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Genetic relationships between blowflies (Calliphoridae) of forensic importance

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Abstract

Phylogenetic relationships among blowfly (Calliphoridae) species of forensic importance are explored using DNA sequence data from the large sub-unit (lsu, 28S) ribosomal RNA (rRNA) gene, the study includes representatives of a range of calliphorid species commonly encountered in forensic analysis in Britain and Europe. The data presented provide a basis to define molecular markers, including the identification of highly informative intra-sequence regions, which may be of use in the identification of larvae for forensic entomology. Phylogenetic analysis of the sequences also provides new insights into the different evolutionary patterns apparent within the family Calliphoridae which, additionally, can provide a measure of the degree of genetic variation likely to be encountered within taxonomic groups of differing forensic utility.

Keywords: Blowflies; Calliphoridae; Phylogenetics; Large sub-unit ribosomal RNA; Forensic entomology

1. Introduction

Saprophagous blowflies of the family Calliphoridae are the most commonly encountered arthropod fauna used in forensic analysis. The adults of individual blowfly species and even age- and sex-classes within any one species, may show specific spatial and temporal differences in abundance and in their responses to corpses at different stages of decomposition [1]. The rates of development of the eggs and larvae are also highly predictable, being strongly determined by temperature and to a lesser extent humidity. A detailed understanding of the biology of various blowfly species and their predictable succession within a decomposing corpse, therefore, can provide important information relating to the place and, particularly, time of death. For such entomologically-based time estimates to be valid and acceptable in law, highly accurate identification of the species present is essential. However, identification of many insect species by morphological methods is often difficult, particularly for poorly preserved specimens and larvae. In such cases, DNA typing of forensic insect specimens offers a quick and reliable alternative; the potential advantages of such an approach have been demonstrated previously for three species of North American blowfly using cytochrome mitochondrial DNA sequences [2].

This paper presents analysis of 28S large sub-unit (lsu) ribosomal RNA (rRNA) sequences for species of calliphorid blowflies commonly encountered in forensic analysis in Britain and Europe. A 'sliding window' approach is adopted to maximize the phylogenetic signal and, hence, the discriminatory power of the technique, while attempting to minimize (for practical purposes) the number and length of DNA fragments to be sequenced. This information can provide a baseline for future sequence-based studies and the development of specific markers for identification purposes.

2. Materials and methods

2.1. Flies and DNA extraction

Blowfly DNA was obtained from the species listed in Table 1. Flies were caught with the aid of liver-baited sticky traps, as described previously [3]. In this study, to avoid possible contamination of fly DNA with DNA from ingested

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

Specimen	Sub-family	Collection location	Date	Accession number
Family Tachinidae				
Tachina grossa (Linnaeus)		Dartmoor, Devon, UK	1999	AJ300130
Family Calliphoridae				
Calliphora vicina Robineau-Desvoidy	Calliphorinae	University of Bristol Colony, UK	1995	AJ300131
Calliphora vicina (Australia)		Adelaide, SA, Australia	1998	AJ300132
Calliphora vomitoria (L.)		Slapton Ley, Devon, UK	1999	AJ300133
Calliphora vomitoria (USA)		Sonoma, California, USA	2000	AJ300134
Cynomya mortuorum (L.)		Durham, Co. Durham, UK	1999	AJ300135
Lucilia illustris (Meigen)	Luciliinae	Langford, Somerset, UK	1994	AJ300136
Lucilia ampullacea Villeneuve		Langford, Somerset, UK	1994	AJ300137
Lucilia caesar (L.)		Langford, Somerset, UK		AJ300138
Lucilia sericata (Mg.)		Langford, Somerset, UK	1994	AJ300139
Lucilia sericata (Denmark)		Hilerod, Sjelland, Denmark	1994	AJ300140
Lucilia sericata (USA)		Los Angeles, California, USA	1994	AJ300141
Protophormia terraenovae (Robineau-Desvoidy)	Chrysomyinae	Exeter, Devon, UK	1999	AJ300142

protein, gut parasites or eggs, only the thoracic flight muscle of flies was used as a source of DNA. However, in practical application, if only other chitinous tissues (e.g. legs) are available, an additional digestion with chitinase may be performed prior to commencing DNA extraction, a previous study [4] describes procedures for processing individual fly tissues (i.e. head, legs, thorax).

