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Description of the puparium and other notes on the morphological and molecular identification of *Phthitia empirica* (Diptera, Sphaeroceridae) collected from animal carcasses

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Abstract

Background: A correct identification of species is fundamental in order to derive all the biological, ecological, and behavioral information useful to reconstruct previous events when insects are used in forensic and funerary archaeological contexts. Little attention was paid in the past to the description of the immature stages of flies, especially of species belonging to family found only occasionally on human cadavers and animal carrion. Sphaeroceridae species belong to this category.

Results: In this work, adult and puparia of *Phthitia empirica* (Hutton, 1901) are illustrated and described. In addition, some observations about the molecular identification of this species are presented and discussed.

Conclusions: This work will allow the identification of puparia of this species providing additional tools in forensic entomological investigation. More in general, the effort of producing morphological descriptions and molecular information of species of potential forensic interest clearly implements the possibility to provide a deeper understanding of the peri- and post-mortem events in violent crimes.

Keywords: Forensic entomology, Funerary archaeoentomology, Phylogeny, Insect colonization, Insect identification

Background

During a study on the necro-microbiome characterizing the decomposition of animals (Tuccia et al. 2019), some specimens of *Phthitia empirica* (Hutton, 1901) (Diptera, Sphaeroceridae) were collected from rabbit carcasses over 2 years after the carcasses' exposure.

Despite decomposition is a continuous process without discrete stages, different communities of arthropods colonize the decomposing matter following a predictable order, with some exceptions depending on the particular

environmental conditions and on the thanatological conditions of the remains.

Flies in the families Calliphoridae, Sarcophagidae, and Muscidae are usually the first colonizers of cadavers and carcasses (Méglin 1894; Smith 1986; Carter et al. 2007), followed by species in the families Piophilidae and Fanniidae, and occasionally Sphaeroceridae and Heleomyzidae. Even though rarely, other species in the families of Trichoceridae, Psychodidae, Milichiidae, Ulidiidae, and Drosophilidae have been found related to dead bodies (Giordani et al. 2018a). As already highlighted (Amendt et al. 2007, Giordani et al. 2018b, Giordani et al. 2018b, 2018c), the correct identification of the specimens collected from human and animal bodies in forensic and archaeological contexts represents one of the most

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important problems to be solved, especially when immature stages are found. This problem is still relevant and sometimes impossible to solve when empty puparia are found and no molecular analysis can be performed on them because of the low amount of DNA, or the DNA degradation and the presence of PCR inhibitors (Scholz et al. 1998; Kemp et al. 2006). The difficulties in puparia morphological identification is due to the paucity of specific identification keys for this developmental stage and to the fact that often some diagnostic characters of larvae are not visible or are deformed in the puparium (Giordani et al. 2018a). Furthermore, the problem of puparia identification is also worst in taxa only occasionally reported from cadavers and carcasses (Giordani et al. 2018b) where often the larvae are not described.

One of the most important contribute on Sphaeroceridae puparia knowledge comes from Skidmore studies on dipterous material from archaeological sites in Greenland, Iceland, and various parts of the British Isles (Skidmore 1992). Previous authors, such as Pitkin (1988) and Okely (1974), gave references for the puparia of Sphaeroceridae but some important features had been overlooked and their descriptions traditionally included trivial details emanating from the process of pupariation (Skidmore 1992).

In order to contribute in filling this gap, this paper reports some illustrations and the description of adults and puparia of *P empirica*. In addition, the molecular analysis based on the sequencing of the gene COI is also presented and discussed. In contrast, any conclusion about the correlation between the appearance of the species *P. empirica* and the decomposition timing was avoided because the sampling was not standardized and designed for this purpose.

