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Non-invasive approaches to morphological and molecular identification of insects from museum, archaeo-funerary and forensic contexts

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Copyright statement

Student Declaration Form

I, Giorgia Giordani, hereby declare that

- while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University of Huddersfield or other academic or professional institution;

- my thesis/dissertation entitled "Non-invasive approaches to morphological and molecular identification of insects from museum, archaeo-funerary and forensic contexts" is the result of my own work. The outcome of work done in collaboration is declared.

I am the main author responsible of the writing of the following publications (Appendix B) results of this thesis

1. **Giordani G.**, Grzywacz A. and Vanin S. (2018) Characterization and identification of puparia of *Hydrotaea* Robineau-Desvoidy, 1830 (Diptera: Muscidae) from forensic and archaeological contexts. Journal of Medical Entomology. https://doi.org/10.1093/jme/tjy142
2. **Giordani G.**, Tuccia F., Zoppis S., Vecchiotti C. and Vanin S. (2018) Record of *Leptometopa latipes* (Diptera: Milichiidae) from a human cadaver in the Mediterranean area. Forensic Sciences Research. https://doi.org/10.1080/20961790.2018.1490473
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The coauthors of the previous publications contributed in the following way:

- Dr A. Grzywacz provided the specimens polish specimens of *Hydrotaea* specimens;
- Dr S. Zoppis and Prof. C. Vecchiotti provided the specimens and the general information about the forensic case;
- Prof. I. Floris provided part of the entomological material from Sant'Antonio Abate Cathedral;
- Tuccia F. provided historical information about the cases or the samplings.
- Dr Vanin S. made available his entomological collection and supervised the work.

Date: Signature:

Print Name:

"I suppose you are an entomologist"
"Not quite so ambitious as that, Sir. I should like to put my eyes on the individual entitled to that name. No man can be truly called an entomologist, Sir; the subject is too vast for any single human intelligence to grasp."

Oliver Wendell Holmes, *The Poet at the Breakfast Table.*

Abstract

Species identification is the starting point for the formulation of any further ipothesis in insect sciences and they are particularly important in forensic entomology and funerary arcaheoentomology. Two main approaches are currently used for identifying insects, the morphological one, preferred by old-school taxonomists and the more recent molecular one, favoured by non-specialists. Both approaches have respectively some limitations: lack of morphological identification keys, especially for the immature stages and the destruction of the specimens during the DNA extraction, as examples. The development of easy-access identification keys and of non-invasive DNA extraction methods permitting the repeatability of the analyses have been the main target of this thesis. To achieve this aims morphological analysis and DNA based methods were integrated and used on samples belonging to different contexts – forensic, museum and archaeological – and different timeframe – 0 to 1000 years old -. The attention was paid to adults and the poor investigate puparia of flies from each context and the different timeframes.

For the DNA analysis commercial kits and homemade digestion buffers were tested on modern and old specimens but applying operational procedure to preserve the morphology of the specimens.

More then 10 puparia belonging to species in the family Heleopmyzidae, Sphaeroceridae, Piophilidae, Muscidae and Calliphoridae have been described and illustrated. Forty new DNA sequences, mainly from poor investigated taxa, were generated and 37 deposited in GenBank despite some problems arised, mainly due to presence of PCR inibithors in and on the cuticle.

The results, reported in this work, will impact in the anlysis of forensic and archaeological material but as well in the study of museum material, that especialy for the species *typus* is particularly precius and can not be destroid or altered during the identification process.

In addition specific question as the separation and identification of Hydrotaea puparia belonging to different species, the identification of poorly investigated species, the systematic of *Sarcophaga* species in the subgenus *Sarcophaga* and the evaluation of pre-Columbian funerary cerimonies have been answered. In addition the pictorial material produced will help entomologists working with fragmented material especially from old

cases or archaeological sites. Several questions are still open and they request further investigations to be done in the future.

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The PhD thesis is the result of a journey in which you have to put yourself personally at stake.

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List of Abbreviations

ADD	Accumulated Degree Days
ADH	Accumulated Degree Hours
AMS	Accelerator Mass Spectrometry
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data System
BSA	Bovine Serum Albumin
BT	Bootstrap
CBOL	Consortium for the Barcode of Life
CI	Colonisation Interval
COI	Cytochrome Oxidase Subunit 1
CRT	Cyclic Reversible Termination
CytB	Cytochrome b
DB	Digestion Buffer
ddNTPs	dideoxinucleotidetriphosphate
DTT	Dithiotreitol
EAFE	European Association for Forensic Entomology
EIP	Early Intermediate Period
Ef1α	Elongation Factor 1 alpha
EtOH	Ethanol
GSR	Gunshot Residue
LH	Late Horizon
LIF	Laser-Induced Fluorescence
LIP	Late Intermediate Period
MH	Middle Horizon
ML	Maximum Likelihood
MP	Maximum Parsimony
mPMI	minimum Post Mortem Interval
mPMSI	minimum Post-Mortem Submergence Interval
mtDNA	mitochondrial DNA
NCBI	National Center for Biotechnology Information

NGS	Next Generation Sequencing
NHMUK	Natural History Museum of London
NJ	Neighbour-Joining
PAI	Pre-Appearance Interval
PCR	Polymerase Chain Reaction
PIA	Period of Insect Activity
PMI	Post Mortem Interval
PPi	Pyrophosphate ions
PVP	Polyvinylpyrrolidone
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TBS	Total Body Score
TEM	Transmission Electron Microscopy
UPC	Universal Product Code

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1 Introduction

In all the disciplines dealing with insect studies, a critical point concerns the species identification. Two mainly approaches are currently used for this purpose, the morphological one (see section 3.5.1), preferred by old-school taxonomists, based on the observation of anatomical characteristics (Gullan & Cranston, 2014) and the more recent molecular one (see section 3.5.2), favoured by non-specialists, and based on sequencing and comparison of specific mitochondrial or nuclear nucleotide regions (Sonet *et al.*, 2013; GilArriortua *et al.*, 2015). Both approaches have respectively some limitations: lack of identification keys, especially for the immature stages, for the first and the total or partial destruction of the specimens for the second. Different works dealing with molecular and morphological identification of insectshave already been published. The majority of them focus onColeoptera from both museum and archaeological contexts (Solomon, 1965; Panagiotakopulu, 2001; Gilbert *et al.*, 2007; Thomsen *et al.*, 2009; Panagiotakopulu & Buckland, 2012). Few data are instead available for the morphological and molecular identification of old Diptera specimens and especially for immature stages there is a quite complete lack of information (Tin *et al.*, 2014). In addition, it is worth mentioning that in the investigation of the cadaveric entomofauna, Diptera, that are the most important taxon (Smith, 1986), are mainly sampled as pupal cage (*puparium*) (Martin-Vega *et al*., 2016). In fact, the active feeding stage of this taxon is the larval stage that perform the metamorphosis in the puparium, a sclerotised structure deriving from the pre-final larval cuticle. As the larvae, and in some cases less than the larvae, puparia have only few visible diagnostic characters that can be used for their specific identification. In addition, in the archaeological context or in old forensic cases puparia are partially destroyed and missing of the part containing the oral sclerites that play an important role for their identification. In recent years, several authors have striven to produce clear and useful descriptions and identification keys for the immature stages of forensically valuable insects. Special interest has been focused on larvae and eggs (Sukontason *et al.*, 2004), whereas puparia are comparatively under-investigated. Despite some larval characters are maintained on the puparium others differ in the two stages or, as previously mentioned not always found (e.g.: oral sclerites). The use of DNA techniques carried out on immature insect stages, which lack important identification features, has become increasingly common in the last 20 years (Stevens & Wall, 2001;

Wallman & Donnellan, 2001; Harvey *et al.*, 2003; Mazzanti *et al.*, 2010). It is worth mentioning that despite the publication of some works concerning the molecular identification of old puparia (Mazzanti *et al.*, 2010), the molecular identification of puparia from archaeological sites is still difficult, it has been poorly investigated and it does not allow for conclusive identification.

Preliminary works have demonstrated that it is possible to extract DNA from puparia, but only of small amounts, and not of high quality (Mazzanti *et al.*, 2010). However, the destructive approach of these DNA extraction techniques has led several scientists to use morphology, breeding immature stages up to the adult fly eclosion as identification keys. This approach can be used only for living specimens, and it cannot be applied in old forensic cases and in the archaeological context. For this reason, analysts have begun to develop DNA extraction buffers and specific protocols in order to both extract DNA, and to preserve the morphological characters of the sample as requested by several judiciary system and for important specimens from museum collection (e.g.: *typus*). The results differ considerably. For example, beetles – thanks to their thick cuticles – have provided particularly good results (Gilbert *et al.*, 2007), while more delicate species such as flies have resulted in more serious damage to the remains (such as wings) (Thomsen *et al.*, 2009; Tin *et al.*, 2014). A key factor actively conditioning the molecular result is the DNA degradation due to the age of the samples. Successful molecular characterisation has been done on 20,000 years old fossil Coleoptera collected from packrat middens, while nothing has been done on Diptera from archaeological contexts. To date, morphological identification remains the principal approach for puparia identification.

1.1 The importance of species identification

A correct species identification is the *conditio sine qua non* for any further reasoning on microbiological, botanic and zoological samples. This is also fundamental in any entomological study where the knowledge about the biology, physiology, ecology, phenology and development of the insect is used to derive important information and reconstruct past event like in Forensic entomology and funerary and non-funerary archaeoentomology.

Giving a name to a specimen, the “identification”, links an organism to all the scientific knowledge available about the species and is thus fundamental for a series of research

and practical applications. Species identification is crucial, for example, in pest management protocols in agricultural contexts, since certain species can be used to identify whether fraud or food adulteration has taken place, and in medico-legal contexts, leading to estimation of the mPMI (see 3.1.1.1). The correct and taxonomical updated identificatio is fundamental: misidentifying a species as a sibling species can result in massive errors in mPMI estimation, with serious consequences for investigative validity such as the erroneus allegation or release of a suspect. Two different species, indeed, at equal temperature, may present a different development rate. This biological variation entails a different result in the calculated minimum Post Mortem Interval, with a consequent temporal shift of the estimated time of death. The significance of the shift depends on the species involved and cannot be estimated in general. As an example the misidentification of *Calliphora vomitoria* (Linnaeus, 1758) with *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera, Calliphoridae) developed a 25℃ has as consequence an error of about 4.6 days if the whole development is considered (Marchenko, 2001) while the misidentification of *Hydrotaea aenescens* (Wiedemann, 1830) with *Hydrotaea capensis* (Wiedemann, 1818) (Diptera, Muscidae) developed at 24 ℃ has as consequence an error of about 4.4 days if the whole development is considered (Lefebvre & Pasquerault, 2004). In the same way, misidentifying a species in a museum context will result in a wrong reference point for further identification. In the agriculture pest management wrong identification deals with wrong insecticide useage that could have important impact in the environement and ineffective costs.

This thesis deals with the identification of species of particular interest in the museum, forensic and archaeological contexts despite the tecniques developed could be applied as well in other entomological framework.

2 Aim of the work

Because the vital importance of a correct species identification in the forensic entomology and archaeology contexts this thesis was performed in order to prop up scientific community in species identification based on morphological and molecular approcahes. In particular the thesis focus on:

1) finalise a non-invasive morphological and molecular method for the identification of Diptera related to animal decomposition from different contexts in order to allow the re-examination of the entomological material and the combination of the morphological and molecular approaches,

2) describe the poor investigate immature stages of diptera development, particularly the puparia of scarcely investigated families of forensic interest;

3)evaluate how time and other environmental and preservation factors can affect the molecular identification of insects of forensic and archaeological interest.in order to understand the factors affecting DNA integrity and suggesting new DNA extraction and amplification protocols/approaches

These problematics were dissected using a temporal scale where samples are considered as a function of their status: forensic, museum, and archaeological. So doing, each chapter of this thesis (Forensic contexts, Museum samples and Archaeological contexts) was able to answer to some specific questions related with the specific case that was analysed.

- **Forensic context:**

-Morphological description and molecular analysis of relatively fresh samples of immature stages and adults of poorly investigated Diptera of forensic interest.

- **Museum context:**

-Development of a DNA extraction method from adults of *Sarcophaga* spp. flies belonging to museum collection without affecting the morphology and the chetotaxi of the specimen.

- **Archaeological context:**

-Resolution of identification problems of puparia of a Muscidae genus of forensic and archaeological interest.

-Molecular characterisation of Diptera puparia and adult collected from mummies dated around XVII century and biodiversity studies of Sardinia (Italy) region in the past.

-Investigation of camelid sacrifice through entomological samples collected from the archaeological site of Pachacamac.

-Evaluation on the possibility of extract DNA from Coleoptera suitable for amplification and Sanger sequencing.

3 State of the art

3.1 Forensic entomology & funerary archaeoentomology

The correlation between dead bodies and insects has long been recognised (Benecke, 2001). Middle Age iconography (Fig. 1) from XV ("Dances of the Death") and XVI centuries (“Skeleton in the Tumba”) shows maggots – somewhat resembling snakes or worms – associated with dead bodies. The skeletonization of the head and the pattern of body mass reduction, focused mainly on the internal organs, suggest furthermore a close observation of the decomposition of human corpses by the authors of the paintings (Benecke, 2001).

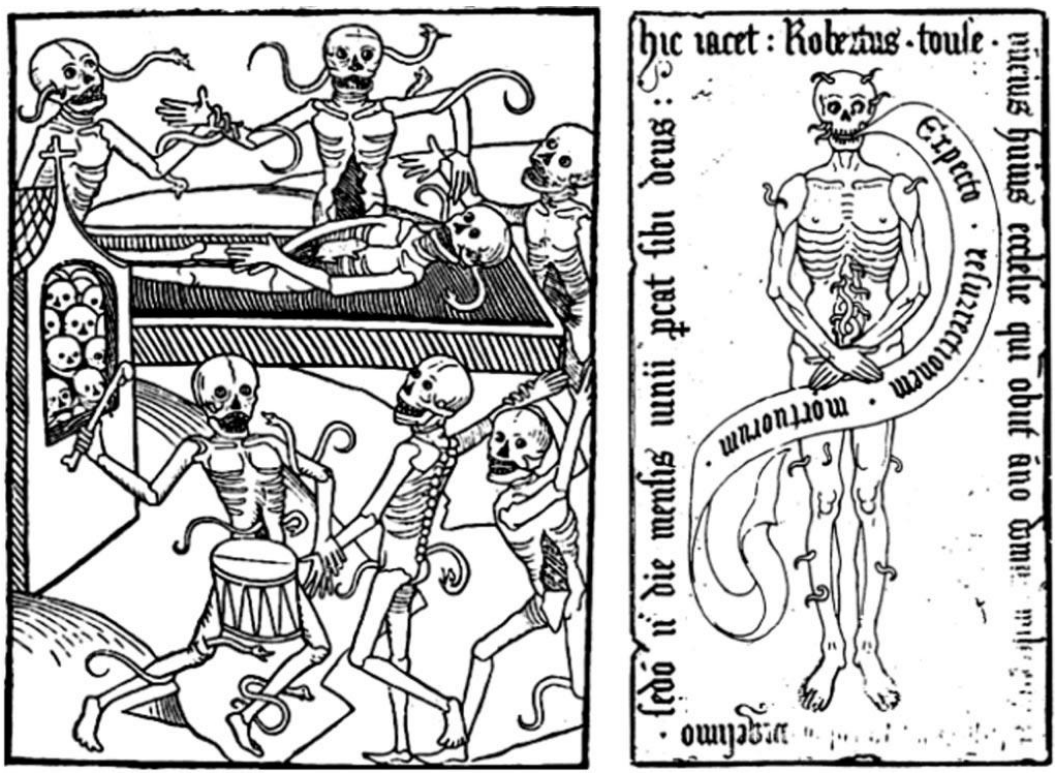

Fig. 1 **Middle Age Iconography.** "Dances of the Death" (left) & “Skeleton in the Tumba” (right) (Benecke, 2001).

The association between insects and mummified bodies of archaeological interest was also reported on as early as the XVIII century. In 1710, a letter from Tommaso Alghisi to his friend and colleague Antonio Vallisneri (1733) illustrated a larva or a puparium of a fly associated with an Egyptian mummy. The analysis and the study of insects associated with archaeological/forensic human remains has made considerable progress and is

burgeoning into a two-pronged research discipline: forensic entomology and funerary archaeoentomology.

3.1.1 Forensic Entomology

Forensic entomology is the branch of forensic science that studies insect lifecycles and behaviour related to legal contexts (Amendt *et al.*, 2004; Wallman, 2004; Joseph *et al.*, 2011). While widely considered to only deal with insect infestation of dead bodies, forensic entomology is in fact involved every time information concerning insects is used in investigative processes linked to both civil and criminal investigations. The origin of forensic entomology has been dated between the XIII and the XIX century (Catts & Goff, 1992; Amendt *et al.*, 2004; Gennard, 2007). The first breakthrough was the controlled experiment done by Redi at the end of the XVII century (1668), which disproved the spontaneous generation theory by demonstrating that larvae develop on meat only from eggs laid by flies. The first use of forensic entomology in a courtroom proceedings dates to 1850 in France where insect evidence was recognised as valid proof in the investigation of an infanticide (Bergeret, 1864; Amendt *et al.*, 2004).

The acceptance of forensic entomology as a fully-fledged forensic discipline arrived only in late XX and early XXI century, when police forces, legal authorities, academics and practitioners started collaborating in order to refine and develop the discipline (Amendt *et al.*, 2004; Gennard, 2007). The divisions within the field are not necessarily fixed; forensic entomology is itself commonly divided into three sub-fields: 1) urban, 2) stored product pests and 3) medico-legal entomology (Lord & Stevenson, 1986; Catts & Goff, 1992).

Urban forensic entomology deals with infestations by unwanted insects of structural environments, whether or not they pose a real threat to humans. The role of the entomologist is to assess the level of infestation, and to provide a potential explanation in term of timing and mode of infestation. Pests like termites may cause structural damage (Scheffrahn *et al.*, 1988), cockroaches and flies could be cause of sanitary problems (El-Sherbini & Gneidy, 2012; Biehler, 2013) whereas bugs (e.g. *Arocatus melanocephalus* Fabricius, 1798) can be considered as aesthetic pest because their unpleasant psychological impact on people (Maistrello *et al.*, 2006).

Stored-product forensic entomology examines commercial food products infestation or contamination once they have been packaged or after they have been harvested and stored. The aim of the entomological investigation is to find evidence relevant for litigation, such as identification of the insects, determination of their development stage and information about when and how the infestation might have occurred.

Medico-legal forensic entomology is the best-known subfield and has seen considerable exposure in public entertainment (e.g. the American "CSI" TV series franchise) and also real-life courtroom processes. Medico-legal forensic entomology focusses on insects colonising and developing on decomposing organic matter of animal origin (wild animals, pets and humans). Using known rates of development, life cycle and biology of insects recovered from dead bodies, entomological analysis can provide information about location and time of the crime. In the last century, forensic entomology has further been subdivided into subdisciplines such as forensic veterinary entomology, entomo-toxicology, and human and insect DNA identification (Byrd & Castner, 2010). However, in the current work, the term 'forensic entomology' refers only to the medico-legal aspect of the discipline.

3.1.1.1 *Estimation of time since death*

The estimation of the time elapsed from death until the discovery of the cadaver (*Post Mortem Interval* [PMI]) is perhaps the most important task facing the forensic entomologist. The time frame imposed on the series of events preceding a body's discovery can lead to the inclusion or exclusion of suspects.

After death a body (human or animal) undergoes putrefactive processes involving the loss of water and chemical reactions such as butyric, glycolytic and ammoniac fermentations (Pless *et al.*, 1997; Campobasso & Introna, 2001; Saukko & Knight, 2015). The arrest of vital functions decreases body temperature (*algor mortis*) via the dispersing of heat by conduction, convection, irradiation and evaporation and the arrest of the immune system.

Under normal conditions, 2-3 hours after death the phenomenon of hypostasis, purple-red skin stains (*livor mortis*), begins to form and then increases by extension and intensity: this phenomenon is due to the gravitational pooling of blood. The rapidity of appearance and the intensity of the hypostases are in relation to the degree of fluidity of the cadaver's blood. In the first 12-15 hours after death, changing the position of the corpse (for

example, from supine to prone) can lead to the disappearance or reduction of the intensity of hypostatic ligatures already formed. Later, the displacements of the body position no longer influence the state of the primitive hypostases.

Postmortal muscular atony gives the corpse a substantial relaxation, such as to allow any passive movement at the level of the different articular districts. After a few hours, however, the establishment of stiffening cadaveric (*rigor mortis*) starts. The stiffness, with a progressive trend towards increasing intensity, induces muscular contracture and results in rigidity of all the joints (culminating period) up to gradual decrease of the phenomenon (period of resolution) eventually terminating in the return of flaccidity to the corpse. While *rigor mortis* is believed to occur simultaneously in all muscles, it is more evident first in the smaller muscle groups (i.e. the jaw and face) then in the muscles of the neck, upper limbs, trunk and – finally – the lower limbs. The maximum intensity of corporeal stiffness is between 36-48 hours after death. Flaccidity typically returns by around the 72nd hour after death (Henßge *et al.*, 2000b; Henßge *et al.*, 2000a; Amendt *et al.*, 2004; Henßge & Madea, 2004).

Historically, all these physical changes were used to estimate the time since death (Pless *et al.*, 1997; Henßge & Madea, 2004; Gennard, 2007), as well as the changes in the *humour vitreous* and other electro/mechanical excitability parameters (Henßge & Madea, 2004; Ding *et al.*, 2017). However, estimating the rate of *post mortem* decay is hampered by intrinsic variables associated with the corpse (i.e. age, weight, cause of death) and extrinsic issues associated with the surrounding environment (i.e. temperature, humidity, ventilation, exposure). Some mathematical tools were developed to include all these variables in the PMI calculation, such as Henssge's nomogram (Campobasso *et al.*, 2001; Henßge & Madea, 2004). In more extreme cases of concealment, dismemberment, explosion and burning, the morphological characteristics of the cadaver are totally unusable, and there is a lack of standardized methods (based on experimental studies) to accurately estimate time of death in such cases (Vanin *et al.*, 2013).

For intermediate range of time (days or weeks) the role of the medico-legal entomologist is of fundamental importance in PMI estimation. The study of cadaveric arthropod-fauna is a well-known and widely-accepted method that can support histological and chemical analysis. The development rate of the communities colonizing the body, together with

study of elapsed time and ambient temperature is the main contribution of entomology to PMI estimates.

Insects have evolved specific mechanisms in order to use the different substrates of decomposition as sources of food. While all insects could be of potential forensic interest, a number of species, mostly among Diptera (i.e. blowflies, flesh flies and house flies) and Coleoptera (beetles), are found more often than others. Necrophagous insects (and parasitoids thereof), predators, omnivores and opportunist species are all considered to be of forensic interest (Smith, 1986; Turchetto & Vanin, 2004b; Gennard, 2007).

The rate of insect appearance and development on decomposing material is species-specific and temperature dependent. The colonisation interval (CI) and the period of insect activity (PIA) on the cadaver refer to the time lapse from eggs or larvae deposition to the discovery of the cadaver. The degree of physical connection between dead body and environment (e.g. outdoor or indoor location, buried or exposed, clothed or unclothed) may hamper estimates (Campobasso *et al.*, 2001). For that reason, forensic entomology aims to evaluate the *minimum Post Mortem Interval*, mPMI, considered as the time passed between eggs or larvae laid by flies and the cadaver's discovery. To date, no experimental tools are available to estimate the PMI (Fig. 2) (Tuccia *et al.*, 2018). Matuszewski et al. (2014) have worked to improve PMI estimation via the pre-appearance interval (PAI) - the time before the appearance of a species on the cadaver/carrion - of forensically useful flies. To date, only *Stearibia nigriceps* (Meigen, 1826) shows the possibility to estimate adult or oviposition PAI from the only temperature data (Matuszewski *et al.*, 2014). The combination of correct species identification and insect developmental stage allows the entomologist to estimate when insect colonisation of the cadaver took place. Because insects are poikilotherms, their development is directly correlated (Fig. 3) with the temperature of the environment; temperature is therefore also considered in this system.

The Accumulated Degree Days (ADD)/Hours (ADH) – the sum of temperature (°C) above the lower development threshold (temperature value below which insects cannot develop; species specific) multiplied by time (days or hours) – is one of the techniques used to estimate insect developmental rate over a period with temperature compensation (Megyesi *et al.*, 2005; Amendt *et al.*, 2007). Experimental laboratory work is required to establish the ADDs or ADHs of each species.

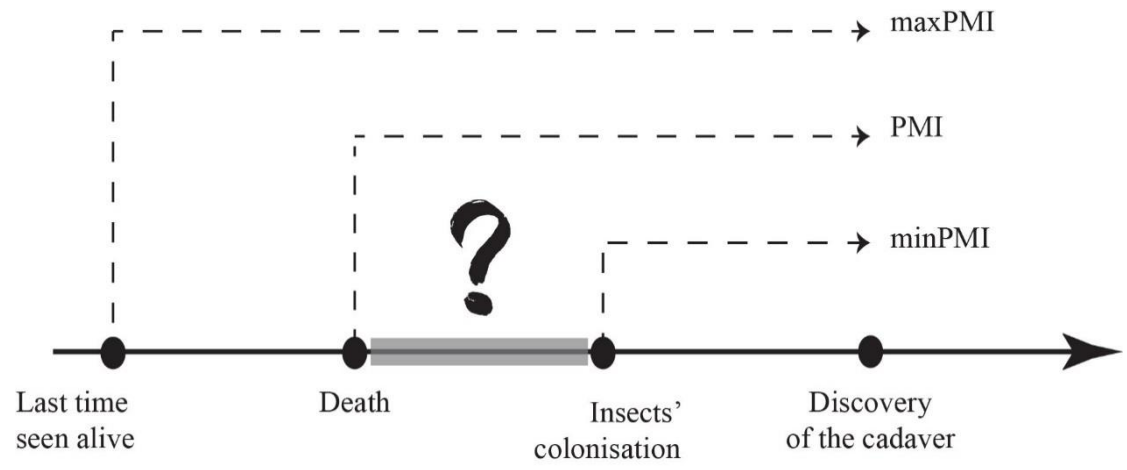

Fig. 2 **Schematic representation of maximum PMI, PMI and minimum PMI**.

PMI estimation is not the only useful information that forensically derived insects can provide. Insects may have specific seasonality; thus, can be used as an indicator of the season when colonisation began. Environmental specificity of insect species means that the presence of a synanthropic species in the forest (or *vice versa*) may be used to support the hypothesis of body transfer (Vanin & Huchet, 2017). Further entomological approaches include identifying the presence of drugs or poisons (entomo-toxicology), and the confirmation of "cadaver presence" (i.e. insect traces testifying to the presence of a corpse removed prior to the arrival of investigators). These approaches are summarised below.

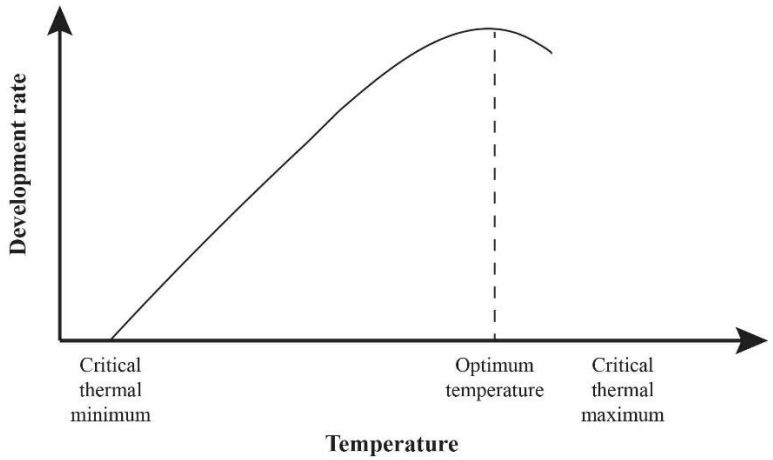

Fig. 3 **Schematic representation of insect developmental rate curve as a function of temperature**.

3.1.1.2 *Estimation of the season of death*

The precision of mPMI estimation decreases with time elapsed since death; however, insects can give useful information months or even years (or millennia, in the case of archaeological remains) after the decease of the individual. While <1 year after death data on arthropod colonisation waves (see 1.2.3) can allow estimates down to the month (Smith, 1986), older remains typically only permit identification of season. A cadaver exposed in the warm season will show a different and richer entomofauna from a corpse deposited in winter (Smith, 1986). The commonest blowflies present on carrion show a fairly long flight period, from early spring to late autumn and almost disappear in winter (except in the tropics) (Smith, 1986).

3.1.1.3 *Estimation of body transfer/concealment*

It often transpires that the body is moved from the scene of the crime or original deposition. Attempts to conceal a cadaver can occur over short or long distances, shortly after death or after a prolonged period (Mozayani & Noziglia, 2010; Charabidze *et al.*, 2017). Cadaver concealment, wrapping and displacement all aim to prevent the discovery of the corpse. Because secondary crime scenes typically differ significantly from that of the original crime (Reibe-Pal *et al.*, 2008), forensic entomology data can be used to identify original crime scenes and derive details of body transfer or concealment processes (Smith, 1986; Catts & Goff, 1992; Byrd & Castner, 2010; Mozayani & Noziglia, 2010; Joseph *et al.*, 2011; Byard & Payne-James, 2015; Archer & Wallman, 2016). The system does have its limitations – namely the wide distribution and dispersal capability of many European necrophagous species. More researches on geographical localisation of different species are needed to strengthen this method (Charabidze *et al.*, 2017).

3.1.1.4 *Human identification*

The advent of modern molecular biology techniques has strongly influenced every aspect of the forensic field. DNA analysis for identification purposes has reached an unprecedented level of refinement, and a similar revolution has occurred in the analysis of human tissues in insect digestive tracts, a reliable proxy for the cadaver in the event

that the latter was moved from the crime scene and later concealed (Coulson *et al.*, 1990; Wells *et al.*, 2001; Campobasso *et al.*, 2005; Gaudry *et al.*, 2007; de Lourdes Chávez-Briones *et al.*, 2013; Charabidze *et al.*, 2017). Marchetti *et al.* (2013) obtained a human STR profile from empty puparia bypassing the problem of the rapid degradation of DNA by gut digestive enzymes (Picard & Wells, 2009; Charabidze *et al.*, 2013).

3.1.1.5 ***Drug extraction (Entomo-toxicology)***

The discipline that investigates the application of toxicological analyses to cadaveric insects to identify drugs, medicines, chemicals and toxins in human and animal tissues is entomo-toxicology (Goff & Lord, 1994; Introna *et al.*, 2001). In the event that the body is in a particularly advanced decomposition stage, biological fluids and solid organs may no longer be available for traditional toxicological analyses. In these cases, insect samples can be used for different types of qualitative toxicological analyses (Goff & Lord, 1994; Introna *et al.*, 2001; Campobasso *et al.*, 2004). Larvae that feed on the carcass can accumulate narcotic and other toxic substances to which the subject was exposed in life. Different types of analyses can be applied to adult insects, puparia fragments (Goff & Lord, 1994; Miller *et al.*, 1994; Goff *et al.*, 1997), exuvia (Miller *et al.*, 1994) and faeces of coleopterans (Introna *et al.*, 2001), all of which enjoy survivorship stretching up to years after the criminal event (Nuorteva & Nuorteva, 1982; Introna *et al.*, 2001; Amendt *et al.*, 2004). For some substances, the toxicological data obtained from insect larvae may be more reliable than those obtained from cadaveric tissues (Kintz *et al.*, 1990). Residues of paracetamol, benzodiazepines, barbiturates and tricyclic antidepressants have all been recovered from cadaveric larvae (Kintz *et al.*, 1990). It has also been demonstrated that ingestion of drugs or toxic substances can influence the development of necrophagous insects, and therefore mPMI calculation, with cocaine and heroin increasing the growth rate while morphine slowing it down (Goff *et al.*, 1989; Goff *et al.*, 1991; Bourel *et al.*, 1999). The manner of narcotic substance accumulation in Diptera is still under investigation (Kharbouche *et al.*, 2008; Parry *et al.*, 2011; Oliveira *et al.*, 2014). It has also been suggested that GSR (gunshot residue) such as lead, antimony and barium may be collected from larval tissues (Roeterdink *et al.*, 2004; Lagoo *et al.*, 2010;). This may prove to be useful, given the overall decrease of GSR in cadaveric tissues through time,

or when the advanced decay of the corpse does not allow the recognition of typical gunshot wounds.

3.1.2 Funerary Archaeoentomology

The study of sub-fossil insect remains (palaeoentomology or archaeoentomology) was developed at the University of Birmingham in the late 1950s by geologist Russell Coope and entomologist Peter Osborne (Ashworth *et al.*, 1997; Reilly, 2003). They elucidated the importance of insect remains as a means of understanding past climate and environment, to develop refined bio-stratigraphical dating for the Quaternary and as a means of understanding ancient biodiversity (Coope *et al.*, 1961; Harrington *et al.*, 1999; Volney & Fleming, 2000; Harrington *et al.*, 2001; Turchetto & Vanin, 2010; Lo Pinto *et al.*, 2017). The development of the paraffin flotation insect remains recovery method (Coope, 1978) moved the field into the study of sub-fossil insects from archaeological contexts, with particular emphasis on ancient human environmental studies (Reilly, 2003). There has been a long emphasis on Coleoptera, but all orders of insects can be used in archaeoentomology. The method was split and refined over time, starting with the application of archaeoentomological techniques to urban archaeology (Reilly, 2003) by analysts such as Kenward (Kenward & Hall, 1995; Carrott & Kenward, 2001), who used insects as markers of occupation intensity and duration (Reilly, 2003). Perhaps the most significant development of the field, however, has been that of funerary archaeoentomology.

Funerary archaeoentomology is the application of forensic entomology techniques to human and animal remains, burials, mummies and any other findings of archaeological origin (Huchet, 1996, 2014b). Forensic entomology and funerary archaeoentomology are two separate disciplines despite having the same core of knowledge about insect colonisation of cadavers and carrion and sharing common techniques for the sampling and the interpretation of the entomological samples. The term ‘forensic’ should not be applied to archaeological remains, given the etymology of the term (from the Latin *forum*: related to the court), and that ‘forensic archaeology’ refers to the application of archaeological techniques (search, excavation, etc.) to forensic contexts and not *vice versa*.

In archaeological contexts, the study of insects associated with burials may provide reliable information about the cultural and social context of the cadaver (Huchet & Greenberg, 2010). The knowledge about the ecology and biology of the species collected from decomposing remains can be particularly important to reconstruct funerary practices (Gilbert & Bass, 1967; Dirrigl & Greenberg, 1995; Nystrom *et al.*, 2005; Huchet & Greenberg, 2010), to describe cadaveric taphonomy (Wood, 1976; Wylie *et al.*, 1987; Vanin *et al.*, 2009; Huchet & Greenberg, 2010; Gaudio *et al.*, 2015) and to understand the hygienic, sanitary and social conditions of ancient human populations (Ewing, 1924; Huchet, 1996; Capasso & Di Tota, 1998; Rick *et al.*, 2002; Reinhard & Buikstra, 2003; Raoult *et al.*, 2006). Given that various extrinsic and intrinsic factors can affect body colonisation by insects (Campobasso & Introna, 2001), archaeological remains necessitate interpretation of cultural context and funerary traditions/practices as part of the entomological findings (Huchet, 2014b). Arthropods found in archaeological contexts can thus be related to body decomposition, offering infestation, parasites, local/environmental biota and connected to secondary contaminations.

Insect carapaces are so robust that they can survive for thousands of years and provide colonisation data to archaeoentomologists. The majority of surviving insect fragments tend to be Coleoptera adult fragments and Diptera puparia, which are particularly common in coffins, burials or other protected environments. Although archaeological mummified (or partially mummified) human and animal bodies are known from all over the world, those that have attracted the most attention from archaeoentomologists are from Europe (Portugal, Italy, France, UK) (Couri *et al.*, 2008; Couri *et al.*, 2009; Huchet, 2010; Panagiotakopulu & Buckland, 2012; Huchet, 2013; Vanin, 2016), North Africa (Egypt) (Panagiotakopulu, 2001; Huchet *et al.*, 2013; Huchet, 2017), and the Americas (Peru, Mexico) (Reinhard & Buikstra, 2003; Nystrom *et al.*, 2005; Huchet & Greenberg, 2010).

3.2 Body decomposition and insect colonisation

3.2.1 Body transformation after death

After death, different modifications occur almost immediately in the body (Dent *et al.*, 2004). Some of them are useful for establishing cause of death, others for

thanatocronology, a fusion of the Greek terms θάνατος (death), χρόνος (time), and λόγος (study or treatise). Decomposition has to be considered a mixed process of internal and external modifications.

The first changes, which are abiotic or negative vital, occur in the body in the transition between life and death, allowing to ascertain the reality of death. Immediate phenomena, in relation to the arrest of the essential functions for the life of the individual are loss of consciousness, of sensitivity, of spontaneous motility as a result of the arrest of the functionality of the central nervous system, breath and cardiovascular activity interruptions. Consecutive phenomena, in relation with the changes that are established in the cadaver while residual vital activities persist, are body cooling, cadaveric rigidity, hypostasis, acidification, dehydration and loss of neuro-muscular excitability (Borri, 1914). The chemical-physical and, therefore, morphological alterations of the corpse, with specific reference to the various factors that can influence its evolution and the relative times of appearance and appreciation is matter of thanatocronology.

The second set of modifications involves transformative phenomena which actively destroy the organism via autolysis, putrefaction and maceration, (Borri, 1914), and its preservation via saponification and mummification (Janssen, 1984; Pinheiro, 2006). These processes are briefly summarised below.

Autolysis is an early destructive transformative process that is closely correlated with putrefaction. It comprises a self-demolition process based on cellular enzymatic activities in the absence of the microorganism intervention. As cells break down, the presence of nutrient-rich fluids permit the commencement of the putrefactive process (Vass, 2001). Putrefaction, meanwhile, describes the decomposition of fat and muscle tissues due to the metabolic activities of saprobiontic organisms. It is triggered by gut-based anaerobic bacterial genera – such as *Clostridium* and *Bacteroides* – expanding through the vessel to all body tissues. The first indicator of putrefaction tends to be discoloration of the skin caused by sulfhemoglobin in the blood, accompanied by gas production and resulting tissue distension (Vass, 2001). Lipid breakdown leads to the formation of complex fatty acids, in turn becoming lower fatty acids (e.g. butyric, propionic, valerian, caproic) and volatile compounds. Protein breakdown leads to the production of carbon dioxide, ptomaine, ammonia and hydrogen sulphide; other cadaveric bases include cadaverine (from lysine), putrescine (from ornithine), skatole and the 3-methylindole (from

tryptophan) (Vass, 2001; Dent *et al.*, 2004). The resulting gases and fluids evacuate through oral, nasal and anal orifices and other skin breaks (Vass, 2001; Dent *et al.*, 2004; Gennard, 2007).

The set of alterations/transformations that the bone undergoes within the soil constitute the complex diagenesis process (Vass, 2001).

Ideally, the decomposition of a human body, lying on the ground, to skeleton is completed within 15–25 years (Fiedler & Graw, 2003). Vass (2001) developed the formula for the estimation of the degree of degradation of soft tissue in relation to temperature. The formula for human body lying on the ground is Y=1285/X, where Y is the number of days to become skeletonised or mummified and X is the average temperature.

In particular condition of very high or very low humidity the transformation of the body follows two different patterns: maceration and mummification.

Maceration is a secondary destructive phenomenon of a corpse submerged in an aseptic liquid environment. Since it primarily involves the fetus in case of death in womb or failure or delayed expulsion, it will not be dealt with further in this thesis.

Saponification (or adipocere formation) is a transformative phenomenon that occurs in corpse submerged in water or buried in moist soil. These environmental conditions hamper standard putrefactive processes, from the water content, the low temperature and the lack of oxygen; these result in a greyish-white tissue envelope called "adipocere". Previous work has attributed adipocere formation to the combination of fatty acids infiltrating the skin with calcium salts and alkaline bases present in the water/soil. Similar conditions occur when the corpse is placed in a tightly closed container (e.g. metal cases, plastic bags), which retain aqueous components exuded by the corpse (Pinheiro, 2006).

Mummification is a conservative process which occurs when the corpse goes towards an intense and fast liquid loss that the tissues are fixed by dehydration. Compared to saponification, there is no biochemical subversion of the cadaveric structures but rather an intense water depletion (Bereuter *et al.*, 1996).

3.2.2 Factors affecting decomposition

Despite the attempt made to standardize the phenomenon (Smith, 1986; Vass, 2001; Fiedler & Graw, 2003; Pinheiro, 2006), decomposition rate varies greatly in different circumstances and climates. The timescale of decomposition can vary even in the same corpse, as skeletonisation may occur in some areas rather than others, depending upon whether the body was partly in the shade or clothed (Saukko & Knight, 2015). Scavenging activity – invertebrate and vertebrate alike – should also be considered; carrion accessibility to insects is the second (after temperature) most important variable in body decomposition rate (Mann *et al.*, 1990; Campobasso *et al.*, 2001). High temperatures result indeed in higher activity, number and type of necrophagous insects found in association with the body (Campobasso *et al.*, 2001), speeding up the decay process. Low temperature, on the contrary, is not favourable for insect development, in particular blowflies, resulting in a slowdown of the process. (Campobasso *et al.*, 2001; Schroeder *et al.*, 2003; Sharanowski *et al.*, 2008). The presence of skin and clothes can act as a protection from adverse meteorological conditions, as well as drop of water. In addition, larvae aggregation are able to increase their habitat temperature by several degrees compared to the surrounding environment (Campobasso *et al.*, 2001; Schroeder *et al.*, 2003; Sharanowski *et al.*, 2008). The location of the body indoor or outdoor, exposed, buried or submerged, provide different accessibility to arthropods (Payne, 1972; Campobasso *et al.*, 2001; Kumara *et al.*, 2012; Bugelli *et al.*, 2015). Only few necrophagous species are indeed able to colonised buried bodies (Smith, 1986; Disney, 1994; Vanin *et al.*, 2009; Giordani *et al.*, 2018a; Giordani *et al.*, 2018b) as well as the aquatic setting generally prevent the oviposition and development of insect terrestrial species (Payne, 1972; Vance *et al.*, 1995).

3.2.3 Arthropod colonisation in exposed bodies

Mégnin (1894) described eight "*Escouades de travailleurs de la mort*": eight waves of arthropods colonising decomposition matter that can be linked to different *post mortem* conditions of the body (Fig. 4). While decomposition is a continuous process without discrete stages, some authors suggested to divide it in six stages (Payne, 1965). The subdivision into waves of colonisation is not of univocal interpretation. Matuszewski *et al.* (2010b) prefer to talk about recurring and non-recurring taxa, the first of which

appears, leaves and reappears, the second which persists on the carcass over a single time interval. It is worth mentioning that from a practical point of view these two theories do not differ.

3.2.3.1 *Fresh Stage*

This stage starts immediately after death, includes the start of autolysis, and continues until the first signs of bloating are evident. The first two waves of necrophagous insects are made up exclusively by Diptera: the first colonisers belong to the genera within Calliphoridae and Muscidae (Mégnin, 1894; Smith, 1986; Carter *et al.*, 2007), whilst flies belonging to Sarcophagidae are usually considered as part as the second wave (Smith, 1986) (Fig. 4). Due to the unclear transaction between the different insect populations, Mégnin's first two waves are often considered to be one (Smith, 1986; Gaudry, 2002).

3.2.3.2 *Bloated Stage*

The putrefactive breakdown of the body tissues results in colour change, odour and bloating of the cadaver (Vass, 2001; Dent *et al.*, 2004), and is the easiest stage to detect. The gases produced in the intestine during putrefaction give the corpse the characteristic swelling aspect, starting from the abdomen and expanding to the whole body. At this time, Calliphoridae, Muscidae and Sarcophagidae flies keep laying eggs, resulting in larvae proliferation. Coleoptera in the families Dermestidae and Staphylinidae, attracted by dried tissue, start the third colonisation waves (Fig. 4), (Mégnin, 1894; Smith, 1986; Gennard, 2007). Outcomes of tissue liquefaction become suitable also for Piophilidae (cheese skipper flies), Fanniidae (little house flies), Syrphidae (hover flies) and Sphaeroceridae (small dung flies) families, which together comprise the fourth invertebrate wave (Smith, 1986) (Fig. 4).

3.2.3.3 *Active Decay*

This stage is characterised by rapid mass loss, due to the evacuation of all the gases and fluids accumulated during putrefaction. Butyric and casein fermentations are here followed by ammoniacal fermentation. This reaction produces a blackish liquefaction of organic matters that attract a different assemblage of insects. The fifth wave usually

includes Diptera (e.g. Phoridae & Muscidae families) and Coleoptera (e.g. Silphidae & Histeridae families) orders (Smith, 1986; Carter *et al.*, 2007) (Fig. 4).

3.2.3.4 *Advanced Decay*

At this stage, mostly of the soft tissues have been removed (Payne, 1965). Advanced decay is characterised by the increase in number of beetles and by migration of Diptera larvae away from the carcass.

3.2.3.5 *Dry and Remains*

Discrimination between dry and remains stages is almost impossible (Payne, 1965). Only dry skin and cartilage, bones and hairs are present at that point. This stage shows a new cohort of insects with the dominant necrophagous ones leaving the body. The sixth wave of colonisers is made up of Arachnida in the order Acarina (Mégnin, 1894; Smith, 1986). Final stages of cadaver decomposition are driven by the seventh and eighth arthropods waves. These include larder beetles (Coleoptera: Dermestidae) and clothes moths (Lepidoptera: Tineidae) (Smith, 1986; Carter *et al.*, 2007) (Fig. 4).

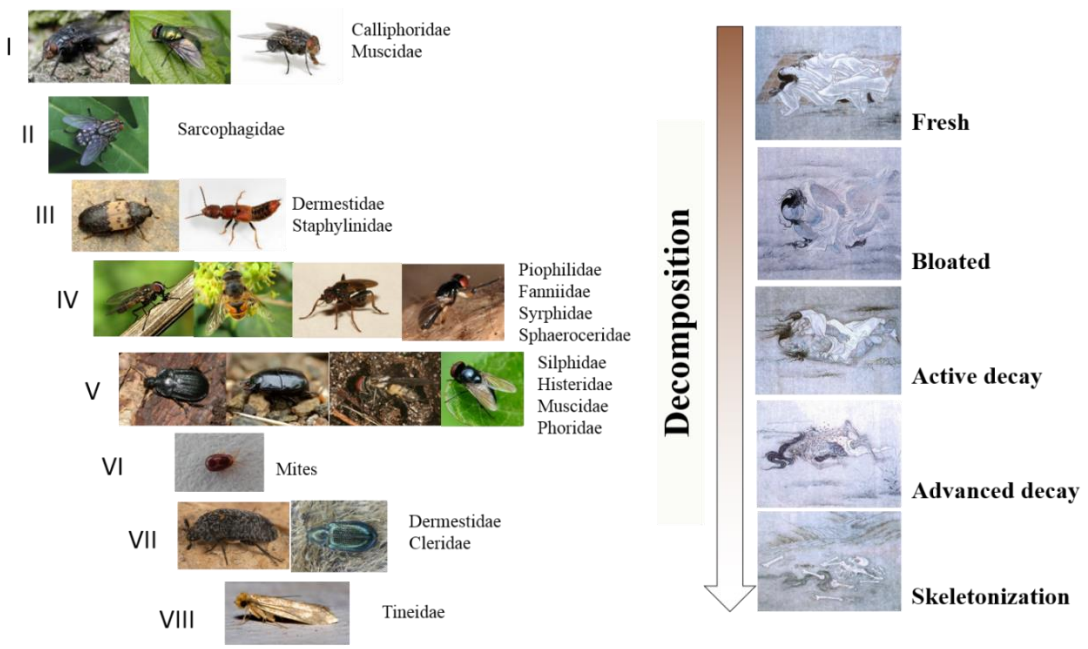

Fig. 4 **Waves of colonisation with decomposition stages.**

Despite in the last years a new approach of the total body score (TBS) has been suggested (Megyesi *et al.*, 2005), the previous mentioned "waves of colonisation" can be still considered a good way to describe insect colonisation on human or animal decomposition as reported as well in the most updated books about Forensic Entomology (Byrd & Castner, 2010; Rivers & Dahlem, 2014).

3.2.4 Arthropod colonisation of buried bodies

Buried corpse decomposition had been extensively researched by forensic entomologists (Breitmeier *et al.*, 2005; Gennard, 2007; Wilson *et al.*, 2007; Gunn & Bird, 2011). Specific sequences of insects are involved in this process, selected by environmental and decomposing conditions such as ground composition, the depth at which the body was buried, sediment temperature and the season of burial (Byrd & Castner, 2010; Breitmeier *et al.*, 2005; Gennard, 2007; Wilson *et al.*, 2007; Gunn & Bird, 2011). Furthermore, the Diptera's ability to colonise (deeply) buried bodies is extremely reduced, the main fly colonisers being Muscidae, Fanniidae, Phoridae and Sphaeroceridae (Vanin & Huchet, 2017). No Calliphoridae flies are found on corpses covered by >5cm of soil (Gennard, 2007; Gunn & Bird, 2011). The main Diptera colonisers of coffin burials are Phoridae flies (Disney, 1994; Martín-Vega *et al.*, 2011a).

3.2.5 Arthropod colonisation in "special cases"

The manner in which insects attack remains that have been burnt (Avila & Goff, 1998), immersed in water (Tomberlin & Adler, 1998) or mummified is of relevance to forensic entomology studies, especially given the difficulties of estimating time since death in such circumstances (Hart *et al.*, 2011; Vanin *et al.*, 2013). A short summary of each of these categories is provided below.

3.2.5.1 *Burnt cadavers*

The value of insects in PMI estimation from burnt bodies is currently well accepted. The colonisation pattern of burnt and unburnt body is the same but with a shift in the timeframe (Avila & Goff, 1998). The major colonisation and oviposition of burnt carcasses by adult Calliphoridae flies occur indeed one day earlier than the colonisation

of unburnt bodies (Avila & Goff, 1998; Vanin *et al.*, 2013). The burning process spreads a wide spectrum of volatile molecules that are attractive for different insect species with a more rapid colonisation of the body. Insect colonisation may also occur before the burning of the corpse. Anderson (2005) demonstrated the capacity of larvae to survive elevated temperature by moving towards the centre of the body. The presence of burnt maggots on the burned body is particularly significant for PMI estimation in cases where the time of the fire is well known (Anderson, 2005).

3.2.5.2 ***Bodies in water***

Dead bodies found in fresh, brackish or salt water can be assessed for minimum *post mortem* submergence interval (mPMSI) using aquatic insects (Catts & Goff, 1992; Haskell, 1993; Sorg *et al.*, 1997; Tomberlin & Adler, 1998). However, this is a comparatively understudied area, and no models for the process have been universally accepted (Merritt & Wallace, 2009; Fenoglio *et al.*, 2010). As aquatic insects have not evolved to develop on decomposing organic matter, the only useful information for a mPMI estimation comes from floating remains (Tomberlin & Benbow, 2015). Some seasonality is apparent; Tomberlin *et al.* (1998) show the absence of Diptera colonisers on carcasses in water during winter, and the presence of *Cochliomyia macellaria* (Fabricius, 1775), *Lucilia sericata* (Meigen, 1826), and *Sarcophaga bullata* (Parker, 1916) during summer. Survivorship of blowflies larvae in water was studied by Singh and Bala (2010), and indicates that 10-hr-old larvae show a low survival rate (<2 hrs) compared to 30/70-hr-old larvae (<4 hrs) (Singh & Bala, 2010). Because the species of flies collected from floating carcasses are the same as those on land carrion, the possibility of colonisation both before and after submersion have to be considered.

3.2.5.3 ***Mummified bodies***

As previously stated, mummification entails the preservation of skin and organs of a dead human or animal. The fast liquid loss by, intentional or accidental, exposure to chemicals, extreme cold, very low humidity, or lack of air entails the dehydration and fixation of tissues. This has implications for insect colonisation and activity. The rapid dehydration of the skin surface hinders blowfly larval infestation, and indeed mummified skin is a generally inhospitable substratum for larval development. Despite the low external

maggot activity, entry holes in skin made by maggots can be frequently observed (Campobasso *et al.*, 2001) (Fig. 5).

Fig. 5 **Mummified hand from Pachacamac site (XIII-XV century).** Red arrows indicate holes produced by insect activity.

Coleoptera play a main and essential role in contribute to the decay of a human body, particularly in the later stages of decomposition, or in mummified remains. Completely dry corpses attract a distinct beetle fauna of mainly Dermestidae, Tenebrionidae and Ptinidae (Smith, 1986). While beetle activity is normally associated with the most advanced stages of the decomposition process, they are among the first groups to infest mummified corpses. Beetles larvae and adults make characteristic holes in the mummified skin (Campobasso *et al.*, 2001). The damages to human remains by insects were clearly illustrated by Haskell *et al.* (1997) and they have been recently summarised by Viero and collaborators (2018).

3.3 Insects of forensic importance

3.3.1 Insects overview

Insects (from the Latin *insectum*) are hexapod invertebrates within the Arthropoda phylum. They represent the largest taxon of animals on earth, numbering more than one million described species, about 75% of the entire animal kingdom. Like other arthropods they are triploblastic, protostome and coelomate animals with a strongly heteronomous metamery, but all share the common characteristic of an exoskeleton divided into three

distinct regions: head, thorax and abdomen. Three pairs of legs and two pairs of wings are articulated to the thorax McAlpine *et al.* 1981) (Fig. 6).

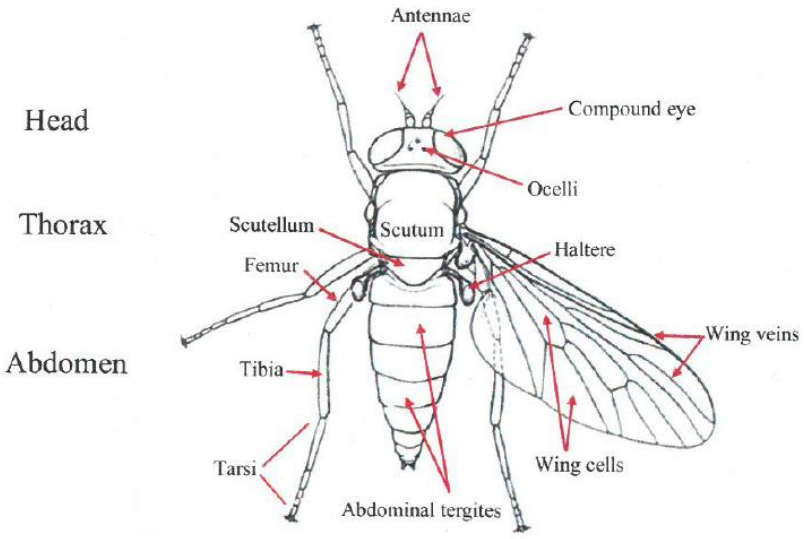

Fig. 6 **Schematic representation of an insect (fly).** In this insect the second pair of wings is transformed in halter. (From Ball, 2015).

3.3.1.1 *Morphological description*

Head

The insect head is formed by the fusion of six segments that have welded together to form a rigid and continuous covering. Structures such as the antennae, the compound eyes, often the ocelli, and the mouthparts are located here (Fig. 7). The antennae are made up of single articulated segments, bear important sense organs, and may assist in species identification. The compound eyes, ovoid or hemispherical, are placed on the sides of the head; the lenses are composed of a mosaic of visual units (ommatidia), each of which gathers a section of the visual field, so the compound eye gathers the complete image. The number of ommatidia ranges from 30,000 in sight-strong insects using sight to capture prey (e.g. the dragonfly), down to <100 in wingless insects; lice and fleas only have one. Ocelli are the only vision organs in larvae. These are primitive, simple eyes, numbering 1-3, or even absent. They are located in the frontal region, in front of the compound eyes, or on the vertex.

Insect mouthparts differ according to the species' feeding adaptation, be it chewing, siphoning, piercing, sucking or sponging. Among insects of forensic relevance, Diptera larvae, Coleoptera larvae, Coleoptera adults and Lepidoptera larvae possess chewing

mouthparts, Diptera adults have a siphoning mouthpart, while Lepidoptera adults have piercing and sucking mouthparts.

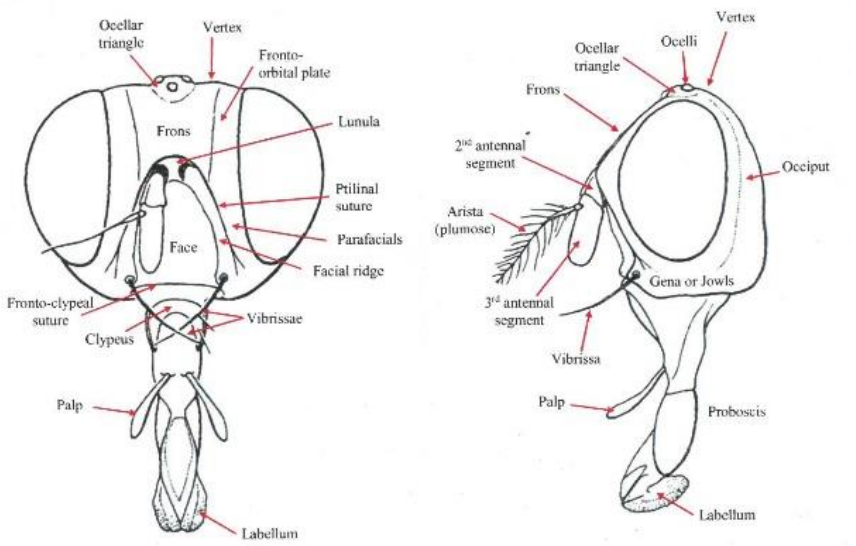

Fig. 7 **Schematic representation of Diptera head** (From Ball, 2015).

Thorax

The thorax is composed of three segments: the prothorax, the mesothorax and the metathorax, each with a pair of legs (Fig. 8).

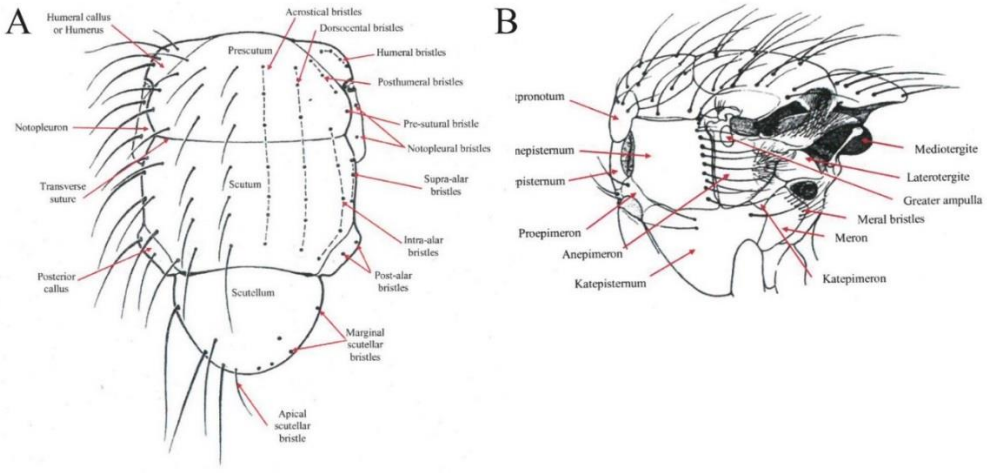

Fig. 8 **Schematic representation of Diptera thorax** (From Ball, 2015).

All the appendices are segmented, including the legs. They are made up of several segments, moving distally from the thorax: coxa, trochanter, femur, tibia and tarsi. The number of tarsal segments is variable, although commonly five per leg (Fig. 9A). At the

end of the last tarsal segment, flies have three thin suction plates, the pulvilli and the empodium, designed to allow walking on smooth surfaces such as glass (Fig. 9B). The first pair of legs (located on the prothorax) are the 'front legs', the second pair (on the mesothorax) are the 'middle legs' and the third pair (on the metathorax) are the 'hind legs'.

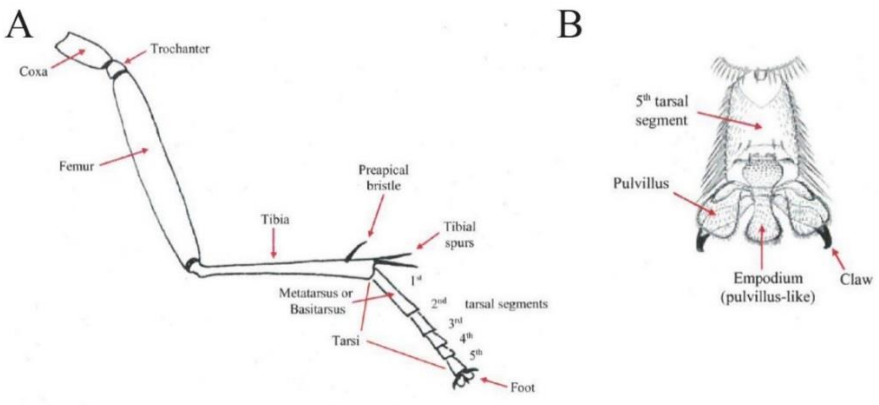

Fig. 9 **Schematic representation of Diptera leg** (From Ball, 2015).

Wings – if present – are located on the mesothorax or the metathorax. The wings are one of the most characteristic traits of the advanced insects, whether they are useful for flying or not. They derive from an expansion of the tegument (Comstock & Needham, 1899). The presence of the wing is accompanied by the thickening and sclerification of the sternum and by the folding of the tegument itself, creating a more rigid structure. Wing morphology varies between insects and may present hairs or scales; wing veins (tubular stiffening structures formed by a fold of the tegument) are useful for identification (Fig. 10). Coleoptera present a pair of modified wings (elytra), not suitable for flight but with a protective function. With few exceptions (e.g. Ephemeroptera larvae (Morihara & McCafferty, 1979)), only adult insects have completely formed wings.

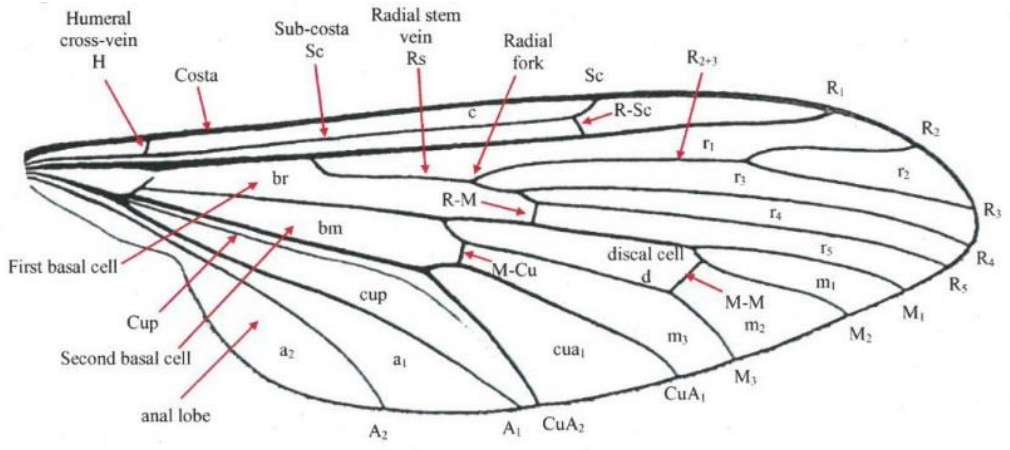

Fig. 10 **Schematic representation of a hypothetical wing** (From Ball, 2015). In nature, no insect presents a wing with this complete venation.

Abdomen

The abdomen has up to 11 segments and – with the exception of some groups (e.g. Dermaptera and Thysanura) – does not carry articulated appendages like the thorax or the head. The presence of cerci is a primitive character; they are typically reduced to rudimentary forms and are absent in the most advanced groups.

3.3.1.2 *Insect life cycle*

Insects are almost all oviparous and only a few (e.g. Aphids among the Homoptera and Sarcophagidae among Diptera) are viviparous. The eggs are equipped with a resistant shell and one or more internal membranes designed to resist various environmental conditions (Fig. 11).

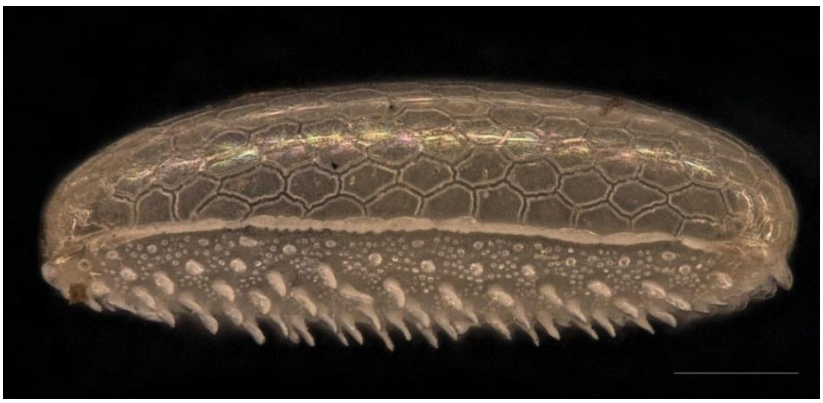

Fig. 11 **Lateral view of *Megaselia scalaris* Loew, 1866 egg** (Scale bar 100µm).

The insect that emerges from the egg rarely resembles the adult, usually lacking wings, being considerably smaller, and lacking reproductive organs. The transformation from immature to adult stages (metamorphosis) may be complete or incomplete (Fig. 12). In incomplete metamorphosis (hemimetaboly), a small version of the adult emerges from the egg (Fig. 12A); these immature forms are called neanids (or nymphs). Their rigid exoskeleton necessitates multiple moultings whenever the previous one becomes too tight. Once shed, the insect is very delicate and pale, until the new cuticle hardens. However, all insects of forensic interest are characterised by a complete metamorphosis (holometaboly) (Fig. 12B), comprising four developmental stages: egg, larva, pupa and adult. The number of larval instars varies according to the taxon. Species of forensic interest belonging to Diptera order are characterised by three larval instars.

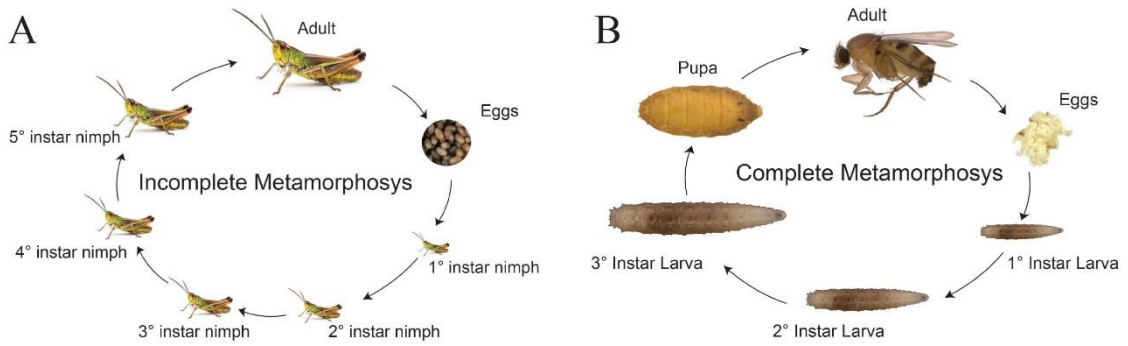

Fig. 12 **Insect life cycle.** Incomplete (A) and complete (B) metamorphosis process.

Adult tissues are represented at first by small undifferentiated cell aggregates that subsequently reorganize themselves to form the adult insect. The adult develops from these groups of cells (imaginal discs) during the pupa stage, what differs in duration between species. The puparium is constituted by the penultimate larval cuticle that becomes hard during the pupariation phase (Martín-Vega *et al.*, 2016) due to a series of chemical reactions in the chitin matrix. The pupa stage is inert, but the internal pupa undergoes profound changes and develops wings before breaking out of the pupal cage. The transformations are caused by hormone secretion from endocrine glands located on the head.

It is worth mentioning that, because of the nomenclature confusion generated in the last years by the inappropriate use of some terms, Daniel Martín-Vega in 2016 suggested the following definitions:

Puparium: hardened cuticle of the third-instar larva;

Prepupa: the insect inside the puparium is still in connection with the last larval cuticle;

Pupa: the epidermal cells of the pupa are completely separated from the last larval cuticle or puparium;

Cryptocephalic pupa: the head of the pupa is not everted;

Phanerocephalic pupa: head, legs and wings of the pupa are completely everted;

Pharate adult: the adult epidermal cells are completely separated from the pupal cuticle;

In this thesis, as commonly reported in most of the forensic entomology literature, the word pupa is used to define all the stages of cryptocephalic pupa, phanerocephalic pupa and pharate adult and the words puparium refer to the external envelope obtained by the hardening of the larval cuticle.

3.3.2 Insects associated with body decomposition

In Europe, the most frequently reported species from forensic and archaeological contexts belong to the order Diptera (families: Calliphoridae, Fanniidae, Muscidae, Phoridae and Sarcophagidae) and Coleoptera (families: Anobiidae [Anobiinae and Ptininae], Cleridae, Cryptophagidae, Rhyzophagidae, Dermestidae, Histeridae and Tenebrionidae) (Smith, 1986; Gennard, 2007; Giordani *et al.*, 2018b; Huchet, 2014b; Morrow *et al.*, 2015). Hymenoptera and Lepidoptera are also reported on occasion (Turchetto & Vanin, 2004b; Giordani *et al.*, 2018b).