High quality DNA was extracted, as total nucleic acid, by the cetyl trimethyl ammonium bromide (CTAB) method [4] and, latterly, by a salt extraction method [5]. Between 1000 and 4000 ng of DNA was obtained separately from individual flies. The yield and quality of DNA extracted from field caught flies was assessed on a 1% Tris–borate/EDTA (TBE) agarose gel prior to PCR amplification of the 28S rRNA gene. Samples (2 μl of total nucleic acid) were run against 10 μl of heat denatured (65°C, 5 min) Lambda Hind III marker. The intensity of the upper band of high molecular weight in the sample DNA was then compared with the 4.36 kb marker band which contained approximately 0.1 μg DNA; this allowed the DNA yield of each sample to be assessed readily. The discreteness of the high molecular weight band and the degree of streaking in each sample lane provided a measure of DNA quality; RNA appeared as an indiscrete broad band near the bottom of the gel.

2.2. Phylogenetic marker

The lsu rRNA gene was selected as the marker for this study. Its inherently diverse rates of genetic evolution 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 [6]. Indeed, over the course of numerous studies the rRNA genes have become the marker of choice for evolutionary analyses of a broad range of organisms, for example [7].

2.3. Choice of outgroup

Definition of the family Calliphoridae and its component sub-families has undergone numerous revisions, for example [8–12]. However, its distinctness from and relationships with the sister families Tachinidae and Sarcophagidae are now generally well accepted, see [12]. Hence, the tachinid, *Tachina grossa*, was used as an outgroup in the current study.

2.4. PCR amplification and DNA sequencing

A fragment of 2.2 kb spanning the D1–D7 regions of the 28S lsu rRNA gene was amplified from 13 species/strains of blowflies (Table 1) by PCR using conserved primers as published [13–15] (Table 2). 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.

PCR amplification mixtures (25 μl volume) were prepared as follows: 50 ng template DNA, reaction buffer (final concentration 75 mM Tris–HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% TWEEN-20, 1.5 mM $MgCl_2$; Hybaid-AGS), dNTP mix at a final concentration of 0.2 mM per dNTP, 2 × primers at a final concentration of 2 μM, 1 U of AGSGold DNA polymerase (Hybaid UK), sterile water to a final volume of 25 μl. Amplification reactions were performed in a Hybaid thermal cycler for an initial denaturation step of 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 1 min (D1–D2 fragment)/1 min 30 s (D3–D7 fragment), followed by a final elongation step of 68°C for 15 min.

Table 2
Amplification and sequencing primers for 28S rRNA regions D1–D7

D1–D2 fragment
Amplification primers
D1.F: 5′-CCCCCTGAATTTAAGCATAT-3′ (20-mer)[a]
D2.R: 5′-GTTAGACTCCTTGGTCCGTG-3′ (20-mer)[b]
Internal sequencing primers
D1.R: 5′-CTCTCTATTCAGAGTTCTTTTC-3′ (22-mer)[a]
D2.F: 5′-GAGGGAAAGTTGAAAAGAAC-3′ (20-mer)[c]
D3–D7 fragment
Amplification primers
D3-5.F: 5′-GACCCGTCTTGAAACACGG-3′ (19-mer)[d]
D7.R: 5′-CGACTTCCCTTACCTACAT-3′ (19-mer)[a]
Internal sequencing primers
D3-5.R: 5′-TTACACACTCCTTAGCGGA-3′ (19-mer)[d]
D3-5.486.R: 5′-TCGGAAGGAACCAGCTACTA-3′ (20-mer)[e]
D3-5.742.F: 5′-TCTCAAACTTTAAATGG-3′ (17-mer)[d]
D7.F: 5′-GACTGAAGTGGAGAAGGGT-3′ (19-mer)[a]

[a] Modified from [14].
[b] From [13].
[c] Modified from [13].
[d] Modified from [15].
[e] From [15].

