]	EF531151 [31]

[I] *Lucilia bufonivora* samples collected from nasal myiasis of a frog (Fig. 1), due to poor condition samples could not be identified morphologically; [II]suspected *Lucilia cuprina* × *Lucilia sericata* hybrid Stevens and Wall (1996*b*); [III]confirmed *Lucilia cuprina* × *Lucilia sericata* hybrid (Tourle *et al*., 2009).

Specimen identification: JRS = J.R. Stevens (Exeter, UK), NW = N. Wyatt (NHM, Lond., UK), JFW = J.F. Wallman (Wollongong, Australia), RDN = R.D. Newcomb (Auckland, New Zealand), LD = L. Davies (Durham, UK), RT = R. Tellman (CSIRO, Australia), MJRH = M.J.R. Hall (NHM, Lond., UK), RLW = R.L. Wall (Bristol, UK), AMLAE = A.M.L. Azeredo-Espin (Campinas, Brazil), OAF = O.A. Fischer (Brno, Czech Rep.), SH-B = S. Hurtrez-Boussès (Montpellier, France), TLW = T.L. Whitworth (Washington State, USA), JDW = J.D. Wells (Florida International, USA), AH = Allen Heath (AgResearch, New Zealand), DMB = D.M. Bishop (AgResearch, New Zealand), MHV = M.H. Villet (Rhodes, South Africa).

Sources used for species identification: [1] Zumpt, 1956; [2] Wallman, 2001; [3] Dear, 1985*a*; [4] Rognes, 1991; [5] Shewell, 1987; [6] Hardy, 1937; [7] Spradbery, 1991; [8] Rognes and Paterson, 2005; [9] James, 1947; [10] Sabrosky *et al*. 1989; [11] James, 1981; [12] Aubertin, 1933; [13] Holloway, 1991*b*; [14] D'Assis Fonseca, 1968.

Published sequences: [15] Harvey *et al*. 2008; [16] Wallman *et al*. 2005; [17] Stevens and Wall, 2001; [18] Wells *et al*. 2001; [19] Wells and Sperling, 1999; [20] Wells and Sperling, 2001; [21] Harvey *et al*. 2003; [22] Kutty *et al*. 2008; [23] Stireman, 2002; [24] Wells *et al*. 2002; [25] Park *et al*. 2009; [26] Wells *et al*. 2007; [27] Stevens *et al*. 2002; [28] Collins and Wiegmann, 2002; [29] Iwasa and Ishiguro, 2010]; [30] Stevens, 2003; [31] Petersen *et al*. 2007.

Table 3. Amplification and internal sequencing primers used to amplify the three genes studied

Gene	Primer	Sequence (5′ to 3′)	Source
COX1	*C1-J-1751a*	GGATCACCTGATATAGCATTCCC	Bogdanowicz *et al*. (1993)
	C1-J-2183	CAACATTTATTTTGATTTTTTGG	Simon *et al*. (1994)
	C1-J-2495	CAGCTACTTTATGAGCTTTAGG	Sperling *et al*. (1994)
	C1-N-1840	AGGAGGATAAACAGTTCAC/TCC	Sperling *et al*. (1995)
	C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	Bogdanowicz *et al*. (1993)
	C1-N-2659	GCTAATCCAGTGAATAATGG	Sperling and Hickey (1994)
	TL2-N-3013	TCCATTACATATAATCTGCCATATTAG	Wells and Sperling (1999)
	TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	Sperling *et al*. (1994)
	UEA7	TACAGTTGGAATAGACGTTGATAC	Lunt *et al*. (1996)
	UEA10	TCCAATGCACTAATCTGCCATATTA	Lunt *et al*. (1996)
EF-1α	*B1*	CCCATYTCCGGHTGGCACGG	McDonagh *et al*. (2009)
	C	GTCTCATGTCACGDACRGCG	McDonagh *et al*. (2009)
	F.In	GGTGGYATCGGHACAGTACC	McDonagh *et al*. (2009)
	R.Int	AGTTTCRACACGACCGACG	McDonagh *et al*. (2009)
	EF1	ACAGCGACGGTTTGTCTCATGTC	McDonagh *et al*. (2009)*
	EF2	CACATTAACATTGTCGTGATTGG	McDonagh *et al*. (2009)*
	EF3	CCGATACCACCGATTTTGTA	McDonagh *et al*. (2009)*
	EF4	CCTGGTTCAAGGGATGGAA	McDonagh *et al*. (2009)*
28S	*D1.F*	CCCCCTGAATTTAAGCATAT	Friedrich and Tautz (1997*a*)
	D2.R	GTTAGACTCCTTGGTCCGTG	Hoelzel and Green (1992)
	D1.R	CTCTCTATTCAGAGTTCTTTTC	Friedrich and Tautz (1997*a*)
	D2.F	GAGGGAAAGTTGAAAAGAAC	Hoelzel and Green (1992)
	D3	GACCCGTCTTGAAACACGG	Friedrich and Tautz (1997*b*)
	D7.R	CGACTTCCCTTACCTACAT	Friedrich and Tautz (1997*a*)
	D3–5.R	TTACACACTCCTTAGCGGA	Friedrich and Tautz (1997*b*)
	D35·486.R	TCGGAAGGAACCAGCTACTA	Friedrich and Tautz (1997*b*)
	D35·742.F	TCTCAAACTTTAAATGG	Friedrich and Tautz (1997*b*)
	D7.F	GACTGAAGTGGAGAAGGGT	Friedrich and Tautz (1997*a*)

* modified from Moulton (2000) primers: *3PC, 5PC, JOM, ShM*.