Methods

Six rabbit carcasses (*Oryctolagus cuniculus* Linnaeus, 1758) (2.75–3.50 kg) were purchased from a pet food company (Kiezebrink, <https://www.kiezebrink.co.uk/>) and placed on the roof of the Science Building at the University of Huddersfield (West Yorkshire, UK, 53°38'36.5'' N 1°46'40.1'' W) between June 2014 and March 2015. No ethical approval was required because the experiment was carried on using rabbit carcasses sold for pet consumption (Tuccia et al. 2019). In September 2017, close puparia of different Diptera species were collected from the completely skeletonized carcasses and stored at room temperature in jars covered with laboratory paper until adult emergence. Adult flies and puparia were observed and photographed using a Leica M60 stereomicroscope (Leica, Germany) and a Keyence VHX-S90BE digital microscope, equipped with Keyence VH-Z250R and VH-Z20R lens and VHX-2000 Ver. 2.2.3.2 software (Keyence, Japan). The morphological

identification of the adults was courteously done by Lorenzo Munari (Laboratory of Entomology, Museum of Natural History of Venice, Italy).

Scanning electron microscopy observations were performed on the puparia in addition to the previous mentioned stereo and digital microscopies because of their reduced size using a FEI Quanta 650 FEG SEM (Thermo Scientific, USA). Air-dried cleaned samples were mounted on metal stubs with conductive adhesive tape and coated with 40–60nm of Au-Pd in a SC7620 Mini Sputter Coater (Quorum Technologies, UK). Pictures were directly digitized from the SEM.

Molecular analyses were performed from adults, closed and empty puparia following two different extraction methods, QIAamp® DNA Mini kit and QIAamp® DNA Investigator kit. Manufacture instructions were followed except for the initial phase in which, to prevent the specimen destruction, each specimen was submerged in the extraction buffer and incubated overnight at 56 °C in a laboratory orbital shaker. The extracted DNA was quantified with the commercial kit “Quant-iT™ Qubit™ dsDNA High-Sensitivity Assay Kit” (Invitrogen, USA) on a Qubit® 3.0 Fluorometer (Invitrogen, USA). The quality of the extracted DNA has been assessed through Agilent Bioanalyzer® 2100 (Agilent, Santa Clara, CA, USA).

The amplification was carried out in the mitochondrial COI barcoding region using the universal primer designed by Folmer et al. (1994) with a temperature of annealing of 49.8 °C as reported in Bortolini et al. (2018).

The new sequences produced during this study are deposited in GenBank (Table 1). Sequences of COI gene from Sphaeroceridae species were downloaded from BOLD online database and included in the phylogenetic analysis (Table 2). Sequences were aligned with Clustal Omega (Sievers et al. 2011). The phylogenetic reconstruction through Neighbour Joining was performed with Kimura 2-parameter model on MEGA 7.0 (Kumar et al. 2016). Fifty sequences of 598 bp were used for the phylogenetic analysis. Two sequences of *Drosophila melanogaster* Meigen, 1830 were used as outgroup (Table 2).

Results

Phthitia empirica (Hutton, 1901) - Sphaeroceridae

Syn: Borborus empirica Hutton, 1901

Limosina cadaverina Duda, 1918

Table 1 GenBank codes of COI sequences of Sphaeroceridae produced in this work

Family	Species	GenBank code
Sphaeroceridae	<i>Phthitia empirica</i> (Hutton, 1901)	MH118267 MH118268 MH825673

Table 2 COI sequences of Sphaeroceridae downloaded from BOLD and included in the analysis

