



Research Paper

First report of Heleomyzidae (Diptera) recovered from the inner cavity of an intact human femur

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ARTICLE INFO

Keywords:

Forensic entomology
Heleomyzidae
Forensic genetics
Forensic investigation

ABSTRACT

One of the main characteristics of the Mafia of Gargano is their use of ritual murders: they shoot their victims in the face and then conceal the corpses in the numerous natural ravines present in the Gargano area. Skeletal remains are often recovered in a poor state of preservation under particular conditions related to the environmental situation. Humidity, temperature and environmental contaminants could be considered very important for forensic examinations and are strictly related to the bone preservation status. One of the most important analyses is the identification of the victim: the success rate is linked to the condition of the bones.

During military investigations in the Gargano area, several bones were recovered and analyzed in a karst ravine about 30 m deep. The forensic examination highlighted the presence of fly puparia from an intact human femur. The colonization of the inner bone cavity by a species of the Heleomyzidae family is described for the first time. Puparia, despite not being identified at the species level, are described and illustrated and their potential role in the degradation of the victim's DNA is discussed. This work increases our knowledge about the effects of Diptera in the taphonomic process underlying the need of a multidisciplinary approach to skeletal investigations.

1. Introduction

The analysis and the interpretation of insects as forensic evidence is currently a common practice carried out in different countries¹ in order to reconstruct the *peri* and *post* crime events and, in so doing, contribute to the crime investigation process. It is worth mentioning that despite the significant increase in our knowledge about the immature stages of cadaver/carrion-breeding insects – including that on secondary taxa not belonging to the first colonizers of a body (eg. Muscidae, Sarcophagidae and Milichidae) - there is a lack of descriptions and illustrations of these stages compared to what is known of the adult species.^{2–6}

The “fifth” Mafia, called Mafia of the Gargano (an area located in

the North of Apulia, also known as “The Spur of Italy”), is involved in many illegal activities, such as drug and arms trafficking, extortion, contract killings, political bribery, prostitution, and car thefts. Therefore, the main characteristics of the Mafia of the Gargano are linked to the particular fashion of their ritual murders: indeed, the Mafia of the Gargano are not only satisfied with killing but they also typically shoot the *coup de grace* in their victims’ faces, in order to thoroughly remove as many somatic traits that might be of potential importance for identification purposes; moreover, this type of ritual murder can make people forget the victims as their corpses are concealed inside the numerous ravines present in the Gargano area.^{7–10} To date, about forty missing individuals in this area could be related to

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Fig. 1. Left femur with the two sections that allowed the discovery of the colonization of the marrow by Diptera of the family Heleomyzidae.

killings by the Mafia of the Gargano. Anthropological and molecular analyses are also difficult when remains are found, due to the poor state of preservation (due to humidity levels and temperatures) in which skeletal remains are often found. Given the environmental conditions, the amount and the quality of the retrieved DNA is also negatively affected by the presence of animal remains (such as wild boar, sheep, goat, etc.), molds and topsoil (the bottom is usually rocky Karst covered with deposits of superficial soil, transported by weathering).

During military investigations in the Gargano area, several bones were recovered and analyzed in a karst ravine about 30 m deep. To obtain the genotyping of the bones, sampling of the femur, rib, and tooth are usually performed.^{11,12} In this particular case, the femur was initially investigated to extract the victim's DNA for identification purposes because of the peculiarity of this murder.

This paper aims to deal with the unusual finding of puparia belonging to the family Heleomyzidae inside a femur of a Mafia victim.

2. Material and methods

2.1. Anthropological analysis

After an initial gross morphological assessment of the whole set of available skeletal remains (preserved, except for the maxilla, right clavicle, left scapula, right radius and ulna, most of the vertebrae, the pubic symphysis and bones of the hands and feet), biological sex was primarily determined from skull and pelvis using standard anthropological methods^{13,14}, whereas age at death was estimated using the Lamendin and the Lovejoy methods.^{15,16} Additionally, stature was estimated adopting the Trotter and Gleser method for limb long bones.¹⁷ Furthermore, a comprehensive radiological examination was carried out.

2.2. Genetic study

For DNA extraction, two sections were made with a sterile saw near the head (*caput femoris*) of victim's left femur. The obtained samples were pre-treated following the protocol of Imaizumi et al.¹⁸ Subsequently, DNA extraction was performed following the QIAmp® DNA Investigator Kit protocol (QIAGEN Vic, AUS), with minor modifications (overnight incubation at 56 °C in an orbital shaker). All samples were amplified both with IdentifierPlus Amplification Kit (Applied Biosystems) and with PowerPlex® ESI 17 Pro System (Promega).

