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IdentiFLY: The Development and Validation of a 15-Plex SNP Assay for Forensic Identification of UK Blowfly Species (Calliphoridae)

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Abstract: Members of the blowfly family (Calliphoridae) are usually the first insect species to arrive at a corpse, using the body as an oviposition site, and, as such, they are the most important group of insects used to estimate the post-mortem interval (PMI). PMI estimations are based on species-specific developmental timings; therefore, accurate species identification is crucial. Current identification methods are based on morphological characteristics, which are time-consuming and difficult to perform on damaged, immature specimens and closely related species. Advances have led to specimens being identified via a host of molecular techniques, mainly DNA sequencing. Although molecular identification is becoming increasingly more common, there is currently a lack of genetic data regarding UK Calliphoridae species. This study aimed to address this issue. We present the development and validation of an identification assay capable of differentiating six UK species (*Calliphora vicina, Calliphora vomitoria, Lucilia sericata, Lucilia illustris, Lucilia caesar,* and *Protophormia terranovae*). The sequencing of six genes, including both nuclear (*28S rRNA* and *Elongation factor 1 alpha*) and mitochondrial markers (*Cytochrome oxidase I* and *II*, *Cytochrome b* and *16S rRNA*) identified 298 species-specific single nucleotide polymorphisms (SNPs). Fifteen SNPs from six genes were chosen for inclusion in a SNaPshot™ multiplex assay. The developed assay is capable of differentiating the species based on between 4 and 12 SNPs. Validation following guidelines by the International Society of Forensic Genetics (ISFG) demonstrated the assay to be accurate, reproducible, sensitive, and specific.

Keywords: Calliphoridae; blowfly; single nucleotide polymorphism; *Lucilia*; *Calliphora*; *Cytochrome oxidase*

Citation: Godfrey, H.; Smith, J.A. IdentiFLY: The Development and Validation of a 15-Plex SNP Assay for Forensic Identification of UK Blowfly Species (Calliphoridae). *Taxonomy* **2024**, *4*, 680–695. https://doi.org/10.3390/taxonomy4040035

Academic Editor: Mathias Harzhauser

Received: 7 August 2024
Revised: 3 September 2024
Accepted: 12 September 2024
Published: 28 September 2024

1. Introduction

Blowfly species (Calliphoridae) are commonly encountered in forensic investigations. Their timely arrival on a corpse, often arriving within minutes of death, makes them a useful tool for estimating the post-mortem interval (PMI) [1]. PMI estimations are based on the succession of insect fauna colonising the corpse and the unique developmental timings of each species [2]. In order for species-specific life cycle data to be applied, it is crucial that the species are identified correctly.

Currently, based on morphological characteristics, species identification is often problematic because of the similar morphological features exhibited by closely related species. This problem can be exacerbated if the forensic evidence is damaged and lacks the necessary diagnostic characteristics needed for identification purposes. Furthermore, taxonomic keys may be subjective and require the user to have detailed knowledge of insect anatomy. The rearing of crime scene collections in the laboratory is time-consuming, and failure of the specimens to reach adulthood can sometimes occur, resulting in the loss of evidence.

Recent advances have led to specimens being identified via a host of molecular biology techniques including RAPD, ISSR and SCAR markers [3–5], allozyme electrophoresis [6], microsatellite analysis [7,8], RFLP-PCR [9–11], AFLP analysis [12], high-resolution melt

PCR [13], antigen-based testing [14], and DNA sequencing [15–17]. Many studies have studies reported the genetic identification of blowflies globally [18–40], with a focus on *Cytochrome Oxidase 1* as a universal barcode for species identification [41,42]; however, there is a lack of data regarding UK species of forensic importance. Many studies conducted so far have been performed on a limited number of samples and species, with most studies conducted on a partial region of a single gene [16,43–45]. Whilst other genes have been used, including *Cytochrome b*, and ribosomal RNA genes, studies rarely take a multi-gene approach. In this study, we report the development and validation of a SNaPshot™ multiplex assay (Applied Biosystems, Warrington, UK) for the identification of UK blowflies commonly encountered in forensic investigations (*Calliphora vicina*, *Calliphora vomitoria*, *Lucilia sericata*, *Lucilia illustris*, *Lucilia caesar*, and *Protophormia terraenovae*). This assay is based on novel sequence data collected from six regions, including the mitochondrial genes *Cytochrome oxidase I* and *II*, *Cytochrome b*, and *16S rRNA* and the nuclear genes *28S rRNA* and *Elongation factor 1 alpha*.

2. Materials and Methods

2.1. Sample Collection

Wild-caught blowfly specimens were collected from 44 different locations across the UK and Ireland. These were collected by various volunteers, suction traps operated by Rothamsted Research, and from the University of Central Lancashire's TRACES facility (Taphonomic Research in Anthropology: Centre for Experimental Studies). Samples were collected from mainland England, Scotland, Wales, and Northern Ireland, along with samples from Fair Isle and the Isle of Skye.

2.2. Preliminary Identification

Samples were preliminarily identified using traditional taxonomic keys [46,47]. In cases where this was problematic, e.g., between *Lucilia illustris* and *Lucilia caesar*, specimens were identified using RFLP-PCR analysis of the *COI* gene [44].

2.3. DNA Extraction and Quantification

Total genomic DNA was extracted from two legs and one wing of each adult fly using the Qiagen® DNeasy® Blood and Tissue Kit (Qiagen, Manchester, UK). The manufacturer's instructions were followed for the isolation of genomic DNA from tissues < 10 mg. The extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Altringham, UK).

2.4. Identification of Species-Specific SNPs

Full-length sequences for six genes (*Cytochrome Oxidase I* and *II* (*COI*/*COII*), *Cytochrome b* (*Cyt b*), *16S rRNA*, *28S rRNA*, and *Elongation factor 1 alpha* (*EF1-α*)) were generated for the six UK blowfly species. Between 5 and 15 samples per species were included for each gene. Sequencing was conducted using Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Sequences were reviewed and edited where necessary and analysed using NCBI's BLAST tool (http://blast.ncbi.nlm.nih.gov/ (accessed on 11 September 2024) [48]. Alignments were created using BioEdit v7.0.9 [49] and analysed for species-specific SNPs. The inclusion of SNPs for a multiplex assay was considered based on the number of species that were distinguished and on the ability to design suitable primers to amplify across all target species.