Family	Species	BOLD code	
Sphaeroceridae	<i>Coproica urbana</i> (Richards, 1960)	JSJUN812-11	JSJUL717-11
		JSJUN972-11	JSDIS133-11
		JSJUN573-11	JSAUG1229-11
		JSJUN430-11	CNEID2176-12
		JSJUN1866-11	CNEID1768-12
		JSJUN1804-11	JSJUL1206-11
	<i>Leptocera caenosa</i> (Rondani, 1880)	BCFOR772-15	
		<i>Leptocera finalis</i> (Collin, 1956)	JWDCG152-10
		JWDCE179-10	JWDCJ330-11
		JWDCH240-10	JWDCJ334-11
		JWDCE279-10	
	<i>Phthitia ovicercus</i> Marshall, 1992	SSBAF3038-13	SSEIB7483-13
		SSEIB11339-13	SSWLB3188-13
		SSEIB6777-13	SSWLB3228-13
	<i>Copromyza equina</i> Fallén, 1820	JSDIP471-10	JSDIP737-10
<i>Copromyza neglecta</i> (Malloch, 1913)	JSDIQ045-10	JSDIQ496-10	
<i>Phthitia plumosula</i> (Rondani, 1880)	BBDEC892-10	CNFDI480-14	
	OPPOA1193-17	GMGMG1040-14	
<i>Phthitia quadricercus</i> Marshall, 1992	SSJAA1739-13	SSBAC3460-12	
	JWDCH112-10	SSBAC3476-12	
	SSBAC3110-12	SSBAC3483-12	
	SSBAC3445-12	SSBAC3485-12	
	SSBAC3446-12	SSJAA1922-13	
	SSBAC3454-12	SSPAB8647-13	
Drosophilidae (outgroup)	<i>Drosophila melanogaster</i> Meigen, 1830	CYTC5404-12	GBDP2877-06

Limosina pectinifera Villeneuve, 1918

Phthitia empirica belongs to the widespread Sphaeroceridae genus *Phthitia* Enderlein, 1938 in the subfamily Limosiniinae. The species is included in the subgenus *Alimosina* Rohacek, 1983 Fig. 1.

Limosiniinae flies are small, generally 1 to 4 mm long (with the exception of the species in the genus *Anatalanta* Eaton, 1875 that exceed 10 mm in length) (Marshall 2012). In the analyzed samples of female *P. empirica*, the body length was 2.1 ± 1.5 mm ($N = 5$) while the length of the wings was 2.3 ± 0.5 mm. No males were sampled, so no measures are available for them

Description of the adult

Phthitia empirica adult is a dull brown fly characterized by the presence of a long distal dorsal bristle in the hind

tibia, and eye height is less than double the genal height (Marshall et al. 2009). The head shows a grey ocellar triangle with two ocellar bristles and 3–5 short central setae, the frons has a dark brown M-shaped pattern surrounding a lighter brown triangle. Two small postvertical setae, two inner vertical bristles, two outer vertical bristles, three pairs of interfrontal bristles plus a minute pair just above ptilinal suture and two strong orbital setae with additional setulae below and inside orbitals are present. Lunula is bare. Antennae are dark brown, arisal hairs are dense and as long as arisal width at base, slightly longer and sparser in distal half. Strong vibrissae and other large setae are present in the light brown gena. The face is concave.

Thorax has a posterior pair of dorso-central bristles. Acrostichal bristles are short with a prescutellar pair enlarged. One humeral bristle, one pre-sutural bristle, two notopleural bristles, and two post-alar bristles are

**Fig. 1** *Phthitia empirica* adults. Dorsal (a), ventral (b), and lateral (c) view (scale bar 500 μ m)

present. Katepisternum has one large posterodorsal bristle and five to six small anterior setulae. Scutellum has four long marginal setae. No apical scutellar bristles are present. The hind tibia shows at least one long distal dorsal bristle and the mid femur has a row of anteroventral spine-like short setae. The mid tibia presents proximally one short anterodorsal and one short posterodorsal seta, and distally one small dorsal seta in front of one long anterodorsal and one posterodorsal setae, and a double row of posteroventral spine-like setae. Wings and wing veins are brownish. Crossveins r-m and dm-cu are separated by less than twice the length of dm-cu. Anal veins are sinuate.

Description of the puparium

Puparia, in the analyzed sample, are yellow to light brown (Fig. 2a–c), 3.39 ± 0.32 -mm long ($N = 11$). Several excrescences surround the posterior region; they are denser and more packed around the anal plate. The late is constituted only by the anal cleft and its crumpled rim with no expansions (wings). All the anal papillae are absent or not discernable (Fig. 3a). The ventral welt of abdominal segment seven is made by two intersegmental inner rows of large and flame-shaped spines enclosed by external lines of smaller and closer spines (Fig. 3b).