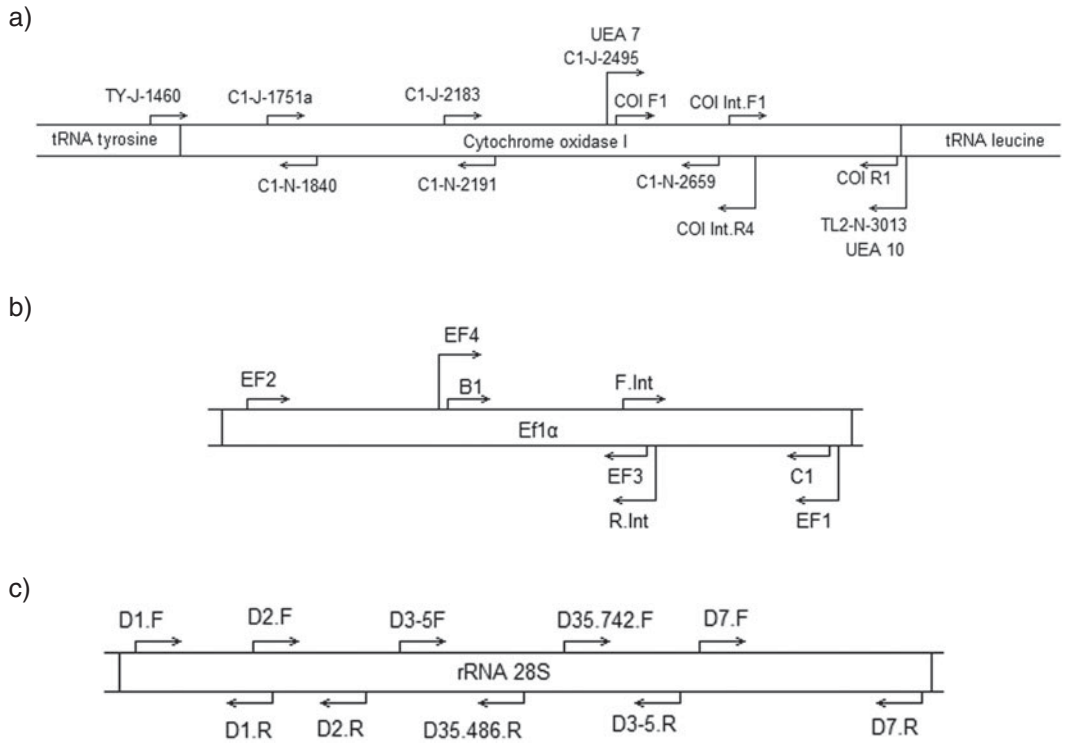

Fig. 2. Maps showing the arrangement of amplification primers and overlapping internal sequencing primers used to amplify the genes analysed in this study; (a) *COX1*, (b) *EF-1α*, (c) *28S rRNA*.

in bees and *Drosophila*, where they have been shown to differ in both intron position and in nucleotide sequence (Danforth and Ji, 1998). As a precaution, BLAST searching and examination of multiple sequence alignments were used to reduce the chance of *EF-1α* paralogs being included in our analyses. Multiple sequence alignment was carried out using the alignment editor SEAVIEW version 2.4 (Galtier *et al*., 1996) implementing the MUSCLE algorithm (Edgar, 2004); see alignments in SEAVIEW format in Supplementary Data files – appendix 1: *EF-1a*, appendix 2: *COX1* and appendix 3: *28S rRNA* – Cambridge Journals On-line, Parasitology. Subsequently, manual corrections by eye and amino acid translation were carried out to ensure parity with appropriate published insect protein-coding gene reading frames for *COX1* (EMBL translation table 5) and *EF-1α* (Kutty *et al*. 2008). The *28S rRNA* sequences were aligned against the framework developed by Stevens (2003); due to the presumably close taxonomic/evolutionary affinity of the taxa analysed, ribosomal RNA sequences were relatively homogeneous and, whilst some major indels were detected (e.g. *Dyscritomyia* spp. and several *Lucilia* spp.), alignment masking was not necessary prior to undertaking phylogenetic analyses.

Phylogenetic analysis

Appropriate nucleotide substitution model parameters were selected by a series of nested hierarchical likelihood-ratio tests using the program MODELTEST Version 3.06, (Posada and Crandall, 1998). Phylogenetic reconstructions were carried out using the program MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). All phylogenetic analyses implemented two independent Metropolis Coupled MCMC (MCMCMC) searches starting from different random trees (nruns = 2). Each search contained three heated chains (using the default heating temperature, temp = 0·2) and one cold chain, with a sampling frequency of 10 generations and selected models and priors applied. The combined-gene analysis implemented a partitioned data set, with model parameters unlinked and variable rate parameters, to allow each gene to evolve under different rates.

Analyses were continued until the convergence diagnostic (standard deviation of split frequencies) fell below the default threshold (stopval = 0·01), indicating sufficient convergence between the two samples of the posterior probability (Ronquist *et al*. 2005). The default convergence diagnostic burn-in fraction of 0·25 was used (burninfrac = 0·25), consequently, a corresponding burn-in of 0·25, relating to the first 25% of samples obtained up until convergence had been reached, was also applied to summarize substitution model parameters (sump) and trees and branch lengths (sumt). Plots of generation versus log probability of the data (log likelihood values) produced by the sump command were also checked to ensure stationarity had been reached (i.e. the plot showed no patterns in the data – data resembled 'white noise') (Ronquist *et al*. 2005). Tree topology was then calculated from the remaining data, after discarding burn-in samples, by constructing a majority-rule consensus tree. In this way the probability that a monophyletic clade was 'true', given the caveats of the model and data, was estimated by the proportion of trees in the MCMC sample in which the clade occurs (Brooks *et al*. 2007).