2.3. Entomological analysis

Insects were collected from the inner cavity of the bone using sterile tweezers. External microscopic examination of the puparia was

performed using 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, Osaka, Japan). In addition, some slides for compound microscope observations (Zeiss Universal microscope with an Olympus 16 Mb camera) were prepared using the following protocol: initial immersion in a cold solution of KOH + Tween 80 + SDS, followed by the removal of tissues with repeated washings using distilled water and Ethanol. Finally, the puparia were immersed in Essig's Aphid Fluid (Lactic acid, liquefied phenol, glacial acetic acid, distilled H₂O) and mounted in PVA (Mowiol N 4-98, Mowiol N 56-98, distilled H₂O, EtOH, Lactic acid).^{19,20} Identification was made using a specific key and illustration²¹ and the comparison with previously identified specimens from one of the authors' (*viz.* SV) own collection.

3. Results and discussion

The analyzed skeleton belonged to a male, aged between 25 and 29 years old at death and with a stature in the range of 168.8–180.4 cm. The left femur, macroscopically and radiologically investigated, did not show any pathological alterations, both at the macroscopic and the radiological observations. On the contrary, taphonomic processes such as the presence of several puparia in the inner cavity (Fig. 1) - previously radiologically undetected- was observed after the bone was sectioned for DNA analysis.

The puparia (9 specimens were analyzed), extracted from the inner cavity of the femur, were light brown, 5–12 mm long and with the anterior spiracles rosette-shaped with 10–12 respiratory bulbs (Fig. 2A–D, Fig. 3A). Posterior spiracles were located in two divergent projections about as tall as basally wide and the plates separated by more than twice the width of the spiracular plate (Fig. 2B and C). The slits were made straight and in a T shape, as long as or slightly longer than the ecdysal scar (Fig. 2C and D). The anal plate was not well defined and surrounded by dense, strong spines not arranged in rows (Fig. 2 B,D).

Part of a cephaloskeleton (Fig. 3A–D) was retrieved showing the club-shaped intermediate sclerite with ventral bridge towards the anterior end and the epipharyngeal plate was slightly sclerotized.

The morphological characteristics of the puparia were important for their identification as belonging to a species of the family Heleomyzidae. The paucity of descriptions of puparia from this family simply makes it impossible to further identify the specimens.^{22,23}

Species of the family Heleomyzidae have been recorded from human and animal remains especially during the cold season (eg. genera *Heleomyza* Fallen, 1810, *Neoleria*, Malloch, 1919 *Suillia* Robineau-Desvoidy, 1830, *Morpholeria* Garrett, 1921 and *Tephrochlamys* Loew, 1862)^{24–27} but in general several species of this family are known to occur in poultry houses, henhouses, bird nests,

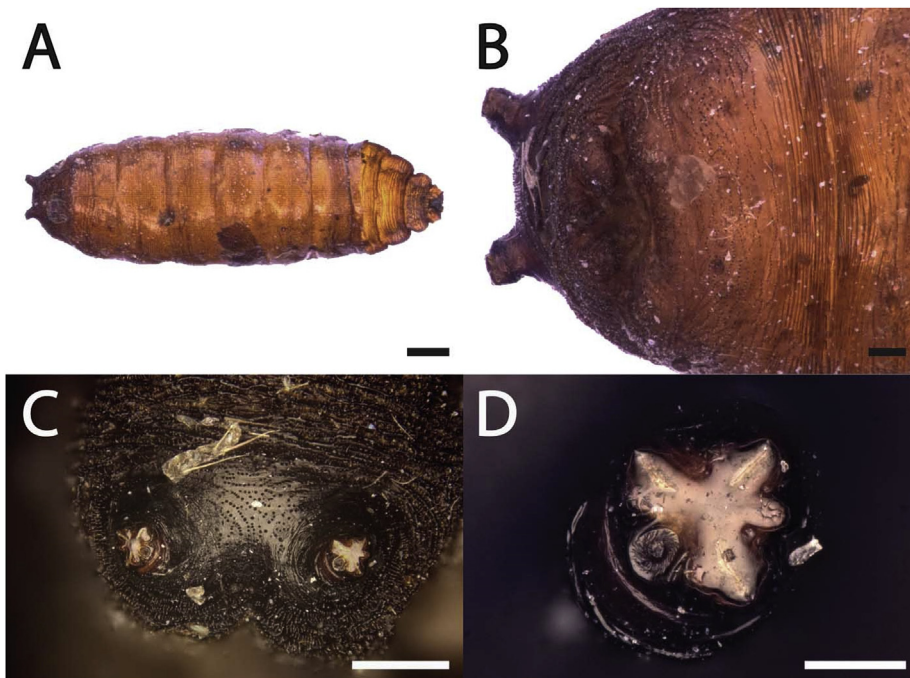


Fig. 2. Pupa of Heleomyzidae found in the inner cavity of the femur (A) open puparium in ventral view; (B) detail of the posterior region: posterior spiracles are located in two divergent projections, the anal plate is not well defined; (C) posterior spiracles as they appear from a posterior view: the area is covered by strong not ordered spines; (D) posterior spiracle: Slits straight and radiate in a T shape, the ecdysal scar is well defined. (A scale bar 500 μ m; B, C scale bar 100 μ m D scale bar 50 μ m).