2.5. PCR Multiplex

Degenerate primers were designed in order to achieve amplification of all six species (Table 1). Multiplex PCR amplifications were performed in a total reaction volume of 10 µL containing 5 µL 2× Platinum® multiplex PCR master mix (Applied Biosystems, Warrington, UK), 1.0 µL primer mix (Table 1), 5.0 ng template, and PCR-grade water (Sigma, Welwyn

Garden City, UK). Amplifications were performed using a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Warrington, UK). PCR parameters were as follows: 95 °C for 2 min, 33 cycles of; 95 °C for 30 s, 54 °C for 90 s, 72 °C for 20 s; and 60 °C for 30 min. Reactions were purified to remove any unincorporated PCR components with the addition of 4.0 µL ExoSAP-IT® reagent (USB®, High Wycombe, UK) and incubated at 37 °C for 15 min followed by deactivation at 80 °C for a further 15 min.

Table 1. Primer sequences for multiplex amplification of 12 fragments from 6 genes incorporating 15 informative SNPs for species identification (standard IUB codes used for degenerate nucleotide positions). SNP positions are based on the nucleotide position from the start codon of the full-length sequence.

Gene (SNP Position)	PCR Primer Sequences (5′-3′)	Product Size (bp)	Concentration (µM)
16S (265)	FOR: AAAAGACGAGAAGACCCTATAAATC REV: AAAAATTACGCTGTTATCCYTAAA	206	0.4
COII (346/418)	FOR: GGTWGATGAAATTAATGAACCTTC REV: GGATAGTTCAWGAATGAATYACATC	239	0.5
Cyt b (155)	FOR: TTCWGCTWGATGAAATTTYGGA REV: GCWCCATTAGCAWGYATAGTTCG	180	0.3
Cyt b (659)	FOR: GGYCCWATAGGATTAAATTCWAATATTG REV: GGAAYTATCATTCTGGTTGRATATG	201	0.4
COI (503)	FOR: GCMGGAATYTCWTCAATTTTAGG REV: AATAGCDCCWGCTAATACTGGTAA	162	0.3
COI (935)	FOR: GGATTAGCTATTGGAYTAYTAGGA REV: GCCRTAAAGAGTTGCTARTCAAC	160	0.3
28S (529)	FOR: GTTAAGCCCGATGAACCTGA REV: TCAACRCTTTATCAAATCAAAAGMA	218	0.4
28S (1535/1620)	FOR: AATGGATGGCGCTTAAGTTG REV: GTCCTCCAAGGTCTCATTCG	209	0.1
28S (1938)	FOR: ATATGGRCCTTGTGCTCATC REV: TTTCAAGGTCCGAGGAGAAA	189	0.1
EF1-α (93)	FOR: CCATCGATATTGCTTTGTGG REV: GATACCRGCTTCGAATTCAC	167	0.5
EF1-α (504)	FOR: GARGCTTCCACCAACATGC REV: GGGTACTGTGCCGATACCMC	183	0.5
EF1-α (723/864)	FOR: CYCCCGCTAACATCACCACT REV: GGGRGTGTAACCGTTGGAGA	236	0.6

2.6. SNaPshot™ Multiplex

Single-base extension primers (SBE primers) were designed for each SNP of interest. Primer lengths were altered by the addition of poly(A) tails to the 5′ end of the primers (Table 2). The multiplex SBE reaction was performed in a total reaction volume of 3 µL containing 1.25 µL SNaPshot™ reaction buffer (Applied Biosystems, Warrington, UK), 0.35 µL primer mix (Table 2), and 1.4 µL purified PCR product. Amplifications were performed in a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Warrington, UK). PCR parameters were as follows: 96 °C for 2 min, 25 cycles of; 96 °C for 10 s; 50 °C for 5 s; and 60 °C for 30 s. Extension products were purified by the addition of 0.5 µL SAP® reagent (USB®, High Wycombe, UK) and incubated at 37 °C for 60 min followed by 75 °C for 15 min.

Table 2. Single-base extension primers for the 15 informative SNPs.

Gene (SNP)	Single-Base Extension Primer Sequence (5′-3′)	SBE Primer Length (bp)	Concentration (µM)
16S (265)	$(A)_{11}$TTTTAATATCACCCCAATAAAATAT	36	0.4
COII (346)	$(A)_{15}$CTACWAAYGAATTATCAATTGATAG	40	0.7
COII (418)	$(A)_{21}$CAARTYCGAATTTTAGTRACWGC	44	0.5
Cyt b (155)	$(A)_{23}$AAATTTTAACTGGWYTATTTTTAGC	48	0.6
Cyt b (659)	$(A)_{27}$CWAATATTGATAAAATTCCATT	52	1.0
COI (503)	$(A)_{29}$CAAGCAAAAATAATTTGAATAAACWTA	88	1.0
COI (935)	$(A)_{36}$TACTCASTAAAGKTTCAAAGTTTA	92	0.2
28S (529)	$(A)_{44}$AACTACTACCACCAAGATCTGT	56	0.2
28S (1535)	$(A)_{46}$AAGAAAAGAAAACTCTTCCGATA	60	0.3
28S (1620)	$(A)_{52}$ATTGATGAAATCTCTSTGACC	64	1.0
28S (1938)	$(A)_{57}$AMGGTAAGACCYTCATCGA	68	1.0
EF1-α (93)	$(A)_{63}$CCAACGTTGTCACCGGG	72	1.0
EF1-α (504)	$(A)_{65}$ACCGTTGGAGATTTGACC	76	1.0
EF1-α (723)	$(A)_{65}$AAGTHATTAATATACGATCWACAGG	80	0.6
EF1-α (864)	$(A)_{68}$AGAGTTGCTARTCAACTAAAAAT	84	0.5

2.7. Capillary Electrophoresis of SNaPshot™ Multiplex Products

A total of 1 µL of the purified SNaPshot™ product was added to 11 µL Hi-Di Formamide™ (Applied Biosystems, Warrington, UK) and 0.3 µL 120-LIZ® size standard (Applied Biosystems, Warrington, UK). The samples were analysed on an ABI 3500 Genetic Analyzer (Applied Biosystems, UK) under the following run conditions: POP-6™ polymer, 50 cm array, protocol; FragmentAnalysis50_POP6_1, size calling; and SNaPshot_PA_Protocol, dye set: E5, run module: FragmentAnalysis50_POP6. The default run parameters of a 10 s injection at 1.6 kV and a run time of 20 min at 60 °C and 15.0 kV were used. On completion of the run, files were imported into GeneMapper® ID-X v1.2 software (Applied Biosystems, UK) for analysis. The peak amplitude thresholds in the software were set to 75 RFUs for each dye, the smoothing and baselining option was set to 'smooth' and the size calling method used was the local southern method.

