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Forensic Science International 222 (2012) 335–339

Contents lists available at SciVerse ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Lucilia silvarum Meigen, 1826 (Diptera: Calliphoridae)—A new species of interest for forensic entomology in Europe

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ARTICLE INFO

Article history:
Received 16 April 2012
Received in revised form 19 June 2012
Accepted 26 July 2012
Available online 18 August 2012

Keywords:
Forensic entomology
Blowflies
Lucilia silvarum
Case report
Cytochrome c oxidase I
Identification key

ABSTRACT

In Europe, the blowfly genus *Lucilia* is represented in Forensic Entomology mainly by the species *L. ampullacea*, *L. caesar*, *L. illustris* and *L. sericata*. In the US, *Lucilia silvarum* is rarely recorded as a carrion breeding species but usually as a more or less exclusive parasite of frogs and toads. We present three forensic cases from different European countries reporting, for the first time, *L. silvarum* on human bodies that were found close to lakes, wetlands, or riversides. To use this species for post-mortem interval estimations, thermal development data is needed. The first step is accurate identification by morphological and molecular means. Therefore, we analysed a 611 bp part of the mitochondrial COI region for 23 specimens of *L. silvarum* from 9 different geographical regions, all of which give the same haplotype. Differences within the haplotype varied by up to 0.2%. Comparison between the haplotype found and those published on GenBank showed up to 1.2% variance. Moreover, we present an updated key for the morphological identification of the third larval instars of European *Lucilia* spp. of forensic importance, adding not only *L. silvarum*, but also *L. cuprina* which was recorded in Europe for the first time about 20 years ago.

1. Introduction

Lucilia is one of the most important blowfly genera in forensic entomology due to their frequent occurrence on human bodies [1]. Beside the carrion colonizing taxa, we find parasites of frogs and toads as well as species causing myiasis [1–6]. So far four species, *L. ampullacea*, *L. caesar*, *L. illustris* and *L. sericata*, are regularly found on human corpses in Europe. *Lucilia cuprina*, despite first being recorded in Spain about 20 years ago [7] and occurring on carrion in the Iberian Peninsula [8], does not play a comparable role, but definitely has the potential to act as a major agent – at least in South Europe.

Unfortunately, our knowledge of the biology and development time for the majority of these species is still incomplete [1,9,10]. This is partly due to the fact that artificial rearing in the laboratory is not easy to conduct for many species and also because an accurate morphological identification of the immature stages is difficult. Sometimes even identification via molecular markers is problematic [11–13].

Adults of *L. silvarum* are found all over Europe and are usually associated with carrion [5,9,14–16]. The adults of the holarctic *Lucilia silvarum* feed on nectar – as do the other species of the genus *Lucilia*. This occurs in areas with high humidity such as riversides, wetlands, lakes etc. Detailed information about the food of the immature stages is remarkably rare in European literature. Groth and Reissmüller [17] bred maggots from different types of small carrion and found that larvae of *L. silvarum* have a strong preference for dead frogs. There are also European records of *L. silvarum* as a parasite of living toads but these records are probably based on misidentifications of the closely related *L. bufonivora* which is a well documented exclusive parasite of living toads [18]. However, from the Nearctic, where *L. bufonivora* is missing, convincing descriptions of *L. silvarum* attacking living frogs and toads are published [19–21]. Furthermore there are individual records of infestations of animals others than amphibians, including a live rat [22] and a dead duck [23].

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http://dx.doi.org/10.1016/j.forsciint.2012.07.013

Adair and Kondratieff [24] described for the first time the infestation of a human corpse by *L. silvarum* in the Rocky Mountains of Colorado (USA). We present in this paper (also for the first time), three cases in Europe where this species was involved. *L. silvarum* is, at least in some parts of Europe, very common and its low incidence in forensic cases is remarkable.

Possible explanations could be:

- The species was present, but not detected because it was not included in the usual keys for identification of forensically important larvae.
- The larvae of *L. silvarum* are not general carrion feeders like *L. ampullacea*, *L. caesar*, *L. illustris* and *L. sericata* but are specialized in an (up till now) unknown substrate. Development in a forensic setting would therefore be an uncommon occurrence.

For estimation of the post-mortem interval it is essential to know as much as possible about the biology and development of necrophagous blowflies. Even more crucial, is the correct identification of sampled individuals. Due to its apparently low relevance in forensic entomology, even recent keys for forensically important Diptera do not include the larval stages of *L. silvarum* [8,25,26]. Nevertheless it was a reasonable assumption - confirmed now by the case reports in this paper – that *L. silvarum* does occur on human corpses. We therefore present a key for the third larval stage of the European forensically important *Lucilia* spp.