The widely used partition homogeneity test (parsimony-based ILD test) in PAUP* (Swofford, 1998) was used to test phylogenetic congruence between the single gene data sets. The test was implemented under parsimony, with random taxa addition, no swapping and 1000 replicates.

The parasitic status of each taxon was mapped onto the phylogenies to facilitate analysis of patterns of evolution of the differing parasitic traits. The status of each taxon was scored as either obligate, primary facultative, secondary facultative or saprophagic.

RESULTS

Single-gene trees

Of the three genes studied, *EF-1α* and *COX1* appeared to resolve relationships within Calliphoridae with the least conflict with existing taxonomy and contemporary evolutionary thinking relating to the family.

As noted, while only a single copy of *EF-1α* has so far been found in members of Oestroidea, two copies of *EF-1α* are known to exist in some insects, including flies (Danforth and Ji, 1998). As a precaution, BLAST searching and examination of multiple sequence alignments were used to reduce the chance of *EF-1α* paralogs being included. The BLAST searches revealed that all *EF-1α* sequences used in this study were of the same origin and not a mixture of different paralogs. Additionally, blowfly sequences were aligned and compared with *Drosophila melanogaster* sequences for *EF-1α* (F1) and the paralog *EF-1α* (F2) described by Hovemann *et al*. (1988) (F1, Accession No. X06869; F2, Accession No. X06870). Our sequences showed complete alignment with the F1 sequence (e.g. at positions 2135–3322 for *Calliphora dubia*), confirming our *EF-1α* sequences to be homologues; however, when aligned with the paralog copy of *EF-1α* (F2) a large number of indels were apparent, showing that our sequences were not paralog copies. Similarly, while no evidence for nuclear copies of *COX1* have been found within Oestroidea, extensive BLAST searching, examination of amino acid translations for stop codons and comparison of multiple sequence alignments suggest that only mitochondrial

orthologs have been included here. Of the three genes analysed, *28S rRNA* appeared to contain the least amount of phylogenetic signal. This was reflected by the relative ease with which sequences were aligned with each other, suggesting that the D1–D7 expansion regions were relatively well conserved across these species. The total number of characters in each sequence alignment was as follows: *COX1*: 1532; *EF-1α*: 1168; *28S rRNA*: 2166.

The best-fit model for all three single-gene data sets was the General Time Reversible model (GTR) with proportion of invariable sites (+I) and gamma distributed rate variation among sites (+*Γ*). The ILD test revealed a significant difference in topology between the three genealogies ($P<0{\cdot}01$; $n=1$; Fig. 3a–c).

Subfamily relationships

Despite incongruence being detected between the three genes, all single-gene trees in this study supported a Calliphorinae-Luciliinae sister-lineage, with respect to Chrysomyinae, although, only one gene (*COX1*) recovered Chrysomyinae monophyletically, with a posterior probability of just 75% (*COX1*; Fig. 3c).

Calliphorinae, on the other hand, was recovered monophyletically by both the *EF-1α* (Fig. 3a) and *28S rRNA* (Fig. 3b) gene trees and, despite significant topological differences between single-gene trees, was also recovered by the multi-gene tree. Within Calliphorinae, genus *Calliphora* was recovered as paraphyletic, with *C. vicina* and *C. vomitoria* grouping with the two *Cynomya* taxa in all three gene trees. *Onesia tibialis* was also found to group with *C. dubia* in both the *EF-1α* (Fig. 3a) and *28S rRNA* (Fig. 3b) trees, with the multi-gene tree (Fig. 4) also recovering *O. tibialis* and *C. dubia* together with high support.

Subfamily Luciliinae was recovered as monophyletic by all three genes, receiving posterior probability values of 100 in the *EF-1α* (Fig. 3a) and *COX1* (Fig. 3c) single-gene trees, as well as the overall multi-gene tree (Fig. 4).

Minor taxa

Within Luciliinae, *Dyscritomyia lucilioides* and *Dyscritomyia robusta* grouped together with high support in the *EF-1α* and *COX1* gene trees, with *28S rRNA* recovering *Dyscritomyia* as a separate sister-lineage to Luciliinae. The multi-gene phylogeny (Fig. 4) also recovered *Dyscritomyia* as a distinct clade, but failed to confirm the precise nature of their relationship to *Lucilia*.

Within Luciliinae, the two *L. bufonivora* specimens were consistently grouped together with high support, being placed in a clade with the UK and USA *L. silvarum* taxa by *EF-1α* (Fig. 3a) and *28S rRNA* (Fig. 3b), and grouped with the USA *L. silvarum* and *L. richardsii* by *COX1* (Fig. 3c).

The positioning of *Bengalia depressa* supports the classification of Bengaliinae as a subfamily within Calliphoridae; in all three single-gene phylogenies *B. depressa* was placed as a sister lineage to Auchmeromyiinae, and it received strong support for sister-taxon status in the multi-gene tree (Fig. 4).