2.8. SNaPshot™ Multiplex Validation

The validation of the multiplex assay was performed following procedures recommended by the International Society of Forensic Genetics (ISFG) regarding the use of non-human DNA in forensic genetics investigations [50]. Validation included a concordance study of the SNPs in a larger sample size (*C. vicina* (32), *C. vomitoria* (34), *L. sericata* (38), *L. illustris* (5), *L. caesar* (50), and *P. terraenovae* (29)), testing the reproducibility, reliability, accuracy precision, specificity, and sensitivity of the assay. The assay's reproducibility was tested by analysing multiple specimens collected from all life cycle stages (egg, instar larvae, pupae, pupal case, and adult). The precision of allele calls was tested by measuring the size range of each fragment, along with the standard deviation for each SNP. Twelve other Oestroidae species were analysed with the assay to test its specificity. These included specimens from the Calliphoridae, Sarcophagidae, Muscidae, and Tachinidae families. The sensitivity was tested using a serial dilution of quantified DNA template ranging from 5 ng to 0.37 pg. All validation assays were performed in triplicate.

3. Results

3.1. Sequencing and the Selection of SNPs

Full-length sequences were generated for four mitochondrial (*COI*, *COII*, *Cyt b*, and *16S rRNA*) and two nuclear markers (*EF1-α* and *28S rRNA*). Sequences were verified in the reverse strand, and the sequence from one specimen per species was submitted to NCBI's GenBank® database (Accession numbers JQ307754–JQ3077812). Analysis of the data revealed 298 species-specific SNPs across the six genes. As expected, intra-species sequence identities were lower than interspecies sequence identities (Table 3).

Table 3. Percentage sequence identity within and between species.

	COI	*COII*	*Cyt b*	*16SrRNA*	*EF-1α*	*28SrRNA*
Intraspecific variation	0–1.2%	0–0.8%	0–1.5%	0–0.9%	0–1.0%	0–0.4%
Interspecific variation	2.2–10.2%	1.7–9.9%	3.0–11.5%	0–3.2%	0–5.0%	0–3.0%
Number of species specific SNPs	128	37	71	11	28	23

Fifteen SNPs in total were selected, one within *16s rRNA*, two SNPs from *COI*, *COII*, and *Cyt b*, and four from both *EF1-α* and *28s rRNA*. All SNPs within coding genes were synonymous, transition variants.

3.2. SNaPshot™ Multiplex Assay

The SNaPshot™ multiplex assay, in which 14–15 single base extension primers (SBE primers) were simultaneously extended, successfully generated unique haplotypes for each species. Based on these 15 SNPs, each species can be differentiated with 4 to 12 SNPs (Table 4, Figure 1a–f).