Even with this larval key, and other currently published keys on adult morphology, identification may be hampered due to badly preserved material or younger larval stages. In such cases molecular methods like DNA sequencing provide a useful option to determine even difficult specimens. In previous years it was shown that the mitochondrial *cytochrome c oxidase I* (*coI*) gene is an appropriate marker to identify forensically important fly species [27–31]. This universal gene is also taken advantage of by DNA barcoding, where so called "universal primers" are used for investigating a standard sequence in all organisms [32]. We chose these to obtain sequence data for this study on *L. silvarum*. Our project provides DNA sequences which may serve as reference data for future identification of *Lucilia* species.

2. Cases

2.1. Case 1

On July 13th 2008, a dead man was found on a shore in an industrial area on the outskirts of a city in central Sweden. One day later the body was autopsied and maggots were collected. The maggots were in early third instar and were reared to adults. 45 *L. silvarum* and 24 *L. illustris* hatched. According to Nuorteva [10], it takes *L. silvarum* 26 days to develop from egg to adult at a mean temperature of 16.6 °C. Mean temperature for this site was 16.9 °C. Assuming that the time to reach third instar is approximately 20% of the total development time (as for most blowflies) this would be a minimum of around 5 days. This seems to fit well with witness observations of when the man was last seen alive. It turned out that he had died from natural causes.

2.2. Case 2

In August 2008 a dead man was found near Amsterdam in the reed-vegetation of a large body of freshwater. The person was a balloon swallower and died from a drug overdose. His head was covered with second and third instar maggots. A selection of sampled specimens was reared to adults and consisted of three different species in roughly the same numbers: *L. silvarum*, *L. illustris* and *L. ampullacea*. This is an unusual combination as *L. illustris* is a typical open area species while *L. ampullacea* is a typical forest species. Despite the fact that *L. silvarum* is very common in the Netherlands, this instance is the first to be recorded in a forensic case. Developmental data for the species found, especially for *L. silvarum*, are very poor and not applicable to the bulk of temperatures. Therefore no reliable estimation of the post mortem interval (PMI) could be made. It was roughly estimated to be 5 days, based on the developmental data for *Lucilia sericata* which require, in larval stage, approximately 5 days at around 20 °C (the mean temperature for this site) to reach the length of the largest maggot found in the sample (12 mm) [33].

2.3. Case 3

In May 2009 the body of a 67-year-old female was found in a grain field near Gütersloh, Germany. She was last seen alive 3 days earlier attending festivities on a farm near the crime scene. The autopsy showed that the woman was strangled to death. The coroner estimated the time of death to be 8–24 h before the discovery of the body. Eggs of *Lucilia ampullacea* and *Calliphora vicina* were found on the face and neck of the victim. In the mouth, two second instar maggots of *L. silvarum* were present, these were reared to the adult stage. Obviously the oldest stages on the corpse indicate a longer PMImin than medical and other entomological findings (PMImin > 24–48 h). However, as previously mentioned, no reliable data is available for the development of *L. silvarum*, the estimations were speculative and based on the time of development for *L. sericata*, at 20 °C [33] – the average ambient temperature at the scene. Nevertheless they were in accordance with the assumptions of the investigating profiler, who found it more likely that the woman was killed on her way home after leaving the festivities. This case is still open.

3. DNA analysis

3.1. Specimens

Flies were collected from human corpses (see cases 1–3), by net or by canopy trap. Mixed bait of beef liver and dimethyl trisulphide was used. Morphological identification was conducted based on Rognes [5]. The genitalia of the males (16 of 23 in total) were adjusted with the line-drawings of Rognes [5] supplementary to the characteristics provided by the original key. Afterwards, the specimens were stored in 95% EtOH. Specimens identified for this study are listed in Table 1. Two legs were taken from each specimen and DNA-extraction was conducted according to a usual phenol–chloroform-protocol. The purified DNA was resuspended in 100 μl of sterile distilled water.

Table 1
List of origin of the specimens analysed in this study, including country, city of origin, date and collection source, as well as number of specimens and variant of haplotype.