PCR products were separated by electrophoresis in 0.5% TBE agarose gels, stained with ethidium bromide and visualized under UV-illumination. The products of 4–8 separate PCR reactions per specimen were excised from the gel with a clean scalpel and pooled prior to purification with Sephaglas BP beads (Amersham Pharmacia Biotech). The pooling of amplicons reduces (dilutes) the possibility that random PCR amplification errors in individual reactions are carried forward and sequenced; in addition, all output traces from automated sequencing (see below) were checked for possible hybrid sites and ambiguities.

Fragments were sequenced in both directions at 350–500 bp intervals, depending on the location of suitable primer sites, using the four original amplification primers and six additional internal primers (Table 2; [13–15]). Between 30 and 90 ng of pooled, purified template were used per sequencing-PCR reaction; reactions were run on a Perkin-Elmer ABI 377 automated sequencer. A consensus sequence was assembled from the individual internal primer sequences for each specimen using the program AutoAssembler (ABI).

2.5. Sequence alignment

All sequences were aligned using the program Clustal W [16], before final adjustments were made by eye. Hypervariable sites (32), where nucleotide changes were saturated and regions where it was not possible to produce a single reliable alignment across all taxa, were excluded from the analysis. The alignment analyzed initially included 2148 (2180 − 32) nucleotide positions (np) and is available on request from JRS.

2.6. Phylogenetic analysis

Phylogenetic analyses were performed using the program PAUP* [17]. Bootstrapped maximum parsimony analyses of 12 Calliphoridae and one Tachinidae 28S lsu rRNA sequences were performed with 100 replicates. The relatively limited number of taxa included allowed the use of the exhaustive branch and bound search strategy to find the most parsimonious tree(s). The default options of PAUP were used: initial upper bound computed stepwise; only minimal trees kept; addition sequence = furthest; zero length branches collapsed.

Maximum-likelihood analysis was also performed; starting trees were derived by both parsimony and neighbour-joining. Transition/transversion ratios were estimated from the data in preliminary runs and then set for full analyses.

The program MacClade [18] 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 map the sites along the length of the D1–D7 fragment of the 28S lsu rRNA gene. The informative regions identified were used as the basis for subsequent assessments of the forensic utility of 200–310 bp sub-sections of the 28S rRNA gene.

3. Results

3.1. Phylogenetic analysis

Parsimony analysis of the 2148 aligned nucleotides yielded 46 informative characters and produced six equally parsimonious trees (length = 97). The analysis yielded two basic tree forms which differed in their arrangement of clades corresponding to sub-families (Fig. 1). In Fig. 1a the arrangement of sub-family clades [[Calliphorinae, Luciliinae] Chrysomyinae] received 47.0% bootstrap support (★ node), while in Fig. 1b, an alternative topology which grouped the sub-families as [[Calliphorinae, Chrysomyinae] Luciliinae] obtained 50.6% bootstrap support (★ node); the third possible topology [[Chrysomyinae, Luciliinae] Calliphorinae] was not represented amongst the most parsimonious trees and received <5% support.

Given the conflicting and approximately equally well supported topologies recovered, a bootstrapped parsimony consensus tree was constructed (Fig. 1c). This tree provides strong support for the integrity of the three sub-families of Calliphoridae included in this study (>96% bootstrap) and for a high degree of relatedness between non-*sericata* *Lucilia* sp. (90% bootstrap). The analysis also indicates a degree of intra-specific genetic variation in *Calliphora*