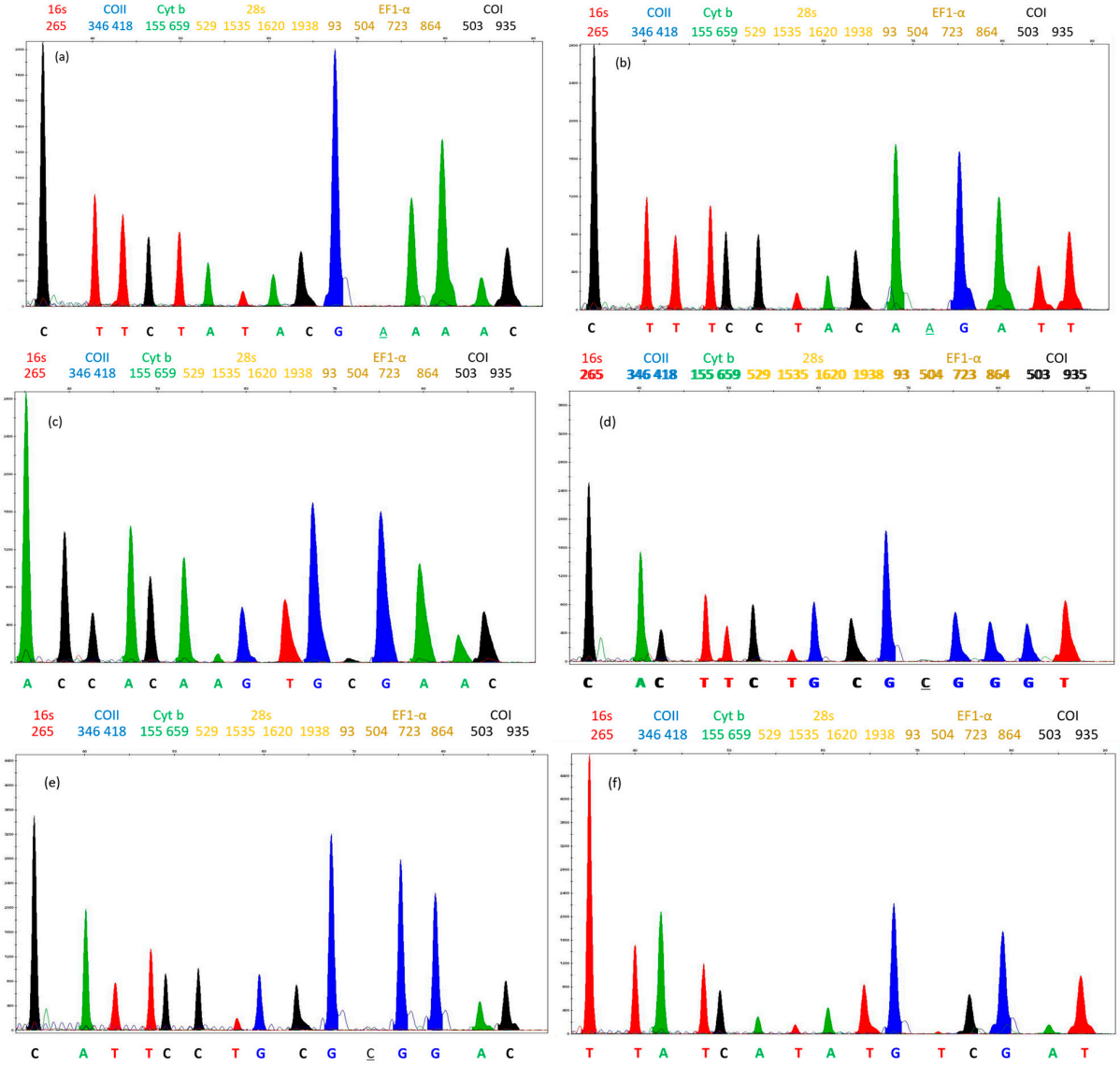

Figure 1. SNaPshot™ electropherograms for (**a**) *C. vicina*, (**b**) *C. vomitoria*, (**c**) L. sericata, (**d**) *L. illustris*, (**e**) *L. caesar*, and (**f**) *P. terraenovae*.

Table 4. Expected haplotypes for 6 common UK blowfly species (SNP positions are based on the nucleotide position from the start codon of the full-length sequence).

Locus	***16S***	***COII***		***Cyt b***		***28S***					***EF1-α***			***COI***	
SNP	**265**	**346**	**418**	**155**	**659**	**529**	**1535**	**1620**	**1938**	**93**	**504**	**723**	**864**	**503**	**935**
C. vicina	C	T	T	C	T	A	T	A	C	G	A	A	A	A	C
C. vomitoria	C	T	T	T	C	C	T	A	C	A	A	G	A	T	T
L. sericata	A	C	C	A	C	A	A	G	T	G	C	G	A	A	C
L. illustris	C	A	C	T	T	C	T	G	C	G	C	G	G	G	T
L. caesar	C	A	T	T	C	C	T	G	C	G	C	G	G	A	C
P. terraenovae	T	T	A	T	C	A	T	A	T	G	T	C	G	A	T

3.3. Validation of the SNaPshot™ Multiplex Assay

The validation for our assay was conducted following guidelines recommended by the International Society of Forensic Genetics (ISFG) regarding the use of non-human DNA in forensic genetics investigations [50]. The assay was validated for its reproducibility, accuracy, precision, sensitivity, specificity, and ability to detect mixed samples.

3.3.1. SNP Concordance Study

Although SNPs were selected as species-specific variants, when tested on a larger sample set intra-species variation was observed at some loci. In *C. vomitoria*, intra-species variation was observed at the SNP *EF1-α* 93, where a 'G' nucleotide was observed in 3 of the 50 samples tested. Variation was also observed in *L. illustris* at *Cyt b* 659 ('C' nucleotide rather than the expected 'T') in 1 in 5 samples and in *L. caesar* at *COII* 418 ('C' nucleotide rather than the expected 'T') and *COI* 935 ('T' nucleotide rather than the expected 'C') in 1 in 50 and 3 in 50 samples, respectively. However, samples were still identified correctly despite the observed variation (see Appendix A Figures A1–A3).

3.3.2. Reproducibility

Replicates of the same sample (one *C. vicina*, one *C. vomitoria*, and one *L. sericata*) gave full profiles, with the expected SNP profile. In addition, eight samples from each of the life stages of *L. sericata* were processed (egg, third instar larvae, pupae, pupal case, and adult), with each sample producing a full and correct profile.

3.3.3. Accuracy and Precision

Differences in the sizing between the observed peak and the expected SBE product have been previously reported and are due to the influence of the dye on the mobility shift in the DNA fragments. This mobility is determined mainly by the length, sequence, and dye used to extend the primer [51]. Slight differences in the size of the same SBE primer could be seen depending on which nucleotide was incorporated. The differences between the estimated and expected extension primer lengths were between 1.6 and 6.6 nucleotides. SBE primers that had a 'T' nucleotide incorporated showed the greatest shift between the expected and observed size. The assay was tested for reproducibility in sizing the peaks, and size calling was extremely precise, with all peaks called within 0.203 nucleotides of the mean (see Appendix A Table A1).

3.3.4. Sensitivity

The assay's sensitivity was assessed by analysing of a serial dilution of quantified DNA template ranging from 5 ng to 0.37 pg. The assay was performed on all species in triplicate. The results show that full profiles (15 SNPs) could be obtained from 100–49 pg DNA template, with partial profiles (up to 14 loci) obtained down to 12 pg template DNA (see Appendix A Table A2).

3.3.5. Specificity

Six Calliphoridae, three Sarcophagidae, five Muscidae, and one Tachinidae samples were tested to determine the specificity of the assay. These samples included *Calliphora loewi*, *Calliphora uralensis*, *Lucilia richardsi*, *Pollenia angustigena*, *Pollenia rudis*, *Sarcophaga incisilobata*, *Sarcophaga melanura*, *Sarcophaga vagans*, *Musca domestica*, *Eudasyphora cyanella*, and one unknown Tachinidae species. Human DNA was also analysed (007 control DNA from Applied Biosystems and the 9947A control DNA from Promega). The results demonstrated that the assay is specific to our species of interest, failing to amplify human DNA and producing unique profiles for the other species tested. A full profile was generated from one of the Calliphoridae species, *L. richardsi*, which was a close match to *L. sericata*, but still differed by four SNPs. The remaining Calliphoridae species gave partial profiles. On the Sarcophagidae species, partial profiles of 13 SNPs in both *S. melanura* and *S. vagans* and 14 SNPs in *S. incisilobata* were obtained. The Muscidae and Tachnidae species detected between 9 and 11 SNPs. None of the profiles in these non-target species could be mistaken for the target species (see Appendix A Table A3).