3.3.2.1 ***Diptera***

Diptera – the true or two-winged flies – are richly represented in almost all environments, from sea-level to high altitude and even marine contexts. They are probably the most ubiquitous of all insects (Marshall, 2012), including about 85,000 species, generally of small and medium size (although larger Diptera have been described). Members of the

order are usually inconspicuous in terms of colour, but some of them may have a metallic sheen. Diptera are anatomically defined by two pairs of membranous wings on the mesothorax and the metathorax. The second (dorsal) pair of membranous wings have been modified into balancers (halters) which may or may not be visible. The head is free and mobile and is generally equipped with two large compound eyes and three ocelli. Antennae can be long and filiform (suborder Nematocera), or short and squat at the base (suborder Brachycera).

Current phylogenetic studies divide the Diptera into two suborders: Nematocera and Brachycera (Fig. 13). The first is characterised by a long slim body, long antennae and a complex wing venation while the second are robust flies with short antennae and less complex wing venation. Brachycera are split in three infraorders: Tabanomorpha, Asilomorpha and Muscomorpha (Marshall, 2012). For the purposes of this thesis, just the third infraorder will be described, because it includes many genera of forensic interest.

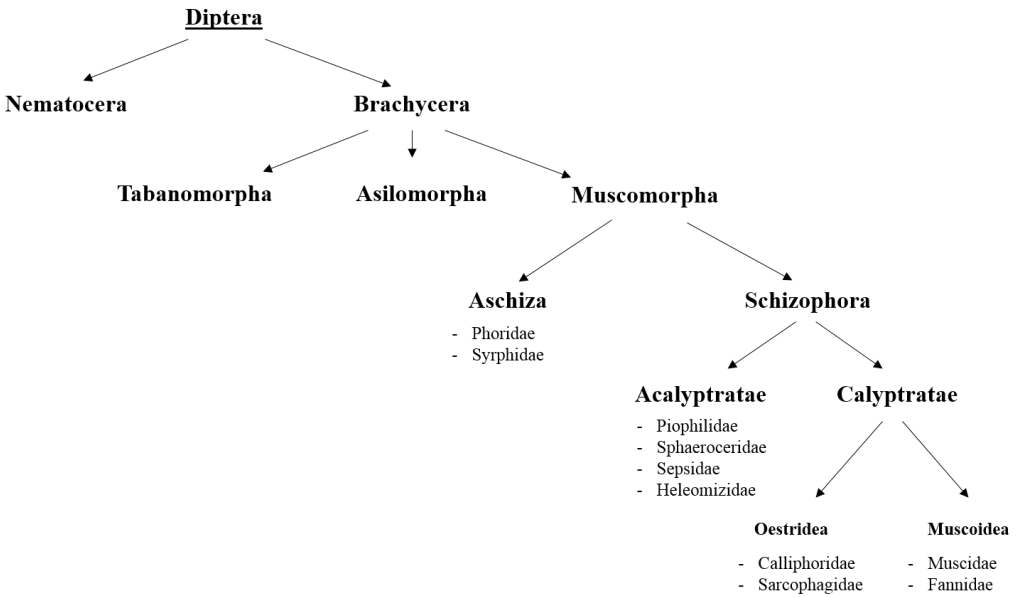

Fig. 13 **Simplified Diptera classification highlighting the most important taxa associated with human decomposition.**

Muscomorpha flies present short three-segmented antennae with a bristle on the dorsal side of the last segment. The three larval stages are characterized by the absence of legs and the absence of morphological distinction between head and body. This group is

subdivided into Aschiza and Schizophora, the last in turn divided into Acalyptratae and Calyptratae. The most important diagnostic characters for Aschiza group are the absence (or indiscernibility) of a depression over the antenna, and the presence of an anal cell in the wing. The Phoridae family of this group is of forensic interest, as are the Piophilidae, Sphaeroceridae and Sepsidae in the second group. Flies in this group have a furrow below the eyes and above the antennae (ptilinal suture), and do not show a complete suture in the thorax nor a slit in the third segment of the antennae. The halteres are exposed. Schizophora Calyptratae include flies where the halteres are concealed by two opaque flaps called the lower calypter (near the body) and upper calypter (far from the body). Calliphoridae, Sarcophagidae, Fanniidae and Muscidae families are part of this group.

Diptera diet is highly differentiated. Among the order there are hematophagous species (e.g. Culicidae, Ceratopogonidae, Tabanidae) that feed on decomposing animals and plants (e.g. Calliphoridae, Muscidae, Sarcophagidae) and species that consume sugary substances such as nectar (Bombilidae). Some species are xylophagous during the larval stage (Xylophagidae, Xylomyidae). In animals they can be vehicles of pathogens; in plants they can cause considerable damages both to crops and wild plants. Other species carry out intense parasitic and predatory activity (e.g.: Syrphidae, Asilidae, Tachinidae) against phytophagous insects.

Family of forensic importance are here listed and described in alphabetical order.

- **Calliphoridae**

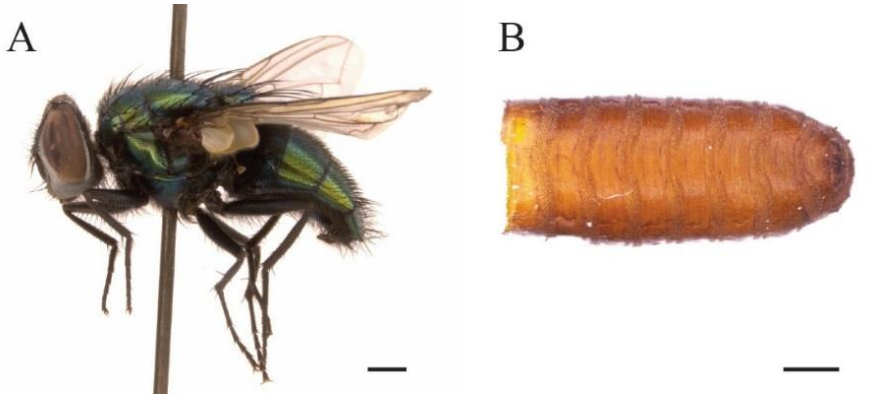

Fig. 14 **Calliphoridae.** (A) Adult, (B) Puparium (Scale bar 1mm).

The Calliphoridae family comprises small to large (4-16mm) robust flies of medical and veterinary interest. These flies are metallic blue, green, or black in colour (Fig. 14A), and noisy in flight. Commonly known as "blow flies", Calliphoridae flies owe their name from an English term for meat that had been 'flyblown' – had eggs laid on it (Marshall, 2012). Calliphoridae puparia are yellow to dark brown in colour, 6-9mm long (Fig. 14B). Larvae of Calliphoridae family, known to fisherman as "gentles", are commonly called maggots. With 1,600 valid species (Marshall, 2012) in 150 genera, Calliphorids are worldwide distributed with the exception of Antarctica (Verves, 2005; Kutty *et al.*, 2010; Singh & Wells, 2013), although they have been recorded in Sub-Antarctica (Convey *et al.*, 2010).

High number of calliphorid subfamilies have been identified: Ameniinae, Aphyssurinae, Bengaliinae, Calliphorinae, Chrysomyiinae, Helicoboscinae, Luciliinae, Melanomyinae, Mesembrinellinae, Phumosiinae, Polleniinae, Prosthetosomatinae, Rhiniinae, and Toxotarsinae (Pont, 1980; Rognes, 1991; Marshall, 2012; Singh & Wells, 2013). The monophyly of Calliphoridae family is a point of contention (Pont, 1980; Rognes, 1991, 2005; Kutty *et al.*, 2010; Singh & Wells, 2013). Several species of calliphorid flies are known to be carrion breeders and hence to be important decomposers and forensic indicators (Smith, 1986), while others are known to be vector of diseases (Greenberg, 1971) as well as responsible of myiases[1] (Stevens, 2003). Although mostly all the meat-

[1] Myasis is the infestation of alive vertebrates by dipterous larvae.

associated species are oviparous, laying hundreds or thousands of eggs depending on the species over their life, some Calliphoridae flies are larviparous or ovoviviparous.

- **Fanniidae**

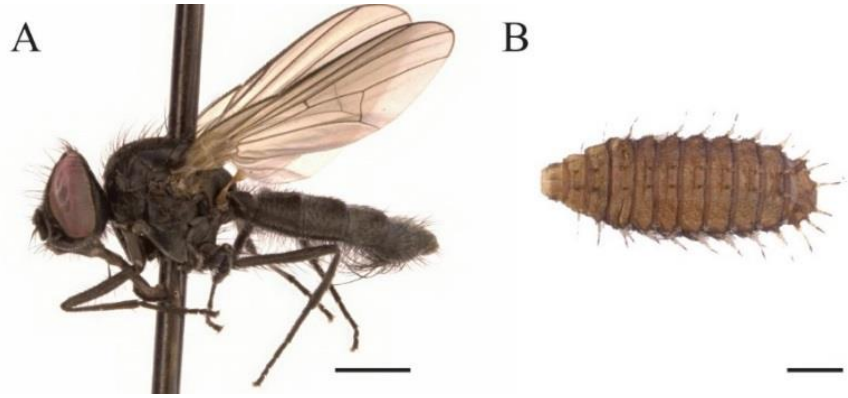

Fig. 15 **Fanniidae.** (A) Adult, (B) Puparium (Scale bar 1mm).

The Fanniidae family include grey to black, small to medium sized flies (2-5mm, some up to 9mm) (Fig. 15A). Males often congregate in small swarms whilst females tend to inhabit lower vegetation (Chillcott, 2012).

Mainly known because of their association with humans and their medical and forensic importance (Domínguez & Pont, 2014), the flies of this family are discernible from the Muscidae by the significant bending of the second anal vein (A2), the vein nearest to the upper calypter (Fig. 16A). Immature (larval and pupal) stages (Fig. 15B) are characterised by a flattened body surrounded by lateral protrusions, the number and position of which is species specific. The family includes about 335 described species (Kutty *et al.*, 2008) in four genera; their habitat is ever increasing, and spans the Holarctic regions down into the Neotropical region, with new species often being described. Adult Fanniidae flies occur mainly in or near forested areas and are relatively rare in open habitats. Some species (e.g. the lesser housefly *Fannia canicularis* (Linnaeus) and the latrine fly *Fannia scalaris* (Fabricius)) have successfully adapted to the human environment (Domínguez & Pont, 2014). Larvae are saprophagous and feed on a range of decaying organic matters including bird nests, mammal dens, rotting wood, old fungi, faeces, animal carrions and human cadavers. Few species are pests or known to cause myiasis (Hall & Smith, 1993; Derraik *et al.*, 2010).

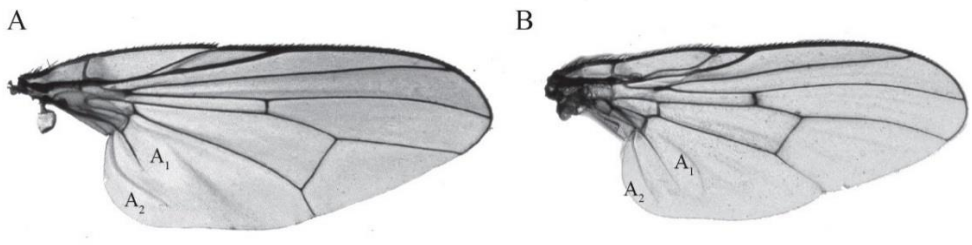

Fig. 16 **Example of differences in wing venation between Fanniidae (A) and Muscidae (B).**

- **Heleomyzidae**

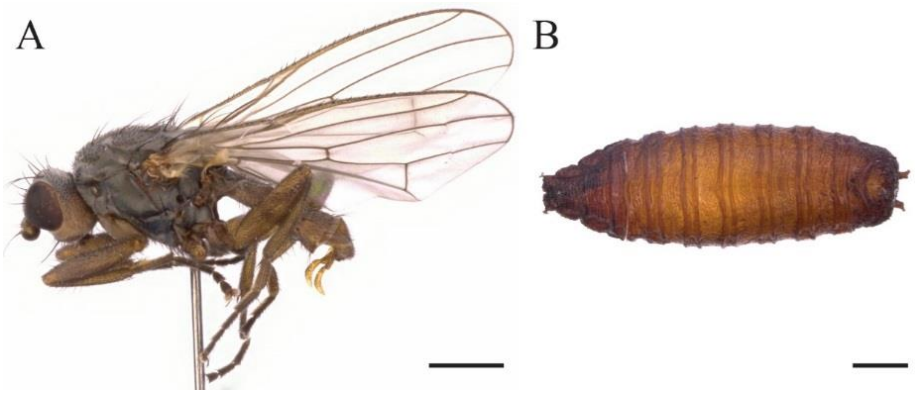

Fig. 17 **Heleomyzidae.** (A) Adult, (B) Puparium (Scale bar 1mm).

The Heleomyzidae family consists of robust flies that range from minute to large (1.2-12mm) and from yellow to dark grey or brown (Fig. 17A). Their wings often present distinctly longer bristles mixed with shorter bristles along the leading edge of the costal vein (Fig. 18). A key character of immature exemplars is the location of the posterior spiracles (Fig. 17B). The family includes approximately 600 species that occur in all biogeographical regions of the world with the exception of Antarctica; 153 European species are known (Woźnica, 2006; Lo Giudice & Józef Woźnica, 2013; Soszynska-Maj & Woznica, 2016). The family is extremely variegated with no obvious common features; however, according to Gorodkov (1984), the Palearctic Heleomyzidae group is divided into three subfamilies: the Suilliinae, Heteromyzinae and Heleomyzinae (Gorodkov, 1984; Papp, 1998; Soszynska-Maj & Woznica, 2016). Flies belonging to Suilliinae subfamily develop on mushrooms, whilst the other two subfamilies are saprophagous, developing on vegetal or animal decomposing matter (Lo Giudice & Józef Woźnica, 2013). Heleomyzidae, especially among the Heleomyzini tribe, are cold-adapted flies, show a boreo-alpine distribution (Soszynska-Maj & Woznica, 2016), and have been recorded on the surface of snow in Poland and Scandinavia (Christian & Spötl, 2010;

Soszynska-Maj & Woznica, 2016). In warmer regions such as Southern Europe, they are active mostly in autumn and winter at high altitude (above 1000m a.s.l.) or in caves (Christian & Spötl, 2010). Members of this family are often found in forests.

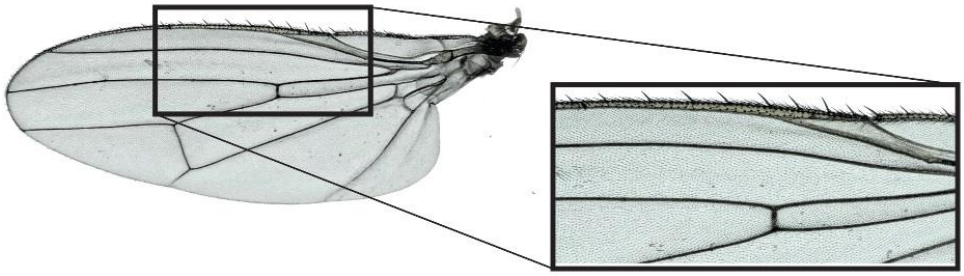

Fig. 18 **Heleomyzidae wing (left) and detail of the costal vein (right).**

- **Muscidae**

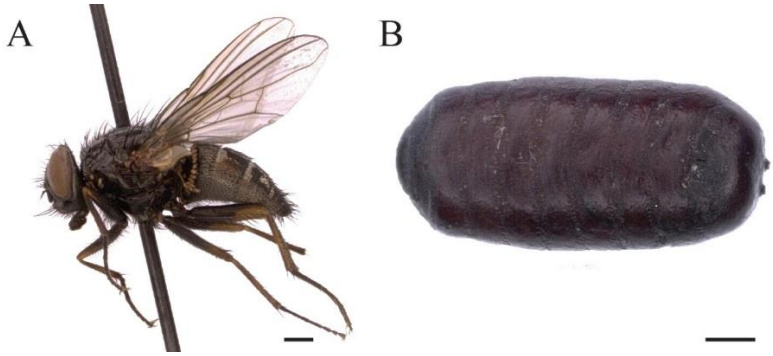

Fig. 19 **Muscidae.** (A) Adult, (B) Puparium (Scale bar 1mm).

Muscidae are small to large (2-18mm) greyish flies characterized by the presence of strips running down the whole thorax and the absence of hypopleural bristles (Gennard, 2007) (Fig. 19A). Wing anal veins are well developed but without reaching the wing border; the second anal vein (A2) is straight (Fig. 16B). The larvae and puparia are barrel-shaped (Fig. 19B), and show a large span of posterior spiracles types with slits range from straight, through sinuate to bowed (Skidmore, 1985; Gennard, 2007). The family contains around 4,500 species in 180 genera (De Carvalho *et al.*, 2005) and represent c. 5% of described Brachycera (Schuehli *et al.*, 2007).

Although Muscid monophyly is generally accepted (Schuehli *et al.*, 2007), different classifications and phylogenetic hypotheses have been proposed. The number of subfamilies ranges from 4 (Hennig, 1955-1964) to 15 (Séguy, 1937). In the most accepted classifications the family is divided in eight subfamilies: Achanthipterinae,

Atherigoninae, Azeliinae, Cyrtoneurininae, Coenosiinae, Muscinae, Mydaeinae, and Phaoniinae (Kutty *et al.*, 2008; Marshall, 2012). Species of this family are worldwide distributed (Skidmore, 1985; Marshall, 2012).

Muscidae flies are innocuous saprophagous (predators) but several genera are hematophagous (Skidmore, 1985; Kutty *et al.*, 2008; Marshall, 2012) and can act as passive vectors of diseases (Watson *et al.*, 2007; Baldacchino *et al.*, 2013). Several synanthropic and ubiquitous species are of medical and forensic interest (Klimešová *et al.*, 2016).

- **Phoridae**

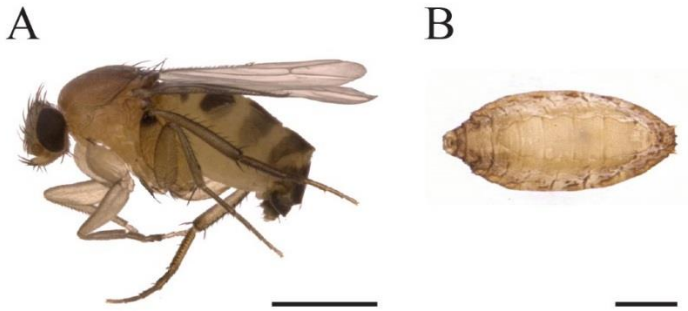

Fig. 20 **Phoridae.** (A) Adult, (B) Puparium (Scale bar 1mm).

The Phoridae are stout yet minute greyish brown or bluish flies (0.5-6mm) (Fig. 20A) characterized by a dorsoventrally flattened puparium (Fig. 20B) and a humped thorax. Wing loss is common in the family, but when wings are present, wing venation is characteristic. Commonly known as "scuttle flies", these species owe their name to their ability to run and jump; 4,000 species in nearly 260 genera are known, plus an estimated 3,000 species yet to be described (Marshall, 2012). The range of ecological backgrounds and different morphological features make this group one of the most diverse insect taxa (Disney, 1994). Most species' phenology lies between April and November, but some species are also active during the winter.

Thanks to their small size, Phoridae flies can pierce narrow gaps and reach buried corpse faster than other groups. Although they feed on a range of organic matter and animal tissues in the wild state, just two species of Phoridae – *Megaselia scalaris* (Disney 2008) and *Conicera tibialis* Schmitz 1925 – are considered to be of forensic interest (Martín-Vega *et al.*, 2011a; Zuha *et al.*, 2017). Recent studies on decomposing carcasses have

highlighted that more Phoridae flies species are likely to appear in forensic cases (Disney, 1994; Thevan *et al.*, 2010; Kumara *et al.*, 2012; Zuha *et al.*, 2014; Zuha *et al.*, 2017). Saprophagous, predators, mycetophagous, phytophagous, parasites or parasitoids species reflect the highest diversity in the family (Disney, 1994; Marshall, 2012).

- **Piophilidae**

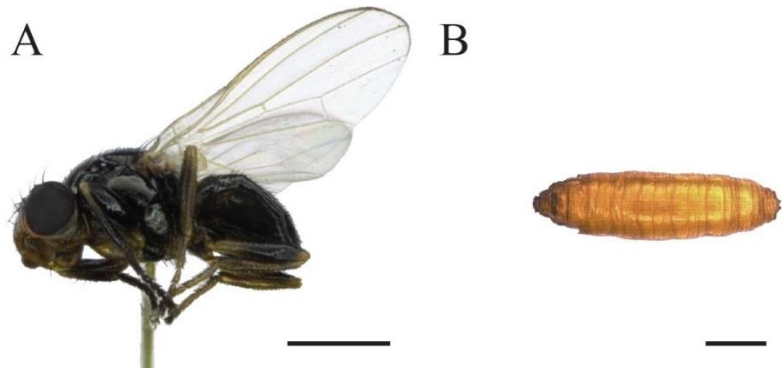

Fig. 21 **Piophilidae.** (A) Adult, (B) Puparium (Scale bar 1mm).

Flies belonging to this family – which are commonly known as "skipper flies", due to the leaping behaviour of their larvae – are small (2.5-6mm long), and yellow or brown to shiny black (Fig. 21A). This group's wings present a subcostal break (Gennard, 2007; Zajac *et al.*, 2016). Puparia are wrinkled, light to dark brown, and generally elongated in shape (Fig. 21B). The Piophilidae family comprises around 80 globally distributed species with around 40 present in the Palearctic region (Rochefort *et al.*, 2015; Zajac *et al.*, 2016).

The systematics of this family is still under discussion. McAlpine (1977) proposed a revised classification of the family with a worldwide identification key to species. Recent phylogenetic research has contributed significantly to the understanding of their systematics (Martín-Vega, 2011; Martín-Vega *et al.*, 2011b; Rochefort *et al.*, 2015).

Most described Piophilidae species are associated with high-protein dead food such as decomposing plant matter, dung, bone, garbage, fungi, bird nests and discarded antlers (Melander & Spuler, 1917; McAlpine, 1977; Bonduriansky & Brooks, 1999) as well as vertebrate animal carrion including human cadavers (Greenberg, 1991; Byrd & Castner, 2010). Several studies on carcasses around the world confirmed the association of Piophilidae flies with advanced decomposition carrion stages (Martín-Vega, 2011; Zajac

et al., 2016), although adult Piophilidae may be found on cadavers as early as 3-4 days after death (Smith, 1986). The Piophilidae family includes food industry pests such as *Piophila casei* Linnaeus, the cheese skipper.

- **Sarcophagidae**

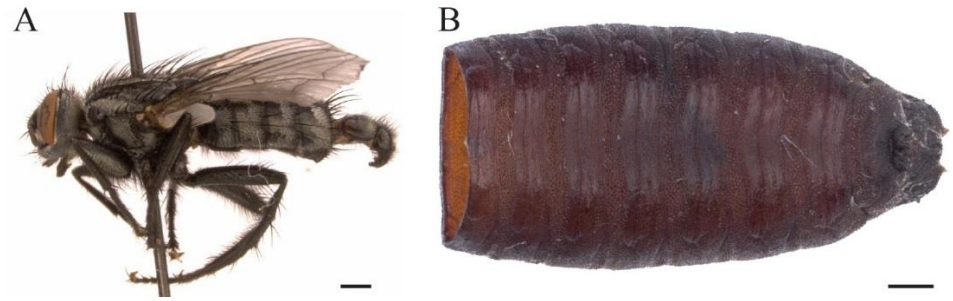

Fig. 22 **Sarcophagidae.** (A) Adult, male with extracted genitalia for identification (B) Puparium (Scale bar 1mm).

Sarcophagidae flies are commonly known as "flesh flies", which describes their dietary niche. Flesh flies are normally 10-25mm long, although smaller species of 5-10mm length are also known (Fig. 22A). Immature individuals are characterised by the posterior spiracles' position in a cavity that may be flat and almost indiscernible in some species (Fig. 22B). The family includes more than 3,000 valid species over 100 genera, and is organised into three subfamilies characterised by significant biological and morphological differences: Miltogramminae, Paramacronychiinae and Sarcophaginae (Pape, 1996; Marshall, 2012). Species of this family have a global distribution (Marshall, 2012). Sarcophaginae adults are easily recognised by the combination of a thorax with three strong black stripes and a tessellated pattern on the abdomen. Miltogramminae lack the three dorsal stripes on thorax and the checker-board pattern (Pape, 1996). Other than this, the Sarcophagidae flies are nearly uniform in appearance; species identification is largely based on the examination of male genitalia (Marshall, 2012). Sarcophagidae are mainly viviparous, and – more rarely – ovoviviparous, producing living larvae from eggs that hatch within the body (Shewell, 1987; Lopes & Leite, 1989; Meier *et al.*, 1999).

- **Sphaeroceridae**

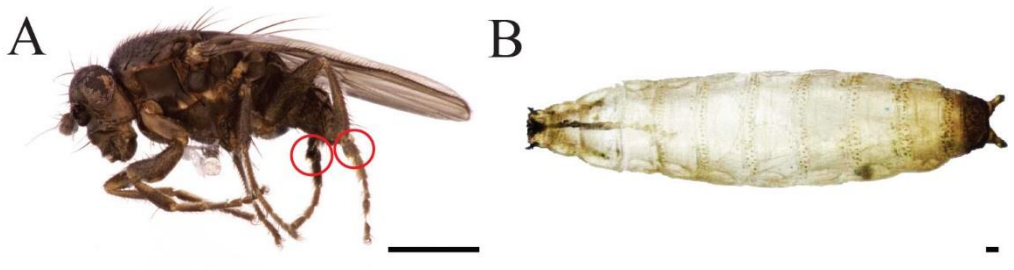

Fig. 23 **Sphaeroceridae.** (A) Adult, (B) Puparium (Scale bar 100μm). Red circles highlight the hind basitarsus, a diagnostic character of this family.

Sphaeroceridae – commonly known as lesser dung flies – are brown to blackish small but robust flies about 0.7-5.5mm long (Fig. 23A). Adults are characterised by the short, dilated hind basitarsus (Fig. 23A) (Roháček & Marshall, 1998). The Sphaeroceridae family includes about 1,500 species and many more species still await description (Marshall *et al.*, 2011). Because the higher classification of this family remains poorly resolved, McAlpine (2007) has suggested that the Heleomyzidae and Sphaeroceridae families should be reclassified as subfamilies of a large "Heteromyzidae" family. Although the Heleomyzidae family is believed to be a paraphyletic group of the Sphaeroceridae, no other taxonomist has accepted McAlpine's proposal (Marshall *et al.*, 2011). Numerous species are synanthropic. Larvae are mainly coprophagous and polysaprophagous, breeding and developing on dung, mud, forest litter, fungi, decaying vegetable matter, seaweed, in caves, in nests of birds, mammals and insects, and on carrion (Pitkin, 1988). Puparia (Fig. 23B) of this family are frequently found in archaeological contexts, but while there is extensive description of subadult members of this group, the morphological identification of archaeological samples is severely understudied. Little is known about the other developmental stages (Pitkin, 1988). Some Sphaeroceridae species can have a potential role as vector of *Salmonella* sp. (Manrique-Saide & Delfín, 1997) and one species, *Poecilosomella angulata* (Thomson, 1869) is a cause of human intestinal myiasis (Warren McKibben & Micks, 1956).

3.3.2.2 ***Coleoptera***

The order Coleoptera – with 380,000 known species – represents the largest group of insects (Zhang *et al.*, 2018) which are widespread in terrestrial, hypogeal and aquatic environments. Their dimensions are extremely variable, from very small forms (about 0.5mm) up to the largest known insects (e.g. *Titanus giganteus* Linnaeus, 1771, at about 20 cm). The order is characterised by tough elytra formation of the first pair of wings (Fig. 24) which ensure protection and guarantee lower dehydration, allowing Coleoptera to survive unfavourable environments (i.e. arid and desert areas).

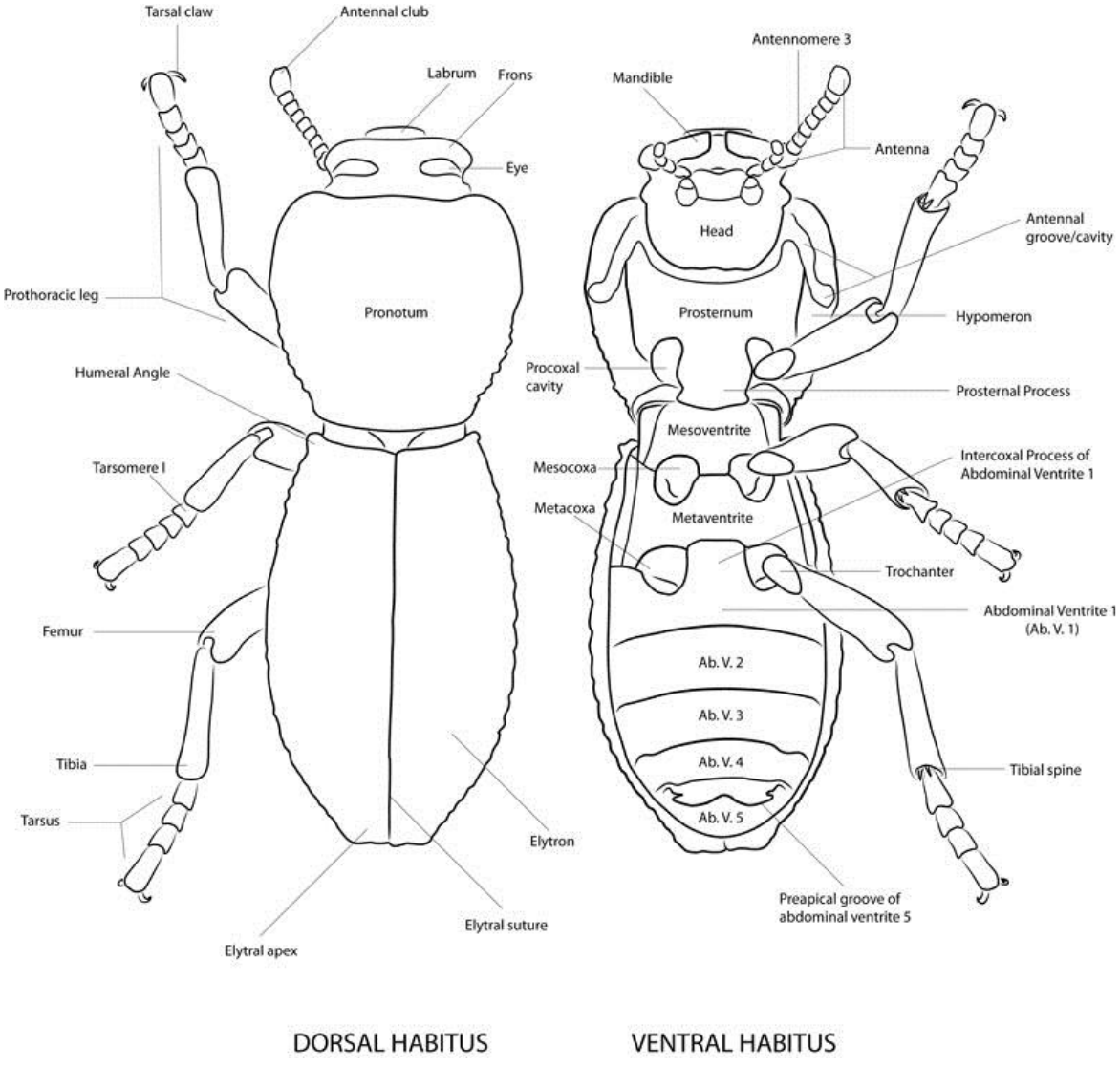

Fig. 24 **Schematic representation of a Coleoptera.** In this insect the first pair of wings is transformed into elytra (From Lord, 2011-2013).

The body is protected by a robust exoskeleton, often adorned with bristles and processes. The livery is extremely diverse and often mimetic with the environment in which they are adapted, or imitating species armed with offensive/defensive organs. The head is usually developed and recessed in the prothorax; it can carry ornaments and different processes such as horns. The buccal apparatus is adapted for chewing, and thus equipped with large, robust jaws. The prothorax is free and generally large, sometimes carrying expansions in addition to various ornamentations. The mesothorax and the metathorax are fused together and are usually covered by the elytra with the exception of the triangular mesoscutellum (Fig. 24). The mesothorax carries the elytra that protect both the second pair of membranous wings and the abdomen; in some families (e.g. Staphylinidae, Histeridae, Meloidae, etc) the elytra are short and leave the last abdominal segments uncovered. In some species the elytra are fused together to form a single protective case. The hind wings, when present, are membranous. The elytra can serve to balance flight, but their main function is typically protective. The legs vary depending on the insect's habitus: cursorial, ski jumping and swimming trips. The abdomen consists of 9-10 urites, some of which may be fused especially in the ventral part. The last abdominal segment is often uncovered and is known as the pygidium (e.g. Scarabaeidae). Reproduction is sexual with some rarely cases of parthenogenesis (e.g.: some Curculionidae), and Coleoptera are generally oviparous. Eggs are laid in isolation or in groups in the soil, on the surface or inside plant or animal tissues and in water. Coleoptera are normally holometabolic insects. The larvae, which have a well-developed head, are endowed with a sturdy chewing apparatus. The shape of the larvae is very varied and is a valid tool for their classification. The nympha usually has free appendages and is sometimes protected by a cocoon.

Coleopteran diet is extremely diverse; many are saprophagous while others are necrophagous and make a major contribution to the decomposition of organic matter; they thus have an important ecological function. Some species are predators of arthropods or other invertebrates including various phytophagous insects, making themselves useful in controlling the phytophagous populations (e.g.: Carabidae, Coccinellidae). Many Coleoptera feed on leaves, flowers, roots, fruits and seeds and woody parts of plants.

Family of forensic importance are here listed and described in alphabetic order.

- **Cleridae**

Fig. 25 **Cleridae.** Adult in dorsal view (Scale bar 1mm).

Clerids (from the Greek "kleros" - κλήρος -) were first mentioned by Aristotle to indicate a larva damaging the beehives (Löbl *et al.*, 2007). Commonly known as "ham beetles" or "checkered beetles", Cleridae comprise small or medium sized species (3-12mm), mostly elongated and with brightly-coloured liveries (Fig. 25) (Gerstmeier, 1998). Over 4,000 species spread in over 300 genera sorted in seven subfamilies are known (Lawrence & Newton, 1995; Gerstmeier, 1998). Recent work demoted three of seven subfamilies to tribe status (Bouchard *et al.*, 2011), while Opitz (2010) has proposed a reclassification into 12 subfamilies.

While possessing worldwide distribution, most species live in tropical regions, with a few species in colder latitudes. Adults are found on flowers, trees, under the barks, in warehouses and in the stores of dried and preserved animal substances as well as on bones and carcasses of dead animals (Gerstmeier, 1998). Adults lay eggs directly on the infested high-protein foods. Larvae are generally carnivorous and can live parasitically in the nests of Hymenoptera. The type of infested food defines the speed of development, which may require from 1.5 to 3+ months at the same temperature (25°C). These species can survive for long periods without food (Hill, 1997; Weston, 2002).

- **Dermestidae**

Fig. 26 **Dermestidae.** Adult in dorsal view (Scale bar 1mm)

Dermestidae beetles - commonly known as skin, larder, leather or carpet beetles - are small to medium beetles (1-10mm), hemi-spherical or hemi-cylindrical in shape, covered in scales or setae (Fig. 26) (Smith, 1986). The body is more or less elongated of dark, uniform or modestly variegated colours, and the head is small and embedded in the thorax. Larvae are characterized by a body completely covered with hairs and bristles. Mature larvae often dig tunnels inside different materials – including wood, paper, textile, masonry and even metal – and can cause serious damages to artefacts in museum collection or archaeological contexts (Charabidze *et al.*, 2014).

Approximately 1,500-1,600 species have been recorded worldwide (Háva, 2007) in six subfamilies: Dermestinae, Thorictinae, Trinodinae, Orphilinae, Attageninae and Megatominae. The name of these beetles literally means "derma feeders"; Dermestes feed on organic substances of animal origin such as skins, leather, fur, feathers and horn (Smith, 1986). Some Dermestidae species are found on animal carcasses while others are found in bird, mammal, bee or wasp nests. Some species are pests and cause extensive damages to natural fibres. Dermestes are active in warm seasons while they spend winter in the pupal chamber in adult stage or inside manufacts in larval stages (Smith, 1986). Traces of Dermestes activities are documented from dinosaurs (Britt *et al.*, 2008) and extinct mammals (Martin & West, 1995; Laudet & Antoine, 2004).

- **Histeridae**

Fig. 27 **Histeridae.** Adult in dorsal view (Scale bar 500μm)

Histerids - commonly known as clown beetles - are small to medium size beetles (Fig. 27), with just few species exceeding 20mm. The majority of genera are stocky, sub-hemispheric and ovoid, but sub-cylindrical and flat species have been reported (Vienna, 1980). The external cuticle is highly sclerified and dark in colour. The dorsal upper part is normally hairless. Both adults and larvae have very robust mouthparts (Vienna, 1980). The family comprises around 330 genera that contained 3900 species distributed worldwide (Kovarik & Caterino, 2001). Since Paykull's work in 1811, the classification has been revised many times. The latest classification records Histeridae divided into two groups: (1) Abraeinae, Trypanaeine and Trypeticinae; and (2) Chlamydopsinae, Dendrophilinae, Haeteriinae, Histerinae, Onthophilinae, Saprininae and Tribalinae (Mckenna *et al.*, 2015). Histeridae beetles live under the barks of dead trees, in tunnels of xylophagous insects, in dung, carrion, rotten mushrooms and other decomposing organic matter as well as in the nests of birds and mammals. Few species are troglobionts, while others are myrmecophilae or termitophilae (Vienna, 1980). Both larvae and adults play an important predatory action against blowfly larvae and pupae (Smith, 1986); few species are mycophagous (Vienna, 1980; Kovarik & Caterino, 2001). Histerids are regarded as a significant factor in dipterous larvae reduction in the decay of putrefactive matter (Holdaway & Evans, 1930).

- **Nitidulidae**

Fig. 28 **Nitidulidae.** Adult in dorsal view (Scale bar 1mm)

The Nitidulidae include small to medium size dark brown beetles (1.1-15mm) (Fig. 28), defined by a prognathous head and antennae located under the lateral margin of the frons. There is high variability of body shape among the genera (Audisio, 1993). The elytra can be short, leaving part of the abdomen uncovered, or long and narrow covering the whole abdomen.

This is a very large family represented by almost 4,500 species classified in ten subfamilies (Cline *et al.*, 2014) distributed in all geographical regions with a propensity for the tropics. Their variability in ecology is echoed in their extremely diverse diets, from predators to frugivores and fungivores, while others are detritivorous, necrophagous, saprophagous and herbivorous (Smith, 1986; Audisio, 1993; Cline *et al.*, 2014). Nitidulids are associated with flowers, carrion and insect nests. A few species are considered pests (Smith, 1986; Audisio, 1993). Feeding on carrion, they prefer moister skin than do the dermestids (Payne & King, 1970).

- **Ptinidae (Anobiidae)**

Fig. 29 **Ptinidae.** Adult in dorsal view (Scale bar 1mm) (From Akotsen-Mensah & Philips, 2009)

Ptinids - death watch or wood borer beetles - are small size Coleoptera about 1-9mm long, with small heads and convex, broad prothoraxes (Fig. 29). Numerous Ptinids show sexual dimorphism in body shape and size, compound eyes, vestiture and shape of the last abdominal ventrite (Arango & Young, 2012). Females (but also males of some species) can be flightless, with the fusion of elytra that completely cover the abdomen. Both adults and larvae have very robust mouthparts. Larvae are short, fleshy, and covered with hairs. The Ptinidae family includes about 3,000 species divided in 250 genera worldwide (Zahradnik, 2013). In 1995, the families Anobiidae and Ptinidae were combined in the only family Anobiidae (Lawrence & Newton, 1995). The name Anobiidae was previously utilised, in contradiction of the International Code of Zoological Nomenclature, and disregarding the "priority rule" (Zahradnik, 2013). Since the publication of the Catalogue of Palearctic Coleoptera (Borowski & Zahradníc, 2007), the name Ptinidae is used by most taxonomists.

Some species infest stored food products (grain, cereals, tobacco), furniture, and museum specimens. Ptinids larvae normally feed on well-seasoned wood (10+ years old) with low starch content as well as on a variety of dry plant (and, rarely, animal) materials, including dry dung, plant stems and dry fungi (Arango & Young, 2012). Eggs are laid on the lumber surface. Larvae burrow through the wood to feed and pupate. Adult typically emerge in warm seasons (Smith, 1986; Arango & Young, 2012).

- **Silphidae**

Fig. 30 **Silphidae.** A) Adult in dorsal view (Scale bar 1mm). Distinction between capitate (B) and clavate (C) antennae (www. thedragonflywoman.com)

Silphids - commonly known as carrion beetles - are medium to large sized (7-45mm) beetles (Fig. 30A) with very variable body shapes and a worldwide distribution (Smith, 1986; Sikes, 2008). Known shape variables include ovate to moderate elongate shape, and flattened to strongly convex, while colour variants include dark with characteristic red-orange-yellow marks on the elytra; the high diversity of this small family is surprising (Sikes, 2005; Dekeirsschieter *et al.*, 2011). The elytra are often shorter than the body, leaving 1 to 5 abdominal segments uncovered. The body length of mature larvae ranges from 12 to 40mm (Dekeirsschieter *et al.*, 2011). The family includes about 200 species divided in two subfamilies: Silphinae and Nicrophorinae (Sikes, 2008). The antennae are often used to discriminate the two subfamilies: they are both eleven-segmented, but are capitate in the Nicrophorinae (Fig. 30B) and clavate in the Silphinae (Fig. 30C) (Hastir & Gaspar, 2001). Additionally, the elytra are punctuate and truncate in *Necrodes* Leach, 1815 (Silphinae) and Nicrophorinae, but not in other Silphinae (Sikes, 2005; Dekeirsschieter *et al.*, 2011).

As their name suggests, most Silphids feed on carrion (Smith, 1986), and predate fly eggs, maggots or other small necrophilous species of beetles (Hastir & Gaspar, 2001; Sikes, 2005, 2008). Despite the common name, not all the species are necrophagous. Some species in the subfamily Silphinae are phytophagous or found in dung or fungi (Sikes, 2008; Dekeirsschieter *et al.*, 2011).

- **Staphylinidae**

Fig. 31 **Staphylinidae**. Adult in dorsal view (Scale bar 1mm).

Staphylinids - commonly known as rove beetles - are elongated in shape (Fig. 31), varying in size from about 1 to 35mm. They show a wide range in colour, from yellow and red to reddish-brown to brown to black to iridescent blue and green (Betz *et al.*, 2018). The family is generally characterized by non-clubbed antenna and very short elytra leaving more than half their abdomens uncovered. Despite the small elytra, most species fly well owing to the large hind wings (Betz *et al.*, 2018).

Currently recognized as the largest family of Coleoptera, the Staphylinidae family counts about 63,000 species, in thousands of genera in almost all habitats and parts of the world (Betz *et al.*, 2018). Mainly predators of other invertebrates, many species are also pollinivore, coprophagous, necrophagous or more generally saprophagous, contributing to animal and vegetal decomposition. Staphylinids (both larvae and adults) are the commonest predators of Diptera larvae and pupae found on cadavers (Smith, 1986). Active both day and night (Payne & King, 1970), subadults arrive to feed on maggots infesting carcasses in experiments with buried pigs (Payne & King, 1970).

- **Tenebrionidae**

Fig. 32 **Tenebrionidae**. Adult in dorsal view (Scale bar 1mm)

Tenebrionids - ground beetles, or darkling beetles - are brownish Coleoptera, either dull or shining, with a well sclerified tegument (Fig. 32). They can be elongated or oval, flat, cylindrical or rounded, from 1.3 to 40mm long (Novák, 2014). The common character that unites the Tenebrionidae family is the heteromerous arrangement of tarsi that follows the pattern 5-5-4 (Novák, 2014). In some species the elytra can be fused together so the insect cannot fly; in others the hind wings are missing. Most Tenebrionids move by walking. The larvae are elongated and cylindrical in shape with a round head that presents short, thick, easily-recognised mandibles (Watt, 1974; Novák, 2014). While found worldwide, they mainly occupy ecological niches in deserts and forests. The Tenebrionidae family comprises approximately 15,000 described species in eight subfamilies: Alleculinae, Coelometopinae, Diaperinae, Lagriinae, Palorinae, Phrenapatinae, Pimeliinae and Tenebrioninae (Watt, 1974).

Commonly associated with bird nests and fungi, the members of this family are also pests that attack stored foodstuffs, especially meal and flour. Most tenebrionids feed on decayed plant and wood materials, and sometimes decomposing animal remains. Tenebrionidae infest human corpses at the same time as Ptinidae, during the eight waves of colonisation (Fig. 4) (Smith, 1986).

3.3.2.3 ***Lepidoptera***

The order Lepidoptera includes 157,000 known species of butterflies and moths (Van Nieukerken *et al.*, 2016). The word Lepidoptera literally means "wings with scales", referring to the minute scales that cover the wings and the body and give them their typical colour. Lepidoptera have two pairs of membranous wings, the anterior generally being bigger than the posterior. The larval stage, the caterpillar, is the only one characterized by a chewing mouthpart (Chinery *et al.*, 1987), while adult butterflies feed on liquids through a proboscis (or haustellum) (Smith, 1986). The haustellum can be as long as the whole body and is normally wrapped in a spiral under the head when it is not in use. Although all Lepidoptera adults are attracted by the products of body decomposition (Payne & King, 1969; Sevastopulo, 1974; Smith, 1986), some species are exclusively carrion feeders at their larval stage (Smith, 1986). Only two families in the order appear to be regularly associated with carrion: Pyralidae and Tineidae (Smith, 1986).

- **Pyralidae**

Pyralids (Latreille, 1802) number around 6,000 species in c.1,000 genera (Robinson, 2009), including numerous small or medium-sized crepuscular and nocturnal species adapted to live in different environments. Despite being widely distributed, Pyralids show a preference for warmer countries (Solis & Mitter, 1992). *Aglossa caprealis* (Hübner, 1809) and *Aglossa pinguinalis* (Linnaeus, 1758) are routinely feed on decomposing carcasses and cadavers, although other members of the family sometimes consume dry animal tissues (Smith, 1986). Larvae of these species normally feed on the damp refuse of wheat stacks, maize and sedge thatch (Smith, 1986). *Aglossa caprealis*, a small reddish/brown moth, has a near universal distribution, while *A. pinguinalis* has been recorded in Europe, North Africa and Cameroon (Smith, 1986).

- **Tineidae**

The Tineids are small or medium sized moths with inconspicuous colours, tending towards grey. The family includes around 2,300 species in more than 350 genera (Robinson, 2009). The larvae are commonly known to be responsible for fabric damages, feeding on substances present in clothing and accessories made of animal origin materials (i.e. leather, wool, feathers) (Antonie & Teodorescu, 2009). Adult Tineids' wings are

arranged in a roof-like form over the body when at rest (New, 2008). Among the most common Tineids, *Tinea pellionella* Linnaeus, 1758 (case-bearing clothes moth), *Tineola bisselliella* (Hummel, 1823) (webbing clothes moth) and *Monopis laevigella* (Denis & Schiffermüller, 1775) (skin moth) are of forensic and archaeological interest (Smith, 1986). The eighth waves of carrion colonisation are made up largely by Tineidae species (Fig. 4).

3.3.2.4 ***Hymenoptera***

The order Hymenoptera (from the ancient Greek ὑμήν, hymèn = membrane and πτερόν, pteròn = wing) counts over 150,000 species (Aguiar *et al.*, 2013), including ants, wasps, bees and bumblebees. The first Hymenoptera fossils are recorded from the Jurassic (Rasnttsyn & Haichun, 2010). The members of this order have four membranous and transparent wings, the posterior ones connected to the bigger front ones through a series of hooks placed under the anterior border to form a single bearing surface (Smith, 1986). Some species can be wingless or can have reduced wings. The front legs are sometimes modified to dig or to grasp; the rear (and sometimes middle) legs are used to collect pollen (Roberts & Vallespir, 1978; Gauld *et al.*, 1988; Müller, 1995). While some species feed on pollen, adult Hymenoptera feed on liquids such as nectar, nectar-like liquids and carrion body fluids. Insect flesh (mostly caterpillars) and spiders are the main nourishment for wasp larvae (Hunt, 1991; Gomes *et al.*, 2007). Although a few species are harmful to agriculture, most Hymenoptera are useful to humans, either directly (the production of honey and wax) or indirectly, as a pollinator of cultivated plants or as predators of harmful insects (Southwick & Southwick Jr, 1992; LaSalle, 1993). The order is traditionally subdivided into two suborders – Symphyta and Apocrita – with 27 superfamilies and 132 families (Aguiar *et al.*, 2013). The most important species in this taxon (insofar as forensics and archaeology are concerned) are the ants in the family Formicidae and the parasitoids in the family Pteromalidae. The Vespidaea family can also play a role predating maggots feeding on the bodies.

- **Formicidae**

The Formicidae is a family of Hymenoptera in the suborder Apocrita that includes around 12,000 species ranging in size from less than one millimetre to over four centimetres,

spread all over the world, and capable of extensive migrations (Gauld *et al.*, 1988; Aguiar *et al.*, 2013). Ants can be found at every stage of carrion decomposition, as predators of necrophagous insects or as direct scavengers feeding on body fluids (Smith, 1986). Members of the Formicidae family represent the dominant colonisation fauna in buried corpses, notably with flies in the families Sphaeroceridae and Phoridae (see 3.3.2.1) (Payne *et al.*, 1968). The feeding action of ants on fresh corpses may result in small gnawed holes that can be misinterpreted as consequences of strong acids, or *ante mortem* injuries (Smith, 1986; Campobasso *et al.*, 2009; Viero *et al.*, 2018).

- **Pteromalidae**

This family includes around 3,500 species (Aguiar *et al.*, 2013) which are very homogeneous in appearance and extremely difficult to differentiate (Gauld *et al.*, 1988). Its members are parasitoids of almost all insect orders, both directly and as hyperparasites. Since parasitism can alter the physiological development of the host, the presence of Pteromalidae Hymenoptera can have forensic significance in mPMI estimation (See 3.1.1.1) (Smith, 1986). The most forensically important species in this taxon is *Nasonia vitripennis* (Walker, 1836) reported from several cases in Europe (Grassberger & Frank, 2003; Turchetto & Vanin, 2004b).

3.4 Arthropods other than insects of forensic importance

Insects are not the only arthropods to be involved in body decomposition. Members of other classes – such as Diplopoda, Arachnida and Crustacea – are sometimes found on organic decomposing matter, and may be of forensic interest (Smith, 1986). Millipedes (Diplopoda) colonise both exposed and buried carrion, mainly within the ultimate stages of "dry" and "remains". *Cambala annulata* (Say, 1821) is also frequently found during the "advanced decay" (Smith, 1986). Mites (Acarina) are also associated with the later stages of decomposition, recorded on human cadavers 6-12 months after death (Mégnin, 1894; Smith, 1986; Perotti *et al.*, 2010). Most mites are wingless (possessing 6 legs as subadults and 8 as adults), and are often transported to the carrion by flies (see 3.3.2.1) and beetles (see 3.3.2.2), or were already present in the vegetation or soil (Smith, 1986). Some Arachnida (spiders = Aranea) are found on carrions, where they mostly predate on maggots and other insects (Smith, 1986).

3.5 Morphological & molecular insect identification

3.5.1 Morphological identification

Morphology has long been the standard means of defining species, and identifying them in palaeontological, taxonomic and forensic cases. It is the primary approach used by most palaeoentomologists and has become more common as analysts avoid destructive and expensive – not to mention often inefficient/inconclusive – molecular approaches. Optical microscopy, and then scanning electron microscopy (SEM) have overcome some limitations linked to the study of small detail or small species, with obvious relevance to palaeoentomology. The morphological method is currently based on ever more refined anatomical scoring, based on dichotomous keys, an analytical tool that allows reliable scoring of organism characteristics. This system progresses through scoring of peculiar characteristics, and the definitions of morphological, macroscopic or microscopic characters. The analyst must 'answer' a series of questions concerning the presence, shape, or colour of a structure, which are presented in the form of choices. This means of proceeding results in the elimination of one option at each step. The terminals in the key may be of any rank: families, genera, or species. It is necessary to check the identification against some form of description (Gullan & Cranston, 2014).

If no identification keys are available, the identification can be done by comparison with the specimen used for the description of the species (taxon) under study, the *typus*, or with specimens preserved in museum and reference collections.

When dealing with archaeological material, the proper use of identification keys become harder as many diagnostic body parts may be missing. For this reason, most archaeological insects are identified only at the genus level, although exceptional preservation (especially in dry or waterlogged sediments[2]) may improve their recovery and identification (Reilly, 2003). Adequate reference collections are of course vital for such investigations.

[2] This will inevitably favour the recovery and identification of certain groups of insects – i.e. those that tend to inhabit such environments

3.5.1.1 ***Microscopy***

Microscopy allows the analyst to view objects that are not within the visible range of the human eye (<0.1mm), and includes optical, electron or scanning probe microscopy. Optical and electron microscopy operates with diffraction, reflection, or refraction respectively of electromagnetic radiation (light) and electron beams (electrons) on the sample. The collection of scattered radiation leads to the creation of the image. Scanning probe microscopy operates on the basis of a scanning probe interacting with the surface of the sample.

- **Optical microscopy**

The theory of microscopes is based on the laws of optics. Optical microscopes use the wavelength range of visible light and a system of lenses to magnify images of small subjects. The simple microscope was the first type of microscope to be created and consists of a lens (or a series of converging lenses) to create an enlarged virtual image of an object placed before the lens. Single-lens observations are hampered by various phenomena, such as colourations and blurred images, which must be totally eliminated.

Zacharias Janssen invented the compound microscope at the beginning of XVII century. The device revolves around a lens that gives an enlarged and inverted real image of the object, observed by means of the eyepiece, which itself acts as a simple optical microscope. The object is placed just beyond the focus. The "compound" type has now reached a high degree of perfection and is of fundamental importance to modern scientific and technical research.

The limit of resolution (d) is a relationship between range of wavelengths (λ), the refraction index of the medium between lens and sample (n) and the aperture angle of the lens (the width of the light cone that enters the lens) (α) ($d = \frac{\lambda}{(n \operatorname{sen}\alpha)}$).

Using an immersion objective, in which the space between the sample and the target is filled with an immersion oil (refraction index lower than air) the minimum attainable limit is about 0.2 micrometres.

In the past, images were captured by photographic film, nowadays digital microscopes using CCD (CMOS and charge-coupled device) camera technology allow the capture of digital images directly on a computer screen without the need for eyepieces.

The stereomicroscope is a variation of the optical microscope, aimed at relatively low magnification observation of a sample. With this device, the natural or artificial light reflects off the surface of an object (rather than passing through it from below), making it suitable for examination of thick specimens. The magnification of some stereo microscopes can deliver up to 100×, although is usually considerably lower.

- **Electron microscopy**

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are valid alternatives to the use of visible light microscopy. The electron microscope is based on a beam of electrons that interact with the sample and bounce back to create the image. The main advantage of this type of instrument is the very high level of attainable resolution, which becomes as large as the wavelength of the light beam used. Visible light has a wavelength whose value averages around 5,000 A, while a beam of electrons accelerated to about 100 kV can boast a wavelength of about 0.05 A. This very powerful tool allows us to observe the smallest details that would be impossible to capture with the use of any optical microscope, aid is of major importance to fields as diverse as biology, metallurgy and medicine. In SEM microscopy, the electron beam that strikes the sample provokes the emission of many particles, including secondary electrons that are picked up by a special detector and transformed into electrical impulses.

In the transmission electron microscope, the electron beam passes through an area containing an artificial vacuum, and only then passes onto the sample under examination.

3.5.2 Molecular identification

Different molecular methods for species identification include classical immunological testing, including precipitation and agglutination reactions (Lowenstein, 1985; Andrasko & Rosen, 1994; Espinoza *et al.*, 1999; Czesny *et al.*, 2000) to more recent molecular genetics based approaches, including the use of mitochondrial DNA (mtDNA) (Zehner *et al.*, 1998; Bataille *et al.*, 1999; Bellis *et al.*, 2003; Branicki *et al.*, 2003; Balitzki-Korte *et al.*, 2005). Molecular biology was revolutionised in 2003 by the invention of "DNA barcoding" as a way to identify species (Hebert *et al.*, 2003), leading onto the launch of the Barcode of Life Project in 2010. The aim is to use a very short genetic sequence from a standard part of the genome as the Universal Product Code (UPC) of each species

(Hebert *et al.*, 2003; Sonet *et al.*, 2013), with obvious advantages for the identification of damaged specimens (where morphological study was impossible) and immature specimens (lacking public identification keys). Barcoding identification is based on the Polymerase Chain Reaction (PCR), the molecular method for sequences amplification conceived by Mullis in 1983 (Mullis *et al.*, 1987). The agreed-upon gene region used as the standard barcode for almost all animal groups is a 648 base-pair region in the mitochondrial Cytochrome Oxidase subunit 1 gene (COI). The highly conserved flanking regions and the high inter-species and low intra-species variability makes COI sequence a good gene to identify species (Hebert *et al.*, 2003; Savolainen *et al.*, 2005; Sonet *et al.*, 2013; GilArriortua *et al.*, 2015). COI is proven to be highly effective in identifying birds (Hebert *et al.*, 2004), butterflies (Lukhtanov *et al.*, 2009), fish (Bhattacharya *et al.*, 2016), flies (Sonet *et al.*, 2013) and many other animal groups (Hebert *et al.*, 2003; Nagy *et al.*, 2012). Due to the slow evolution rate, COI is not considered to be an effective barcode region in plants (Hollingsworth *et al.*, 2009), although the combination of two gene regions in the chloroplast (*matK* and *rbcL*) are accepted as valid barcode regions for land plants (Hollingsworth *et al.*, 2009). While useful, it should be noted that barcoding should not – indeed, must not – replace standard taxonomy, but can serve as a new tool in the taxonomist's toolbox to supplement extant knowledge as well as being an innovative device for non-experts to obtain a quick identification (especially when traditional taxonomy is not possible).

3.5.2.1 *Polymerase Chain Reaction (PCR)*

PCR is the standard method for producing a large number of copies of a specific DNA sequence and allows *in vitro* synthesis of bi-catenary DNA segments and high number amplification of the target sequence in a short amount of time. The amplification reaction starts from the enzymatic capacity of DNA polymerase I to synthesize a second filament starting from a denatured mould of DNA. The fundamental components of the reaction mixture include four deoxyribonucleotides, suitable salt and pH concentrations and oligonucleotides to act as primers. Typically, thermostable forms of DNA polymerases are used (such as the Taq polymerase, extracted from the bacterium *Thermus aquaticus* Brock & Freeze, 1969), which allows the reaction in cyclic repeats. The polymerase chain reaction is typically composed of 30-50 cycles, each made up of three steps:

I. **Denaturation:** the bi-catenary DNA is split into two separate mono-catenary filaments by heating at temperatures close to 95°C.
II. **Annealing:** the two primers are bound to the portions of mono-catenary DNA complementary to them, through the formation of hydrogen bonds.
III. **Elongation phase:** the Taq polymerase binds to the primers and uses the free nucleotides to complete the synthesis, thus determining the polymerization of new complementary chains.

The optimization of different parameters of the amplification reaction – including the DNA template, the primers and the salts concentration, the number of cycles and the choice of the annealing temperature – can improve reaction results (Mullis *et al.*, 1986; Wolcott, 1992). Other PCR settings parameters include the length of the primers, as well as the adenine/thymine (A/T) and guanine/cytosine (G/C) ratios (Hemmer, 1997). The optimisation of primer sequences for PCR is nowadays facilitated by the use of digital software (Meyer, 1995; Hemmer, 1997). The choice of a positive and negative control is fundamental to control the efficiency of PCR reaction (Mullis *et al.*, 1986; Wolcott, 1992; Karch *et al.*, 1995). Recent use of biological and genetic approaches for identification purposes has led to the proliferation of new primers targeting the same gene. The final result of this process is the existence of online datasets of different size sequences randomly positioned among the gene, leading to ever more complicated bioinformatics analyses, as in the case of the mitochondrial gene COI (Tuccia *et al.*, 2016b).

Standardisation of protocols is as important as using the same reagents if the scientific community can aspire towards results reproducibility (Mahony *et al.*, 1994; Hemmer, 1997), yet the enormous variability of scientific programmes and research programmes has hampered attempts to globally standardise the molecular identification process (Tuccia *et al.*, 2016b). Quality of the DNA used in PCR is an ever-present concern. The average size of DNA fragments represents the main discriminant of DNA "quality" (Hemmer, 1997); significantly smaller average length size of the DNA fragments in the test sample than the target sequence (amplicon length) will likely lead to negative PCR results. Further possible DNA damage caused by chemical, physical or enzymatic processes should also be considered (Hemmer, 1997), and results validity may be affected

if any such factors are overlooked. Equally, extraction methods should ensure the absence of PCR inhibitors in the DNA solution, as the presence of co-extracted inhibitors is one of the major problems in subsequent sample analyses. PCR inhibitors include various components of body fluids, organic and phenolic compounds, glycogen, fats, collagen and environmental compounds such as humic acids and heavy metals (Wilson, 1997).

3.5.2.2 ***Sequencing***

The term 'sequencing' describes the decoding of the exact nucleotide sequence of a nucleic acid (DNA or RNA), which was first done in 1977 by Maxam and Gilbert, who published their sequencing (or chemical sequencing) method for the identification of about 100 nucleotides (Maxam & Gilbert, 1977). Their work was immediately followed by Sanger's first entire sequenced genome of the bacteriophage ΦX174 (over 5,000 bases) (Sanger *et al.*, 1977a), and a methodological paper describing a new sequencing method (or enzymatic method) based on chain terminator nucleotides (Sanger *et al.*, 1977b).

The Maxam and Gilbert (1977) method was based on chemical modifications of the DNA and on the consequent cut in specific nucleotide positions but was abandoned due to methodological complexity and chemical toxicity. It was largely replaced by the Sanger method, which uses an enzyme (DNA polymerase) and modified nucleotides (dideoxinucleotidetriphosphate, ddNTPs) – lacking the hydroxyl group on the carbon 3' of the sugar – to stop the reaction in specific positions. The conformation of these artificial nucleotides prevents phosphodiesterics bonds with additional nucleotides. Four separate reactions are set up, each of which contains – in addition to the DNA template – a primer, the DNA polymerase and all four dNTPs. For each of these reactions only one of the four ddNTPs (labelled with radioactive isotope) is added in stoichiometrically lower amounts than normal dNTPs, to allow filament elongation sufficient for analysis (Sanger *et al.*, 1977b). The incorporation of a dideoxynucleotide during the elongation phase causes a premature termination of the process. The four reactions were run on polyacrylamide gels, where the DNA fragments of different length could be discriminated, even of a single base difference and detected by autoradiography. The relative positions of the bands in the four lanes were used to read the sequence. This method for nucleic acid sequencing led to Frederick Sanger receiving the second Nobel

Prize in chemistry of his career in 1980. The GenBank project was launched in 1982, a database created by the NCBI (National Center for Biotechnology Information) to gather and publish all the sequences in the public domain (Strasser, 2011). The method was increasingly refined, substituting the radioactive isotope with fluorophores, and relative detection through capillary-based electrophoresis (Heather & Chain, 2016) in automated systems. In 1986 the Applied Biosystems Incorporated marketed the first automatic sequencer, invented by Leroy Hood (Hunkapiller *et al.*, 1991).

Technologies were then created to allow the simultaneous sequencing of large numbers of DNA fragments. The so-called platforms for Next Generation Sequencing (NGS) generate high numbers of sequential parallel reads, spatially separated in a flow cell. Such platforms allow sequencing from hundreds of millions to billions of DNA base pairs in a single analytical session (Ronaghi *et al.*, 1998). Second-generation sequencing was developed by Pål Nyrén and colleagues who developed pyrosequencing: the combination of micro-compartment DNA amplification, and sequencing based on chemiluminescent detection of pyrophosphate released during the dNTP incorporation by DNA polymerase (Nyrén *et al.*, 1993; Ronaghi *et al.*, 1998; Tawfik & Griffiths, 1998). The method became commercially available in 2005, with the GS20 platform (developed by 454 Life Sciences), while the second version of 454 technology (GS FLX) appeared in 2007 (Voelkerding *et al.*, 2009).

In following years, different platforms were developed based on different sequencing methods. All NGS platforms are characterized by a first library preparation, clonal amplification, and sequencing of the sample and a second computer phase of analysis of the data obtained. The sample preparation is a step common to all platforms and consists of fragmentation of genomic DNA and on the ligation of specific adapters necessary for the following amplification phase (Mardis, 2008). PCR in emulsion or on solid phase are the two strategies used in NGS. The PCR in emulsion, described for the first time by Tawfik and Griffiths (1998), establishes the clonal amplification of a single strand DNA in micro-compartments consisting of water and oil mixtures through a marble technology able to covalently binds DNA. Solid phase amplification, as well as the PCR in emulsion, provides for DNA fragments to bind complementary sequences of the adapter based, in this case, on a solid surface similar to a slide. The amplification of the fragments occurs through the formation of a bridge due to the folding DNA strands that hybridize to an

adjacent anchor nucleotide in the slide. The principle on which the sequencing and the acquisition of the image is based are the peculiar steps that characterise the different platforms on the market (Metzker, 2010).

- **Sequence for cyclic reversible termination (CRT)**. CRT uses reversibly labelled terminator nucleotides. Each cycle of sequencing is made up of three steps: 1) the incorporation of the nucleotide, 2) the acquisition of the fluorescence and 3) the nucleotide cutting. The addition – by DNA polymerase – of a modified nucleotide complementary to the template, causes the interruption of the extension phase. Subsequent washing steps ensure the elimination of all non-incorporated nucleotides. After the identification of the inserted nucleotide followed by the cleavage of the terminator group, the polymerase can continue the extension reaction and tie to the second nucleotide. Two current commercial platforms – Illumina and Helicos – use this process. While Illumina uses fragments amplified clonally on a solid surface, Helicos is currently the only one commercial platform able to use single non-amplified DNA molecules (Milos, 2008; Metzker, 2010). The Illumina platform uses simultaneously the four reversible terminators labelled with different fluorochromes, while the Helicos platform labels the nucleotides with the same fluorochrome, which are released in a determined hierarchical order (Metzker, 2010).

- **Sequencing by ligation.** This method differs from the previous one in the use of DNA ligase and for the use of coding probes. Each probe consists of 8bp in which, in the 3 '→ 5' direction, two bases are specific nucleotides (consisting of one of 16 possible 2-base combinations of A, T, C, D), three degenerated nucleotides and three bases for the fluorochrome binding. The reaction is made up of a thermostable ligase and of the 16 probes representing all combinations of two possible bases at 3' position. After the first ligation, a washing step ensures the elimination of the non-hybridized probes. After fluorescence optical detection, the labelled portion of the probe is cleaved to regenerate a free phosphate group at 5' and restart the cyclic reaction. This type of sequencing is characteristic of the Applied Byosistem Solid Platform, which uses an emulsion PCR as amplification method (Voelkerding *et al.*, 2009).

- Pyrosequencing. This is a technique based on the detection of pyrophosphate ions (PPi) released during DNA synthesis. The PPi released after the incorporation of a nucleotide is converted into ATP by the ATP sulfurylase. The energy produced is then used by the luciferase for the oxidation of a luciferin molecule via light generation (Ronaghi, 2001). Although the light emitted is directly proportional to the amount of nucleotides incorporated, the incorporation of homopolymers can increase the sensitivity of the detector, causing errors in sequencing (Mardis, 2008). The input signal is the same for all four nucleotides. The sequence of the template can be determined based on the known nucleotide dispensed in the reaction. After each cycle the apyrase removes all unincorporated nucleotides in the reaction (Ronaghi, 2001). This technology, as already mentioned, was the first to be placed on the market, combining pyrosequencing to PCR amplification in emulsion, and is characteristic of the Roche 454 platform.

- Sequencing by semiconduction. This technique – specific to the Ion platform Torrent (Applied Biosystem) – is part of the third-generation technology, allowing a further reduction in cost and time. It entails the transformation of chemical into digital signals, thanks to the presence of a semiconductor chip. The incorporation of each base releases hydrogen ions. The release of the proton causes weak pH alteration of the solution, causing small differences in potential (ΔV) that can be measured. The detected signal is proportional to the number of embedded nucleotides. This methodology also consists of wash-and-scan steps (Rothberg *et al.*, 2011).

The amount of data produced by the NGS platforms (around one terabyte) presents issue for data storage and analysis. The necessary software differs according to the NGS technology used in the sequencing, but all follow a system of "pipeline" data analysis that converts acquired luminescence or fluorescence in nucleotide sequences ("reads"). This process is known as base calling and entails the assignment of a quality score to each nucleotide, which indicates the probability of error associated with it. "Quality scores" are an important tool to eliminate the background noise from the analysis process (Li *et al.*, 2008).

