

Do longer sequences improve the accuracy of identification of forensically important Calliphoridae species?

Sara Bortolini¹, Giorgia Giordani², Fabiola Tuccia², Lara Maistrello¹ and Stefano Vanin²

¹Department of Life Sciences, University of Modena and Reggio Emilia, Reggio Emilia, Italy

²School of Applied Sciences, University of Huddersfield, Huddersfield, United Kingdom

ABSTRACT

Species identification is a crucial step in forensic entomology. In several cases the calculation of the larval age allows the estimation of the minimum Post-Mortem Interval (mPMI). A correct identification of the species is the first step for a correct mPMI estimation. To overcome the difficulties due to the morphological identification especially of the immature stages, a molecular approach can be applied. However, difficulties in separation of closely related species are still an unsolved problem. Sequences of 4 different genes (COI, ND5, EF-1 α , PER) of 13 different fly species collected during forensic experiments (*Calliphora vicina*, *Calliphora vomitoria*, *Lucilia sericata*, *Lucilia illustris*, *Lucilia caesar*, *Chrysomya albiceps*, *Phormia regina*, *Cynomyia mortuorum*, *Sarcophaga* sp., *Hydrotaea* sp., *Fannia scalaris*, *Piophilina* sp., *Megaselia scalaris*) were evaluated for their capability to identify correctly the species. Three concatenated sequences were obtained combining the four genes in order to verify if longer sequences increase the probability of a correct identification. The obtained results showed that this rule does not work for the species *L. caesar* and *L. illustris*. Future works on other DNA regions are suggested to solve this taxonomic issue.

Subjects Entomology, Taxonomy

Keywords ND5, COI, PER, Diptera, EF-1 α , Maximum-likelihood, Phylogeny

Submitted 19 March 2018
Accepted 17 October 2018
Published 17 December 2018

Corresponding author
Stefano Vanin,
stefano.vanin@hud.ac.uk

Academic editor
Ilaria Negri

Additional Information and
Declarations can be found on
page 13

DOI 10.7717/peerj.5962

© Copyright
2018 Bortolini et al.

Distributed under
Creative Commons CC-BY 4.0

OPEN ACCESS

INTRODUCTION

Species identification is a crucial step in forensic entomology. In particular, the calculation of the age of the insects collected from a cadaver or a crime scene allows the estimation of the time of oviposition that, except in case of myiasis, can be considered as the minimum Post-Mortem Interval (mPMI) (*Erzinclioğlu, 1983; Marchenko, 1982; Smith, 1986; Amendt et al., 2007; Vanin et al., 2017*). This method is particularly precise when insects of the first colonization wave—mainly Diptera in the family Calliphoridae, Sarcophagidae and Muscidae—are considered. Insect development is temperature dependent and each species has a different growth rate. Thus, the correct identification of the species leads to an accurate mPMI estimation. Species identification is classically performed by morphological analysis of the specimens, but the lack of complete identification keys for immature stages represents a limitation to this approach. In the last twenty years, to overcome this limit, several authors have suggested a DNA-based identification method which is frequently

used today because of the new sequencing technologies and the relative reduction of the costs (Benecke, 1998; Sperling, Anderson & Hickey, 1994; Stevens & Wall, 1996). Most of the publications about identification of forensically important species focused on the analysis of the genes coding for the cytochrome c oxidase subunit I (COI) as summarized by Tuccia and co-workers (Tuccia, Giordani & Vanin, 2016) and cytochrome c oxidase subunit II (COII) (Sperling, Anderson & Hickey, 1994; Aly, Wen & Wang, 2013; Boehme, Amendt & Zehner, 2012; Malgorn & Coquoz, 1999; Wallman & Donnellan, 2001; Xiong et al., 2012). Tested target markers other than COI and COII were: Cytb (GilArriortua et al., 2013; GilArriortua et al., 2014; GilArriortua et al., 2015; Giraldo, Uribe & Lopez, 2011), ND1 (Giraldo, Uribe & Lopez, 2011), ND5 (Zaidi et al., 2011; Zehner et al., 2004), 28S rDNA (Gibson et al., 2011; McDonagh & Stevens, 2011; Stevens & Wall, 2001; Tourle, Downie & Villet, 2009), mt16S rDNA (Guo et al., 2014; Li et al., 2010), CAD (Gibson et al., 2011; Meiklejohn et al., 2013; Schnell Schühli, Barros de Carvalho & Wiegmann, 2007), EF-1 α (Gibson et al., 2011; Schnell Schühli, Barros de Carvalho & Wiegmann, 2007; McDonagh, García & Stevens, 2009), ITS1 (Zaidi et al., 2011), ITS2 (GilArriortua et al., 2014; GilArriortua et al., 2015; Zaidi et al., 2011; Ferreira et al., 2011; Nelson, Wallman & Dowton, 2008; Song, Wang & Liang, 2008; Yusseff-Vanegas & Agnarsson, 2017), PER (Guo et al., 2014) and Bicoid (Park et al., 2013). Analysis of mitochondrial DNA (mtDNA), in particular COI gene, seems to provide good species identification among Diptera, although a correct identification is still problematic for closely related species (Tourle, Downie & Villet, 2009; Harvey et al., 2008; Sonet et al., 2012). Nuclear DNA, especially ITS2 gene, presents a lack of intra-specific genetic divergence but high inter-specific variation in the genus *Lucilia* Robineau-Desvoidy, 1830, leading to a better resolution of closely related species (GilArriortua et al., 2014; GilArriortua et al., 2015). ITS2 was able to fully resolve the relationship within the species in the genus *Cochliomyia* Townsend 1915 (Yusseff-Vanegas & Agnarsson, 2016), otherwise, the same gene appeared to be not useful for *Chrysomya* Robineau-Desvoidy, 1830 genus studies (Nelson, Wallman & Dowton, 2008).

Previous works indicate that the combination of nuclear and mitochondrial markers is a much more accurate approach for species identification. In a recent paper, the study of Caribbean blow-flies through DNA markers highlights the possibility to resolve phylogenetic relations using a combination of COI and ITS2 genes. In fact, COI failed in demonstrating a monophyly in recently diverged species, leading to uncertain identification. The addition of a second nuclear marker, such as ITS2, increases certainty in species identification (Yusseff-Vanegas & Agnarsson, 2017). McDonagh and co-worker tested a multi-loci approach (28S rRNA, COI and EF-1 α) finding that multiple-gene phylogenies permit the use of genes that have evolved at different rates, and also allow the identification of experimental errors in species identification and sequencing (McDonagh & Stevens, 2011). Zaidi et al. (2011) based the identification of Diptera species on five genes and demonstrated that such a multi-gene approach allows to overcome and clarify the misdiagnosis given by a single gene identification.

We focused our attention on dipteran specimens morphologically identified in order to evaluate the accuracy of the molecular approach in the identification of forensically important species. Sequences of four different markers, two mitochondrial (COI and

ND5) and two nuclear (EF-1 α and PER) were used. According to literature, identification based on a single gene had showed discordant outcomes compared with morphological results (*Meier et al., 2006; Vilgalys, 2003*) especially in the case of closely related species. In order to clarify the accuracy of a molecular multiple-loci approach in the identification of forensically important species, we built concatenated sequences using the four different markers.