4. Discussion

To date, the choice of a marker for species identification across a wide range of taxa has largely focused on COI [41]. The ideal choice of a marker has a non-overlapping range of variation within and between species, and mtDNA are typically ideally suitable as the mitochondrial genome has a higher mutation rate than nuclear DNA. Together with the uniparental mode of inheritance and low effective population size, this means variation is quickly fixed within species. However, identification relies on good-quality reference data, and the best marker choice may vary among taxa. Focusing on species-specific SNPs circumvents the large amount of redundant sequence data generated through the standard sequencing approaches to species identification. Building a multi-gene approach allows for a level of redundancy to be built into the system to allow for the detection of new mutations or rare alleles; it also allows for dropout if dealing with poor-quality samples yet still provides sufficient data for positive identification. Our sequence analysis supports the use of COI as the most suitable marker for species identification because this marker was the most polymorphic, containing 128 species-specific SNPs; however, in our study, *COII* and *Cyt b* were also capable of differentiating all seven species. Some studies have proposed that a simplified or short amplicon COI barcode would be sufficient for identification [52,53]; however, others suggested a wider multi-locus approach including both nuclear and mtDNA markers [54,55]. Other mitochondrial genes are often overlooked but may hold potential for specific species. Whole mtDNA sequencing of *L. illustris* and *L. caesar* suggests that *ND6* may have more species-specific SNPs when comparing these two species, yet this gene has rarely been considered [56]. The results of our sequencing are mostly concordant with the previously published literature with similar levels of intra- and interspecies variation observed; however, there is still a paucity of data for many genes that may still be useful targets [45,57]. In our study, each of the nuclear markers failed to distinguish between *L. illustris* and *L. caesar*. It is not surprising that the nuclear markers failed to differentiate all the target species. Nuclear DNA is reported to mutate 2–9 times slower than mitochondrial DNA [58], and closely related species have not been separated long enough for species-specific mutations to be observed as frequently in their nuclear DNA. The identification of closely related species can be problematic; in our assay, three genes can reliably differentiate all species including the two closest related species, *L. illustris* and *L. caesar*. The identification of these two species is problematic because of low sequence divergence and only subtle morphological differences; in a forensic context, this could be problematic if these species have differing developmental timings and overlapping geographical ranges or habitat preferences, which are poorly understood. Other studies have found it difficult to differentiate these two species with shared haplotypes being observed across COI and other genes [54]. In our study, the 16s rRNA gene also failed to differentiate between *L. illustris* and *L. caesar*; however, other studies were able to

separate the two species even across the same 250 bp region of the 16s rRNA gene [48]. A study on specimens from China found differences between the species in their *EF1-α* and 28s rRNA results [59]. Both of these markers successfully differentiated the *L. illustris* and *L. caesar* samples in that study (although only one sequence for each species was examined); yet, our study failed to find any sequence variation between *L. illustris* and *L. caesar*. This suggests biogeographical differences within species and that the best choice of marker may vary globally. However, comparisons are difficult to perform; some studies have shown geographical differences either using a traditional Sanger sequencing approach or whole genome-based SNP typing [57,60,61], which may need to be considered when validating any SNP panel for species identification. A multigene approach, such as the one we used here, may help given that species are identified through a number of different SNPs across a multigene panel. As the rapid identification of species is advantageous in forensic investigations, the identification tool presented here has several advantages over the more commonly used molecular identification method of Sanger sequencing, mainly the ability to analyse more than one locus at a time. Others have taken a SNaPshot™ approach [62] but only looking at SNPs within COI or a multigene approach using qPCR and species-specific primers [63]. However, SNapShot™ has the advantage of a much higher multiplexing capacity than a qPCR approach, with allele detection being based on both length and colour. Incorporating both nuclear and mitochondrial markers has the potential to investigate potential hybridisation. Because of the inability of the nuclear genes to differentiate *L. illustris* and *L. caesar* in this study, it is not possible to determine if hybridisation occurs between these two species; however, hybridisation has been seen in other closely related species [64]. Sub-species *L. cuprina cuprina* and *L. cuprina dorsalis* interbreed readily in the laboratory, intermediate forms are believed to be common in parts of Australia [3], and whole genome sequencing has revealed interspecific hybridisation between *Calliphora hilli* and *C. styiga* in New Zealand (both species being recent introductions from Australia) [65]. In a forensic context, hybrid individuals may have altered developmental timings that could impact on the accuracy of PMI estimations. Although the sensitivity of our assay is not as low as some assays that have been developed [66,67], it is not envisaged that the level of sensitivity will be a problem. This assay was developed to identify samples with very few morphologically distinct characteristics, i.e., egg and larvae samples. These samples are usually abundant at a crime scene, so low DNA templates are not likely to be an issue. However, reduced levels of DNA are likely to be found if specimens are degraded or damaged, as well as in pupal cases (puparia) that may be used for analysis. The assay presented offers the ability to identify the key UK blowfly species of forensic interest, it is quicker and cheaper to perform, and the results are easy to interpret. The assay is also more amendable to degraded DNA because of the smaller loci size.

5. Conclusions

The presented assay correctly identifies its target species; however, amplification in non-target species indicates this approach could be adapted and utilised in other taxa. As the focus of this study was species of forensic importance in the UK, further validation would be required to assess the applicability of the assay globally, testing a larger number of specimens from a wider geographical distribution to ensure specificity is maintained across different populations. Future work could also include assessing the assay's performance with mixtures (that might simulate possible heteroplasmy) or applications for investigating dynamic changes and interspecies competition in maggot masses. Whilst the costs of molecular identification mean these approaches may not become routine practice in forensic entomology, these approaches still hold merit in terms of the speed of identification in comparison with rearing specimens to adulthood. More broadly, this approach has applications beyond the identification of blowflies of forensic interest. For example, in biogeography and species monitoring, in the detection of invasive species or new introductions, in veterinary entomology for the identification of species involved in myiasis, or in understanding the interactions among species in multi-species habitats.