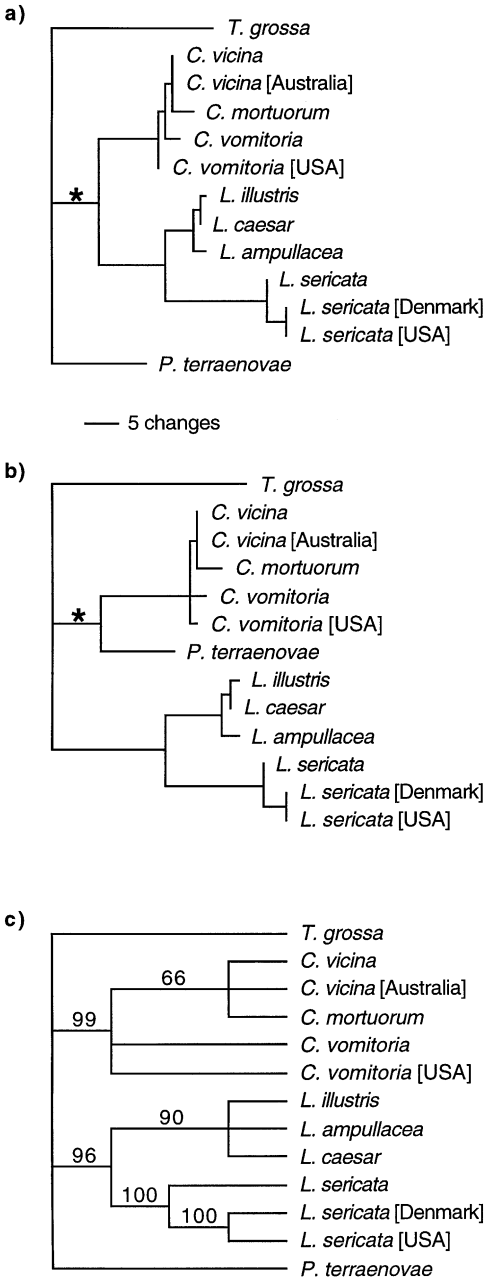

Fig. 1. Phylogenetic trees contructed by maximum parsimony analysis of 2148 aligned nucleotides. Trees (a) and (b) summarize the two forms of the six equally parsimonious trees (length = 97) recovered. In tree (a) the relationship of sub-family clades is [[Calliphorinae, Luciliinae] Chrysomyinae], while in tree (b) the alternative topology [[Calliphorinae, Chrysomyinae] Luciliinae] is presented. See text for details of bootstrap values at critical (★) nodes. The bootstrapped (100 repetitions) maximum parsimony consensus tree is presented in (c); nodes receiving bootstrap support below 50% are shown as polytomies.

vomitoria (*C. vomitoria*) across wide geographical areas, and provides evidence of the genetic similarity of *Cynomya mortuorum* and *Calliphora vicina* at this gene; the implications of these findings for using molecular approaches to distinguish these flies for forensic purposes are discussed (see below).

Maximum-likelihood analysis (not shown) confirmed the results of the parsimony analysis, with the most likely tree ($\ln L = 3554.59$) having the same basic topology as Fig. 1a; otherwise, only minor variations in the positions and branching order of certain terminal taxa were apparent (irrespective of starting tree). All parameters were estimated from the data in preliminary runs and then set for the full analysis; base frequencies were unequal and the HKY85 two parameter model was selected, transition/transversion ratio = 0.6800, gamma-distribution shape parameter for variable sites = 0.0688; starting trees were derived by both parsimony and neighbour-joining.

3.2. Identification of informative regions

The distribution of phylogenetically informative characters (nucleotide changes which correspond to steps on a tree) was explored in MacClade. In the absence of a single most parsimonious tree, trees with the highest likelihood score ($\ln L = 3554.64$) were analyzed. The resulting chart (Fig. 2) shows that 39 informative characters (i.e. 85% of those included in the full analysis) are localized in two distinct regions of the gene fragment, between nucleotide positions: 511–710 and np: 1521–1830. Such short, discrete regions — provided they yield sufficient phylogenetic signal to reliably differentiate taxa — are obvious candidates for forensic identity markers. The suitability of these 'short' regions for this task was explored further (see below).

The overall absolute nucleotide differences between taxa are summarized in a matrix (Table 3), where it can be seen that absolute character differences do not always equate to informative differences, e.g. *C. vomitoria* (USA).

3.3. Analysis of 'short' sub-regions within the 28S gene

3.3.1. Analysis of sub-regions np: 511–710 and np: 1521–1830

Analysis of these two short (200 and 310 bp) regions included 483 characters, of which 39 were phylogenetically informative. As in the full analysis (2148 characters), six equally parsimonious trees were recovered (length = 74) which, as before, yielded two basic tree forms differing in their arrangement of clades corresponding to sub-families (see Fig. 1a and b). Moreover, while some differences (reduction) in relative branch lengths were apparent in the reduced data trees, the branching structure of the six trees was identical to that produced in the full analysis. This result indicates that analysis of these regions alone is sufficient for reliable phylogenetic identification of blowflies of forensic importance.