MATERIALS AND METHODS

Eighty specimens ([Table 1](#)) were collected between 2011 and 2014 in Italy (Emilia Romagna, Veneto and Calabria), England (West Yorkshire) and Belgium, and preserved in absolute ethanol. The specimens were observed under the microscope and identified using taxonomic keys ([Table 2](#)). DNA extraction from adult insects was performed on abdominal tissues carefully dissected, to prevent external contaminations and to preserve the external structure of the insect for future examination. Full puparia and larvae were instead entirely processed, after a photographic documentation to allow further observations. DNA was extracted using the QIAamp DNA Mini Kit[®] (QIAGEN, Germantown, MD, USA), following the manufacture protocol “DNA Purification from Tissue” (QIAGEN). Sterile deionized water was used to elute the DNA. The amplification of DNA was carried out on selected regions of four genes. In particular the barcoding region of the COI gene, and portions within ND5, EF-1 α and PER genes were amplified. COI gene was selected as mainstream component of the analysis, and conversely ND5, EF-1 α and PER genes were selected because only a little information is available on these DNA portions. A list of the used primers and their specifications are reported in [Table 3](#). PCR was performed using 4 μ l of the DNA extract as template for a 40 μ l reaction final volume, using 0.5 μ l of GoTaq[®] Flexi DNA Polymerase (Promega, Madison, WI, USA) per reaction. Each 40 μ l reaction consisted of 8 μ l of 5X Colorless GoTaq[®] Flexi Buffer (Promega), 4 μ l of MgCl₂ (25 mM), 1 μ l of each of the two primers (10 pmol/ μ l), 1 μ l of 10 mM nucleotide mix (Promega), and 20.5 μ l sterile distilled water. Thermal cycler program used for the amplification consisted of an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of 1 min at 95 °C, 1 min at the annealing temperature and 1 min at 72 °C; with a final extension at 72 °C for 10 min. Annealing temperatures were 49.8 °C for COI, 53 °C for ND5, 55 °C for EF-1 α and 58 °C for PER. Amplifications were confirmed by standard gel electrophoresis, using 2% w/v agarose/TBE gels, stained with ethidium bromide. Thirty-five μ l of PCR products were purified using QIAquick PCR Purification kit[®] (QIAGEN, Germantown, MD, USA) following the manufacturer protocol and were sequenced by Eurofins Genomics (Ebersberg, Germany). Sequences were considered for species identification purposes using nBLAST[®] (*Altschul et al., 1990*) to confirm the previous morphological identification. A total of 309 sequences were analysed, from them 257 were sequenced in this work ([Table 4](#)), and 52 were downloaded from GenBank ([Table 5](#)). Analyses based on the phylogenetic relationships between the studied species were carried out to confirm the identification. It is worth mentioning that in order to obtain consistent blocks of nucleotides for all the species, the sequences were processed with Gblock and manually checked ([Talavera &](#)

Table 1 List of species analysed, with the number of samples, stage of development (A, adult; P, pupae; III L, third larval instar) and country of origin (B, Belgium; UK, United Kingdom; I, Italy).

Species	Nr. of samples	Stage of development	Country of origin
<i>Calliphora vicina</i> Robineau-Desvoidy, 1830	28	A	B, UK, I
<i>Calliphora vomitoria</i> (Linnaeus 1758)	10	A	UK, I
<i>Lucilia sericata</i> (Linnaeus 1758)	22	A	B, UK, I
<i>Lucilia illustris</i> (Meigen 1826)	4	A	I
<i>Lucilia caesar</i> (Meigen 1826)	3	A	I
<i>Chrysomya albiceps</i> (Wiedemann 1819)	3	A, III L	I
<i>Phormia regina</i> (Meigen 1826)	1	P	I
<i>Cynomya mortuorum</i> (Linnaeus 1761)	1	A	B
<i>Sarcophaga africa</i> (Wiedemann, 1824)	1	A	I
<i>Sarcophaga</i> sp. Meigen 1826	1	III L	UK
<i>Hydrotaea</i> sp. Robineau-Desvoidy 1830	2	P	UK
<i>Fannia scalaris</i> (Fabricius 1794)	2	III L	I
<i>Piophilina</i> sp. Fallen 1810	1	P	UK
<i>Megaselia scalaris</i> (Loew 1866)	1	A	I

Table 2 Taxonomical keys used for morphological identification of the specimens.

Family	Identification key
Calliphoridae	Rognes (1991), Szpila (2010)
Sarcophagidae	Pape (1996)
Muscidae	Skidmore (1985)
Fanniidae	Skidmore (1985)
Phoridae	McAlpine (1987)
Piophilidae	McAlpine (1987)

Castresana, 2007; Castresana, 2000). Subsequently, sequences were aligned using Clustal Omega (*Sievers et al., 2011*) and concatenated with FASconCAT v1.0 (*Kück & Meusemann, 2010*). Trees were built using the Neighbour Joining and the Maximum Likelihood methods on MEGA 7.0 (*Kumar, Stecher & Tamura, 2016*) using Kimura 2-parameter (K2P) evolutionary model (*Čandek & Kuntner, 2015*). A bootstrap of 1,000 replicates was used for the phylogenetic reconstructions. Trees were visualised with ITOL (*Letunic & Bork, 2016*). In the trees reconstruction Piophilidae and Muscidae species were considered as outgroups.

RESULTS

The analysed specimens belonged to fourteen species, with *Calliphora vicina* Robineau-Desvoidy, 1830 and *Lucilia sericata* (Meigen, 1826) (Diptera, Calliphoridae) as the most abundant taxa representing 29.8 and 27.4% respectively. The first analysis step was based on a local alignment using GenBank BLAST (*Altschul et al., 1990*) and the percentage of correct identification was evaluated. In particular, the molecular one-gene identification

Table 3 List of primers used in this study.

Gene	Primer name and sequence	Reference
COI	LCO1490 5'- GGTC AACAAATCATAAAGATATTGG -3'	<i>Folmer et al. (1994)</i>
	HCO2198 5'- TAAACTTCAGGGTGACCAAAAAATCA -3'	
ND5	ND5(a) 5'- CCAAAATATTTCWGATCAHCCYTG -3'	<i>Zehner et al. (2004)</i>
	ND5(b) 5'- GGATTA ACTGTTTGTATWCTTTTCG -3'	
EF-1 α	B1 5'- CCCATYTCCGGHTGGCAGCG -3'	<i>McDonagh, García & Stevens (2009)</i>
	C1 5'- GTCTCATGTACGDACRGCG -3'	
PER	PERFW 5'- CTR GAR YTR CCC AAT GAA -3'	This paper
	PERRV 5'- TSR CCC TCC CAH GAA TG -3'	

was compared with the morphological identification obtaining a percentage value match of 87.5% for COI, 72.5% for ND5, 77.1% for EF-1 α and 67.9% for PER. Concerning Calliphoridae, the percentages were 77.5, 64.1, 71.2 and 64.2% respectively. A phylogenetic approach was used to verify the molecular identification efficiency, however, the sequencing of EF-1 α and PER regions was successful only in 72.6% and 69.1% of the specimens respectively. Independent analysis of COI (Fig. 1A, Fig. S1) recovered the monophyly of all families. All the subfamilies (Calliphorinae, Luciliinae and Chrysomyinae) are separated with robust bootstrap values ranging from 0.8 to 1 in a scale between 0 and 1. Among the genus *Lucilia*, *L. sericata* sequences cluster together and are clearly distinct from the other co-generic species (bootstrap 1), while the pattern of *Lucilia illustris* Meigen, 1826 and *Lucilia Caesar* (Linnaeus, 1758) is not clearly resolved with *L. illustris* showing a paraphyletic pattern. The genus *Calliphora* Robineau-Desvoidy, 1830 was also recovered as paraphyletic, in this case *C. vicina* branches with *Cynomya mortuorum* (Linnaeus, 1761), but with a weak support, instead of branching with *C. vomitoria*.