Author Contributions: Individual contributions are as follows: conceptualisation, J.A.S.; methodology, H.G.; validation, H.G.; formal analysis, H.G.; writing—original draft preparation, H.G.; writing—review and editing, J.A.S.; supervision, J.A.S.; project administration, J.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in this study are included in this articles/Appendix A, further inquiries can be directed to the corresponding author.

Acknowledgments: We would like to thank all the volunteers who collected specimens and the University of Central Lancashire for internal funding.

Conflicts of Interest: The authors declare no conflicts of interest. The authors received no commercial funding, and this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A

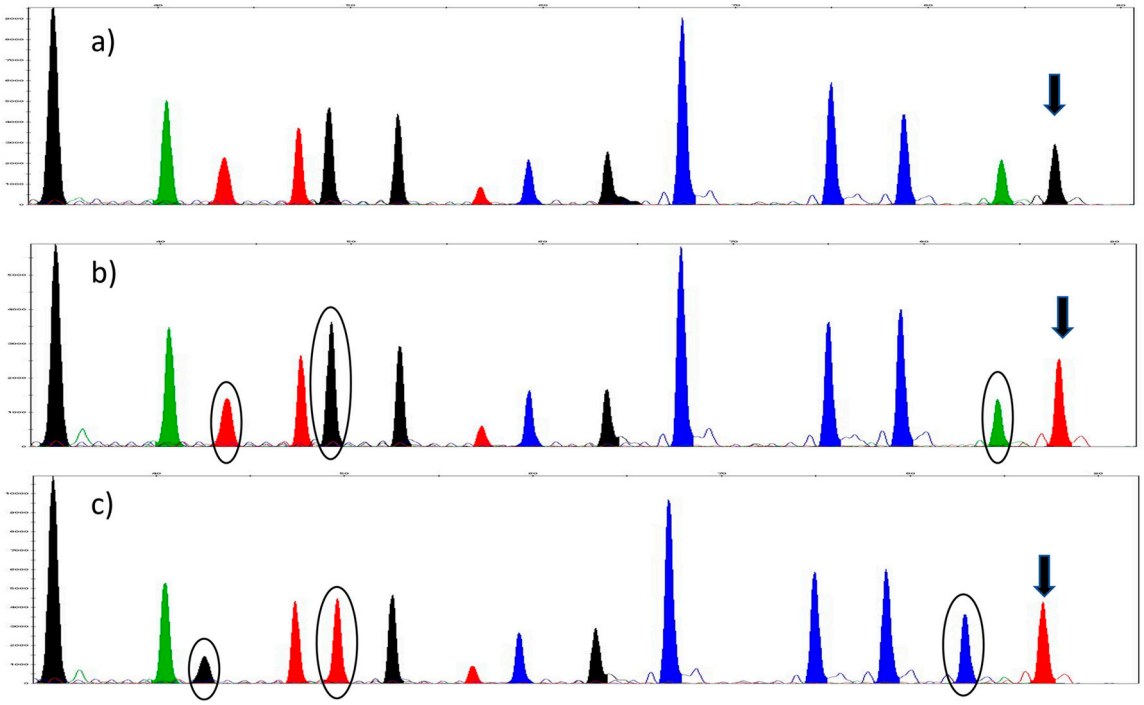

Figure A1. Concordance study—Intra-species variation detected during the concordance study. The arrow denotes the COI 935 SNP. (**a**) The expected C allele in *L. caesar*, and (**b**) the T allele observed in *L. caesar*, which corresponds to the known allele in (**c**) *L. illustris*. All species are identified by multiple SNPs (circled), allowing corrected species identification in the event of mutation or the presence of a rare allele.

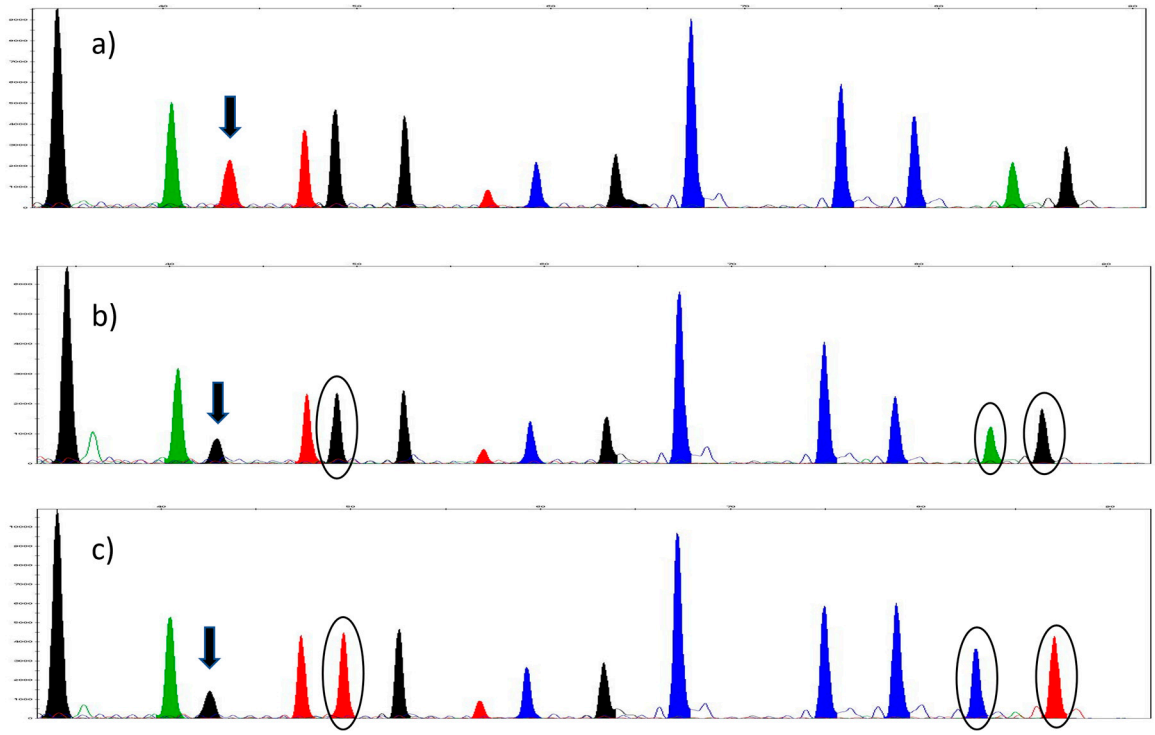

Figure A2. Concordance study—Intra-species variation detected during the concordance study. The arrow denotes the COII 418 SNP. (**a**) The expected T allele in *L. caesar*, and (**b**) the C allele observed in *L. caesar*, which corresponds to the known allele in (**c**) *L. illustris*. All species are identified by multiple SNPs (circled), allowing corrected species identification in the event of mutation or the presence of a rare allele.