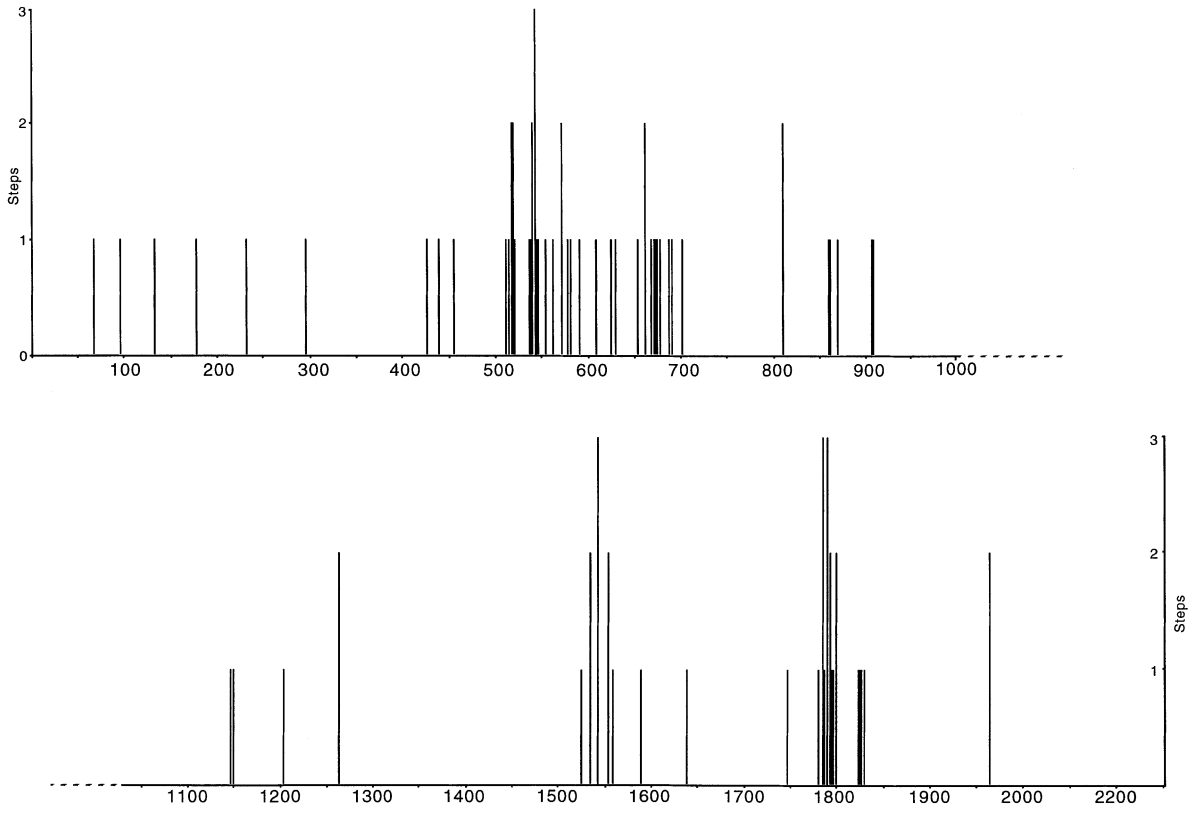

Fig. 2. Diagram showing the distribution of phylogenetically informative sites (as determined from the phylogenetic trees constructed in Fig. 1) across the D1–D7 fragment of the 28S rRNA gene.