Phylogenetic reconstruction based on the ND5 marker (Fig. 1B, Fig. S2) shows an unresolved topology with problems of determination at all taxonomic levels (family, subfamily, genus and species). *Lucilia caesar* and *L. illustris* are not clearly distinct and in addition *Protophormia terraenovae* Robineau-Desvoidy, 1830 sequence clusters with *L. sericata* sequences. A small number of sequences was available for both EF-1 α (Fig. 1C, Fig. S3) and PER gene (Fig. 1D, Fig. S4). Both phylogenetic reconstructions obtained using these markers showed the same problems reported for COI and ND5, with *L. illustris* and *L. caesar* not clearly distinct.

In order to increase the molecular information three concatenated sequences were generated using the previous genes (*DeSalle, Egan & Siddall, 2005*). The phylogenetic reconstruction based on the chimeric sequence generated on the two mitochondrial genes (COI and ND5) (Fig. 2A, Fig. S5) does not provide a better resolution for *L. illustris/L. caesar* species as well as for the position of *C. mortuorum* among the Calliphorinae. These two points are not better clarified when the nuclear sequences are included and two more chimeric sequences with three (COI, ND5 and EF-1 α) (Fig. 2B, Fig. S6) and four (COI, ND5, EF-1 α and PER) (Fig. 2C, Fig. S7) genes are generated. Table 6 summarizes the information of the sequences used in the phylogenetic reconstructions.

Table 4 New sequences with geographical origin and GenBank code listed by gene.

Morphological identification	Sequence ID	Geographical origin	Genbank code			
			COI	ND5	EF1a	PER
<i>Calliphora vicina</i>	2BGCvi	Belgium - B	MH401768	MH401920	MH401958	MH401876
	ITMA8Cvi	Italy - I	MH401769	MH588583		
	ITMO3Cvi	Italy - I	MH401773	MH401924	MH401961	MH401879
	1ITCvi	Italy - I	MH401777	MH588588		
	2ITCvi	Italy - I		MH588592	MH588602	MH588607
	3ITCvi	Italy - I	MH401780	MH588589	MH588601	
	4ITCvi	Italy - I	MH401782	MH401915	MH401963	MH401866
	5ITCvi	Italy - I	MH401784	MH401916	MH401965	MH401863
	6ITCvi	Italy - I	MH401786	MH401917	MH401966	MH401869
	7ITCvi	Italy - I	MH401788	MH588584	MH588603	
	8ITCvi	Italy - I	MH401790	MH588593		
	9ITCvi	Italy - I	MH401792	MH401918	MH401967	MH401873
	10ITCvi	Italy - I	MH401776	MH588585	MH588604	
	ITMACvi1	Italy - I	MH401803	MH588590		
	ITMACvi2	Italy - I	MH401804	MH588591		
	ITMOCvi3	Italy - I	MH401805	MH401904	MH401946	MH401861
	ITMOCvi4	Italy - I	MH401806	MH401902	MH401944	MH401859
	ITMOII1Cvi	Italy - I	MH401807	MH401884	MH401928	MH401839
	ITMOII2Cvi	Italy - I	MH401809	MH401885	MH401929	MH401840
	ITMOII3Cvi	Italy - I	MH401834	MH401886	MH401930	MH401841
	ITMOII4Cvi	Italy - I	MH401810	MH401887	MH401931	MH401842
	ITMOII5Cvi	Italy - I	MH401811	MH401888	MH401932	MH401843
	ITTVCvi1	Italy - I	MH401818	MH588582		MH588605
	ITTVCvi2	Italy - I	MH401819	MH588586		
	ITTVCvi3	Italy - I	MH401820	MH401901	MH401943	MH401858
	BOX4UKPrt	United Kingdom - UK	MH401798	MH588587		
	CvoUK	United Kingdom - UK	MH401764	MH401923	MH401960	MH401878
	ITMO1Cvo	Italy - I	MH401767	MH401925	MH401964	MH401881
	ITMA1Cvo	Italy - I	MH401775	MH588580		
	BOX3UKCvo	United Kingdom - UK	MH401795	MH401898		MH401855
ITTVCvo1	Italy - I	MH401821	MH401899	MH401969	MH401856	
ITTVCvo2	Italy - I	MH401822	MH588579			
ITTVCvo3	Italy - I	MH401823	MH401900	MH401942	MH401857	
UKCvo	United Kingdom - UK	MH401832	MH401893	MH401938	MH401849	
70UKCvo	United Kingdom - UK		MH588581	MH588599	MH588606	
99UKCvo	United Kingdom - UK		MH588578	MH588600	MH401882	
<i>Chrysomya albiceps</i>	ITVVChalbA	Italy - I	MH401800	MH588568	MH588596	
	ITVVChalbL	Italy - I	MH401801	MH588567		
	ITChalb	Italy - I	MH401833	MH588569	MH605069	

(continued on next page)

Table 4 (continued)