Posterior spiracles are allocated on two inclined protuberances. The angle formed by their projection is greater than 90° . The slits follow the external shape of the spiracle in a “U-like” pattern. Filaments emanating from perispiracular glands are well visible (Fig. 3c, d). Anterior spiracles has 6–7 prospiracular lobes (Fig. 3e, f).

Biology and distribution

This cosmopolitan species, probably originated from Australia, was defined by Richards (1930) as a “domestic fly” because of its synantropic attitude. Reported from different locations, *P. empirica* is a common species in UK (Marshall et al. 2011). The species was reported feeding on animal excrements (Rohacek 1983), animal carriages, and human dead bodies (Marshall et al. 2009; Anton et al. 2011; Carles-Tolrá et al. 2012).

Molecular analysis

DNA extraction yielded positive results from all the entomological samples: adult, close, and empty puparia (Table 3). PCR amplification was positive for all adult

and close puparium samples while negative results were obtained from empty puparia (Table 3).

In order to interpret the negative result of PCR amplification from some puparia, the dimensions of the extracted DNA fragments were assessed with Agilent Bioanalyzer. The result pattern excludes a potential degradation as reason of the negative amplification (data not shown).

To confirm the morphological identification, the phylogenetic reconstruction based on the amplification of the COI barcoding region was used.

The lack of sequences of *P. empirica* in GenBank prevented the directly identification of the species through the local alignment of the sequences using the online system BLASTn°. The output of the comparison resulted in a 92% identity with *Phthitia ovicecus* Marshall, 1992.

The NJ analysis of 52 sequences of 598 bp belonging to different genera (*Coproica* Rondani, 1861, *Copromyza* Duda, 1923, *Leptocera* Olivier, 1813, and *Phthitia* Enderlein, 1938) in the Sphaeroceridae family (Fig. 4) generated a phylogenetic tree with well-defined species clusters. The sequences obtained from the puparia collected from the rabbit carcasses (MH118267, MH118268, and MH825673) group together and appear to be closely related with *P. ovicecus*. In the reconstructed tree, the genera *Leptocera* and *Copromyza* appear to be monophyletic while *Phthitia* genus is found as polyphyletic. However, because of the weak bootstrap values at the basal nodes, no conclusions can be made about the relationship among and within the genera.

Discussion and Conclusions

In forensic cases, it is currently accepted that insects can provide the investigators important information about the time of death and the environment where the crime occurred (Amendt et al. 2007; Gennard 2007; Amendt et al. 2011; Hart et al. 2011; Tomberlin and Benbow 2015). The critical step to obtain this kind of information is the species identification. The literature about the morphology of adult insects of forensic interest is extensive (Barros de Carvalho and Antunes de Mello-Patiu 2008; Rochefort et al. 2015; Szpila et al. 2015) as though a great effort has been made by several authors to provide identification keys for larvae species of sanitary, medical, veterinary, and agricultural importance (Szpila



Fig. 2 *Phthitia empirica* puparium. Dorsal (a), ventral (b), and lateral (c) view (scale bar 500 μ m)