3.3.2. Analysis of sub-region np: 511–710 only

Analysis of the 200 bp np: 511–710 region included 200 characters, of which 22 were informative. Two equally parsimonious trees were recovered (length = 40), both of which, apart from some loss of definition at the most terminal branches and, thus, within sub-families, were of the same basic topology (Fig. 3a) as that presented in Fig. 1a; the three sub-families were identified unequivocally (>76% bootstrap) and the distinction between *Lucilia sericata* (*L. sericata*) and non-*sericata Lucilia* species was

Table 3
Distance matrix of nucleotide differences in the D1–D7 region of the 28S rRNA gene in blowflies

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Tachina grossa*	–												
2	*Calliphora vicina*	42	–											
3	*Calliphora vicina* (Australia)	42	0	–										
4	*Calliphora vomitoria*	42	3	3	–									
5	*Calliphora vomitoria* (USA)	40	2	2	3	–								
6	*Cynomya mortuorum*	45	3	3	6	5	–							
7	*Lucilia illustris*	44	24	24	23	22	27	–						
8	*Lucilia ampullacea*	43	26	26	25	24	29	4	–					
9	*Lucilia caesar*	43	23	23	22	21	26	1	3	–				
10	*Lucilia sericata*	47	36	36	37	34	39	21	19	20	–			
11	*Lucilia sericata* (Denmark)	50	37	37	38	37	36	24	22	23	3	–		
12	*Lucilia sericata* (USA)	50	37	37	38	37	36	24	22	23	3	0	–	
13	*Lucilia terraenovae*	38	21	21	22	21	24	31	32	30	37	38	38	–

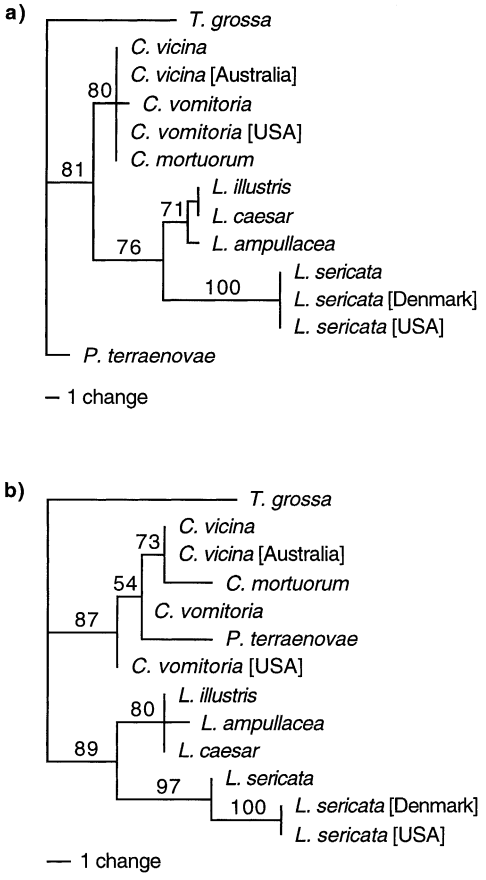

Fig. 3. Phylogenetic trees based on the 'short' informative sub-regions identified within the 28S rRNA gene. Tree (a) represents a consensus of the two equally parsimonious trees recovered (length = 40) from analysis of the 200 bp np: 511–710 region (see Fig. 2). Tree (b) is the single most parsimonious tree recovered (length = 31) from analysis of the 310 bp np: 1521–1830 region (see Fig. 2). See text for further details.

maintained. This result suggests that analysis of this single region may be sufficient for reliable phylogenetic blowfly identification.

3.3.3. Analysis of sub-region np: 1521–1830 only

Analysis of the 310 bp np: 1521–1830 region included 283 characters, of which 17 were informative. A single most parsimonious tree was recovered (length = 31, Fig. 3b), which, while of a similar form to Fig. 1b, did not unequivocally identify sub-family Calliphorinae. Instead, *Protophormia terraenovae* (*P. terraenovae*) (Chrysomyinae) was placed integral to the Calliphorinae clade, indicating that, while bootstrap support for this apparent misclassification was low (54%), some specimens of *C. vomitoria* and *P. terraenovae* may not be unequivocally distinguished on the basis of this region alone.