Morphological identification	Sequence ID	Geographical origin	Genbank code			
			COI	ND5	EF1a	PER
<i>Cynomya mortuorum</i>	3BGCym	Belgium - B	MH401763	MH401921	MH401959	MH401877
<i>Fannia</i> sp.	FanniaL	Italy - I	MH401835	MH588563		
	FanniaP	Italy - I	MH401836	MH588564		
<i>Hydrotaea</i> sp.	BOX4UKH	United Kingdom - UK		MH588560	MH588595	
	BOX6UKH	United Kingdom - UK	MH401799	MH588559		
<i>Lucilia caesar</i>	ITTVLc1	Italy - I	MH401824	MH401926	MH401941	MH401853
	ITTVLc2	Italy - I	MH401825	MH588572	MH401968	MH401854
	ITTVLc3	Italy - I	MH401826	MH588573		MH605070
<i>Lucilia illustris</i>	ITMO2Li	Italy - I	MH401766	MH401922	MH401962	MH401880
	ITNOai15Li	Italy - I	MH401771	MH588571		
<i>Lucilia sericata</i>	ITMOLi1	Italy - I	MH401816	MH588570		MH588608
	ITTVLill1	Italy - I	MH401827	MH588574		
	ITMA3Lse	Italy - I	MH401765	MH588575	MH588598	
	1BGLs	Belgium - B	MH401772	MH401919	MH401957	MH401875
	ITMO1Ls	Italy - I	MH401774	MH588577		
	1ITLs	Italy - I	MH401778	MH401906	MH401948	MH401864
	2ITLs	Italy - I	MH401779	MH401907	MH401949	MH401862
	3ITLs	Italy - I	MH401781	MH401908	MH401950	MH401865
	4ITLs	Italy - I	MH401783	MH401909	MH401951	MH401867
	5ITLs	Italy - I	MH401785	MH401910	MH401952	MH401868
	6ITLs	Italy - I	MH401787	MH401911	MH401953	MH401870
	7ITLs	Italy - I	MH401789	MH401912	MH401954	MH401871
	8ITLs	Italy - I	MH401791	MH401913	MH401955	MH401872
	9ITLs	Italy - I	MH401793	MH401914	MH401956	MH401874
	BOX3UKLs	United Kingdom - UK		MH588576	MH588597	
	ITVVLs	Italy - I	MH401802	MH401905	MH401947	MH401848
	ITMOII10Ls	Italy - I	MH401808	MH401883	MH401927	MH401838
ITMOII6Ls	Italy - I	MH401812	MH401889	MH401933	MH401844	
ITMOII7Ls	Italy - I	MH401813	MH401890	MH401934	MH401845	
ITMOII8Ls	Italy - I	MH401814	MH401891	MH401935	MH401846	
ITMOII9Ls	Italy - I	MH401815	MH401892	MH401936	MH401847	
ITTVLs1	Italy - I	MH401828	MH401894	MH401937	MH401850	
ITTVLs2	Italy - I	MH401829	MH401895	MH401939	MH401851	
ITTVLs3	Italy - I	MH401830	MH401896	MH401940	MH401852	
<i>Megaselia scalaris</i>	Ms	Italy - I		MH588562		MH401837
<i>Phormia regina</i>	ITQC2Phr	Italy - I	MH401770	MH588566		
<i>Piophilha</i> sp.	UKPio	United Kingdom - UK	MH401817	MH588561	MH588594	
<i>Sarcophaga africa</i>	ITTVSA	Italy - I	MH401831	MH401903	MH401945	MH401860
<i>Sarcophaga</i> sp.	BOX1UKSL	United Kingdom	MH401794	MH588565		

Table 5 Sequences selected from GenBank listed by gene. Country of origin and its abbreviation used in the cladograms are reported.

Gene	Species	GenBank #	Country	Abbreviation
COI	<i>C.vicina</i>	JX438024	Portugal	P
		KC617807	USA	USA
	<i>C.vomitorea</i>	JX438025	Portugal	P
		KC775967	Portugal	P
	<i>C. albiceps</i>	JX438026	Portugal	P
		HE814059	Germany	D
	<i>P. regina</i>	KM569886	Canada	CDN
		KM569803	Canada	CDN
	<i>P. terraenovae</i>	KM570349	Canada	CDN
		KF908116	Belgium	B
	<i>L. sericata</i>	JX438041	Portugal	P
		KC776060	Portugal	P
	<i>L. illustris</i>	KM571189	Canada	CDN
		KM570007	Canada	CDN
	<i>L. caesar</i>	KJ635700	Spain	E
		KJ635701	Spain	E
	<i>S. africa</i>	JQ413455	Kenya	EAK
	<i>S. melanura</i>	JQ413457	Belgium	B
	<i>H. dentipes</i>	HM891630	Sweden	S
	<i>F. canicularis</i>	JX438029	Portugal	P
KC617819		USA	USA	
<i>M. scalaris</i>	KC407774	Korea	ROK	
	JQ941746	China	RC	
ND5	<i>C.vicina</i>	NC_019639	France	F
		JX_913760	France	F
	<i>C. albiceps</i>	NC_019631	Zambia	Z
	<i>P. regina</i>	NC_026668	USA	USA
	<i>P. terraenovae</i>	NC_019636	France	F
		FJ614877	China	RC
	<i>L. sericata</i>	FJ614876	China	RC
		KM571189	China	RC
	<i>L. illustris</i>	KM570007	China	RC
	<i>S. africa</i>	NC_025944	China	RC
	<i>M. scalaris</i>	NC_023794	China	RC
	PER	<i>L. sericata</i>	JN792856	USA
JN792853			South Africa	ZA
<i>L. illustris</i>		KF839550	USA	USA
		KF839549	USA	USA
<i>L. caesar</i>		KF839532	France	F
		JN792858	France	F
<i>S. africa</i>		KC966442	China	RC
	KC966441	China	RC	

(continued on next page)

Table 5 (continued)

Gene	Species	GenBank #	Country	Abbreviation
EF 1 alfa	<i>M. scalaris</i>	KC178059	USA	USA
	<i>C.vomitorea</i>	JQ307782	United Kingdom	UK
		FJ025666	Singapore	SGP
	<i>P. terraenovae</i>	JQ307784	United Kingdom	UK
	<i>L. sericata</i>	JQ307785	United Kingdom	UK
	<i>L. illustris</i>	JQ307786	United Kingdom	UK
	<i>L. caesar</i>	JQ307787	United Kingdom	UK
		JQ307787	United Kingdom	UK
	<i>H. dentipes</i>	FJ025679	China	RC
<i>F. canicularis</i>	AJ871202	Canada	CDN	

DISCUSSION

The results obtained with a local alignment demonstrate that the match of the molecular identification with the morphological identification of the specimens was never higher than 90% also considering COI gene (87.5%), currently used for species identification (DNA Barcoding Project (<http://www.barcodeoflife.org/>)). The analysis of ND5 gene, a mitochondrial gene, was difficult for *Calliphora vomitoria* (Linnaeus, 1758) due to a complete lack of ND5 sequences from this species in the database (GenBank) at the moment of the analysis. The molecular analysis of the closely related though morphologically well distinct species, *L. illustris* and *L. caesar*, does not allow a unambiguous identification of them, as already reported in previous works where different phylogenetic approaches (e.g., Maximum Parsimony) were also used (GilArriortua *et al.*, 2015; Wells, Wall & Stevens, 2007). In fact, GilArriortua and co-workers (GilArriortua *et al.*, 2015) indicated that *L. caesar* and *L. illustris* species appear to share mitochondrial genomes with a divergence value lower than the minimum inter-specific threshold value for mitochondrial loci. ND5 gene showed the same problem in the discrimination of the close *Lucilia* species. To our knowledge, the analysis of closely related species in blowflies using ND5 gene was only reported by Zaidi and co-workers who showed a good identification performance using this gene (Zaidi *et al.*, 2011). In addition, the same mitochondrial region was used to analyse the evolutionary relationship between flesh flies with a good resolution (Zehner *et al.*, 2004). The analysis of EF-1 α gene is in agreement with a previous study (McDonagh & Stevens, 2011) that demonstrated a good ability of this gene to separate blowflies according to morphological classification. However, in our reconstruction both the position of the *Lucilia* species and *Cynomya*, within Calliphorinae, are not well resolved. To our knowledge, PER gene was studied for identification purposes only in flesh flies (Guo *et al.*, 2014). This work showed the possibility to use successfully PER gene for identification purposes, although public datasets might be enriched with further DNA sequences belonging to different family of Diptera.