human excrement, fungi, mammal burrows and caves.²³

It is reasonable to think that the larvae may have penetrated the bone through the several vascular foramina with a diameter larger than 1 mm present on the neck of the femoral head (Fig. 4). This hypothesis is also supported by the lack of evidence of any external injuries on the femoral surface (Fig. 1).

To the best of our knowledge, the presence of the larvae inside intact long bones is very unusual with only scant reports in scientific papers, where the most common cited taxon is the family Piophilidae and only occasional species in other families (eg. Calliphoridae and Sarcophagidae).^{28,29} Species of the family Heleomyzidae have not been

reported so far from the inner cavity of bones. This makes this finding a novelty improving our knowledge about the taphonomic processes where insects are involved.

The paucity of data on this topic can be related to the lack of external evidence indicating the presence of larvae in the bone marrow and the fact that, as previously mentioned, the presence of larvae is not detectable by X-ray analysis of the bone. Only the preparation of the bones for DNA extraction, routinely performed by forensic geneticists, may reveal the presence of an internal colonization of the bone as mentioned by Skowronek and colleagues²⁹ and confirmed by our work.

In addition, it is worth mentioning that the quality of the DNA

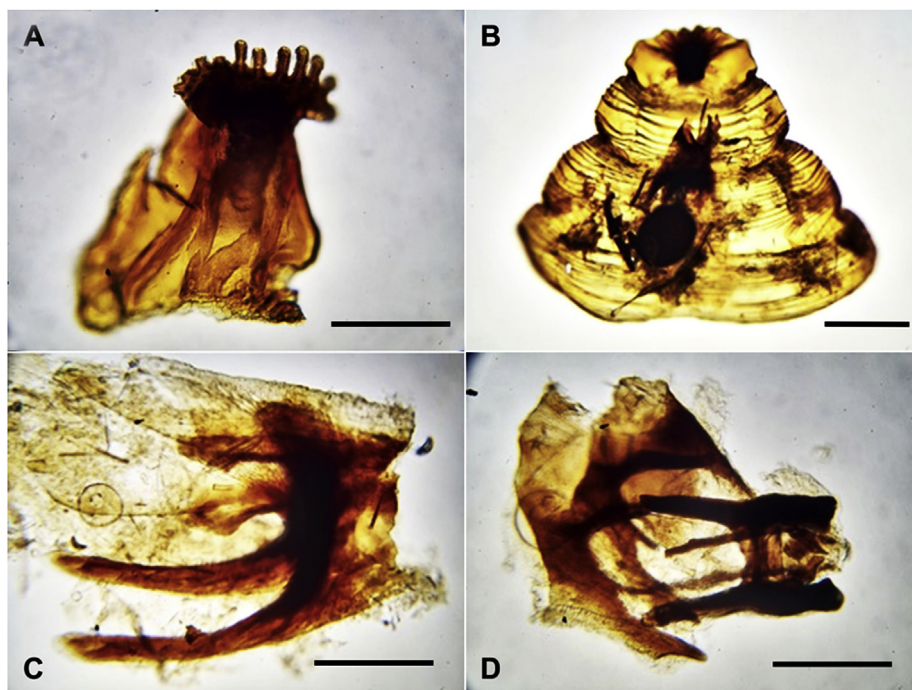


Fig. 3. (A) Anterior spiracles, transverse rosette with 11 respiratory bulbs. (B–C) Head skeleton, mandible with a pin-hole window; and (D) Intermediate sclerite, club-shaped with the ventral bridge towards the anterior end (B scale bar 50 μ m; A, C, D scale bar 30 μ m).



Fig. 4. Vascular foramina on the neck of the femoral head.

extracted here described was not high enough to achieve any successful victim profiling. The presence of insects feeding on the marrow, could be one of the reasons of the poor DNA quality, however, other factors such as the environmental conditions where the skeleton was found cannot be excluded.

In conclusion, the inner examination of the bones during forensic autopsy may provide useful entomological evidence, and for this reason the analysis of skeletal remains should, as regularly as possible, be performed by a multidisciplinary team capable of producing a complete reconstruction of *peri* and *postmortem* events.

Acknowledgements

The authors thank A. Dinisi and B. Tomaiuolo for technical assistance. Moreover, the authors wish to thank the Scientific Bureau of the University of Catania for language support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jflm.2019.05.021>.

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