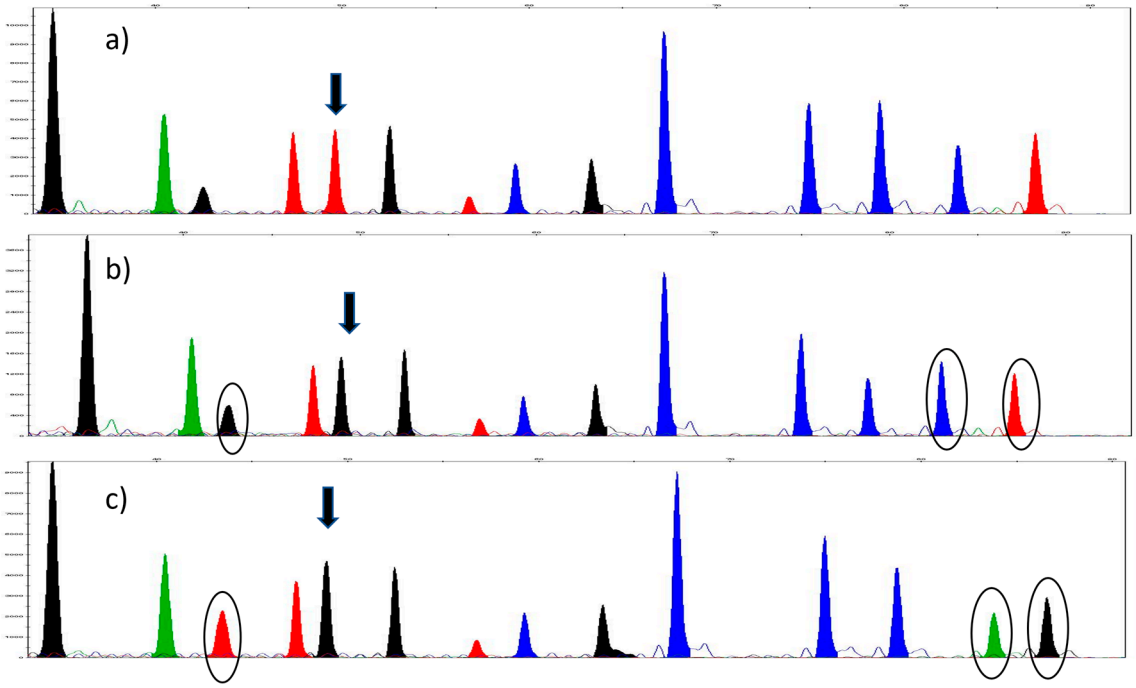

Figure A3. Concordance study—Intra-species variation detected during the concordance study. The arrow denotes the Cytb 659 SNP. (**a**) The expected T allele in *L. illustris*, and (**b**) the C allele observed in *L. illustris*, which corresponds to the known allele in (**c**) *L. caesar*. All species are identified by multiple SNPs (circled), allowing corrected species identification in the event of mutation or the presence of a rare allele.

Table A1. Precision of allele sizes—Sizing data for the SNaPshot® multiplex assay, run using POP-6™ on an ABI Prism® 3500 Genetic Analyser. The sizing of the multiplex was extremely reproducible, with all peaks being called ≤ 0.203 bp from the mean for each allele.

Gene	***16S***	***COII***	***COII***	***Cytb***	***Cytb***	***28S***	***28S***	***28S***	***28S***	***EF1-a***	***EF1-a***	***EF1-a***	***EF1-a***	***COI***	***COI***
SNP	265	*346*	418	*155*	659	529	1535	1620	1938	93	504	723	864	503	935
Expected size	36	40	44	48	52	56	60	64	68	72	76	80	84	88	92
Nucleotide	A	A	A	A		A	A	A		A		A	A	A	
Size range	35.06–35.47	40.12–40.19	42.67–42.9	46.55–46.96		52.68–53.06	56.5–56.88	60.27–60.53		67.73–68.12		76.02–76.18	79.45–79.69	83.71–84.06	
Average	35.21	40.15	42.76	46.88		52.9	56.66	60.37		67.96		76.13	79.56	83.35	
StdDev	0.14	0.022	0.09	0.075		0.075	0.091	0.065		0.1		0.042	0.064	0.115	
n	25	37	10	25		53	23	66		25		12	32	96	
Nucleotide	T	T	T	T	T		T		T		T			T	T
Size range	35.0–35.18	39.91–40.46	43.41–43.57	47.17–47.43	49.45–49.78		56.7–57.04		63.96–64.42		72.02–72.1			83.59–84.21	86.91–87.56
Average	35.05	40.33	43.49	47.31	49.52		56.84		64.25		72.05			83.78	87.17
StdDev	0.055	0.157	0.037	0.054	0.065		0.119		0.114		0.033			0.172	0.203
n	10	64	82	77	31		102		33		7			30	40
Nucleotide	C	C	C	C	C	C			C		C	C			C
Size range	34.29–34.72	39.39–39.71	42.41–42.74	46.27–46.39	48.83–49.17	52.47–52.74			63.26–63.63		71.37–71.55	75.04–75.59			86.46–86.98
Average	64.55	39.51	42.58	46.31	48.97	52.63			63.4		71.46	75.38			96.62
StdDev	0.105	0.106	0.09	0.026	0.087	0.097			0.12		0.054	0.151			0.181
n	103	25	29	30	85	55			93		18	10			86
Nucleotide								G		G		G	G	G	
Size range								59.14–59.54		67.13–67.55		74.94–75.27	78.8–79.17	83.19–83.26	
Average								59.42		67.32		75.14	78.99	83.22	
StdDev								0.11		0.13		0.07	0.102	0.026	
n								59		95		68	44	5	

Table A2. Sensitivity assay—Average peak heights (RFU + Std Dev) for fifteen SNPs amplified with between 50 ng and 12 pg starting material. The peak heights for most loci decrease as the DNA template is reduced, though sensitivity is locus-specific with peak heights for *COI* 503 and *COI* 935 remaining fairly consistent regardless of the DNA template amount and EF1-α 503 dropping out below 49 pg.

