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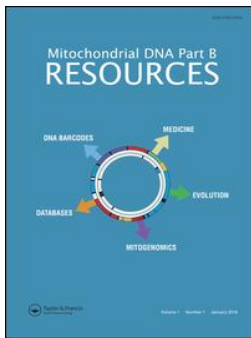
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Analysis of the complete mitochondrial genomes of two forensically important blowfly species: *Lucilia caesar* and *Lucilia illustris*

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ABSTRACT

Blowfly species of the family Calliphoridae can be used in forensic investigations to estimate the minimum post-mortem interval (PMI_{min}). *Lucilia caesar* and *Lucilia illustris* (Diptera: Calliphoridae) are closely related and phenotypically similar, making reliable identification difficult. To identify potential genetic markers to distinguish these species, five complete mitochondrial genomes were sequenced: three for *L. caesar* (KM657111–KM657113) and two for *L. illustris* (KM657109, KM657110). The ND6 gene contained the most species-specific SNPs (1.71%), followed by the ND5 gene (1.68%) and the COI gene (1.56%), identifying ND6 and ND5 as valuable loci for differentiating *L. caesar* and *L. illustris* specimens.

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Species from the family Calliphoridae are typically the first to colonize a corpse (Rodriguez and Bass 1983) and their development can be used to determine the time since oviposition (Amendt et al. 2007), and hence an estimation of the PMI_{min}, which can aid legal investigations (Easton and Smith 1970; Erzinclioglu 1983; Kulshrestha and Chandra 1987). The two closely related greenbottles *Lucilia caesar* and *Lucilia illustris* are both members of the family Calliphoridae and are commonly found in the UK. These species are very similar and morphological identification is a challenging task even for experienced entomologists (Stevens and Wall 1996; Wallman and Donnellan 2001).

mtDNA-based identification has been explored in previous studies, most frequently looking at COI, to distinguish species, including *L. caesar* and *L. illustris* (Malgorn and Coquoz 1999; Wells and Sperling 2000; Wallman 2001; Harvey et al. 2003; Sonet et al. 2012). The COII gene, the 16S ribosomal gene and the *cyt b* gene also showed insufficient interspecific variation between *L. caesar* and *L. illustris* (Sonet et al. 2012).

The samples were all collected from a site in Burnley UK (53.7661140 N, -2.2366190 W), which is at an altitude of 268 m. DNA was extracted using the DNeasy[®] Blood and Tissue Kit (Qiagen, Hilden, Germany). The genomes of five specimens were amplified with 21 custom-made primer pairs and Sanger sequenced (KM657109.1, KM657110.1, KM657111.1, KM657112.1, KM657113.1). DNA extracts are stored at the University of Central Lancashire, UK.

Genes were identified using ORF Finder (Wheeler et al. 2002), tRNAscan-SE (v 1.21) (Lowe and Eddy 1996), and alignment with other complete *Lucilia* mitochondrial genomes (*L. sericata* AJ422212, *L. porphyrina* JX913758, *L. cuprina*

JX913744) (Stevens et al. 2008; Nelson et al. 2012). Newly sequenced mitochondrial genomes of both species consisted of 13 protein-coding genes, 22 tRNA genes and two rRNA genes: 23 are located on the heavy strand (tRNA^{-Ile}, tRNA^{-Met}, ND2, tRNA^{-Trp}, COI, tRNA^{-Leu(UUA)}, COII, tRNA^{-Lys}, tRNA^{-Asp}, ATP8, ATP6, COIII, tRNA^{-Gly}, ND3, tRNA^{-Ala}, tRNA^{-Arg}, tRNA^{-Asn}, tRNA^{-Ser(AGC)}, tRNA^{-Glu}, tRNA^{-Thr}, ND6, *cyt b*, and tRNA^{-Ser(UCA)}). The other 14 genes are located on the light strand. This genome organization is consistent with other *Lucilia* species AJ422212, JX913758, and JX913744 (Stevens et al. 2008; Nelson et al. 2012).