3.5.2.3 ***Phylogenetic analysis***

The mutational change of genes has long been recognized as a characteristic of DNA sequence evolution (Fitch & Margoliash, 1967; Fitch & Markowitz, 1970; Kocher & Wilson, 1991; Wakeley, 1993), and are therefore used in the construction of phylogenetic trees (Hillis *et al.*, 1993). Substitutions among the four nucleotides (A, T, C, G) are commonly used in phylogenetic studies to compare any group of organisms (Nei & Kumar, 2000). Nucleotide substitutions are divided into transition, substitution among purines (A↔G) or pyrimidines (T↔C), transversion, and substitution of a purine with a pyrimidine and *vice versa*. Transitional substitutions are more frequent than transversional substitutions (Fitch & Margoliash, 1967; Kocher & Wilson, 1991; Nei & Kumar, 2000). Nucleotide insertions and deletions are commonly known as frameshift mutations, occurring particularly in non-coding DNA sequences (Nei & Kumar, 2000). All phylogenetic reconstruction methods use evolutionary models that describe the nucleotide substitution process underlying the observed differences between sequences. The measure of the genetic divergence between species is called genetic distance and is usually expressed in graphic form as a phylogenetic tree. The topology of a tree is the set of relationships described by the nodes and branches of the tree itself. Clustering methods (or distance methods [i.e. Neighbour-Joining and UPGMA]) and optimisation methods (or character method [i.e. Maximum Parsimony and Maximum Likelihood]) according to the type of algorithm used, allow the reconstruction of phylogenetic trees from molecular data and clustering algorithms. Different models of distance have been developed. Some – such as Jukes-Cantor – consider mutations equally likely, while others (i.e. the Kimura-2-parameters) assign different mutation rates for transitions and transversions (Nei & Kumar, 2000). In optimisation methods, nucleotide sites are considered to be direct descriptors of the taxonomic entities being analysed (Nei & Kumar, 2000).

- Neighbour-Joining (NJ). NJ (Saitou & Nei, 1987) is a method conceptually related to traditional group analysis (cluster analysis) but does not assume that nucleotide replacement rates are the same for all evolutionary lines (the ‘molecular clock’ concept). The phylogenetic tree is obtained by an iterative mathematical process comprising subsequent addition of taxa. Starting from the initial matrix of genetic distances, "closer" sequences are grouped to construct a new matrix that contains the two taxa merged into

a single element. This process of linking closer taxonomic units continues until all the nodes are resolved (Rzhetsky & Nei, 1993; Nei, 1996; Nei & Kumar, 2000). This method creates a tree with the minimum value of the length of the arms. Based on the principle of "minimum evolution", the best tree is the one with the minimum length of the branches (Cavalli-Sforza & Edwards, 1967).

- **Maximum Parsimony (MP).** MP (Fitch, 1971) is a method that uses all the information contained in the data operating directly on the individual characters rather than on distance matrices. Introduced by Cavalli-Sforza and Edwards (1967), the application algorithms were developed by Eck and Dayhoff (1966), Kluge and Farris (1969) and, finally, by Fitch (1971). These methods are based on the principle of maximum parsimony (Occam's razor) and operate by selecting the trees that contain the least number of evolutionary events or nucleotide substitutions. In other words, the best hypothesis is the one that requires the least number of assumptions. Parsimony analysis considers only informative sites, defined as a site where at least two different types of nucleotide substitution are detectable, each of which present at least twice in the whole set of studied sequences.

- **Maximum likelihood (ML).** Unlike previous methods that use a hypothetical-deductive point of view, ML (Felsenstein, 1981) method is based on a probabilistic model, treating the estimation of phylogeny as a statistical problem and the construction of a tree through methods of statistical inference (Siebert, 1992). It is statistically consistent by definition, and allows the use of sophisticated and realistic evolutionary models (Nei & Kumar, 2000). However, it cannot be used for large numbers of taxa (Izquierdo-Carrasco *et al.*, 2011). The likelihood (L) of a hypothesis (H) is the probability (P) of the data (D) given the hypothesis [L = P (D | H)]. In the context of phylogenetic studies, the topology under examination and its parameters represent the hypothesis, while the nucleotide or aminoacidic sequences correspond to the data observed. Thus, the likelihood is the probability of either nucleotide sequences or amino acids supporting a given topology, or a phylogenetic tree. Obviously, different hypotheses will have different values of likelihood. The ML method is able to evaluate different hypotheses and select those with higher values of likelihood. The topology that gives maximum likelihood is assumed to represent the phylogenetic tree (Nei, 1996).

Distance methods, parsimony and maximum likelihood respectively require increasing amounts of data, and increased computer power. The historic trend in phylogenetic research has therefore been from distance methods (used in late 1960s until early 1980s) to parsimony (used in late 1970s until 1990s) to an increasing use of maximum likelihood through the 1990s and the 21st century.

The degree of confidence in the results coming from the analysis of a dataset can be assessed using the bootstrap method (Efron, 1982). This technique is applied in phylogenetic studies to generate several simulated multi-alignments. The sequence resampling allows the construction of different distances matrices and phylogenetic trees (Felsenstein, 1985). The topologies of these new trees are compared to that of the original. A score of 1 (identity value) is assigned to each node of the original phylogenetic tree that corresponds to that of the tree obtained by bootstrapping. The resampling procedure results in the construction of new trees and is reiterated several times. The bootstrap index is calculated as the percentage of times in which each internal node of the original tree obtained the identity value. Nodes with bootstrap higher than 95% are considered to be statistically highly supported (Felsenstein, 1985), although node values up to a threshold of 70% reflect good confidence (Hillis & Bull, 1993). Node values between 50 % and 70% indicate moderate support (Hillis & Bull, 1993).

4 Material & Methods

4.1 Samples

A variety of insects from different contexts (forensic cases, museums, and archaeological excavations) were used to carry out the different analyses reported in this thesis. Diptera adults, open and closed Diptera puparia and Coleoptera adults of different "ages" were studied using both morphological and molecular approaches. All the specific information about the samples is reported in depth in each chapter of this thesis.

4.2 Microscopy

4.2.1 Adult samples

Observations were done using a Leica M60 stereomicroscope (Leica, Germany) or the 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). Identification was performed using specific identification keys (Table 1). A taxonomic specialist was consulted when taxonomic keys were not available. The names of the consulted specialists are reported in the relevant chapters.

Table 1 **List of identification keys used for morphological identification.**

Order	Family	Identification Key
Diptera	Calliphoridae	(Szpila, 2010)
	Sarcophagidae	(Szpila *et al.*, 2015)
	Muscidae	(Skidmore, 1985) (Grzywacz *et al.*, 2017a)
	Fanniidae	(Lyneborg, 1970) (Domínguez & Pont, 2014)
	Piophilidae	(McAlpine, 1977) (Rochefort *et al.*, 2015)
	Phoridae	(Disney, 1994)
Coleoptera		(Porta, 1923)

4.2.2 Puparia samples

A cleaning process based on a water and soap solution and a fine detail paintbrush, and a sonication for 30 seconds using a sonicator bath (QH. Kerry Ultrasonic Limited, f = 50 Hz, Guyson, UK) were applied to the puparia before any other microscopical observation. In a preliminary experiment hot 20% NaOH treatment did not improve the clarity of the observations and for this reason was not used in this thesis. Samples were air-dried and photographed 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, Japan). Puparia were places with the anal region (Fig. 33A,B) in ventral position in order to allow the comparison of the pictures of the spiracles with previous publications and to enhance visibility of identification characters. The spines mentioned on the species descriptions refer to the ventral abdominal welt of segment 7 (Fig. 33A,C).

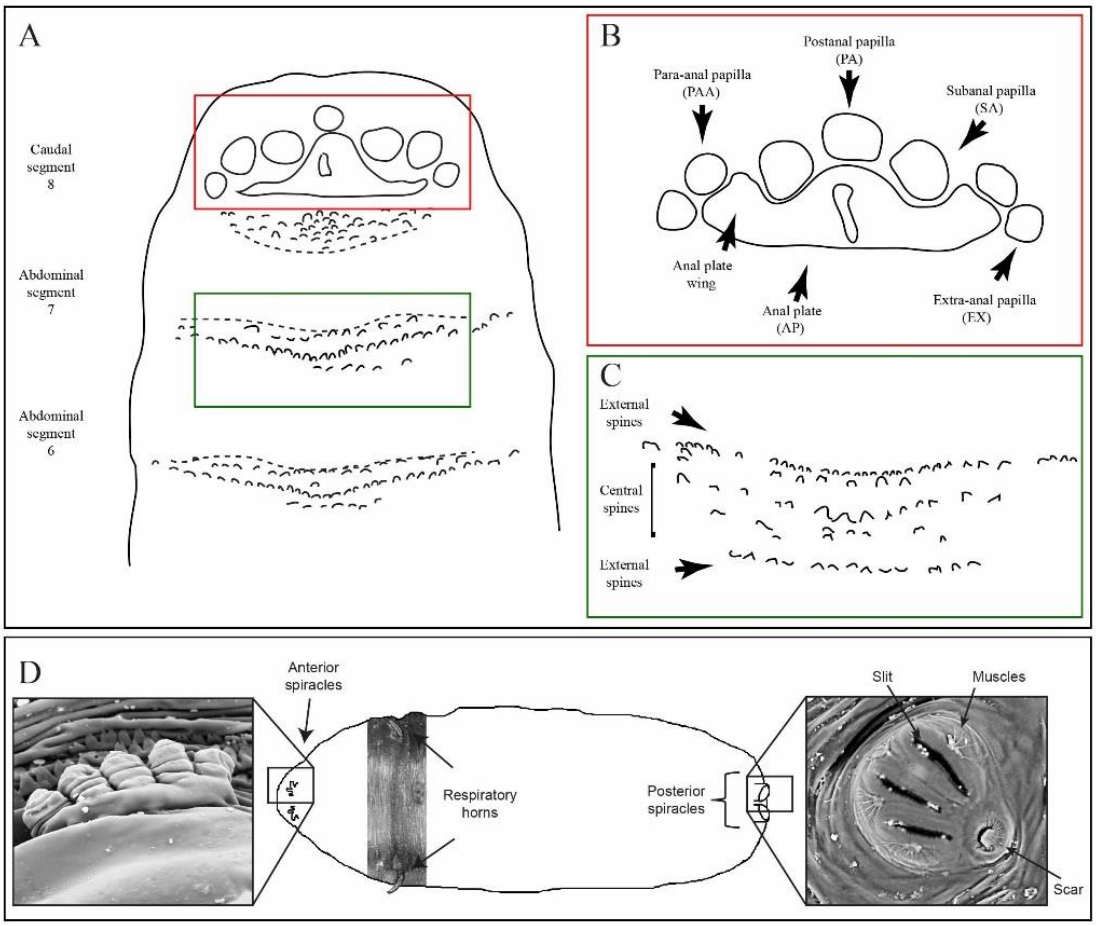

Fig. 33 **Puparium nomenclature.** A) Posterior region (segments 6-8) B) Anal region nomenclature according to Skidmore (1985) and Grzywacz *et al.* (2017a), C) nomenclature used in the description of cuticular spinose bands. (From Giordani et al. 2018a)

In addition to digital microscopy, scanning electron microscopy (SEM) observations were performed on some samples. Dried, cleaned specimens were mounted on stubs with conductive adhesive tape and coated with Au-Pd in a SC7620 Mini Sputter Coater (Quorum Technologies, UK) and observed with a FEI QUANTA 650 FEG SEM (Thermo Scientific, USA). Pictures were directly digitized from the SEM.
Terminology of morphological characters in the puparia description follows recent works on the topic (Skidmore, 1985; Martín-Vega *et al.*, 2016; Grzywacz *et al.*, 2017a; Giordani *et al.*, 2018a).

All the images and graphs presented on this thesis, if not different cited, belong to the author.

4.3 DNA extraction & quantification

Five different extraction strategies (4.3.1, 4.3.2, 4.3.3, 4.3.4, 4.3.5) were performed in order to achieve the best results in term of DNA quantity and quality. Puparia samples were carefully washed in warm water (≈60°C) with a wet tiny paintbrush. In each chapter of this thesis one or more DNA extraction methods have been applied and compared. Deeper information will follow in each section. The extracted DNA was quantified via Qubit® 3.0 Fluorometer (Thermo Scientific, Waltham, Massachusetts, USA) and analysed through 2100 Agilent Bioanalyzer (Agilent, California).

4.3.1 QIAamp® DNA Mini kit

The QIAamp® DNA Mini kit (QIAGEN, Redwood City, CA, USA) is a fast spin-column procedure designed for DNA extraction from human tissue samples, and insect samples (Rubink *et al.*, 2003; Goto *et al.*, 2006; Mazzanti *et al.*, 2010). The silica-gel membrane aims to bind the DNA that can then be used in PCR. QIAamp® DNA Mini kit was used following manufacturer protocols. The amount of proteinase K was increased from 20µl to 40µl and the incubation time was increased to 24 hours. These changes increased the total DNA yield. Two hundred microliters of buffer AE were used to elute DNA.

4.3.2 PrepFiler™ Forensic DNA Extraction Kit

The PrepFiler® Forensic DNA Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) is a magnetic particles system optimized to improving DNA yield from both routine and challenging samples while facilitating PCR inhibitors removal. The eluted DNA is ready to use in PCR. The PrepFiler® Forensic DNA Extraction Kit was used to extract DNA from challenging archaeological samples. All manufacturer procedures were followed. The extracted DNA was eluted in 50µl of PrepFiler Elution Buffer.

4.3.3 Homemade Digestion Buffer + commercial kits

Different homemade digestion buffers obtained from the specific literature were used for a 20h lysis incubation step followed by several commercial kits silica-gel membrane based as reported in Table 2. Elution was done in 40µl of EB solution.

Table 2 **Combination of homemade digestion buffer & commercial kits.**

Homemade Buffer	Commercial Kit
Gilbert *et al.* (2007) - 3mM $CaCl_2$ - 2% sodium dodecyl sulphate (SDS) - 40mM dithiotreitol (DTT) - 100mM Tris buffer (pH 8.0) - 100mM NaCl Store at room temperature Secondary add: - 250µg/ml Proteinase K	QIAquick PCR Purification Kit® (See paragraph 4.7)
Campos and Gilbert (2012) - 5mM $CaCl_2$ - 2% sodium dodecyl sulphate (SDS) - 10mM Tris buffer (pH 8.0), - 10mM NaCl - 2.5mM EDTA (pH 8.0) Store at 4°C Secondary add: - 200µg/ml Proteinase K - 40µl*ml(of digestion buffer) DTT 1M	QIAquick PCR Purification Kit® (See paragraph 4.7)
Santos *et al.* (2018) - 0.5% sodium dodecyl sulphate (SDS)	QIAamp® DNA Mini kit (See paragraph 4.3.1)

- 200mM Tris buffer pH 8 - 250mM NaCl - 25mM EDTA Store at room temperature	QIAamp® DNA Investigator kit (See paragraph 4.3.4) QIAquick PCR Purification Kit® (See paragraph 4.7)

Secondary add:
- 400µg/ml Proteinase K

4.3.4 DNeasy® PowerSoil® Kit

The DNeasy® PowerSoil® Kit (QIAGEN, Redwood City, CA, USA) aims to optimize DNA extraction from difficult environmental samples. The Inhibitor Removal Technology (IRT) removed humic acid content and other PCR inhibitors found in challenging sample types. The eluted DNA is ready to use in PCR. DNeasy® PowerSoil® Kit was used to extract DNA from samples covered by a thick layer of soil. All manufacturer procedures were followed. One hundred microliters of buffer C6 were used to elute DNA.

4.3.5 QIAamp® DNA Investigator kit

The QIAamp® DNA Investigator kit (QIAGEN, Redwood City, CA, USA) allows DNA extraction from a wide range of forensic samples. The combination of silica-gel membrane column with flexible elution volumes results in high quality genomic and mitochondrial DNA extraction from small volumes or small sizes sample. The eluted DNA is ready to be used in PCR. QIAamp® DNA Investigator kit was used to extract DNA from difficult samples with a likely low amount of DNA. All manufacturer procedures were followed. The amount of proteinase K was increased from 20µl to 40µl. Fifty to 100µl of buffer ATE were used to elute the DNA.

4.4 Purification of the extracted DNA

In order to clean the samples prior to PCR, two strategies were applied: 1) use of the OneStep™ PCR Inhibitor Removal Kit and 2) DNA precipitation with Sodium Acetate and Ethanol. The OneStep™ PCR Inhibitor Removal Kit was used following manufacturers protocol, while precipitation with Sodium Acetate and Ethanol was carried out using 0.1 volumes of 3M Sodium Acetate solution pH 5.2, 1µl Glycogen and 2

volumes of 95% EtOH added to 1 volume of DNA sample. The solution was centrifuged at maximum speed for 15 minutes. The supernatant was discarded. For the washing of the pellet, 1 ml of 70% EtOH was added and let sit for 5 minutes. The samples were centrifuged at maximum speed for 5 minutes. The supernatant was discarded, and the pellet was air-dried for 10-15 minutes at room temperature until the complete evaporation of any liquid. The DNA was re-suspended in 100μl of ultra-pure water.

4.5 Qubit assay

The Qubit® 3.0 Fluorometer (Invitrogen, USA) was used with the commercial kit " Quant-iT™ Qubit™ dsDNA High-Sensitivity Assay Kit" (Invitrogen, United States) to accurately quantify DNA. In order to minimize the effects of contaminants, the fluorescence emission occurs specifically when the dye bounds to the target molecule. The integrated design of the instrument and assays results in a quantification that is very sensitive. The kit provides concentrated assay reagents, dilution buffer, and prediluted standards. The selected kit is highly selective for double-stranded DNA, able to quantify double-stranded DNA in a range between 0.2 (200pg) and 100 ng.

4.6 Agilent Bioanalyzer® 2100

Agilent Bioanalyzer® 2100 (Agilent, Santa Clara, California, USA) allows the determination of the profile of the molecules present in a mixture of nucleic acids or proteins. It is used for quality control of DNA and RNA samples and of biomolecular reactions during the preparation of sequencing libraries. The capillary electrophoresis technique applied on a microchip device permits the evaluation of nucleic acid or protein integrity, providing a characteristic electropherogram. The small amount of sample needed (1-100ng), the digitalized output and the ability to analyse 11-12 samples in around 40 minutes are other advantages of this instrument. For this study, the Agilent Bioanalyzer 2100 and Agilent High Sensitivity Chip were used to test the integrity of DNA after the extraction process. Small amounts of charged DNA micelles were separated by electrophoresis within the microchannels, driven by a voltage gradient according to their molecular weight. The intercalation of dye molecules between the nitrogen bases of the nucleotides allows the laser-induced fluorescence detection (LIF).

This system, opposite to the traditional gel electrophoresis, leads to the detection of even small differences in concentration in the sample. A ladder made up of known DNA size and concentration is provided as a molecular weight marker and as an inner quality control of the analysis. Two DNA fragments, the lower and upper marker, are run with each of the samples.

For all DNA assays, quantification is done in relation to the upper marker. For the Agilent High Sensitivity Chip, the size range of detection is between 50–7,000bp. The 2100 Expert software automatically compares analysed samples with the upper marker and the ladder to determine the concentration and size of the fragments. The results are presented both as separate bands on the gel and as a Cartesian graph with the fluorescence unit on ordinate axis. These reflect the concentration of the sample, and the size of the DNA molecules expressed in bp on the abscissa (Fig. 34). The result pattern determines the suitability of the sample for further processing.

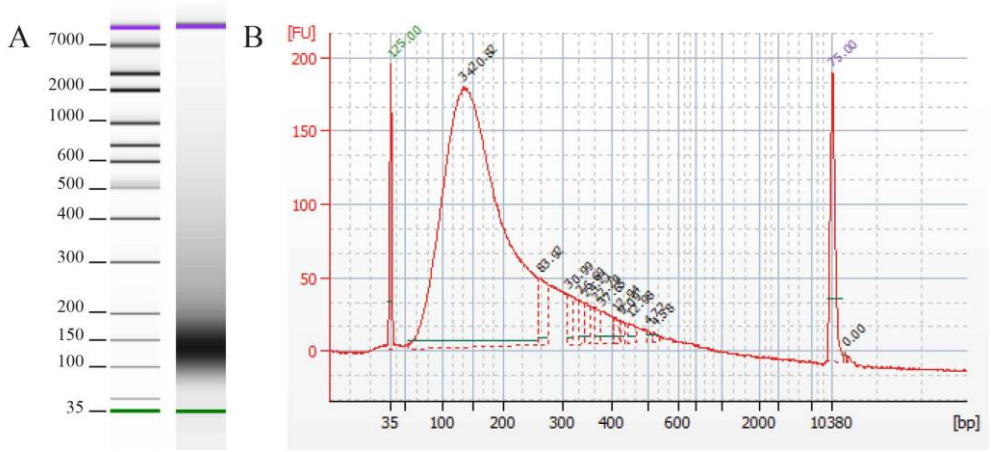

Fig. 34 **Bioanalyzer electrophoresis results**. A) Gel and B) Cartesian graph.

4.7 PCR

LCO/HCO universal primers (Folmer *et al.*, 1994) were used for the amplification of a region within COI gene, chosen by the Consortium for the Barcode of Life (CBOL). Forward and reverse primers were specifically designed in order to amplify smaller and partially overlapped portions of the gene (see 5.2.3 and 5.3.4.2). Homo-dimer and hetero-dimer analysis of oligo sequences were performed on IDT OligoAnalyzer® Tool. The targeted fragments together cover the 658bp 'DNA Barcoding' (Fig. 35).

Mcb398/mcb869 (Verma & Singh, 2003) were used for the amplification of the CytB gene.

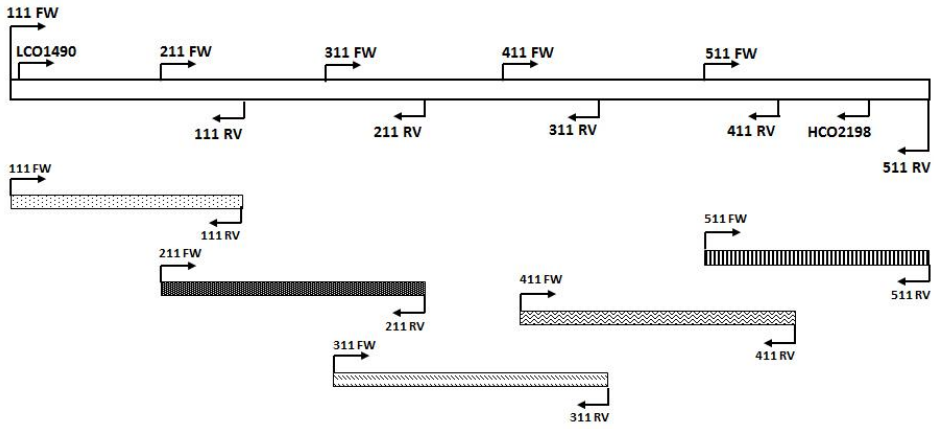

Fig. 35 **Primers scheme for concatenated sequences.** The whole sequence length corresponds to 658bp of the COI mitochondrial gene.

PROMEGA GoTaq® Flexi Polymerase protocol was followed in order to prepare a master mix reaction of 20µl final volume: 4µl of Colourless GoTaq Flexi Buffer (5×), 4 µl of $MgCl_2$ (50mM), 0.5µl of each primer (10pmol/µl), 0.5µl of Nucleotide Mix (10mM), 0.25µl GoTaq DNA Polymerase (5u/µl) and 2 to 4 µl of DNA (5-6ng).

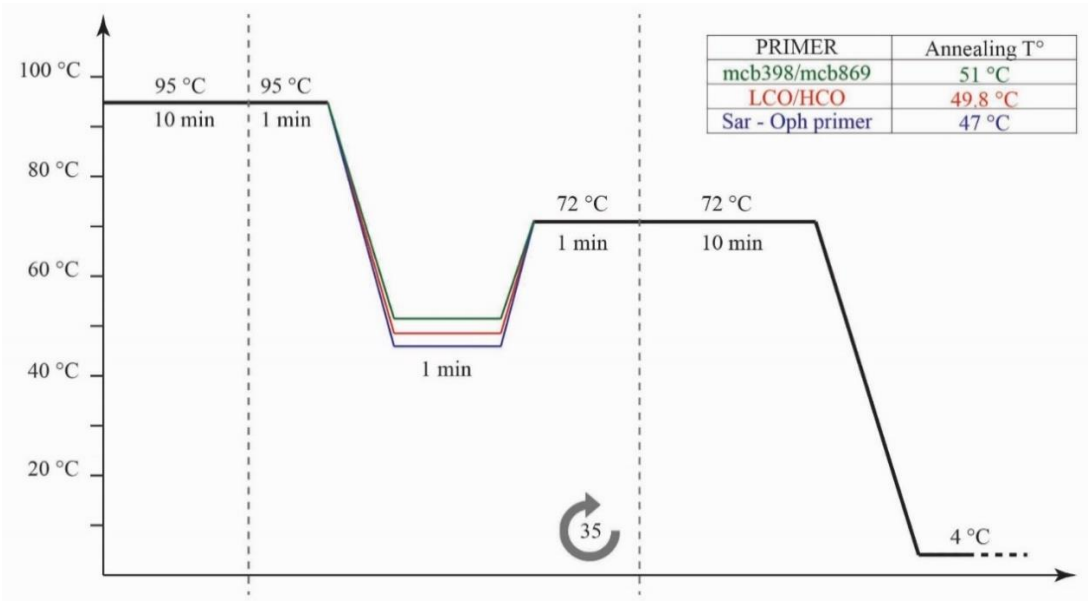

Fig. 36 **PCR conditions for the amplification of two mitochondrial regions.** Sar and Oph primers are illustrated in section 4.2.3 and 4.3.4.2.

The following amplification programme was set up on BioRad C1000 Thermal Cycler (Bio-Rad Laboratories, California, USA.): initial heat activation step at 95 °C for10 min, 35 cycles of 95 °C for 1 min, annealing temperature for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min (Fig. 36).

A positive and negative control reaction were included in each run.

For samples from archaeological contexts, the PCR master mix reaction (see section 4.7) was added of 1.6µl BSA (10mg/ml) and the amount of each primer (10pmol/µl) and of the nucleotide mix (10mM) was increased at 1µl. Up to 5µl of DNA (1-6ng) per reaction were used.

Each reaction was visualised on 1.5% w/v agarose gel previously stained with Midori Green Advanced DNA Stain (Geneflow, Elmhurst, UK).

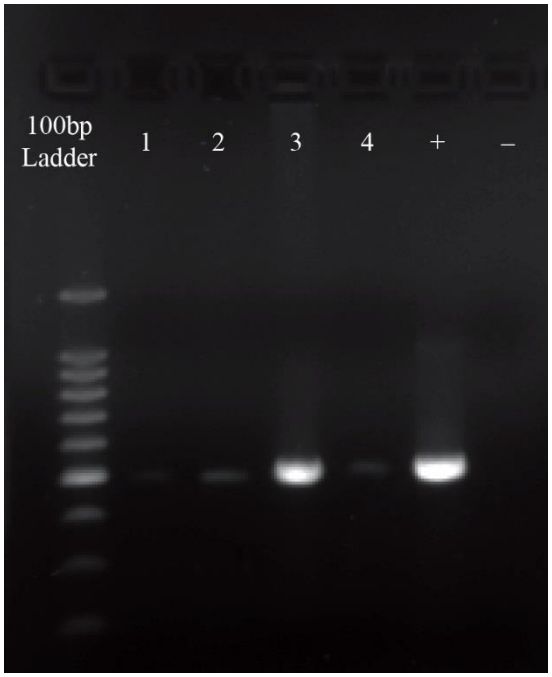

Fig. 37 **Agarose gel.** Band as reported in samples 2 and 4 were standard for archaeological samples and were purified. Band in sample 3 reflect the amplification of fresh material. Positive and negative controls are indicated as + and -.

Samples that showed a clear band (Fig. 37) of the appropriate molecular weight were purified using QIAquick PCR Purification Kit® (QIAGEN), following the manufacturer's instructions. This silica-membrane technology aims to remove nucleotides, primers, enzymes and other impurities present in the DNA samples. Purified amplicons were eluted in 40µl of sterile/deionized water and sequenced by an external company (Eurofins

Operon MWG, Ebersberg, Germany) using a one-directional sequencing process based on the standard Sanger method. To avoid the loss of information, both forward and reverse primer were used for the sequencing process. Results were obtained from the company as a FASTA file.

4.8 Analysis of sequences

The sequence editing was done with ChromasPro software (Technolysium Ltd., Queens land, Australia), version 2.1.8 or EMBOSS merger program (Rice *et al.*, 2000). The sequences given by the amplification of genomics with the new designed primers (see paragraph 4.7) were aligned in pairs to find the overlap points and recreate the consensus sequence of the COI gene. A manual check of the chromatograms is essential to guarantee the goodness of the concatenated sequence. The online system BLASTn® (Altschul *et al.*, 1990) provided by NCBI was used for species identification based on the % of identity with those available in online gene banks. In addition to the % of identity, further analyses based on a phylogenetic approach were carried out. The reconstructed sequences, supplemented with sequences available from GenBank, were imported into the integrated tool (MEGA Version 7, (Kumar *et al.*, 2016)) and phylogenetic trees were inferred through Neighbour Joining (see 4.8). Clustal Omega (Sievers et al., 2012) was used to obtain the alignments in MEGA software. Gap opening penalty was set at 15, gap extension penalty at 6.66 and transition weight at 0.5. The phylogeny reconstruction through Neighbour Joining was performed with Kimura 2-parameter model. The goodness of the phylogenetic reconstructions was calculated at 1,000 replicates with the Bootstrap method, providing a measure of robustness for each hypothesis. Trees were visualised with ITOL (Letunic & Bork, 2016). All the specific information related to the sequences is examined and reported in depth in each chapter of this thesis.

4.9 Statistical analysis

All the descriptive and analytical statistics reported in the thesis were performed using Excel or SPSS version 22 (IDM). Statistics are reported as average ± standard deviation. N indicates the number of specimens analysed. Significance was considered at 95%.

5 Results

5.1 Forensic contexts

5.1.1 Introduction

The estimation of the time since death, the *post mortem* interval (PMI), is one of the main questions addressed to the forensic entomologist. Data on the development rate of species belonging to Calliphoridae, Sarcophagidae and Muscidae families, usually the first colonisers, and occasionally species in the family Stratiomyidae and Phoridae are used to provide such information. However, species among other necrophagous families can provide information about the corpse relocation or the season of the death.

Flies are, indeed, among insects, the primary coloniser of cadavers and animal carcasses. Despite the fact that fly species involved in colonization of cadavers and carrion may differ between locations, different studies have pointed out that the first species arriving on the corpse belong to a relatively restricted number of families: Calliphoridae, Sarcophagidae and Muscidae (Smith, 1986; Amendt *et al.*, 2004; Gennard, 2007). At a later time of decomposition, other species in the families of Stratiomyidae, Fanniidae, Piophilidae and Phoridae have been frequently sampled from corpses. Species belonging to Syrphidae, Sphaeroceridae, Heleomyzidae and Sepsidae families are only sporadically collected. Other species, in the families Trichoceridae, Psycodidae, Milichiidae, Ulididae and Drosophilidae are very rare on human cadavers and animal carcasses and their presence depend on very specific seasonal, geographical or environmental contexts in addition to the decay stage of the body. Despite the lack of development data and their unforeseeable arrival on the corpse makes these latter species useless for the mPMI estimation, they could provide exhaustive information about the *peri mortem* events such as body transfer or concealment. The lack of a correct identification may justify the scarcity of records for these species. In fact, for these "secondary" species, from a morphological point of view, no detailed identifications keys of immature stages are available and, from a molecular point of view, GenBank or BOLD are incomplete (Vanin, 2008; Tuccia *et al.*, 2016b, 2016a).

The importance of correct species identification in all the disciplines dealing with insects has been extensively discussed in this thesis. Original species description and

identification keys for "secondary families" of forensic interest, are not always of easy access. Old books with hand drawings, the consultation of taxonomic experts or the comparison with the *typus* are often the only ways to reach the identification and most of the time just for mature stages, the adults. The development of pictorial archives for all the stages of insect species of forensic interest is of fundamental importance to avoid misidentification.

Samples belonging to an experiment of carcasses decomposition in UK (Paragraph 5.1.3) and a forensic case in a Maghreb country (Paragraph 5.1.4) are here analysed from a morphological and molecular point of view.

5.1.2 Aim

The aim of this chapter is the morphological description and molecular analysis of relatively fresh samples of immature stages and adults of poorly investigated Diptera of forensic interest. The lack of morphological descriptions and molecular data can results in wrong conclusions due to species misidentification. The availability of new molecular data and high resolution fully-colour pictures of distinctive morphological characters will facilitate non-taxonomic experts in the identification of under investigated species.

5.1.3 Molecular and morphological characterization of puparia of *Phthitia empirica* (Hutton, 1901) (Diptera: Sphaeroceridae), *Heleomyza serrata* (Linnaeus, 1758) (Diptera: Heleomyzidae) and *Parapiophila vulgaris* (Fallén, 1820) (Diptera: Piophilidae) species of potential forensic interest.

5.1.3.1 *Introduction*

During studies carried out in Huddersfield regarding the microbiological decomposition, species of Sphaeroceridae, Heleomyzidae and Piophilidae were collected from rabbit carcasses (Zurgani, 2018). Some illustrations and morphological description of adults and puparia are here represented to contribute to the knowledge of these species. General conclusion about the correlation of species and decomposition timing were avoided because the sampling was not standardized. Molecular analyses are also presented and discussed.

5.1.3.2 ***Materials and methods***

Six rabbit carcasses (*Oryctolagus cuniculus* Linnaeus, 1758) (2.75-3.50Kg) were purchased from a pet food company (Kiezebrink, https://www.kiezebrink.co.uk/) in Huddersfield, West Yorkshire, United Kingdom (53°38'36.5'' N 1°46'40.1''W) and placed on the roof of Science Building at the University of Huddersfield between June 2014 and March 2015. No ethical approval was required because the experiment was carried on using rabbit carcasses sold for pet consumption and with purposes of studying animal decomposition. In September 2017, pupae of different fly species were collected from the carcasses and stored at room temperature in glass jars covered with some laboratory-paper until adult eclosion. Adult and puparia samples were treated as described in Material and Methods (Chapter 4).

Morphological identifications were performed using specific identification keys or descriptive articles (see 4.2). Specimens belonging to the Sphaeroceridae family were courteously identified by Lorenzo Munari (Laboratory of Entomology, Museum of Natural History of Venice - Italy). The confirmation of the correct morphological identification of Heleomyzidae and Piophilidae specimen was provide respectively by Dr. Andrzej J. Woźnica (Instytut Biologii, Uniwersytet Przyrodniczy we Wrocławiuand - Poland) and Dr. Daniel Martín-Vega (Facultad de Ciencias, Universidad de Alcalá - Spain).

SEM observations were performed on the puparia in addition to the stereomicroscope because of the reduced size of the investigated puparia (3.39-5.24mm). Air-dried cleaned samples were mounted on metal stubs with conductive adhesive tape and coated with 40-60nm of Au-Pd. Pictures were directly digitized from the SEM.

Molecular analyses were performed from adults, pupae and empty puparia following two different extraction methods, QIAamp® DNA Mini kit and QIAamp® DNA Investigator kit (Table 5). To prevent morphological destruction, each specimen has been submerged into the extraction buffer and incubated overnight at 56°C. The extracted DNA has been quantified with a Qubit Assay. The amplification was carried out in the mitochondrial COI Barcoding region using the universal primer designed by Folmer *et al.* (1994).

For more detail information about material and methods refer to Chapter 4.

The new sequences produced during this work have been deposited in GenBank (Table 3). Sequences of COI gene respectively from Sphaeroceridae, Piophilidae and Heleomyzidae species were downloaded from BOLD online system and included in the phylogenetic analyses (Table 4). Sequences were aligned with Clustal Omega (Sievers *et al.*, 2011). The phylogenetic trees were built using the Neighbour Joining method on MEGA 7.0 (Kumar *et al.*, 2016) (See 4.8). Fifty sequences of 598bp, 563bp and 480bp were used respectively for the phylogenetic analysis of the three species identified: *Phthitia empirica* (Hutton, 1901, Sphaeroceridae), *Heleomyza serrata* (Linnaeus, 1758, Heleomyzidae) and *Parapiophila vulgaris* (Fallén, 1820, Piophilidae).
Sequences of *Drosophila melanogaster* Meigen, 1830 were used as outgroup.

Table 3 **GenBank codes of COI sequences of Sphaeroceridae, Heleomyzidae and Piophilidae produced in this work.**

Family	Species	GenBank code
Sphaeroceridae	*Phthitia empirica* (Hutton, 1901)	MH118267
		MH118268
		MH825673
Heleomyzidae	*Heleomyza serrata* (Linnaeus, 1758)	MH825674
		MH825675
		MH825676
Piophilidae	*Parapiophila vulgaris* (Fallén, 1820)	MH825677
		MH825678
		MH825679
		MH921575

Table 4 **COI sequences of Sphaeroceridae, Heleomyzidae and Piophilidae downloaded from BOLD and included in the analyses.**

Family	Species	BOLD code	
Sphaeroceridae	*Coproica urbana* (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
		JSJUN1792-11	
	Leptocera caenosa (Rondani, 1880)	BCFOR772-15	
	Leptocera finalis (Collin, 1956)	JWDCG152-10	JWDCE291-10
		JWDCE179-10	JWDCJ330-11
		JWDCH240-10	JWDCJ334-11
		JWDCE279-10	
	Phthitia ovicercus Marshall, 1992	SSBAF3038-13	SSEIB7483-13
		SSEIB11339-13	SSWLB3188-13
		SSEIB6777-13	SSWLB3228-13
	Copromyza equina Fallén, 1820	JSDIP471-10	JSDIP737-10
	Copromyza neglecta (Malloch, 1913)	JSDIQ045-10	JSDIQ496-10

	Phthitia plumosula (Rondani, 1880)	BBDEC892-10 OPPOA1193-17	CNFDI480-14 GMGMG1040-14
	Phthitia quadricercus Marshall, 1992	SSJAA1739-13 JWDCH112-10 SSBAC3110-12 SSBAC3445-12 SSBAC3446-12 SSBAC3454-12	SSBAC3460-12 SSBAC3476-12 SSBAC3483-12 SSBAC3485-12 SSJAA1922-13 SSPAB8647-13
Heleomyzidae	*Tephrochlamys flavipes* (Zetterstedt, 1838)	GMGMF066-14 GMGMG599-14 GMGMF169-14 GMGMF070-14 GMGMF067-14	GMGMF048-14 GMGMF118-14 GMGME1399-14 GMGME593-14
	Tephrochlamys rufiventris (Meigen, 1830)	NSMTP094-15 NSMTP095-15 MAMTA1456-12 CDFD034-12	GMGMH242-14 GMGMG513-14 GMGMG530-14
	Tephrochlamys tarsalis (Zetterstedt, 1847)	SSBAA5552-12 SSBAC1964-12 CNTNK076-14	JWDCE454-10 SMTPM2063-15
	Borboropsis puberula (Zetterstedt, 1838)	SSDWA6374-15	SSECC323-15
	Heleomyza bisetata (Garrett, 1922)	SMTPB143-13 SSKOA1062-15 NGNAA2145-13 SMTPM1735-15	CNFDL185-14 SMTPM1173-15 JSDIP575-10
	Heleomyza brachypterna (Loew, 1873)	PHDIP677-11	
	Heleomyza serrata (Linnaeus, 1758)	MOBIL1324-16 ARTYT100-12 ARTYT111-12 SMTPB133-13 SMTPM6945-15	SMTPM6375-15 GMGMI1145-14 GMGMG448-14 HEAPR1900-12
	Suillia apicalis (Loew, 1862)	ARTYT128-12	
	Suillia quinquepunctata (Say, 1823)	RRMFC2068-15	
	Suillia barberi (Darlington, 1908)	SSECC023-15	
	Suillia nemorum (Meigen, 1830)	ARTYT129-12	ARTYT131-12
	Trixoscelis fumipennis Melander, 1913	SSWLB7408-13	SSWLB7503-13
Piophilidae	*Parapiophila vulgaris*[3] (Fallén, 1820)	GBDP18775-15 CNSIB1275-15 CNSIA084-15 ZMUCG055-12 BCFOR623-15	GBDP18776-15 GBDP15922-15 GBDP9366-11 GBDP9367-11 GBDP9368-11
	Parapiophila flavipes (Zetterstedt, 1847)	CNIVA120-14	
	Parapiophila atrifrons (Melander & Spuler, 1917)	CNMIE1076-14	CNMIE425-14
	Parapiophila pectiniventris (Duda, 1924)	SSBAC1728-12 SSWLB1864-13	SSJAB1302-13
	Parapiophila Kugluktuk Rochefort & Wheeler, 1977	CNTMA1522-14 CNTMA1539-14	CNBAD288-12
	Allopiophila luteata (Haliday, 1833)	CNYOK251-15 SSEID826-13	CNWAD2099-14
	Parapiophila fulviceps (Holmgren, 1883)	DARC180-10 DARC167-10	DARC393-11
	Liopiophila varipes (Meigen, 1830)	GMGMC1167-14	BCFOR397-15

[3] *Allopiophila* Hendel is considered a synonymus of *Parapiophila* McAlpine (Ozerov, 2004).

		BCFOR398-15	GMGMB893-14
	Piophila megastigmata McAlpine, 1978	GBDP18876-15	GBDP18877-15
	Piophila casei (Linnaeus, 1758*)*	DIQT130-08 GBDP18872-15	GBDP18870-15
	Protopiophila litigata Bonduriansky, 1995	CNFNS554-14 CNFNR866-14	CNTNE764-14
	Protopiophila latipes (Meigen, 1838)	NSMTP002-15 GBDP18947-15	TTMDI1244-10
	Centrophlebomyia furcata (Fabricius, 1794)	GBDP17534-15	
	Stearibia nigriceps (Meigen, 1826)	GBDP18900-15 GBDP18901-15 BCFOR400-15	GBDP18898-15 GBDP18899-15
Drosophilidae (Outgroup)	*Drosophila melanogaster* Meigen, 1830	CYTC5404-12	GBDP2877-06

5.1.3.3 ***Results***

- **Morphological analysis**

According to the morphological analysis, three families of flies (Diptera) were identified: Sphaeroceridae, Heleomyzidae and Piophilidae. Adults fly of *Phthitia empirica* (Hutton, 1901), *Heleomyza serrata* (Linnaeus, 1758) and *Parapiophila vulgaris* (Fallén, 1820) were identified respectively for the three families after the adults emerged from the puparia. These three species have been scarcely investigated to date and little information is available about their immature stages. In addition, their puparia were not or only superficially described. Here a detailed analysis of the species is reported.

***Phthitia empirica* (Hutton, 1901) - Sphaeroceridae**

Syn*: Borborus empirica Hutton, 1901*

Limosina cadaverina Duda, 1918

Limosina pectinifera Villeneuve, 1918

Phthitia empirica belongs to the widespread sphaerocerid genus *Phthitia* Enderlein, 1938 in the subfamily Limosininae. The species is included in the subgenus *Alimosina* Roháček, 1983.

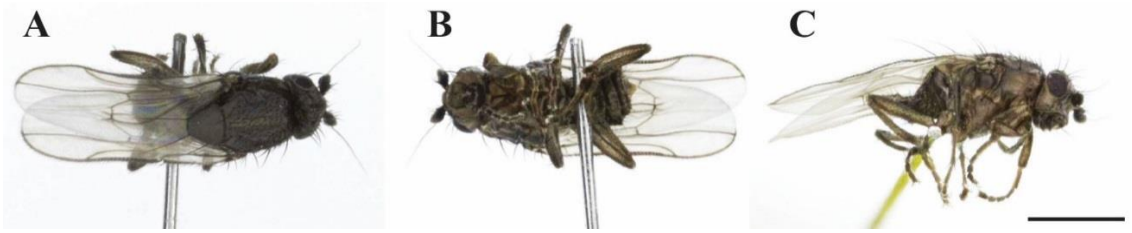

Fig. 38 ***Phthitia empirica* adult.** Dorsal (A), ventral (B) and lateral (C) view (Scale bar 500 µm).

Limosininae flies are small, generally 1 to 4mm long (with the exception of the species in the genus *Anatalanta* Eaton, 1875 that exceed 10mm in length) (Marshall, 2012). In the analysed samples of females of *P. empirica*, the body length is 2.1±1.5mm (N=5) while the length of the wings is 2.3±0.5mm**.** No males were sampled, so no measurements are available for this sex.

Phthitia empirica adult is a dull brown fly characterised by the presence of a long distal dorsal bristle in the hind tibia, 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, aristal hairs are dense and as long as aristal width at base, slightly longer and sparser in distal half. Strong vibrissae and other large setae are present in the light brown gena. Face is concave.

Thorax has a posterior pair of dorsocentral 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 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 presents at least one long distal dorsal bristle. The mid femur shows a row of anteroventral spine-like short setae. The mid tibia presents proximally one short anterodorsal and one short posterodorsal setae, distally one small dorsal seta in front of one long anterodorsal and one posterodorsal setae, and double rows of posteroventral spine-like setae. Wing and vein are brownish, crossveins r-m and dm-cu separated by less than twice the length of dm-cu. Anal veins are sinuate (Fig. 10).

Puparia are yellow to light brown (Fig. 39A,B,C), 3.39±0.32mm long (N=11) in the analysed samples. Several excrescences surround the posterior region becoming denser and more packed around the anal plate. The later is mainly made only on the anal cleft and its crumpled rim with no expansions (wings). All the anal papillae are absent or not discernible (Fig. 40A). The ventral welt of abdominal segment seven shows intersegmental large and flame shape spines disposed in two lines. External lines are made smaller and closer spines, enclose the central ones (Fig. 40B).

Posterior spiracles are allocated on two-inclined protuberance. The angle formed by the projection is greater than 90º. The slits follow the external shape of the spiracle in a "U-like" pattern. Filaments emanating from perispiracular glands are well visible (Fig. 40C,D). In all the analysed samples, anterior spiracles showed 7 prospiracular lobes (Fig. 40E,F).

Fig. 39 ***Phthitia empirica* puparium**. Dorsal (A), ventral (B) and lateral (C) view (Scale bar 500μm).

Biology and distribution

This cosmopolitan species, probably originated from Australia, was defined by Richards (Richards, 1930) as "domestic fly" suggesting its synantropic attitude. Reported from different places, it's a common species in UK. The species was reported feeding on animal excrements (Rohacek, 1983), animal carrions and human dead bodies (Marshall *et al.*, 2009; Anton *et al.*, 2011; Carles-Tolrá *et al.*, 2012).

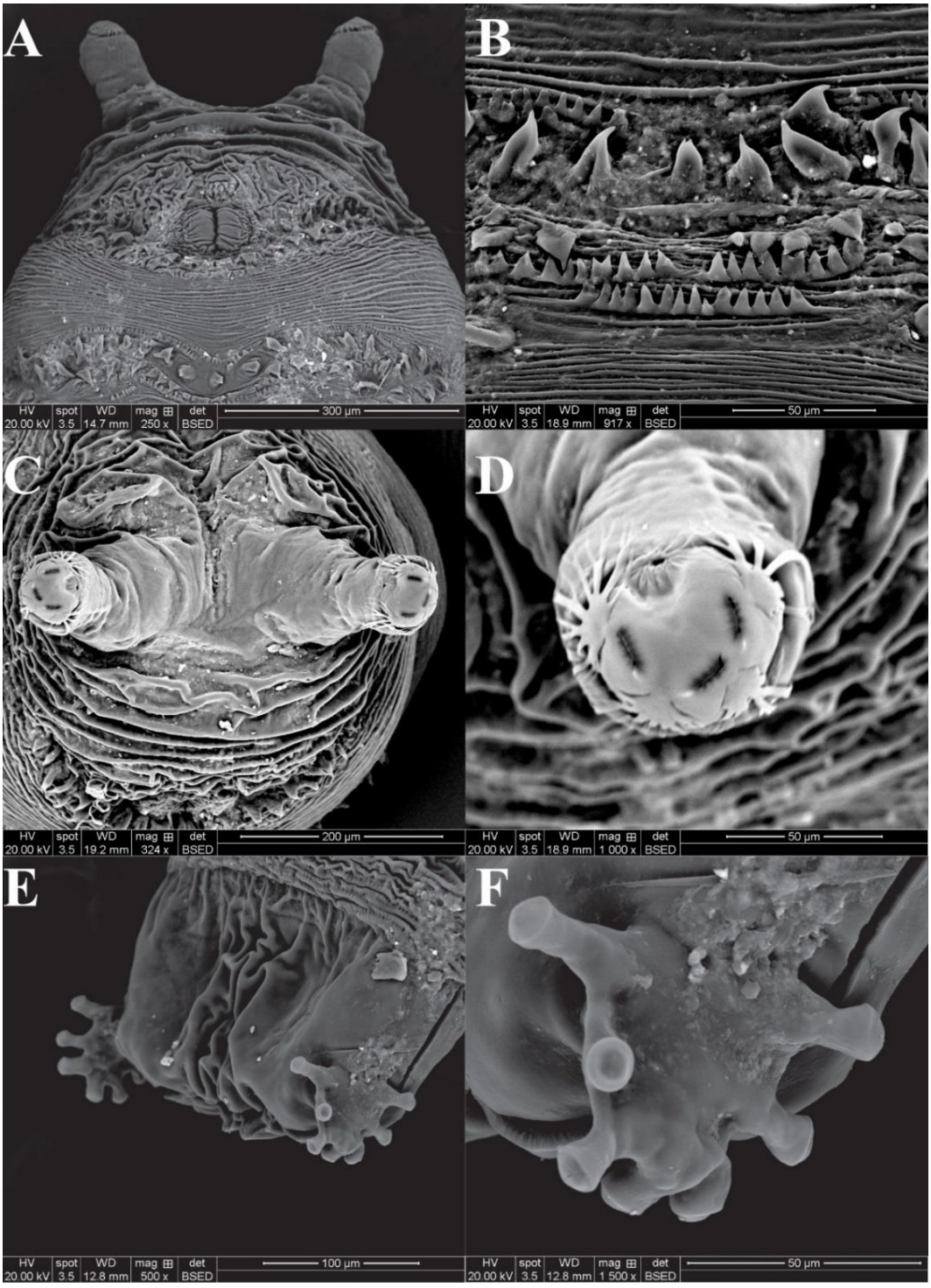

Fig. 40 ***Phthitia empirica*** **puparium details.** Anal plate (A), intersegmental spicules (B), posterior spiracle (C,D) and anterior spiracle (E,F) (Scale bar is reported in each frame).

***Heleomyza serrata* (Linnaeus, 1758) - Heleomyzidae**

***Syn**: Musca serrata* Linnaeus, 1758

Helomyza geniculata Zetterstedt, 1838

The genus *Heleomyza* Fallen, 1810 counts more than 30 species with a Holarctic distribution but with a couple of Afrotropical species. The genus belongs to the subfamily Heleomyzini.

In the analysed samples of *Heleomyza serrata* adults, the body length is 4.3±03mm (N=7) while the length of the wings is 4.9±0.3mm (Fig. 41A). *Heleomyza serrata* adults are grey flies with an orange frons, eye height is more than double the genal one. The head shows a grey ocellar triangle with two divergent ocellar bristles and two converging postvertical bristles (Fig. 41C,D). Two outer vertical bristles, three pairs of fronto-orbital bristles and numerous minute disorganized bristles in the frons are present. Lunula is bare. The hind part of the head is grey (Fig. 41D). The lower part of the head and the gena are yellowish-light brown with one strong black vibrissa and two additional bristles little smaller than the above one (Fig. 41C,G). Thorax has four dorsocentral bristles, one short prescutellar acrostichal bristle, one humeral bristle, one posthumeral bristle, two notopleural bristles and two post-alar bristles (Fig. 41E). Prosternum is shiny grey with 4 to six lateral bristles (Fig. 41F). Katepisternum has one strong posterodorsal black bristle and six small anterior setulae (Fig. 41G,H). Scutellum has four long and strong black marginal setae. No apical scutellar bristles are present.

In the analysed samples, the length of the puparia was 5.24±1.14mm (N=20). Puparia are yellowish-brown (Fig. 39A,B,C) wrinkled and covered by spines. The anal plate is light brown and has short expansions upward directed (wings). The post- and subanal papillae are present (Fig. 42D, Fig. 43A). The posterior spiracles are allocated on two parallel projections. The slits are radiate, the scar is in upper position (Fig. 42E,F, Fig. 43C). Dorsal muscle scars are well defined and visible in the SEM image (Fig. 43C). Anterior spiracles in the analysed samples have a branch shape with 11-13 prospiracular lobes (Fig. 42G, Fig. 43D). The ventral welt of abdominal segment seven is made up of small flame-shape spines, some of which bifid, placed in disorganized rows. Some rows are directed toward the anal plate, some other toward the anterior part.

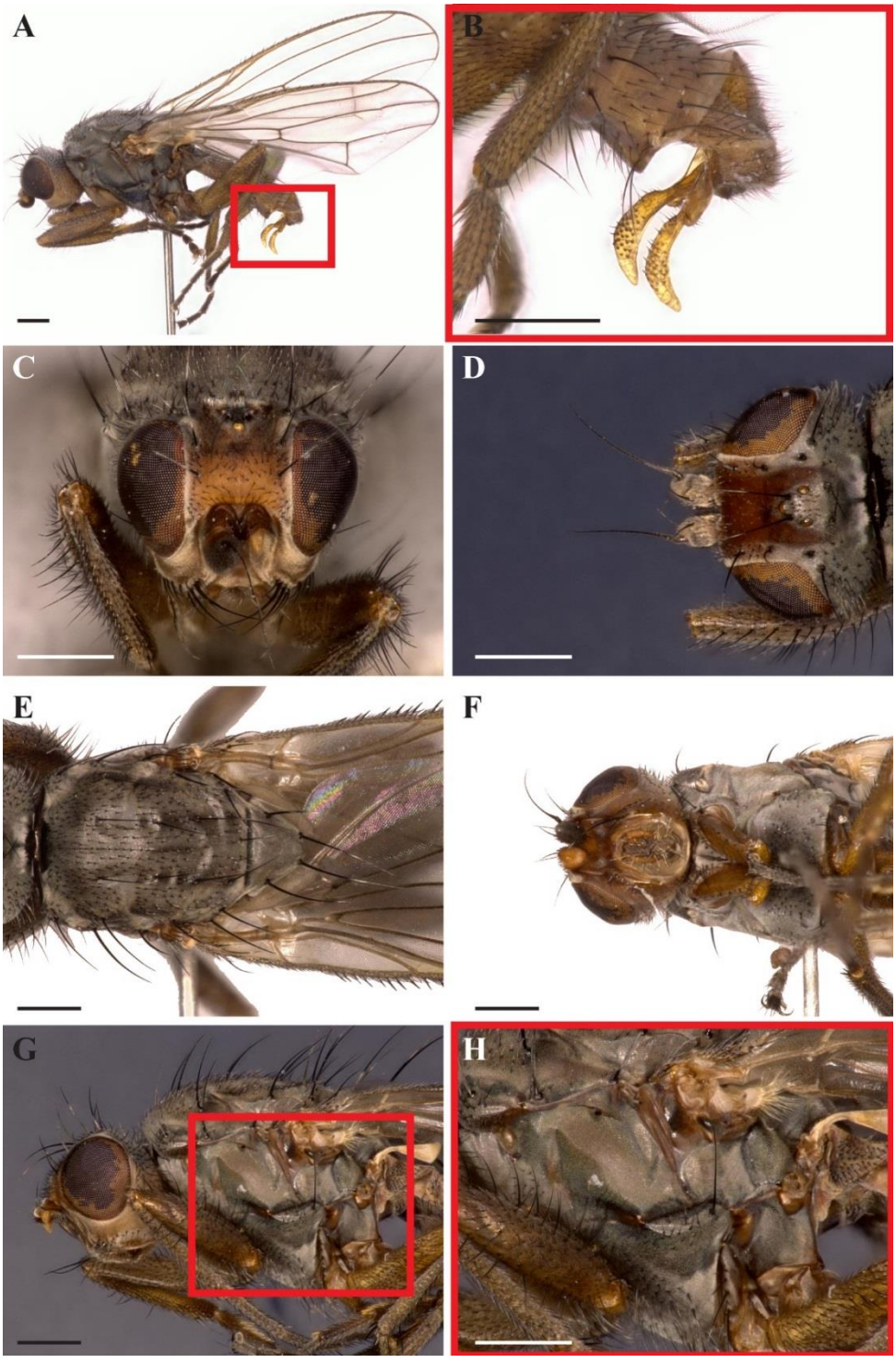

Fig. 41 ***Heleomyza serrata* adult.** Lateral View (A), magnification of male genitalia (B), head chaetotaxy in frontal view (C), head chaetotaxy in dorsal view (D), thorax chaetotaxy in dorsal view (E), prosternum (F), thorax chaetotaxy in lateral view (G) and magnification of the katepisternum (H) (Scale bar 500µm).

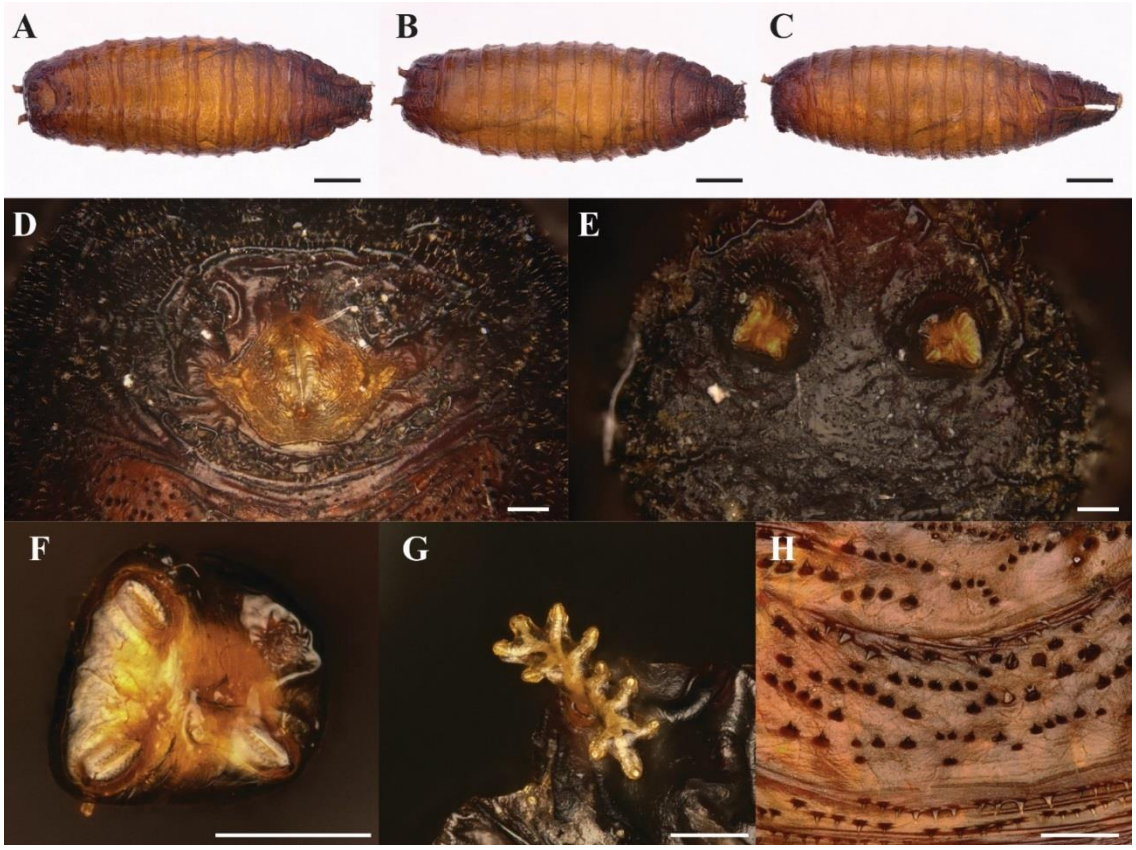

Fig. 42 ***Heleomyza serrata*** **puparium**. Ventral (A), dorsal (B) and lateral (C) view (Scale bar 500μm); anal plate (D), posterior spiracles (E,F) anterior spiracle (G) and intersegmental spicules (H). (Scale bar 100μm).

Biology and distribution

Coprophagous and saprophagous species (Tolrá, 2011), *H. serrata* was often recorded from caves in Norway, Austria and Poland (Kjærandsen, 1993; Christian & Spötl, 2010; Østbye & Lauritzen, 2013). This species was also found in excrements, bird nests, fungi, dung, chicken manure, decomposing plant remains and cadaver (Gill, 1962; Skidmore, 1962; Garnett & Foote, 1966; Krivosheina, 2008; Tolrá, 2011). *Heleomyza serrata*, as many other heleomyzids, is a cold-adapted species, active in winter and often recorded on the surface of snow in Poland (Soszynska, 2004; Soszyńska-Maj & Woźnica; Soszynska-Maj & Woznica, 2016) and Scandinavia (Hågvar & Greve, 2003). Adult species are often misidentify with *Heleomyza captiosa* Gorodkov, 1962 (Chandler, 1998), from which can be discriminate by male genitalia (Fig. 41A,B) or by the shape of spermathecae (Gorodkov, 1962; Soszyńska-Maj & Woźnica).

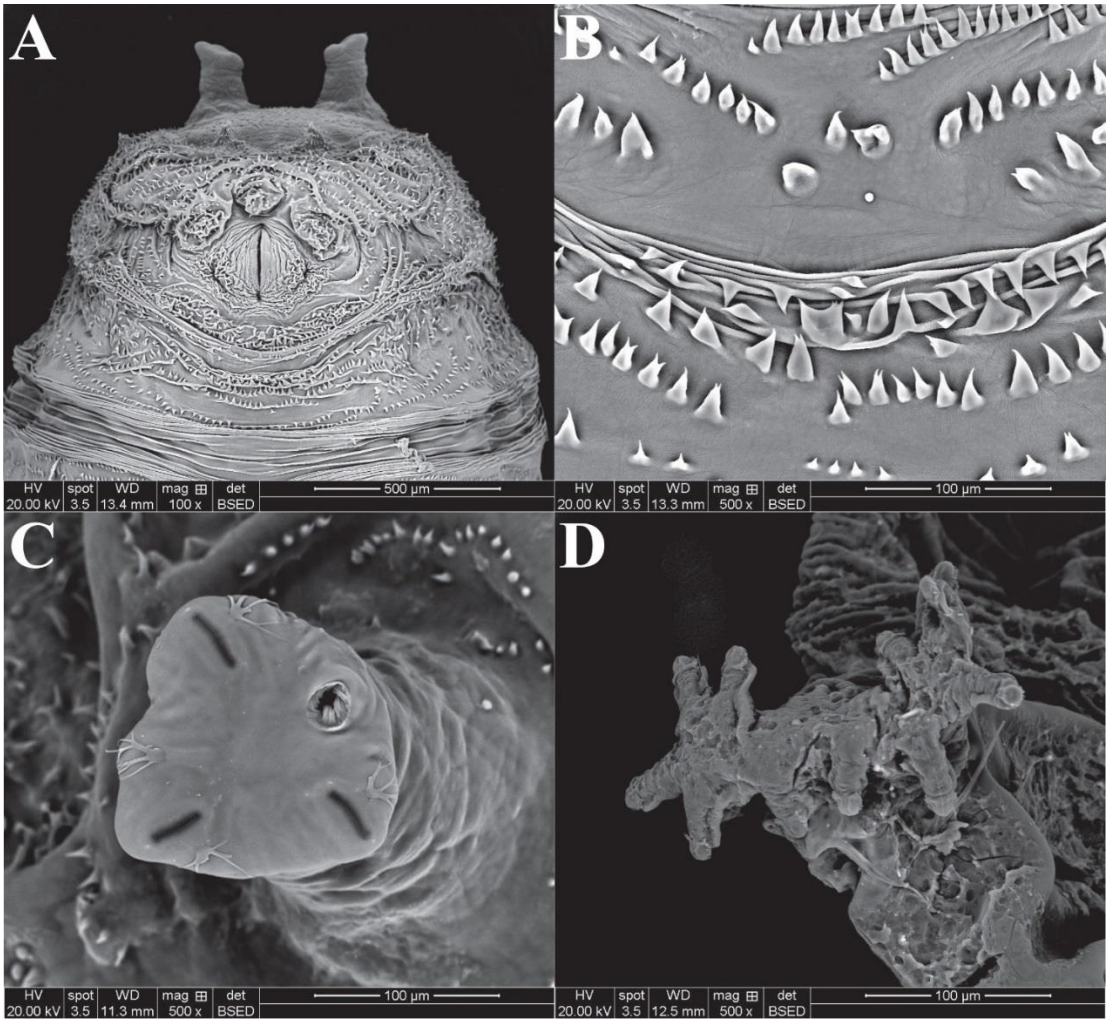

Fig. 43 ***Heleomyza serrata* puparium details**. Anal plate (A), intersegmental spicules (B), posterior spiracle (C) and anterior spiracle (D) (Scale bar is reported in each frame).

Parapiophila vulgaris (Fallén, 1820) - Piophilidae

***Syn**: Piophila vulgaris* Fallen, 1820

The identification keys for forensically important Piophilidae (Rochefort *et al.*, 2015) allowed the identification of the adult piophilids as *Parapiophila vulgaris*.

Parapiophila vulgaris adult body length, in the analysed samples, is 2.9±0.2mm (N=4) while the wings length is 2.9±0.4mm (Fig. 44), these data are in accordance with Rochefort *et al.* (2015).

Adults are glossy black and yellow flies with eyes occupying mostly all the height of the head. The frons is yellow while the ocellar triangle and the hind part of the head are black.

Thorax has one postpronotum bristle and one dorsocentral bristle. The fore leg is entirely black. Mid and hind femur and tibia are yellow at the extremities (Fig. 44)

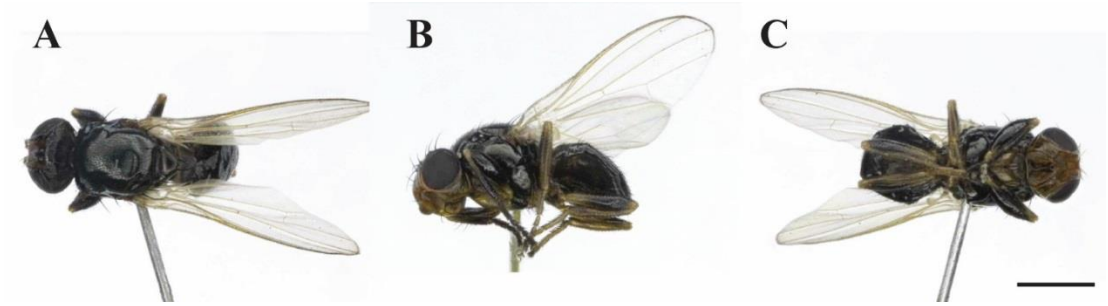

Fig. 44 ***Parapiophila vulgaris* adult.** Dorsal (A), lateral (B) and ventral (C) view (Scale bar 500µm).

Some illustrations and morphological description of puparia of *P. vulgaris* are here presented to contribute to the knowledge of this species.

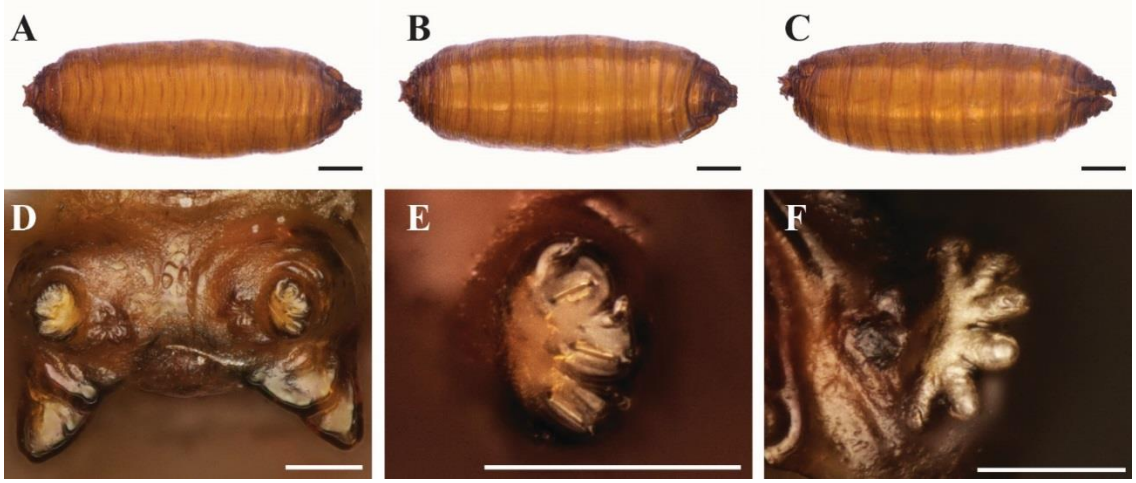

Fig. 45 ***Parapiophila vulgaris* puparium**. Ventral (A), dorsal (B) and lateral (C) view; posterior spiracles (D,E) and anterior spiracle (F). (Black scale bar 500µm, white scale bar 50µm).

Puparia of *P. vulgaris* are light brown, darker at the extremities (Fig. 45A,B,C), 3.40±0.29mm long (*N*=3). The puparium is characterized by wrinkles folded as the bellows of an accordion. The anal plate is not visible because of its invagination in the several excrescences of the posterior region (Fig. 46A). All the anal papillae are absent or, as the anal plate, not discernible (Fig. 46A).

Posterior spiracles are located on two slightly projected processes. The three slits occupy half of the surface of the projection and are convergent to the scar. The latter is situated in a middle-low position outside the protrusion (Fig. 45D,E; Fig. 46C). In the analysed

samples, the anterior spiracles showed a hand-like shape with six or seven prospiracular lobes (Fig. 45F, Fig. 46D). The intersegmental spicules of ventral welt of abdominal segment seven are small and sharp, distributed in organised rows (Fig. 46D).