Finally, *L. sericata* and *L. cuprina* were recovered as two distinct clades by *EF-1α* (Fig. 3a), with posterior probabilities of over 0·95. In contrast, *COX1* (Fig. 3c) grouped the South African *L. cuprina*×*L. sericata* hybrid and the Hawaiian suspected hybrid taxa together, forming a sister-lineage to the *L. sericata* clade. The *28S rRNA* gene tree (Fig. 3b), however, while grouping the two hybrid taxa within other *L. cuprina* taxa, failed to recover either *L. cuprina* or *L. sericata* monophyletically. Analysing the multi-gene data set (Fig. 4) resulted in a sub-grouping of the South African and Hawaiian hybrid taxa within a monophyletic *L. cuprina* clade, confirming mixed hybrid signals between genes.

Analysis of inter-gene phylogenetic congruence

The level of phylogenetic congruence between single gene data sets assessed using the partition homogeneity test (ILD test) in PAUP* (Swofford, 1998) revealed a significant difference ($P<0{\cdot}01$, $n=1$) in topology between the three genealogies. Surprisingly, perhaps, subsequent querying of the gene partitions failed to highlight any one gene as being obviously aberrant from the other two, though this could be due to less robust resolution at key upper nodes, rather than any indication that the evolutionary histories described by the three genes are equally different.

Differing forms of parasitism within Calliphoridae

In all single-gene trees (Fig. 3a–c) and the multi-gene phylogeny (Fig. 4), the distribution of taxa demonstrating obligate parasitism, facultative parasitism and saprophagy is approximately equal throughout, probably reflecting both the multiple independent evolution of these life-history traits and the non-random taxonomic sampling effort applied in order to best represent the range of parasitic styles found within Calliphoridae. The blowfly species used in this study include eight saprophagic taxa, representing the subfamilies Luciliinae (*L. papuensis*, *H. fergusoni*, *H. fernandica*), Calliphorinae (*C. cadaverina*), Chrysomyinae (*C. fulvicrura*), and Helicoboscinae (*E. palpalis*). Four of the seven blowfly subfamilies include obligate parasitic species, namely: Auchmeromyiinae (*A. luteola* and

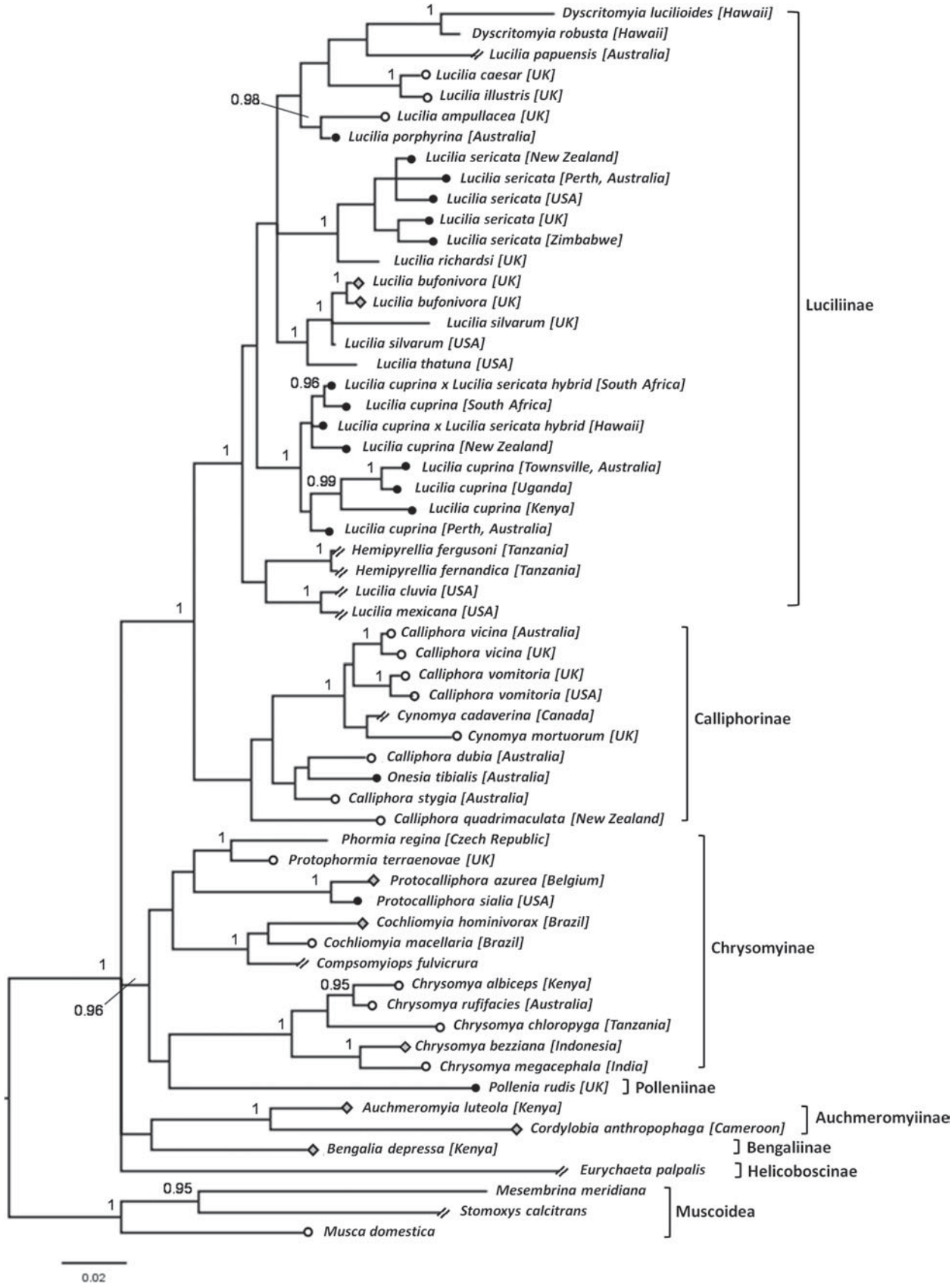

Fig. 3a. *EF-1α*. For legend see Fig. 3c.