Country	City of origin	Date	Collection source	*N*	Variant of ht1
Germany	Frankfurt a. M.	09/2008	Canopy Trap	2	1
Germany	Frankfurt a. M.	05/2009	Canopy Trap	1	1
Germany	Guetersloh	05/2009	Human corpse	2	1
Germany	Würzburg	05/2007	Flowers	6	1, 2
Netherlands	Herwen	08/2008	Net	6	1
Poland	Dobiegniewo	06/2010	Flowers	1	1
Poland	Grudziądz	09/2010	Flowers	1	1
Portugal	Coimbra	06/2004	Pig cadaver	1	2
Sweden	Karlskoga	07/2008	Human corpse	2	1
Ukraine	Askania Nova	07/2008	Flowers	1	1

3.2. PCR amplification

For molecular analysis, the so called barcoding region of the mitochondrial genome encoding Cytochrome C Oxidase subunit I was amplified using the "universal primers" LCO1490 and HCO 2198, as described by Folmer et al. [32]. The PCR system was set up in a reaction volume of 25 μl including 160 μM dNTPs (GE Healthcare), 10 pmol of each primer (Biospring, Frankfurt), 0.3 mg/ml BSA (Sigma Aldrich), 1.5 U Taq DNA Polymerase (Sigma Aldrich) and buffer provided by the manufacturer which included $MgCl_2$ and 1 μl DNA.

For PCR amplification, a T3000 thermal cycler (Biometra) was used. The parameters for amplification were applied according to Boehme et al. [34].

The success of each amplification was confirmed by gel-electrophoresis in a 3% agarose gel, stained with GelRed (Biotium, Darmstadt) and visualized under UV light.

3.3. Sequencing analysis

Both forward and reverse strands were sequenced with the amplification primers using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The 20 μl reaction mix contained 1.5 μl Big Dye, 2.5 μl 5× sequencing buffer, 10 pmol primer and 1 μl PCR product. The sequencing reaction was conducted under the following conditions: 30 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 55 °C.

Sequencing products were purified using DyeEx 2.0 Spin Kit (Qiagen) and run on an automated ABI3130 genetic analyzer using POP 4 and a capillary length of 30 cm (Applied Biosystems).

Sequences were edited and aligned using Sequence analysis software (Applied Biosystems, Version 2.1.2) and Sequence Navigator software (Applied Biosystems, Version 1.0.1).

4. Results and discussion

For a total of 23 specimens of *L. silvarum*, a 611 bp fragment of the barcoding region was successfully analysed corresponding to bases 57–667 in the published *coI*-sequence of *L. sericata* (L14947, [35]). Those sequences were assigned to the same haplotype since only one SNP in the whole fragment occurred. This SNP was independent of the geographic origin of the flies (Table 1). Since variant 1 occurred most frequently (in 21 individuals, i.e. 91.3%; Accn No. JQ801750), it was chosen to serve as the reference sequence in this study. Variant 2 occurred only twice (8.7%; Accn No. JQ801751).

Additionally, four other previously published nucleotide sequences were used for comparison. However, due to differential amplification of sequence contigs, a complete comparison of all nucleotide positions was not possible. Nevertheless, an alignment of the overlapping areas was performed and 2 additional haplotypes were detected (haplotypes 2 and 3, Table 2). One nucleotide sequence (GenBank Accession No. FR719176) showed significant differences to the other nucleotide sequences, primarily at the 3′ end of the COI gene. In addition, sequence alignments (data not shown) revealed undetermined nucleotides in FR719176. This prevents a reliable comparison with the reference sequence (Accn No. JQ801750) at this time. Accordingly, only the remaining GenBank sequences were used for interpreting intraspecific variation. JN257230 showed no polymorphisms in comparison to variant 1 of haplotype 1.

Altogether we found 9 variable positions (1.5%) (Table 2). With reference to variant 1 of haplotype 1, variant 2 showed only one SNP (0.2%) with the least polymorphism. Nevertheless, intraspecific variation of up to 1.2% was detected (in haplotype 3).