3.4. Comparative rates of sequence evolution

The phylogenetic analysis (Fig. 1a and b) provides evidence of very different rates of evolution within the Calliphorinae and Luciliinae sub-family clades. Comparison of intra-family branch lengths suggests that, on the basis of these 28S rRNA gene sequences, rates of molecular evolution within the Luciliinae may be more than three times as fast as that within the Calliphorinae. Insufficient taxa from sub-family Chrysomyinae were included in the study for valid comparison.

4. Discussion

This study provides the first comprehensive molecular-based phylogenetic analysis of blowfly species of forensic importance within Britain and Europe. Variation in 28S rRNA gene sequences of 13 specimens places the two ubiquitous blowfly sub-families Calliphorinae and Luciliinae unequivocally in two distinct clades, both of which are reliably separated from *P. terraenovae*, the single representative of a third sub-family, Chrysomyinae.

On the basis of the 28S rRNA sequences analyzed, two distinct patterns of evolution are supported (Fig. 1a and b). The relationships supported in Fig. 1b [[Calliphorinae, Chrysomyinae] Luciliinae] agree with the classical morphologically-based phylogeny of blowflies [12], while the relationships defined in Fig. 1a [[Calliphorinae, Luciliinae] Chrysomyinae] suggest an alternative sequence of evolutionary events in which sub-family Chrysomyinae diverged earliest. Analysis of additional genes will be required to resolve this question. Within the genus *Lucilia*, our results support the findings of previous studies [19,20] and confirm the monophyly of the *Lucilia ampullacea* (*L. ampullacea*)/*Lucilia caesar* (*L. caesar*)/*Lucilia illustris* (*L. illustris*) clade and their separation from *L. sericata*. Within the genus *Calliphora*, our data confirm the global genetic homogeneity of *C. vicina* and highlight previously unexplored inter-continental level variation within *C. vomitoria*.

For the purposes of the current study, it is important to note that (a) the three sub-families can be reliably distinguished on the basis of sequence data, and (b) that different patterns and levels of intra-specific and intra-sub-family sequence variation are apparent, which may have a bearing on the level of sequence variation accepted in sequences from unknown specimens for forensic typing.

4.1. Sub-family identity

With the approach developed here, the sequences allow confident allocation of sub-family status to insect material. Clearly, the ability to distinguish between the Calliphorinae, Luciliinae and Chrysomyinae using molecular methods may have forensic value, particularly where small quantities of badly preserved insect material are

recovered. Since the Calliphorinae and Luciliinae, for example, have strongly divergent habitat preferences, the distinction between these two groups of calliphorid is particularly valuable in helping to determine whether a corpse has been killed on site or moved there from another geographical location.

4.2. Species identity

Within the Luciliinae and Chrysomyinae species level identification is possible for the majority of species using the 'short' sequence fragments (Fig. 3a and b) and for all species using the full D1–D7 28S rRNA fragment (Fig. 1; Table 3). Only *L. illustris* and *L. caesar* require more than 'short' sequence analysis for establishing definitive identification, while *L. sericata* exhibits notable intra-specific variation within sub-region np: 1521–1830 which, with appropriate calibration, may potentially serve a useful role in localized specimen identification. *P. terraenovae* is readily identified using the 'short' sequence from sub-region np: 511–710 (Fig. 3a), although, its classification is ambiguous if sub-region np: 1521–1830 only is used (Fig. 3b). Specimens of *Calliphora* sp. and *Cynomya* sp. require full D1–D7 fragment analysis for definitive identification and the genetic heterogeneity of *C. vomitoria* cannot be discounted when the possible presence of this species is anticipated; evidently, further refinement of these techniques, to allow species and sub-species-specific resolution of all groups of forensic importance, is required to allow the use of molecular sequence markers to be fully realized.

The forensic utility of any method will also be dependent on a number factors, of which the most important may be accuracy, speed and cost. The technical accuracy of DNA sequence analysis is now well documented and appropriate choice of specific primers can readily circumvent common criticisms concerning problems of bacterial and other forms of DNA contamination. DNA extraction, PCR amplification of target fragment(s), particularly high copy number genes such as rRNA sub-units and sequencing, is routinely completed in less than 48 h, particularly where short, definitive fragments can be identified and, thus, sequenced in one run, for example the definitive 200 bp np: 511–710 region. Similarly, the cost to double strand sequence such a short fragment is less than US$ 25. Hence, this study suggests that short well-conserved rRNA-based markers such as the 28S np: 511–710 region may provide the basis for a reliable, quick and inexpensive molecular marker for the identification of forensically important blowflies.