C. anthropophaga), Bengaliinae (*B. depressa*), Chrysomyinae (*C. hominivorax* and *P. azurea*), and Luciliinae (*L. bufonivora*). The remaining taxa are all facultative parasites, with larvae being capable of feeding on dead or living host tissue. The facultative parasitic taxa were also sub-divided into those

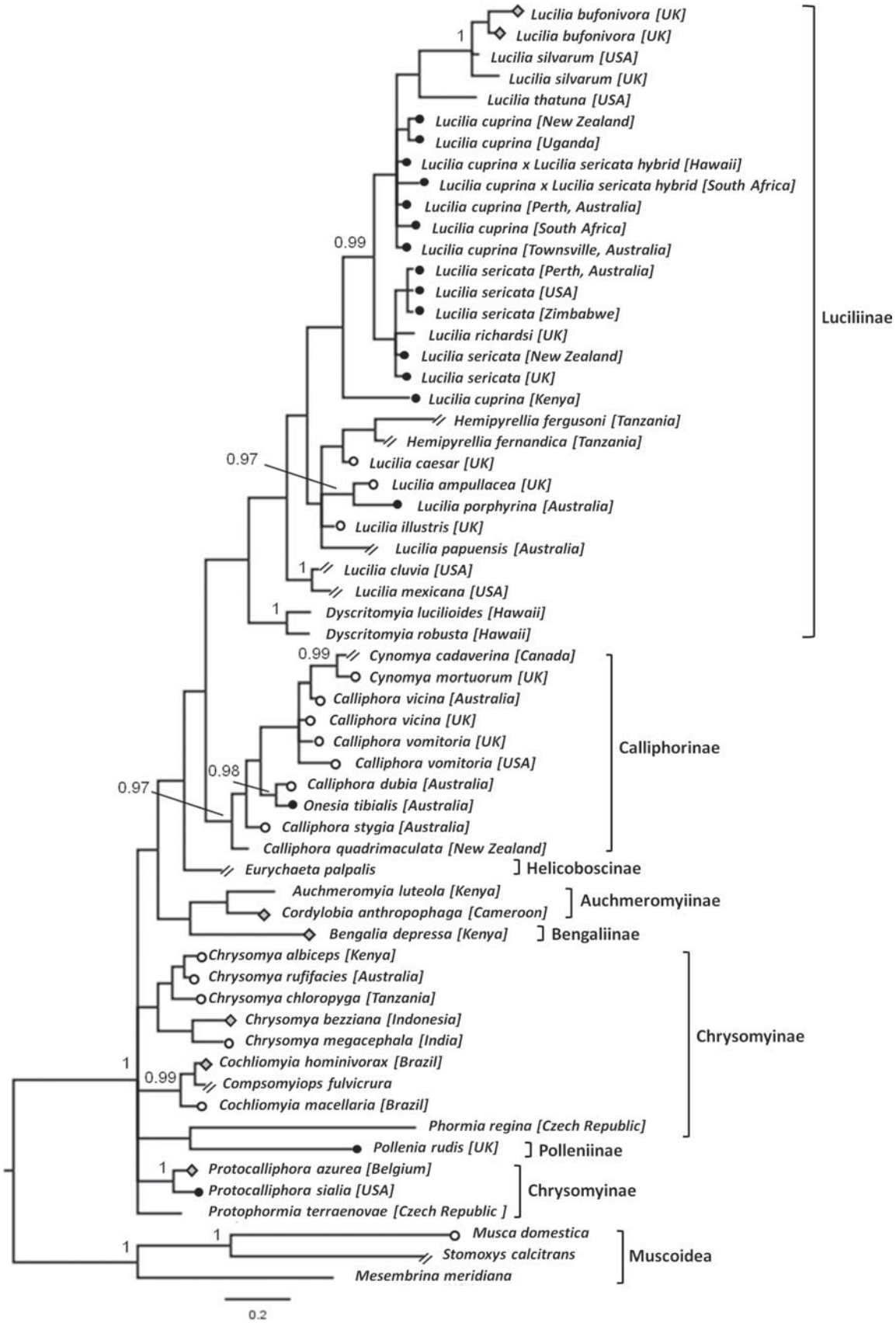

Fig. 3b. *28S rRNA*. For legend see Fig. 3c.

capable of initiating myiasis in an otherwise healthy host (primary facultative parasites), and those capable only of infesting existing wounds (secondary facultative parasites). However, a lack of information or conflicting reports, regarding whether a species is capable of initiating myiasis or not, have prevented