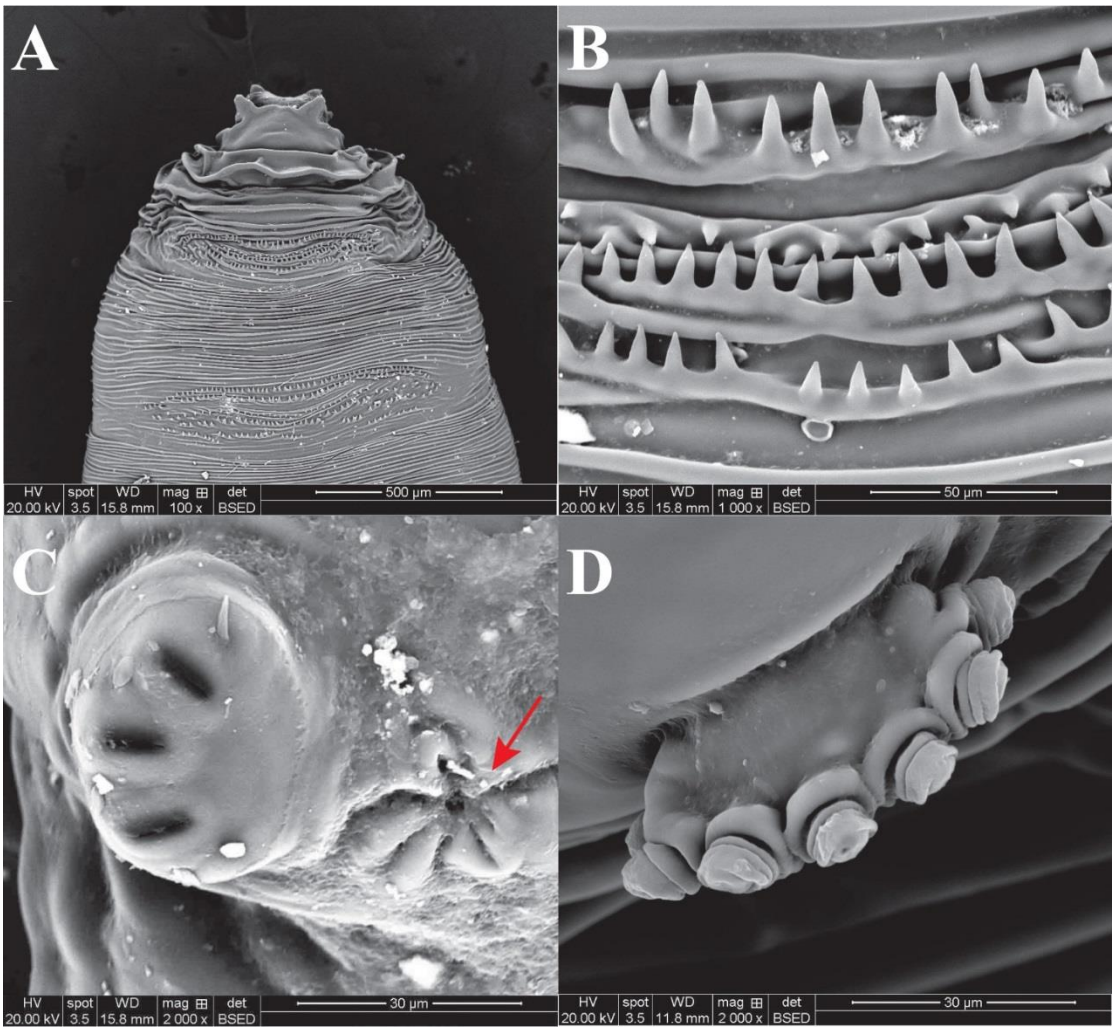

Fig. 46 ***Parapiophila vulgaris*** **puparium.** Anal plate (A), intersegmental spicules (B), posterior spiracle (C) and anterior spiracle (D). (Scale bar is reported in each frame). The red arrow shows the posterior spiracle scar.

Biology and distribution

Wide distributed in the Nearctic (Duda, 1924; Steyskal, 1964; Rochefort *et al.*, 2015) and Palearctic regions (Zuska, 1965; Lohm, 1978; Martín-Vega *et al.*, 2010), this species has been often found on decomposing matter, human cadavers and animal carcasses (Fiedler *et al.*, 2008; Matuszewski *et al.*, 2008; Martín-Vega, 2011; Baumjohann & Rudzinski,

2013). In addition, *P. vulgaris* has been collected on meat and on rotten fruit, confirming its association with food industry (Zuska & Laštovka, 1969).

- **Molecular analysis**

DNA extraction yielded positive results from all the entomological samples: adult, pupa and puparium (Table 5). PCR amplification was positive for all adult and pupa samples and for *P. vulgaris* puparia while negative results were obtained from *H. serrata* and *P. empirica* puparia (Table 5).

Table 5 **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. Standard deviation is reported. ✓positive PCR, *X* negative PCR result.

Species	Sample	Method	Elution (µl)	Average DNA quantification (ng/µl)	Total amount of extracted DNA (ng) per specimen	PCR success
P. empirica	***1 adult***	Investigator Kit	100	2.19±0.02	219.33±2.31	✓
P. empirica	***2 adults***	Mini kit	200	0.93±0.02	92.73±2.41	✓
P. empirica	***3 pupae***	Mini kit	200	4.99±0.02	332.44±2.04	✓
P. empirica	***5 puparia***	Investigator Kit	100	1.16±0.01	46.40±0.40	*X*
H. serrata	***1 adult***	Investigator Kit	100	2.32±0.03	232.00±3.46	✓
H. serrata	***1 adult***	Mini kit	200	3.26±0.00	652.00±0.00	✓
H. serrata	***1 adult***	Mini kit	200	6.46±0.02	1292.00±4.00	✓
H. serrata	***5 puparia***	Investigator Kit	100	11.50±0.00	230.00±0.00	*X*
H. serrata	***2 puparia***	Investigator Kit	100	1.63±0.01	81.33±0.58	*X*
P. vulgaris	***1 adult***	Investigator Kit	100	2.66±0.09	266.00±10.58	✓
P. vulgaris	***1 adult***	Mini kit	200	1.38±0.00	276.00±0.00	✓
P. vulgaris	***1 adult***	Mini kit	200	4.21±0.10	842.67±25.40	✓
P. vulgaris	***2 puparia***	Investigator Kit	100	1.32±0.02	66.00±1.00	✓

Despite the internal variability of each specimen, the dimension of the adult seems to control the extraction kit efficiency, influencing the DNA yield. The QIAamp® DNA Mini kit appeared to give a better result for relatively big specimen as *H. serrata* (1292.00±4.00 vs 232.00±3.46ng/specimen) while QIAamp® DNA Investigator kit was revealed to be the best for small specimens such as *P. empirica* (219.33±2.31 vs 92.73±2.41ng/specimen).

In order to explain the negative result of PCR amplification from some puparia, the dimensions of the extracted DNA fragments were assed with Agilent Bioanalyzer (see 4.6). The result pattern was comparable between unsuccessful puparia (e.g. *P. empirica*)

and successful puparia (e.g. *P. vulgaris*), excluding the size as reason of the negative amplification.

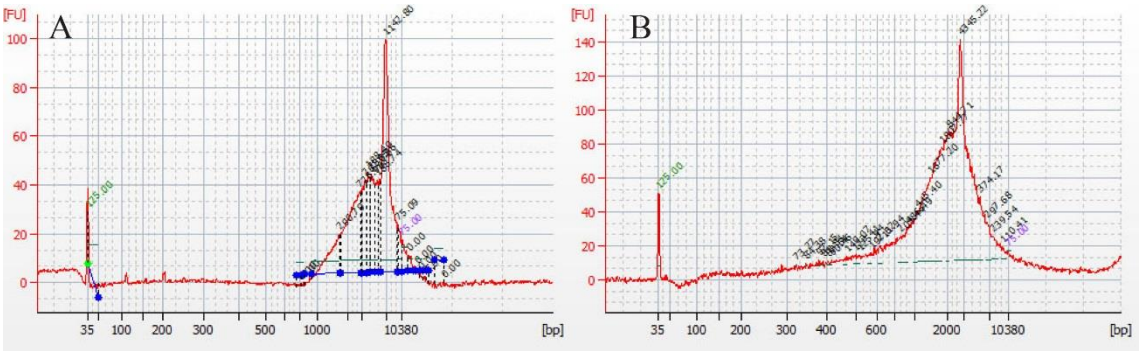

Fig. 47 **Comparison of BioAnalyser results between *P. empirica* and *P. vulgaris* puparia.**

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

The lack of database sequences of *Phthitia empirica* prevented the direct identification of the Sphaeroceridae species through the local alignment of the sequences on the online system BLASTn®. The output of the comparison resulted in a 92 % identity with *Phthitia ovicercus* Marshall, 1992. The NJ analysis of 52 sequences of 598bp belonging to different genera (*Coproica* Róndani, 1861, *Copromyza* Duda, 1923, *Leptocera* Olivier, 1813 and *Phthitia* Enderlein, 1938) in the Sphaeroceridae family (Fig. 48) generated a phylogenetic tree with well-defined species clusters. The sequences obtained from the puparia collected from the rabbit carcasses (MH118267, MH118268 & MH825673) group together and appear to be closely related with *P. ovicercus* (Bootstrap = 79/100). In the reconstructed tree, the genera *Coproica*, *Leptocera* and *Copromyza* appear to be monophyletic while *Phthitia* genus seems not. However, because the very weak value of bootstrap at the basal nodes no conclusion can be made about the relationship among the genera.

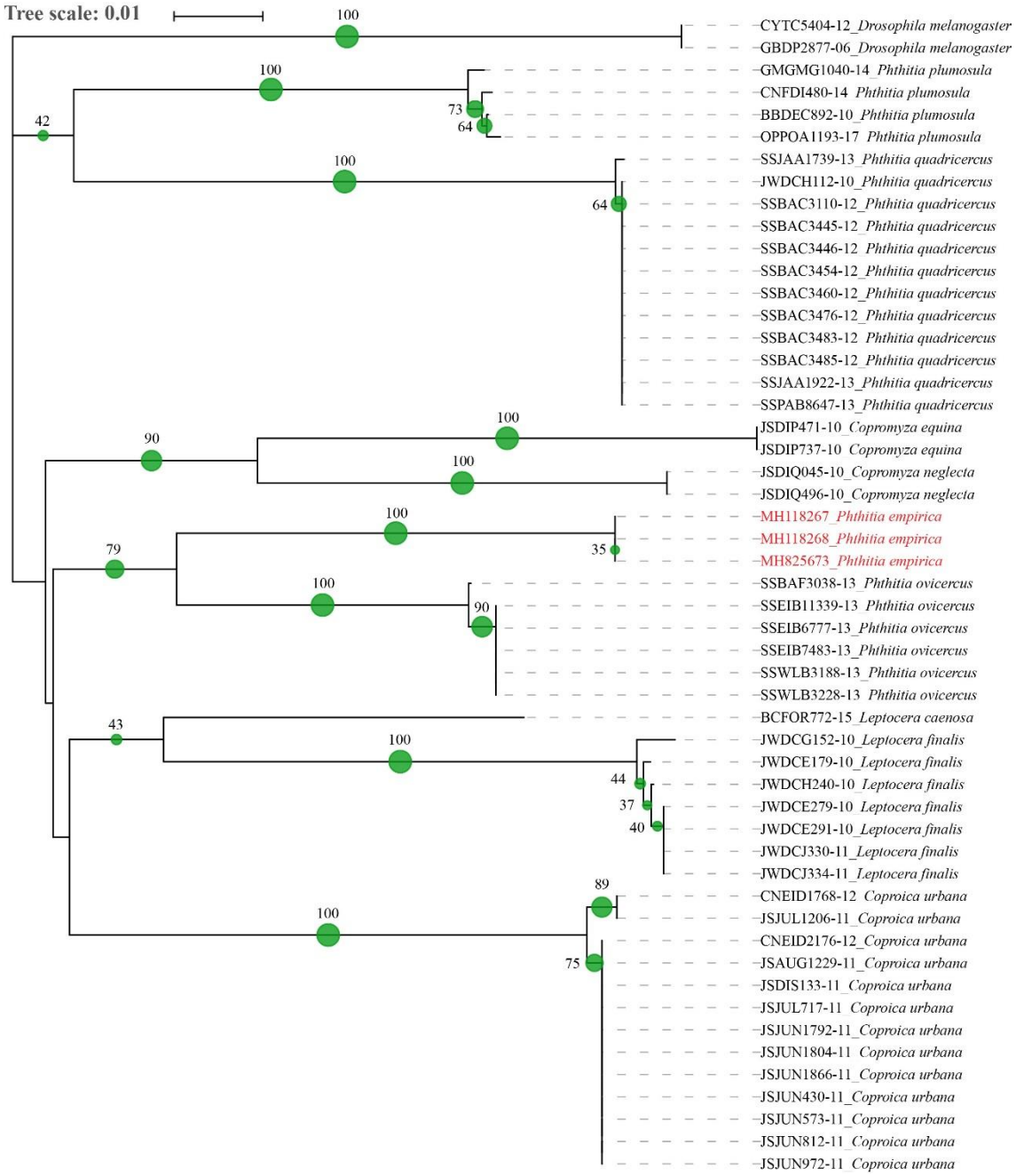

Fig. 48 **Phylogenetic tree of Sphaeroceridae family.** Neighbour Joining method analysis of 598bp sequence of the COI gene. The green spots and the number at each node indicate the bootstrap support. Sequences from this study are reported in red.

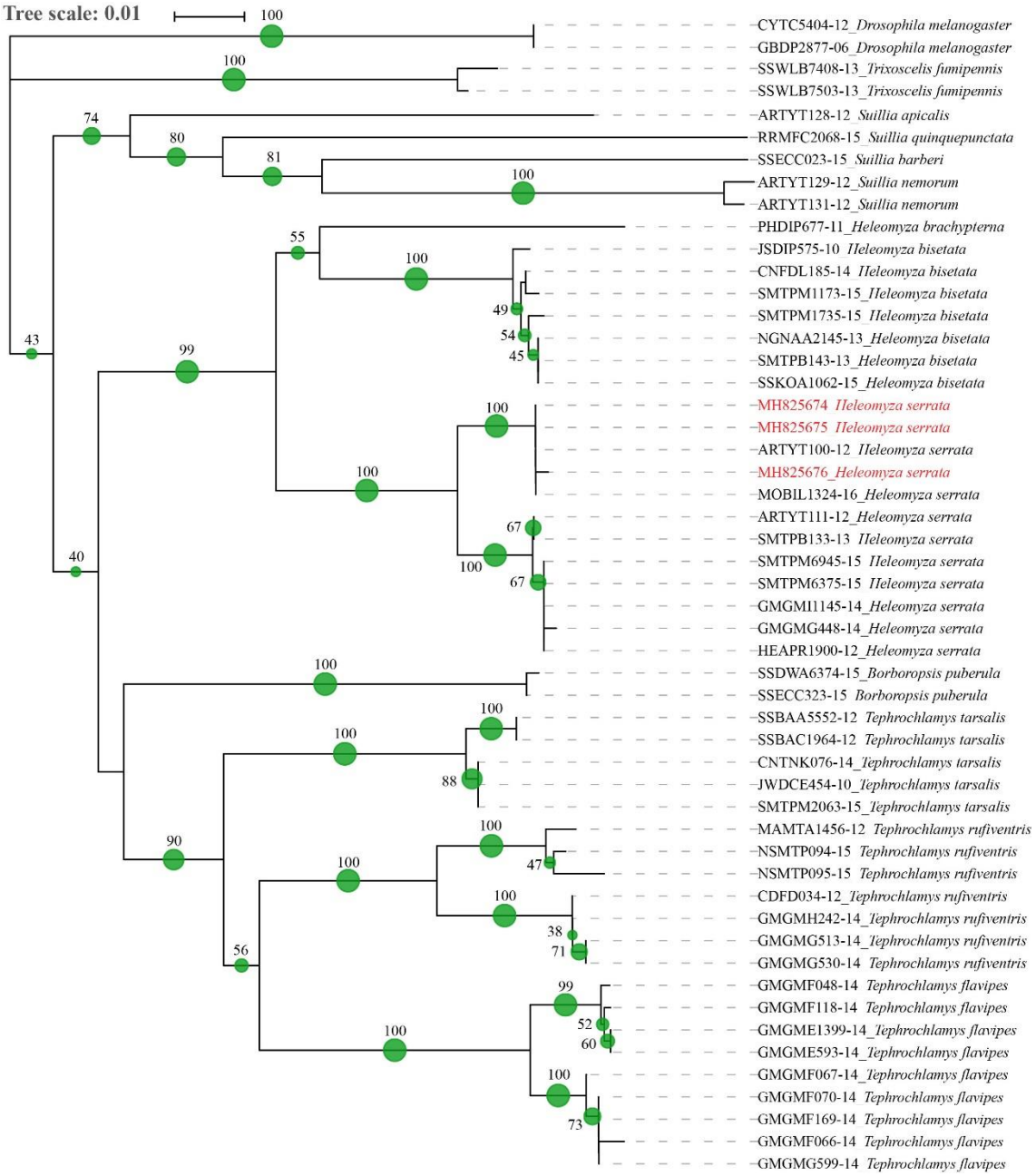

Fig. 49 **Phylogenetic tree of Heleomyzidae family**. Neighbour Joining method analysis of 563bp sequence of the COI gene. The green spots and the number at each node indicate the bootstrap support. Sequences from this study are reported in red.

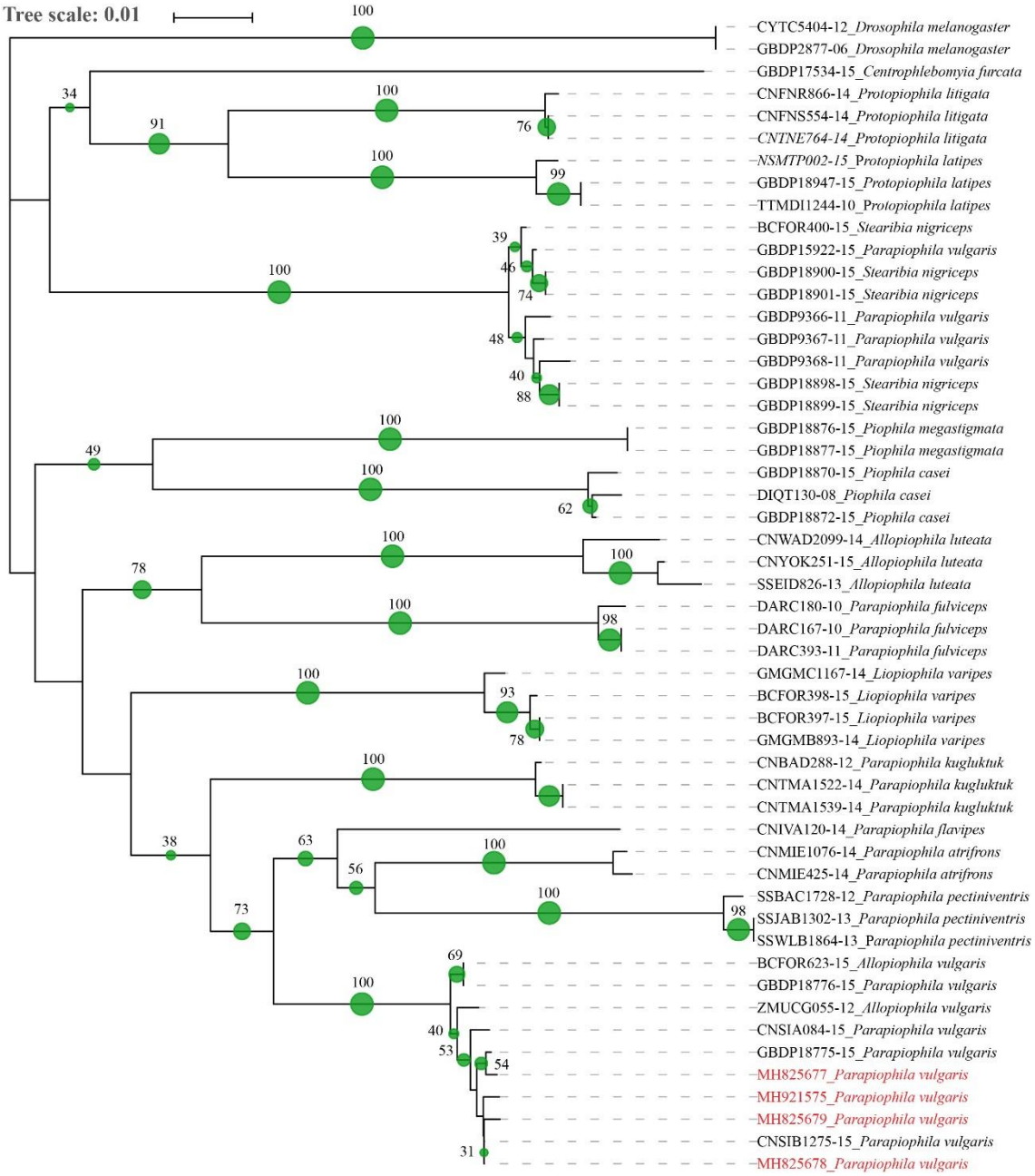

Fig. 50 **Phylogenetic tree of Piophilidae family**. Neighbour Joining method analysis of 480bp sequence of the COI gene. The green spots and the number at each node indicate the bootstrap support. Sequences from this study are reported in red.

The analysis of the sequences obtained from the Heleomyzidae specimens confirmed the morphological identification as *Heleomyza serrata*. The NJ analysis of 52 sequences of 563bp belonging to different genera (*Borboropsis* Czerny, 1902, *Heleomyza* Fallen, 1810, *Suillia* Robineau-Desvoidy, 1830, *Tephrochlamys* Loew, 1862 and *Trixoscelis* Rondani,

1856) in the Heleomyzidae family brought about a phylogenetic tree with well-supported clusters at both genus and species levels (Fig. 49). The sequences obtained from this study (MH825676, MH825675 and MH825674) branch together with two sequences of *H. serrata* coded in BOLD as MOBIL1324-16 and ARTYT100-12 from Alaska and Canada. A second cluster of *H. serrata* is closely related to the previous one but significantly separated with a 100-bootstrap value. *Heleomyza* genus seems to be monophyletic. However, the low level of bootstrap at the basal nodes of the tree does not allow for any statement about the relationship between and among genera.

The Piophilidae sequences comparison revealed a 99% identity with two sequences coded in GenBank respectively as *Piophila nigriceps*[4] Meigen, 1826 KU876233 and *Parapiophila* sp. KR520884, with a 0.0 E value. The NJ analysis of 52 sequences of 480bp belonging to different genera (*Allopiophila* Hendel, 1917, *Centrophlebomyia* Hendel, 1903, *Liopiophila* Duda, 1924, *Parapiophila* McAlpine, 1977, *Piophila* Fallen, 1810, *Protopiophila* Duda, 1924, and *Stearibia* Lioy, 1864) in the Piophilidae family results in a phylogenetic tree where the sequences obtained from this study (MH825679, MH825678, MH825677, and MH921575) cluster together to other *P. vulgaris* and *A. vulgaris* present in the BOLD System (Fig. 50). Dr. Daniel Martín-Vega, specialist of Piophilidae, suggested that *Allopiophila vulgaris* is considered as synonymous of *Parapiophila vulgaris*, consistently with the structure of the tree here presented (personal communication). Some *Parapiophila vulgaris* sequences (GBDP9366-11, GBDP9367-11, GBDP9368-11 & GBDP15922-15) cluster with *Stearibia nigriceps* Meigen, 1826. Unfortunately, it is not possible to verify the correct identification of these specimens, but it is a current opinion that they belong to the genus *Stearibia* and not to *Parapiophila* (Dr. Daniel Martín-Vega, personal communication).

5.1.3.4 ***Discussion***

In forensic cases, it is currently accepted that insects can give investigator high number of information related to environmental conditions and the time of the death (Amendt *et al.*, 2007; Gennard, 2007; Amendt *et al.*, 2011; Hart *et al.*, 2011; Tomberlin & Benbow,

[4] *Piophila nigriceps* Meigen 1826 is considered a synonymus of *Stearibia nigriceps* Meigen, 1826.

2015). A critical step to get this information is the species identification. The literature about the morphology of adult insects of forensic interest is extensive (Barros de Carvalho & 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, 2010; Szpila *et al.*, 2015; Grzywacz *et al.*, 2017a). Puparia, due to the hardness of their external sclerotised cuticle are the most abundant insect remains found in forensic and archaeological contexts but are still an under studied topic. The evaluation of the general shape, dimension and presence of projections, although allowing the identification at family level, is not sufficient to reach the species level. Furthermore, in the 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 morphological identification. Some authors do not feel comfortable in the use of morphological characters identification method considering the necessity of a specialized taxonomic knowledge (Amendt *et al.*, 2011; Grzywacz *et al.*, 2017a). Molecular approaches have been developed to meet scientific necessity. Gene databases, because of the lack of information or the incorrectness of the uploaded information, do not yet allow an identification of all the taxa of forensic interest. Therefore, the support of the morphological identification approach remains uncontested. The preparation of open access well-illustrated keys allow non-taxonomic specialist to rely on easily recognizable characters (Grzywacz *et al.*, 2017a).

The DNA extraction, in particular for what concern puparia samples, was in most of the cases challenging. The chemical composition of the puparium and its role as toxic compounds storage (Wigglesworth, 1972), that may differ among families, can negatively affect the DNA exctraction itself as well as downstream reactions such as PCR.

Molecular analysis here performed clearly included the sequences in the correct genus. Because of the lack of available sequences, some species are still not identifiable by only 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. Focusing on the three families Sphaeroceridae, Heleomyzidae, and Piophilidae, only for the last, because its economical (food pest) and forensic interest, some important and inclusive molecular studies have been performed (Zajac *et al.*, 2016). In contrast, few papers for specific genera have been published for Sphaeroceridae (Kits *et al.*, 2013;

Marshall *et al.*, 2015) and, to the knowledge of the author of the thesis, no one for Heleomyzidae.

The correct identification of *Phthitia empirica* based on molecular data was not possible due to the lack of sequences uploaded. The 92% identity revealed by the comparison on GenBank with *Phthitia ovicercus* is congruent with their close relation in the phylogenetic tree (Fig. 48). It is worth mentioning that the aims of this approach were only to verify which species/genus the analysed samples were more related with. Its location close to *Phthitia* species confirms the morphological analysis. In addition, because the sequences are now deposited in NCBI GenBank and available online, they will be the reference for further works.

A high number of sequences are available in GenBank for the Heleomyzidae family but unfortunately referring to few species, reflecting the absence of phylogenetical works. The lack of sequences of *H. captiosa* (Gorodkov, 1962) in BOLD system and NCBI GenBank prevents the possibility to discriminate the two species and it underlines the actual weakness of the molecular approach in poorly investigated families.

When working with the phylogeny of Piophilids, the amount of synonyms used worldwide have to be considered (Prado e Castro & García, 2010; Martín-Vega, 2011; Mei *et al.*, 2013; Kirinoki *et al.*, 2015). The Holarctic and Neotropical *Stearibia nigriceps*, called also *Piophila nigriceps*, is considered to be the same species of the east European Russian *Piophila foveolata* (McAlpine, 1977). The genera *Arctopiophila* Duda, 1924 and *Allopiophila* Hendel, 1917 are used as synonyms of *Parapiophila* McAlpine, 1977 (Martín-Vega, 2011) while, according to Prado e Castro and García (2010), *Liopiophila* is a synonym for *Prochyliza*. These results (Fig. 50) support the synonymy of *Parapiophila vulgaris* and *Allopiophila vulgaris*. The clusterisation of some sequencing of *Parapiophila vulgaris* with *Stearibia nigriceps* suggest a mistake in morphological identification. In order to carry out molecular analysis the researchers that deposited these questioned sequences, sacrificed the whole specimen with the consequent impossibility of further checking of the morphology identification. Further studies need to be done in order to better understand the phylogeny of this family and clarify the synonymies of the species in this family. The usage of DNA extraction method that prevents the morphological destruction of the specimen is of fundamental importance to allow in every

moment the check of the sequences found on the databases. A new method to reduce this risk has been recently published by Tuccia *et al.* (2016a) on 3rd instar larvae of Calliphoridae, Muscidae and Phoridae, but nothing has been published yet about pupae and puparia.

5.1.4 Record of *Leptometopa latipes* (Diptera: Milichiidae) from a human cadaver in the Mediterranean area

5.1.4.1 *Introduction*

In this chapter, the finding of several specimens of a fly species in the family Milichiidae, identified as *Leptometopa latipes* (Meigen, 1830) from a cadaver are discussed. The lack of descriptions and pictorial plates of the immature stages of species in this family can justify the lack of records from forensic cases. For the purpose of providing support in species of forensic interest identification, a detailed description of the puparium and the adult of *L. latipes* are here presented. A molecular approach is also shown and discussed. Small black acalyptratae flies, Milichiidae are sometimes called "free loader flies" due to their kleptoparasitic habits. Larvae are mostly saprophagous, generally developing on decomposing organic substrata. Immature stages of this species can be also collected from animal and human faeces and biological waste inside ant nests (Smith, 1986).

5.1.4.2 *Case description*

In 2006, inside an apartment in a Maghreb country[5], in Northern Africa, the corpse of a dead Italian young man was found. Carbon monoxide intoxication has been addressed as cause of death according to the first autopsy report. The body was moved to Italy for a second *post mortem* examination. The conditions of the remains made impossible the determination of the cause of death. In 2013, following demands of the relatives of a new investigation to confirm the body identity, the corpse was exhumed. Samples from the bones were collected and the kinship genetic tests performed. However, the identity matching was not possible due to the poor conservative conditions of the remains. High

[5] More specific data cannot be presented because the legal aspect of the case.

number of small flies and puparia were collected from the bone surface, underlain a heavily colonisation by insects. The sampling, preparation and analysis of the entomological samples have been done according to the standards and guidelines of the European Association for Forensic Entomology (EAFE) (Amendt *et al.*, 2007) in order to obtain information useful for the investigation. Samples were stored in the collection of Dr. S. Vanin (Huddersfield) till being analysed by the author of this thesis.

5.1.4.3 *Materials and methods*

Before microscopic observation, puparia specimens went through a carefully cleaning in a water-soap solution and then air-dried. The observation of the diagnostic characters did not require any sonication. Both adults and puparia samples were observed and photographed under a Keyence VHX-S90BE digital microscope (For further details see 4.2). In addition, puparia samples where further visualized under a FEI QUANTA 650 FEG (Thermo Scientific, USA) scanning electron microscope (SEM). Stubs with conductive adhesive tape were used to mount dried cleaned specimens successively coated with Au-Pd in a SC7620 Mini Sputter Coater (Quorum Technologies, UK). Pictures were directly digitized from the SEM. Terminology of the diagnostic characters useful for puparia identification are in accordance with the most recent works on the topic (Skidmore, 1985; Martín-Vega *et al.*, 2016; Grzywacz *et al.*, 2017a; Giordani *et al.*, 2018a). A molecular approach was used to further confirm the morphological identification. DNA extraction with both the QIAamp® DNA Mini kit and the QIAamp® DNA Investigator kit (Table 8) were performed from adult flies and empty puparia.

Destruction of the specimens was substituted with the less invasive submersion in the extraction buffer and incubation for 20 hours at 56°C. The extracted DNA was quantified with a Qubit Assays (Table 8).

The mitochondrial COI barcoding region was amplified using the universal primers designed by Folmer *et al.* (1994).

The percentage identity between the obtained sequences and those available on the online gene banks has been evaluated on the online system BLASTn® (Altschul *et al.*, 1990). The obtained sequences are deposited on GenBank with accession number MH069729, MH825680, and MH921582. NCBI and BOLD genbanks were used as sources of

Milichiidae species sequences to include in the analysis (Table 6). To maximize the number of investigate species, a region of only 407bp was taking in account for the phylogenetic reconstruction. Sequences were aligned with Clustal Omega (Sievers *et al.*, 2011). Molecular evolutionary genetics analysis version 7.0 (MEGA 7.0) (Kumar *et al.*, 2016) was used to build a phylogenetic tree using the Neighbour Joining method with a 1,000 replications bootstrap to test the robustness of the phylogenetic reconstruction. Interactive tree of life (ITOL) (Letunic & Bork, 2016) was used to visualise the tree. Sequences of *Drosophila melanogaster* were used as outgroup.

Table 6 **Sequences of Milichiidae downloaded from NCBI and BOLD and included in the analysis (From Giordani *et al.*, 2018c).**

		NCBI	BOLD
Milichiidae	*Leptometopa latipes* (Meigen, 1830)	KR755741.1 KR434028.1 KR671912.1 KP045736.1 KT619898.1	BARSL464-16 BARSM1099-17 BARSM1282-17
	Leptometopa halteralis (Coquillett, 1900)	KR968305.1 KR968352.1	
	Milichiella arcuata (Loew, 1876)	KR658784.1	OPPFQ2054-17 OPPQQ542-17
	Neophyllomyza quadricornis Melander, 1913		CNWBB606-13 JWDCA911-10 JWDCA913-10
	Pholeomyia indecora (Loew, 1869)	KT116944.1 KR519912.1 KR671964.1	
	Paramyia nitens (Loew, 1869)	KR520034.1 KR519577.1 KR518018.1	
	Madiza glabra Fallén, 1820	KR667790.1 KM936729.1 KR652358.1	
	Desmometopa sordida (Fallen, 1820)	KR756711.1 KR972336.1 KR770933.1	
Drosophilidae (Outgroup)	*Drosophila melanogaster* Meigen, 1830	KJ767244.1 KJ767243.1	

5.1.4.4 ***Results***

Hydrotaea capensis (Wiedemann, 1818) in the family Muscidae and *Leptometopa latipes* (Meigen, 1830) in the family Milichiidae were identified among the numerous puparia and small flies collected over the remains. While the finding of *H. capensis* was not

surprising due to the common recording of this species from decomposing cadaver or carrion and buried remains, the finding of the latter was unexpected. Molecular analysis also confirmed the identification of the latter species as *L. latipes*. DNA quantification is reported in Table 8. The description and analysis of *L. latipes* are reported in the following paragraphs together with a briefly description of *H. capensis*. This latter species and the genus *Hydrotaea* in general are the main subject of the sections 5.3.3.

Hydrotaea capensis

Hydrotaea capensis is a synanthropic species hailing from the old world. It has been recorded from different habitats (Grzywacz *et al.*, 2017c) mainly in warm seasons. The minimum developmental temperature and thermal constant of this species have been calculated by Lefebvre and Pasquerault (2004) to be respectively 12.8°C and 237.05±22.73 ADD. This species, able to colonise both exposed and buried bodies, has been recorded during the active decay stage of decomposition in the first case (Smith, 1986; Giordani *et al.*, 2018a) while as one of the first colonisers in concealed bodies (Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Huchet, 2013). Puparia of *H. capensis* were collected from both forensic and archaeological contexts in France, Germany, Italy, Portugal, Spain and other European countries (Greenberg, 1985; Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Vanin *et al.*, 2011; Huchet, 2013). A detailed puparium description of this species is illustrated in chapter 5.3.1.

Leptometopa latipes[6]

Adults flies are shiny black, and their diagnostic characters are mainly related to the frons, the thorax and the legs. *Leptometopa latipes* is a small fly showing reddish margin frons, silvery microtomentose median longitudinal stripe and a bare mesopleuron. Fore and mid tibia of male and female specimens have yellow basal and medial rings. Hind tibia in male is enlarged and it is considered a good taxonomic character. Body length in female is 1.68±0.06mm (N=3) while in male is 1.22±0.07mm (N=5). The average length of wings is 1.74±0.14 and 1.31±0.25mm respectively for females and males**.** The light grey ocellar triangle on the head presents a distinctive microtomentum, two ocellar setae and

[6] Puparium and adult descriptions follow Giordani *et al*., 2018c

three to five short central setae. The frons is rectangular, longer than wider. The anterior margin of the frons is red and silvery microtomentose longitudinal stripes are present next to the eye margin. Four orbital and one lower fronto-orbital pairs of setae are presents on the frons. Lunula is yellow and bare triangular-shaped. Antennae are dark with the first flagellomere irregularly rounded and a pubescent arista. Para-facial is yellow with microtomentum, gena are thick, 1/4 of eye height and yellowish. At the level of lower eye margin there are strong vibrissae. Palpi are yellow, slightly sickle-shaped in lateral view with black setulae in the ventral margin. The proboscis is geniculate, darkish brown, with sparse setulae in the margin (**Error! Reference source not found.**).

Fig. 51 ***Leptometopa latipes* adults head details.** Female ♀ oral region (A), male ♂ bristles (B) and male ♂ antennae (C) (Scale bar 100µm) (From Giordani *et al*., 2018c).

The convex thorax has one postpronotal, one notopleural, one posterior dorsocentral, one supra- and two post-alar (in intra-alar position) pairs of setae. The scutellum shows two pairs of marginal bristles. Apical scutellar bristles are not present (**Error! Reference ource not found.**A). As in other species of the same family the wing present two costal breaks, once near humeral cross-vein and once near apex of vein R_1. Anal vein is extremely reduced, detectable only as shadow (**Error! Reference source not found.**B). oreleg and midleg present a yellowish ring and light colour tarsi. Tibiae do not have any dorsal preapical bristle. In the male specimen, the diagnostic feature is the strongly broaden and flat hind tibia (**Error! Reference source not found.**) (Giordani *et al*., 018c).

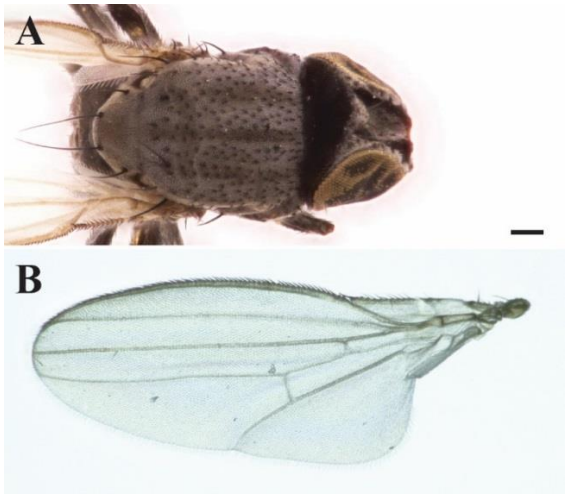

Fig. 52 ***Leptometopa latipes* adult details.** Thorax (A) and wing (B) (Scale bar 100µm) (From Giordani *et al*., 2018c).

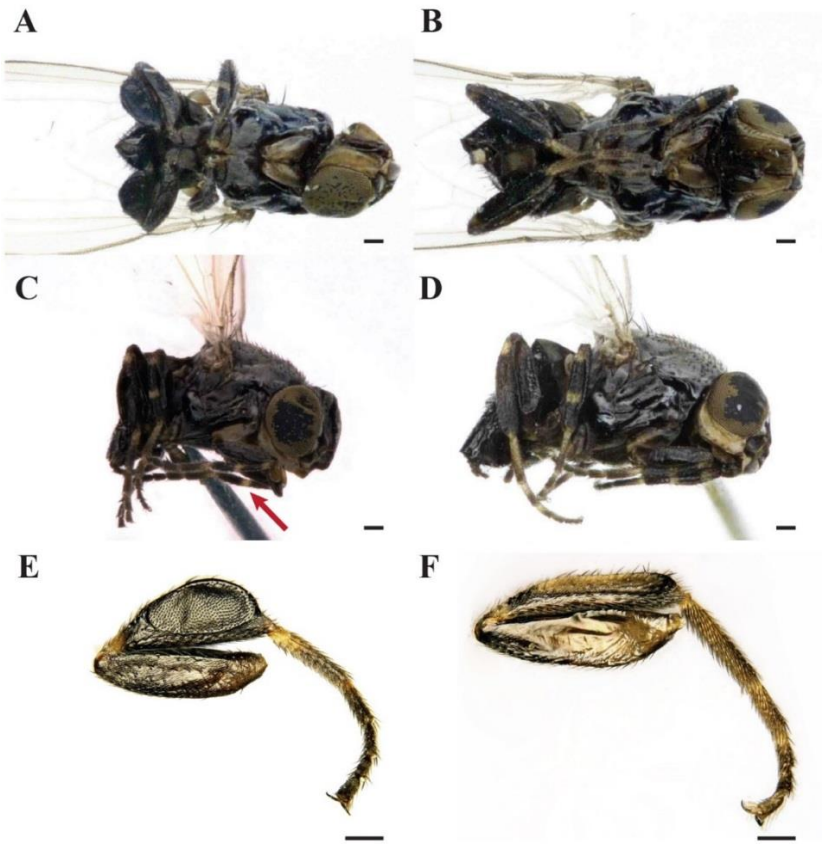

Fig. 53 ***Leptometopa latipes* adults.** Male in ventral (A) and lateral (C) view. The arrow shows the yellowish ring in the foreleg. Female in ventral (B) and lateral (D) view. *Leptometopa latipes* hind tibia: male (E) and female (F) (Scale bar 100µm) (From Giordani *et al*., 2018c).

Puparia of *L. latipes* are yellow to light brown (Fig. 55A,B,C). The average length of the close puparium in the studied sample is 0.32±0.05mm long (*N*=5). Several excrescences

surrounding a smooth anal plate with no expansions (wings) cover the posterior anal region. All the anal papillae are absent or not discernible (Fig. 54A; Fig. 55D, E). The intersegmental spines of ventral welt of abdominal segment seven are small and differently oriented, with the top half directed toward the anal plate and the bottom half directed on the opposite direction. Spines in the external lines are smaller and closer than the central ones and ending with sharp tips (Fig. 54B; Fig. 55F).

Posterior spiracles are situated on two strongly projected processes. The three slits are allocated each one on a different expansion kept closed to the puparium by filaments emanating from perispiracular glands (Fig. 54D,E,F; Fig. 55G,H). In the studied specimens, star shape anterior spiracles showed five to six prospiracular lobes (Fig. 54C; Fig. 55I) (Giordani *et al*., 2018c).

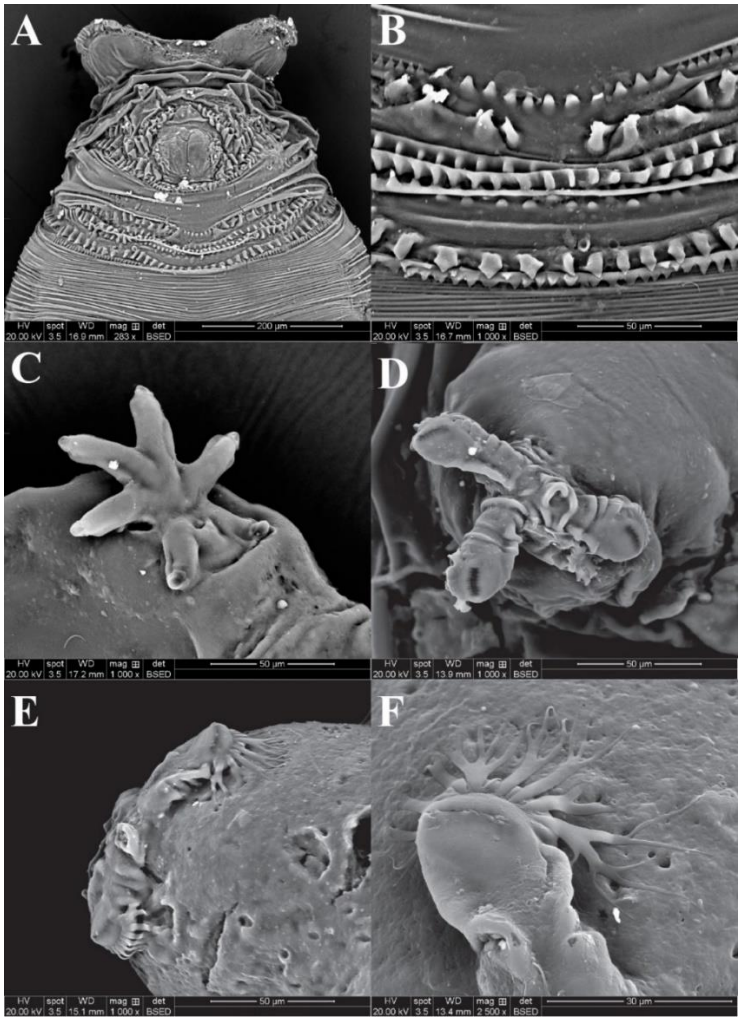

Fig. 54 ***Leptometopa latipes*** **puparium details.** Anal plate (A), intersegmental spicules (B), anterior spiracle (C), posterior spiracle (D,E) and filaments emanating from perispiracular glands (F) (Scale bars are reported in each frame) (From Giordani *et al*., 2018c).

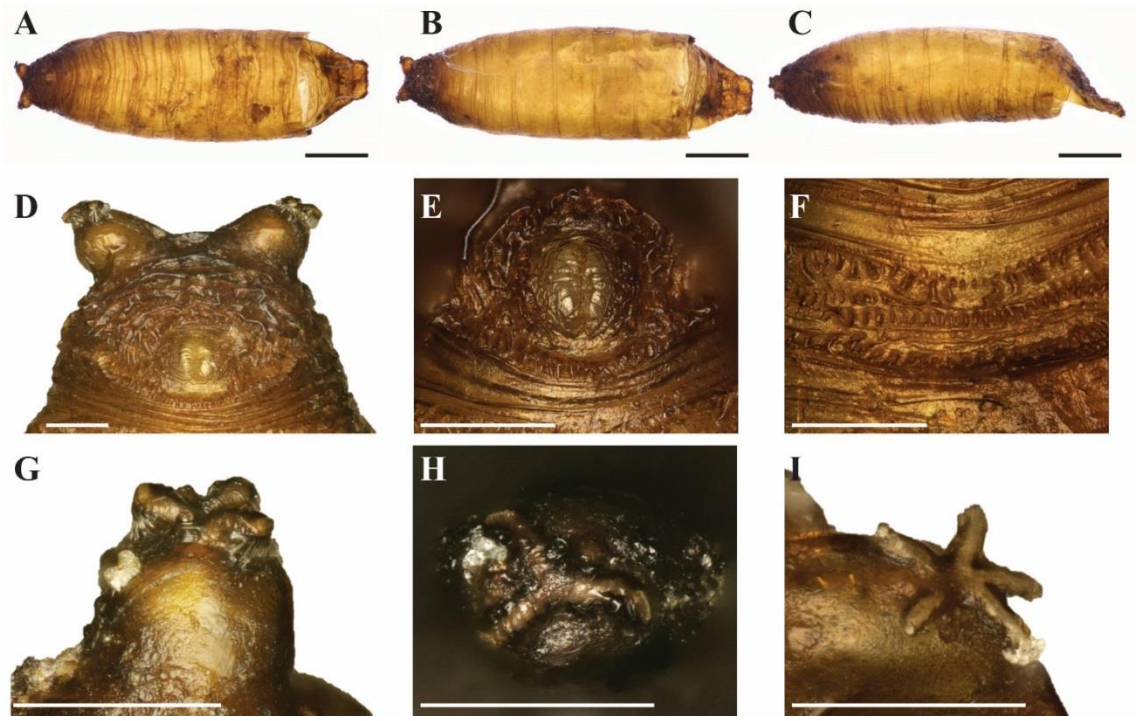

Fig. 55 ***Leptometopa latipes* puparium.** Ventral (A), dorsal (B) and lateral (C) view (Scale bar 500µm). Puparium details: Posterior anal region (D), anal plate (E), intersegmental spicules (F), posterior spiracle (G,H) and anterior spiracle (I) (Scale bar 100µm) (From Giordani *et al*., 2018c).

Biology and distribution

Leptometopa latipes shows a widespread distribution in Palearctic, African, Nearctic and Neotropical regions (Papp, 1984; Papp & Wheeler, 1998; Leto Barone *et al.*, 2002). Larvae are saprophagous and coprophagous, feeding on decomposing biological matter and consuming and re-digesting large animals excrements (Papp & Wheeler, 1998; Leto Barone *et al.*, 2002). Both field traps and animal carcasses allowed the collection of this species (Braack, 1986; Carles-Tolrá *et al.*, 2012). The only archaeological record of *L. latipes* refers to puparia and an adult fragment collected from the sarcophagus of Federico II in Palermo, Italy (Leto Barone *et al.*, 2002) (Table 7).

Table 7 **Published records of *L. latipes* from decomposing organic matters, animals and excrements** (From Giordani *et al*., 2018c).

Location	Context	Reference
EUROPE		
Czech Republic	Decaying Pig foot	(Barták & Roháček, 2011)
Hungary	Poultry houses	(Farkas & Papp, 1989)
Italy (Lampedusa Island)	Sarcophagus of Federico II	(Raspi *et al.*, 2009)
Spain	Decaying Pig	(Carles-Tolrá *et al.*, 2012)
ASIA		
India	Fish infestation	(Esser, 1988)
AFRICA		
South Africa	Carcasses of freshly killed *Aepyceros melampus* (Lichtenstein, 1812)	(Braack, 1986)
North AMERICA		
Indiana	Excrements of *Nycticeius humeralis* (Rafinesque, 1818)	(Whitaker *et al.*, 1991)
New York	Nests of A*sio otus* (Linnaeus, 1758) and *Falco sparverius* (Linnaeus, 1758)	(Philips & Dindal, 1990)

The identification of the *L. latipes* was confirmed as well by molecular analysis. DNA quantification is reported in Table 8.

Table 8 **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. Standard deviation is reported.

Species	Sample	Method	Elution (µl)	Average DNA quantification (ng/µl)	Total amount of extracted DNA (ng) per specimen
L. latipes	***5 adults***	Investigator kit	100	13.83±0.05	276.67±1.15
L. latipes	***5 adults***	Mini kit	200	2.68±0.00	107.20±0.00
L. latipes	***10 puparia***	Investigator kit	100	0.72±0.00	7.20±0.00

The local alignment on BLASTn® of the sequence obtained from this case with those recorded in GenBank resulted in a 99-100% identity with a sequence coded as *Leptometopa latipes* KR671912, with a 0.0 E value. The quality of the molecular result was evaluated through a phylogenetic approach based on 33 sequences of 407bp in the COI gene. The reconstructed tree (Fig. 56) visibly strengthens the morphological identification. The genus *Leptometopa* appears to be monophyletic even though the low bootstrap value at basal nodes does not allow any final consideration at genus level. All the sequences of *L. latipes* downloaded from literature and included in this study belong to American specimens. No Palearctic specimen sequences were available at the time of the analysis. So, this work allowed the uploading in GenBank of the first Palearctic datum.

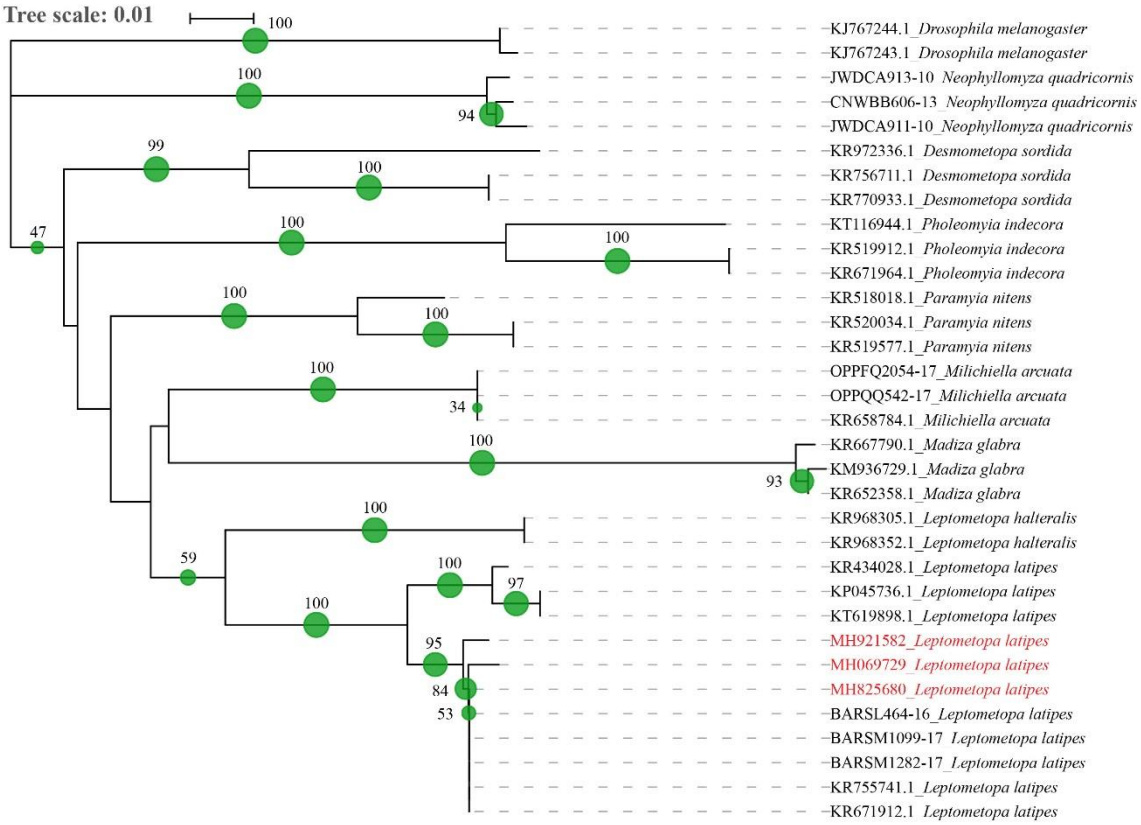

Fig. 56 **Phylogenetic tree of Milichiidae family.** Neighbour Joining method analysis of 407bp sequence of the COI gene. The green spots and the number at each node indicate the bootstrap support. Sequences from this study are reported in red (Modified from Giordani *et al*., 2018c).

5.1.4.5 ***Discussion***

After death, the lack of vital functions in the body activates a series of physical and biochemical reactions involving the bacterial communities residing on and inside the body which releases volatile molecular organic compounds (VOCs) and creates the conditions for insect colonisation. Species in the families Calliphoridae, Muscidae and Sarcophagidae are, among the necrophagous flies, usually the first colonisers, followed by other taxa in the Diptera order. These species are followed by members of the Coleoptera and Lepidoptera orders. Predators and parasitoids of other insects, mainly in the order Hymenoptera can also be present on the body. In the Mediterranean region, *Calliphora* Robineau-Desvoidy, 1830, *Chrysomya* Robineau-Desvoidy, 1830 and *Lucilia* Robineau-Desvoidy, 1830 species are commonly among the first colonizers of exposed bodies and their presence is mainly related to the season (Vanin *et al.*, 2008; Díaz-Aranda *et al.*, 2018). In the case presented here, the finding of the corpse a few hours after death

can justify the absence of blowflies. However, nothing can be said about when the insect colonisation actually started due to the fact that the body was stored in unknown conditions in a very warm country and furthermore it was subjected to repeated exhumations and autopsies.

Muscidae species are often found on cadavers in urban areas (Smith, 1986; Lo Pinto *et al.*, 2017) with some species typically found indoor, in underground crypts and other hypogeal environments (Giordani *et al.*, 2018b). Typical of corpses buried in crypts or underground (Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Huchet, 2013), the Muscidae species, *H. capensis*, found on this body is also reported from active decomposing exposed cadavers (Skidmore, 1985; Smith, 1986).

Leptometopa latipes, is known to feed on decomposing matter, animal faeces and insect nests and, as other species in the family Milichiidae, it is only rarely recovered from decomposing human bodies (Smith, 1986). The findings of the species in this context is worth mentioning and could be related to the initial storage conditions of body as previously mentioned (e.g. morgue, repeated autopsies, etc).

According to the geographical distribution of both species which do not exclude the Mediterranean basin, it's not surprisingly that *H. capensis* and *L. latipes* have been found in this forensic case. From a forensic point of view this point was important to confirm the potential origin of the remains, which the police were still investigating. The morphological identification for the less common *L. latipes* was confirmed by molecular analysis.

Despite the phylogenetic analysis showing the sequences in the correct cluster of species, it would be necessary to increase the number of sequences and their size in order to strengthen the Bootstrap values of the tree basal node. In fact, it is worth reporting that the sequences used in this reconstruction are the first Palearctic sequences deposited in NCBI GenBank and available online.

The observations reported in this chapter underline the need of an increased and detailed knowledge of the species phenology, habitat preference, development and distribution to permit a better application of the entomological analyses. In this way, insect analysis can contribute to a more complete scenario of a crime scene together with data from other forensic disciplines like forensic pathology and anthropology.

Part of this chapter has been published in Giordani *et al.* 2018c, attached at the end of this thesis (Appendix B).

5.1.5 Conclusions

In the studies presented in 1.1.3 and 1.1.4, distinct morphological characters of puparia and adults of species of forensic interest, allowing for precise species identification, have been described. Insects, based on their biology and ecology, can provide a different set of information about the *peri mortem* events. The availability of easy access digitalised pictures may overturn the paucity of records of poorly investigated forensic species.

The molecular approach was used to confirm the identification. The non-invasive technique of DNA extraction by submersion provided good quality results for both adult and immature stages allowing the preservation of morphological characters. The dimension of the specimen can influence the commercial kit efficiency and have to be consider at the experimental settings stage. The chemical composition of the puparium can differ among species and could contain inihibitors that can affect downstream results such as PCR. The calcium carbonate crystals ($CaCO_3$) deposition in Stratiomyidae and Xylomyidae puparia (Woodley, 1989) is a well studied example. Despite no clear evidence of high carbonate deposition in other families, the phenomenon of other salt deposition on the puparia cannot be excluded. Furthermore, the puparium acts as the storage of toxic substances accumulated by the larval life as reflection of their biology and ecology (Wigglesworth, 1972). Further studies on the biochemistry and biosynthesis of the puparium in different families could help in understanding the effect of these components on the DNA extraction and amplification. It is worth mentioning that the number of cells in a puparium is low and in order to obtain a positive molecular result it could be necessary to combine more than one specimen. Commercial kit might be re-designed to allow the use of high lysis buffer volume, able to cover the whole samples, and the final DNA concentration in a small volume.

On the other hand, pooling together different puparia with similar features can lead to a non-monospecific sample and might increase the inhibition rate. The cleaning of the external surfaces and the accurate morphological identification will minimise these contraindications.

The pictorial tables and the descriptions reported in this chapter could facilitate the identification of immature stages of Diptera from forensic and archaeological contexts. They represent as well a model that could be applied to other taxa of forensic and archaeological interest.

5.2 Museum samples

5.2.1 Introduction

Sarcophagidae, with almost 3,000 described species, is the second-largest family of Diptera Oestroidea (Courtney *et al.*, 2009; Pape *et al.*, 2011). In the current, broadly-followed classification, *Sarcophaga* Meigen 1826, is by far the largest genus in the family, with over 900 valid species described to date (Pape, 1996; Buenaventura *et al.*, 2017). This genus has greatly diversified in the Old World, occupying a large diversity of ecological niches. Species of *Sarcophaga* are important ecosystem service providers as decomposers, pollinators and biocontrol agents. Several species are forensic indicators, providing information on mPMI (Szpila *et al.*, 2015). However, due to high morphological similarity between closely-related species and to the lack of clear-cut diagnostic features at the species level, the identification of both the larvae and adults of these flies is difficult, particularly for non-experts. An accurate morphological identification of species relies on close analysis of male or female genitalia or of small details of the larval morphology. Additionally, the classification of the genus has traditionally heavily depended on male genitalia morphology (Whitmore *et al.*, 2013), and knowledge of female and larval morphology is still far behind that of males. Due to these difficulties in their identification, females and larvae of *Sarcophaga* spp., despite being abundant in samples, are often underrepresented in reference collections and therefore not readily available for study and have been left undescribed or are unknown. The issue of the identification of females and larvae of *Sarcophaga* spp. can be solved by matching of DNA sequences against sequences in molecular libraries, but such libraries are still far from complete and molecular identification using COI barcodes is still problematic in some groups. At the same time, in many species-groups, even in comparatively well-studied faunas, knowledge of the males is far from complete and comprehensive studies are required before taxonomic issues can be resolved. This is the case of the subgenus *Sarcophaga sensu stricto*, whose larvae are well-known predators of earthworms and macrolepidopteran pupae (Povolny & Verves, 1997), and which currently comprises just under 35 valid species distributed in the West Palaearctic (Pape, 1996). Despite including some of the most common and widespread sarcophagids in

Europe, the group has a history of misidentifications, including that of the type species of the family, due to the great morphological similarity of its species. Recent studies have found that even COI barcodes are not able to properly separate some of the most closely-related species (Jordaens *et al.*, 2013). Three species of *Sarcophaga sensu stricto* occur in Britain: *S. carnaria* Linnaeus, 1758, *S. variegata* (Scopoli, 1763) and *S. subvicina* Baranov, 1937. The males of these three species are well-characterised morphologically, presenting no particular identification problems. For this reason, and because they are very abundant in collections, in particularly in the NHMUK's British collection, they represent excellent models for testing DNA extraction techniques from old specimens collected over a time-span of about 100 years.

5.2.2 Aim

This study explored the possibility of a molecular analysis on Diptera flies preserving the appearance of the external characters useful for the identification of the specimens. The aim is the development of a DNA extraction method from adult flies belonging to museum collections without affecting the morphology and the chaetotaxy of the sample.

5.2.3 Materials and Methods

Eighty-one dry specimens of Sarcophagidae flies collected in the period 1892-2016 were tested for the non-invasive DNA extraction protocol (Table 9). All the adult specimens belong to private collection (S. Vanin) or from the Natural History Museum, London. Two samples of *Calliphora vomitoria* (Linnaeus, 1758) (Diptera: Calliphoridae) and one adult of *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae) from UK (Huddersfield, West Yorkshire) were included in the analysis. The morphological identification was performed in a blind way by Dr. Daniel Whitmore (Senior Curator Insects Division, Natural History Museum, London), one of the most important European experts on Sarcophagidae (Table 9).

DNA extraction was performed from the abdomen of each sample that, after being gently removed from the rest of the body using tweezers, was submerged into the extraction solution (Fig. 57). Different DNA extraction protocols were tested.

- Twenty-five specimens were tested for the QIAamp® DNA Mini kit (see 4.3.1). DNA was eluted in 200µl; half of the abdomen of other three specimens were tested for the same commercial kit.
- Fifteen samples were tried for the homemade digestion buffer suggested by Gilbert *et al.* (2007) followed by QIAquick PCR Purification Kit® (see 4.3.3). DNA was eluted in 40µl half of the abdomen of other three specimens were tested for the same commercial kit.
- Five specimens were tested with the homemade digestion buffer proposed in Campos and Gilbert (2012) followed by QIAquick PCR Purification Kit® (see 4.3.3). DNA was eluted in 40µl.
- Twelve samples were verified for the homemade digestion buffer suggested by Santos *et al.* (2018) followed by different commercial kit. In detail, eight abdomens were processed using the QIAquick PCR Purification Kit®, two the QIAamp® DNA Mini kit and two the QIAamp® DNA Investigator kit (see 4.3.3). DNA was eluted in 40µl for the Purification Kit, 200µl for the DNA Mini kit and 100µl for the Investigator kit.
- Twenty-four specimens were tested for the QIAamp® DNA Investigator kit (see section 4.3.5). Different elution volumes were tested. The DNA belonging to 8 specimens was eluted in 100µl while for the rest of the samples the elution was in 50µl.

The extracted DNA was quantified by Qubit® 3.0 Fluorometer.

After the lysis step, each abdomen was removed from the buffer, dried on an absorbent paper, placed for some hours in Absolute Ethanol to stop further digestion and passed in Ethyl Acetate before being re-attached to the rest of the pinned specimen in order to restore the original adult fly (Fig. 57). The very fast evaporation of the Ethyl Acetate avoids the alteration of the microtomentum and of the hairs. Each sample was photographed before and after the treatment using Keyence VHX-S90BE digital microscope (Fig. 58) (see 4.2 for more details).

Table 9 **List of the specimens used in the extraction experiment.** The year of collection, the extraction method used, and the morphological identification are reported.

Sample	Year	Extraction method	Morphological ID
1	2015	QIAamp® DNA Mini kit	*S. variegata*
2	2011	QIAamp® DNA Mini kit	*S. crassipalpis*
3	2015	QIAamp® DNA Mini kit	*S. baranoffi*
4	2011	QIAamp® DNA Mini kit	*S. crassipalpis*
5	2015	QIAamp® DNA Mini kit	*C. vomitoria*
6	2011	QIAamp® DNA Mini kit	*S. crassipalpis*
7	2011	QIAamp® DNA Mini kit	*S. crassipalpis*
8	2002	QIAamp® DNA Mini kit	*S. variegata*
9	2013	QIAamp® DNA Mini kit	*S. variegata*
10	2013	QIAamp® DNA Mini kit	*S. variegata*
11	2004	QIAamp® DNA Mini kit	*S. variegata*
12	2003	QIAamp® DNA Mini kit	*S. carnaria*
13	2004	QIAamp® DNA Mini kit	*S. carnaria*
14	2002	QIAamp® DNA Mini kit	*S. emdeni*
15	2002	QIAamp® DNA Mini kit	*S. lehmanni*
16	2002	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
17	2002	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
18	2002	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
19	2003	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
20	2003	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. lehmanni*
21	2006	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. carnaria*
22	2007	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. croatica*
23	2005	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. croatica*
24	2015	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
25	2013	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
26	2013	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. variegata*
27	2012	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*C. vomitoria*
28	2011	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. crassipalpis*
29	2011	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. crassipalpis*
30	2011	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. crassipalpis*
31	2003	QIAamp® DNA Mini kit	*S. crassipalpis*
32	2004	QIAamp® DNA Mini kit	*S. crassipalpis*
33	2004	QIAamp® DNA Mini kit	*S. crassipalpis*
34	2004	QIAamp® DNA Mini kit	*S. crassipalpis*
35	2004	QIAamp® DNA Mini kit	*S. crassipalpis*
36	2004	QIAamp® DNA Mini kit	*S. argyrostoma*
37	2003	QIAamp® DNA Mini kit	*S. argyrostoma*
38	2017	QIAamp® DNA Mini kit	*T. molitor*
39a	2006	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. croatica*
39b	2006	QIAamp® DNA Mini kit	*S. croatica*
40a	2005	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. croatica*
40b	2005	QIAamp® DNA Mini kit	*S. croatica*
41a	2016	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	*S. carnaria*
41b	2016	QIAamp® DNA Mini kit	*S. carnaria*
42	2002	QIAamp® DNA Mini kit	*S. variegata*
43	2003	QIAamp® DNA Mini kit	*S. lehmanni*
44	2002	QIAamp® DNA Investigator kit	*S. variegata*
45	2002	QIAamp® DNA Investigator kit	*S. lehmanni*
46	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. croatica*
47	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. variegata*
48	2002	DB Santos et al (2018) + QIAamp® DNA Mini kit	*S. variegata*
49	2002	DB Santos et al (2018) + QIAamp® DNA Mini kit	*S. croatica*

50	2002	DB Santos et al (2018) + QIAamp® DNA Investigator kit	*S. variegata*
51	2004	DB Santos et al (2018) + QIAamp® DNA Investigator kit	*S. variegata*
52	2004	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. variegata*
53	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. variegata*
54	1983	QIAamp® DNA Investigator kit	*S. carnaria*
55	1983	QIAamp® DNA Investigator kit	*S. subvicina*
56	1983	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. carnaria*
57	1983	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. subvicina*
58	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. variegata*
59	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	*S. lehmanni*
60	1967	QIAamp® DNA Investigator kit	*S. carnaria*
61	1966	QIAamp® DNA Investigator kit	*S. subvicina*
62	1946	QIAamp® DNA Investigator kit	*S. carnaria*
63	1942	QIAamp® DNA Investigator kit	*S. carnaria*
64	2004	Campos and Gilbert 2012 + ppK	*S. carnaria*
65	2005	Campos and Gilbert 2012 + ppK	*S. croatica*
66	2005	Campos and Gilbert 2012 + ppK	*S. croatica*
67	1962	Campos and Gilbert 2012 + ppK	*S. carnaria*
68	1948	Campos and Gilbert 2012 + ppK	*S. carnaria*
69	1977	QIAamp® DNA Investigator kit	*S. carnaria*
70	1977	QIAamp® DNA Investigator kit	*S. carnaria*
71	1977	QIAamp® DNA Investigator kit	*S. carnaria*
72	1971	QIAamp® DNA Investigator kit	*S. carnaria*
73	1954	QIAamp® DNA Investigator kit	*S. carnaria*
74	1954	QIAamp® DNA Investigator kit	*S. carnaria*
75	1953	QIAamp® DNA Investigator kit	*S. carnaria*
76	1951	QIAamp® DNA Investigator kit	*S. carnaria*
77	1932	QIAamp® DNA Investigator kit	*S. carnaria*
78	1931	QIAamp® DNA Investigator kit	*S. carnaria*
79	1930	QIAamp® DNA Investigator kit	*S. carnaria*
80	1936	QIAamp® DNA Investigator kit	*S. carnaria*
81	1920	QIAamp® DNA Investigator kit	*S. carnaria*
82	1919	QIAamp® DNA Investigator kit	*S. carnaria*
83	1902	QIAamp® DNA Investigator kit	*S. subvicina*
84	1898	QIAamp® DNA Investigator kit	*S. subvicina*

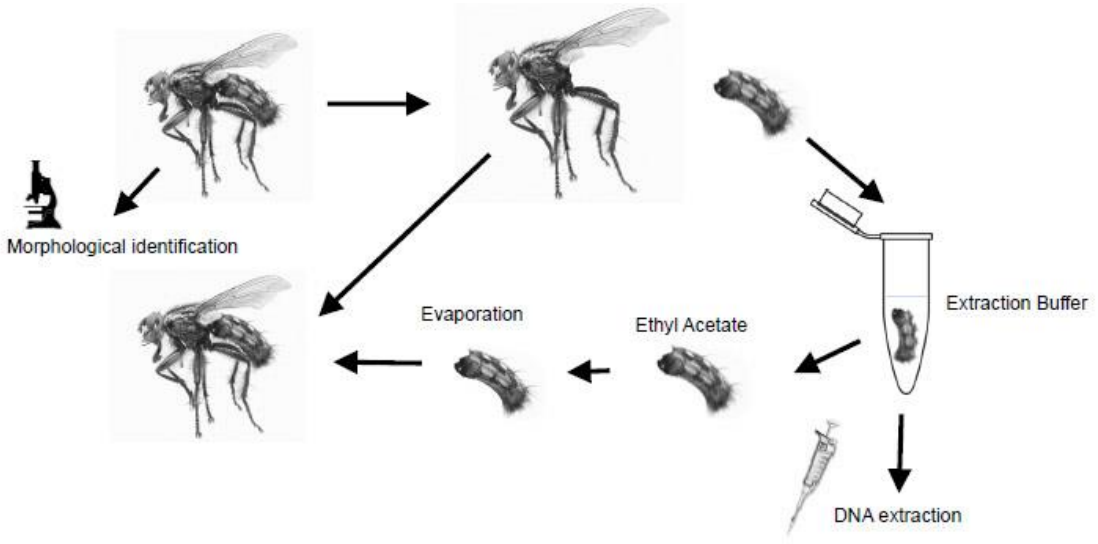

Fig. 57 **Schematic representation of the followed protocol.**

The size of the extracted DNA was evaluated with Agilent Bioanalyzer® 2100 (see 4.6). The 658bp barcoding region of the COI gene was targeted with the universal LCO/HCO primers designed by Folmer *et al.* (1994) or through the amplification of smaller overlapping region with new primers designed specificly for Sarcophagidae flies (Table 10). The total overlapping of the targeted fragments covers the 658bp (Fig. 35).