Gene	***16S***	***COII***	***COII***	***Cytb***	***Cytb***	***28S***	***28S***	***28S***	***28S***	***EF1-a***	***EF1-a***	***EF1-a***	***EF1-a***	***COI***	***COI***
SNP	**265**	***346***	**418**	***155***	**659**	**529**	**1535**	**1620**	**1938**	**93**	**504**	**723**	**864**	**503**	**935**
50 ng	2441 (666)	971 (229)	438 (115)	1441 (367)	849 (181)	665 (159)	191 (44)	1110 (245)	690 (168)	1785 (414)	110 (25)	1308 (391)	2057 (624)	200 (45)	559 (134)
25 ng	3108 (78)	1152 (55)	531 (25)	1777 (81)	860 (204)	753 (105)	224 (8)	1413 (107)	877 (36)	2212 (178)	136 (12)	1757 (222)	2855 (234)	230 (13)	676 (54)
12.5 ng	2254 (767)	895 (281)	402 (121)	1326 (390)	796 (236)	681 (191)	179 (50)	1025 (349)	678 (208)	1706 (505)	97 (36)	1461 (347)	2233 (698)	172 (47)	462 (141)
6.25 ng	2921 (684)	1294 (126)	568 (55)	1699 (341)	1002 (141)	922 (149)	237 (249)	1538 (181)	964 (116)	2348 (199)	129 (20)	2008 (144)	2926 (516)	226 (43)	695 (89)
3.1 ng	2909 (284)	1196 (74)	535 (34)	1674 (100)	834 (101)	817 (71)	217 (19)	1447 (78)	927 (30)	2166 (64)	116 (7)	1810 (44)	2707 (112)	207 (7)	652 (13)
1.5 ng	2545 (891)	1096 (392)	494 (156)	1666 (474)	1058 (215)	920 (252)	234 (63)	1364 (561)	847 (332)	1908 (823)	93 (42)	1545 (614)	2446 (960)	227 (63)	599 (237)
0.75 ng	2954 (106)	1500 (152)	650 (54)	1909 (161)	1292 (162)	1120 (97)	260 (29)	1865 (247)	1078 (108)	2414 (318)	108 (18)	1836 (232)	2671 (207)	260 (29)	812 (137)
0.375 ng	2692 (252)	1156 (139)	540 (63)	1616 (113)	833 (51)	915 (33)	223 (15)	1471 (176)	873 (85)	1521 (215)	66 (11)	1333 (133)	2054 (192)	209 (14)	589 (74)
0.2 ng	2295 (647)	1107 (421)	519 (173)	1565 (487)	983 (445)	940 (338)	219 (78)	1450 (499)	873 (273)	1282 (440)	47 (15)	1121 (172)	1768 (476)	219 (77)	605 (227)
0.1 ng	2847 (109)	1408 (124)	644 (54)	1878 (59)	1054 (63)	1146 (65)	262 (5)	1837 (228)	1040 (94)	1346 (174)	38 (5)	868 (126)	1565 (68)	264 (23)	756 (110)
0.049 ng	1880 (668)	1115 (246)	497 (97)	1286 (402)	920 (48)	831 (15)	185 (36)	1326 (364)	737 (227)	943 (295)	13 (10)	561 (234)	915 (432)	187 (43)	609 (110)
0.024 ng	1687 (736)	1083 (198)	506 (83)	1134 (487)	733 (179)	720 (262)	164 (48)	1226 (288)	727 (212)	794 (167)	0	468 (121)	769 (339)	168 (56)	633 (51)
0.012 ng	2308 (366)	1337 (208)	613 (81)	1691 (228)	1017 (120)	1172 (139)	219 (30)	1573 (321)	916 (192)	928 (186)	0	447 (197)	898 (288)	247 (32)	767 (108)

Table A3. Specificity assay—Profiles produced by non-target species. Whilst amplification and SNP detection occurred in non-target species, no profiles resulted in the misidentification of the target species (x = no amplification).

Gene	***16S***	***COII***	***COII***	***Cytb***	***Cytb***	***28S***	***28S***	***28S***	***28S***	***EF1-a***	***EF1-a***	***EF1-a***	***EF1-a***	***COI***	***COI***
SNP	**265**	***346***	**418**	***155***	**659**	**529**	**1535**	**1620**	**1938**	**93**	**504**	**723**	**864**	**503**	**935**
L. ampullacae	A	A	T	T	C	C	T	G	C	G	G	A	G	A	T
C. loewi	C/T	T/A	T/A	T	C	C/A	T	G/A	C/T	G	x	x	x	A	C/T
C. uralensis	C/A	C/T	T/G	T	C	G/A	T	G/A	C/T	G	x	x	x	G	C/T
L. richardsi	T/A	C	T	A	T	A	A	G	T	G	C	G	A	G	T
P. angustgena	T	T/A	T	A	C	x	x	A	G	A	x	T	A	T	T
P. rudis	T/A	T/G	T/G	C	C	T	G/A	C	G/A	x	x	A	A	T	C/T
S. incisilobata	A	A	T	C	x	C	T	A	T	A	C	T	A	A	C
S. melanura	A	x	C	x	C	C	T	A	T	A	C	T	A	A	C/T
S. vagans	T/A	C/T	C/T	x	C/T	C	T	A	T	G/A	x	T	A	A	C
M. domestica	T	x	T	A	C	x	T	A	T	G	x	G/A	G/A	x	C/T
E. cyanella	x	T	A	x	x	x	T	G	T	A	x	A	A	x	T
Tachinidae sp.	T	x	x	C/T	C/T	x	x	A	C	A	x	A	G/A	A	T

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