The analysis of the concatenated sequences generated with COI, ND5, EF-1 α and PER markers unfortunately does not improve the resolution of the investigation despite previous works indicate that the combination of nuclear and mitochondrial genes for

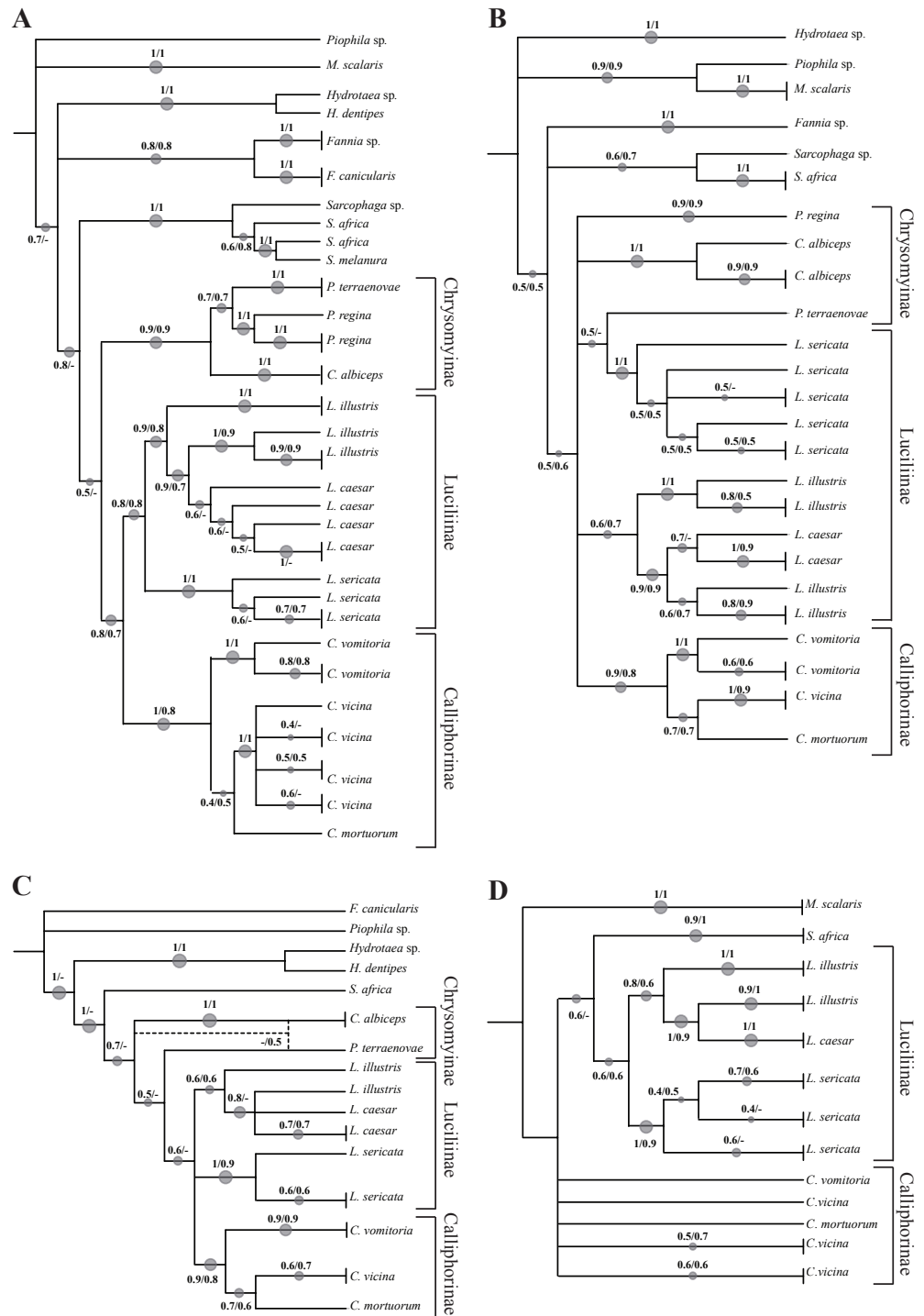


Figure 1 Simplified phylogenetic trees. Simplified representation of the phylogenetic trees obtained using COI (A), ND5 (B), EF-1 α (C) and PER (D) genes. Original trees are reported in the [Supplemental Information](#).

Full-size [DOI: 10.7717/peerj.5962/fig-1](https://doi.org/10.7717/peerj.5962/fig-1)

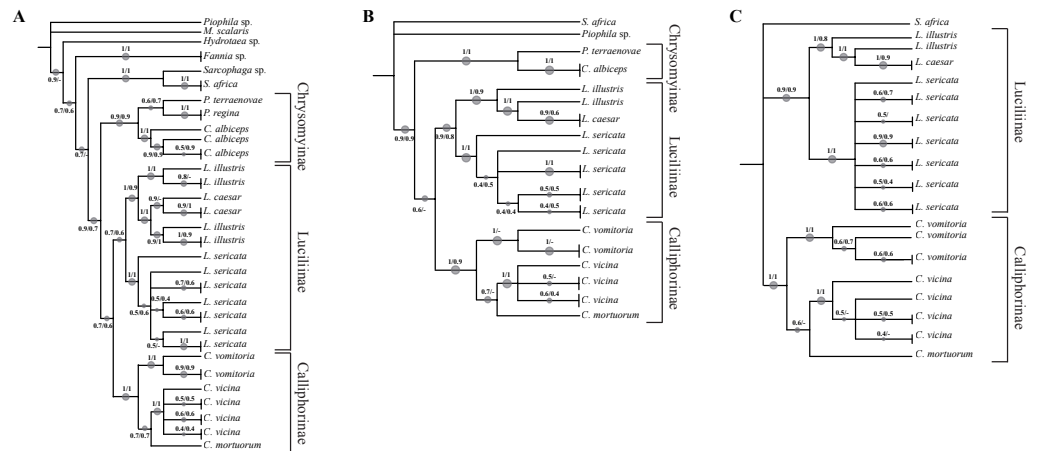


Figure 2 Simplified phylogenetic trees. Simplified representations of the phylogenetic trees obtained using COI and ND5 (A), COI, ND5 and EF-1 α (B) and COI, ND5, EF-1 α and PER (C) genes. Original trees are reported in the [Supplemental Information](#).

Full-size [DOI: 10.7717/peerj.5962/fig-2](https://doi.org/10.7717/peerj.5962/fig-2)

Table 6 Size (bp) and number of sequences analysed.

	Gene				Concatenated sequences		
	COI	ND5	EF-1 α	PER	COI+ND5	COI+ND5+ EF-1 α	COI+ND5+ EF-1 α +PER
Length (bp)	605	329	309	327	934	1,243	1,570
Nr. of sequences	72 + 23*	78 + 11*	55 + 9*	52 + 9*	72 + 10*	50 + 3*	43 + 2*

Notes.

*indicates the sequence from GenBank.

species identification is a much more accurate approach. In fact the combination of markers that have different evolutionary histories, fast and slow evolving genes, allows a better resolution of the phylogenies. In particular, the multi-loci analysis of COI, EF-1 α , and 28S rRNA genes and the combined analysis of COI, CYTB, ND5, and ITS1 and ITS2 genes has demonstrated to be more successful compared to the single-locus phylogeny, leading to a better grouping of species belonging to the same family (Zaidi et al., 2011; McDonagh & Stevens, 2011; Grzywacz, Wallman & Piwczynski, 2017). However, as underlined by Sonet et al. (2012), not always the addition of more genes with different evolutionary histories resolves the monophyly of closely related species such as *L. illustris* and *L. caesar*. The monophyly of these two species was clearly demonstrated only by two research groups: one working with the gene Bicoid (Park et al., 2013) and another one using the AFLP (Amplified Fragment Length Polymorphism) approach (Picard et al., 2018). In both cases the two species were well resolved with a strong basal support, confirming the conclusions obtained from the morphological analysis of male and female specimens of both species.