Table 10 **New designed degenerated primers for *Sarcophaga* genus.**

Oligo name	**Sequence (5' -> 3')**
Sar111_FW	TCGCAACAATGGTTATTCTCT
Sar111_RV	TCARTTTCCAAAYCCTCCAAT
Sar211_FW	GTAATTGTTACAGCYCATGC
Sar211_RV	TTCCAGCTCCRTTTTCTACT
Sar311_FW	CYCGAATRAAYAATATAAGTTTTTG
Sar311_RV	CCTAAAATTGAAGAAATTCCWGCTA
Sar411_FW	CTAATATTGCYCATGGRGGAGC
Sar411_RV	CGRTCAGTTAATARTATRGTRATWGC
Sar511_FW	GGWATTACHTTTGAYCGAAT
Sar511_RV	GAYTCTTGRCTAATAATGTGAG

The master mix reported in section 4.7 was used for the amplification of samples 1 to 63 (Table 14) with 2 to 4µl of DNA template. A PCR master mix added of 1.6µl BSA (10mg/ml) and with 1µl of each primer (10pmol/µl) and 1µl of the nucleotide mix (10 mM) was used for the amplification of samples 67 to 84 (Table 14). For the lasts, up to 5µl of DNA per reaction were used.

Sarcophaga sp. COI gene sequences belonging to this work (Table 11), to the personal archive of Dr. Daniel Whitmore (Table 12) and sequences downloaded from BOLD and NCBI genbanks were used for the phylogenetic analysis (Table 13). Sequences were aligned with Clustal Omega (Sievers *et al.*, 2011). The phylogenetic trees were built using the Neighbour Joining method on MEGA 7.0 (Kumar *et al.*, 2016) (see 4.8).

Table 11 **GenBank unique codes of sequences of *Sarcophaga* spp. belonging to this work.**

Morphological identification	Sequence_ID	Country	GenBank code
Sarcophaga variegate (Scopoli, 1763)	1	Italy	MH118172
	9	Italy	MH118174
	10	Italy	MH118175
	16	Italy	MH118179
	17	Italy	MH118180
	19	France	MH118181
	24	Italy	MH118185
	25	Italy	MH118186
	26	Italy	MH118187
Sarcophaga baranoffi Rohdendorf, 1937	3	Italy	MH118173
Sarcophaga carnaria Linnaeus, 1758	12	France	MH118176
	21	France	MH118182
Sarcophaga lehmanni Mueller, 1922	15	Greece	MH118178
Sarcophaga croatica Baranov, 1941	22	Italy	MH118183
	23	Italy	MH118184
Sarcophaga crassipalpis Macquart, 1839	30	Italy	MH118188
	31	Italy	MH118189
Sarcophaga argyrostoma (Robineau-Desvoidy, 1830)	36	Italy	MH118190
	37	Italy	MH118191

Table 12 **GenBank unique codes of sequences of *Sarcophaga* spp. belonging to Dr. D. Whitmore archive.**

Morphological identification	Sequence_ID	Country	GenBank code
Sarcophaga variegate (Scopoli, 1763)	SeqDW-SD-03	England	MH118200
	SeqDW-SD-05	Romania	MH118201
	SeqDW-SD-06	Czech Republic	MH118202
	SeqDW-SD-09	Romania	MH118203
	SeqDW-SD-139	Czech Republic	MH118204
	SeqDW-SD-140	Czech Republic	MH118205
	SeqDW-SD-144	Romania	MH118206
	SeqDW-SD-156	Italy	MH118207
	SeqDW-SD-23	Czech Republic	MH118208
	SeqDW-SD-126	England	MH118209
	SeqDW-SD-128	England	MH118210
	SeqDW-SD-70	Italy	MH118211
	SeqDW-SD-58	Italy	MH118219
	SeqDW-SD-68	Italy	MH118220
	SeqDW-SD-69	Italy	MH118221
	SeqDW-SD-71	Italy	MH118222
	EB151	Croatia	MH118261
	EB159	Croatia	MH118262
	EB175	Croatia	MH118263
	EB110	Croatia	MH118264
	EB119	Croatia	MH118265
	EB120	Croatia	MH118266
Sarcophaga subvicina Baranov, 1937	DW1	Austria	MH118192
	DW3	Austria	MH118193
	DW4	Austria	MH118194
	DW5	Austria	MH118195
	DW6	Austria	MH118196
	DW8	Austria	MH118197

	DW9	Austria	MH118198
	DW10	Austria	MH118199
	SeqDW-SD-18	Czech Republic	MH118223
	SeqDW-SD-116	Italy	MH118224
	SeqDW-SD-117	Italy	MH118225
	SeqDW-SD-143	Czech Republic	MH118226
	SeqDW-SD-19	England	MH118227
	SeqDW-SD-118	Italy	MH118228
	SeqDW-SD-119	Italy	MH118229
Sarcophaga carnaria Linnaeus, 1758	SeqDW-SD-147	Italy	MH118252
	SeqDW-SD-148	Italy	MH118253
	SeqDW-SD-149	Italy	MH118254
	SeqDW-SD-150	Italy	MH118255
	SeqDW-SD-136	Czech Republic	MH118256
	SeqDW-SD-132	England	MH118257
	SeqDW-SD-131	England	MH118258
	SeqDW-SD-130	England	MH118259
	SeqDW-SD-133	England	MH118260
Sarcophaga lehmanni Mueller, 1922	SeqDW-SD-20	Czech Republic	MH118212
	SeqDW-SD-30	Italy	MH118213
	SeqDW-SD-43	Italy	MH118214
	SeqDW-SD-35	Italy	MH118215
	SeqDW-SD-37	Italy	MH118216
	SeqDW-SD-40	Italy	MH118217
	SeqDW-SD-46	Italy	MH118218
Sarcophaga croatica Baranov, 1941	SeqDW-SD-112	Italy	MH118231
	SeqDW-SD-107	Italy	MH118232
	SeqDW-SD-111	Italy	MH118233
	SeqDW-SD-115	Italy	MH118234
	SeqDW-SD-99	Italy	MH118235
	SeqDW-SD-87	Italy	MH118236
	SeqDW-SD-90	Italy	MH118237
	SeqDW-SD-88	Italy	MH118239
	SeqDW-SD-89	Italy	MH118240
	SeqDW-SD-93	Italy	MH118241
	SeqDW-SD-95	Italy	MH118242
	SeqDW-SD-96	Italy	MH118243
	SeqDW-SD-97	Italy	MH118244
	SeqDW-SD-98	Italy	MH118245
	SeqDW-SD-106	Italy	MH118246
	SeqDW-SD-108	Italy	MH118247
	SeqDW-SD-86	Italy	MH118248
Sarcophaga cfr. *croatica*	SeqDW-SD-77	Italy	MH118230
	SeqDW-SD-13	Italy	MH118238
Sarcophaga jeanleclercqi Lehrer, 1975	SeqDW-SD-124	Italy	MH118249
	SeqDW-SD-125	Italy	MH118250
	SeqDW-SD-145	Italy	MH118251

Table 13 **Sequences of *Sarcophaga* spp. downloaded from GenBank and BOLD and included in the analysis.**

Species	NCBI	BOLD		
Sarcophaga crassipalpis Macquart, 1839		AUSFF196-11	BBDIT842-11	GBDP6303-09
Sarcophaga argyrostoma (Robineau-Desvoidy, 1830)		GBDP0543-06	GBDP12526-12	SASI001-15
Sarcophaga matilei Blackith, Richet, Pape & Andrei-Ruiz, 2001	KU746507.1			
Sarcophaga lehmanni Mueller, 1922	KU746506.1	GBDP13967-13 GBDP12542-12 GBDP12450-12	GBDP12537-12 GBDP12558-12	GBDP12559- 12GBDP12557-12
Sarcophaga variegata (Scopoli, 1763)	KU746512.1	GMGMI106-14 GMGRB1542-13 GBDP125518-12 GBDP12532-12 GBDP12543-12	GBDP12533-12 GBDP12544-12 GBDP12547-12 GBDP12549-12 GMGMM217-14	GMGRF6346-13 GBDP12540-12 GBDP12545-12 GBDP12551-12
Sarcophaga carnaria Linnaeus, 1758	KU746611.1 KR752510.1 KR752826.1 KR753328.1 KR755286.1	GMGRB1541-13 GBDP12444-12	GBDP12536-12 GBDP12555-12	
Sarcophaga jeanleclercqi Lehrer, 1975	JQ582059.1			
Sarcophaga pyrenaica Seguy, 1941	KU746510.1	GBDP12515-12		
Sarcophaga bergi Rohdendorf, 1937	KU746610.1 KU746612.1			
Sarcophaga croatica Baranov, 1941	JQ582068.1 KU746505.1			
Sarcophaga pagensis (Baranov, 1939)	KU746509.1	GBDP12451-12	GBDP12514-12	
Sarcophaga ornatijuxta Richet, Pape, Blackith & Blackith, 1995	KU746508.1	GBDP12512-12	GBDP12513-12	
Sarcophaga subvicina Baranov, 1937		GBDP12517-12 GBDP12453-12 TTMDJ658-10 TTMDJ650-10 TTMDJ602-10 TTMDJ600-10 TTMDJ589-10 TTMDJ575-10 RRSSA234-15 PHSEP149-11 PHMTV181-10 PHMTV127-10 PHMTV084-10 GMGMJ729-14 TTMDI1227-10 TTMDI065-08 TTMDI006-08 TTDBW262-09 TTDBW225-09	PHMAL808-10 PHMAL818-10 PHMAL822-10 PHMTV022-10 PHMTV019-10 PHMTV018-10 PHMTV017-10 PHMTV016-10 PHMTV015-10 PHMTV014-10 PHMTV013-10 PHMTV012-10 PHMTV011-10 PHMTV010-10 PHMTV009-10 PHMTV008-10 PHMTV007-10 PHMTV006-10 PHMAL990-10	JSDIQ813-10 JSDIP486-10 JSDIP538-10 JSDIP607-10 JSDIP612-10 JSDIP626-10 JSDIP724-10 JSDIP755-10 JSDIP783-10 JSDIR115-11 JSDIR116-11 JSDIR125-11 JSDIR128-11 JSDIR146-11 JSDIR153-11 JSJUL165-11 JSSEP1095-11 PAJUN1126-12 PHAUG1241-11

		TTDBW047-08	PHMAL985-10	PHAUG1253-11
		TTDBW043-08	PHMAL980-10	PHAUG1799-11
		JSAUG1688-11	PHMAL940-10	PHJUL1217-11
		PHDIP018-11	PHMAL913-10	PHJUL1220-11
		PHDIP1058-11	PHMAL910-10	PHJUL1226-11
		PHDIP1287-11	PHMAL907-10	PHJUL2489-11
		PHDIP1290-11	JSDIQ279-10	PHJUL2503-11
		PHDIP192-11	JSDIQ285-10	PHJUL2562-11
		PHDIP330-11	JSDIQ288-10	PHJUN2400-11
		PHDIP333-11	JSDIQ295-10	PHJUN2401-11
		PHDIP351-11	JSDIQ298-10	PHJUN2402-11
		PHDIP352-11	JSDIQ302-10	PHJUN2403-11
		PHDIP368-11	JSDIQ313-10	PHJUN2404-11
		PHDIP385-11	JSDIQ321-10	PHJUN2406-11
		PHDIP601-11	JSDIQ324-10	PHJUN2408-11
		PHDIP849-11	JSDIQ333-10	PHMTV069-10
		PHDIP972-11	JSDIQ794-10	JSDIQ817-10
		PHMAL806-10	JSDIQ811-10	JSDIQ834-10
Sarcophaga adriatica (Bottcher, 1913)	KU746608.1			
Sarcophaga baranoffi Rohdendorf, 1937	KU746609.1			
Musca domestica Linnaeus, 1758 **(Outgroup)**	KM570270.1 KM570927.1	BBDIT1074-11	AGIRI228-17	

5.2.4 Results & Discussion

The specimens' key identification characters, such as the checkerboard pattern or the genitals structures, are conserved after the DNA extraction, as shown in Fig. 58. Although some hairs/setae are lost after the DNA extraction, their original position can be detected observing the position of their alveoli, which are easily visible at the appropriate magnification.

Two samples of *Calliphora vomitoria* (Diptera: Calliphoridae) abdomen (Samples 5 and 27) and one adult of *Tenebrio molitor* (Coleoptera: Tenebrionidae) (sample 38) were included in the analysis as inner control of the extraction methods. DNA was positively extracted from all the samples with a maximum amount of 2,513.33±11.55ng (sample 6, 2011) and a minimum of 9.37±0.21ng (sample 68, 1948) (Table 14).

A comparison of the "average DNA extracted per specimen" on the whole range of specimens tested is complicated because of the combination of the following aspects 1) different extraction methods, 2) different year of collection and 3) possible different preservation between collection and preparation. From specimens collected in the last 15 years (2003-2018), the QIAamp® DNA Mini kit allowed a recovery of 421±571ng (N=19) of DNA, with a minimum of 36ng and a maximum of 2,500ng, while the average

DNA extracted with the homemade digestion buffer by Gilbert *et al.* (2007) followed by the QIAquick PCR Purification Kit® was 594±494ng (N=11) with a minimum of 170ng and a maximum of 1,780ng. The homemade digestion buffer proposed by Campos and Gilbert (2012) allowed the recovery of a similar DNA amount, 435±136ng (N=3). The amount of DNA yielded with the homemade buffer proposed by Santos *et al.* (2018) varies based on the commercial kit used. The combination with the QIAquick PCR Purification Kit® allowed the recovery of 892±17ng and with the QIAamp® DNA Investigator kit of 67±0ng. The slight differences between the average DNA extracted on "fresh" material do not allow one method to be preferred on the other.

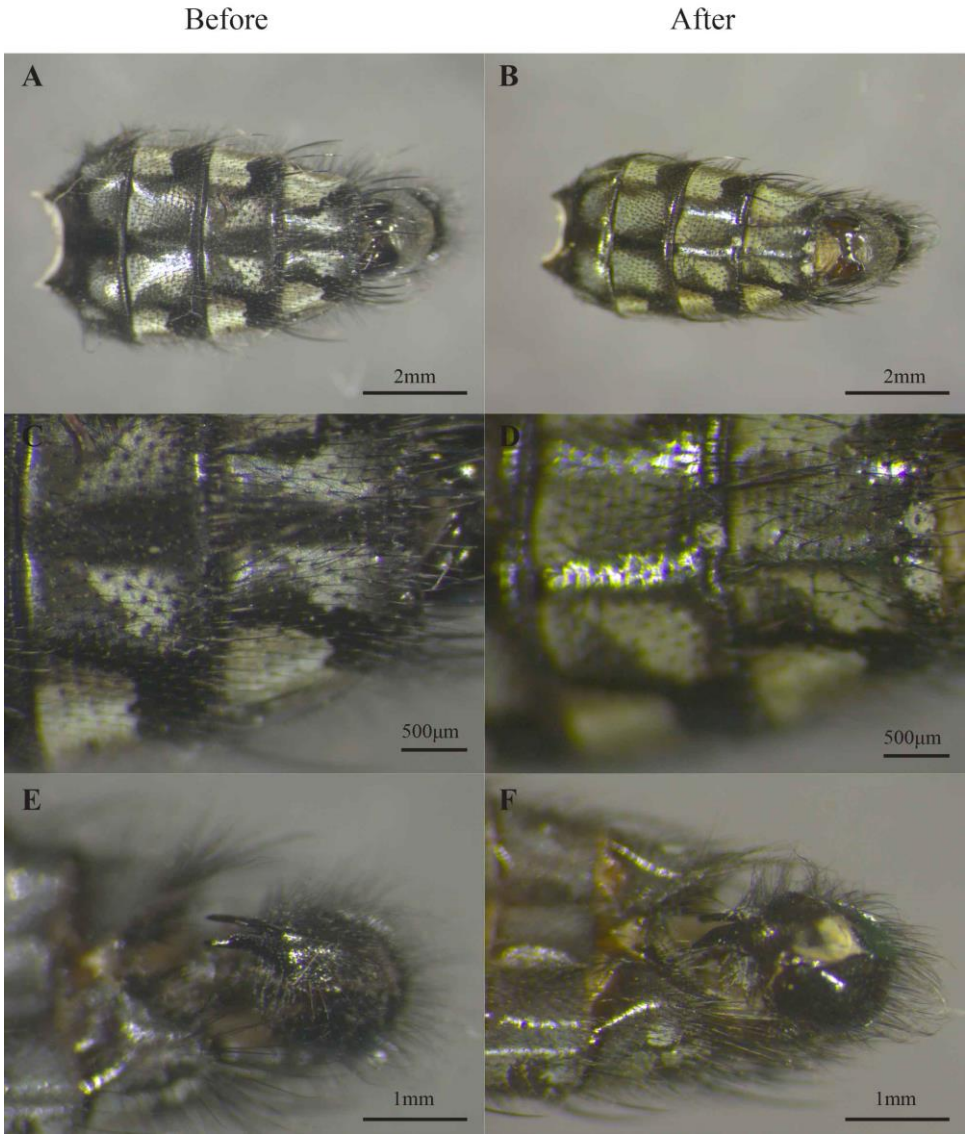

Fig. 58 **Morphological characters before (A,C,E) and after (B,D,F) the DNA extraction**: A,B) Abdomen; C,D) checkboard pattern; E,F) male genitalia.

The average DNA yielded for samples collected in 1980s was 619±906ng, in 1970s 918±630ng, in 1960s 372±286ng, in 1950s 448±341ng, in 1940s 73±57ng, in 1930s 168±192ng, in 1910-1930s 96±6g. The two oldest samples, 1902 and 1898, allowed the recovery of respectively 26.40±1.04 and 13.47±0.61ng of DNA. The high variability of amount of DNA extracted from specimens collected in the same year underline how a positive extraction result is subject to the dimension of the specimens, to its history and to its nature. A statistical analysis was done to understand the relation between the total DNA extracted amount and the years. Despite the presence of a trend (Fig. 59), the correlation coefficient is not robust (r=0.182). A two ways ANOVA was used to verify the effect of the year (grouped by 10 years), the extraction method and the interaction between these two factors on the amount of DNA extracted. Whereas the kit used for the extraction is not statistically significant (F=1.651, df=7, p=0.123) (Fig. 60), the years has a significant impact in the DNA extraction (F=5.072, df= 12, p=0.000) the interaction between the two parameters (years*extraction kit) does not have any significant effect (F=1.833, df=7, p=0.083).

Three *Sarcophaga* abdomens (Samples 39-41) were split in two and tested in parallel for two extraction methods (Homemade Digestion Buffer by Gilbert *et al.* (2007) followed by QIAquick PCR Purification Kit® and QIAamp® DNA Mini kit for a reliable comparison of the extraction methods. The quantification of the extracted DNA (Table 14) from the same specimen show a better efficiency of the Home-made Digestion Buffer compare to the commercial kit. The length of the DNA fragments obtained from samples collected in 2002 was compared for the different extraction methods (Fig. 61). The use of the homemade buffer proposed by Gilbert *et al.* (2007) or Campos and Gilbert (2012) gave the same pattern. These two methods and the QIAamp® DNA Investigator kit provided the best results.

Table 14 **DNA extraction methods and quantification.** Average amount of extracted DNA (ng/µl) is shown. Standard deviation is reported. The specimens in green boxes are the controls: 5 & 27 *Calliphora vomitoria*, 38 *Tenebrio molitor*. The quantification in yellow boxes refer to half specimen (see 5.2.3).

Sample	Year	Elution µl	Average DNA quantification (ng)
1	2015	QIAamp® DNA Mini kit	328.00±3.46
2	2011	QIAamp® DNA Mini kit	102.13±0.61
3	2015	QIAamp® DNA Mini kit	39.20±0.40
4	2011	QIAamp® DNA Mini kit	708.00±0.00
5	2015	QIAamp® DNA Mini kit	76.40±0.40
6	2011	QIAamp® DNA Mini kit	2513.33±11.55

7	2011	QIAamp® DNA Mini kit	837.33±2.31
8	2002	QIAamp® DNA Mini kit	45.33±0.46
9	2013	QIAamp® DNA Mini kit	36.67±0.23
10	2013	QIAamp® DNA Mini kit	130.27±0.46
11	2004	QIAamp® DNA Mini kit	82.80±0.00
12	2003	QIAamp® DNA Mini kit	60.40±0.00
13	2004	QIAamp® DNA Mini kit	160.27±0.23
14	2002	QIAamp® DNA Mini kit	24.53±0.23
15	2002	QIAamp® DNA Mini kit	772.00±0.00
16	2002	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	97.07±0.46
17	2002	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	518.67±2.31
18	2002	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	101.60±0.00
19	2003	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	309.33±0.46
20	2003	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	424.00±0.00
21	2006	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	213.60±0.80
22	2007	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	1016.00±16.00
23	2005	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	406.67±2.31
24	2015	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	306.40±0.80
25	2013	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	294.40±3.20
26	2013	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	384.80±0.80
27	2012	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	128.80±0.80
28	2011	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	1784.00±8.00
29	2011	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	1229.33±4.62
30	2011	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	169.87±0.46
31	2003	QIAamp® DNA Mini kit	1044.00±4.00
32	2004	QIAamp® DNA Mini kit	212.00±2.00
33	2004	QIAamp® DNA Mini kit	363.33±3.06
34	2004	QIAamp® DNA Mini kit	116.80±0.40
35	2004	QIAamp® DNA Mini kit	460.00±0.00
36	2004	QIAamp® DNA Mini kit	248.00±2.00
37	2003	QIAamp® DNA Mini kit	368.67±5.03
38	2017	QIAamp® DNA Mini kit	3093.33±30.55
39a	2006	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	469.00±5.66
40a	2005	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	385.50±3.54
41a	2016	DB Gilbert et al (2007) + QIAquick PCR Purification Kit®	85.50±0.71
39b	2006	QIAamp® DNA Mini kit	118.60±0.85
40b	2005	QIAamp® DNA Mini kit	68.00±1.13
41b	2016	QIAamp® DNA Mini kit	38.00±1.13
42	2002	QIAamp® DNA Mini kit	91.60±1.70
43	2003	QIAamp® DNA Mini kit	70.80±6.22
44	2002	QIAamp® DNA Investigator kit	114.00±1.41
45	2002	QIAamp® DNA Investigator kit	622.00±14.14
46	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	22.96±0.45
47	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	43.20±0.00
48	2002	DB Santos et al (2018) + QIAamp® DNA Mini kit	23.20±2.83
49	2002	DB Santos et al (2018) + QIAamp® DNA Mini kit	36.80±0.00
50	2002	DB Santos et al (2018) + QIAamp® DNA Investigator kit	22.60±0.00
51	2004	DB Santos et al (2018) + QIAamp® DNA Investigator kit	67.20±0.00
52	2004	DB Santos et al (2018) + QIAquick PCR Purification Kit®	892.00±16.97
53	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	36.36±1.19
54	1983	QIAamp® DNA Investigator kit	2180.00±28.28
55	1983	QIAamp® DNA Investigator kit	240.00±0.00
56	1983	DB Santos et al (2018) + QIAquick PCR Purification Kit®	20.56±0.11
57	1983	DB Santos et al (2018) + QIAquick PCR Purification Kit®	34.12±0.40
58	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	30.00±6.34

59	2002	DB Santos et al (2018) + QIAquick PCR Purification Kit®	70.00±1.13
60	1967	QIAamp® DNA Investigator kit	86.30±0.85
61	1966	QIAamp® DNA Investigator kit	657.33±34.95
62	1946	QIAamp® DNA Investigator kit	147.67±2.08
63	1941	QIAamp® DNA Investigator kit	61.20±0.72
64	2004	Campos and Gilbert 2012 + ppK	550.00±12.49
65	2005	Campos and Gilbert 2012 + ppK	511.33±1.15
66	2005	Campos and Gilbert 2012 + ppK	245.33±2.31
67	1962	Campos and Gilbert 2012 + ppK	407.67±0.58
68	1948	Campos and Gilbert 2012 + ppK	9.37±0.21
69	1977	QIAamp® DNA Investigator kit	591.67±36.17
70	1977	QIAamp® DNA Investigator kit	336.33±63.54
71	1977	QIAamp® DNA Investigator kit	1970.00±130.00
72	1971	QIAamp® DNA Investigator kit	775.00±18.03
73	1954	QIAamp® DNA Investigator kit	452.67±13.01
74	1954	QIAamp® DNA Investigator kit	973.33±24.66
75	1953	QIAamp® DNA Investigator kit	338.67±2.08
76	1951	QIAamp® DNA Investigator kit	26.93±0.25
77	1932	QIAamp® DNA Investigator kit	63.50±2.60
78	1931	QIAamp® DNA Investigator kit	497.33±3.06
79	1930	QIAamp® DNA Investigator kit	19.13±0.15
80	1936	QIAamp® DNA Investigator kit	90.83±5.77
81	1920	QIAamp® DNA Investigator kit	92.00±7.76
82	1919	QIAamp® DNA Investigator kit	99.67±0.29
83	1902	QIAamp® DNA Investigator kit	26.40±1.04
84	1898	QIAamp® DNA Investigator kit	13.47±0.61

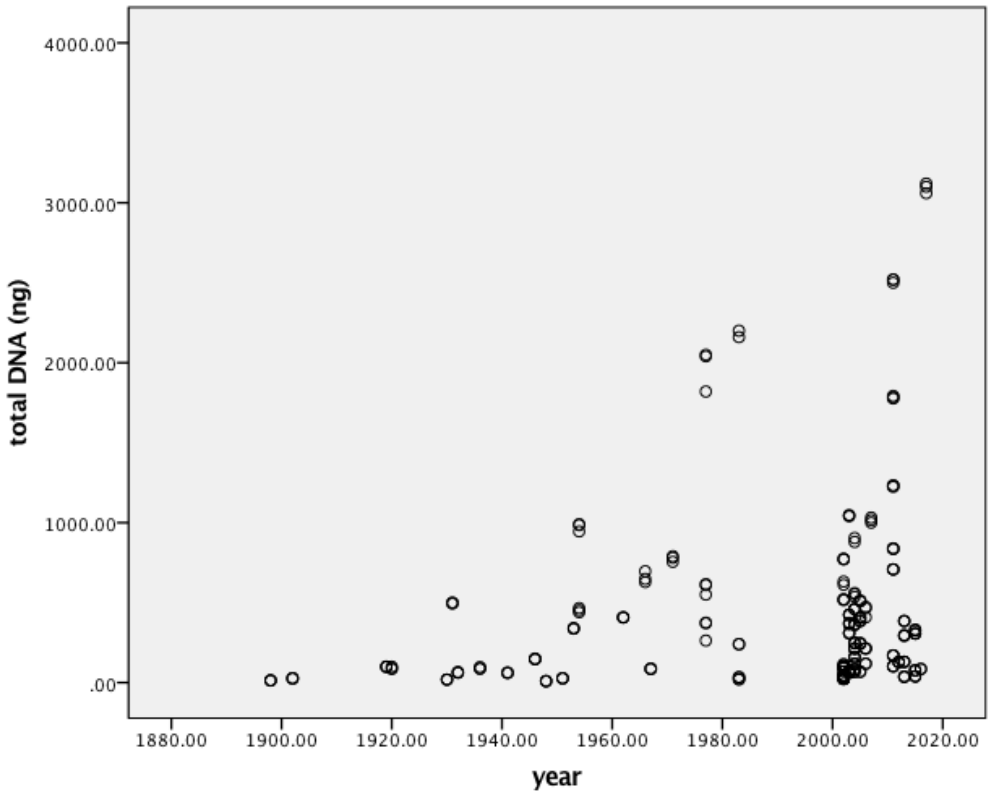

Fig. 59 **Statistical correlation between total DNA extracted and time (year)**.

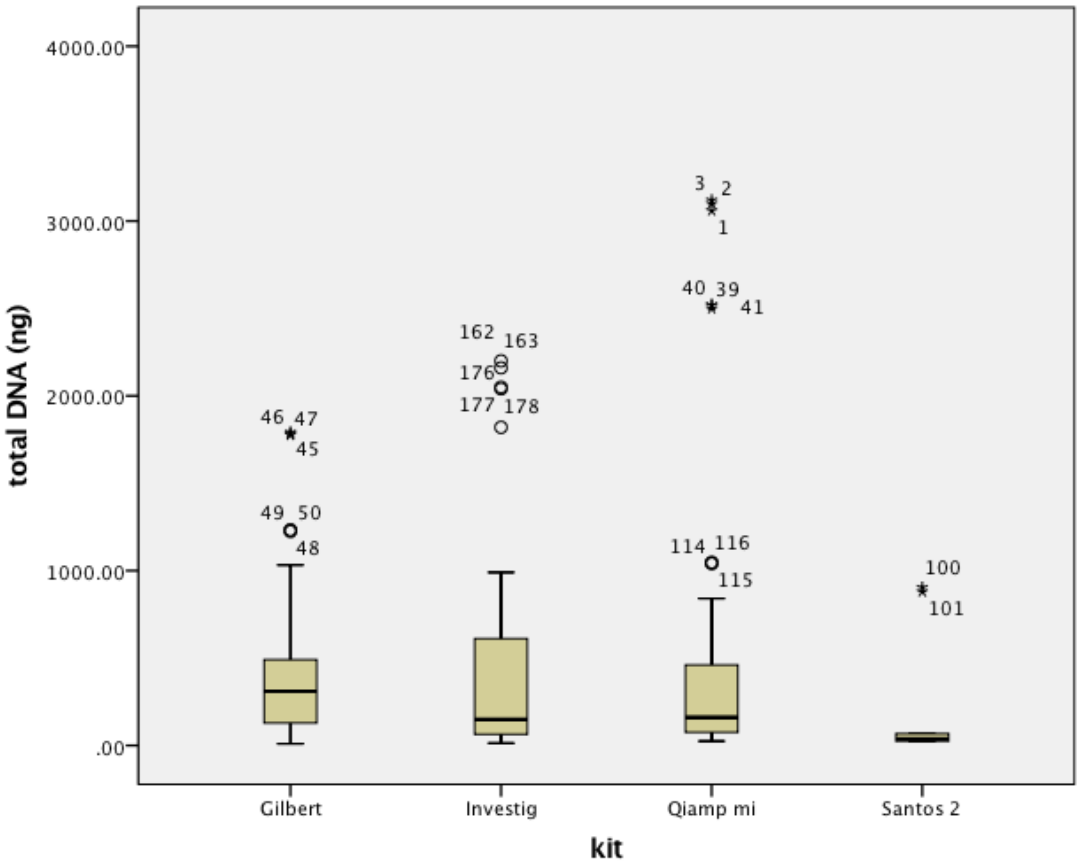

Fig. 60 **Correlation between total DNA extracted and kit.**

PCR amplification targeted the "barcoding region" of the mitochondrial gene COI. The amplification with the universal primer LCO/HCO (Folmer *et al.*, 1994) gave positive results only for fresh samples (2015-2017) (Data not shown). The new designed primers (Table 10) allowed the positive amplification of around 200bp for a high number of samples (Table 15). Due to the dominant presence of A + T composition, which is characteristics of insect DNA (Sharma *et al.*, 2015), and the presence of some inner repetitions, the amplifications of the COI portion with the 511 FW and RV (Table 10) primers were often problematic, producing weak results.

The molecular identification, for the single fragments and for the reconstructed COI gene, was carried out on BLASTn® (Altschul *et al.*, 1990). The positive amplification of a 151bp fragment allowed the correct identification at species level of a 98 years old specimen. The amplification of older specimens allowed the identification only at genus level.

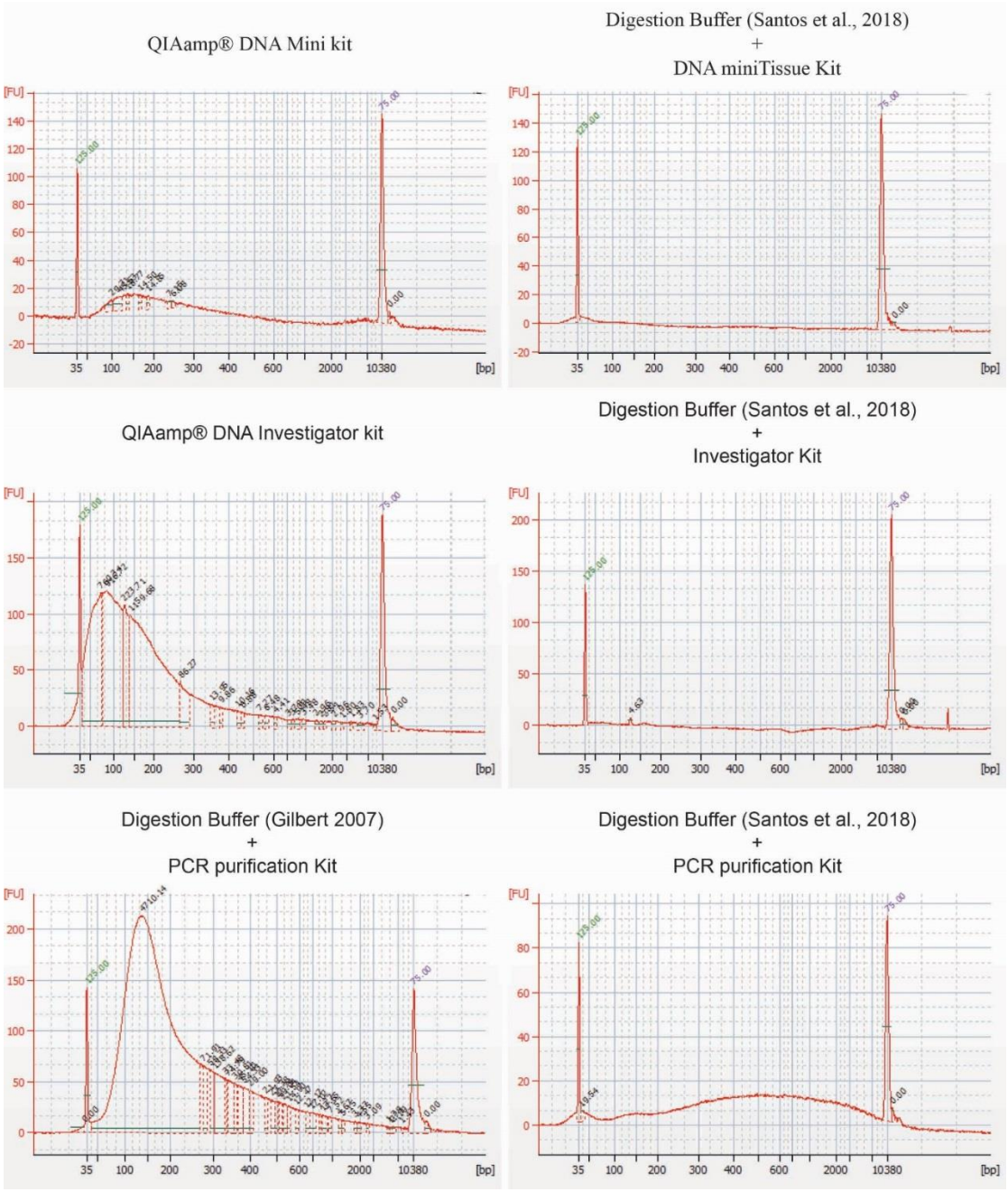

Fig. 61 **DNA fragments obtained from samples collected in 2002.** Six different methods are illustrated. The fluorescence scale is automatically made by the software and cannot be manipulated from the operator. For this reason, a direct visual comparison cannot be performed at the quantification level.

On 80 sequences of around 200bp of *S. variegata*, 70 resulted in a positive identification at species level. Among these, 59% shared the same identity percentage and score with *S. lehmanni*. The concatenated gene, around 700bp, decreased this percentage to 7%. This data is in accordance with Jordaens *et al.* (2013), according to whom a 200bp fragment is not sufficient for species identification in Sarcophagidae family. On the contrary, on 20

short sequences of *S. lehmanni*, 17 resulted in a positive identification at species level where 75% shared the same identity percentage and score with *S. variegata*. The concatenated gene did not give any positive identification result. On 43 positive identification of around 200bp sequences of *S. carnaria*, 100% showed the same identity percentage and score with *S. pyrenaica*. The concatenated gene decreased this percentage only to 79%.

In order to better understand if the identification issues were due to the length of the fragments, a phylogenetic analysis on 548bp sequences of the COI gene was carry out.

Table 15 **Results of the molecular identifications.** Legend: ✓ ✓ (Bright green): Correct identification at species level; ✓ ✓* (Dark green): Correct identification at species level but same score and identity percentage with other species; ✓ (Yellow): Correct identification at genus level; - (Red): positive PCR amplification but wrong identification; X (Grey): negative PCR amplification. The empty boxes indicate reactions that were not performed.

Sample	Year	Sar111	Sar211	Sar311	Sar411	Sar511	Concatenated gene	Morphological ID
1	2015	✓✓	✓✓*	✓✓	✓✓*	✓✓*	*S. variegata*	*S. variegata*
2	2011	✓	✓	✓✓	✓✓	✓✓	*S. crassipalpis*	*S. crassipalpis*
3	2015	✓	✓	✓	✓	✓	*S. subvicina*	*S. baranoffi*
4	2011	✓	✓✓	✓✓	✓✓	✓✓	*S. crassipalpis*	*S. crassipalpis*
6	2011	✓✓	✓✓	✓✓	✓✓	✓✓	*S. crassipalpis*	*S. crassipalpis*
7	2011	✓✓	✓✓	✓✓	✓✓	✓✓	*S. crassipalpis*	*S. crassipalpis*
8	2002	✓✓*	✓✓*	-	-	-	*C. vomitoria*	*S. variegata*
9	2013	✓✓	✓✓*	✓✓	✓✓*	✓✓*	*S. variegata*	*S. variegata*
10	2013	✓✓*	✓✓*	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
11	2004	✓✓*	✓✓	-	✓✓*	✓✓*	*S. variegata*	*S. variegata*
12	2003	✓✓*	✓✓*	✓	✓✓*	✓✓*	*S. carnaria*	*S. carnaria*
13	2004	✓✓*	✓✓*	✓✓*	✓✓*	✓✓*	*S. carnaria*	*S. carnaria*
14	2002	✓✓	✓✓	✓✓*	✓✓*	✓	*S. teretirostris*	*S. emdeni*
15	2002	✓✓*	✓✓*	✓✓*	✓✓*	✓✓*	*S. variegata*	*S. lehmanni*
16	2002	✓✓*	✓✓*	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
17	2002	✓✓*	✓✓*	✓✓	✓✓*	✓✓*	*S. variegata*	*S. variegata*
18	2002	✓✓*	✓✓*	✓	✓	✓✓*	*S. lehmanni*	*S. variegata*
19	2003	✓✓*	✓✓*	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
20	2003	✓✓*	✓✓*	✓✓	✓✓	✓✓*	*S. variegata*	*S. lehmanni*
21	2006	✓✓*	✓	✓✓*	✓✓*	✓✓*	*S. carnaria*	*S.carnaria*
22	2007	✓✓	✓✓	✓✓	✓✓	✓	*S. subvicina*	*S. croatica*
23	2005	✓✓	✓✓	✓	✓	✓	*S. subvicina*	*S. croatica*
24	2015	✓✓*	✓✓*	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
25	2013	✓✓*	✓✓*	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
26	2013	✓✓*	✓✓	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
28	2011	X	✓✓	✓✓	✓✓	X	*S. crassipalpis*	*S. crassipalpis*
29	2011	X	✓✓	✓✓	✓✓	X	*S. crassipalpis*	*S. crassipalpis*
30	2011	✓✓*	✓✓	✓✓	✓✓	✓✓	*S. crassipalpis*	*S. crassipalpis*
31	2003	✓	✓✓	✓✓	✓✓	✓✓	*S. crassipalpis*	*S. crassipalpis*
32	2004	✓	✓✓	✓	✓	-	*S. argyrostoma*	*S. crassipalpis*

33	2004	✓✓	✓✓	✓	✓	-	*S. cultellata*	*S. crassipalpis*
34	2004	-	✓✓	✓	✓	-	*S. argyrostoma*	*S. crassipalpis*
35	2004	✓✓	✓✓	✓	✓	-	*S. argyrostoma*	*S. crassipalpis*
36	2004	✓	✓✓	✓✓	✓✓	-	*S. argyrostoma*	*S. argyrostoma*
37	2003	✓✓	✓✓	✓✓	✓✓	-	*S. argyrostoma*	*S.argyrostoma*
39	2006							*S. croatica*
40	2005							*S. croatica*
41	2016							*S. carnaria*
42	2002							*S. variegata*
43	2003	✓✓*	✓✓*	✓✓*	✓✓*	✓✓*	*S. variegata*	*S. lehmanni*
44	2002	✓✓*	✓✓	✓✓	✓✓	✓✓*	*S. variegata*	*S. variegata*
45	2002	✓✓*	✓	✓	✓	✓✓*	*S. variegata*	*S. lehmanni*
46	2002	✓✓	✓✓	-	-	✓✓*	*S. lehmanni*	*S. croatica*
47	2002	✓✓*	✓	-	-	✓✓*	*S. variegata*	*S. variegata*
48	2002	X	X	X	X	X		*S. variegata*
49	2002	X	X	X	X	X		*S. croatica*
50	2002	X	X	X	X	X		*S. variegata*
51	2004	X	X	X	X	X		*S. variegata*
52	2004	✓✓	✓✓	✓✓	✓✓	✓✓	*S. variegata*	*S. variegata*
53	2002	✓✓*	✓✓	✓✓	✓✓*		*S. variegata**	*S. variegata*
54	1983	✓✓*	✓	✓	✓		*S. carnaria**	*S. carnaria*
55	1983	✓	✓	✓✓*	✓		*S. variegata*	*S. subvicina*
56	1983	✓	✓	✓	✓		*S. variegata*	*S. carnaria*
57	1983	✓	✓	-	-		*S. variegata*	*S. subvicina*
58	2002							*S. variegata*
59	2002							*S. lehmanni*
60	1967	✓✓*	X	✓	✓			*S. carnaria*
61	1966	✓✓*	X	✓	✓✓*			*S. subvicina*
62	1946	✓✓*	X	✓✓*	✓✓*			*S. carnaria*
63	1942	X	X	X	✓			*S. carnaria*
64	2004							*S. carnaria*
65	2005							*S. croatica*
66	2005							*S. croatica*
67	1962		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
68	1948		X	X	X			*S. carnaria*
69	1977		-	-	✓✓*			*S. carnaria*
70	1977		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
71	1977		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
72	1971		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
73	1954		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
74	1954		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
75	1953		✓✓*	✓✓*	✓✓*		*S. carnaria**	*S. carnaria*
76	1951	X	-	-	✓	X		*S. carnaria*
77	1932	X	-	✓✓*	✓	X	*S. carnaria**	*S. carnaria*
78	1931		-	✓✓*	X		*S. carnaria**	*S. carnaria*
79	1930	*X*	*X*	-	✓	*X*	*S. carnaria**	*S. carnaria*
80	1936	*X*	-	✓	*X*	*X*		*S. carnaria*
81	1920	*X*	*X*	✓✓*	✓	*X*	*S. albiceps*	*S. carnaria*
82	1919	*X*	-	-	✓	*X*		*S. carnaria*
83	1902	*X*	-	-	✓	*X*	*S. carnaria*	*S. subvicina*
84	1898	*X*	*X*	-	✓	*X*		*S. subvicina*

The Neighbour Joining analysis involved 266 sequences of *Sarcophaga* sp. from this study, from Daniel Whitmore's archive and NCBI and Bold genbanks.

The phylogenetic tree underlined how the systematic of this group is far from being clear and resolved (Fig. 62). Some *S. carnaria* Linnaeus, 1758 sequences from Canada (KR755286.1, KR753430.1, KR753328.1, KR752826.1 and KR752510.1) cluster with *S. subvicina* Baranov, 1937. It is a current opinion that they are a misidentification of *S. subvicina*, which is the only species of that group that is present in Canada (Ontario), whereas *S. carnaria* is not reported from Canada and from any other North American country (Daniel Whitmore, personal communication).

The sequence (JQ582068.1) deposited as *Sarcophaga croatica* clusters with the sequences of *S. matilei*. A secondary check of the specimens performed by Daniel Whitmore confirmed the identification error, so the sequences has to be considered as belonging to *S. matilei* (Daniel Whitmore, personal communication).

The two species *S. lehmanni* and *S. variegata* clusters together as well as *S. carnaria, S. pyrenaica* and *S. jeanleclercqi.* Another unresolved group concern *S. subvicina*, *S. baranoffi* and *S. adriatica*. The COI gene appears not to be enough informative to discriminate at species level. According to Zehner *et al.* (2004), the use of the mitochondrial gene ND5 (NADH dehydrogenase subunit 5) to analyse the evolutionary relationship between flesh flies provides a good resolution. Furthermore PER (period) gene appeared to be useful for identification purposes in flesh flies (Guo *et al.*, 2014) although public datasets might be enriched with further DNA sequences. Additional work using mtDNA in association with other genetic markers (Bortolini, 2014; Grzywacz *et al.*, 2017c) could clarify and resolve the relationships among the *Sarcophaga* genus as well as other close related species.

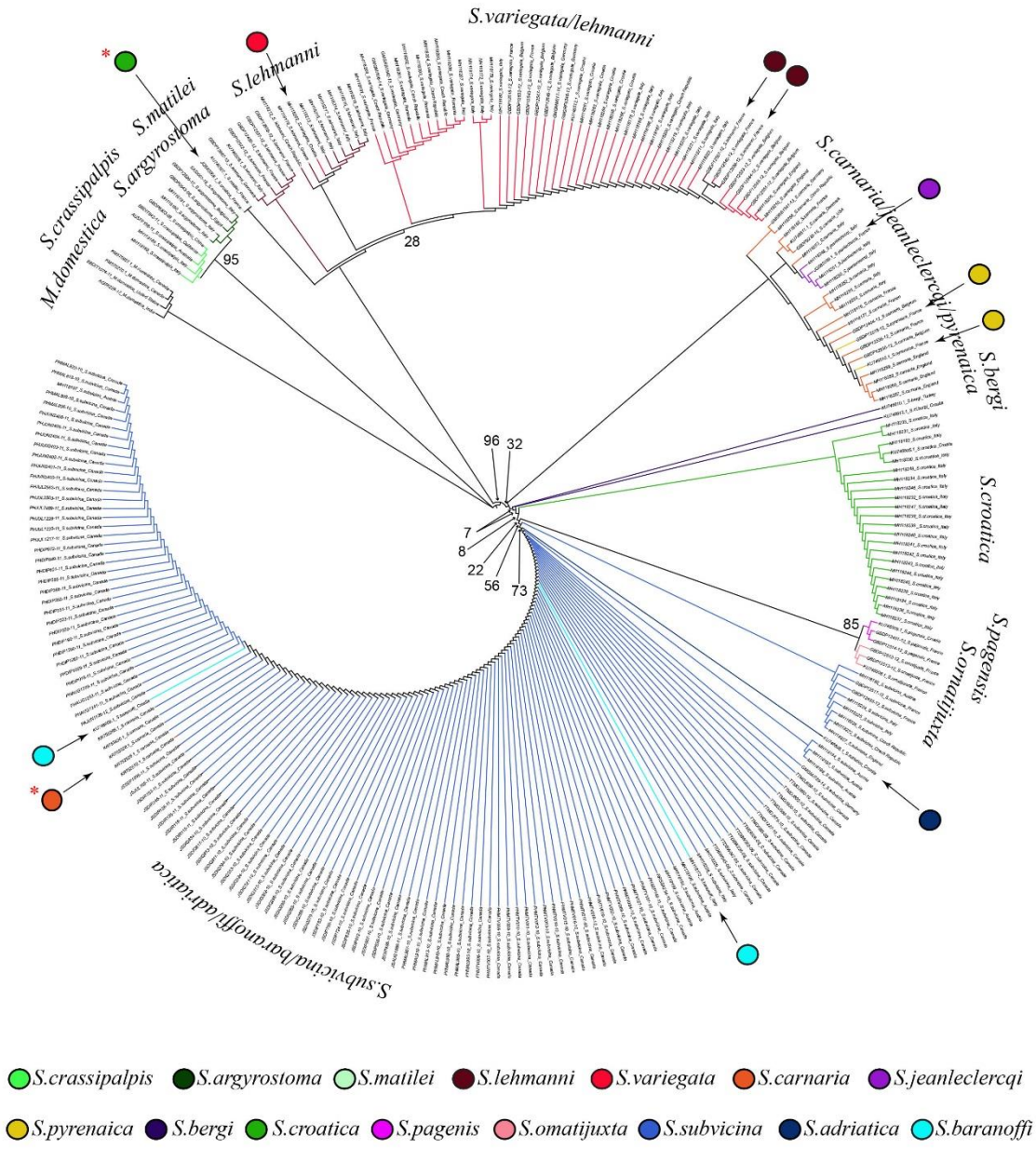

Fig. 62 **Phylogenetic tree of *Sarcophaga* genus.** Neighbour Joining method analysis of 548bp sequence of the COI gene. The coloured spot underlain the unresolved group. The * indicates a high probable misidentification.

5.2.5 Conclusion

From the perspective of a museum collection, maintaining the morphological characteristics of entomological samples is crucial. This study explored a non-invasive species identification method through their DNA.

In literature, previous studies have described non-destructive extraction methods for insect specimens (Phillips & Simon, 1995; Gilbert *et al.*, 2007; Rowley *et al.*, 2007).

However, as stated above, most of these methods deal with adult beetles (Coleoptera), which have fairly robust exoskeletons, or fresh samples. Based on the results here presented, molecular analysis on Sarcophagidae abdomens allows the correct identification of the specimens without impairing their delicate morphological structures fundamental for further taxonomic studies.

DNA extraction and amplification cannot necessarily be expected from all specimens, indeed a variation in collection and storage conditions may adulterate the DNA survival rate (Gilbert *et al.*, 2007). Six different extraction methods, based on commercial kits and on home-made digestion buffers, were used in the analysis. The latter being the best for fresh samples (>15years old) while QIAamp® DNA Investigator kit being the best for old samples where the DNA amount is lower. In most of the samples, the quality of the extracted DNA was good for the amplification of short fragments of around 200bp in the COI Barcoding Region and not for the amplification of the entire 658bp region (data not shown).

Despite 200bp fragments not being sufficient for identification, the results of this study clearly demonstrated the possibility of extracting DNA without altering morphological characters useful for the identification. The COI gene appeared not to be helpful in clearly solving the phylogeny of *Sarcophaga* genus. The use of concatenated mitochondrial and nuclear genes and an extended taxon sampling used in the analysis could provide more complete results. The necessity of a database improvement is the first step for taxonomic research advancement.

For future works, a field that will be usefull to investigate in order to improve the DNA extraction and amplification from Museum samples is the sequencial treatment at which flies are exposed from the collection to the storage (e.g. killing method, preservative chemical, etc). Although different studies dealt with the effect of preserving solution on DNA integrity (Post *et al.*, 1993; Day & Wallman, 2008; Moreau *et al.*, 2013), few studies investigated the effect of the killing agents on DNA damages (Moreau *et al.*, 2013).

5.3 Archaeological contexts

5.3.1 Introduction

The study of the insect remains found in archaeo-funerary contexts can provide information on the funerary practices (Huchet & Greenberg, 2010), on the events that occurred after the death (Vanin *et al.*, 2009), on the hygiene condition of the populations in the past. Furthermore, the comparison between distribution of the insects in the past and nowadays can provide important information about biodiversity changes due to the climatic changes and/or to the increase of urbanisation and commercial traffic.

Insects remains, due to their highly resistant chitinous cuticle, can still be detected on cadavers long after the death, such in the case of skeletonized or on mummified bodies. (Vanin & Huchet, 2017). Beetle adult remains, thanks to their thick hardened bodies, are very resistant to mechanical decomposition. Hence, they are the main entomological samples collected in those cases (Panagiotakopulu, 2004). However, even though Diptera puparia are less resistant compared to beetles fragments, they were found in large numbers in sheltered or concealed environments, such as coffins, burials, and crypts, due to their peculiar physical and chemical composition (Panagiotakopulu, 2004; Huchet, 2014a).

In Europe, insects belonging to Diptera (families: Calliphoridae, Fanniidae, Muscidae, Phoridae and Sarcophagidae) and Coleoptera (families: Anobiidae [Anobiinae and Ptininae], Cleridae, Cryptophagidae, Rhyzophagidae, Dermestidae, Histeridae and Tenebrionidae) order are reported to be the most collected species from archaeological contexts (Huchet, 2014b; Morrow *et al.*, 2015).

As stressed above, the identification of insects, in particular for immature stages is problematical due to the limited literature available concerning the morphological distinctive characters. In a context, like the archaeological one, where thanatological phenomena and mechanical pressures can occur, the problem is exacerbated by the presence of filth or broken entomological samples.

5.3.2 Aim

The aim of the work reported in this chapter deals with the morphological and molecular identification of entomological samples found in archaeological contexts. In addition, in

case of a lack of data, immature stages were described in detail. In fact, the different environments in which the specimens are located can affect their preservation state and the integrity of the samples from both a morphological and molecular point of view. The identification of external features accessible in puparia fragments is the key point for the morphological comparison between species. The establishment of a molecular approach, from extraction till amplification, suitable for difficult sample will allow the synergy of the two identification methods.

5.3.3 Description of *Hydrotaea* Robineau-Desvoidy, 1830 (Diptera: Muscidae) puparia of forensic and archaeological importance.

5.3.3.1 *Introduction*

Puparia of the necrophagous species in the genus *Hydrotaea* Robineau-Desvoidy, 1830 are, among the family Muscidae, the most difficult to be identified due to the lack of species-specific diagnostic characters on the external surface and the absence of informative literature (Skidmore, 1985).

The *Hydrotaea* genus, widely distributed mainly in warmer regions, counts around 150 species described so far (Marshall, 2012). Skidmore (1985) had suggested that some specific species have become cosmopolitan thanks to the increasing of worldwide commerce. The development of larval stages occurs during the active decay, when high bacterial fermentation conditions have finished. This genus can often copresent with the other early colonisers such as blowflies (Calliphoridae), flesh flies (Sarcophagidae) and some other Muscidae (e.g. *Muscina* spp.).

In the literature, some species of *Hydrotaea* have been collected or reported from both human cadavers and animal carcasses (Couri & Araújo, 1990; Couri *et al.*, 2009; Huchet & Greenberg, 2010; Grzywacz *et al.*, 2014).

Species belonging to this genus can be collected during the early stages of decomposition even though they are more commonly collected or reported to arrive during ammoniac fermentation (Wyss & Cherix, 2013). Moreover, they are known to belong to a restricted group of flies able to colonise buried remains, usually in high numbers (Huchet & Greenberg, 2010).

Due to the absence of identification keys specifically for puparia of this genus, and due to the lack of recent and updated monographic paper on this topic, a description and an illustrated key of the most commonly found puparia of *Hydrotaea* species are presented. The aim of this work is to create a tool to allow a more precise peri- and post-mortem events reconstruction (Couri *et al.*, 2009; Vanin, 2012; Huchet, 2013; Morrow *et al.*, 2015; Grzywacz *et al.*, 2017c; Giordani *et al.*, 2018b).

Puparia of six species were available for investigation. They belong to the most common *Hydrotaea* collected or reported in forensic and archaeological contexts (Table 16). Four species were collected from different forensic cases and archaeological sites in Europe and South America while the remaining two were obtained during laboratory breeding from material collected on animals' decomposition experiments performed in Central-Eastern Europe.

Table 16 **Geographical origin and collection context of the samples (Modified from Giordani *et al.*, 2018a).**

Species	General context	Geographical origin
Hydrotaea aenescens (Wiedemann, 1830)	Forensic and Archaeological (X–XV cent.)	Europe, Italy, Pont Saint Martin South America, Peru, Pachacamac
Hydrotaea capensis (Wiedemann, 1818)	Forensic and Archaeological (XV–XVIII cent.)	Europe, Italy, Firenze Europe, Italy, Roccapelago
Hydrotaea ignava (Harris, 1780)	Forensic and experimental (on rabbit carrion)	Europe, Italy, Milan Europe, England, Huddersfield
Hydrotaea dentipes (Fabricius, 1805)	Archaeological (XV–XVIII cent.) and experimental (on rabbit carrion)	Europe, Italy, Roccapelago Europe, England, Huddersfield
Hydrotaea similis Meade, 1887	Experimental (on pig carrion)	Europe, Poland, Toruń
Hydrotaea pilipes Stein, 1903	Experimental (on pig carrion)	Europe, Poland, Poznan

5.3.3.2 *Material and Methods*

Samples were collected by Dr. Vanin or provided by forensic pathologists for forensic purposes. EAFE guidelines were followed for the collection and the storage in dry conditions of the specimens as in Amendt *et al.* (2007). Per each specimen, the geographic origin and the sampling context are listed in Table 16.

Clearing techniques, such as soaking the specimens in a 20% NaOH solution for several days (Sukontason *et al.*, 2007) can be applied on the study of the puparia in addition to conventional and electron microscopy. The soaking technique is extremely efficient, but it is time consuming as it requires several days of preparation. However, the hot 20%

NaOH treatment did not improved the clarity of the observations of the samples. The samples were treated as described in Material and Methods (see Chapter 4). The spiracles were photographed placing the anal region in ventral position to allow a comparison with previous published data and to better visualise all the characters required for identification. The spines of the ventral welt of abdominal segment seven were used to describe the species.

Terminology of morphological features follows Skidmore (1985) and Grzywacz *et al.* (2017a). Nomenclature is reported in Fig. 33. Synonyms were derived from the specific literature reported on the main text and from the British National Biodiversity Network's Gateway (https://data.nbn.org.uk).

To confirm the morphological identification, DNA extractions from 1 single pupa of *H. aenescens* (Poland, 2016), *H. capensis* (Italy, 1600), *H. ignava* (UK, 2017), *H. dentipes* (UK, 2017), *H. similis* (Poland, 2016) and *H. pilipes* (Poland, 2016) were performed with the QIAamp® DNA Mini kit and quantify with Qubit assay (Table 17).

Table 17 **DNA extraction methods and quantification.** Number and type of samples are here reported with the extraction method used. Average amount of extracted DNA (ng/µl) is shown. Standard deviation is reported.

Species	**Sample**	**Method**	**Elution (µl)**	**Average DNA quantification (ng/µl)**
H. aenescens	1 pupa	Mini kit	200	1.64±0.01
H. capensis	1 pupa	Mini kit	200	0.25±0.00
H. ignava	1 pupa	Mini kit	200	3.89±0.03
H. dentipes	1 pupa	Mini kit	200	0.37±0.00
H. pilipes	1 pupa	Mini kit	200	1.41±0.01
H. similis	1 pupa	Mini kit	200	0.26±0.00

The mitochondrial cytochrome oxidase c subunit 1 gene was amplified using the universal primer LCO/HCO designed by Folmer *et al.* (1994) and *Hydrotaea*-specific primers designed by Dr. Sara Bortolini (Table 18). The editing of all the sequences obtained was done with EMBOSS *merger* (Rice *et al.*, 2000) and Chromas Pro (Technolysium Ltd.) programs. The new sequences produced during this work have been deposited in GenBank (Table 19). Sequences of *Hydrotaea* species of forensic interest were downloaded from BOLD online system and included in the phylogenetic analysis (Table 20). Sequences were aligned with Clustal Omega (Sievers *et al.*, 2011). The phylogenetic

trees were built using the Neighbour Joining method on MEGA 7.0 (Kumar *et al.*, 2016) (see 4.8). *Drosophila melanogaster* sequences were used as outgroup.

Table 18 **Degenerated primers designed for *Hydrotaea* genus by Dr. Sara Bortolini.**

Oligo name	**Sequence (5' -> 3')**
Oph111_FW	TCGCAACAATGGTTATTCTCT
Oph111_RV	TCAATTACCRAATCCTCCAAT
Oph211_FW	GTAATTGTWACAGCTCATGC
Oph211_RV	AACCWGTWCCAGCYCCRTTT
Oph311_FW	CWCGAATAAATAATATAAGTTTYTG
Oph311_RV	ATTCCWGCTAARTGWARAGA
Oph411_FW	TCTAAYATTGCWCATGGAGG
Oph411_RV	TWGTAATWGCWCCAGCTAWAC
Oph511_FW	GGAATYACWTTTGAYCGAAT
Oph511_RV	YCCTGATTCTTGRCTAATAAT

Table 19 **GenBank unique codes of sequences of *Hydrotaea* spp. belonging to this work.**

Family	***Species***	**GenBank code**
Muscidae	*H. aenescens* (Wiedemann, 1830)	MH921579
	H. capensis (Wiedemann, 1818)	MH921578
	H. ignava (Harris, 1780)	MH921576
	H. dentipes (Fabricius, 1805)	MH921580
	H. similis Rothschild, 1909	MH921577
	H. pilipes Stein, 1903	MH921581

Table 20 **Sequences of *Hydrotaea* spp. of forensic interest downloaded from BOLD and included in the analysis.** *Drosophila melanogaster* sequences used as outgroup are reported.

Family	**Species**	**BOLD**		
Muscidae	*Hydrotaea aenescens* (Wiedemann, 1830)	FFECU141-14 FFECU142-14 FFECU125-14	FFECU138-14 FFECU139-14	FFECU137-14 FFECU140-14
	Hydrotaea capensis (Wiedemann, 1818)	AGIRI256-17 CDFD012-12	GBMIN59025-17 GBMIN59026-17	GBMIN59024-17
	Hydrotaea ignava (Harris, 1780)	BCFOR404-15 BCFOR395-15 BCFOR420-15	BCFOR441-15 BCFOR445-15 BCFOR422-15	BCFOR421-15 BCFOR439-15 BCFOR446-15
	Hydrotaea dentipes (Fabricius, 1805)	GBDP15308-14 GBDP15309-14 GBDP15310-14	GBDP15314-14 GBDP15315-14 GBDP15316-14	GBDP15311-14 GBDP15312-14 GBDP15317-14
	Hydrotaea pilipes Stein, 1903	GBMIN59050-17		
	Hydrotaea similis Meade, 1887	GBDP9371		
	Hydrotaea albuquerquei (Lopes, 1985)	GBDP12063-12 GBDP12064-12	GBDP12065-12 GBDP12067-12	
	Hydrotaea armipes (Fallen, 1825)	CNTMC1022-14 CNTGD629-15	CNTMA1641-14 CDFD011-12	
	Hydrotaea cyrtoneurina (Zetterstedt, 1845)	GMGME1284-14 GMGMD1245-14	GMGMD076-14 GBMIN59040-17	
Drosophilidae (Outgroup)	*Drosophila melanogaster* Meigen, 1830	CYTC5404-12	GBDP2877-06	

For further information about the phylogenetic reconstruction refer to section 4.

5.3.3.3 *Results and Discussion*

- **Morphological analysis**

A deep cleaning of the sample is mandatory for a reliable observation of these features, in particular of puparia collected from archaeological and forensic cases. Good results in a short timeframe were obtained using the cleaning technique described in the material and methods section. In the case of the anal plate missing due to the bad preservation, its shape can still be recognisable by the surrounding area.

Hydrotaea puparia, 6–7mm long if closed or 4–5mm long if missing the operculum, are intermediate size between the bigger Sarcophagidae and Calliphoridae and the smaller Piophilidae and Phoridae (Fig. 63).

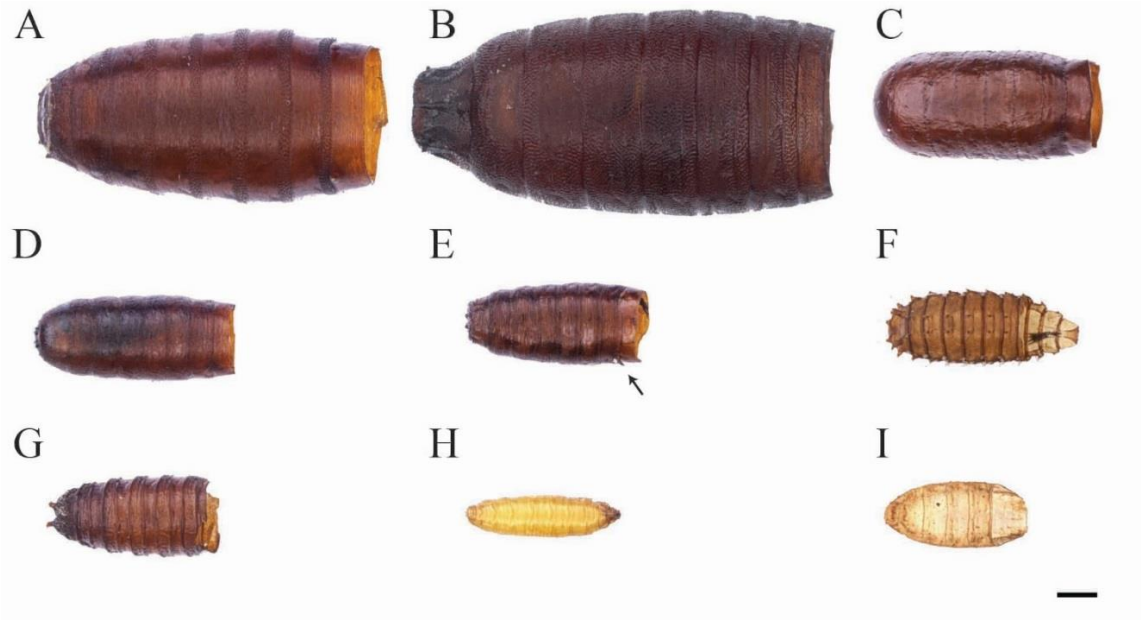

Fig. 63 **Dorsal view of open puparia of the main carrion-breeding taxa**. A) Calliphoridae, B) Sarcophagidae, C) Muscidae (*Musca*), D) Muscidae (*Muscina*), E) Muscidae (*Hydrotaea*) with respiratory horns (arrow), F) Fanniidae, G) Heleomyzidae, H) Piophilidae and I) Phoridae (Scale bar 500µm) (From Giordani *et al*., 2018a).

While other Muscidae of forensic interest (genera *Musca* Linnaeus, 1758, *Muscina* Robineau-Desvoidy, 1830 and *Synthesiomyia* Brauer and Bergenstamm, 1893) are characterized by posterior spiracles with bowed respiratory slits, species in the genus *Hydrotaea* show quite linear slits (Fig. 64,Fig. 66). The slight protrusion and the more

rounded shape of posterior spiracles of *Hydrotaea* can be used as a diagnostic character to distinguish these puparia from species of the families Calliphoridae and Sarcophagidae (Fig. 64,Fig. 65).

The barrel shape *Hydrotaea* puparia are characterized by the presence of respiratory horns and slightly protruding posterior spiracles (Fig. 63E, Fig. 65). The environmental conditions at which the puparia have been exposed, in particular from archaeological contexts, can affect the protrusion of the posterior spiracles. As well, the soil in burial condition can impair the puparium anal region and foster its collapse. Puparium colour depends on the species but it can also be affected by the environmental conditions (e.g., soil, light, humidity, presence of organic matter, etc.) and the preservation state (Vanin S. & Huchet J.B., personal communication).