Table 2
Comparison of haplotypes, listing the occurring SNPs as well as fragment length, length of coverage and relative variance in relation to haplotype 1, variant 1. Position in *coI* refers to the position in the complete *coI*-gene as published by Sperling et al. [35] for *Lucilia sericata*. Ht, haplotype; v, variant; n.a., not available.

	ht1, v1	ht1, v1	ht1, v2	ht2	ht3
	GenBank accession number				
	JQ801750	JN257230	JQ801751	FR719175	FJ650564
Position in coI					
221	G			A	n.a.
227	A			G	n.a.
239	T			C	n.a.
272	T			C	n.a.
353	G			A	A
452	A				G
470	A			G	G
590	T		A		
602	G			A	A
Fragment length (bp)	611	511	611	1233	1198
Length of coverage (bp)		511	611	611	345
% variance in relation to ht1, v1			0.2	1.1	1.2

Despite this observation, there is a widespread assumption that intraspecific variation is usually lower than 1% [36] and congeneric species reveal a variation generally greater than 2% [37]. This rule, the so called "barcoding gap", initially leads to the question of whether the degree of difference observed with GenBank sequence, FR719176 (origin Durham, UK), could be the result of a poor sequence quality. This seems to be the most likely explanation, since the correct identification of the original specimen is confirmed by other nucleotide sequence markers, e.g. 28S rRNA [27, Stevens, personal communication]. Additionally there are other, more general concerns regarding the utility of the seemingly arbitrary 2% rule and there is now a broad opposition in the literature, which calls the so-called 'barcoding gap' into question, since intraspecific variation may overlap interspecific divergence [38,39]. Wiemers and Fiedler [40] state, e.g. in the blue butterflies (Lepidoptera: Lycaenidae), that the "barcoding gap" is just an artefact of insufficient sampling across taxa and that a high percentage of well-differentiated species have similar or even identical *coI* sequences, that would be overlooked in an isolated DNA barcoding approach. Whitworth et al. [39] present a similar argument in their recent study of genetic variation in bird blowflies, where they demonstrated that individuals from four different *Protocalliphora* species had identical *coI* barcodes.

These controversies demonstrate the need for a multidisciplinary approach like this one (including morphology and DNA analysis), when identifying rare (from a forensic point of view) taxa like *L. silvarum*. Further studies should also include close relatives of the target species, e.g. in this case the sister species, the toad parasite *L. bufonivora*. So far there are no reports of association of this species with a human corpse, but this may change in the future, as in the case of *L. silvarum*. However, Brumpt [41] demonstrated experimentally that *L. bufonivora* only develop in living toads and not in toad cadavers. Therefore human corpses seem highly unlikely to act as substrate for development.

5. Key

5.1. Revised key for identification of the third instars of European species of Lucilia spp. of forensic importance

This key for the larvae of *Lucilia* is an upgrade (by addition *L. cuprina* and *L. silvarum*) to a recently published key for identifica-