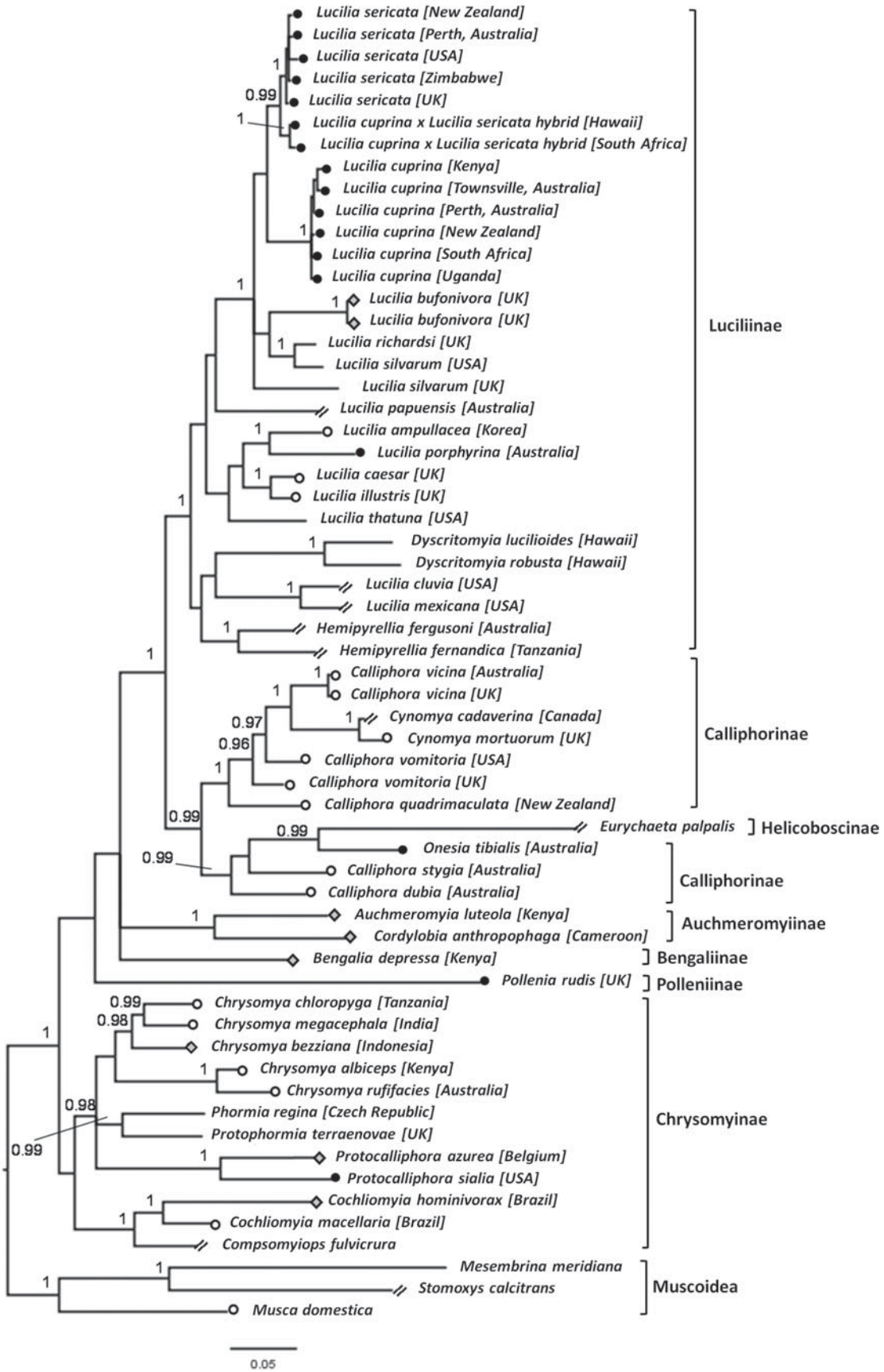

Fig. 3c. Phylogenetic relationships within Calliphoridae (ingroup) and representatives of Muscoidea (outgroup), based on a Bayesian analysis of nucleotide data from (a) *EF-1α*, (b) *28S rRNA* and (c) *COX1*. All branches supported with a Bayesian posterior probability of ⩾0·95 are labelled. ● Primary facultative parasite; ○ Secondary facultative parasite; ◇ Obligate parasite; // Saprophagic.