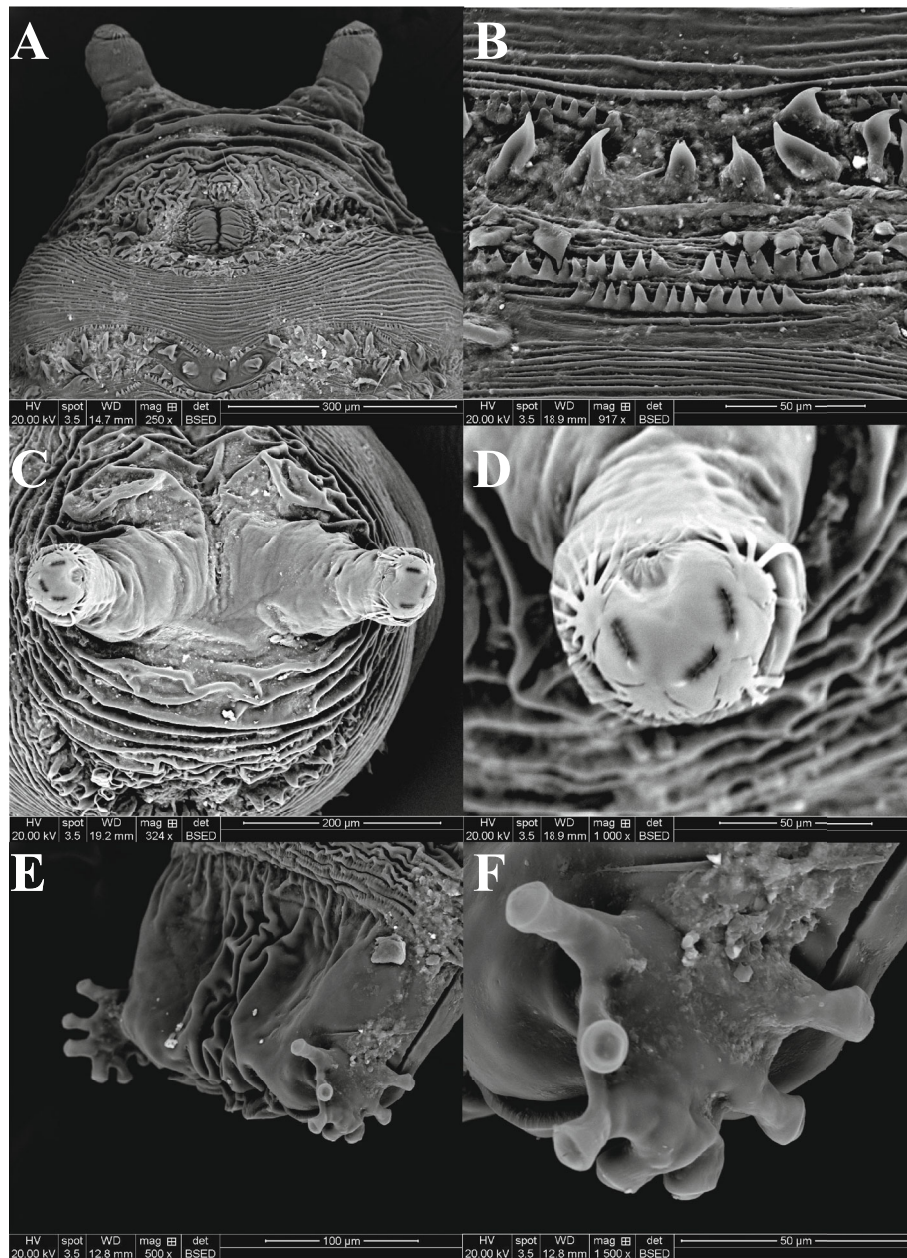