Start and stop codons were also similar to other Diptera *Elodia flavipalpis* (Zhao et al. 2013) and *Chrysomya chloropyga* (Junqueira et al. 2004): the most used start codon was ATG (6), but ATT (4) and ATA (2) were also present; the COI start codon is TCG (serine), which is not a typical start codon, but has been observed in other Diptera (including *L. cuprina* and *L. sericata*) – the ATTTAA sequence adjacent to the start codon is involved in translation initiation (Junqueira et al. 2004). Incomplete termination codons (T) were observed for COII, ND4, and ND5; it is thought that the termination codon is completed by polyadenylation (Lessinger et al. 2000).

Two extra copies of tRNA^{-Leu} and tRNA^{-Ser} were found, which is typical for most Diptera. Some Diptera contain more duplicate copies of tRNAs, but this was not the case for *L. caesar* and *L. illustris* (Lessinger et al. 2000; Harvey et al. 2003; Junqueira et al. 2004; Duarte et al. 2008). Anti-codons of the tRNAs were identified and showed to be consistent with other Diptera (*L. sericata* AJ422212, *L. porphyrina*, JX913758, *L. cuprina* JX913744). Based on tRNAscan-SE results and RNAfold (Lorenz et al. 2011), all identified tRNAs successfully folded

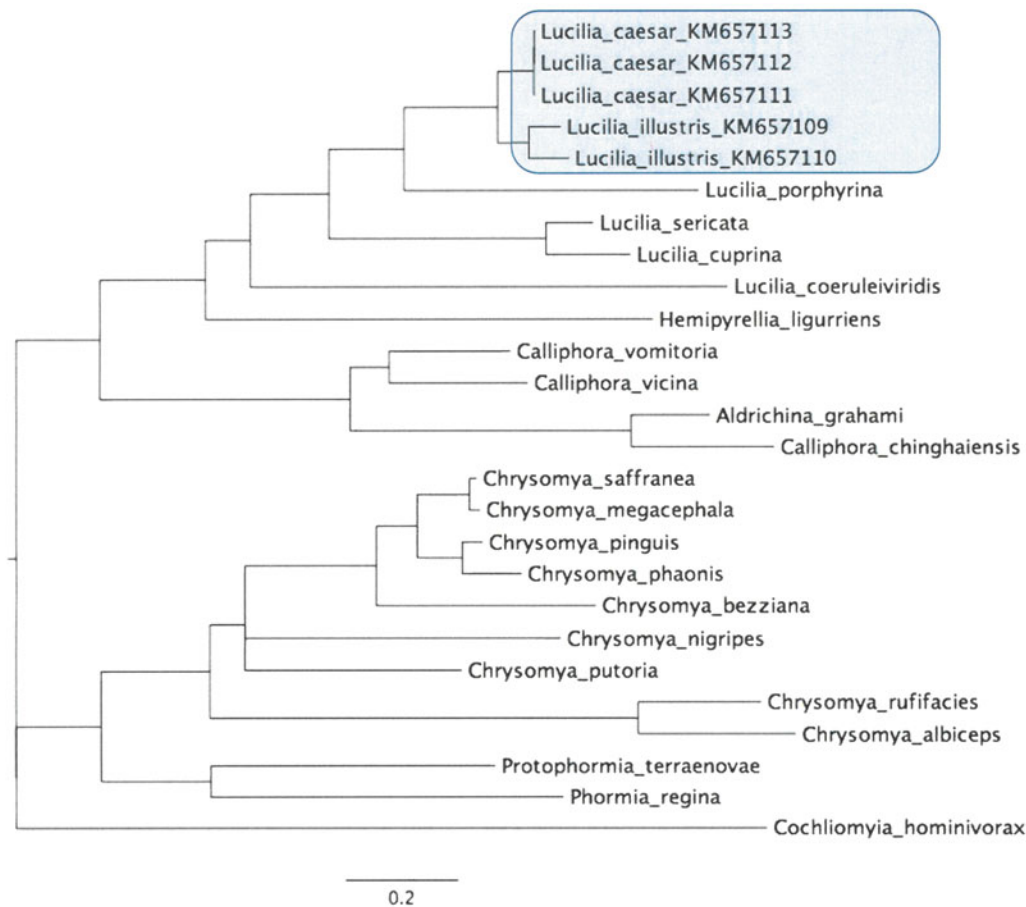


Figure 1. The Calliphoridae (NC_019636, NC_026668, NC_009733, NC_019637, KM657109-KM657113, NC_019573, NC_029486, NC_019638, NC_002660, NC_019635, NC_019634, NC_002697, NC_025338, NC_031381, NC_028412, NC_019633, NC_019632, NC_019631, NC_028411, NC_019639, NC_029215, NC_026996) phylogenetic summary tree inferred using the Bayesian approach implemented in MrBayes 3.2 (Ronquist et al. 2012) with Sarcophagidae: *Sarcophaga crassipalpis* (NC_026667) and *Ravinia pernix* (NC_026196) as an outgroup (not shown). Mitogenomes sequenced in this study are highlighted. All nodes are well-supported (PP > 0.9). Four independent runs were performed for 10 million generations and sampled every 1000 generations. Convergence of replicates was verified in TRACER v. 1.6 (Rambaut et al. 2014) by ensuring unimodality of posterior distributions and effective sample size (ESS > 200) for all parameters. Trees were summarized using the 50% majority rule method, after discarding the first 25% of the sample as burnin, and visualized using FIGTREE 1.4.2 (Rambaut 2012).

into typical cloverleaf structures, except for tRNA^{Ser(AGC)}, which missed the D stem. However, this has been observed in other species as well, including Diptera (Junqueira et al. 2004).