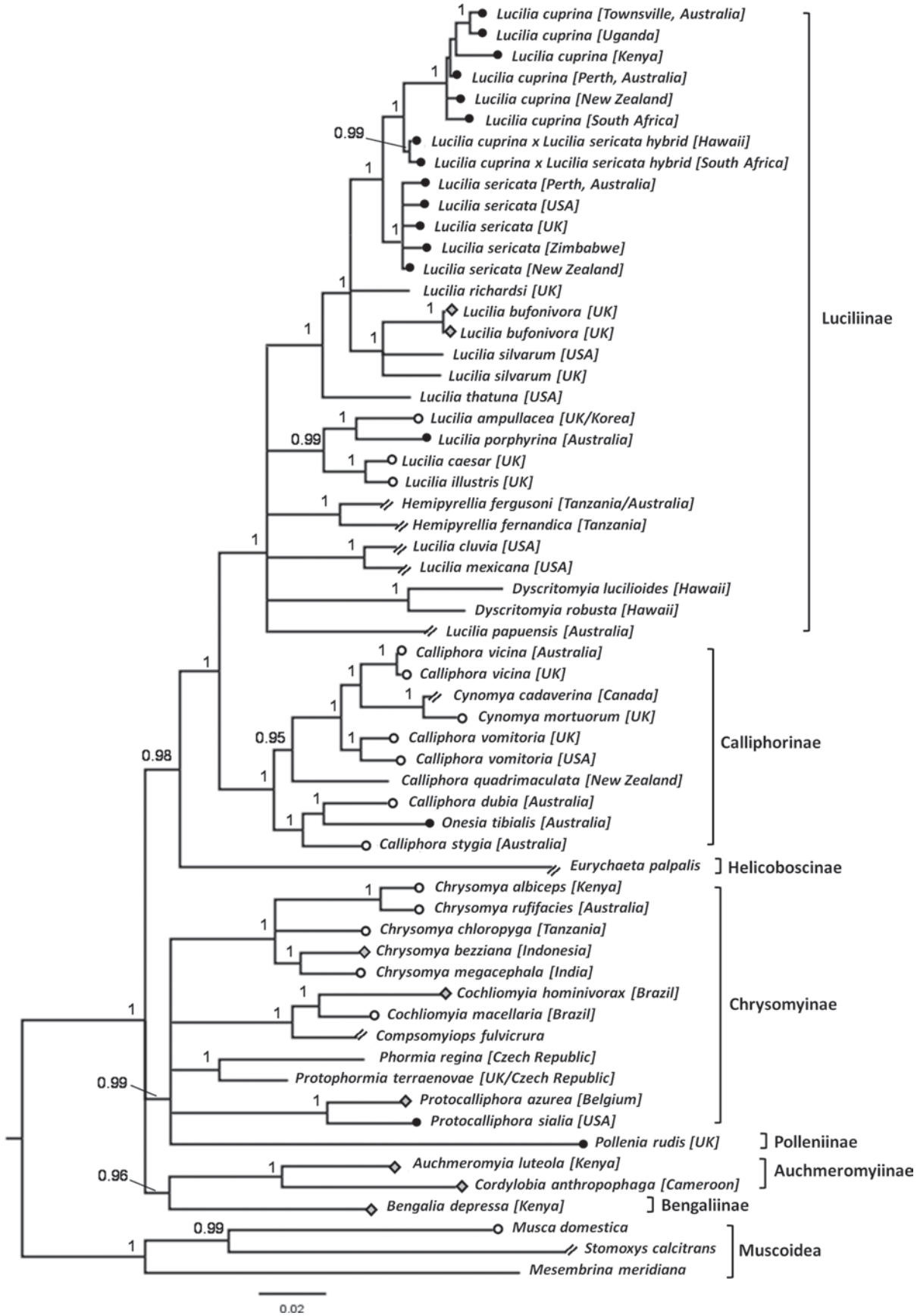

Fig. 4. Phylogenetic relationships within Calliphoridae (ingroup) and representatives of Muscoidea (outgroup), based on a partitioned Bayesian analysis of the combined gene (*EF-1α, 28S rRNA, COX1*) data set. All branches supported with a PP $\geqslant 0{\cdot}95$ are labelled. The ILD test indicated significant phylogenetic conflict between the three genes; consequently, this multi-gene-based topology should not be used to represent accurate phylogenetic relationships within Calliphoridae, but instead can be taken as a guide to areas of congruence and conflict between the single gene phylogenies. ● Primary facultative parasite; ○ Secondary facultative parasite; ◇ Obligate parasite; // Saprophagic.

some taxa from being unequivocally classified in this way (Table 1). While the presence of each of these forms of parasitism in each of the subfamilies within Calliphoridae appear to suggest either multiple origins or multiple losses of parasitism, in reality it is likely that parasitism has both evolved and possibly been lost at several points within the family, and that ancestral larval forms were already predisposed to be able to exploit proteinaceous material, e.g. carrion.

DISCUSSION

Of the largest (most taxon-rich) subfamilies included in this study – Calliphorinae, Luciliinae, Chrysomyinae – support for monophyly of each has varied depending on the gene(s) analysed and phylogenetic method employed (Stevens 2003; Wallman *et al*. 2005; Stevens and Wallman, 2006). While the current study found strong support for Calliphorinae and Luciliinae, Chrysomyinae was recovered as a monophyletic grouping by only one of the three genes employed (*COX1*). This may reflect the differing evolutionary history of these three genes or it may (at least in part) be an artefact of unbalanced sampling; for example, while all Luciliinae and most Calliphorinae genera were represented by two or more taxa, three Chrysomyinae genera were represented each by only a single taxon. While this study has been limited to those taxa available for DNA extraction and to those for which sequence data are published, future detailed sampling effort focusing on Chrysomyinae appears much needed. Similarly, additional Helicoboscinae taxa also need to be analysed to allow the true evolutionary position of this under-studied subfamily to be unequivocally determined.

Multiple-gene phylogenies not only permit the use of genes that have evolved at different rates, but also allow the identification of experimental errors in species identification and sequencing (Monteiro and Pierce, 2001). For example, despite *Onesia* traditionally being classified as a separate genus within Calliphorinae, two of the three genes included in this study recovered *O. tibialis* within the genus *Calliphora*. Whether this incorrect genus classification is true for other, or all, *Onesia* species is unclear, and to date only a small number of *Onesia* species have been included in molecular phylogenetic studies.