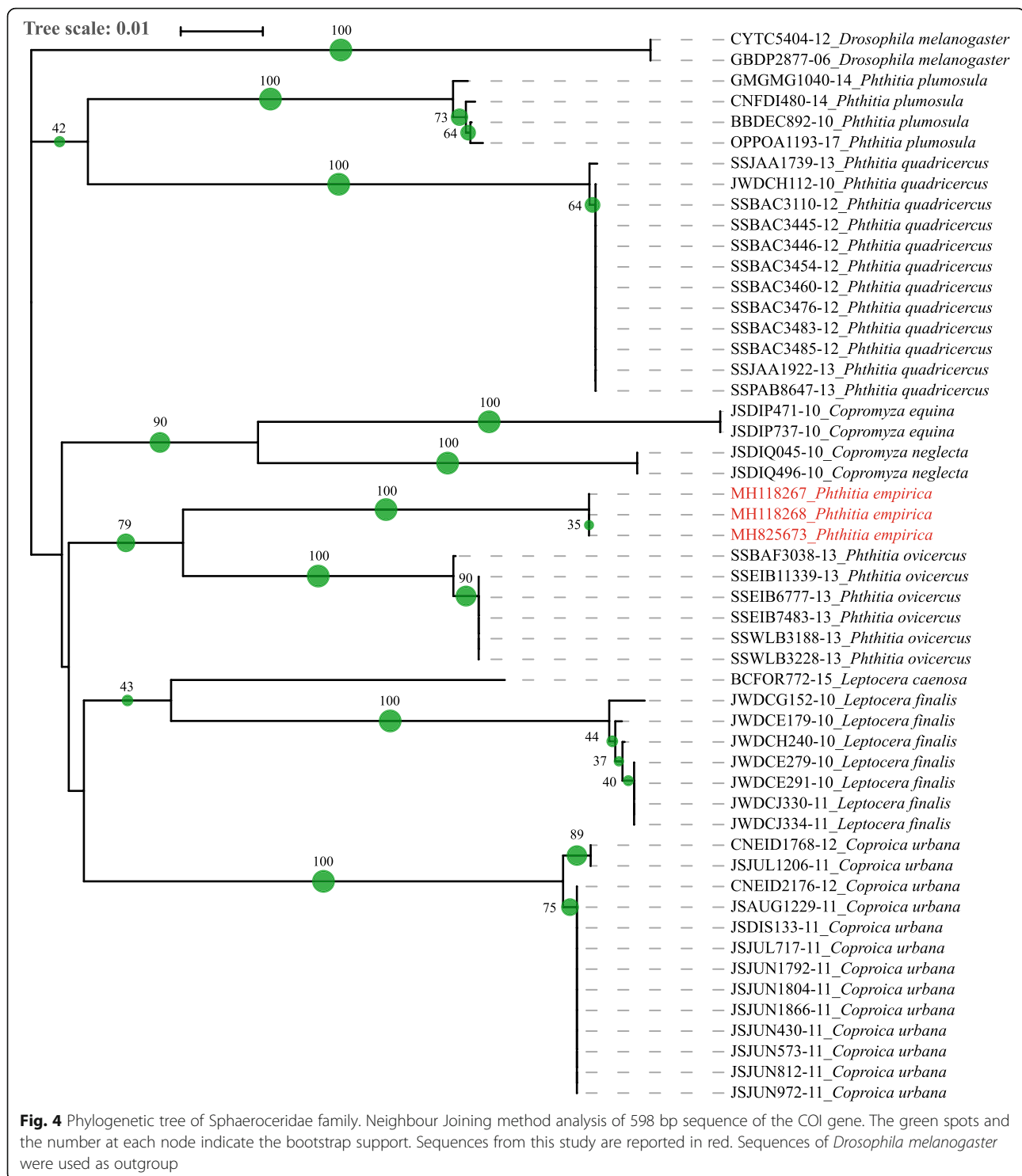