The length of the ribosomal RNAs, 1327 bp for the 16S subunit and 787 bp for the 12S subunit was the same as other *Lucilia* species (*L. sericata* AJ422212, *L. porphyrina* JX913758, and *L. cuprina* JX913744). The control regions of *L. caesar* and *L. illustris* were 1122 bp and 1121 bp, respectively; this is shorter than *L. sericata* (1125 bp) and *L. cuprina* (1136), but larger than *L. porphyrina* (1047 bp).

Besides finding possible new genetic markers, the complete mitochondrial genomes provide insight into the evolution of Calliphoridae. To infer phylogenetic relationships among Calliphoridae, we performed Bayesian phylogenetic analysis based on complete mitochondrial genome sequences (Figure 1). PartitionFinder (Lanfear et al. 2012) was run prior to phylogenetic analysis to find the most supported data partitioning and associated substitution models based on the Bayesian Information Criterion (BIC). Fourteen partitions were identified for the alignment of the complete mitochondrial

genomes of analysed Calliphoridae. All tested *Lucilia* species formed a monophyletic group. Phylogenetic analysis confirmed *L. illustris* and *L. caesar* as sister species. There were two distinct clades among tested Calliphoridae species, one included *Lucilia* spp., *Calliphora* spp., *Hemipyrellia* sp., *Aldrichina* sp. while the other clade included *Chrysomya* spp., *Protophormia* sp., and *Phormia* sp. A polytomy was present in the final tree, represented by *Cochliomyia* sp.

Most studies for genetic identification of *L. caesar* and *L. illustris* have been based on the COI gene. Other mitochondrial genes that have been used are the COII gene, the 16S ribosomal gene and the *cyt b* gene, but these show insufficient interspecific variation between these two Calliphoridae indicating that additional genetic markers need to be developed (Sonet et al. 2012). In this study, the ND6 and ND5 genes contained the most species specific SNPs (1.71% and 1.68%, respectively), followed by the COI gene (1.56%). Although these results are based on only few mitochondrial genomes, the results are consistent with the study of Nelson et al. (2012), where the ND6 gene showed the highest interspecific genetic variation within Calliphoridae (and

Chrysomyinae), when compared to other mitochondrial genes. Further work, including population studies, is needed to evaluate the value of ND6 and NR5 as markers for differentiating between *L. caesar* and *L. illustris* in forensic casework.

Disclosure statement

The authors report no conflict of interest and are responsible for the content and writing of this article. The University of Central Lancashire's Ethics Committee approved all experimental procedures.

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