At the moment, because of the small number of sequences available for these two species, we cannot exclude phenomena of hybridization at least in some parts of the distribution area of the species, but this point needs further investigations and a larger dataset to be analysed.

The importance to have complete and correct dataset is a crucial point to reach a correct species identification, with both local alignment systems and/or phylogenetic methods. Molecular approach is strongly related to the quality of information stored in databases, and the possibility to improve the amount of genetic markers from different specimens from different geographical locations is important to recover the best resolution in phylogenetic trees. The availability of genetic data from different populations allows to have information about the intraspecific variability that, in closely related species can affect the phylogenetic reconstruction. The use of a single gene approach to identify animal species is an open argument, especially for closely related species. In particular, mtDNA does not seem to be significantly different from any other marker group revealing an overall success rate of 71% (Dupuis, Roe & Sperling, 2012). In fact, the mitochondrial evolution reduces its applicability for detailed systematic or taxonomic analysis for closely related species (Dupuis, Roe & Sperling, 2012; Will, Mishler & Wheeler, 2005; De Carvalho *et al.*, 2008). Dupuis and co-workers (Dupuis, Roe & Sperling, 2012) highlighted two main results: (i) marker classes (mtDNA, ribosomal DNA, autosomal loci, sex-linked loci, and anonymous loci) were moderately successful to delimit closely related species, if used as unique identifier, and (ii) multi-locus power analysis data support investigation and use of multiple markers for species delimitation. Several papers have discussed multi-locus analysis as species identification methods for animal kingdom. In particular, sex-linked markers showed a high success ratio in delimiting closely related species in Diptera and Lepidoptera (Coyne & Orr, 1989; Roe & Sperling, 2007). The improvement of genetic datasets and the concatenation of different mitochondrial and nuclear loci could improve the capability of molecular approach to identify closely related species but this aspect has to be further explored considering as well the taxon's specificity.

It is worth mentioning as well that in this kind of studies the species choice and intra-specific sampling scheme can strongly affect the level of resolution of the analysis. In our study, a further investigation including a larger sequence dataset of species in the genus *Lucilia* from different geographical contexts would better clarify the results here reported and the derived conclusions.

CONCLUSIONS

Nowadays, in forensic entomology, the morphological identification approach for some species is not completely replaceable by the molecular one if based on a single gene. The two methodologies can complement each other. In addition, because of the lack of information in databases, a phylogenetic approach can increase the ability of species identification when the molecular approach is used. The analysis of mitochondrial genes is considered the best approach because of the peculiarity of this kind of DNA, in terms of haploidy, high copy numbers, low recombination and lack of introns (Hebert *et al.*, 2003). However, considering the nature of mitochondrial evolution and the results of this and previous studies, the use of mtDNA does not provide a good level of resolution for some of the *Lucilia* species. In addition, the analysis of nuclear genes, such as EF-1 α and PER, cannot improve this point. Additional work using mtDNA in association with other genetic markers (i.e.,

sex-linked loci) could clarify and resolve the relationships among the *Lucilia* genus as well as other close related species. It is worth mentioning that the investigation for the best marker has to be done at the genus level, in fact some markers that have been suggested in addition to COI (e.g., ITS2) work for the resolution of certain taxa but not for others. In addition, given the problems in the resolution of several genera/species in the family Calliphoridae as highlighted as well in this paper, an approach based on NGS technologies (e.g., WGS –whole genome shotgun) will probably provide enough information to distinguish the taxa.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Giorgia Giordani and Fabiola Tuccia were supported by the Leverhulme trust scholarship. Sara Bortolini was supported by the “Finanziamento di azioni di mobilita’ nell’ambito del programma di collaborazione scientifica e culturale dell’Universita’ di Modena e Reggio Emilia con universita’ straniere convenzionate 2013–2014”. There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

Leverhulme trust scholarship.

Finanziamento di azioni di mobilita’ nell’ambito del programma di collaborazione scientifica e culturale dell’Universita’ di Modena e Reggio Emilia con universita’ straniere convenzionate 2013–2014.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Sara Bortolini performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper.
- Giorgia Giordani and Fabiola Tuccia analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper.
- Lara Maistrello contributed reagents/materials/analysis tools.
- Stefano Vanin conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The following information was supplied regarding data availability:

The downloaded GenBank sequences: [JX438024](#), [KC617807](#), [JX438025](#), [KC775967](#), [JX438026](#), [HE814059](#), [KM569886](#), [KM569803](#), [KM570349](#), [KF908116](#), [JX438041](#),

KC776060, KM571189, KM570007, KJ635700, KJ635701, JQ413455, JQ413457, HM891630, JX438029, KC617819, KC407774, JQ941746, NC_019639, JX_913760, NC_019631, NC_026668, NC_019636, FJ614877, FJ614876, KM571189, KM570007, NC_025944, NC_023794, JN792856, JN792853, KF839550, KF839549, KF839532, JN792858, KC966442, KC966441, KC178059, JQ307782, FJ025666, JQ307784, JQ307785, JQ307786, JQ307787, JQ307787, FJ025679, AJ871202

The sequences used to create all the phylogenetic trees are available in the [Supplemental File](#) and at Genbank: [MH401763–MH401969](#) and [MH588559–MH588609](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.5962#supplemental-information>.

REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990.** Basic local alignment search tool. *Journal of Molecular Biology* **215**:403–410
DOI [10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
- Aly SM, Wen J, Wang X. 2013.** Identification of forensically important Sarcophagidae (Diptera) based on partial mitochondrial cytochrome oxidase I and II genes. *American Journal of Forensic Medicine and Pathology* **34**:159–163
DOI [10.1097/PAF.0b013e31828c390e](https://doi.org/10.1097/PAF.0b013e31828c390e).
- Amendt J, Campobasso CP, Gaudry E, Reiter C, LeBlanc HN, Hall MJR. 2007.** Best practice in forensic entomology—standards and guidelines. *International Journal of Legal Medicine* **121**:90–104 DOI [10.1007/s00414-006-0086-x](https://doi.org/10.1007/s00414-006-0086-x).
- Benecke M. 1998.** Random amplified polymorphic DNA (RAPD) typing of necrophagous insects (Diptera, Coleoptera) in criminal forensic studies: validation and use in practice. *Forensic Science International* **98**:157–168
DOI [10.1016/S0379-0738\(98\)00150-9](https://doi.org/10.1016/S0379-0738(98)00150-9).
- Boehme P, Amendt J, Zehner R. 2012.** The use of COI barcodes for molecular identification of forensically important fly species in Germany. *Parasitology Research* **110**:2325–2332 DOI [10.1007/s00436-011-2767-8](https://doi.org/10.1007/s00436-011-2767-8).
- Čandek K, Kuntner M. 2015.** DNA barcoding gap: reliable species identification over morphological and geographical scales. *Molecular Ecology Resources* **15**:268–277
DOI [10.1111/1755-0998.12304](https://doi.org/10.1111/1755-0998.12304).
- Castresana J. 2000.** Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**:540–552
DOI [10.1093/oxfordjournals.molbev.a026334](https://doi.org/10.1093/oxfordjournals.molbev.a026334).
- Coyne JA, Orr HA. 1989.** Two rules of speciation. In: Otte D, Endler JA, eds. *Speciation and its consequences*. Sunderland: Sinauer Associates, 180–207.
- De Carvalho MR, Bockmann FA, Amorim DDS, Brandão CRF. 2008.** Systematics must embrace comparative biology and evolution, not speed and automation. *Evolutionary Biology* **35**:150–157 DOI [10.1007/s11692-008-9018-7](https://doi.org/10.1007/s11692-008-9018-7).