Fig. 3 *Phthitia empirica* puparium details. Anal plate (a), intersegmental spicules (b), posterior region with spiracles (c), detail of the posterior spiracle (d), anterior region with anterior spiracles (e), and detail of the anterior spiracle (f) (scale bar is reported in each frame)

Table 3 DNA extraction methods and quantification. Number and type of samples are here related with the extraction method used. Average amount of extracted DNA (ng/μl and per specimen) is shown

Sample	Method	Elution (μl)	Average DNA quantification (ng/μl)	PCR success
1 adult	QIAamp® DNA Investigator Kit	100	2.19 ± 0.02	✓
2 adults	QIAamp® DNA Mini kit	200	0.93 ± 0.02	✓
3 close puparia	QIAamp® DNA Mini kit	200	4.99 ± 0.02	✓
5 empty puparia	QIAamp® DNA Investigator Kit	100	1.16 ± 0.01	X

Standard deviation is reported. ✓positive PCR, X negative PCR result



2010; Szpila et al. 2015; Grzywacz et al. 2017). Puparia, due to the hardness of their external sclerotized cuticle, are often the most abundant and Diptera remains associated with cadavers found in forensic—especially in old

cases—and archaeological contexts (Gaudio et al. 2015; Giordani et al. 2018a; Pradelli et al. 2019), but they are still an under studied topic. Skidmore (1992) suggested the length of the puparium, the projections of the

posterior spiracles, and the prospiracular type as the diagnostic characters of sphaerocerid puparia. Although there is a intraspecific variability in size in most sphaerocerids, there are nevertheless considerable interspecific differences within the family (Skidmore 1992). Despite this, the taxonomic importance of the posterior spiracles has been largely overlooked over time, the dimension and the length of the exerted tubercles as though as the position of the slits vary among the sphaerocerids. The characters always recognized of great taxonomic importance are the anterior spiracles, which range from short stellate or palmate structures to very long dendriform types (Skidmore 1992). On the contrary, the spiculation of the ventral abdominal belts appears to be unvarying throughout the family (Skidmore 1992).

The evaluation of the puparium general shape, size and presence of projections, although allowing the identification at family level, is not sufficient to reach the species level (e.g., Sessa et al. 2019). Furthermore, in case of empty puparia or dead pupae, the impossibility to rear the specimens to adults make the study of external characters the only way for identification. Some authors do not feel comfortable in the use of morphological character identification method considering the necessity of a specialized taxonomic knowledge (Amendt et al. 2011; Grzywacz et al. 2017). Molecular approaches have been developed to meet this scientist necessity. Gene database, because of the lack of information or the incorrectness of the uploaded information, do not allow yet an identification of all the taxa recognised of forensic interest (Bortolini et al. 2018; Garg et al. 2019). Therefore, the support of the morphological identification approach remains uncontested.

This study revealed that DNA extraction without impairing the morphology of the sample is possible for all the developmental stages investigated. The DNA amplification via PCR was successful for both adult and close puparia while was not efficient for empty puparia.

The reason of this negative result cannot be related with a too low amount of DNA or with a DNA degradation. Also, the presence of PCR inhibitors cannot be taken fully in account being the PCR of the closed puparia positive, despite a potential higher concentration of inhibitor co-extracted from the puparia cuticle. In this work, five puparia were used and the extracted DNA diluted in 100 µl. Further investigation needs to be performed to solve this technical issue.

Molecular analysis here performed clearly included the *P. empirica* sequences in a cluster with another species of the same genus with which it shows an identity, based on the BLAST analysis, of 92%. It is worth to mention that the aim of the phylogenetic approach was only to verify with which species/genus the analyzed samples were more related. No further comparison or discussion

about the position of the species/genus among and within the Sphaeroceridae family based on molecular data can be done because of the paucity of the literature on this topic and the small dataset analyzed (Kits et al. 2013; Marshall et al. 2015).

In conclusion, because of the lack of available sequences, currently, some species are still not identifiable using only the molecular analysis. The synergy between the molecular and the morphological analysis is nowadays the only way to provide a reliable identification result for many species considered of potential forensic/archaeological interest. The sequences here produced are now deposited in NCBI GenBank and available online, for further works. Moreover, the illustration and description of the puparium of the species will help scientists in dealing with identification of samples from carcasses and cadavers despite the absence of adult, till now considered as the easiest or the only instar to identify.

Abbreviations

BLAST: Basic Local Alignment Search Tool; BOLD: Barcode of Life Data System; COI: Cytochrome c oxidase I; NCBI: National Center for Biotechnology Information; PCR: Polymerase chain reaction; SEM: Scanning electron microscope; UK: United Kingdom

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Adherence to national and international regulations

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Authors' contributions

SV contributed to the idea of the research, the study design, and the writing of the manuscript. GG performed the samples analysis and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting our findings can be found with the corresponding author. Please contact the author for data requests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

This study does not include publishing of personal data.

Competing interests

The authors declare that they have no competing interests.

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