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References

[1] K.G.V. Smith, A Manual of Forensic Entomology, The British Museum (Natural History), London and Cornell University Press, London, 1986, pp. 204.
[2] F.A.H. Sperling, G.S. Anderson, D.A. Hickey, A DNA-based approach to the identification of insect species used for postmortem interval estimation, J. Foren. Sci. 39 (1994) 418–427.
[3] J. Stevens, R. Wall, Species, sub-species and hybrid populations of the blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae), Proc. R. Soc. London, Ser. B 263 (1996) 1335–1341.
[4] J. Stevens, R. Wall, The use of random amplified polymorphic DNA (RAPD) analysis for studies of genetic variation in populations of the blowfly *Lucilia sericata* in Southern England, Bull. Entomol. Res. 85 (1995) 549–555.
[5] S.M. Aljanabi, I. Martinez, Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques, Nucl. Acids Res. 25 (1997) 4692–4693.
[6] A. Larson, Evolutionary analysis of length-variable sequences: divergent domains of ribosomal RNA, in: M.M. Miyamoto, J. Cracraft (Eds.), Phylogenetic Analysis of DNA Sequences, Oxford University Press, New York, 1991, pp. 221–248.
[7] M.L. Sogin, H.J. Elwood, J.H. Gunderson, Evolutionary diversity of eukaryotic small-sub-unit rRNA genes, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 1383–1387.
[8] D.G. Hall, The Blowflies of North America, Thomas Say Foundation, Lafayette, Indiana, 1948, pp. 477.
[9] R.W. Crosskey, A systematic revision of the Ameniinae (Diptera: Calliphoridae), Bull. Brit. Mus. Natl. Hist. (Entomol.) 16 (1965) 33–140.
[10] G.E. Shewell, Calliphoridae, in: J.F. McAlpine, B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, D.M. Wood (Eds.), Manual of Nearctic Diptera. 2. Monogr. Res. Br. Agric., Vol. 28, Ottawa, Canada, 1987, pp. 1133–1145.
[11] K. Rognes, Blowflies (Diptera, Calliphoridae) of Fennoscandia and Denmark, Fauna Entomol. Scand. 24 (1991) 1–272.
[12] K. Rognes, The Calliphoridae (Blowflies) (Diptera: Oestroidea) are not a monophyletic group, Cladistics 13 (1997) 27–66.
[13] A.R. Hoelzel, A. Green, Analysis of population-level variation by sequencing PCR-amplified DNA, in: A.R. Hoelzel (Ed.), Molecular Genetic Analysis of Populations: A Practical Approach, Oxford University Press, Oxford, 1992, pp. 159–187.
[14] M. Friedrich, D. Tautz, Evolution and phylogeny of the Diptera: a molecular phylogenetic analysis using 28S rDNA sequences, Syst. Biol. 46 (1997) 674–698.
[15] M. Friedrich, D. Tautz, An episodic change of rDNA nucleotide substitution rate has occurred during the

emergence of the insect order Diptera, Mol. Biol. E 14 (1997) 644–653.

[16] J.D. Thompson, D.G. Higgins, T.J. Gibson, Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, Nucl. Acids Res. 22 (1994) 4673–4680.

[17] D.L. Swofford, PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), Version 4, Sinauer Associates, Sunderland, MA, 1998.

[18] W.P. Maddison, D.R. Maddison, MacClade: Analysis of Phylogeny and Character Evolution, Version 3.0, Sinauer Associates, Sunderland, MA, 1992.

[19] J. Stevens, R. Wall, Classification of the genus *Lucilia* (Diptera: Calliphoridae): a preliminary parsimony analysis, J. Natl. Hist. 30 (1996) 1087–1094.

[20] J. Stevens, R. Wall, The evolution of ectoparasitism in the genus *Lucilia* (Diptera: Calliphoridae), Int. J. Parasitol. 27 (1997) 51–59.