- DeSalle R, Egan MG, Siddall M. 2005.** The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society of London B Biological Sciences* **360**:1905–1916 DOI [10.1098/rstb.2005.1722](https://doi.org/10.1098/rstb.2005.1722).
- Dupuis JR, Roe AD, Sperling FAH. 2012.** Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology* **21**:4422–4436 DOI [10.1111/j.1365-294X.2012.05642.x](https://doi.org/10.1111/j.1365-294X.2012.05642.x).
- Erzinclioglu YZ. 1983.** The application of entomology to forensic medicine. *Medicine, Science and the Law* **23**:57–63 DOI [10.1177/002580248302300110](https://doi.org/10.1177/002580248302300110).
- Ferreira S, Oliveira AR, Farinha A, Rebelo MT, Dias D. 2011.** Forensic entomology: nuclear and mitochondrial markers for Diptera and Coleoptera identification. *Forensic Science International: Genetics Supplement Series* **3**:e174–e175.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994.** DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**:294–299.
- Gibson JF, Kelso S, Jackson MD, Kits JH, Miranda GFG, Skevington JH. 2011.** Diptera-specific polymerase chain reaction amplification primers of use in molecular phylogenetic research. *Annals of the Entomological Society of America* **104**:976–997 DOI [10.1603/AN10153](https://doi.org/10.1603/AN10153).
- GilArriortua M, Saloñá-Bordas MI, Cainé LM, Pinheiro F, De Pancorbo MM. 2015.** Technical note: mitochondrial and nuclear DNA approaches for reliable identification of *Lucilia* (Diptera, Calliphoridae) species of forensic interest from Southern Europe. *Forensic Science International* **257**:393–397 DOI [10.1016/j.forsciint.2015.10.010](https://doi.org/10.1016/j.forsciint.2015.10.010).
- GilArriortua M, Saloñá-Bordas MI, Köhnemann S, Pfeiffer H, De Pancorbo MM. 2014.** Molecular differentiation of Central European blowfly species (Diptera, Calliphoridae) using mitochondrial and nuclear genetic markers. *Forensic Science International* **242**:274–282 DOI [10.1016/j.forsciint.2014.07.018](https://doi.org/10.1016/j.forsciint.2014.07.018).
- GilArriortua M, Saloñá-Bordas MI, Cainé LM, Pinheiro F, De Pancorbo MM. 2013.** Cytochrome b as a useful tool for the identification of blowflies of forensic interest (Diptera, Calliphoridae). *Forensic Science International* **228**:132–136 DOI [10.1016/j.forsciint.2013.02.037](https://doi.org/10.1016/j.forsciint.2013.02.037).
- Giraldo HPA, Uribe SSI, Lopez RA. 2011.** Analysis of mitochondrial DNA sequences (Cytb and ND1) in *Lucilia eximia* (Diptera: Calliphoridae). *Revista Colombiana de Entomologia* **37**:273–278.
- Grzywacz A, Wallman JF, Piwczyński M. 2017.** To be or not to be a valid genus: the systematic position of *Ophyra* R.-D. revised (Diptera: Muscidae). *Systematic Entomology* **42**:714–723 DOI [10.1111/syen.12240](https://doi.org/10.1111/syen.12240).
- Guo Y, Zha L, Yan W, Li P, Cai J, Wu L. 2014.** Identification of forensically important sarcophagid flies (Diptera: Sarcophagidae) in China based on COI and period gene. *International Journal of Legal Medicine* **128**:221–228 DOI [10.1007/s00414-013-0923-7](https://doi.org/10.1007/s00414-013-0923-7).
- Harvey ML, Gaudieri S, Villet MH, Dadour IR. 2008.** A global study of forensically significant calliphorids: implications for identification. *Forensic Science International* **177**:66–76 DOI [10.1016/j.forsciint.2007.10.009](https://doi.org/10.1016/j.forsciint.2007.10.009).

- Hebert PDN, Cywinska A, Ball SL, DeWaard JR. 2003.** Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* **270**:313–321 DOI [10.1098/rspb.2002.2218](https://doi.org/10.1098/rspb.2002.2218).
- Kück P, Meusemann K. 2010.** FASconCAT: convenient handling of data matrices. *Molecular Phylogenetics and Evolution* **56**:1115–1118 DOI [10.1016/j.ympev.2010.04.024](https://doi.org/10.1016/j.ympev.2010.04.024).
- Kumar S, Stecher G, Tamura K. 2016.** MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**:1870–1874 DOI [10.1093/molbev/msw054](https://doi.org/10.1093/molbev/msw054).
- Letunic I, Bork P. 2016.** Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research* **44**:W242–W245 DOI [10.1093/nar/gkw290](https://doi.org/10.1093/nar/gkw290).
- Li X, Cai JF, Guo YD, Wu KL, Wang JF, Liu QL, Wang XH, Chang YF, Yang L, Lan LM, Zhong M, Wang X, Song C, Liu Y, Li JB, Dai ZH. 2010.** The availability of 16S rRNA for the identification of forensically important flies (Diptera: Muscidae) in China. *Tropical Biomedicine* **27**:155–166.
- Malgorn Y, Coquoz R. 1999.** DNA typing for identification of some species of Calliphoridae: an interest in forensic entomology. *Forensic Science International* **102**:111–119 DOI [10.1016/S0379-0738\(99\)00039-0](https://doi.org/10.1016/S0379-0738(99)00039-0).
- Marchenko MI. 1982.** Trends in using entomological and botanical methods for establishing the time of death. *Sudebno-Meditsinskaia Ekspertiz* **25**:29.
- McAlpine JF. 1987.** *Manual of Nearctic Diptera. Monograph/Research Branch, Agriculture Canada*, vol. 2. Ottawa: Research Branch, Agriculture Canada, 675.
- McDonagh L, García R, Stevens JR. 2009.** Phylogenetic analysis of New World screw-worm fly, *Cochliomyia hominivorax*, suggests genetic isolation of some Caribbean island populations following colonization from South America. *Medical and Veterinary Entomology* **23**:14–22 DOI [10.1111/j.1365-2915.2008.00777.x](https://doi.org/10.1111/j.1365-2915.2008.00777.x).
- McDonagh LM, Stevens JR. 2011.** The molecular systematics of blowflies and screw-worm flies (Diptera: Calliphoridae) using 28S rRNA, COX1 and EF-1 α : insights into the evolution of dipteran parasitism. *Parasitology* **138**:1760–1777 DOI [10.1017/S0031182011001089](https://doi.org/10.1017/S0031182011001089).
- Meier R, Shiyang K, Vaidya G, Ng PKL. 2006.** DNA Barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Systematic Biology* **55**:715–728 DOI [10.1080/10635150600969864](https://doi.org/10.1080/10635150600969864).
- Meiklejohn KA, Wallman JF, Pape T, Cameron SL, Dowton M. 2013.** Utility of COI, CAD and morphological data for resolving relationships within the genus *Sarcophaga* (sensu lato) (Diptera: Sarcophagidae): a preliminary study. *Molecular Phylogenetics and Evolution* **69**:133–141 DOI [10.1016/j.ympev.2013.04.034](https://doi.org/10.1016/j.ympev.2013.04.034).
- Nelson LA, Wallman JF, Dowton M. 2008.** Identification of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2). *Forensic Science International* **177**:238–247 DOI [10.1016/j.forsciint.2008.01.009](https://doi.org/10.1016/j.forsciint.2008.01.009).
- Pape T. 1996.** *Catalogue of the Sarcophagidae of the world (Insecta: Diptera)*. Gainesville: Associated Publishers, 558.