This study also attempted to resolve the position of the endemic Hawaiian saprophagous group *Dyscritomyia* within Calliphoridae. While all three genes recovered *Dyscritomyia* within Luciliinae, the position of the genus within this subfamily differed between phylogenies. While, in agreement with Wells *et al*. (2002), *Dyscritomyia* were recovered as a separate sister lineage to *Lucilia* by nuclear *28S rRNA*, *COX1* placed *Dyscritomyia* away from the main clade of *Lucilia* species, instead grouping it with *L. mexicana*, *L. cluvia* (both North American species), and two *Hemipyrellia* taxa. In contrast, *EF-1α* recovered *Dyscritomyia* within the main *Lucilia* clade. These findings highlight the complicated genetic history of *Dyscritomyia*, with a nuclear gene phylogeny (*28S rRNA*) clearly placing *Dyscritomyia* as a sister-clade to *Lucilia*, while protein-coding gene phylogenies suggest more recent shared genetic ancestry with *Lucilia* spp. and, indeed, the possibility that the *Dyscritomyia* evolved from within genus *Lucilia*. Such a result accords with examples of phylogenetic incongruence between nuclear and mitochondrial phylogenies seen previously in Calliphoridae (Stevens and Wall, 1996*b*; Stevens *et al*. 2002; Nelson *et al*. 2007; Toure *et al*. 2009) and highlights the importance of using multiple genes to establish true evolutionary relationships in these Diptera. Clearly, more work using additional nuclear genetic markers will be required to resolve the evolutionary history of this enigmatic Hawaiian genus.

This study also included, for the first time, two *L. bufonivora* samples from a parasitised common toad. The consistent separation of these taxa from *L. silvarum*, the only other *Lucilia* species implicated in toad myiasis (Hall, 1948; Zumpt, 1965), suggests that they are separate sister species. Additionally, two out of three single-gene phylogenies (*28S rRNA* and *COX1*) placed the *L. bufonivora*/*L. silvarum* clade as a sister group to *L. cuprina* and *L. sericata*, suggesting a possible northern Hemisphere origin for these flies and the toad/frog parasitic habit.

The present study also revisited the topic of hybridisation within Calliphoridae, focusing on the two economically significant blowflies, *L. cuprina* and *L. sericata*. If hybridization is rare, the few viable hybrids that do occur may still potentially have significant evolutionary consequences; additionally, hybridization between morphologically similar species can often be very cryptic (Mallet, 2005). By comparing molecular data from three different genes, this study has extended the findings of previous studies that have demonstrated the introgression of *L. sericata* mitochondrial *COX1* haplotypes into *L. cuprina* morphotype specimens. In terms of adult phenotype, *L. cuprina* seems to be dominant over *L. sericata*, as previously indicated by the findings of Ullyett (1945) and Stevens *et al*. (2002). Additionally, backcrossing hybrids are often very difficult to distinguish morphologically from parent species, in consequence, rates of backcrossing can easily be underestimated (Mallet, 2005). An important implication of hybridization between *L. sericata* and *L. cuprina* is the potential introgression of insecticide resistance (Stevens *et al*. 2002; Tourle *et al*. 2009) which has already been documented between other fly species (Boakye and Meredith, 1993). Recent advances towards the development

of a female killing (FK) system for the control of *L. cuprina* in Australia (Scott *et al*. 2004) could also come under threat if hybridization proves more common than expected. In this FK system, modified male *L. cuprina* carrying wild type alleles on a Y-linked translocation and alleles for a recessive eye colour mutation on a normal set of autosomes, pass the Y-linked translocation onto their male offspring only, and the mutation onto their female offspring. When heterozygous females mate with modified males, half of all female offspring will be homozygous for the mutation, making them functionally blind with white eyes, thereby greatly reducing their chance of surviving to maturity (Whitten *et al*. 1977; Foster *et al*. 1988). Field trials of this FK system have proved to reduce wild *L. cuprina* populations by both the semi-sterility caused by the translocation itself, and through the eventual increase in proportion of homozygotic individuals through sustained release (Whitten *et al*. 1977; Whitten, 1979; Foster *et al*. 1985; Foster, 1989). While genetic death rates of up to 94% have been obtained using this FK system (Foster, 1991) hybridization could potentially affect success rates.

Finally, this study has demonstrated that taxa exhibiting obligate parasitism, facultative parasitism and saprophagy are spread approximately equally across multi-gene phylogenies. Of course, there are many species which have not been included and, despite the breadth and taxon coverage of the current study, taxon coverage remains biased towards those species of veterinary, medical and economic importance. Nonetheless, the study confirms and extends the findings of many previous studies (see Stevens and Wallman, 2006; Stevens *et al*. 2006 for full details) and highlights the multiple and in some cases probably relatively recent origins of the parasitic habit within this group of Diptera (Wiegmann *et al*. 2011). Our findings also indicate that the origins of the group probably lie with an ancestral form that was already pre-adapted to utilise proteinaceous matter as a key resource in its development.

ACKNOWLEDGEMENTS

We thank L. M. Evans (Exeter) for help with additional sequencing, N. Wyatt (Natural History Museum, London) for assistance in confirming the identity of *Bengalia depressa* and Mr M. Porter (Suffolk) for specimens and photographs of larvae infesting a live toad. We thank also the many colleagues who have helped in providing specimens for this and previous studies which have contributed to this work; chief among these are: R. Wall (Bristol), J. Wallman (Wollongong), R. Newcomb (Auckland), L. Davies (Durham), R. Tellman (CSIRO, Australia), M. Hall (NHM, Lond.), A.M.L. Azeredo-Espin (Campinas), O. A. Fischer (Brno, Czech Rep.), S. Hurtrez-Boussès (Montpellier), T. L. Whitworth (Washington State), J.D. Wells (Florida International), A. Heath (AgResearch, New Zealand), D. M. Bishop (AgResearch, New Zealand), M. H. Villet (Rhodes, S. A.).

FINANCIAL SUPPORT

This work was funded by the University of Exeter and the Natural Environment Research Council, UK (Grant No. NER/M/S/2003/00081).

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