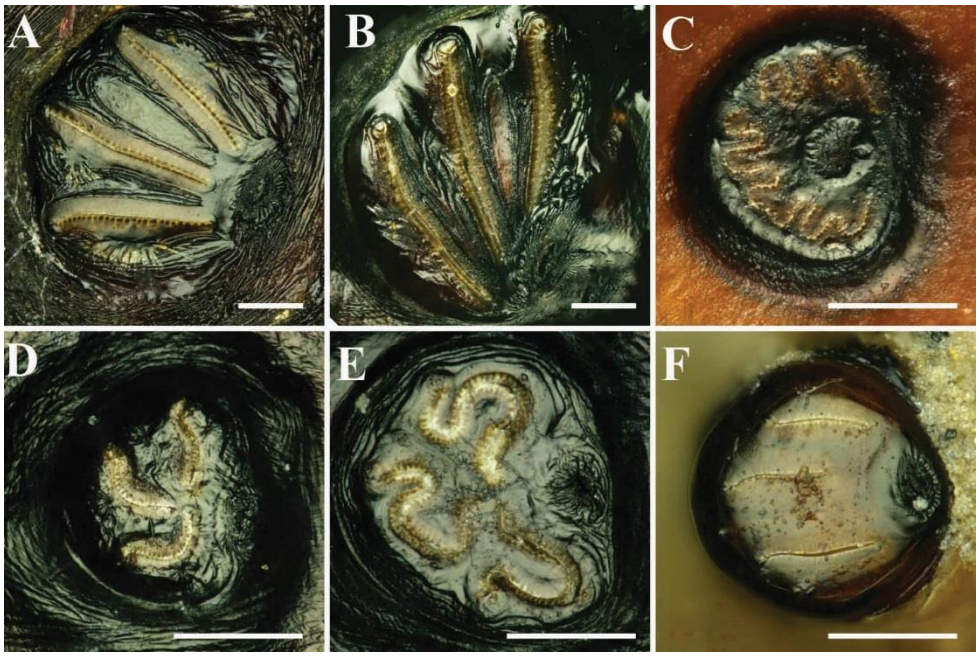

Fig. 64 **Comparison of the posterior spiracle slits** of A) Calliphoridae, B) Sarcophagidae, C) Muscidae (*Musca*), D) Muscidae (*Muscina*), E) Muscidae (*Synthesiomyia*) and F) Muscidae (*Hydrotaea*) (Scale bar 100µm) (From Giordani *et al*., 2018a).

Hydrotaea species differ in the position of the spiracular scars (inferior or median) and respiratory slits (parallel, sub-parallel or radial), although high similarity are recognised between close related species (e.g., *H. capensis* and *H. aenescens*) (Fig. 66A, B, D, E). The anal plate shape, the anal plate papillae shape and position and the structure of the intersegmental spines (Fig. 66, Fig. 67) are, in addition to the orientation and shape of the posterior spiracle slits, important diagnostic characters.

Fig. 65 **Direct comparison of six *Hydrotaea* species**: *H. aenescens* (A,B), *H. capensis* (C,D), *H. ignava* (E,F), *H. dentipes* (G,H), *H. similis* (I,J) and *H. pilipes* (K,L). Dorsal view (A,C,E,G,I,K) and lateral view (B,D,F,H,J,L) of the posterior spiracles (Scale bar 100µm) (From Giordani *et al*., 2018a).

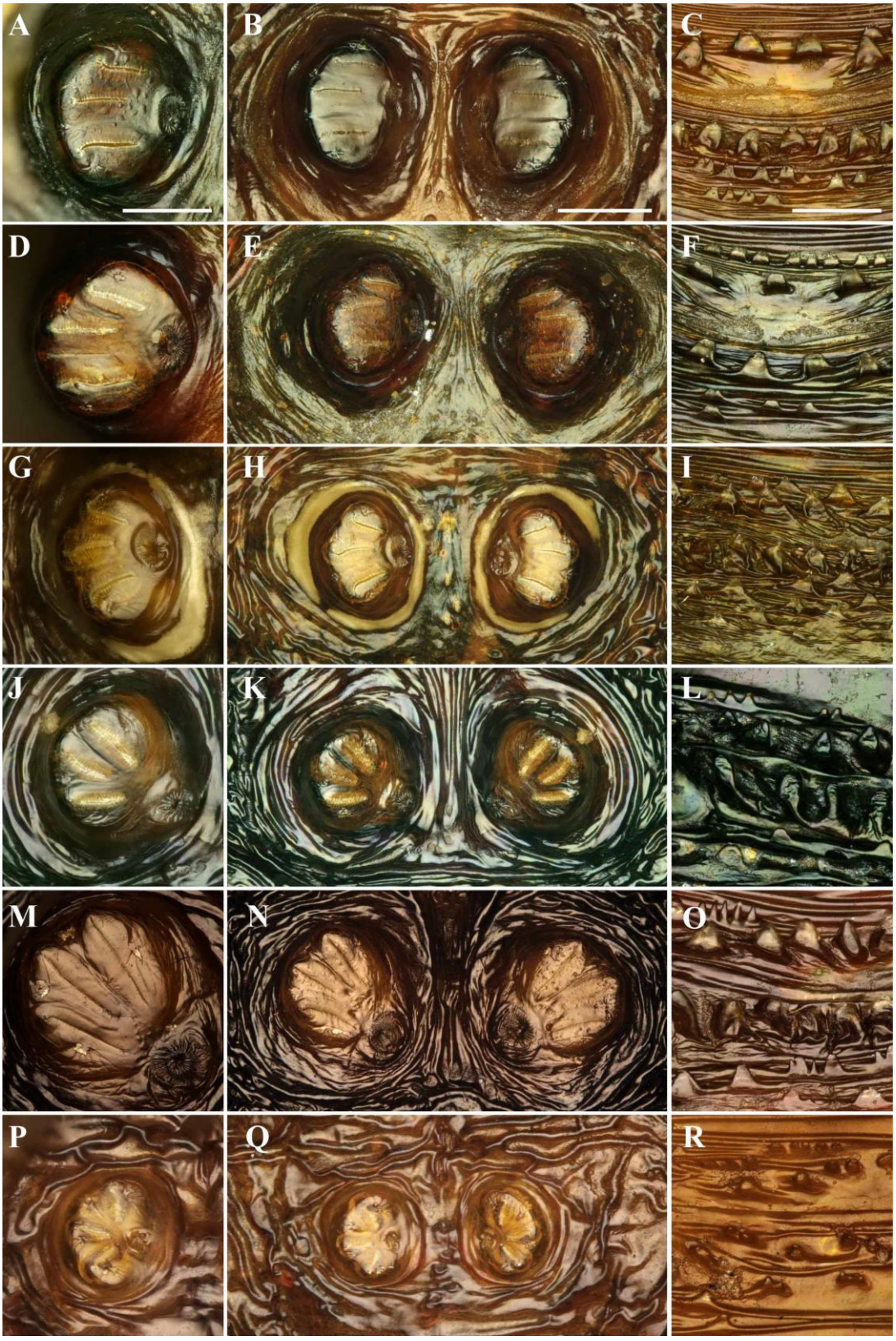

Fig. 66 **Direct comparison of six *Hydrotaea* species:** *H. aenescens* (A,B,C), *H. capensis* (D,E,F), *H. ignava* (G,H,I), *H. dentipes* (J,K,L), *H. similis* (M,N,O) and *H. pilipes* (P,Q,R). Single spiracle (A,D,G,J,M,P), posterior spiracles distance (B,E,H,K,N,Q) and intersegmental spines (C,F,I,L,O,R) (Scale bar 100µm) (From Giordani *et al.*, 2018a).

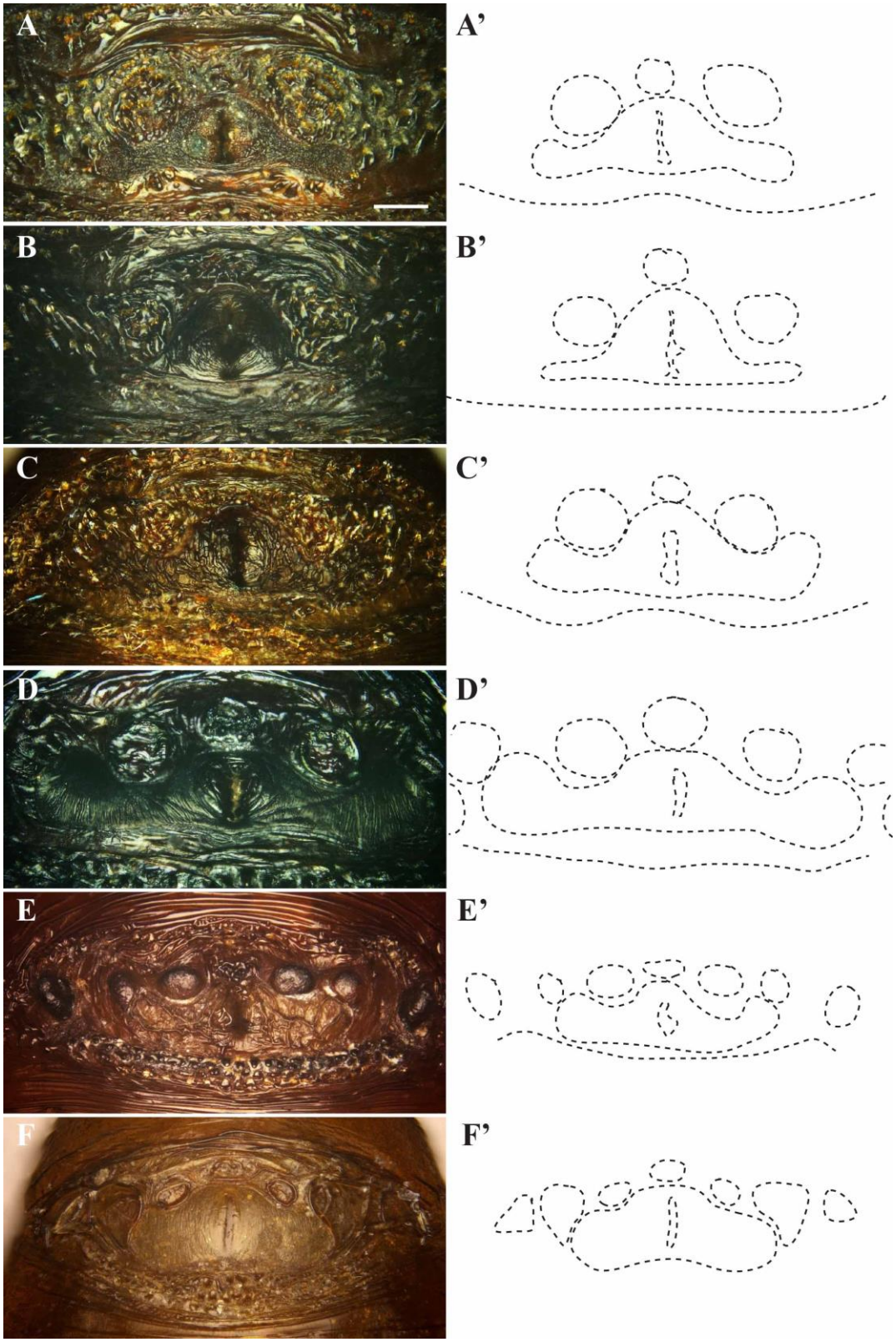

Fig. 67 **Direct comparison of the anal region of four *Hydrotaea* species with schematic representation of the most important features useful for species identification**: *H. aenescens* (A, A'), *H. capensis* (B, B'), *H. ignava* (C, C'), *H. dentipes* (D, D'), *H. similis* (E, E') and *H. pilipes* (F, F') (Scale bar 100µm) (From Giordani *et al*., 2018a).

Seven species of *Hydrotaea* were reported by Grzywacz *et al.* (2017a) (*H. capensis, H. dentipes, H. similis, H. aenescens, H. ignava, H. armipes* and *H. pilipes*). The presence on carrions/cadavers of other species such as *H. cyrtoneurina* (Zetterstedt, 1845) (Grzywacz, 2013), *H. albuquerquei* (Lopes, 1985) (Barros de Carvalho & Antunes de Mello-Patiu, 2008) and *H. obscrifrons* (Sabrosky, 1949) have been reported respectively from Europe, South America and Asia (South Korea) (Shin *et al.*, 2015). In addition, from archaeological contexts, species within *Hydrotaea* genus have been samples from cadavers (Couri *et al.*, 2009; Vanin, 2012; Huchet, 2013), cesspits (Smith, 2013), Roman and Medieval wells (Buckland *et al.*, 2018) and from a hayfield in Norse Greenland (Panagiotakopulu & Buckland, 2012).

The systematics and taxonomy of the genus *Hydrotaea* has been recently reviewed by many authors (Savage & Wheeler, 2004; Schuehli *et al.*, 2004, 2007; Haseyama *et al.*, 2015; Grzywacz *et al.*, 2017c). In several cases the same species have been given different names increasing the difficulty in the study of *Hydrotaea* species (Savage & Wheeler, 2004; Grzywacz *et al.*, 2017b). In order to clarify the literature search, a section of the available nomenclature, including the existing synonymous and name combinations is here reported for four species out of six.

***Hydrotaea aenescens* (Wiedemann, 1830)**[7]

***Syn**: aenescens* Wiedemann, 1830 (*Anthomyia*)
virescens Macquart, 1843 (*Ophyra*)
setia Walker, 1849 (*Anthomyia*)
argentina Bigot, 1885 (*Ophyra*), comb. *Hydrotaea argentina*
carbonaria Shannon and Del Ponte, 1926 (*Ophyra*)
trochanterata Malloch, 1932 (*Ophyra*)

Hydrotaea aenescens puparia are deep reddish to black (Fig. 65A, B), about 6 mm long with short respiratory horns on the dorsal side (Fig. 33D). A high variability in size and coloration was observed between and among samples (inter and intra variability). The specimens analysed in this thesis were sampled from Northern Italy and have a full puparium length of 6.27±0.16mm (N = 7). Cortinhas *et al.* (2016) indicate a length of 6.68±0.91mm while Skidmore (1985) a measure in the range of 5.2-5.9mm. In the analysed sample, the open puparium length was 4.83±0.25mm (N = 4).

[7] Synonymic list and puparium description follow Giordani *et al.*, 2018a

The spines of segment seven, and the ventral creeping welt, are large and smoothed and arranged in several crowded lines, rather similar to *H. capensis*. Spines in the external lines are smaller than in the central, and they have a bifid or trifid shape (Fig. 66C).

The anal plate has well-defined wings narrower than the median area and broadly rounded apically, sometimes folded upward. The anal region around the plate is densely and coarsely spiculate. Subanal papillae are strong and double the diameter of the postanal papilla. Distinct para-anal and extra-anal papillae are absent; however, this area is covered by distinct spines (Fig. 67A). The posterior spiracles are raised. The angle formed by the spiracular surfaces as reported by Turchetto and Vanin (2004a) is greater than 90º (Fig. 65A, B). The respiratory slits are parallel, and the scar is in a median position. Dorsal muscle scars are well defined and clearly detectable (Fig. 66A, B).

The anterior spiracle of the pupa has four/five lobes. Schumann (1982) indicates that the third larval stage of this species is different from the cogeneric *H. ignava* and *H. capensis* in the number of anterior spiracles lobes. Five to six lobes were described in *H. aenescens*, whereas four lobes were reported in the other two species. Other taxonomists indicated a quite high variability in this character, with four to six lobes listed by Skidmore (1985) and seven by Couri and Araújo (1990).

Hydrotaea aenescens is known to be attracted to vertebrate carrions (Vikhrev, 2008) and, as reported by Byrd and Castner (2010), this species may colonize human bodies during the late active decay stage of the decomposition. The species originating from America was introduced to the Old World in 1960 (Saccà, 1964). Huchet and Greenberger (2010) reported the finding of *H. aenescens* from burials in Peru. In this archaeological context several puparia of this species were found together with puparia of other Muscidae, such as *Synthesiomyia nudiseta* (van der Wulp, 1883), and Calliphoridae, such as *Cochliomyia macellaria* (Fabricius, 1775) and *Compsomyiops verena* (Walker, 1849).

The effect of temperature on the developmental rate of *H. aenescens* was studied by Lefebvre and Pasquerault (2004) who calculated a minimum developmental temperature of 8.9° C and a thermal constant of 276.10±21.50 ADD (Lefebvre & Pasquerault, 2004).

***Hydrotaea capensis* (Wiedemann, 1818)[8]**

Syn: *capensis* Wiedemann, 1818 (*Anthomyia*)

Anthomyia anthrax Meigen, 1826 (*Anthomyia*), comb. *Hydrotaea anthrax*

rutilans Robineau-Desvoidy, 1830 (*Ophyra*)

viridescens Robineau-Desvoidy, 1830 (*Ophyra*)

cadaverina Curtis, 1837 (*Anthomyia*) *nomen nudum*

cadaverina Megnin, 1894 (*Ophyra*), comb. *Ophyra cadaverina*, comb. *Hydrotaea cadaverina*

caerulea Brunetti, 1913 (*Ophyra*)

villosa Aldrich, 1928 (*Ophyra*)

Hydrotaea capensis puparia are light brown to black (Fig. 65C, D). The puparia length is 6–7mm and they present strong arcuate respiratory horns on the dorsal side. Size and coloration showed a high variability between and among the samples (inter- and intra-variability). The whole length of puparia collected from Central Italy, and here reported, was measured as 6.23±0.28mm (N = 18). The open puparium was, in the analysed specimens, 4.86±0.34mm long (N = 4). The intersegmental spines are large and smoothly rounded and disposed in several packed lines. Spines in the external lines are smaller than the central ones and end with serrated tips. Tips can be bifid or trifid (Fig. 66F). The anal plate wings are narrow and thin at the extremities and reach the external border of the subanal papillae. The extremities of the wings are not upward folded. Distinct para-anal and extra-anal papillae are absent. Subanal papillae are made up of several excrescences, without well-defined borders (Fig. 67B). Posterior spiracles are strongly projected. The angle formed by the spiracles surfaces is ≥ 90º (Fig. 65C, D). Slits are almost straight and parallel. Spiracular muscles are quite well defined and visible (Fig. 33D, Fig. 66D, E). The lateral ones are bigger and more defined than the central ones (Fig. 66D, E). Anterior spiracles are clearly detectable in the close puparia before adult emergence and, as reported in the analysed sample, have four to five lobes. Literature lists a high variability in this feature [four reported by Skidmore (1985) and five/six drown in a schematic sketch by Turchetto and Vanin (2004b)].

Hydrotaea capensis is a synanthropic species and it has been recorded from a wide spectrum of different habitats (Grzywacz *et al.*, 2017c), principally in the warmer seasons. Originating from the Europe, it has been reported from several archaeological and

[8] Synonymic list and puparium description follow Giordani *et al.*, 2018a

forensic cases from all over the continent (France, Portugal, Italy, Germany, Spain, etc.) (Greenberg, 1985; Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Vanin *et al.*, 2011; Huchet, 2013).

This fly usually colonises exposed corpses and carcasses in advanced decomposition stages but it is also one of the first colonisers of buried or concealed cadavers (Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Huchet, 2013). In the latter case, *H. capensis* is potentially one of the most important taxon colonizing the body (Greenberg, 1985; Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Vanin *et al.*, 2011; Huchet, 2013). In 15 exhumations out of 22 carried out in Northern France *H. capensis* was found in high quantity with *Conicera tibialis* Schmitz, 1925 (Diptera: Phoridae) and *Leptocera caenosa* (Rondani, 1880) (Diptera: Sphaeroceridae) (Bourel *et al.*, 2004). It has also been reported in association with mummified bodies (Couri *et al.*, 2009; Vanin, 2012) and with WWI soldier remains (Huchet, 2013) from several archaeological contexts (France, Portugal and Italy).

Lefebvre and Pasquerault (2004) investigated the temperature effect on the developmental rate of *H. capensis* calculating a minimum developmental temperature of 12.8° C and a thermal constant of 237.05±22.73 ADD. Therefore, a longer developmental time is needed by *H. capensis* compared to *H. aenescens* raised at the same temperature conditions.

***Hydrotaea ignava* (Harris, 1780)[9]**

Syn: *ignava* Harris, 1780 (*Musca*)

leucostoma Wiedemann, 1817 (*Anthomyia*), comb. *Hydrotaea leucostoma*, comb. *Ophyra leucostoma*

nitida Robineau-Desvoidy, 1830 (*Ophyra*)

pubescens Robineau-Desvoidy, 1830 (*Ophyra*)

spoliata Zetterstedt, 1846 (*Aricia*)

opalia Walker, 1849 (*Anthomyia*)

Hydrotaea ignava puparia are yellow to brown (Fig. 65E, F) with large arcuate respiratory horns, which are reddish or blackish, on the dorsal side. Puparium length is about 7mm

[9] Synonymic list and puparium description follow Giordani *et al*., 2018a

long (Skidmore, 1985). The open puparia were analysed from a forensic case in Northern Italy, in a mountainous area, and were 4.33±0.51mm in length (N = 3).

Segmental edges are very clearly defined, and the cuticle is roughly cross-striate; large and sharp spines disposed in several crowded lines (Fig. 66I). The anal region is coarsely thorny, with the anal plate and the lateral wings being hardly narrower than the median area, and the enlarged extremities upward folded. Peripheral papillae are indiscernible. Subanal papillae are composed of several excrescences and without well-defined borders (Fig. 67C). Posterior spiracles are broadly rounded and strongly projected. Spiracles surfaces form, in this species, an angle of about 180º (Fig. 65E, F). Respiratory slits are distinctly convergent towards the median scar. The spiracular lateral muscles are well defined and clearly discernable (Fig. 33D, Fig. 66G, H), and the anterior spiracle of the pupa has four to five lobes.

Hydrotaea ignava is an Old World species with three annual appearances observed in UK, the first occurring in spring when the soil reaches a temperature of 14°C, the second in August and the third in October (Skidmore, 1985).

***Hydrotaea dentipes* (Fabricius, 1805)[10]**

***Syn**: dentipes* Fabricius, 1805 (*Musca*)
flavifacies Robineau-Desvoidy, 1830 (*Hydrotaea*)
monacantha Robineau-Desvoidy, 1830 (*Hydrotaea*)
obscuripennis Macquart, 1835 (*Hydrotaea*)
brunnipennis Macquart, 1835 (*Hydrotaea*)

Hydrotaea dentipes puparia are dark brown to black (Fig. 65G, H) and their length is of 7mm in length (Skidmore, 1985). Puparia have very short respiratory horns on the dorsal side. The specimens measured in this work were collected from Yorkshire (UK) and Central Italy and show a full puparium length of 6.67±0.44mm (N = 3). The open puparium is, in the analysed sample, 4.82±0.23mm long (N = 4).

Body segments have two to three rows of large spines which are preceded by scattered minute ones and followed by one to two ranks of short-arcuate toothed rows (Fig. 66L).

The anal plate is clearly visible, and it presents lateral expansions reaching the bases of the para-anal papillae. The anal region around the plate does not have any spine. All papillae are of almost the same size and they have well-defined borders. The postanal

[10] Synonymic list and puparium description follow Giordani *et al*., 2018a

papilla is spiculate (Fig. 67D) and the posterior spiracles are strongly projected and ovoid (Fig. 65G, H). Respiratory slits are straight and diagonally convergent towards the inferior scar. Dorsal muscle scars are faintly impressed (Fig. 66J, K). The anterior spiracle of the puparia of the analysed specimens shows six to eight lobes (more often seven), which is in agreement with the literature (Skidmore, 1985). This species is commonly found in rural and forest habitats as the cogeneric *H. similis* (Matuszewski *et al.*, 2010b). Within archaeological contexts, puparia of *H. dentipes* were reported only from a Roman well excavated in Sherwood forest (UK) (Buckland *et al.*, 2018).

***Hydrotaea similis* Meade, 1887**[11]

Hydrotaea similis puparia are yellow to brown (Fig. 65I, J) and they are similar to the puparia of *H. dentipes.* The studied sample belonged to eggs oviposited by females, collected in Poland (Toruń), during summer 2010. The open puparium is, in the analysed specimens, 6.30±0.41mm long (N = 5). As in *H. dentipes*, body segments have two to three rows of massive spines preceded and followed by one to two ranks of short-arcuate toothed rows (Fig. 5O). This is in agreement with the description of the larvae published by Grzywacz *et al.* (2014).

The W-shaped anal plate presents wings reaching the bases of the para-anal papillae. Subanal, para-anal and extra-anal papillae are present with well-defined borders, and the postanal papilla is spiculated and the external borders less defined. As reported by Grzywacz *et al.* (2014), the region behind the anal papillae is coarsely spiculated (Fig. 67E), in contrast to *H. dentipes* where it appears completely smooth. Posterior spiracles are broadly oval and slightly raised (Fig. 65I, J). Straight respiratory slits are directed diagonally towards the inferior scar. Dorsal muscle scars are well defined and visible (Fig. 66M, N). Anterior spiracles of the close puparia in the analysed material present seven to eight prospiracular lobes, whereas Grzywacz *et al.* (2014) listed six lobes.

Hydrotaea similis is typically spread in the Palearctic region including Central and Western Europe, Israel, China and Japan (Shinonaga, 2003; Pont, 2012; Grzywacz *et al.*, 2014), where the species colonises and develops in environments having a moderate or well-balanced supply of moisture (Gregor *et al.*, 2002; Hwang & Turner, 2005; Grzywacz *et al.*, 2014). *H. similis* was collected from a human body in Sweden (Grzywacz *et al.*,

[11] Puparium description follow Giordani *et al*., 2018a

2014), as only forensic record, while it has never been found from archaeological contexts.

As the same as *H. dentipes* and *H. ignava, t*he arrival of adults of *H. similis*, on exposed pigs correlates with the temperature. This observation allows the estimation of the pre-appearance interval (PAI) (Matuszewski *et al.*, 2014).

***Hydrotaea pilipes* Stein, 1903**[12]

Hydrotaea pilipes puparia are small and light brown (Fig. 65K, L). The whole length of the close puparium is 5.91±0.18mm (N=5), whereas the open puparium average length is 5.16±0.92mm (N=3).

The intersegmental spines are small and smoothly rounded with a concentric disposition, closer at the extremities and increasingly sparse internally (Fig. 66R). The anal region is well detectable with a wide, high and completely smooth anal plate. Papillae are well defined: para-anal papillae are larger compared to subanal and extra-anal once. The postanal papilla is small and spiculate (Fig. 67F). Posterior spiracles are minute rounded and slightly projected (Fig. 65K, L). As reported for *H. ignava*, respiratory slits are distinctly convergent towards the median scar (Fig. 66P, Q). The spiracles lateral muscles are well impressed. The anterior spiracle of the puparium has four lobes.

Hydrotaea pilipes has a Holarctic distribution, and has been described from North and Central Europe (Rognes, 1986; Kahanpää & Haarto, 2014), North America (Huckett, 1954, 1977), and Japan (Shinonaga & Kano, 1983).

In contrast with the observation done on previous species, the appearance of *H. pilipes* adults on carcasses seems not to be useful for the PAI estimation (Matuszewski *et al.*, 2014).

Based upon the above-mentioned descriptions, and literature data (e.g.: Skidmore, 1985; Grzywacz *et al*., 2017a), an identification key of the most common species of archaeological and forensic importance has been designed and reported below. Posterior spiracles, anal plate and intersegmental spines have been considered and used as good diagnostic characters for the identification of these puparia.

[12] Puparium description follow Giordani *et al*., 2018a

Key for the identification of the most common puparia of *Hydrotaea* collected on human and animal remains of forensic and archaeological interest (Giordani *et al*., 2018a)[13]

1	Posterior spiracles with scar in lower position	2
	Posterior spiracles rounded with scar in median position	3
2	The region behind the anal papillae devoid of spines (Fig. 67D)	*H. dentipes*
	The region behind the anal papillae coarsely spiculated Fig. 67E)	*H. similis*
3	Anal papillae distinct and surface surrounding papillae without spines (Fig. 67F)	*H. pilipes*
	Surface of the anal papillae covered with distinct spines (Fig. 67A, B, C)	*4*
4	Upper and lower respiratory slits in posterior spiracles convergent towards the spiracular scar (Fig. 66G, H)	*H. ignava*
	Respiratory slits in posterior spiracles parallel, at most slightly convergent (Fig. 66A, B, D, E)	*5*
5	Anal plate wings rounded (Fig. 67A, A`)	*H. aenescens*
	Anal plate wings narrowed (Fig. 67B, B`)	*H. capensis*

- **Molecular analysis**

The amplification of the 658bp region of the COI gene with the universal primer LCO/HCO designed by Folmer *et al.* (1994) gave positive results only for the pupa of *H. ignava* and *H. similis*. Despite the result was predictable for the 400 years old *H. capensis* pharate adult, it was unexpected for *H. aenescens, H. pilipes* and *H. dentipes*. A possible explanation for the latter, collected from rabbit decomposing carcasses in UK, is the long-

[13] Attention has to be paid because not all the species in the genus have been considered in this key. The species here reported refer in particular to Europeand South America (for the only *H. aenescens*)

time hovering in wet environment due to the climatic condition, resulting in high DNA damages rate. Primers targeting smaller region of the COI gene were used to amplify the DNA extracted from pupae of *H. aenescens*, *H. capensis*, *H. pilipes* and *H. dentipes*. The merge of the three fragments obtained from the different amplifications carried out with the primers presented in Table 18 gave 695bp sequences for *H. aenescens*, *H. capensis*, 704bp for *H. pilipes* and 590bp for *H. dentipes*.

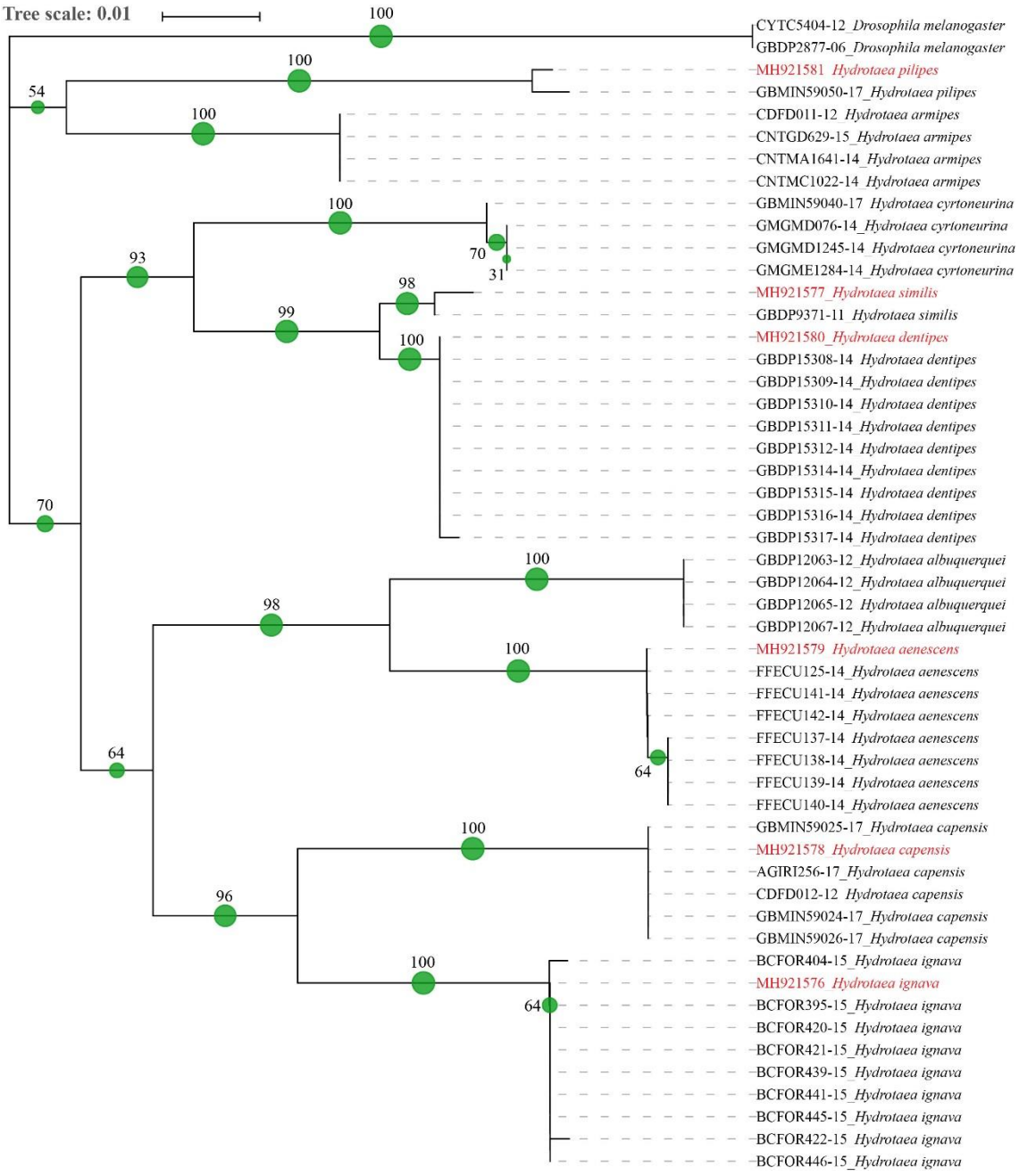

Fig. 68 **Phylogenetic tree of *Hydrotaea* genus.** Neighbour Joining method analysis of 516 bp sequence of the COI gene. The green spots and the number at each node indicate the bootstrap support. Sequences from this study are reported in red.

The local alignment of each sequences on the online system BLASTn® revealed a 97-99% identity with sequences coded in GenBank for the predicted species. A phylogenetic approach was used to verify the goodness of the molecular results. Each sequence of the investigated specimen groups in the correct species cluster. The NJ analysis, based on 52 sequences 516bp long (Fig. 68,Fig. 56), highly supported the morphological identification by good bootstrap value at all tree nodes.

The systematic position of the former genus *Ophyra* Robineau-Desvoidy, 1830, including 16 species, in relation to the genus *Hydrotaea* Robineau-Desvoidy, 1830 has been long investigated from a morphological and molecular point of view with contradictory results (Savage & Wheeler, 2004; Schuehli *et al.*, 2004, 2007; Haseyama *et al.*, 2015; Grzywacz *et al.*, 2017c). The phylogenetic tree resulted from this analysis, despite the low number of species included, supported the former *Ophyra* genus to be monophyletic but nested within *Hydrotaea*, in agreement with what reported by Grzywacz *et al.* (2017c). The systematic positions of the species included in the analysis are in congruence with what reported by Grzywacz *et al.* (2017) by the analysis of three concatenated gene (COI, CytB and Ef1α) with the exception of *H. pilipes*, clustering in an external position with *H. armipes*. This data suggests the use of the only COI gene is functional for identification purposes, but it is not sufficient for phylogenetic investigations.

5.3.3.4 ***Conclusion***

Species in the genus *Hydrotaea* are often found in forensic and archaeological cases. However, to date, only few studies have been taken to validate whether morphological characters of the immature stages allow for species discrimination of *Hydrotaea* puparia. The present study highlights distinct morphological differences of the external morphology of examined puparia, providing their accurate and precise identification. The molecular analysis was used to confirm and support the morphological identification. The sequences were deposited in NCBI GenBank, contributing to databases extension.

Results of this study will facilitate the identification of puparia collected during legal investigations on the crime scenes and archaeological excavations and can be applied as well on other taxa. The produced pictorial archive will help entomologists working with fragmented material often representing the only source of study when they deal with old cases or archaeological sites.

Part of this chapter has been published in Giordani *et al.* 2018a, attached at the end of this thesis (Appendix B).

5.3.4 Description of the entomofauna from the mummies discovered under the Sant'Antonio Abate Cathedral of Castelsardo: *Phormia regina* (Meigen, 1826) (Diptera: Calliphoridae) new record from, Sardinia, Italy.

5.3.4.1 *Introduction*

The studies of the entomological fauna related to archaeological contexts provides an important supplement of evidence, allowing speculation on past events, climate and environmental conditions (Huchet & Greenberg, 2010). Furthermore, the comparison between the reconstructed entomofauna and the current list of species present in the same area, can provide important information about biodiversity changes.
In this chapter the entomological findings collected from mummified bodies discovered during the restoration works of the crypt of the Sant'Antonio Abate Cathedral of Castelsardo (Sardinia, Italy) are illustrated and discussed. This archaeoentomological study records, for the first time, the presence of *Phormia regina* (Meigen, 1826) in Sardinia, which is no longer reported from this Mediterranean island.

- **Geographical and Historical context**

The Sant'Antonio Abate Cathedral is located in the old town center of Castelsardo, a small village in the Northern coast of Sardinia (Italy), 114m a.s.l, as part of Sassari province (40°54′52″N 8°42′46″E) overlooking the Asinara gulf (Fig. 69). The Mediterranean climate has been classified as Csa type (Köppen & Geiger, 1930; Peel *et al.*, 2007). The annual average temperature is 16.1°C, with a maximum of 28.9°C in warmer seasons and a minimum of 6-7°C in colder seasons (Meteorological station of Alghero Fertilia, Sassari, Sardinia; (Pinna, 1954)). The average annual rainfall is 588mm (https://it.climate-data.org).
The Sant'Antonio Abate Cathedral was initially built as a Romanic Church and it was the seat of the bishop of Ampurias in 1503. Between 1597 and 1606 reconstruction works bring to the transformation of the original Romanic Church into Cathedral. A mixture of

Romanic and Renaissance architectonic elements defines the style of the Cathedral. The sepulchral crypts derive from the old basements of the Church left unburied.

Fig. 69 **Map of the Mediterranean Basin.** The black dot indicates the location of the sampling in Castelsardo. The black triangle indicates the location of the modern specimens sampling in Alghero (From Giordani *et al.*, 2018b).[14]

[14] Maps used for the plate retrieved from:
http://d-maps.com/carte.php?num_car=3130& lang=en;
http://d-maps.com/carte.php?num_car=4828&lang=en;
http://d-maps.com/carte.php?num_car=22838&lang=en.

In 2011, excavations took place in the underground crypts in order to the expand the Diocesan museum area. During the renovation works, human remains were found in the so called "Room 3". An abundance of bones and mummified corpses have been found in different locations and position of the crypt, permitting the reconstruction of the time line of the depositions.

The conventionally called "Bob and Mary", two individuals found close to the Cathedral floor at the top of the excavation, are believed to be the most recent inhumations. Napoleon's Saint Cloud Edict, which became law in Italy in 1806, established that burials inside the city walls were no longer permitted. Based on that, "Bob and Mary" can be considered with high probability the most recent and the last inhumations. Despite the uncertain chronology of the previous burials, the depositions in the Sant'Antonio Abate Cathedral can be dated between 1597 and 1806.

A multidisciplinary team of scientists including anthropologists, archaeologists, biologists, and immunologists was involved in the study of the archaeological samples in order to determine the sanitary condition of the population and to better investigate the burial practices carried out in Sardinia.

5.3.4.2 *Material and Methods*

The archaeological excavation of the crypts of Castelsardo, brought to light some insect fragments associated with the human remains buried there.

Entomological samples were collected from the remains of the two individuals previously mentioned surrounded by several other burials. Sterile tweezers and paintbrushes were used to recover the specimens then stored in sterile plastic vials. The collection and the study of the archaeological material was undertaken upon agreement with the Ufficio Culturale – Diocesi di Tempio Ampurias in March 2011.

Puparia samples were treated as described in Material and Methods (see Chapter 4). The morphological study of the samples was performed using identification keys (Vienna, 1980; Skidmore, 1985; Smith, 1986; Whitaker, 2007; Giordani *et al.*, 2018a) and by comparison with already identified specimens as part of the breeding or private collection of Dr Vanin. The shape and position of the posterior spiracles, anal plate and intersegmental spines were used as diagnostic characters for fly puparia identification. The identification of Lepidoptera cocoons was not possible at a deeper taxonomic level than family level. The identifications of flea specimens were confirmed by Maurizio Mei

(Department of Biology and Biotechnology Charles Darwin, University of Rome "La Sapienza", Italy).

Due to the low amount of starting material, DNA extraction was performed from both immature stage and adult samples with the QIAamp® DNA Investigator kit on modern and archaeological samples. D1AA and D2AA refer to modern Dolichopodidae adults collected from inside Cathedral of Alghero by Dr Vanin in May 2018 (Fig. 69), D4AC refer to adults Dolichopodidae flies collected in the crypt of Castelsardo. One single specimen was used for the DNA extraction from fresh sample while 12 adult fragments were used for the old specimens. Due to their size and state of preservation, all the samples were sacrificed for the molecular analysis except for the male genitalia that were conserved for further morphological analysis.

The DNA extraction from immature stages was performed on one (S1P1I, S1P1D), two (S1P2) or three (S1P3) pharate adults of *H. capensis* inside the puparia; on one pharate adult of *H. capensis* without the puparia (S1F1I, S1F1D) and on five empty puparia (S1Pu5D). Except samples S1P1I and S1F1I that were preserved for further analysis the other specimens were sacrificed for the molecular analysis. The extracted DNA has been quantified with Qubit assay. The size of the extracted DNA fragments from old specimens was evaluated with the Agilent Bioanalyzer 2100 (For further information refer to 4.6).

The DNA amplification was carried out in the Barcoding region (COI) with the universal primer LCO/HCO (Folmer *et al.*, 1994) and in the Cytochrome b gene (CytB) with the universal primer mcb398/mcb869 (Verma & Singh, 2003). In case of negative result in the COI gene, shorter fragments were amplified with degenerated primers shown in Table 18 and merged together to reconstruct the gene. For further information refer to section 4. The PCR master mix reaction (see 4.7) was added of 1.6µl BSA (10mg/ml) and the amount of each primer (10pmol/µl) and of the nucleotide mix (10mM) was increase at 1µl. Up to 5µl of DNA (1-6ng) per reaction were used.

Sequences downloaded from GenBank were included in the phylogenetic analysis (Table 21). Sequences of *Drosophila melanogaster* were used as outgroup.

Table 21 **COI sequences of Dolichopodidae downloaded from BOLD and included in the analysis. Drosophila melanogaster sequences used as outgroup are reported.**

Family	Morphological identification	GenBank code	
Dolichopodidae	*Dolichopus plumipes* (Scopoli, 1763)	KM913022	KM913477
		KM913404	KM913141
	Dolichopus hastatus Loew, 1864	MF820326	MF820112
	Dolichopus lamellipes Walker, 1849	MF820626	MF820600
		MF820605	MF820561
	Dolichopus brevipennis Meigen 1824	KM909416	KM917509
		KM902985	
	Hercostomus unicolor Loew, 1864	KM911249	KM910610
		KM911731	KM907127
	Neurigona tenuis (Loew, 1864)	KR746617	KR747089
		KR746820	KR748228
	Medetera vockerothi Bickel, 1985	KR720762	
	Medetera signaticornis (Loew, 1857)	KR518088	KR516978
		KM910455	KR521727
	Medetera vittata Van Duzee, 1919	KR695103	KR694205
		KR687935	KR726082
	Medetera apicalis (Zetterstedt, 1843)	KT113222	
	Chrysotimus delicates Loew, 1861	KR670314	KR672682
		KR673220	KR671028
	Sympycnus lineatus Loew, 1861	KR516543	KR522140
		KR519973	KR518593
Drosophilidae (outgroup)	*Drosophila melanogaster* Meigen, 1830	KP843843	KP843844

5.3.4.3 *Results and Discussion*

- **Morphological analysis**

Specimens belonging to Diptera, Coleoptera, Lepidoptera and Siphonaptera were identified among the material (Table 22).

Diptera

Ninety five percent of the entire entomological samples was composed by Diptera fragments. The Puparia were collected and sampled overwhelmingly from the area near the inhumations known as "Bob & Mary". The significant amount of puparia fragments clearly support a colonisation of the bodies during early stages of decomposition excluding any sporadic event of later contamination. It was impossible to determine whether the puparia belong to Bob's or to Mary's body due to the specific archaeological context. Additionally, post-feeding maggots' leave the body searching a sheltered and protected environment to pupariate. Thus, puparia are not usually found on the body itself but nearby (Lewis & Benbow, 2011; Martín-Vega *et al.*, 2016).

Table 22 **List of the samples collected from the mummies found in the crypts of the Sant'Antonio Abate Cathedral in Castelsardo, Sardinia, Italy.** (Fragment count: *=1-10, ** 10-100, ***>100) (Modified from Giordani *et al*., 2018b).

	Complete puparia	**Puparia Posterior spiracles**	**Puparia Fragments**	**Adult fragment**	**Cocoons**
DIPTERA					
n.d.				*	
Muscidae					
Hydrotaea capensis (Wiedemann, 1818)	***	***	***		
Calliphoridae					
Phormia regina Meigen, 1826	*	**	**		
Calliphora vicina Robineau-Desvoidy, 1830		**	**		
Sarcophagidae					
Sarcophaga sp.		*	*		
Dolichopodidae					
Gen. sp.				**	
COLEOPTERA					
Histeridae					
Saprinus semistriatus (Scriba, 1790)				*	
Tenebrionidae					
Gen. sp.				*	
LEPIDOPTERA					
Tineidae					
Gen. sp.					*
SIPHONAPTERA					
Pulicidae					
Nosopsyllus cfr. *fasciatus* (Bosc, 1800)				*	
Echidnophaga cfr. *murina* (Tiraboschi, 1903)				*	
Leptopsylla segnis (Schönherr, 1811)				*	
Pulex irritans Linnaeus, 1758				*	

Among the analysed puparia, four morphotypes were isolated and identified.

The three biggest morphotypes were identified as members of Calliphoridae family (Calliphorinae and Chrysomyiinae) and Sarcophagidae family. The last morphotype, the smallest compared to the others, was recognized as members of Muscidae family.

Morphological identification was performed trough an accurate observation of diagnostic features present on the external surface of the puparia such as posterior spiracles shape and orientation, anal plate structure and intersegmental spines distribution.
Puparia in the family Calliphoridae were identified as *P. regina* (Fig. 70) and *Calliphora vicina* (Robineau-Desvoidy, 1830) (Fig. 71), species known to be first colonizers of exposed bodies (Smith, 1986).

Phormia regina puparia (Fig. 70) are characterised by the peculiar shape of the posterior region where spiracles, big and rounded, are placed in a superficial invagination. Puparia external cuticle is rough and covered by minute, sharp intersegmental spines arranged in short rows. Puparia of this species can be differentiated by the close related species *Protophormia terraenovae* (Robineau-Desvoidy, 1830) by the lack of big and robust anal papillae with a crow shape. The two species have been often found together (e.g.:Vanin *et al.*, 2009).
The forensically important fly *P. regina* belongs to the subfamily Chrysomyiinae. It is a hemisynanthropic[15] cosmopolitan species associated with decaying matter (dung and carrion) (Greenberg, 1985; Byrd & Allen, 2001; Nabity *et al.*, 2006). The preference of warmer seasons, make this species particularly abundant in tepid latitudes during spring and summer, while its number consistently decrease during cooler season or in areas where low temperature are present all year around, such as glaciers (Hall, 1948; Goddard & Lago, 1985; Greenberg, 1985). Previous studies have assessed the minimum and maximum thresholds for this species range from 10-12.7°C to 35°C (Deonier, 1942; Haskell, 1993) assessing that *P. regina* is relatively tolerant to cold temperature. Despite oviposition is mostly observed and recorded during daylight in natural environments, nocturnal oviposition has been as well recorded even if only in rare cases (Berg & Benbow, 2013).
Widespread all over Europe, *P. regina* has not yet been recorded in just a few countries (Iberian Peninsula, the Denmark, the Hellenic Peninsula and the Italian big island, Sardinia and Sicily) (http://www.fauna-eu.org). The lack of information on the presence or absence of this species in certain European area (e.g. Sardinia) has to be carefully evaluated. Indeed, the absence of this species could be linked to a misidentification with *P. terraenovae*, a very similar species.

[15] Hemisynantropic species usually thrive on the edges of towns, with greater or lesser affinity to humans.

The finding of *P. regina* in this set of archaeoentomological samples is particularly important in the history record of Sardinia region. In fact, this species does not appear either among modern specimens in museum collections in Italy neither has it been mentioned in the specific literature from this region. In contrast, *P. regina* is well known to be present in the rest of Italy, recorded from both forensic and archaeological sites (Vanin *et al.*, 2009).

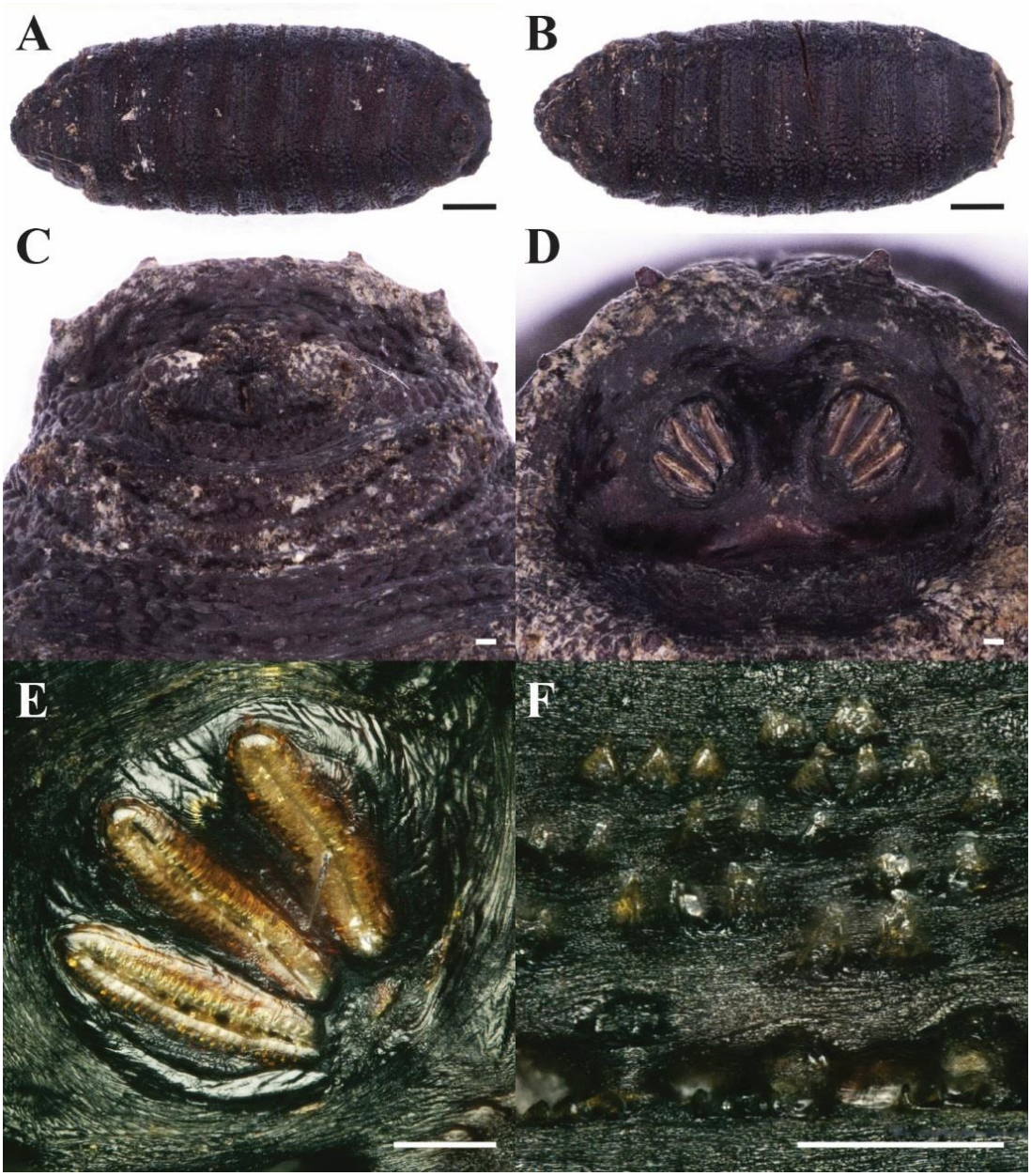

Fig. 70 ***Phormia regina***. Puparium in ventral (A) and dorsal (B) view (Black scale bar: 1mm). Puparium details: anal plate (C), posterior spiracles (D, E), segmental spiculae (F) (White scale bar: 100μm) (From Giordani *et al*., 2018b).

Phormia regina has a long history of records in forensic contexts both from North America and from Central Europe (Italy, Germany, Poland, etc.) considering specimens collected during animal carcasses (pigs and fishes) decomposition experiments and during real cases on human bodies (Goddard & Lago, 1985; Greenberg, 1985; Cianci & Sheldon, 1990; Schroeder *et al.*, 2003; Matuszewski *et al.*, 2008; Sharanowski *et al.*, 2008; Matuszewski *et al.*, 2010b, 2010a; Anton *et al.*, 2011; Matuszewski *et al.*, 2011; Vanin *et al.*, 2011). Less numerous are the records of it in archaeological contexts: one from a WWI soldier remains near the Italian front (North-Eastern Italy) (Vanin *et al.*, 2009) and one from pre-historic graves in Canada by Teskey and Turnbull (1979). In both cases the idea suggested by the author is a long time elapsed between death and the burial (Teskey & Turnbull, 1979; Vanin *et al.*, 2009).

Puparia of *C. vicina* are externally smooth and light brown in colour (Fig. 71). By a superficial observation they can be easily confused with the puparia of the cogeneric *Calliphora vomitoria* (Linnaeus, 1758). However, the ratio between the major lenght of the spiracle and the distance between the two spiracles, can be used to differentiate the two species. In fact *C.vomitoria* ratio is <1 (big spiracles, small interspiracle distance) and *C. vicina* ratio is >1 (smaller spiracle, bigger interspiracle distance) in (Emden, 1965). The forensically important blowfly *Calliphora vicina* belongs to the subfamily Calliphorinae. Cosmopolite and very common in urban habitat, it is normally found in close association with humans (Wallman, 2001) but it has also been collected in hedgerows and forests (Smith & Wall, 1997b). Recorded over the whole year (Schroeder *et al.*, 2003), in torrid regions, this species presents spring and fall peaks where the temperatures are around 13-24˚C (Zumpt, 1965; Greenberg, 1985; Salvetti *et al.*, 2012). The sudden responsiveness of *C. vicina* to decomposing bodies makes this species one of the first coloniser and one of the most important fly species in forensic entomology (Davies, 1990; Isiche *et al.*, 1992; Smith & Wall, 1997b, 1997a). Its arrival on dead corpses or animal carrion has been worldwide (North America, Europe, Asia and Africa) intensely studied (Greenberg, 1985; Bonacci *et al.*, 2009; Dupont *et al.*, 2011; Bugelli *et al.*, 2015; Moemenbellah-Fard *et al.*, 2015; Keshavarzi *et al.*, 2016).

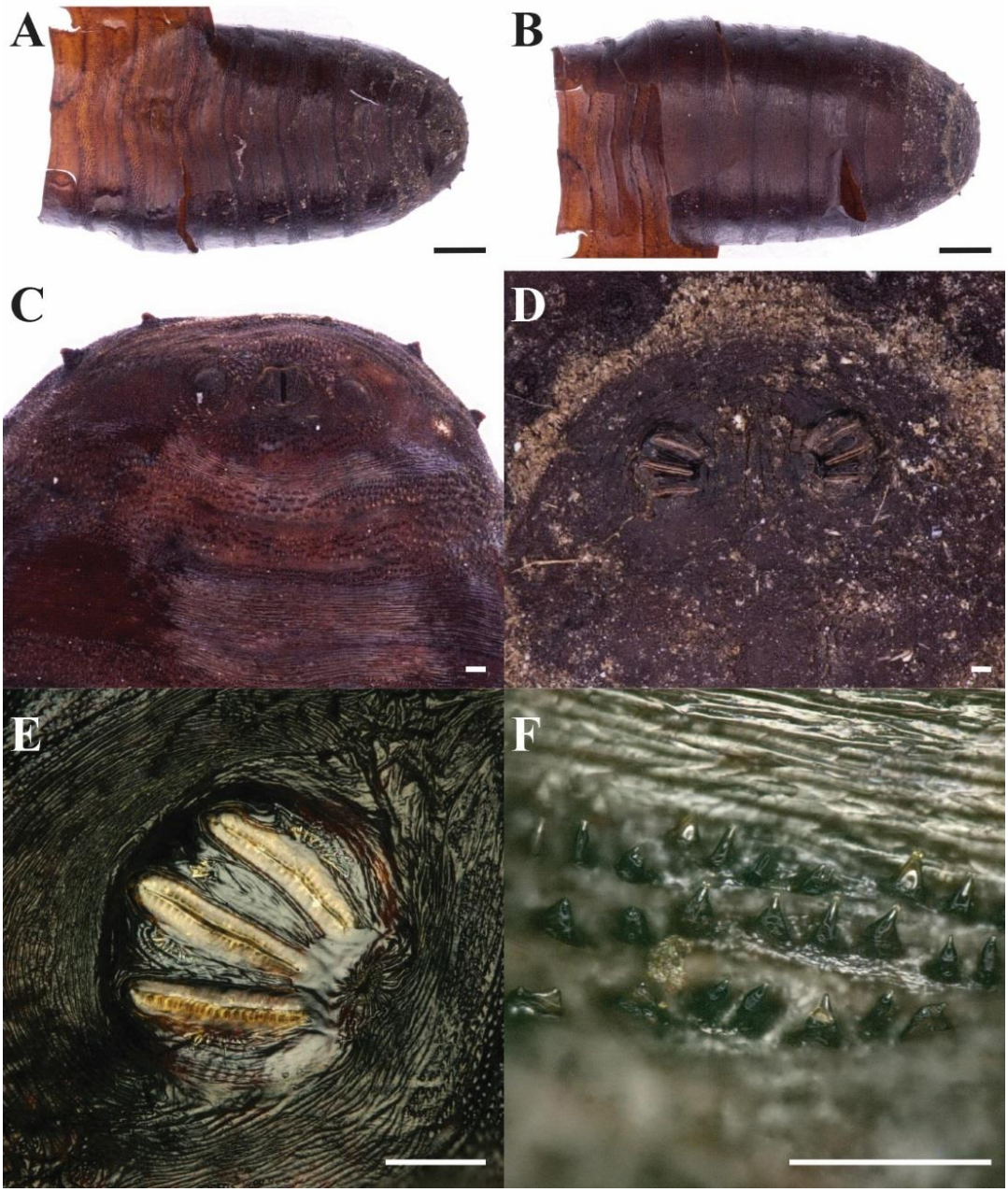

Fig. 71 ***Calliphora vicina***. Puparium in ventral (A) and dorsal (B) view (Black scale bar: 1mm). Puparium details: anal plate (C), posterior spiracles (D, E), segmental spiculae (F) (White scale bar: 100μm) (From Giordani *et al*., 2018b).

This species has been recorded from an archaeological site in Central Italy, on a partially mummified body concealed inside an underground crypt of a Church (Vanin, 2012) in a circumstance close to the one described in this work. However in that particular case several openings (small windows) of the crypt could have allowed the insect access to colonise the body. The capability to colonise concealed bodies in hypogeic context has been also confirmed by Faucherre *et al.* (1999), who collected *C. vicina* eggs from a human cadaver found in a 10 m deep cave in the Swiss Jura mountains at an altitude of

1260 m a.s.l. Nevertheless, previous colonisation experiments showed *C. vicina* aptitude for colonise buried remains is limited. In fact, this species seems not to be able to colonise corpses buried deeper than 10cm (Gunn & Bird, 2011) and it has never been previously reported on buried human remains. In general, the presence of this species indicates a colonisation of exposed bodies which could have happened before the burial or during the transfer into the crypt.

Among the largest puparia, three fragments (Table 22, Fig. 72) were identified, in the family Sarcophagidae, as belonging to a species in the genus *Sarcophaga* Meigen, 1826. The posterior spiracles hidden in a crateriform-shaped cavity located in the upper part of the last abdominal segment are with the large dimension (bigger compare to Calliphoridae puparia) discriminating characters of these puparia. The shape and position of the posterior spiracle slits and the open peritreme are other diagnostic features for Sarcophagidae family identification (Fig. 72).

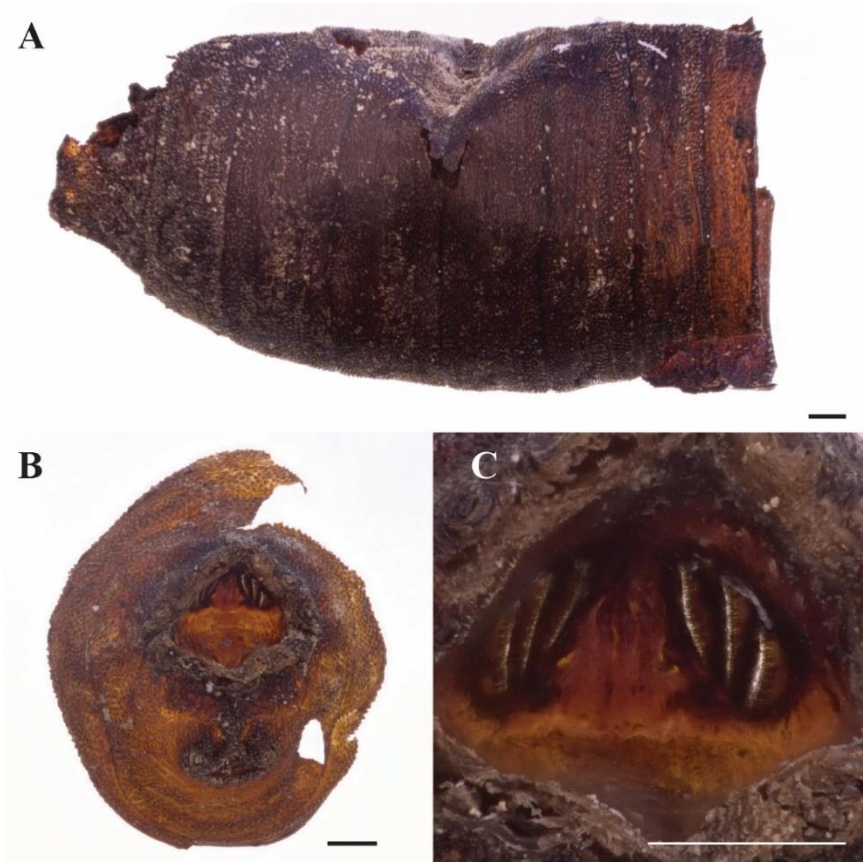

Fig. 72 ***Sarcophaga* sp.** Puparium in later view (A). Puparium details: posterior region with the characteristic crateriform-shaped cavity located in the upper part of the last abdominal segment (B) where the posterior spiracles are located (C) (Black scale bar: 1mm; White scale bar: 100µm) (From Giordani *et al.*, 2018b).

This family represent about 2600 species worldwide (Pape *et al.*, 2006). Females in *Sarcophaga* genus directly depose first instar living larvae (larviparous) on human cadavers and animal carrions. Species in the family Sarcophagidae have been reported from both exposed and buried remains and are commonly considered to arrive on decomposing matter sligthly after Calliphoridae flies (Pastula & Merritt, 2013; Bugelli *et al.*, 2015; Lo Pinto *et al.*, 2017). In Italy, species in the *Sarcophaga* genus were collected from forensic cases occurred during the warmer season of the year (Vanin *et al.*, 2008; Bugelli *et al.*, 2015; Lo Pinto *et al.*, 2017). From archaeological contexts, few records are available for this family and mainly refer to undetermined species (Huchet, 2010, 2013).

The overwhelmingly of the analysed puparia were smaller than the Calliphoridae ones (Giordani *et al.*, 2018a). The presence of respiratory horns, the shape and structure of the anal plate and the weakly protrusion from the posterior region of spiracles showing three subparallel slits (Fig. 73) allowed the identification of the puparia as belonging to the Muscidae fly, *Hydrotaea capensis* (Wiedemann, 1818).

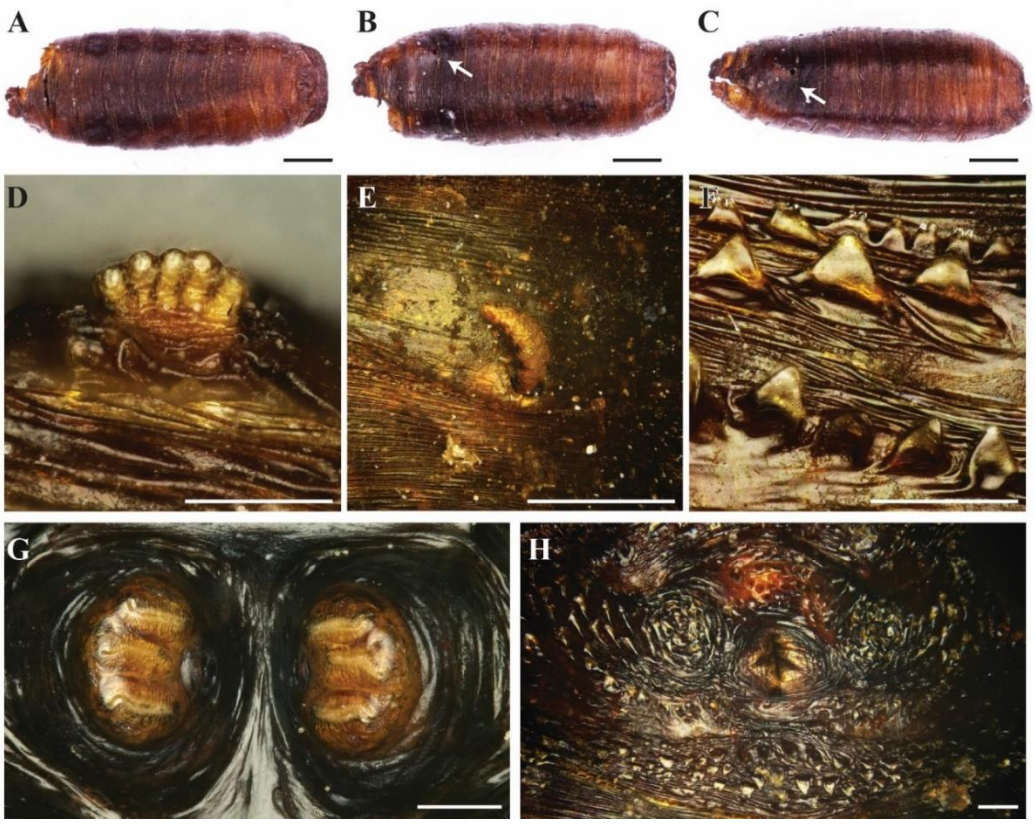

Fig. 73 ***Hydrotaea capensis.*** Puparium in ventral (A), dorsal (B) and lateral (C) view (Black scale bar: 1mm). White arrow indicates the position of the respiratory horns. Puparium details: anterior spiracle (D), horn (E), segmental spiculae (F), posterior spiracles (G), anal plate (H) (White scale bar: 100μm) (From Giordani *et al.*, 2018b).

Puparia of this species can be easily misidentified with the very similar *Hydrotaea aenescens* (Wiedemann, 1830), a South American species introduced in Europe after the XV century and widely spread in the Mediterranean region (Giordani *et al.*, 2018a). Despite sharing many characters, the two species can be distinguished by an accurate observation of the anal plate and the posterior spiracle slits (Skidmore, 1985; Giordani *et al.*, 2018a).

The synanthropic species *H. capensis* has been reported from a wide range of environments with the exception of arid habitats (Grzywacz *et al.*, 2017a). Reported from all over the continent (France, Portugal, Italy, Germany, Spain, etc.), *H. capensis* arrives during the advanced stages of decomposition in exposed bodies whereas it tends to be one of the first coloniser of buried or concealed corpses (Smith, 1986; Greenberg, 1991; Greenberg & Kunich, 2005; Huchet *et al.*, 2013). In archaeological contexts, this species has been described in Europe on mummified bodies in religious burials (Couri *et al.*, 2009; Vanin, 2012; Morrow *et al.*, 2015) and from remains of WWI soldiers (Huchet, 2013).

A high number of adult Diptera were collected from the same area. The shining green colour and the prominent male genitalia slung under the body allow the identification of this small flies as belonging to the Dolichopodidae family (Marshall, 2012) (Fig. 74). Dolichopodidae family includes around 7000 worldwide distributed species (Marshall, 2012) abundant in moist habitat such as log, bark, rocks and mudflats (Bickel & Dyte, 1989; Marshall, 2012).

Fig. 74 **Dolichopodidae Gen. sp.** Adult collected in the crypt of the Sant'Antonio Abate Cathedral of Castelsardo, Sardinia (Scale bar: 1mm).

All adult Dolichopodids are predators of soft-bodied arthropods such as mites (Bickel & Dyte, 1989; Marshall, 2012) and some of them are considered important as biological control agents (Negrobov & Naglis, 2016). Dolichopodids have been rarely collected from decomposing experiments and only as adults (Azwandi & Abu Hassan, 2009; Prado e Castro *et al.*, 2012; Díaz-Martín & Saloña-Bordas, 2015) where they probably act as predators of necrophagous arthropoda. Their role in decomposition has never been assessed.

Lepidoptera

Among the entomological samples a few moth cocoons were also collected (Fig. 75, Table 22). The shape, structure and composition of the cocoon allow the identification in the family Tineidae. *Tinea pellionella* (Linnaeus, 1758) and *Tineola bisselliella* (Hummel, 1823) are the most frequent species among this family found in association with carcasses. These species are known to be involved as well in textiles biodegradation and to have some implications in cultural heritage conservation. Because of their propensity in feeding on dry tissues, Tineidae moths typically occur during the last phases of the human decomposition (Smith, 1986; Mazzarelli *et al.*, 2015).

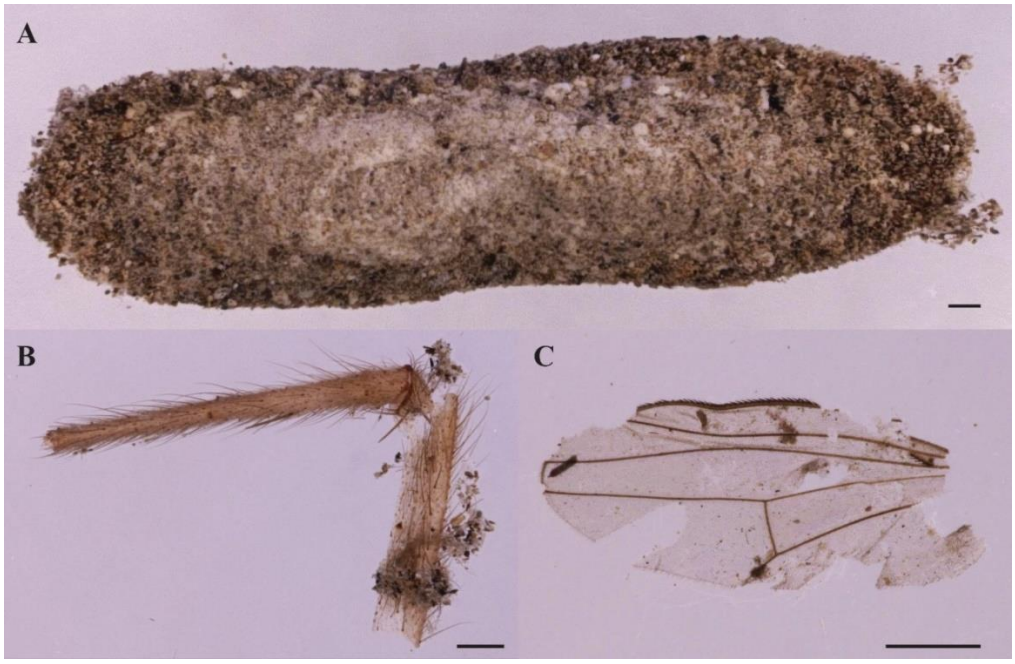

Fig. 75 **Cocoon of Tineidae moths** (A). The cocoon was recovered by moth frass and insect fragments as fly leg (B) and wing (C). This indicates that moth arrival on the human remains was subsequent to the Diptera (Scale bar: 500µm).