- Park SH, Park CH, Zhang Y, Piao H, Chung U, Kim SY, Ko KS, Yi C-H, Jo T-H, Hwang J-J. 2013. Using the developmental gene bicoid to identify species of forensically important blowflies (Diptera: Calliphoridae). *BioMed Research International* 2013:538051 DOI 10.1155/2013/538051.
- Picard CJ, Wells JD, Ulyot A, Rognes K. 2018. Amplified fragment length polymorphism analysis supports the valid separate species status of *Lucilia caesar* and *L. illustris* (Diptera: Calliphoridae). *Forensic Sciences Research* 3:60–64 DOI 10.1080/20961790.2017.1398286.
- Roe AD, Sperling FAH. 2007. Population structure and species boundary delimitation of cryptic *Dioryctria* moths: an integrative approach. *Molecular Ecology* 16:3617–3633 DOI 10.1111/j.1365-294X.2007.03412.x.
- Rognes K. 1991. *Blowflies (Diptera, Calliphoridae) of Fennoscandia and Denmark*. *Fauna Entomologica Scandinavica*, vol. 24. Leiden: Brill.
- Schnell Schühli G, Barros de Carvalho CJ, Wiegmann BM. 2007. Molecular phylogenetics of the Muscidae (Diptera: Calyptratae): new ideas in a congruence context. *Invertebrate Systematics* 21:263–278 DOI 10.1071/IS06026.
- Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* 7:539 DOI 10.1038/msb.2011.75.
- Skidmore P. 1985. *The biology of the Muscidae of the world*. *Series Entomol*, vol. 29. London: Springer Netherlands, 550.
- Smith KGV. 1986. *A manual of forensic entomology*. London: The Trustees of British Museum (Natural History), 205.
- Sonet G, Jordaens K, Braet Y, Desmyter S. 2012. Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic? *Forensic Science International* 223:153–159 DOI 10.1016/j.forsciint.2012.08.020.
- Song Z, Wang X, Liang G. 2008. Species identification of some common necrophagous flies in Guangdong province, southern China based on the rDNA internal transcribed spacer 2 (ITS2). *Forensic Science International* 175:17–22 DOI 10.1016/j.forsciint.2007.04.227.
- Sperling FA, Anderson GS, Hickey DA. 1994. A DNA-based approach to the identification of insect species used for postmortem interval estimation. *Journal of Forensic Sciences* 39:418–427.
- Stevens J, Wall R. 1996. Species, sub-species and hybrid populations of the blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae). *Proceedings of the Royal Society of London B Biological Sciences* 263:1335–1341 DOI 10.1098/rspb.1996.0196.
- Stevens J, Wall R. 2001. Genetic relationships between blowflies (Calliphoridae) of forensic importance. *Forensic Science International* 120:116–123 DOI 10.1016/S0379-0738(01)00417-0.
- Szpila K. 2010. Key for the identification of third instars of European blowflies (Diptera: Calliphoridae) of forensic importance. In: Amendt J, Campobasso CP, Goff ML,

- Grassberger M, eds. *Current concepts in forensic entomology*. Dordrecht: Springer, 43–56.
- Talavera G, Castresana J. 2007.** Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56**:564–577 DOI [10.1080/10635150701472164](https://doi.org/10.1080/10635150701472164).
- Tourle R, Downie DA, Villet MH. 2009.** Flies in the ointment: a morphological and molecular comparison of *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae) in South Africa. *Medical and Veterinary Entomology* **23**:6–14 DOI [10.1111/j.1365-2915.2008.00765.x](https://doi.org/10.1111/j.1365-2915.2008.00765.x).
- Tuccia F, Giordani G, Vanin S. 2016.** A general review of the most common COI primers for Calliphoridae identification in forensic entomology. *Forensic Science International: Genetics Supplement Series* **24**:e9–e11.
- Vanin S, Bonizzoli M, Migliaccio ML, Tadini Buoninsegni L, Bugelli V, Pinchi V, Focardi M. 2017.** A case of insect colonization before the death. *Journal of Forensic Sciences* **62**:1665–1667 DOI [10.1111/1556-4029.13481](https://doi.org/10.1111/1556-4029.13481).
- Vilgalys R. 2003.** Taxonomic misidentification in public DNA databases. *New Phytologist* **160**:4–5 DOI [10.1046/j.1469-8137.2003.00894.x](https://doi.org/10.1046/j.1469-8137.2003.00894.x).
- Wallman JF, Donnellan SC. 2001.** The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in southeastern Australia. *Forensic Science International* **120**:60–67 DOI [10.1016/S0379-0738\(01\)00426-1](https://doi.org/10.1016/S0379-0738(01)00426-1).
- Wells JD, Wall R, Stevens JR. 2007.** Phylogenetic analysis of forensically important *Lucilia* flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. *International Journal of Legal Medicine* **121**:229–233 DOI [10.1007/s00414-006-0147-1](https://doi.org/10.1007/s00414-006-0147-1).
- Will KW, Mishler BD, Wheeler QD. 2005.** The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology* **54**:844–851 DOI [10.1080/10635150500354878](https://doi.org/10.1080/10635150500354878).
- Xiong F, Guo Y, Luo B, Zhang L, Cai J, Li X, Yang Z. 2012.** Identification of the forensically important flies (Diptera: Muscidae) based on cytochrome oxidase subunit I (COI) gene in China. *African Journal of Biotechnology* **11**:10912–10918.
- Yusseff-Vanegas SZ, Agnarsson I. 2016.** Molecular phylogeny of the forensically important genus *Cochliomyia* (Diptera: Calliphoridae). *ZooKeys* **609**:107–120 DOI [10.3897/zookeys.609.8638](https://doi.org/10.3897/zookeys.609.8638).
- Yusseff-Vanegas SZ, Agnarsson I. 2017.** DNA-barcoding of forensically important blow flies (Diptera: Calliphoridae) in the Caribbean Region. *PeerJ* **5**:e3516 DOI [10.7717/peerj.3516](https://doi.org/10.7717/peerj.3516).
- Zaidi F, Wei S, Shi M, Chen X. 2011.** Utility of multi-gene loci for forensic species diagnosis of blowflies. *Journal of Insect Science* **11**:59 DOI [10.1673/031.011.5901](https://doi.org/10.1673/031.011.5901).
- Zehner R, Amendt J, Schütt S, Sauer J, Krettek R, Povolný D. 2004.** Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). *International Journal of Legal Medicine* **118**:245–247.