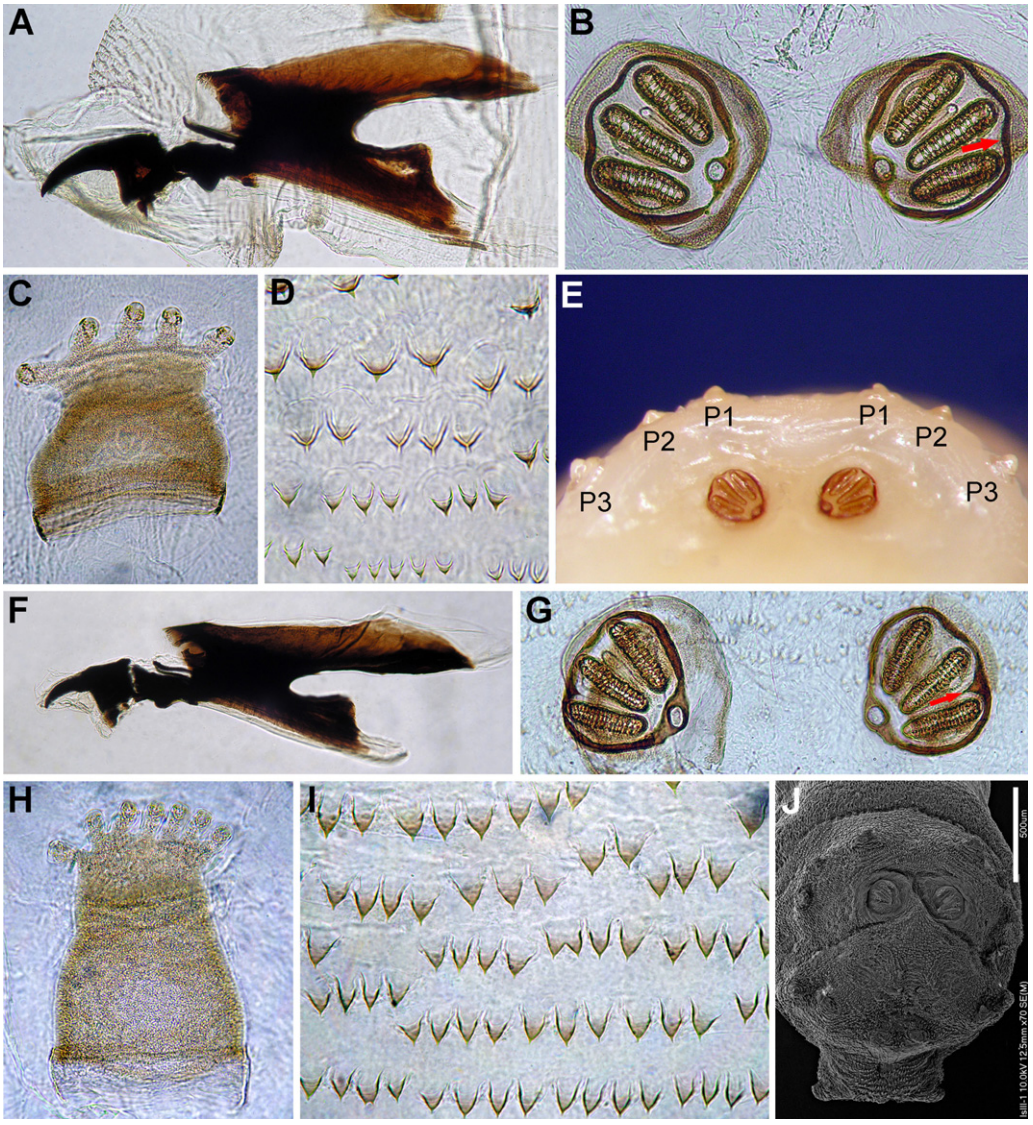

Fig. 1. Third instars of *Lucilia cuprina* (A–D) and *Lucilia silvarum* (E–J): A – cephaloskeleton, lateral view; B – posterior spiracles; C – anterior spiracle; D – thoracic segment III, spines; E – anal division, upper half of spiracular field; F – cephaloskeleton, lateral view; G – posterior spiracles; H – anterior spiracle; I – thoracic segment III, spines; J – anal division, posterior view.

tion of the third instars of forensically important European blowflies [26]. Users can begin identification of larval material with the aforementioned key but from point 10 text should be replaced by the text below. Illustrations of larval characters other than *L. cuprina* and *L. silvarum* are available in Szpila [26].

10. – distance between each P1 similar to distance between P1 and P2 . . . *Lucilia sericata*

    – distance between each P1 larger than distance between P1 and P2 (Fig. 1E) . . . 11

11. – distance between P1 and P2 smaller than distance between P2 and P3 (Fig. 1E), peritreme between outer and median spiracular slits not projecting inward (Fig. 1B) . . . *Lucilia cuprina*

    – distance between P1 and P2 larger than distance between P2 and P3, peritreme between outer and median spiracular slits projecting inward (Fig. 1G) . . . 12

12. – cephaloskeleton without sclerotized area below the posterior tip of ventral cornua (Fig. 1F), posterior spiracles rather widely apart (SDF > 1) (Fig. 1G), almost whole surface of anal division covered with small spines (Fig. 1J) . . . *Lucilia silvarum*

    – cephaloskeleton with sclerotized area below the posterior tip of ventral cornua, posterior spiracles more closely set (SDF < 0.9), at least parts of lateral surfaces of anal division without spines . . . 13

13. – posterior spinose band on abdominal segment VI interrupted dorsally, postero-dorsal angle of the basal part of the mouthhook with process directed postero-dorsally . . . *Lucilia caesar*

    – posterior spinose band on abdominal segment VI complete, postero-dorsal angle of the basal part of the mouthhook with process directed posteriorly . . . *Lucilia illustris*

Acknowledgments

We thank Catarina Prado e Castro, Dep. Biologia Animal Faculdade de Ciências da Universidade de Lisboa, Portugal, for providing one *Lucilia silvarum* specimen for DNA-Analysis. Chris Freeman and Melissa Anderton, Portsmouth, UK, kindly checked and improved the language of the manuscript. Jamie Stevens, University of Exceter, UK, for a helpful discussion regarding the "Barcoding gap". Anders Lindström is funded through AniBioThreat with financial support from the Prevention of and Fight Against Crime

Programme of the European Union. The present work was financially supported by the Polish Ministry of Science and Higher Education (grant no. N N303 470838 to Krzysztof Szpila).

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