Coleoptera

Among the Coleoptera remains, a single specimen of Histeridae beetle and a single elytron, potentially belonging to Tenebrionidae, were collected (Table 22). The hister beetle (Fig. 76) was identified as *Saprinus* (*Saprinus*) *semistriatus* (Scriba, 1790). This Palearctic species, reported also from India and Taiwan, is commonly found beneath animal carrions and decomposing fish, manure heaps and rubbish dumps (Vienna, 1980). *Saprinus semistriatus* was reported from both small and big animal carrions in Europe where it represents the most frequent coloniser among Histeridae species during warmer seasons (Kočárek, 2003; Bajerlein *et al.*, 2011). In Northern Italy this species was reported from burnt and unburnt swine carcasses during hot season but not in cold period (Vanin *et al.*, 2013). Matuszewski and Szafalowicz (2013) found out that *S. semistriatus* and other taxa can be used to evaluate the PAI based upon temperature data. The close relationship between *S. semistriatus* PAI and the environmental temperature allows this approach to be used.

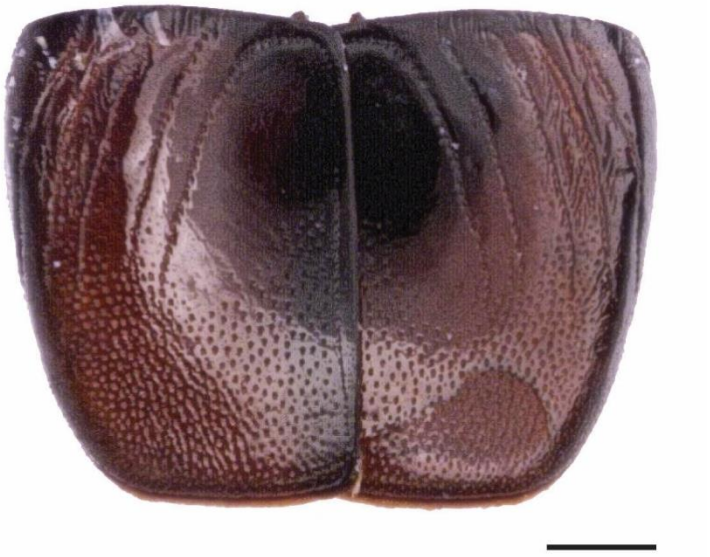

Fig. 76 ***Saprinus semistriatus.*** Elytra. (Scale bar: 500μm).

Siphonaptera/Aphaniptera

A few fragments of flea adults were found from the "Mary" body. Adult specimens of flea are obligate ectoparasites feeding on the blood of the host. Despite the decreasing in

the host body temperature due to the stop of vital function prompt the adult flea to move away, the presence of clothes as obstruction and the presence of rats (common flea host) that were probably living in the crypt can explained the finding of a high number of fleas. The specimens were identified as *Nosopsyllus* cfr. *fasciatus* (Bosc d'Antic, 1800), *Echidnophaga* cfr. *murina* (Tiraboschi, 1903), *Leptopsylla segnis* (Schönherr, 1811) and *Pulex irritans* Linnaeus, 1758 (Fig. 77).

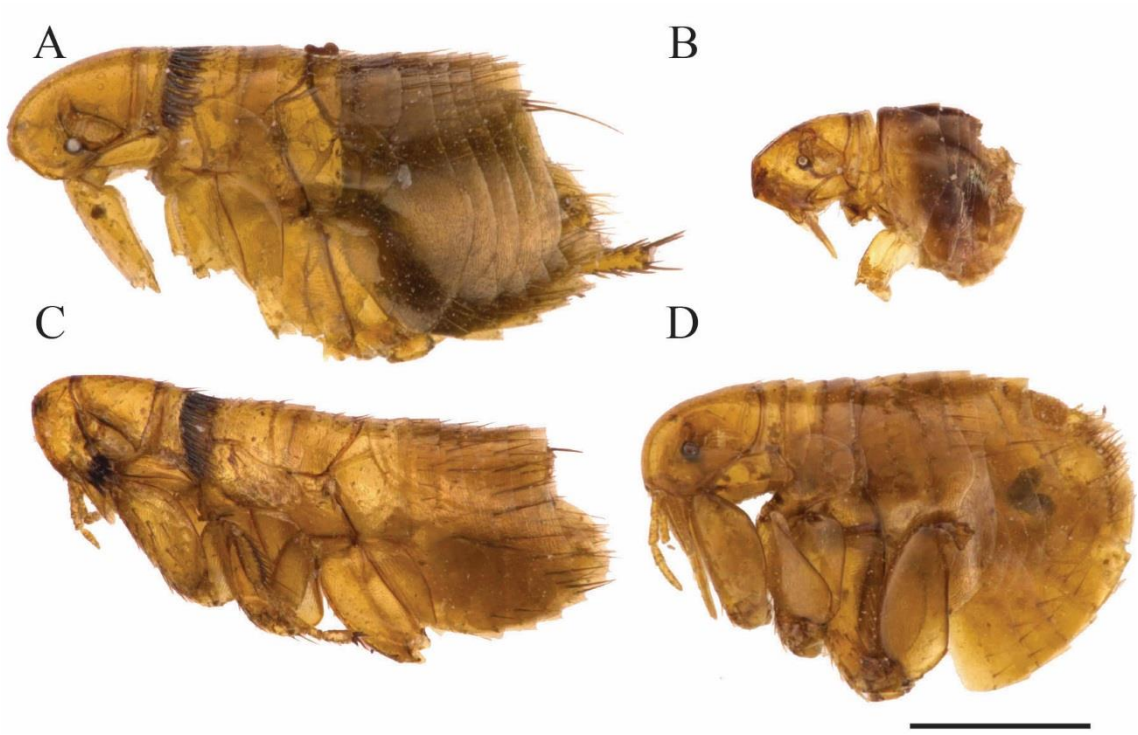

Fig. 77 **Fleas.** A) *Nosopsyllus* cfr. *fasciatus*, B) *Echidnophaga* cfr. *murina*, C) *Leptopsylla segnis* and D) *Pulex irritans* (Scale bar: 500µm).

The external morphology of fleas is closely correlated with their biological cycle and the typology of their host (Whitaker, 2007).

One flea specimen was identified as belonging to the *Nosopsyllus* Jordan, 1933 genus, potentially *N. fasciatus*, commonly known as the Northern rat flea (Fig. 77A). The provisional identification is likely to become definitive after comparing with reference material. (Fig. 77A). This 3-4mm flea can be a minor vector of black plague (Eskey *et al.*, 1949; Bitam *et al.*, 2010), native of Europe is now worldwide distributed (Chick & Martin, 1911).

As in the case of *Nosopsyllus* cfr. *fasciatus*, also for the species identified in the genus *Echidnophaga* Olliff, 1886 the identification at species level is not certain. *Echidnophaga gallinacea* (Westwood, 1875) and *Echidnophaga murina* share the same hosts (birds and

mammals) and can be distinguished by minimal differences in the chaetotaxy of tergite and tarsi. Despite *E. gallinacea* is widly distributed, it is not common in Europe (de Jong *et al.*, 2014), on the contrary *E. murina* is present in the Mediterranean basin and has already been recorded in Sardinia (http://www.faunaitalia.it/checklist/introduction.html) (de Jong *et al.*, 2014) (Fig. 77B). Despite its association with rat, it is unlikely that *E. murina* can transmit rickettsiae (the etiologic agents of typhus) to humans (Traub & Wisseman, 1978; Mumcuoglu *et al.*, 2001). Recognised as vector of the murine typhus is the mouse flea *Leptopsylla segnis* (Azad & Traub, 1987) (Fig. 77C). This worldwide distributed species has been previously reported in Sardinia (Whitaker, 2007) (http://www.faunaitalia.it/checklist/introduction.html). The human flea *Pulex irritans* was furthermore collected from the body (Fig. 77D). Human are generally considered secondary host of this flea, while primary host can be a wide range of animals such as pig (*Sus scrofa* Linnaeus, 1758), dog (*Canis familiaris* Linnaeus, 1758) and cat (*Felis catus* Linnaeus, 1758). *Pulex irritans* acts as a vector of many etiological agents of human diseases such as *Yersinia pestsis* (Lehmann & Neumann, 1896) van Loghem, 1944, the agent of the black plague, *Rickettsia typhi* (Wolbach & Todd, 1920) Philip, 1943, the agent of murine typhus and *Rickettsia felis* Bouyer et al., 2001 emend. La Scola et al., 2002, the agent of flea-borne spotted rickettsiosis (Azad, 1990; Azad *et al.*, 1997; Ruiz, 2001; Brouqui & Raoult, 2006).

These findings of several fleas on the bodies and some preliminary studies carried out on the sediments of the crypts by the University of Sassari can support the hypothesis of the cause of death of the people deposited into the crypt. In fact, the cause of death can be related to the epidemic plague that affected Italy, and Sardinia in particular, between XVI and XVII centuries. After the epidemic "Black Death" that affected Europe between the 1347 and the 1352, probably as part of a wider pandemic, a second plague epidemy began in Sardinia in 1652 arriving from Spain (Alfani, 2013; Bianucci *et al.*, 2013).

- **Molecular analysis**

Molecular analyses have been carried out on the entomological material collected from the crypts of the church of Castelsardo (Sassari). All the tested specimens produced a high DNA yield (Table 23).

The size of the DNA fragments was ranging from 100 to 10,000bp. The D4AC sample showed a weak peak between 1,200 and 10,000bp while S1P3 appeared to be significantly

degraded with a high peak around 100bp. Despite this, longer sequences were present in the S1P3 sample (Fig. 78).

Table 23 **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. Standard deviation is reported.

Sample code	Origin	Analysed material	Elution (µl)	Average DNA quantification (ng/µl)	Total amount of extracted DNA (ng) per specimen
D1AA	Alghero	1 adult	100	4.68±0.11	468.00±13.86
D2AA	Alghero	1 adult	100	3.19±0.05	638.67±11.55
S1P1I	Castelsardo	1 pharate adult with puparium	100	0.26±0.01	26.27±1.62
S1P1D	Castelsardo	1 pharate adult with puparium	100	0.24±0.01	23.67±1.45
S1P2	Castelsardo	2 pharate adult with puparium	50	1.44±0.01	36.00±0.25
S1P3	Castelsardo	3 pharate adult with puparium	50	2.35±0.06	39.22±1.26
S1Pu5D	Castelsardo	5 puparia	100	0.51±0.02	10.25±0.37
S1F1I	Castelsardo	1 pharate adult	100	0.24±0.01	24.00±1.00
S1F1D	Castelsardo	1 pharate adult	100	0.27±0.01	27.27±1.81
D4AC	Castelsardo	12 adults	50	0.85±0.01	3.53±0.05

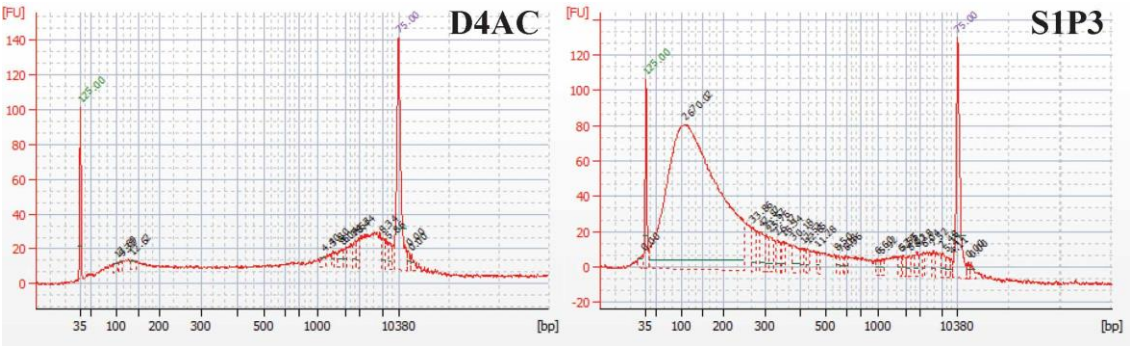

Fig. 78 **Fragment size of extracted DNA for D4AC (left) and S1P3 (right).**

The phylogenetic reconstruction based on the amplification of the COI barcoding region and/or the CytB gene was used to confirm the species identification.

The amplification of the DNA extracted from adult Diptera flies gave positive results for both archaeological and fresh material for both the investigated genes. The local alignment of the sequences was performed using the online system BLASTn® (Table 24). The low percentage of identity (81-87%) did not allow the identification at species level but clearly confirmed the belonging to Dolichopodidae family.

Table 24 **Results of the local alignment of the Dolichopodidae sequences on the online system BLASTn.**

Sequence information			GenBank result		
Specimen code	**Gene**	**length (bp)**	**Species and GenBank code**	**Identity %**	**E value**
D1AA	COI	689	*Medetera vittata* Van Duzee, 1919 KR694205	87	0.0
	CytB	456	*Neurigona* sp. FJ808473	82	2e-89
D2AA	COI	690	*Medetera vittata* Van Duzee, 1919 KR694205	87	0.0
	CytB	456	*Hercostomus brevidigitalis* Zhang, Yang & Grootaert 2008 FJ808458	83	7e-94
D4AC	COI	586	Dolichopodidae Gen. sp. KR383052	87	4e-177
	CytB	386	*Neurigona* sp. FJ808473	81	4e-66

A phylogenetic approach was used to verify the goodness of the molecular result. The alignment of 44 sequences of 565bp in the COI gene belonging to different genera (*Dolichopus* Latreille, 1796, *Neurigona* Róndani, 1856, *Hercostomus* Loew, 1857, *Medetera* Fischer von Waldheim, 1819, *Chrysotimus* Loew, 1857 and *Sympycnus* Loew, 1857) in the Dolichopodidae family generated a phylogenetic tree with well-defined species clusters (Fig. 79). The sequences belonging to this study group together in the *Medetera* Fischer von Waldheim, 1819 genus. The bootstrap support value of 58 suggests the monophyly of the genus despite, considering the 180 species included in the genus (Negrobov & Naglis, 2016), the presence in GenBank of only four *Medetera* species is a limitation in the analysis. The alignment of 64 sequences of 331bp in the CytB gene was not informative. The sequences of specimens belonging to Sardinia cluster in *Medetera* genus, with a high bootstrap support a species level but a low bootstrap support at genus. The characteristic of *Medetera* adult to feed on soft invertebrates such as mites and to settle on vertical surfaces (Negrobov & Naglis, 2016) is not in disagreement with the condition in which the archaeological and fresh samples were collected. Mites include about 50,000 species spread in almost all the environment (Amrine Jr & Manson, 1996). The moulds growing on the wall of the church as well as decomposing organic matter are an attraction for mites and, as consequence, for their predators. From a geographical point of view, different species of flies in Dolichopodidae family have been collected from Sardinia; *Medetera dendrobaena* Kowarz, 1877 is the only species in the *Medetera* genus recorded from the region (http://www.faunaitalia.it/checklist/introduction.html). The sequences reported in this thesis (Appendix A) are not yet officially deposited because

the identification of the specimens has not yet been confirmed by a Dolichopodidae expert. When the final identification will be confirmed the sequences will be deposited, improving the molecular knowledge of this taxon.

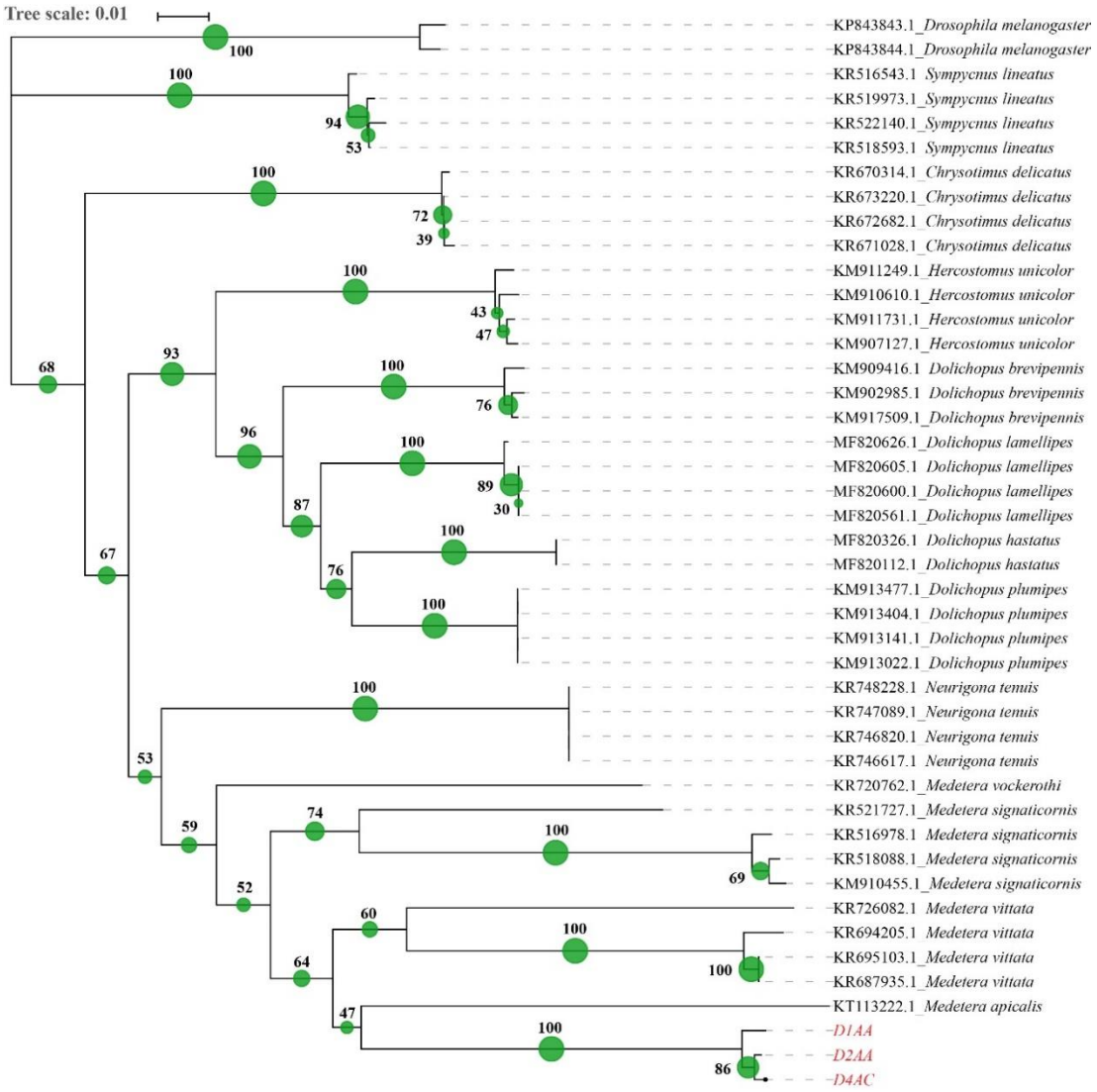

Fig. 79 **Phylogenetic tree of Dolichopodidae family**. Neighbour Joining method analysis of 565bp sequence of the COI gene. The green spots and the number at each node indicate the bootstrap support. Sequences from this study are reported in red.

The molecular extraction was performed also on pupae collected in close proximity to the "Bob and Mary" bodies. Previous work on pupae and puparia of *Chrysomia albiceps* Wiedemann, 1819 showed the possibility to extract DNA from 20 years old big puparia (Bortolini, 2014). DNA was extracted from one, two and three pupae, five puparia and one pharatus of *H. capensis*. This species, commonly collected from archaeological contexts, is among the difficult muscids to identify at immature stages, because of the close similarity in characters with the sister species *H. aenescens*. The problem of the

identification of the species of this genus based on morphological differences and molecular analysis and the biology of these two species are reported in section 5.3.3.

The amount of DNA recovered per pharate with puparium was extremely variable, from 23.67±1.45ng to 39.22±1.26ng (Table 23). The DNA yielded from an empty puparia was 10.25±0.37ng while from each single pharatus was 24.00±1.00ng and 27.27±1.81ng (Table 23).

The DNA amplification with the universal primer LCO/HCO were negative for all the samples while positive results were obtained for sample S1P3 for the amplification of one shorter fragment of COI gene. The local alignment of the 141bp amplified with the primer Oph311FW-Oph311RV result in 98% identity with *H. capensis*, confirming the morphological identification. The result of the PCR might be subjected to the stochastic effect of the unbalance amount of short and long DNA fragments, with the consequence of poor reproducibility result. The high degradation of DNA and the presence of PCR inhibitors, due to the environmental conditions related to decomposition (high rate of bacterial) and to the crypt (cold temperature, high level of humidity), could be an additional explanation for the weak molecular results.

5.3.4.4 ***Conclusion***

The entomological remains sampled from an archaeological context often represent only a portion of the whole necrophagous fauna colonising the cadaver: in fact, some insects move away from the body after their development and others have so fragile structures to not survive the taphonomic processes. However, such insect remains allow a good reconstruction of the diptero-fauna in association with the corpse decomposition since they represent the feeding stage on the body (larval stage) and because they do not have any motility. Based upon their different biology, ecology and ability to reach buried and concealed corpses, Diptera can provide information about the post-mortem events. In the above reported case, as in Vanin (2012), the list of the species associated with the cadaver reveal that the colonization initially occurred in an open, exposed environment, mainly during warm season (as stated by the presence of *P. regina* and *Sarcophaga* spp.). In contrast, the co-presence of *C. vicina* indicates a colonisation occurred in a cooler season as reported by Vanin et al. (2008) and Bugelli et al. (2015). It generally happens either in exposed condition or inside the crypt but, specifically in this case, the arrival of this species into the crypt after the *P. regina* colonisation can be speculated. In support of this, *Calliphora* species have been previously reported from human bodies buried in

underground crypts (Vanin, 2012) and caves (Faucherre *et al.*, 1999). A co-occurrence of the two species in late spring, on the other hand when adults of both species could lay their eggs on exposed bodies, cannot be excluded. In addition, considering that there were additional burials in the surrounding environment, a cross-contamination cannot be excluded.

This research allows the reporting of the presence of *P. regina* from Sardinia. In fact, this species is not included in the list of the Sardinian species derived from modern field samplings (Rognes, 2011). Furthermore no records of *P. regina* in the island are available referring to Sardinian entomological public collections. In contrast, the species is reported from mainland Italy and the other countries in the Mediterranean area (www.faunaeur.org). Several hypotheses can be made in order to explain the lack of records of this species in recent years. Local extension is one of them, but further samplings are needed to provide better insights to the presence/distribution of the species in Sardinia. One of the reasons for this potential "extinction" could be related with the change in the space sharing between people and livestock that occurred in the last century. *Phormia regina*, in fact, was known to cause sheep myiasis in North America and sheep rearing has been an important part of the Sardinian income. In addition, only the morphological identification of the Dolichopodidae specimens can provide a better understanding of their ecological role in the crypt. The identification will also confirm that a species that was present two-three centuries ago in the crypts is still present in the same kind of environments nowadays.

Among the entomological material fragments of fleas (Siphonaptera) were also collected from the mummified corpses. Fleas are well known to be vector of etiological agent of important human diseases. Further molecular studies could confirm the transmission of pathogens from the fleas and potentially identify "Bob & Mary" among the victims of the epidemic plague that affected Sardinia in the XVI-XVII centuries.

Part of this chapter has been published in Giordani *et al.* 2018b, attached at the end of this thesis (Appendix B).

5.3.5 Funerary archaeoentomology on the Peruvian Central Coast: entomological approaches to mummified camelids at the site of Pachacamac during the Late Intermediate Period (AD1100-1450).

5.3.5.1 *Introduction*

Funerary archaeoentomology (as defined by Huchet, 1996) comprises the formalisation of guidelines concerning insect remains in archaeological funerary contexts. Although anecdotal observations on the subject date to 1710 when Tommaso Alghisi described and illustrated a fly puparium associated with an Egyptian mummy (Vallisneri, 1733) (Fig. 1). The discipline has been applied to both human and animal remains in order to elucidate funerary practices in the ancient past (Huchet, 1996, 2014b). Forensic entomology and funerary archaeoentomology are intellectually distinct, but are allied methodologically in their collection and analysis of insect remains from archaeologically- or forensically-derived bodies (Giordani *et al.*, 2018b).

Archaeoentomological approaches provide data not obtainable by any other means, which can be related with archaeothanatology to better understand the evolution of burial contexts. These methods have been extensively used in order to understand burial sequences and funerary practices (Huchet & Greenberg, 2010). While focusing primarily on mummies and other cadaveric remains, archaeoentomological methods have been applied occasionally to offerings made during – or after – burial ceremonies, or even in the absence of funerary remains (Zaidi & Chen, 2011). In the wider field of bio/archaeology, there has been a very strong scientific focus on burial structure, physical anthropology and – particularly – the grave goods associated with interments. However, there has been relatively little work carried out on archaeoentomological materials connected with ancient animal remains. Notable exceptions include the study of an Egyptian dog mummy by Huchet *et al.* (2013) and Otranto *et al.* (2014), and analyses of guinea pig mummies in South America (Dittmar, 2000; Dittmar *et al.*, 2003). In both of these cases, the central emphasis was on parasitological aspects of arthropods associated with animal mummies, including the brown dog tick *Rhipicephalus sanguineus* (Latreille, 1806), the louse fly *Hippobosca longipennis* Fabricius, 1805, and fleas of the genus *Pulex* Linnaeus, 1758. In his work on the dog mummy Huchet *et al.* (2013) also noted the presence of sarcosaprophagous fly puparia belonging to the families Sarcophagidae and Calliphoridae.

Several works have been published on South American mummies and the coastal area represents an important source of findings where Huchet and Greenberg (2010) identified a series of fly puparia in Moche tombs, with various implications for North Coast funerary behaviour. The first study of human funerary archaeoentomology on the Central Coast was carried out at Pachacamac (Peru), where Owens *et al.* (2015) located some puparia of Calliphoridae (Diptera) and some fragments of dermestid beetles (Coleoptera, Dermestidae) in a series of mummified human remains. A number of pseudoscorpions were also recovered from the same mummy series (Morrow *et al.*, 2017), but to my knowledge there are no data concerning archaeoentomological analysis of animal remains in the region. Furthermore, there are no published data from anywhere on the South American continent concerning archaeoentomology of camelid remains. This has to be considered as a surprising omission given both the importance of camelids to ancient Andeans, and archaeoentomology's potential to elucidate the manner in which these evidently valuable resources were exploited and utilised through time and space.

The study, reported in this chapter of the thesis, entails the analysis of camelid mummies from structure B15 at the large multiperiod coastal site of Pachacamac. The study was carried out in order to provide independent evidence for the manner of camelid sacrifice in the Ychsma, with further implications for zooarchaeological/palaeoentomological studies in the Andean area in general. This study is part of a bigger collaboration with the Project Ychsma, directed by Prof. Eeckhout (Université libre de Bruxelles).

- **Geographical Context**

Pachacamac is situated on the Pacific coast some 40km south of modern Lima. It is located directly adjacent to the Lurin River on a dry promontory overlooking the fertile river plain and the coastline (Fig. 80). The site has various pedological and climatic characteristics that promote excellent preservation of even the most fragile organic evidence, such as that discussed here (Eeckhout, 1999).

- **Archaeological and Historical Context**

From South to North, the archaeological site of Pachacamac can be divided into three precincts. The first sector, where the main temples are located, is the Sacred Precinct. The second area includes the pyramids with ramps (elite residences), plazas, streets and other

structures of different sizes and functions, while the last precinct is an almost empty (and possibly residential) area currently covered by sand (Uhle, 1903).

The occupation of the site was estimated to cover from the Early Intermediate Period (EIP) to the Late Horizon (LH) (Table 25) when it was the capital of the local/regional Ychsma polity (Eeckhout, 2013). Pachacamac shares a similar architectural structure with the smaller sites situated around, particularly in the Lurín and neighbouring valleys (Eeckhout, 2003; Lopez-Hurtado Orjeda, 2011).

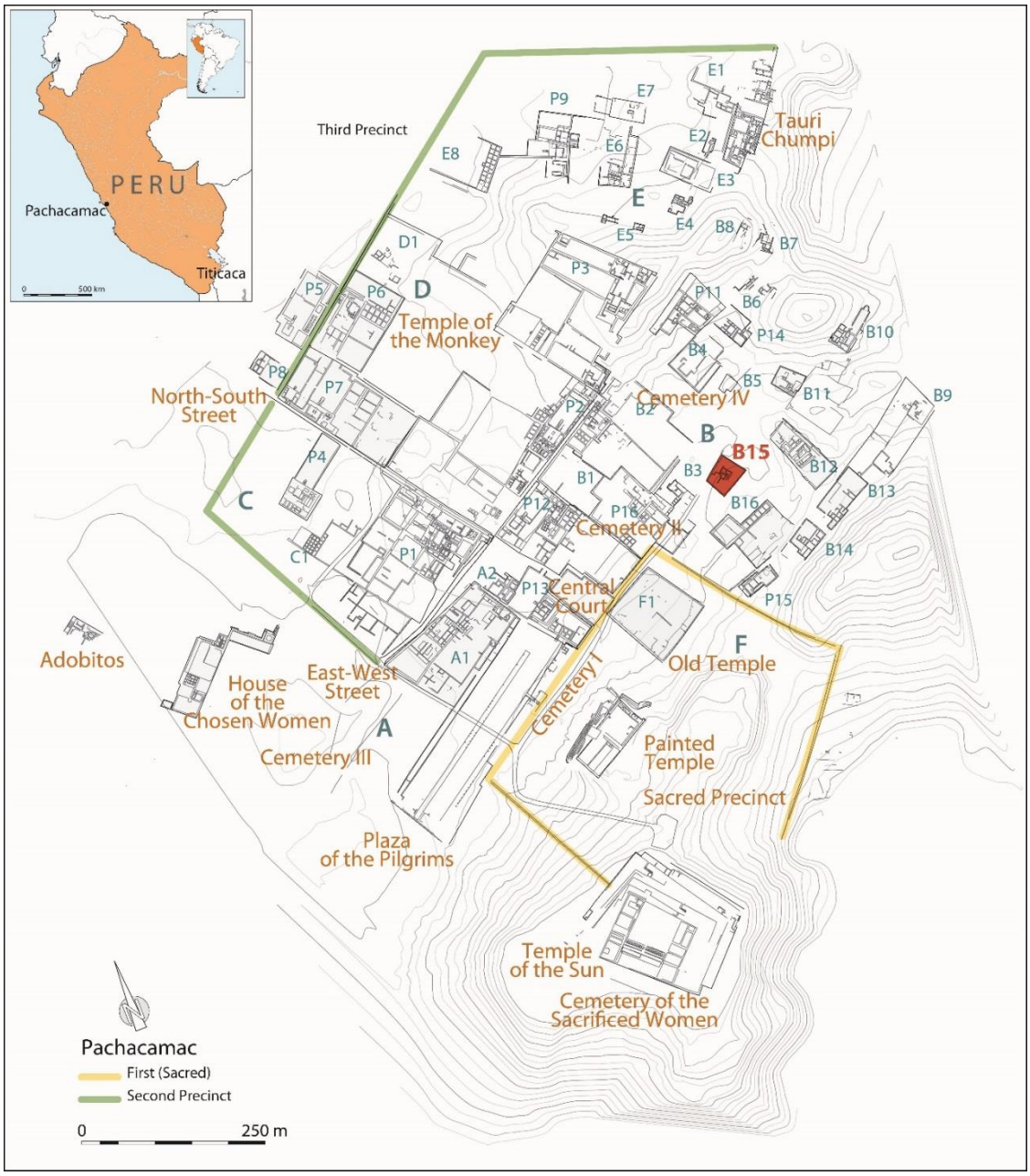

Fig. 80 **Building B15 at Pachacamac** (120 15’ 24” South, 760 54’ 01”West) (courtesy P. Eeckhout).

Table 25 **Cultural sequence at Pachacamac**

Culture	Lima	Wari	Ychsma	Inca
Period	Early Intermediate (EIP)	Middle Horizon (MH)	Late Intermediate (LIP)	Late Horizon (LH)
Approximative dates	BC 200-AD 650	AD 650-1000	AD 1000-1470	AD 1470-1533

- **Archaeological Context: Temple B15**

The camelids were discovered during the excavations of building B15. This temple, roughly trapezoidal in shape, covers an area of approximately 1,400m^2 (35x40m) and is surrounded by a wall. Test excavations were carried out in several units within the complex, exposing around 300m^2 of the central structure (Fig. 81). Several orthogonal rooms and narrow corridors, divided by 1.5m tall walls, made up the building. The presence of specific potholes allows the hypothesis of the original presence of a roof made of organic materials. In contrast to the majority of other buildings at the site, large and with visually impactive structures, this building appears labyrinthine, with reduced internal spaces and extensive polychrome decoration (Eeckhout, 2003; Eeckhout, 2013). Archaeological excavations between 2014 and 2018 have thrown light on occupation sequencing in this complex. This sequence is summarised in Table 26.

Table 26 **Building and occupation sequence of the central area U124 in B15[16].**

Phase	**Description**	**Date AD**
1	Late Lima; low walls and an intrusive child burial	800-1,000
2	Sacred wooden pole and related offerings in Room 2	900-1,000/1,150?
3A	Foundation of Room 4	1,150/1,200
3B	Transformation of Room 4 and stone ancestor cult in Room 2	1,410-1,500
4	Inca remodeling of the entire building	1,500-1,534
5	Major abandonment ceremony during the Transition Period	1,533-1,561
6	Looting and definitive abandonment	1,561- present

The current chapter focus upon the phases 3A and 3B of this sequence. Particular attention was put on the main room of B15's central area. This zone is denoted Unit 124/Room 4.

[16] Information provided by Prof. P. Eeckhout (Université libre de Bruxelles)

Fig. 81 **Sacrificed camelids under excavation in Building B15** (photos courtesy P. Eeckhout).

- **Camelid sacrifice in the Andean area**

Although the most analysed remains from archaeological contexts belong to humans, a long history of sacrifice of camelids, guinea pigs, birds, carnivores and cultural artefacts has been reported by archaeologist from all over the Andean area (Rofes, 2003; Swenson, 2003). Skeletal remains, historical (contact period) sources, ethnohistorical records and iconography can testify the sacrifice. In this context, all the sources of information have

to be combined in order to identify a sacrifice due to the fact that often the sacrificial process does not leave unambiguous traces on the remains. An extensive use of camelid sacrifices has been attested in ritual contexts, both as funerary offerings and foundation offerings (Goepfert, 2011, 2012; Alaica, 2018). As described by Rowe (1946), the animal sacrifices had important social roles and they were selectively performed. Although with some exceptions (Strong & Evans, 1952; Wheeler *et al.*, 1995), the preponderance of camelid sacrifices in Peru was found in association with human burials or in contexts of human sacrifice (Rowe, 1946; Donnan & Foote, 1978; Rodriguez Loredo, 2001; Lozada *et al.*, 2004; Wake, 2007; Goepfert, 2008; Lozada *et al.*, 2009; Prieto *et al.*, 2014; Goepfert & Prieto, 2016; Kent *et al.*, 2016; Dufour *et al.*, 2018). In Pachacamac archaeological site, camelid sacrifices are less frequent compare to other species, such as guinea pigs and dogs. A strong correlation have been outlined between sacrificed camelids and contexts of group burials, although there is at least one record of a camelid being used as a foundation offering (Franco & Paredes, 2001; Eeckhout, 2004).

For many Andean groups, death – usually the human one – could be used to get social, spiritual or divine advantage. Based on that, the display or burial of sacrificial victims is considered a key element (Korpisaari, 2006; Verano, 2014; Eeckhout & Owens, 2015). The use of animals for these purposes have never been investigated but is likely to prove informative about ancient funerary practices. The intention behind this entomological investigation is to use the B15 camelids as a case study to demonstrate the potential of the entomological approaches in clarify the role of camelid sacrificial and depositional habits in the Late Intermediate Period (LIP). Because of the main local deity, called exactly Pachacamac, is a telluric divinity, two possible scenarios can be hypothesized: an immediate burial of the remains after their sacrifice or, in contrast, an exposure of the sacrificial remains at the oracular site to increase social and visual impact.

As this is the first time this approach has ever been attempted in the Andean area, this will form the benchmark for further analyses of similar remains in an effort to determine how the utilisation/sacrifice/deposition sequence differs across time and space, and whether societies can be distinguished on the basis of their sacrificial habits.

The specific aim of this part of the research was to test the possibility of extracting DNA useful for species identification of “archaeological” insects from the very peculiar environment of Pachacamac characterized by high temperature.

5.3.5.2 *Material and Methods*

The materials were located, excavated and recorded in May 2016 by an international team headed by Prof. Eeckhout using standard archaeological procedures, which have been described elsewhere (Eeckhout & Lujan Davila, 2016). Tweezers and paintbrushes were used to manually collect the entomological samples from the camelid remains by the author (Fig. 82) of this thesis and her supervisor Dr. Vanin during the 2016 excavation. The sediments surrounding the animals were also picked through manually, and were then sieved at three resolutions (2mm, 1mm and 355µm) to recover all elements of entomological interest.

Fig. 82 **Working moments on the field in Pachacamac archaeological site**.

The finds were identified and sorted using a stereomicroscope Leica M60. Puparia were treated as described in section 3.2.2. Preliminary treatment with a heated 20% NaOH solution did not improve the clarity of the observations. Coleoptera samples were photographed before cleaning, to prevent the collapse of the specimens.

Identification was performed using specific identification keys and descriptions (Lyneborg, 1970; McAlpine, 1977; Skidmore, 1985; Szpila, 2010; Domínguez & Pont, 2014; Grzywacz *et al.*, 2017a) and by comparison with a reference collection of previously identified modern samples (belonging to Dr. S. Vanin).

Accelerator Mass Spectrometry (AMS) has been done by the archaeologist to date the historical period.

- **Molecular analysis of the entomological samples**

Different molecular extraction strategies have been tested on Coleoptera sample in the family Ptinidae (Anobiidae) (Fig. 83).

Fig. 83 *Tricorynus* sp. Adult in dorsal (A) and ventral (B) view (Scale bar 1mm).

- Six batches (PI1, PI2, PI3, PN1, PN2, PN3) of 30 adult Coleoptera per vial were tested with the QIAamp® DNA Mini kit (4.3.1). Three batches were incubated by immersion in the extraction buffer, the other three were first smashed in liquid nitrogen. The fragility of the specimens prevented the possibility of a cleaning before the extraction. The extracted DNA was eluted in 200µl of nuclease free water.

  Three independent strategies of DNA purification were applied previous to the PCR amplification using: 1) OneStep™ PCR Inhibitor Removal Kit, 2) DNA precipitation with Sodium Acetate and Ethanol (see 4.4 for further information about the techniques) and 3) PrepFiler™ Forensic DNA Extraction Kit following "Repurification protocol" (see 4.3.2). The active carbon filtering and the addition of dichloromethane were used to decolourize the solution. Each purification step has been done on 100µl of extracted DNA.

- Two batches (PI4, PI5) of 30 adult Coleoptera per vial were tested with the PrepFiler™ Forensic DNA Extraction Kit (see 4.3.2 for further information about this technique). Magnetic particles were activated at 37°C for 30 minutes. The extracted DNA was eluted in 50µl of PrepFiler Elution Buffer.
- Two batches (Ptc3, Ptc4) of 30 beetles per vial were tested with the homemade digestion buffer suggested by Gilbert *et al.* (2007) followed by QIAquick PCR Purification Kit® (see 4.3.3). The extracted DNA was eluted in 40µl of EB solution.

- Three batches (P1, P2, P3) of 40 adult Coleoptera (≈0.25g) per vial were tested with the DNeasy® PowerSoil® Kit (see 4.3.4 for further information about this technique). The extracted DNA was eluted in 100µl of C6 solution.
- Two batches (Ptc1, Ptc2) of 10 beetles per vial were tested with the QIAamp® DNA Investigator kit (see section 4.3.5 for further information about this technique). The extracted DNA was eluted in 50µl of ATE solution.

The extracted DNA was quantified via Qubit® 3.0 Fluorimeter (Thermo Scientific, Waltham, Massachusetts, USA) and via Nanodrop 2000c (Thermo Scientific, Waltham, Massachusetts, USA).
The size of the extracted DNA was evaluated with Agilent Bioanalyzer® 2100 as reported in section 4.6.
The mitochondrial cytochrome oxidase c subunit I (COI) gene was used for identification purposes. Amplification of the barcoding region was done using degenerated new designed primers as describe in section 5.2.3 (Table 10).

5.3.5.3 ***Results***

The carcasses of three camelids that were identified and analysed during the excavation of the main room (Unit 124/Room 4) of the Temple B15, originated from two different contexts. Context 29 yielded remains of two juvenile individuals; the estimated age was less than one year for one individual and 1-2 years for the second. The remains, from this context, were incomplete, although the body parts recovered – front leg, ribs and vertebrae – were articulated. Organic preservation was excellent with a large amount of brown hair still covering the bones. Context 30 contained an almost complete camelid skeletonized carcass, lying on its left side with the legs folded inwards. All the bones except for the head, the cervical vertebrae and some bones from the right side, were well preserved and in articulation, along with extensive beige/red hair covering the legs and the left side. The age of the animal at death was estimated between 2 and 3 years. A review of the animal size and proportions allowed to identify it as a "large camelid": *Lama glama*/*Lama guanicoe*. Further observations recognise it as a llama: *Lama glama* (Erauw *et al.*, 2017).

Accelerator Mass Spectrometry (AMS) dating performed on both contexts gave the results reported in Table 27.

Table 27 **AMS dating of samples associated with the camelids identified during the excavation of the Temple B15 in the archaeological area of Pachacamac (Peru)**[17].

Lab Code	Field Code	Sample	Date	Cal. 2 sigma	Arch. Phase	note
RICH-24038	Ych16-Cx29	Assd. plant remains	896±28BP	AD1140-1270 (95,4%)	Phase 3A	
RICH-24044	Ych16-Cx30	Skin & hair	422±30BP	AD1440-1520 (62.0%)	Phase 3B	Cal. 1 most probable
				AD1540-1630 (33.4%)		

The dating evidence suggests that there were two distinct phases in the construction and infilling of B15/Room4. Both events included the deposition of fill and 'special' offerings – including camelids – separated by a period of at least two centuries. The first deposition/sacrifice (Cx29) took place around AD1200 (i.e. when Pachacamac was under the control of the Early-Middle Ychsma polity), while the deposition process that led to the formation of Cx30 took place under late Ychsma or even Inca rule.

- **Entomological findings**

More than one thousand fragments were recovered directly from the camelid carcasses themselves, and hundreds more from sieving the associated sediments. The sample was primarily comprised of insects and scorpions, Coleoptera and Diptera being the most abundant taxa. Summaries of these findings are presented below.

Diptera

The Diptera sample is composed of fragments of puparia. These are particularly taphonomically resistant owing to their chemical and mechanical makeup, and regularly survive in archaeological contexts dating back centuries or millennia (Vanin & Huchet, 2017). The dry conditions at Pachacamac seem to have assisted in this regard.

Taxa were identified and differentiated by observation of the posterior spiracles and the anal plate. This allowed the positive identification of specimens belonging to six different families: Calliphoridae, Sarcophagidae, Muscidae, Fanniidae, Piophilidae and Phoridae (Fig. 84,

[17] (SOUTHERN HEMISPHERE NEMEN\oxcal\shcal13.14COxCal v3.10 Bronk Ramsey (2005); cub r:20 sd:12 prob usp[chron])

Table 28). The most abundant taxon was Phoridae, with thousands of tiny puparia present between the fur and the bones of the two carcasses. Further taxonomic identification (i.e. at the species level) was not attempted on these remains. A single as-yet unidentified puparium fragment was also recovered (Fig. 84H) and is currently under investigation.

Muscidae belonging to *Hydrotaea aenescens* and *Synthesiomyia nudiseta* (van der Wulp, 1883) were recovered from both the carcasses and the surrounding sediments. The highest concentration of the puparia of both these species was found in the soil under and around the carcasses, although specimens were also recovered from both the fur and the skeletal remains.

Only one species of Calliphoridae – identified as *Cochliomyia macellaria* (Fabricius, 1775) – was recovered from both carcasses. Interestingly, while puparia were recovered from the sediments under and around the carcasses, none were found on the fur or the bones. A similar finding was made in the identification of Sarcophagidae remains. Identified only at the genus level as *Sarcophaga*, several specimens were collected from the soil surrounding only one carcass, yet none were found on the remains themselves. Single puparia of Piophilidae (n=1) and Fanniidae (n=1) were collected from a single carcass (
Table 28).

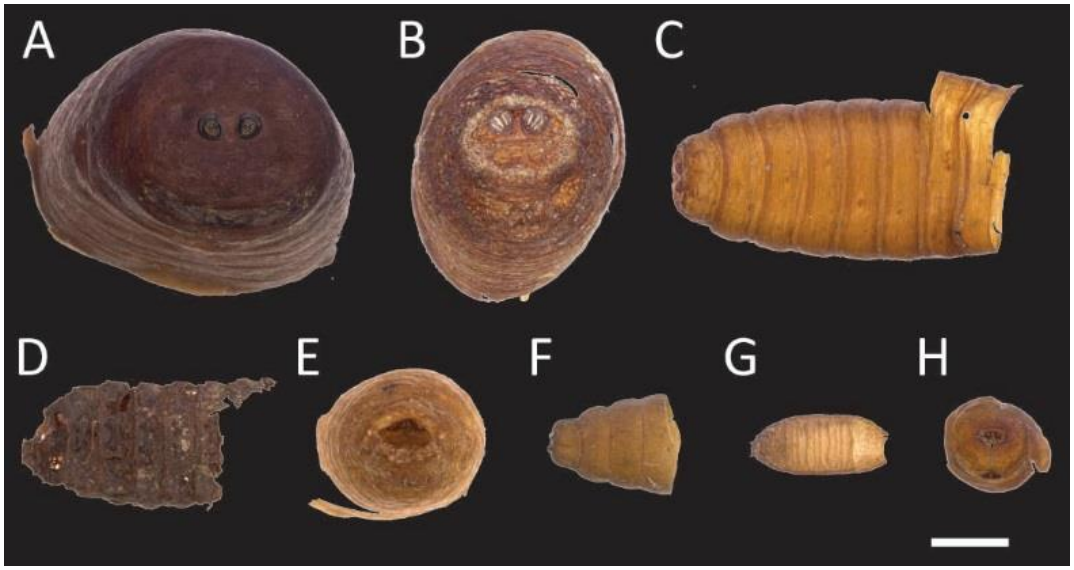

Fig. 84 **Fragments of puparia collected from the camelids excavated at the archaeological site of Pachacamac.** The well-preserved posterior spiracles and anal regions allowed a good species identification. A: *Synthesiomyia nudiseta*; B: *Cochliomyia macellaria*; C: *Hydrotaea aenescens*; D: *Fannia* sp.; E: *Sarcophaga* sp.; F: Piophilidae Gen. sp.; G: Phoridae, Gen. sp.; H: TBC (scale bar: 1mm).

Table 28 **List of Diptera puparia collected from the camelid carcasses**. Fragment count: *=1-10, ** 10-100, ***>100).

		CX29		CX30	
Family	**Species**	**carcass**	**soil**	**carcass**	**soil**
Calliphoridae	*Cochliomyia macellaria*	*	*	*	*
Sarcophagidae	*Sarcophaga* sp.			*	
Muscidae	*Hydrotaea aenescens*	*	**	*	**
Muscidae	*Synthesiomyia nudiseta*	*	*	*	**
Fanniidae	*Fannia* sp.			*	
Phoridae	Gen. sp.	***	**	***	**
Piophilidae	Gen. sp.			*	

Coleoptera

Adult beetles are fairly common in most archaeological contexts, and the material collected in the current case showed excellent preservation. Few complete specimens were found, and most of the sample comprised individual elytra (Fig. 83, Fig. 85, Table 29). Beetle remains were particularly concentrated in the soil surrounding the carcasses. Dermestidae and Tenebrionidae were the most common taxa among the remains collected from the carcasses and the soil. Samples belonging to Dermestidae were identified as belonging to the genus *Dermestes* Linnaeus, 1758. One elytron had a distal spine that permitted the identification of *Dermestes maculatus*, a species and on stored product of animal origin. The sole representative of the Ptinidae[18] – a specimen of *Trigonogenius globulus* Solier, 1849 – was collected from the carcasses. Two elytra belonging to a species in the family Bostrichidae were also recovered. A few fragments of Histeridae and Carabidae adults were also identified among the material.

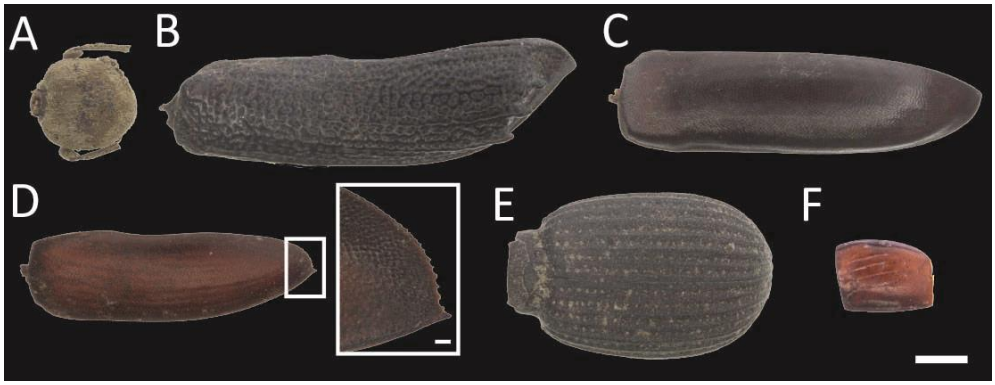

Fig. 85 **Fragments of beetles collected from the camelids at Pachacamac**. A: *Trigonogenius globulus*; B: Bostrichidae Gen. sp.; C: *Dermestes* sp.; D: *Dermestes maculatus*; E: Tenebrionidae Gen. sp; F: Histeridae Gen. sp. (scale bar: 1mm, scale bar detail D= 100μm).

[18] The nomenclature of this family changed a lot in the last years with Ptinidae it is considered here the old Ptinidae+Anobiidae, now Ptininae and Anobinae

Table 29 **List of Coleoptera fragments collected from the camelid carcasses.** Fragment count: *=1-10, ** 10-100, ***>100).

Coleoptera		**CX29**		**CX30**	
Family	**Species**	**carcass**	**soil**	**carcass**	**soil**
Dermestidae	*Dermestes* spp.	*	*	*	**
Ptinidae (=Anobiidae)	*Trigonogenius globulus* Solier, 1849	*	*		*
	Tricorynus sp.		***		
Histeridae				*	*
Tenebrionidae		*	*	*	**
Bostrichidae					*
Carabidae			*		

Scorpiones

Several fragments of scorpion exoskeletons were recovered from the sediments surrounding both the carcasses, but more precise identifications were not possible. These arthropods do not have any relationship with the carcasses *per se* but are very common in the Pachacamac area (Fig. 86).

Fig. 86 **Fragments of scorpions collected from the camelids at Pachacamac.** A: metasoma segment; B: Telson with the characteristic aculeus (scale bar: 1mm).

- **Molecoular analysis**

The DNA extraction solution showed a yellowish/brownish colour, probably due to the presence of insects/soil dyes, in all the samples except for the once tested with the DNeasy® PowerSoil® Kit. Understanding the nature of these pigments, that apparently have the same polarity as DNA (they share the same phases), could help in future DNA extraction protocol development.

The fluorometric quantification via Qubit gave positive results for the majority of the samples. For the extraction with QIAamp® DNA Mini kit, the average DNA extracted was 85.01±0.77ng, with a minimum of 29ng and a maximum of 146.07ng. The simple

immersion of the samples in the buffer yielded a better result (Max: 0.74ng/µl – Min: 0.57ng/µl) in term of extracted DNA compare to the destruction in liquid nitrogen (Max: 0.28ng/µl – Min: 0.14ng/µl). The average DNA extracted with the PrepFiler™ Forensic DNA Extraction Kit was 77.42±1.08ng. DNeasy® PowerSoil® Kit, despite leading to a colourless solution, allow the recovery of only 32.27±7.53ng while the QIAamp® DNA Investigator kit allowed a recovery of approximately 49±9ng.

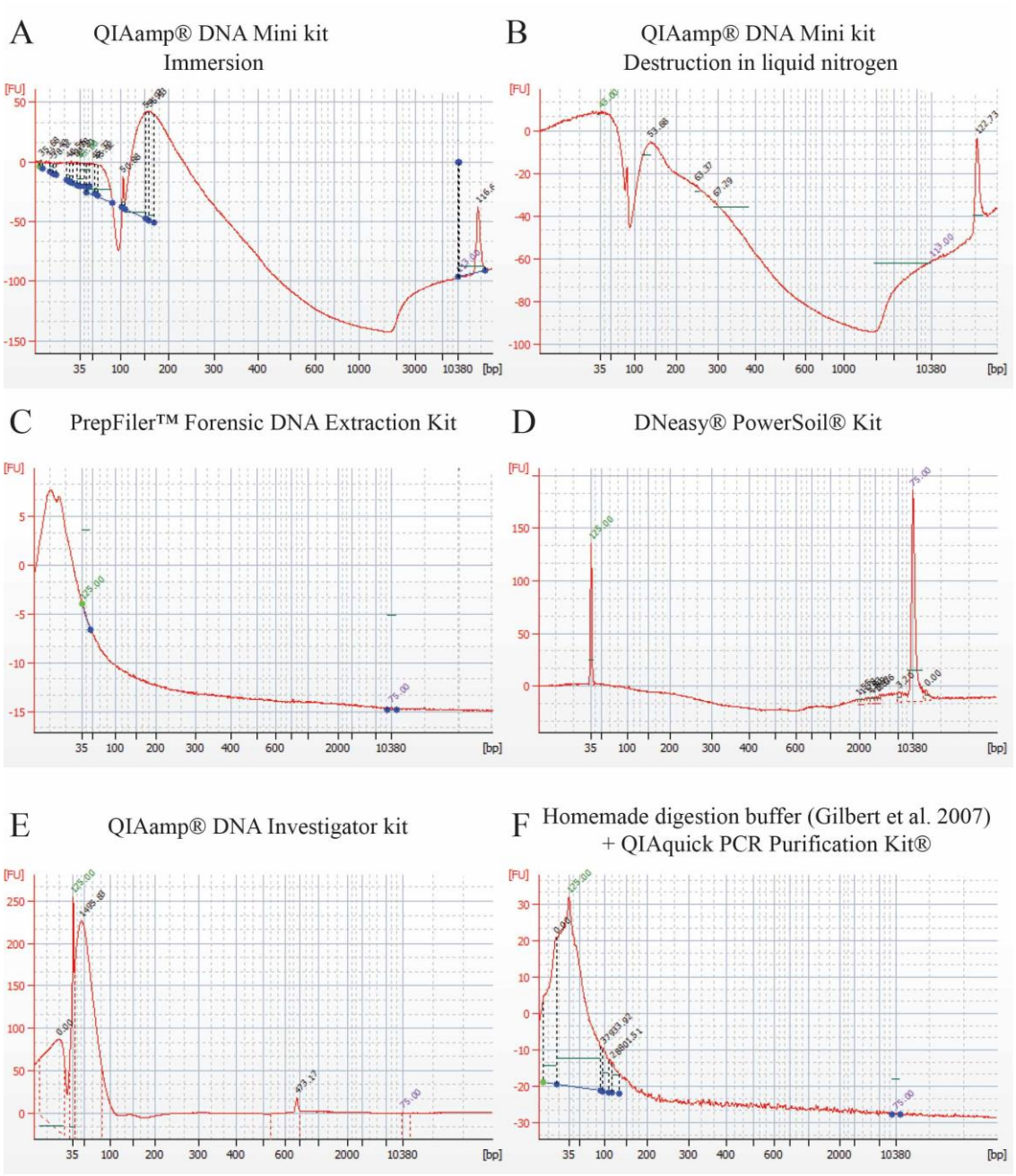

Fig. 87 **DNA extraction fragment sizes**: A) QIAamp® DNA Mini kit, immersion of the sample; B) QIAamp® DNA Mini kit, destruction of the sample; C) PrepFiler™ Forensic DNA Extraction Kit; D) DNeasy® PowerSoil® Kit; E) QIAamp® DNA Investigator kit; F) homemade digestion buffer suggested by Gilbert *et al.* (2007) followed by QIAquick PCR Purification Kit® .

The environment where samples have been collected, the ages of the samples and the presence of pigments/dyes are only some of the reasons that led to the use of a strategy aimed to clean the extracted DNA. Approximately two third of DNA were lost during the process with the easy to use and fast OneStep™ PCR Inhibitor Removal Kit while almost the same reading with Qubit has been obtained with the Sodium Acetate and Ethanol precipitation. No DNA was detected by the Qubit assay after the purification through PrepFiler™ Forensic DNA Extraction Kit.

The quantification with Nanodrop 2000c resulted negative for all the samples.

The pigments contained in the tissues (which were originally brownish) remained in the fraction containing the nucleic acids during the whole procedure. The pellets of DNA resulted coloured at every step, and the final elution maintained the same colour. The purification with powdered activated carbon yielded a colourless solution. Unfortunately, this technique results in a great loss of DNA, too high dealing with archaeological material.

The Bioanalyzer results showed unusual curves (Fig. 87). The presence of dyes in the DNA extracted solution may be the cause of negative values of fluorescence (Fig. 87A,B) and the low hanging of the internal markers (Fig. 87A, B, C, E, F). The quantity of DNA extracted with DNeasy® PowerSoil® Kit seems to be under the lower detectable threshold of the kit (Fig. 87D).

The better results came out with the QIAamp® DNA Investigator kit and the homemade digestion buffer suggested by Gilbert *et al.* (2007) followed by QIAquick PCR Purification Kit®. The average fragments length was 46±11bp confirming the high degradation level of the samples DNA. None of the specimens could be amplified.

5.3.5.4 ***Discussion***

While there are various historical and ethnographic data concerning sacrificial methods, the preservation of these particular remains is imperfect, and it is thus inadvisable to postulate on exactly what happened to the living animals prior to their deposition. While both of the heads were missing, this is insufficient to claim decapitation as a cause of death, as taphonomy and bioturbation may have moved the remains following their interment.

The arthropodo-fauna associated with the carcasses is primarily comprised of sarco-saprophagous insects belonging to Diptera and Coleoptera, and in direct association with

plentiful local species within Coleoptera and Scorpiones. There are at least six species of Diptera, belonging to 5 families that are characterised by both early and late colonisation of decaying bodies. Primary colonisers are represented by Calliphoridae, Sarcophagidae and one Muscidae species.

Diptera remains are comparatively rare archaeologically, especially when compared with the usually high Coleoptera prevalence (Panagiotakopulu, 2004); however, when recovered, they can be informative about past animal usage and disposal methods. The Calliphoridae species *Cochliomyia macellaria* (Huchet & Greenberg, 2010) is known for being a primary coloniser of a body in synanthropic environments, and there is no evidence that this species is able to colonise buried remains.

The camelid data summarised here also suggest that the Calliphorid *Cochliomyia macellaria* was followed by the *Sarcophaga* species, accompanied – or followed shortly after – by the synanthropic *Synthesiomyia nudiseta*. Controversy surrounds this species, which was believed to be a 2nd phase coloniser but is now proposed to be a primary coloniser, especially in indoor environments (Huchet & Greenberg, 2010; Lo Pinto *et al.*, 2017). The other species as *Hydrotaea aenescens*, *Fannia* sp. and the Piophilidae and Phoridae ones are typical of later phases of the colonisation and possibly able to reach buried bodies (Smith, 1986; Disney, 1994; Vanin *et al.*, 2009; Giordani *et al.*, 2018a; Giordani *et al.*, 2018b). In either case, these species were possibly concurrent with *Dermestes* beetles, which are considered to be saprophagous, and colonise bodies when dry fleshes are present (Charabidze *et al.*, 2014).

Based on the previous observations, the entomofauna collected from the two camelids permits the following hypothetical sequential scenario:

A) Initial colonisation of the two carcasses (when still exposed) by *Cochliomyia macellaria*, *Sarcophaga* sp., *Synthesiomyia nudiseta* and *Dermestes* beetles.

B) Secondary burial of the carcasses, later colonised by other species.

However, if the sacrifice and interment took place in a covered environment such as the temple in which they were found – and/or perhaps at a warm time of year – then it is not impossible that all the colonisations took place simultaneously.

The molecular analysis was not able to provide any information. The possibility to extract DNA from archaeoentomological material could help in confirming the species identification, at least at genus level. Despite the Qubit assay gave positive results for the DNA quantification of the extracted samples, the Agilent Bioanalyzer 2100 clearly showed an absence of significant DNA curves for mostly of the samples.

Insect cuticle contains various pigment molecules that provide them the characteristic colouration. The combination of absorbed and dissipate wavelength of light, based on the molecule structures, give the pigment colour. These pigments, anthraquinones, aphins, pterins, tetrapyrroles, ommochromes, melanins, carotenoids and flavonoids may be water or lipid solution soluble (Shamim *et al.*, 2014), some of them are even soluble in organic solvents and in strong acids and bases (Umebachi, 1975; Kayser, 1985; Burghardt *et al.*, 2001; Shamim *et al.*, 2014). Campos and Gilbert (2012) stated that melanin pigments often copurify with the DNA resulting in a brown DNA pellet and a brown solution after resuspension. Despite the presence of other dyes cannot be excluded, the presence of melanin pigments, occurring in Diptera and Coleoptera cuticle, is reliable.

Melanin is a phenolic compound, negatively charged and able to make links with nucleic acid (Manoj *et al.*, 2007; Shamim *et al.*, 2014). This irreversibly interaction results in the inhibition of different downstream reactions (Eckhart *et al.*, 2000; Manoj *et al.*, 2007; Campos & Gilbert, 2012). The addition of over 15µg BSA for 25µl has been demonstrate to effectively revert the inhibition (Giambernardi *et al.*, 1998). The addition of Polyvinylpyrrolidone (PVP) and β-mercaptoethanol is conventionally used in plant sciences to chelate phenolic compounds before their interaction with nucleic acids (Couch & Fritz, 1990; Lodhi *et al.*, 1994; Zidani *et al.*, 2005) but was not tested in this thesis.

The 50bp fragments, recovered with the QIAamp® DNA Investigator kit, did not allow a successful amplification of the COI gene for Sanger sequencing. Based on these results, once the problem of the dyes has been solved, next generation sequencing (NGS) could be a strategy to obtain positive results.

5.3.5.5 ***Conclusion***

The archaeological and chronometric evidence suggests a multiphase deposition process, with two main events that took place at the end of 12th and 15th centuries AD. Each event encompassed the partial construction and destruction of temple B15. Despite the temporal lapse between the two events, they are extraordinarily similar, from an archaeological

point of view, in as far as can be determined from the current evidence. Focusing on the animal sacrifices, three successive stages can be hypothesised:

1) Sacrifice of the animals
2) Exposure of the caracsses or carcass parts for several days
3) Burial of the carcasses in the construction fill

It seems that camelid sacrifice and burial followed – at least in part – the same ritual that it is currently known about human burial practice at Pachacamac. Entomological evidence indicates a certain delay between death and burial, resulting in significant decomposition of the carcasses. It is of course impossible to make any assumption if such a result was the aim of exposure, or only a tolerated consequence of it. In the case of human burials, a mythical scenario involving flying insects metaphorically identified as the "soul" of the deceased was well described and summarized for the Moche (ca. 100-900AD) on the basis of ethno-historical data and entomological findings by Huchet and Greenberg (2010) and suggested as well for the Ychsma by Owens *et al.* (2015). Whether the animals were thus considered remains unclear, although the recovery of such findings allows to make some hypothesis about the role of animals in Ychsma religious pratctices.

There is not a great deal of information concerning this point among other human civilisations/societies in the Andean region, although Alaica (2018) has suggested that the Moche perceived in a different way wild and domestic species in both representation and ceremonial practices.

The observations in this context are in agreement with this statement and with fragmented data concerning wild animals in Ychsma contexts at the site. The role in ritual activities entailed a carefully planification of the architectural structure of Room 4 and the related construction fill in B15 which lasted at least several days, and maybe weeks, as demonstrated by the insect development. A much longer time in comparison to what would be necessitated by a simple filling process. In fact, filling of the excavations of Room 4 by 10 workmen took only 3-4 days, as observed during the field work, and there is no doubt that the Ychsma had more people available to perform this kind of activity (Prof. Eeckhout personal communication).

The previous observations about insect colonisation and the temple building indicate that all the processes had a religious character and that probably it followed specific rules.

The inability to achieve a molecular result from the investigated entomological material does not affect the validity of the reconstructed hypothesis. Physical preservation of the sample does not always correlate with DNA preservation. Further studies will demonstrate if the application of NGS technologies could support researches in species identification.

This study marks a watershed in the development of entomological analyses, for the purpose of understanding the way in which ancient groups utilised, sacrificed and disposed of their animals. It provides an added dimension to established means of ascertaining such behavior in Andean groups and comprises a baseline for comparison with animal remains from other areas and periods of history. These data can also be correlated with information from insect remains collected from human sacrifices and burials at Pachacamac and elsewhere, to develop a holistic view of sacrificial and burial tradition that would not otherwise understand based only on traditional archaeological findings.

5.3.6 Conclusion

Time can affect the preservation of the morphological and molecular characteristics of entomological samples. A perfectly preserved insect does not necessarily correlate with good preserved DNA. From a morphological point of view, the sample can be structurally damaged, missing of portions or simply be dirty, covered by sand or encrusted with decomposing liquid. From a molecular point of view, problems of contamination, DNA damages and PCR inhibitors have to be considered. Despite both the techniques being subject to limitations, the morphological approach is currently considered the most reliable method to reach species identification.

Different archaeological contexts, a crypt and a desert, on a different temporal line, 300 to 1,000 years old, were considered here.

The morphological preservation of the samples was substantial in all the archaeological sites. External cleaning did not affect in any case the diagnostic structures. The molecular analysis gave positive DNA extraction results for 300 years old adult Diptera and negative results for 1,000 years old Coleoptera. Unfortunately, the differences in archaeological contexts, age and insect order did not allowed any kind of comparison. No adult Diptera were collected from the 1,000 years entomological material as not enough Coleoptera were collected from the 300 years old crypt. The comparison of different archaeological

contexts of the same age, as the comparison of the same context on different age could provide a better understanding in the efficiency of the molecular approach for identification from old insect specimens.

This research offered a wide view of the information that archaeo-entomological studies can provide: 1) evidences of biodiversity changing, 2) presence of human diseases vectors and 3) funerary practices reconstruction.

6 Discussion

Insects are the richest animal group existing on Earth, with only 1 million described species out of the 7.2 million estimated (Stork, 2018). The peculiar species-specific distribution and seasonality of insects entail finding them in a wide range of different environments, along the whole year.

Insects are currently used in a range of applied disciplines from biological pest-control to human disease model system. Forensic entomology and funerary archaeoentomology are among them. Despite the relevance of these disciplines being accepted, further studies are necessary in order to overcome the critical step of species identification.

Although the literature regarding the morphology of insects of forensic interest is extensive (Barros de Carvalho & Antunes de Mello-Patiu, 2008; Rochefort *et al.*, 2015; Szpila *et al.*, 2015), numerous species are still poorly investigated. Furthermore, extended attention has been put on adult and larva description while puparia, the most abundant insect remains found in forensic and archaeological contexts, are still an under studied topic, even of the most common species. Despite immature stages sharing some common characteristics, others can differ or not be visible in the puparium.

This thesis shows how the importance of a deep cleaning of the external features is usefull for the identification of the puparia. In fact, in case of soil incrusted samples, indeed, minimal details can be misinterpreted or not be visible (Personal Observation) and performances of molecular methods can be altered because the presence of PCR inhibitors. The only submersion in warm water was not enough to remove all the encrustation. A mechanically force applied with a paintbrush appeared to be optimum even for the cleaning of small puparia as the *P. empirica* and *L. latipes* ones. The combination with sonication allow the removal even of persistent salt crystals, that can be embedded within the puparium spiculation. It is worth mentioning that archaeoentomological samples are more fragile when compared to fresh material. Dirt can act as adhesive, helping to keep the structure together. Sonication can be too strong for samples with cracks. The use of the soap was avoided in puparia marked for DNA extraction in order to avoid alteration of the buffers' efficiency. This interaction has not been addressed yet but cannot been excluded.

In order to make the morphological identification method accessible to non taxonomists, I started the production of digitalized pictures that can, in addition to already available hand-drawings, act as a reference point in identification. This will not undermine the taxonomist figure; the role of whom is far more complicated than the single specimen identification. Close cooperation with taxonomists, who mainly focus on adult identifications, is furthermore essential in order to resolve identification problems of immature stages and the progression of insect sciences. High-resolution microscopes and cameras have been revealed to be necessary to provide good quality colour digital photos while electron scanning microscopy may help dealing with small puparia (few mm long). It is worth mentioning that in last years computer-based keys have been developed for different taxa. Some of them are available online whereas others are available on CD. The use of computer-assisted keys with the possibility to skip an answer where the character is not present or the answer is not clear, can be particular usefull in case of partial/damaged samples overcoming the problem concerning dichotomous keys of following a specific path. The use of morphological characters for identification purposes has been erroneous considered by many researcher to be exclusive to specialized taxonomist (Amendt *et al.*, 2011; Grzywacz *et al.*, 2017a) introducing the idea that only the molecular analysis can bypass this problem, but without considering the problems (e.g.: lack of deposited sequences, incorrectness of some deposited sequences, problems in DNA extraction and amplification) that this thesis underlined. This research further demonstrated that DNA extraction without damaging the morphology of the sample is possible for adults, pupae and puparia, despite some limitations. Many molecular approaches have been developed to help scientists in specimen identification but these methodologies often complete or partially destroied the characters useful for the identification of the specimen. The provision of a non-invasive DNA extraction with the ability to check the morphology of the specimen is essential in all the disciplines dealing with species identification, independently from the context (legal, museum, etc.). The quantity and quality of the DNA extracted appeared to depend on different variables such as the dimension of the sample and the time lapse before the collection of the entomological sample. Physical integrity of the specimen does not directly correlate with DNA integrity. DNA fragment length appeared to be correlated with the extraction method used. The not-equivalent results of both DNA extracted amount and DNA

fragment sizes from comparable samples in term of year of collection and dimension suggest an intrinsic variability among specimens. It is worth to mention that this project evaluates a possible bias in the use of the fluorimetric quantification. The the fluorometric assay with Qubit of DNA extracted from old Coleoptera specimens gave a positive signal where the nanodrop gave a negative result. An artefact signal due to the presence of pigments, with high probability melanin, can be a possible explanation. The insect cuticle pigments that give them the characteristic colouration may enter solution during the lysis step. The presence of pigments in the solution is known to negatively affect downstream reactions (Yoshii *et al.*, 1993; Akane *et al.*, 1994; Bélec *et al.*, 1998; Eckhart *et al.*, 2000). Activated carbon is used to remove pigments and other contaminants during DNA extraction (Thomas, 2008; Barbarić *et al.*, 2015), but it caused DNA loss. The dark colour of Diptera and Coleoptera is due to melanin pigments which are able to absorb wavelengths in all the visible spectrum (Shamim *et al.*, 2014). The irreversible interaction between melanin pigments and nucleic acid (Manoj *et al.*, 2007; Shamim *et al.*, 2014) is reliable and could explain the inability to obtain uncoloured solution with the majority of the DNA extraction techniques. The addition of 16µg of BSA to the 20µl PCR reaction did not increase the efficiency of PCR amplification. The addition of Polyvinylpyrrolidone (PVP) and β-mercaptoethanol can be a further strategy to chelate phenolic compounds before their interaction with nucleic acids (Couch & Fritz, 1990; Lodhi *et al.*, 1994; Zidani *et al.*, 2005). While the presence of pigments/dyes in fresh Tenebrionidae seems not to be a stumbling block, preliminary results on relatively fresh Meloidae species (3 to 10 years old) (in collaboration with University of Rome 3) revealed inhibition. Further studies on insect cuticle composition could allow the development of more suitable DNA extraction commercial kits. Although the presence of pigments is indisputable, a negative PCR result can follow a high level of DNA degradation. DNA is indeed subject to breakage, cross-linkage and modification of nitrogenous bases that make PCR incompetent (Reiss, 2006). The concatenation of amplified small segments (≈200bp) allowed to a large extent to provide good quality results. In order to minimise artefacts, 50bp were considered the average overlapping zone between consequent sequences, with the exclusion of primers. Smaller fragments were not tested. The difficulties in amplification of DNA extracted from fresh empty puparia is not connected to DNA fragment sizes (Fig. 47). The physical dimension of the

analysed sample did not affect the result even if the amount of DNA is undoubtedly a limitation that requires the use of more than one sample per extraction. Further element that may affect DNA extraction and PCR is the chemical composition of the puparium - possibly different among families – that can alter the buffer solutions. Significant amount of Ca and P are present in the cuticle of the puparium of Muscidae (Grodowitz & Broce, 1983) as calcium carbonate crystals ($CaCO_3$) are reported in Stratiomyidae and Xylomyidae puparia (Woodley, 1989). Furthermore, the fact that the puparium is an "excretions storage compartment" (Wigglesworth, 1972) might increase the amount of inhibitors present in/on it. For what concern DNA extraction from adult specimens, killing agents and preservation factors are other variables that can negatively affect DNA integrity or PCR reaction. The killing methods of insects for collection are multiple. The use of ethyl acetate, *Prunus laurocerasus* leaves, pure ethanol and cryotechniques are just examples of the different possibility for specimens preparation. In the museum collection it is often impossible to know which methodology was used to kill the specimen. The killing agents and preservation factors may influence downstream reactions. The impossibility to recover such information from the tested samples, the divergences in used techniques and starting material (families, dimension and number of samples) do not permit a close comparison. Additionally, different compound can co-purify with DNA resulting in the inhibition of the *Taq* DNA polymerase activity. Salts, protease, phenolic compounds and sodium dodecyl sulphate, reagents commonly used to nucleic acids extraction, constitute other PCR inhibitors. Different commercial kits and homemade buffer were tested to minimise the effect of these variables. The use of purification systems did not produce a better result.

Overcame the problem of DNA fragment length and PCR inhibitors, tThe positive amplification of the target sequence does not mean to be able to achieve the correct species identification. This thesis demonstates that the direct identification of many species through the local alignment of sequences via BLASTn® is prevented by omissions in the databases. This work evaluates that the use of sequences longer than the 658bp COI barcoding region reduce the efficiency of the identification. Furthermore, online genbanks have been proved to contain erroneous misidentifications. The use of a phylogenetic approach is indeed highly recommended to confirm the morphological identification. From the analysis and discussion of the results, based on the aims of this

thesis new ideas and problems have been detected. Because of the limited time, they have not been investigated, however in order to provide new ideas for the future they are listed in the chapter 8 “Future works”.

7 Conclusion

This research strongly contributed, with morphological and molecular results, to the identification of immature and mature stages of insects from different contexts - museum, forensic and archaeological - but in the majority of the cases associated with carrion decomposition. The identification of immature stages was achieved by the development of well-illustrated morphological identification keys and descriptions, in particular for the puparia. From a molecular point of view, the environment, the preservation factors and the killing agents appeared to be affect the identification more than sample age. After this study, 37 new sequences were deposited in GenBank (Table 30), a substantial and important improvement in a field were no or just few sequences are available for many species. Additional three sequences will be uploaded when the Dolichopodidae specimens has been identified by an expert.

Table 30 **Sequences belonging to this research deposited on GenBank.**

Family	Species	GenBank code	
Sphaeroceridae	*Phthitia empirica* (Hutton, 1901)	MH118267	MH825673
		MH118268	
Heleomyzidae	*Heleomyza serrata* (Linnaeus, 1758)	MH825674	MH825676
		MH825675	
Piophilidae	*Parapiophila vulgaris* (Fallén, 1820)	MH825677	MH825679
		MH825678	MH921575
Milichiidae	*Leptometopa latipes* (Meigen, 1830)	MH069729	MH921582
		MH825680	
Sarcophagidae	*S. variegate* (Scopoli, 1763)	MH118172	MH118181
		MH118174	MH118185
		MH118175	MH118186
		MH118179	MH118187
		MH118180	
	S. baranoffi Rohdendorf, 1937	MH118173	
	S. carnaria Linnaeus, 1758	MH118176	MH118182
	S. lehmanni Mueller, 1922	MH118178	
	S. croatica Baranov, 1941	MH118183	MH118184
	S. crassipalpis Macquart, 1839	MH118188	MH118189
	S. argyrostoma (Robineau-Desvoidy, 1830)	MH118190	
Muscidae	*H. aenescens* (Wiedemann, 1830)	MH921579	
	H. capensis (Wiedemann, 1818)	MH921578	
	H. ignava (Harris, 1780)	MH921576	
	H. dentipes (Fabricius, 1805)	MH921580	
	H. similis Rothschild, 1909	MH921577	
	H. pilipes Stein, 1903	MH921581	

An additional observation of this thesis is that not only environmental and time factors can affect a molecular identification of the insects. In fact, as discussed, intrinsic factors

like the presence of pigments in the cuticle and extrinsic factors and the method used to kill and preserve the specimens can affect the DNA extractability and quality.

Concluding, in this thesis, for each context highlighted in the aims, a specific answer was obtained:

- **Forensic context:**

0-3 ybp

- Fresh samples of immature stages and adults of poorly investigated Diptera of forensic interest were morphologically described. Molecular analysis were carried out preserving the morphology of the samples. DNA extracted from adults appeared to be of good quality for all the species evaluated. Different puparia species entailed a different DNA usability in PCR reaction.

- **Museum context:**

0-120 ybp

- A no-invasive DNA extraction method was developed on adult *Sarcophaga* spp. flies belonging to museum collection. The method has been demonstrated not to affect the morphology and the chaetotaxi of the specimen.

- **Archaeological context:**

0-1,000 ybp

- Identification problems of puparia of a Muscidae genus of forensic and archaeological interest were resolved by the development of well-illustrated morphological identification keys.

200-400 ybp

- Diptera adult and puparia samples collected from mummies dated around XVII century were molecular characterized. A species, *P. regina*, was reported to be, at least in the past, present in Sardinia (Italy) providing important information on the biodiversity of this region in the past. The presence of several flea species can be used to further investigate the black plague of the XVII century in Sardinia. The collection of some Dolichopodidae could provide interesting ecological information.

1,000 ybp

- Camelid sacrifice was investigated through entomological samples collected from the archaeological site of Pachacamac and supported the hypothesis of a first exposure of the carcasses followed by a secondary interment.
- The extraction of DNA from Coleoptera appeared not to be suitable for amplification and with traditional sequencing (Sanger) but could provide potential results using new sequences approaches.

All the works, related to this topic, published during the PhD have been attached at the end of this thesis (Appendix B, C).

8 Future Works

This thesis, despite providing interesting and useful results pinpointed also some problems that, in my opinion, can be addressed with the following approaches:

- *To provide a better understanding of the influence of sample age on the molecular results*, shorter timeframe have to be considered. In order to reduce the number of variables, samples should belong to the same family and have been killed, prepared and preserved in similar conditions;

- *To provide a better understanding of the influence of killing agents on the molecular results*, a specific study on specimens of the same age and killed with different methodology has to be performed. Because of a combination of the two factors - age and killing agent - cannot be excluded, the study should have a discrete timeframe.

- *To provide a solution for phenolic compounds* (e.g. Melanin) present on the insect exoskeleton that can inhibit PCR amplification, the addition of β-Mercaptoethanol and PVP needs to be tested on fresh and old specimens.

- *To improve the GenBanks databases*, an international project of DNA extraction from fresh specimens of forensic interest could be useful in order to provide the sequences needed for a reliable molecular identification. For this purpose, a collaboration with medical doctors is suggested to facilitate the collection and study of interesting species.

- *To achieve positive molecular results from samples with high degraded DNA*, new sequencing approach need to be carried out.

Future works will be furthermore aimed, with the collaboration of taxonomist specialists, to provide pictorial identification keys for other taxa of forensic interest, with particular consideration to immature stages.

9 References

Aguiar, A. P., Deans, A. R., Engel, M. S., Forshage, M., Huber, J. T., Jennings, J. T., *et al.* (2013) Order Hymenoptera. *Zootaxa,* **3703**, 51-62.

Akane, A., Matsubara, K., Nakamura, H., Takahashi, S. & Kimura, K. (1994) Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. *Journal of Forensic Science,* **39**, 362-372.

Akotsen-Mensah, C. & Philips, T. K. (2009) Description of a new genus of spider beetle (Coleoptera: Ptinidae) from South Africa. *Zootaxa,* **2160**, 51-67.

Alaica, A. K. (2018) Partial and complete deposits and depictions: Social zooarchaeology, iconography and the role of animals in Late Moche Peru. *Journal of Archaeological Science: Reports.* In press. DOI: 10.1016/j.jasrep.2018.02.002.

Alfani, G. (2013) Plague in seventeenth-century Europe and the decline of Italy: an epidemiological hypothesis. *European Review of Economic History*, **17**, 408-430.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403-410.

Amendt, J., Campobasso, C. P., Gaudry, E., Reiter, C., LeBlanc, H. N. & Hall, M. J. (2007) Best practice in forensic entomology-standards and guidelines. *International Journal of Legal Medicine,* **121**, 90-104.

Amendt, J., Krettek, R. & Zehner, R. (2004) Forensic entomology. *Naturwissenschaften,* **91**, 51-65.

Amendt, J., Richards, C. S., Campobasso, C. P., Zehner, R. & Hall, M. J. (2011) Forensic entomology: applications and limitations. *Forensic Science, Medicine and Pathology*, **7**, 379-392.

Amrine Jr, J. W. & Manson, D. C. M. (1996) Preparation, mounting and descriptive study of eriophyoid mites. In *World Crop Pests* (ed. by E. E. Lindquist, M. W. Sabelis & J. Bruin), pp. 383-396. Elsevier, New York.

Anderson, G. (2005) Effects of Arson on forensic entomology evidence. *Canadian Society of Forensic Science Journal* **38**, 49-67.

Andrasko, J. & Rosen, B. (1994) Sensitive identification of hemoglobin in bloodstains from different species by high performance liquid chromatography with combined UV and fluorescence detection. *Journal of Forensic Science*, **39**, 1018-1025.

Anton, E., Niederegger, S. & Beutel, R. G. (2011) Beetles and flies collected on pig carrion in an experimental setting in Thuringia and their forensic implications. *Medical and Veterinary Entomology*, **25**, 353-364.

Antonie, I. & Teodorescu, I. (2009) European and exotic insect pest species in Brukenthal museum (Sibiu county, Romania). *Romanian Journal of Biology*, **54**, 139-149.

Arango, R. A. & Young, D. K. (2012) Death-watch and spider beetles of Wisconsin-Coleoptera: Ptinidae. Forest Products Laboratory, Madison.

Archer, M. S. & Wallman, J. F. (2016) Context effects in forensic entomology and use of sequential unmasking in casework. *Journal of Forensic Sciences*, **61**, 1270-1277.

Ashworth, A. C., Buckland, P. C. & Sadler, J. P. (1997) *Studies in Quaternary Entomology: An Inordinate Fondness for Insects*. Wiley, Chichester.

Audisio, P. (1993) *Coleoptera Nitidulidae-Kateretidae*. Calderini, Bologna.

Avila, F. W. & Goff, M. L. (1998) Arthropod succession patterns onto burnt carrion in two contrasting habitats in the Hawaiian Islands. *Journal of Forensic Science*, **43**, 581-586.

Azad, A. (1990) Epidemiology of murine typhus. *Annual Review of Entomology*, **35**, 553-570.

Azad, A. F., Radulovic, S., Higgins, J. A., Noden, B. & Troyer, J. M. (1997) Flea-borne rickettsioses: Ecologic considerations. *Emerging Infectious Diseases*, **3**, 319.

Azad, A. F. & Traub, R. (1987) Transmission of murine *Typhus rickettsiae* by *Leptopsylla segnis* (Siphonaptera: Leptopsyllidae). *Journal of Medical Entomology*, **24**, 689-693.

Azwandi, A. & Abu Hassan, A. (2009) A preliminary study on the decomposition and dipteran associated with exposed carcasses in an oil palm plantation in Bandar Baharu, Kedah, Malaysia. *Tropical Biomedicine*, **26**, 1-10.

Bajerlein, D., Matuszewski, S. & Konwerski, S. (2011) Insect succession on carrion: Seasonality, habitat preference and residency of histerid beetles (Coleoptera: Histeridae) visiting pig carrion exposed in various forests (Western Poland). *Polish Journal of Ecology*, **59**, 787-797.

Baldacchino, F., Muenworn, V., Desquesnes, M., Desoli, F., Charoenviriyaphap, T. & Duvallet, G. (2013) Transmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): A review. *Parasite*, **20**, 26.

Balitzki-Korte, B., Anslinger, K., Bartsch, C. & Rolf, B. (2005) Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene. *International Journal of Legal Medicine*, **119**, 291-294.

Ball, S. (2015) Introduction to the families of British Diptera. Part 1: Naming of the parts and glossary. Dipterist Forum. http://www.dipteristsforum.org.uk.

Barbarić, L., Bačić, I. & Grubić, Z. (2015) Powdered activated carbon: An alternative approach to genomic DNA purification. *Journal of Forensic Sciences*, **60**, 1012-1015.

Barros de Carvalho, C. J. & Antunes de Mello-Patiu, C. (2008) Key to the adults of the most common forensic species of Diptera in South America. *Revista Brasileira de Entomologia,* **52**, 390-406.

Barták, M. & Roháček, J. c. (2011) Records of interesting flies (Diptera) attracted to meat baited pyramidal trap on sapping stump of European walnut (*Juglans regia*) in Central Bohemia (Czech Republic). *Casopis Slezského Zemského Muzea (A),* **60**, 223–233.

Bataille, M., Crainic, K., Leterreux, M., Durigon, M. & de Mazancourt, P. (1999) Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. *Forensic Science International,* **99**, 165-170.

Baumjohann, K. & Rudzinski, H.-G. (2013) Bemerkenswerte Fliegen-Funde (Diptera) im Rahmen einer forensisch entomologischen Freilandstudie in Nordrhein-Westfalen (Deutschland). *Studia Dipterologica,* **19**, 9-16.

Bélec, L., Authier, J., rôme, Eliezer-Vanerot, M. C., Piédouillet, C., Mohamed, A. S., *et al.* (1998) Myoglobin as a polymerase chain reaction (PCR) inhibitor: A limitation for PCR from skeletal muscle tissue avoided by the use of *Thermus thermophilus* polymerase. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine,* **21**, 1064-1067.

Bellis, C., Ashton, K., Freney, L., Blair, B. & Griffiths, L. R. (2003) A molecular genetic approach for forensic animal species identification. *Forensic Science International,* **134**, 99-108.

Benecke, M. (2001) A brief history of forensic entomology. *Forensic Science International,* **120**, 2-14.

Bereuter, T. L., Lorbeer, E., Reiter, C., Seidler, H. & Unterdorfer, H. (1996) Post-mortem alterations of human lipids — part I: evaluation of adipocere formation and mummification by desiccation. In *Proceedings of the Human Mummies* (ed. by K. Spindler, H. Wilfing, E. Rastbichler-Zissernig, D. zur Nedden & H. Nothdurfter), pp. 265-273. Springer, Vienna.

Berg, M. C. & Benbow, M. E. (2013) Environmental factors associated with *Phormia regina* (Diptera: Calliphoridae) oviposition. *Journal of Medical Entomology,* **50**, 451-457.

Bergeret, L. F. É. (1864) *Infanticide, Momification Naturelle du Cadavre*. Impr. de E. Javel, Arbois.

Betz, O., Irmler, U. & Klimaszewski, J. (2018) *Biology of Rove Beetles (Staphylinidae): Life History, Evolution, Ecology And Distribution*. Springer International Publishing, Berlino.

Bhattacharya, M., Sharma, A. R., Patra, B. C., Sharma, G., Seo, E. M., Nam, J. S., *et al.* (2016) DNA barcoding to fishes: Current status and future directions. *Mitochondrial DNA Part A,* **27**, 2744-2752.

Bianucci, R., Benedictow, O. J., Fornaciari, G. & Giuffra, V. (2013) Quinto Tiberio Angelerio and new measures for controlling plague in 16th-Century Alghero, Sardinia. *Emerging Infectious Diseases,* **19**, 1478-1483.

Bickel, D. & Dyte, C. (1989) Family Dolichopodidae. In *Catalog of Diptera of the Australasian and Oceanian Regions* (ed. by N. L. Evenhuis), pp. 393-418. Bishop Museum, Honolulu.

Biehler, D. D. (2013) *Pests in the City: Flies, Bedbugs, Cockroaches, and Rats*. University of Washington Press, Seattle.

Bitam, I., Dittmar, K., Parola, P., Whiting, M. F. & Raoult, D. (2010) Fleas and flea-borne diseases. *International Journal of Infectious Diseases,* **14**, e667-676.

Bonacci, T., Vercillo, V., Brandmayr, P., Fonti, A., Tersaruolo, C. & Zetto Brandmayr, T. (2009) A case of *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera, Calliphoridae) breeding in a human corpse in Calabria (Southern Italy). *Legal Medicine,* **11**, 30-32.

Bonduriansky, R. & Brooks, R. J. (1999) Why do male antler flies (*Protopiophila litigata*) fight? The role of male combat in the structure of mating aggregations on moose antlers. *Ethology Ecology & Evolution,* **11**, 287-301.

Borowski, J. & Zahradníc, P. (2007) Ptinidae. In *Catalogue of Palaearctic Coleoptera* (ed. by I. Lobl & A. Smetana), pp. 328-362. Apollo Books, Stenstrup.

Borri, L. (1914) Delle indagini sul cadavere e del valore dei fenomeni cadaverici sotto il rispetto medico-legale (tanatologia forense). In *Trattato di Medicina Legale Conforme al Diritto Italiano Costituito*. Villardi, Milano.

Bortolini, S. (2014) Molecular characterisation of Diptera that colonise organic matter of animal origin. University of Modena and Reggio Emilia, Modena. PhD Thesis.

Bouchard, P., Bousquet, Y., Davies, A., Alonso-Zarazaga, M. A., Lawrence, J., Lyal, C. H. C., *et al.* (2011) Family-Group names in Coleoptera (Insecta). *ZooKeys,* **88**, 1-972.

Bourel, B., Hédouin, V., Martin-Bouyer, L., Bécart, A., Tournel, G., Deveaux, M., *et al.* (1999) Effects of morphine in decomposing bodies on the development of *Lucilia sericata* (Diptera: Calliphoridae). *Journal of Forensic Science,* **44**, 354-358.

Bourel, B., Tournel, G., Hedouin, V. & Gosset, D. (2004) Entomofauna of buried bodies in Northern France. *International Journal of Legal Medicine,* **118**, 215-220.

Braack, L. E. O. (1986) Arthropods associated with carcasscs in the Northern Kruger National Park. *South African Journal of Wildlife Research,* **16**, 91-98.

Branicki, W., Kupiec, T. & Pawlowski, R. (2003) Validation of Cytochrome b sequence analysis as a method of species identification. *Journal of Forensic Science,* **48**, 1-5.

Breitmeier, D., Graefe-Kirci, U., Albrecht, K., Weber, M., Tröger, H. D. & Kleemann, W. J. (2005) Evaluation of the correlation between time corpses spent in in-ground graves and findings at exhumation. *Forensic Science International,* **154**, 218-223.

Britt, B. B., Scheetz, R. D. & Dangerfield, A. (2008) A suite of dermestid beetle traces on dinosaur bone from the upper Jurassic Morrison Formation, Wyoming, USA. *Ichnos,* **15**, 59-71.

Brouqui, P. & Raoult, D. (2006) Arthropod-borne diseases in homeless. *Annals of the New York Academy of Sciences,* **1078**, 223-235.

Buckland, P. C., Buckland, P. I. & Panagiotakopulu, E. (2018) Caught in a trap: Landscape and climate implications of the insect fauna from a Roman well in Sherwood Forest. *Archaeological and Anthropological Sciences,* **10**, 125-140.

Buenaventura, E., Whitmore, D. & Pape, T. (2017) Molecular phylogeny of the hyperdiverse genus *Sarcophaga* (Diptera: Sarcophagidae), and comparison between algorithms for identification of rogue taxa. *Cladistics,* **33**, 109-133.

Bugelli, V., Forni, D., Bassi, L. A., Di Paolo, M., Marra, D., Lenzi, S., *et al.* (2015) Forensic entomology and the estimation of the minimum time since death in indoor cases. *Journal of Forensic Science,* **60**, 525-531.

Burghardt, F., Proksch, P. & Fiedler, K. (2001) Flavonoid sequestration by the common blue butterfly *Polyommatus icarus*: Quantitative intraspecific variation in relation to larval hostplant, sex and body size. *Biochemical Systematics and Ecology*, **29**, 875-889.

Byard, R. & Payne-James, J. (2015) *Encyclopedia of Forensic and Legal Medicine*. Academic Press, Cambridge.

Byrd, J. H. & Allen, J. C. (2001) The development of the black blow fly, *Phormia regina* (Meigen). *Forensic Science International,* **120**, 79-88.

Byrd, J. H. & Castner, J. L. (2010) *Forensic Entomology the Utility of Arthropods in Legal Investigations*. CRC Press, Boca Raton.

Campobasso, C. P., Di Vella, G. & Introna, F. (2001) Factors affecting decomposition and Diptera colonization. *Forensic Science International,* **120**, 18-27.

Campobasso, C. P., Gherardi, M., Caligara, M., Sironi, L. & Introna, F. (2004) Drug analysis in blowfly larvae and in human tissues: A comparative study. *International Journal of Legal Medicine,* **118**, 210-214.

Campobasso, C. P. & Introna, F. (2001) The forensic entomologist in the context of the forensic pathologist's role. *Forensic Science International*, **120**, 132-139.

Campobasso, C. P., Linville, J. G., Wells, J. D. & Introna, F. (2005) Forensic genetic analysis of insect gut contents. *The American Journal of Forensic Medicine and Pathology,* **26**, 161-165.

Campobasso, C. P., Marchetti, D., Introna, F. & Colonna, M. F. (2009) Postmortem artifacts made by ants and the effect of ant activity on decompositional rates. *The American Journal of Forensic Medicine and Pathology,* **30**, 84-87.

Campos, P. F. & Gilbert, T. M. (2012) DNA extraction from keratin and chitin. In *Ancient DNA. Methods and Protocols* (ed. by B. Shapiro & M. Hofreiter), pp. 43-49. Springer, Berlin.

Capasso, L. & Di Tota, G. (1998) Lice buried under the ashes of Herculaneum. *The Lancet,* **351**, 992.

Carles-Tolrá, M., Díaz, B. & Saloña, M. (2012) Algunos dípteros necrófilos capturados sobre cadáveres de cerdos en el País Vasco (España) (Insecta: Diptera: Brachycera). *Heteropterus Revista de Entomología,* **12**, 213–222.

Carrott, J. & Kenward, H. (2001) Species associations among insect remains from urban archaeological deposits and their significance in reconstructing the past human environment. *Journal of Archaeological Science,* **28**, 887-905.

Carter, D. O., Yellowlees, D. & Tibbett, M. (2007) Cadaver decomposition in terrestrial ecosystems. *Naturwissenschaften,* **94**, 12-24.

Catts, E. P. & Goff, M. L. (1992) Forensic entomology in criminal investigations. *Annual Review of Entomology,* **37**, 253-272.

Cavalli-Sforza, L. L. & Edwards, A. W. (1967) Phylogenetic analysis: Models and estimation procedures. *Evolution,* **21**, 550-570.

Chandler, P. J. (1998) Checklists of insects of the British Isles. *Handbooks for the Identification of British Insects,* **12**, 1-234.

Charabidze, D., Colard, T., Vincent, B., Pasquerault, T. & Hedouin, V. (2014) Involvement of larder beetles (Coleoptera: Dermestidae) on human cadavers: a review of 81 forensic cases. *International Journal of Legal Medicine,* **128**, 1021-1030.

Charabidze, D., Gosselin, M. & Hedouin, V. (2017) Use of necrophagous insects as evidence of cadaver relocation: Myth or reality? *PeerJ* **5**, e3506.

Charabidze, D., Hedouin, V. & Gosset, D. (2013) Discontinuous foraging behavior of necrophagous *Lucilia sericata* (Meigen 1826) (Diptera Calliphoridae) larvae. *Journal of Insect Physiology,* **59**, 325-331.

Chick, H. & Martin, C. (1911) The fleas common on rats in different parts of the world and the readiness with which they bite man. *Epidemiology & Infection,* **11**, 122-136.

Chillcott, J. G. (2012) A Revision of the Nearctic species of Fanniinae (Diptera: Muscidae). *Memoirs of the Entomological Society of Canada,* **92**, 5-295.

Chinery, M., Manicastri, C. & Infanti, O. (1987) *Guida degli Insetti d'Europa: Atlante Illustrato a Colori*. Franco Muzzio, Padova.

Christian, E. & Spötl, C. (2010) Karst geology and cave fauna of Austria: A concise review. *International Journal of Speleology,* **39**, 71-90.

Cianci, T. J. & Sheldon, J. K. (1990) Endothermic generation by blow fly larvae *Phormia regina* developing in pig carcasses. *Bulletin of the Society for Vector Ecology,* **15**, 33-40.

Cline, A. R., Smith, T. R., Miller, K., Moulton, M., Whiting, M. & Audisio, P. (2014) Molecular phylogeny of Nitidulidae: assessment of subfamilial and tribal classification and formalization of the family Cybocephalidae (Coleoptera: Cucujoidea). *Systematic Entomology,* **39**, 758-772.

Comstock, J. H. & Needham, J. G. (1899) The wings of insects. *The American Naturalist,* **33**, 845-860.

Convey, P., Key, R. S. & Key, R. J. D. (2010) The establishment of a new ecological guild of pollinating insects on sub-Antarctic South Georgia. *Antarctic Science*, **22**, 508-512.

Coope, G. (1978) Constancy of insect species versus inconstancy of Quaternary environments. In *Diversity of Insect Faunas* (ed. by L. A. Mound & N. Waloff), pp. 176-187. Blackwell Scientific Publications London, Oxford.

Coope, G. R., Shotton, F. W. & Strachan, I. (1961) A late Pleistocene fauna and flora from Upton Warren, Worcestershire. *Philosophical Transactions of the Royal Society B*, **244**, 379-421.

Cortinhas, L. B., Mendonca, P. M., Barbosa, R. R. & Queiroz, M. M. (2016) Ultrastructure of immature stages of the black dump fly: *Ophyra aenescens* (Wiedemann, 1830) (Diptera: Muscidae: Azeliinae). *Acta Tropica*, **158**, 125-129.

Couch, J. A. & Fritz, P. J. (1990) Isolation of DNA from plants high in polyphenolics. *Plant Molecular Biology Reporter*, **8**, 8-12.

Coulson, R. M., Curtis, C. F., Ready, P. D., Hill, N. & Smith, D. F. (1990) Amplification and analysis of human DNA present in mosquito bloodmeals. *Medical and Veterinary Entomology*, **4**, 357-366.

Couri, M. S. & Araújo, P. F. (1990) Contribuição ao conhecimento das fases imaturas de *Ophyra aenescens* (Wiedemann, 1830) e *Ophyra chalcogaster* (Wiedemann, 1824) (Diptera-Muscidae). *Revista Brasileira de Zoologia*, **7**, 525-529.

Couri, M. S., Cunha, A. M., Souza, S. M. F. M. d. & Laeta, M. (2009) *Ophyra capensis* (Wiedemann) (Diptera, Muscidae) found inside the esophagus of a mummy in Lisbon (Portugal). *Papéis Avulsos de Zoologia (São Paulo)*, **49**, 87-91.

Couri, M. S., Souza, S. M. d., Cunha, A. M., Pinheiro, J. & Cunha, E. (2008) Diptera Brachycera found inside the esophagus of a mummified adult male from the early XIX century, Lisbon, Portugal. *Memorias do Instituto Oswaldo Cruz*, **103**, 211-213.

Courtney, G. W., Pape, T., Skevington, J. H. & Sinclair, B. J. (2009) Chapter 9. Biodiversity of Diptera. In *Insect Biodiversity: Science and Society, 1st edition* (ed. by R. Foottit & P. Adler), pp. 185-222. Blackwell Publishing, Hoboken.

Czesny, S., Dabrowski, K., Christensen, J. E., Van Eenennaam, J. & Doroshov, S. (2000) Discrimination of wild and domestic origin of sturgeon ova based on lipids and fatty acid analysis. *Aquaculture*, **189**, 145-153.

Davies, L. (1990) Species composition and larval habitats of blowfly (Calliphoridae) populations in upland areas in England and Wales. *Medical and Veterinary Entomology*, **4**, 61-68.

Day, D. M. & Wallman, J. F. (2008) Effect of preservative solutions on preservation of *Calliphora augur* and *Lucilia cuprina* larvae (Diptera: Calliphoridae) with implications for post-mortem interval estimates. *Forensic Science International*, **179**, 1-10.

De Carvalho, C., Couri, M., Pont, A., Pamplona, D. & Lopes, S. (2005) A catalogue of the Muscidae (Diptera) of the Neotropical Region. *Zootaxa*, **860**, 1-282.

de Jong, Y., Verbeek, M., Michelsen, V., de Place Bjørn, P., Los, W., Steeman, F., *et al.* (2014) Fauna Europaea - all European animal species on the web. *Biodiversity Data Journal,* **2**, e4034.

de Lourdes Chávez-Briones, M., Hernández-Cortés, R., Díaz-Torres, P., Niderhauser-García, A., Ancer-Rodríguez, J., Jaramillo-Rangel, G., *et al.* (2013) Identification of human remains by DNA analysis of the gastrointestinal contents of fly larvae. *Journal of Forensic Sciences,* **58**, 248-250.

Dekeirsschieter, J., Verheggen, F., Lognay, G. & Haubruge, E. (2011) Large carrion beetles (Coleoptera, Silphidae) in Western Europe: A review. *Biotechnologie, Agronomie, Société et Environnement,* **15**, 425-437.

Dent, B. B., Forbes, S. L. & Stuart, B. H. (2004) Review of human decomposition processes in soil. *Environmental Geology,* **45**, 576-585.

Deonier, C. C. (1942) Seasonal abundance and distribution of certain blowflies in Southern Arizona and their economic importance. *Journal of Economic Entomology,* **35**, 65-70 pp.

Derraik, J. G., Heath, A. C. & Rademaker, M. (2010) Human myiasis in New Zealand: Imported and indigenously-acquired cases: The species of concern and clinical aspects. *New Zealand Medical Journal,* **123**, 21-38.

Díaz-Aranda, L. M., Martín-Vega, D., Gómez-Gómez, A., Cifrián, B. & Baz, A. (2018) Annual variation in decomposition and insect succession at a periurban area of central Iberian Peninsula. *Journal of Forensic and Legal Medicine,* **56**, 21-31.

Díaz-Martín, B. & Saloña-Bordas, M. I. (2015) Arthropods of forensic interest associated to pig carcasses in Aiako Harria Natural Park (Basque Country, Northern Spain). *Ciencia Forense,* **12**, 207-228.

Ding, Y., Li, X., Guo, Y., Duan, W., Ling, J., Zha, L., *et al.* (2017) Estimation of postmortem interval by vitreous potassium evaluation with a novel fluorescence aptasensor. *Scientific Reports,* **7**, 1868.

Dirrigl, F. J. & Greenberg, B. (1995) The utility of insect remains to assessing human burials: A connecticut case study. *Archaeology of Eastern North America,* **23**, 1-7.

Disney, R. H. L. (1994) *Scuttle Flies: The Phoridae*. Chapman & Hall, London.

Dittmar, K. (2000) Evaluation of ectoparasites on the guinea pig mummies of El Yaral and Moquegua Valley, in Southern Peru. *Chungara, Revista de Antropología Chilena,* **32**, 123-125.

Dittmar, K., Mamat, U., Whiting, M., Goldmann, T., Reinhard, K. & Guillen, S. (2003) Techniques of DNA-studies on prehispanic ectoparasites (*Pulex* sp., Pulicidae, Siphonaptera) from animal mummies of the Chiribaya Culture, Southern Peru. *Memorias do Instituto Oswaldo Cruz,* **98**, 53-58.

Domínguez, M. C. & Pont, A. C. (2014) Fanniidae (Insecta: Diptera). *Fauna of New Zealand,* **71**, 1-91.

Donnan, C. B. & Foote, L. J. (1978) Appendix 2. Child and llama burials from Huanchaco. In *Ancient Burial Patterns of the Moche Valley, Peru* (ed. by C. B. Donnan & C. Mackey), pp. 399-408. University of Texas Press, Austin.

Duda, O. (1924) Revision der europäischen u. grönländischen sowie einiger südostasiat. *Arten der Gattung Piophila Fallén (Dipteren). Konowia,* **3**, 97-113.

Dufour, E., Goepfert, N., Le Neün, M., Prieto, G. & Verano, J. W. (2018) Life history and origin of the camelids provisioning a mass killing sacrifice during the Chimú Period: Insight from stable isotopes. *Environmental Archaeology*, 1-14.

Dupont, F. Y. F., Champlain D.L., Cyrille A.A.J. & Félix B.B.C. (2011) A preliminary study of arthropod associated with carrion in Yaounde, Cameroon: A first step in forensic entomology in Central Africa. *Journal of Ecology and the Natural Environment* **3(6)**, 215-220.

Eck, R. V. & Dayhoff, M. O. (1966) Atlas of protein sequence and structure. *Systematic Biology,* **16**, 262–263.

Eckhart, L., Bach, J., Ban, J. & Tschachler, E. (2000) Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. *Biochemical and Biophysical Research Communications,* **271**, 726-730.

Eeckhout, P. (1999) Pachacamac durant l'Intermédiaire récent: Étude d'un site monumental préhispanique de la Côte centrale du Pérou. *British Archaeological Reports International Series,* **747**.

Eeckhout, P. (2003) Ancient monuments and patterns of power at Pachacamac, central coast of Peru. *Beiträge zur Allgemeinen und Vergleichenden Archäologie,* **23**, 139-182.

Eeckhout, P. (2004) Relatos míticos y prácticas rituales en Pachacamac. *Bulletin de l'Institut Français d'Études Andines,* **33**, 1-54.

Eeckhout, P. (2013) Change and permanency on the coast of ancient Peru: The religious site of Pachacamac. *World Archaeology,* **45**, 137-160.

Eeckhout, P. & Lujan Davila, M. (2016) Proyecto Ychsma. Investigaciones arqueológicas en el sitio de Pachacamac, undecima temporada. Unofficial report to the Ministry of Culture of Peru, Lima.

Eeckhout, P. & Owens, L. S. (2015) The impossibility of death: Introduction to funerary practices and models in the ancient Andes. In *Funerary Practices and Models in the Ancient Andes: The Return of the Living Dead* (ed. by P. Eeckhout & L. S. Owens), pp. 1-11. University Press, Cambridge.

Efron, B. (1982) *The jackknife, the bootstrap, and other resampling plans*. Society for Industrial and Applied Mathematics, Philadelphia.

El-Sherbini, G. T. & Gneidy, M. R. (2012) Cockroaches and flies in mechanical transmission of medical important parasites in Khaldyia Village, El-Fayoum, Governorate, Egypt. *Journal of the Egyptian Society of Parasitology,* **42**, 165-174.

Emden, F. I. v. (1965) *Diptera: Muscidae*. Zoological Survey of India, Calcutta.

Erauw, C., Pigière, F. & Eeckhout, P. (2017) Zooarchaeological analysis of faunal remains from the ritual site of Pachacamac: First insights into the sacrifice of camelids. In *Proceedings of the 4th Young Natural History Scientists Meeting. Paris*.

Eskey, C., Prince, F. M. & Fuller, F. B. (1949) Transmission of *Salmonella enteritidis* by the rat fleas *Xenopsylla cheopis* and *Nosopsyllus fasciatus*. *Public health reports (Washington, DC: 1896),* **64**, 933-941.

Espinoza, E. O., Lindley, N. C., Gordon, K. M., Ekhoff, J. A. & Kirms, M. A. (1999) Electrospray ionization mass spectrometric analysis of blood for differentiation of species. *Analytical Biochemistry,* **268**, 252-261.

Esser, J. R. (1988) Assessment and reduction of insect infestation of cured fish in South East Asia, with laboratory studies on *Chrysomya raegacephala* (Fab.), a principal causative agent. Durham University, Durham. PhD Thesis.

Ewing, H. E. (1924) Lice from human mummies. *Science,* **60**, 389-390.

Farkas, R. & Papp, L. (1989) Species composition and breeding sites of fly communities (Diptera) in caged-layer houses in Hungary. *Parasitologia Hungarica,* **22**, 93-97.

Faucherre, J., Cherix, D. & Wyss, C. (1999) Behavior of *Calliphora vicina* (Diptera, Calliphoridae) under extreme conditions. *Journal of Insect Behavior,* **12**, 687-690.

Felsenstein, J. (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution,* **17**, 368-376.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783-791.

Fenoglio, S., Bo, T., Cammarata, M., Malacarne, G. & Del Frate, G. (2010) Contribution of macro-and micro-consumers to the decomposition of fish carcasses in low-order streams: An experimental study. *Hydrobiologia*, **637**, 219-228.

Fiedler, A., Halbach, M., Sinclair, B. & M., B. (2008) What is the edge of a forest? A diversity analysis of adult Diptera found on decomposing piglets inside and on the edge of a Western german woodland inspired by a courtroom question. *Entomologie Heute,* **20**, 173-191.

Fiedler, S. & Graw, M. (2003) Decomposition of buried corpses, with special reference to the formation of adipocere. *Naturwissenschaften,* **90**, 291-300.

Fitch, W. M. (1971) Toward defining the course of evolution: Minimum change for a specific tree topology. *Systematic Biology*, **20**, 406-416.

Fitch, W. M. & Margoliash, E. (1967) A method for estimating the number of invariant amino acid coding positions in a gene using cytochrome c as a model case. *Biochemical Genetics,* **1**, 65-71.

Fitch, W. M. & Markowitz, E. (1970) An improved method for determining codon variability in a gene and its application to the rate of fixation of mutations in evolution. *Biochemical Genetics,* **4**, 579-593.

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.

Franco, R. & Paredes, P. (2001) El templo viejo de Pachacamac: Nuevos aportes al estudio del Horizonte Medio. *Boletín de Arqueología PUCP,* **4**, 607-630.

Garnett, W. & Foote, B. (1966) Notes on the biology of certain heleomyzid flies of eastern North America (Diptera: Heleomyzidae). *Journal of the Kansas Entomological Society,* **39**, 552-555.

Gaudio, D., Betto, A., Vanin, S., De Guio, A., Galassi, A. & Cattaneo, C. (2015) Excavation and study of skeletal remains from a World War I mass grave. *International Journal of Osteoarchaeology,* **25**, 585-592.

Gaudry, E. (2002) Eight squadrons for one target: The fauna of cadaver described by P. Mégnin. In *Proceedings of the First European Forensic Entomology*, pp. 23.

Gaudry, E., Dourel, L., Chauvet, B., Vincent, B. & Pasquerault, T. (2007) L'entomologie légale lorsque insecte rime avec indice. *Revue Francophone des Laboratoires,* **2007**, 23-32.

Gauld, I. D., Bolton, B., Huddleston, T., Fitton, M., Shaw, M. R., Noyes, J., *et al.* (1988) *The Hymenoptera*. Oxford University Press, Oxford.

Gennard, D. (2007) *Forensic Entomology: An introduction*. Wiley, Chichester.

Gerstmeier, R. (1998) *Checkered beetles illustrated key to the Cleridae and Thanerocleridae of the Western Palaearctic illustrierter Schlüssel zu den Cleridae und Thanerocleridae der West-Paläarktis*. Margraf Verlog, Weikersheim.

Giambernardi, T. A., Rodeck, U. & Klebe, R. J. (1998) Bovine serum albumin reverses inhibition of RT-PCR by melanin. *BioTechniques,* **25**, 564-566.

GilArriortua, M., Salona-Bordas, M. I., Caine, L. M., Pinheiro, F. & M, M. d. P. (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.

Gilbert, B. M. & Bass, W. M. (1967) Seasonal dating of burials from the presence of fly pupae. *American Antiquity,* **32**, 534-535.

Gilbert, M. T., Moore, W., Melchior, L. & Worobey, M. (2007) DNA extraction from dry museum beetles without conferring external morphological damage. *PLoS One,* **2**, e272.

Gill, G. D. (1962) The heleomyzid files of America north of Mexico (Diptera: Heleomyzidae). *Proceedings of the United States National Museum,* **113**, 495-603.

Giordani, G., Grzywacz, A. & Vanin, S. (2018a) Characterization and identification of puparia of *Hydrotaea* Robineau-Desvoidy, 1830 (Diptera: Muscidae) from forensic and archaeological contexts. *Journal of Medical Entomology.* In press. DOI: 10.1093/jme/tjy142.

Giordani, G., Tuccia, F., Floris, I. & Vanin, S. (2018b) First record of *Phormia regina* (Meigen, 1826) (Diptera: Calliphoridae) from mummies at the Sant'Antonio Abate Cathedral of Castelsardo, Sardinia, Italy. *PeerJ,* **6**, e4176.

Giordani, G., Tuccia, F., Zoppis, S., Vecchiotti, C. & Vanin, S. (2018c) Record of *Leptometopa latipes* (Diptera: Milichiidae) from a human cadaver in the Mediterranean area. *Forensic Sciences Research.* In press. DOI: 10.1080/20961790.2018.1490473.

Goddard, J. & Lago, P. K. (1985) Notes on blow fly (diptera: Calliphoridae) succession on carrion in Northern Mississippi. *Journal of Entomological Science,* **20**, 312-317.

Goepfert, N. (2008) Ofrendas y sacrificio de animales en la cultura Mochica: El ejemplo de la Plataforma Uhle, complejo arqueológico Huacas del Sol y de la Luna. In *Moche Archaeology: New Approaches* (ed. by L. J. Castillo, H. Bernier, G. Lockard & J. Rucabado), pp. 231-244. IFEA, PUCP, Lima.

Goepfert, N. (2011) Frayer la route d'un monde inversé. Sacrifice et offrandes animales dans la culture Mochica (100–800 apr. J.-C.), cote nord du Pérou. *British Archaeological Reports International Series*, **2278**, 244-248.

Goepfert, N. (2012) New zooarchaeological and funerary perspectives on Mochica culture (AD 100–800), Peru. *Journal of Field Archaeology,* **37**, 104-120.

Goepfert, N. & Prieto, G. (2016) Offering llamas to the sea: The economic and ideological importance of camelids in the Chimú society, North Coast of Peru. In *The Archaeology of Andean Pastoralism* (ed. by J. M. Capriles & N. Tripcevich), pp. 197-210. University of New Mexico Press, Albuquerque.

Goff, M. L., A. Brown, W., A. Hewadikaram, K. & I. Omori, A. (1991) Effect of heroin in decomposing tissues on the development rate of *Boettcherisca peregrina* (Diptera, Sarcophagidae) and implications of this effect on estimation of postmortem intervals using arthropod development patterns. *Journal of Forensic Sciences,* **36**, 537-542.

Goff, M. L., I Omori, A. & R Goodbrod, J. (1989) Effect of cocaine in tissues on the development rate of *Boettcherisca peregrina* (Diptera: Sarcophagidae). *Journal of Medical Entomology*, **26**, 91-93.

Goff, M. L., L. Miller, M., D. Paulson, J., D. Lord, W., Richards, E. & I. Omori, A. (1997) Effects of 3,4-Methylenedioxymethamphetamine in decomposing tissues on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and detection of the drug in postmortem blood, liver tissue, larvae, and puparia. *Journal of Forensic Sciences,* **42**, 276-280.

Goff, M. L. & Lord, W. D. (1994) Entomotoxicology. A new area for forensic investigation. *American Journal of Forensic Medicine and Pathology,* **15**, 51-57.

Gomes, L., Gomes, G., Oliveira, H. G., Morlin Junior, J. J., Desuo, I. C., Queiroz, M. M. C., *et al.* (2007) Occurrence of Hymenoptera on *Sus scrofa* carcasses during summer and winter seasons in southeastern Brazil. *Revista Brasileira de Entomologia,* **51**, 394-396.

Gorodkov, K. (1962) Revision of the Palaearctic species of the genus Leria R.-D.(Diptera, Helomyzidae). *Entomologicheskoe Obozrenie,* **41**, 643-671.

Gorodkov, K. B. (1984) Family Heleomyzidae. In *Catalogue of Palaearctic Diptera* (ed. by S. A. P. L.), pp. 15-45. Elsevier Science, Amsterdam.

Goto, S., Anbutsu, H. & Fukatsu, T. (2006) Asymmetrical interactions between Wolbachia and Spiroplasma endosymbionts coexisting in the same insect host. *Applied and Environmental Microbiology,* **72**, 4805-4810.

Grassberger, M. & Frank, C. (2003) Temperature-related development of the parasitoid wasp *Nasonia vitripennis* as forensic indicator. *Medical and Veterinary Entomology,* **17**, 257-262.

Greenberg, B. (1971) *Flies and Disease: Ecology, classification, and biotic associations*. Princeton University Press, New Jersey.

Greenberg, B. (1985) Forensic entomology: Case studies. *Bulletin of the Entomological Society of America,* **31**, 25-28.

Greenberg, B. (1991) Flies as forensic indicators. *Journal of Medical Entomology,* **28**, 565-577.

Greenberg, B. & Kunich, J. C. (2005) *Entomology and the law: Flies as forensic indicators*. Cambridge University Press, New York.

Gregor, F., Rozkošný, R., Barták, M. & Vaňhara, J. (2002) *The Muscidae (Diptera) of Central Europe.* Masaryk University, Brno.

Grodowitz, M. J. & Broce, A. B. (1983) Calcium storage in face fly (Diptera: Muscidae) larvae for puparium formation. *Annals of the Entomological Society of America,* **76**, 418-424.

Grzywacz, A. (2013) Third instar larva morphology of *Hydrotaea cyrtoneurina* (Zetterstedt, 1845) (Diptera: Muscidae) – a species of forensic interest. *Polish Journal of Entomology,* **82**, 303-315.

Grzywacz, A., Hall, M. J., Pape, T. & Szpila, K. (2017a) Muscidae (Diptera) of forensic importance-an identification key to third instar larvae of the Western Palaearctic region and a catalogue of the muscid carrion community. *International Journal of Legal Medicine,* **131**, 855-866.

Grzywacz, A., Lindstrom, A. & Hall, M. J. (2014) *Hydrotaea similis* Meade (Diptera: Muscidae) newly reported from a human cadaver: a case report and larval morphology. *Forensic Science International,* **242**, e34-43.

Grzywacz, A., Ogiela, J. & Tofilski, A. (2017b) Identification of Muscidae (Diptera) of medico-legal importance by means of wing measurements. *Parasitology Research,* **116**, 1495-1504.

Grzywacz, A., Wallman, J. F. & Piwczyński, M. (2017c) To be or not to be a valid genus: the systematic position of *Ophyra* R.-D. revised (Diptera: Muscidae). *Systematic Entomology*, **42**, 714-723.

Gullan, P. J. & Cranston, P. S. (2014) *The Insects: An Outline of Entomology*. John Wiley & Sons, Hoboken.

Gunn, A. & Bird, J. (2011) The ability of the blowflies *Calliphora vomitoria* (Linnaeus), *Calliphora vicina* (Rob-Desvoidy) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and the muscid flies *Muscina stabulans* (Fallen) and *Muscina prolapsa* (Harris) (Diptera: Muscidae) to colonise buried remains. *Forensic Science International*, **207**, 198-204.

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.

Hågvar, S. & Greve, L. (2003) Winter active flies (Diptera, Brachycera) recorded on snow-a long-term study in south Norway. *Studia Dipterologica*, **10**, 401-421.

Hall, D. G. (1948) *The Blowflies of North America*. Thomas Say Foundation, Baltimore.

Hall, M. J. & Smith, K. G. (1993) Diptera causing myiasis in man. In *Medical insects and arachnids* (ed. by R. P. Lane & R. W. Crosskey), pp. 429-469. Springer, Dordrecht.

Harrington, R., Fleming, R. A. & Woiwod, I. P. (2001) Climate change impacts on insect management and conservation in temperate regions: can they be predicted? *Agricultural and Forest Entomology*, **3**, 233-240.

Harrington, R., Woiwod, I. & Sparks, T. (1999) Climate change and trophic interactions. *Trends in Ecology & Evolution*, **14**, 146-150.

Hart, A. J., Hall, M. J. R. & Whitaker, A. P. (2011) The use of forensic entomology to assist the Criminal Justice System. In *The Barrister Magazine*. Media Management Corporation Ltd, London.

Harvey, M. L., Dadour, I. R. & Gaudieri, S. (2003) Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in western Australia. *Forensic Science International*, **131**, 134-139.

Haseyama, K. L. F., Wiegmann, B. M., Almeida, E. A. B. & Carvalho, C. J. B. d. (2015) Say goodbye to tribes in the new house fly classification: A new molecular phylogenetic analysis and an updated biogeographical narrative for the Muscidae (Diptera). *Molecular Phylogenetics and Evolution*, **89**, 1-12.

Haskell, N. H. (1993) *Factors Affecting Diurnal Flight and Oviposition Periods Of Blow Flies (Diptera: Calliphoridae) in Indiana*. Purdue University, West Lafayette.

Haskell, N. H., Hall, R. D., Cervenka, V. J. & Clark, M. A. (1997) On the body: Insect's life-stage presence and their postmortem artifacts. In *Forensic Taphonomy: The Postmortem Fate of Human Remains* (ed. by W. D. Haglund & M. A. Sorg), pp. 415–448. CRC Press, Boston.

Hastir, P. & Gaspar, C. (2001) Diagnose d'une famille de fossoyeurs: Les Silphidae. *Notes fauniques de Gembloux*, **44**, 13-25.

Háva, J. (2007) Dermestidae. In *Catalogue of Palaearctic Coleoptera. Volume 4. Elateroidea, Derodontoidea, Bostrichoidea, Lymexyloidea, Cleroidea and Cucujoidea* (ed. by I. Löbl & A. Smetana), pp. 299-320. Apollo Books, Stenstrup.

Heather, J. M. & Chain, B. (2016) The sequence of sequencers: the history of sequencing DNA. *Genomics,* **107**, 1-8.

Hebert, P. D., Cywinska, A., Ball, S. L. & deWaard, J. R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences,* **270**, 313-321.

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S. & Francis, C. M. (2004) Identification of birds through DNA Barcodes. *PLoS Biology,* **2**, e312.

Hemmer, W. (1997) *Foods Derived from Genetically Modified Organisms and Detection Methods*. Agency BATS, Basel.

Hennig, W. (1955-1964) Muscidae. In *Die Fliegen der Palaearktischen Region* (ed. by L. E.), pp. 1110. Schweizerbart, Stuttgart.

Henßge, C., Althaus, L., Bolt, J., Freislederer, A., Haffner, H. T., Henßge, C. A., *et al.* (2000a) Experiences with a compound method for estimating the time since death. I. Rectal temperature nomogram for time since death. *International Journal of Legal Medicine,* **113**, 303-319.

Henßge, C., Althaus, L., Bolt, J., Freislederer, A., Haffner, H. T., Henßge, C. A., *et al.* (2000b) Experiences with a compound method for estimating the time since death. II. Integration of non-temperature-based methods. *International Journal of Legal Medicine,* **113**, 320–331.

Henßge, C. & Madea, B. (2004) Estimation of the time since death in the early post-mortem period. *Forensic Science International*, **144**, 167-175.

Hill, D. S. (1997) *The Economic Importance of Insects*. Chapman & Hall, New York.

Hillis, D. M., Allard, M. W. & Miyamoto, M. M. (1993) Analysis of DNA sequence data: Phylogenetic inference. *Methods in Enzymology,* **224**, 456-487.

Hillis, D. M. & Bull, J. J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology,* **42**, 182-192.

Holdaway, F. G. & Evans, A. C. (1930) Parasitism a stimulus to pupation: *Alysia manducator* in relation to the host *Lucilia sericata*. *Nature*, **125**, 598–599.

Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., *et al.* (2009) A DNA barcode for land plants. *Proceedings of the National Academy of Sciences,* **106**, 12794-12797.

Huchet, J. B. (1996) L'Archéoentomologie funéraire: une approche originale dans l'interprétation des sépultures. *Bulletins et Mémoires de la Société d'anthropologie de Paris,* **3-4**, 299-311.

Huchet, J. B. (2010) Archaeoentomological study of the insects remains found within the mummy of Namenkhet Amon, San Lazzaro Armenian Monastery, (Venice/Italy). *Advances in Egyptology,* **1**, 59-80.

Huchet, J. B. (2013) L'archéo-entomologie: les insectes nécrophages associés aux soldats de Carspach. In *A l'est du nouveau! Archéologie de la Grande Guerre en Alsace et en Lorraine.* (ed. by B. Schnitzler & M. Landolt), pp. 109-110. Musée de Strasbourg, collection Archéologie, Strasbourg.

Huchet, J. B. (2014a) Insect remains and their traces: Relevant fossil witnesses in the reconstruction of past funerary practices. *Anthropologie (Brno),* **52**, 329–346.

Huchet, J. B. (2014b) L'archéoentomologie funéraire. In *Insectes, Cadavres et Scènes de Crim. Principes et Applications de l'Entomologie Médico-Légale* (ed. by D. Charabidze & M. Gosselin), pp. 201-224. De Boeck Superieur, Paris.

Huchet, J. B. (2017) Archéoentomologie et archéoparasitologie d'une momie égyptienne. *Techné,* **44**, 79-83.

Huchet, J. B., Callou, C., Lichtenberg, R. & Dunand, F. (2013) The dog mummy, the ticks and the louse fly: Archaeological report of severe ectoparasitosis in Ancient Egypt. *International Journal of Paleopathology,* **3**, 165-175.

Huchet, J. B. & Greenberg, B. (2010) Flies, Mochicas and burial practices: A case study from Huaca de la Luna, Peru. *Journal of Archaeological Science,* **37**, 2846-2856.

Huckett, H. C. (1954) A review of the North American species belonging to the genus *Hydrotaea* Robineau-Desvoidy (Diptera, Muscidae). *Annals of the Entomological Society of America,* **47**, 316-342.

Huckett, H. C. (1977) The Anthomyiidae and Muscidae of the Presidential Range in New Hampshire (Diptera). *Journal of the New York Entomological Society,* **85**, 130-142.

Hunkapiller, T., Kaiser, R., Koop, B. & Hood, L. (1991) Large-scale and automated DNA sequence determination. *Science,* **254**, 59-67.

Hunt, J. H. (1991) Nourishment and the evolution of the social Vespidae. In *The Social Biology of Wasps* (ed. by K. Ross & R. W. Matthews), pp. 426-450. Cornell University Press, Ithaca.

Hwang, C. & Turner, B. D. (2005) Spatial and temporal variability of necrophagous Diptera from urban to rural areas. *Medical and Veterinary Entomology,* **19**, 379-391.

Introna, F., Campobasso, C. P. & Goff, M. L. (2001) Entomotoxicology. *Forensic Science International,* **120**, 42-47.

Isiche, J., Hillerton, J. E. & Nowell, F. (1992) Colonization of the mouse cadaver by flies in Southern England. *Medical and Veterinary Entomology,* **6**, 168-170.

Izquierdo-Carrasco, F., Smith, S. A. & Stamatakis, A. (2011) Algorithms, data structures, and numerics for likelihood-based phylogenetic inference of huge trees. *BMC Bioinformatics,* **12**, 470-470.

Janssen, W. (1984) *Forensic Histopathology*. Springer-Verlag, Berlin.

Jordaens, K., Sonet, G., Richet, R., Dupont, E., Braet, Y. & Desmyter, S. (2013) Identification of forensically important *Sarcophaga* species (Diptera: Sarcophagidae) using the mitochondrial COI gene. *International Journal of Legal Medicine,* **127**, 491-504.

Joseph, I., Mathew, D. G., Sathyan, P. & Vargheese, G. (2011) The use of insects in forensic investigations: An overview on the scope of forensic entomology. *Journal of Forensic Dental Sciences,* **3**, 89-91.

Kahanpää, J. & Haarto, A. (2014) Checklist of the families Scathophagidae, Fanniidae and Muscidae of Finland (Insecta, Diptera). *ZooKeys*, **441**, 347-367.

Karch, H., Schwarzkopf, A. & Schmidt, H. (1995) Amplification methods in diagnostic bacteriology (selected examples). *Journal of Microbiological Methods*, **23**, 55-73.

Kayser, H. (1985) Pigments. In *Comparative Insect Physiology, Biochemistry, and Pharmacology*. (ed. by G. A. Kerkut & L. I. Gilbert), pp. 367–415. Pergamon Press, Oxford.

Kent, J. D., Tham, T. R., Sánchez, V. V., Gaither, C. M. & Jonathan, D. (2016) The Camelid Sacrifices of Santa Rita B: An agropastoral site in the Chao Valley, north coastal Peru. In *The Archaeology of Andean Pastoralism* (ed. by J. M. Capriles & N. Tripcevich), pp. 183-196. University of New Mexico Press, Albuquerque.

Kenward, H. K. & Hall, A. R. (1995) *Biological evidence from Anglo-Scandinavian deposits at 16-22 Coppergate*. Council for British Archaeology, York.

Keshavarzi, D., Moemenbellah-Fard, M. D., Zarenezhad, M. & Gholamzadeh, S. (2016) First forensic record of blowfly, *Calliphora vicina*, larvae on an indoor human corpse in winter, South of Iran. *International Journal of Forensic Science & Pathology*, **2**, 218-220.

Kharbouche, H., Augsburger, M., Cherix, D., Sporkert, F., Giroud, C., Wyss, C., ... & Mangin, P. (2008) Codeine accumulation and elimination in larvae, pupae, and imago of the blowfly *Lucilia sericata* and effects on its development. *International journal of legal medicine*, **122**(3), 205-211.

Kintz, P., Tracqui, A. & Mangin, P. (1990) Toxicology and fly larvae on a putrefied cadaver. *Journal of the Forensic Science Society*, **30**, 243-246.

Kirinoki, M., Hitosugi, M., Kato-Hayashi, N., Iwasa, M. & Chigusa, Y. (2015) Discovery of Liopiophila varipes and Protopiophila contecta (Diptera: Piophilidae) from human cadavers. *Forensic Science International*, **248**, e8-e12.

Kits, J. H., Marshall, S. A. & Skevington, J. H. (2013) Phylogeny of the Archiborborinae (Diptera: Sphaeroceridae) based on combined morphological and molecular analysis. *PloS One*, **8**, e51190.

Kjærandsen, J. (1993) Diptera in mines and other cave systems in southern Norway. *Entomologica Fennica*, **4**, 151-160.

Klimešová, V., Oleksakova, T., Bartak, M. & Šuláková, H. (2016) Forensically important muscidae (Diptera) associated with decomposition of carcasses and corpses in the Czech Republic. In *MendelNet*, pp. 784-789, Brno.

Kluge, A. G. & Farris, J. S. (1969) Quantitative phyletics and the evolution of anurans. *Systematic Biology*, **18**, 1-32.

Kočárek, P. (2003) Decomposition and Coleoptera succession on exposed carrion of small mammal in Opava, the Czech Republic. *European Journal of Soil Biology*, **39**, 31-45.

Kocher, T. D. & Wilson, A. C. (1991) Sequence evolution of mitochondrial DNA in humans and chimpanzees: Control region and a protein-coding region. In *Evolution of Life* (ed. by S. Osawa & T. Honjo), pp. 391-413. Springer, Tokyo.

Köppen, W. P. & Geiger, R. (1930) *Handbuch der Klimatologie*. Gebrüder Borntraeger.

Korpisaari, A. (2006) Death in the Bolivian high plateau: Burials and Tiwanaku society. Oxford University Press, Oxford.

Kovarik, P. & Caterino, M. (2001) Histeridae. *American Beetles,* **1**, 212–227.

Krivosheina, N. (2008) Macromycete fruit bodies as a habitat for dipterans (Insecta, Diptera). *Entomological Review*, **88**, 778–792.

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.

Kumara, T. K., Disney, R. H., Abu Hassan, A., Flores, M., Hwa, T. S., Mohamed, Z., *et al.* (2012) Occurrence of oriental flies associated with indoor and outdoor human remains in the tropical climate of north Malaysia. *Journal of Vector Ecology*, **37**, 62-68.

Kutty, N. S., Pape, T., Wiegmann , M. B. & Meier, R. (2010) Molecular phylogeny of the Calyptratae (Diptera: Cyclorrhapha) with an emphasis on the superfamily Oestroidea and the position of Mystacinobiidae and McAlpine's fly. *Systematic Entomology*, **35**, 614-635.

Kutty, S. N., Pape, T., Pont, A., Wiegmann, B. M. & Meier, R. (2008) The Muscoidea (Diptera: Calyptratae) are paraphyletic: Evidence from four mitochondrial and four nuclear genes. *Molecular Phylogenetics and Evolution*, **49**, 639-652.

Lagoo, L. Schaeffer, L.S., Szymanski, D.W. & Smith, R.W. (2010) Detection of gunshot residue in blowfly larvae and decomposing porcine tissue using inductively coupled plasma mass spectrometry (ICP-MS). *Journal of Forensic Sciences*, **55**(3):624-32.

LaSalle, J. (1993) Parasitic Hymenoptera, biological control and biodiversity. In *Hymenoptera and Biodiversity* (ed. by R. K. Upadhyay, K. G. Mukerji & B. P. Chamola), pp. 197-215. Springer, Boston.

Laudet, F. & Antoine, P.-O. (2004) Des chambres de pupation de Dermestidae (Insecta : Coleoptera) sur un os de mammifère tertiaire (phosphorites du Quercy) : implications taphonomiques et paléoenvironnementales. *Geobios*, **37**, 376-381.

Lawrence, J. F. & Newton, A. (1995) Families and subfamilies of Coleoptera (with selected genera, notes, references and data on family-group names). In *Biology, Phylogeny, and Classification of Coleoptera* (ed. by J. Pakaluk & S. S.A.), pp. 779-1006. Museum i Instytut Zoologii PAN, Warszawa.

Lefebvre, F. & Pasquerault, T. (2004) Temperature-dependent development of *Ophyra aenescens* (Wiedemann, 1830) and *Ophyra capensis* (Wiedemann, 1818) (Diptera, Muscidae). *Forensic Science International,* **139**, 75-79.

Leto Barone, G., Liotta, G. & Raspi, A. (2002) Indagini entomologiche. Metodi e scelte. In *Il Sarcofago dell'imperatore. Studi, Ricerche e Indagini sulla Tomba*

di Federico II nella Cattedrale di Palermo, pp. 107-113. Regione Siciliana. Assessorato dei Beni Culturali ed Ambientali e della Pubblica Istruzione, Palermo.

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**, W242-245.

Lewis, A. & Benbow, M. (2011) When entomological evidence crawls away: *Phormia regina* en masse larval dispersal. *Journal of Medical Entomology*, **48**, 1112-1119.

Li, H., Ruan, J. & Durbin, R. (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Research*, **18**, 1851-1858.

Lo Giudice, G. & Józef Woźnica, A. (2013) An updated checklist of the Italian Heleomyzidae (Diptera: Sphaeroceroidea). *International Journal of Invertebrate Taxonomy*, **24**, 439–458.

Lo Pinto, S., Giordani, G., Tuccia, F., Ventura, F. & Vanin, S. (2017) First records of *Synthesiomyia nudiseta* (Diptera: Muscidae) from forensic cases in Italy. *Forensic Science International*, **276**, e1-e7.

Löbl, I., Rolčík, J., Kolibáč, J. & Gerstmeier, R. (2007) Cleridae. *Catalogue of Palaearctic Coleoptera*, **4**, 367-384.

Lodhi, M. A., Ye, G.-N., Weeden, N. F. & Reisch, B. I. (1994) A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Plant Molecular Biology Reporter*, **12**, 6-13.

Lohm, U. (1978) *Piophila vulgaris* Fall. (Dipt., Piophilidae) new to Spitsbergen, Svalbard. *Norwegian Journal of Entomology*, **25**, 224.

Lopes, H. d. S. & Leite, A. C. R. (1989) Morphology of the egg of *Sarcodexia lambens* (Diptera: Sarcophagidae). *Memorias do Instituto Oswaldo Cruz*, **84**, 497-500.

Lopez-Hurtado Orjeda, L. E. (2011) Ideology and the development of social hierarchy at the site of Panquilma, peruvian central coast. University of Pittsburgh, Pittsburgh. PhD Thesis.

Lord, N. P. (2011-2013) Ironclad ID: Tool for Diagnosing Ironclad and Cylindrical Bark Beetles (Coleoptera: Zopheridae) of North America north of Mexico. The University of New Mexico and Center for Plant Health Science and Technology, USDA, APHIS, PPQ, Albuquerque.

Lord, W. D. & Stevenson, J. R. (1986) *Directory of Forensic Entomologists*. Defense Pest Management Information Analysis Center, Walter Reed Army Medical Center, Washington.

Lowenstein, J. M. (1985) Molecular Approaches to the Identification of Species: Immunological analysis of proteins from living or fossil organisms can be used to identify unrecognizable species and to obtain new information on taxonomic relationships and dates of evolutionary divergence. *American Scientist*, **73**, 541-547.

Lozada, M. C., Buikstra, J. E., Rakita, G. & Wheeler, J. C. (2009) Camelid herders: the forgotten specialists in the coastal senorio of Chiribaya, southern Peru. In *Andean civilization: A tribute to Michael E. Moseley* (ed. by J. Marcus & P. R. Williams), pp. 351-364. Cotsen Institute of Archaeology, Los Angeles.

Lozada, M. C., Rakita, G., Buikstra, J. E., Leónardini, N., Rodriguez, D. & Cabanillas, V. (2004) Dando la bienvenida a la muerte: El rol de los camélidos en las festividades funerarias chiribaya en la costa del Perú. In *Proceedings of the Imagen de la Muerte. Primer Congreso Latinoamericano de Ciencias Sociales y Humanidades (UNMSM, 1–4 de noviembre 2004). Lima: Universidad Nacional Mayor de San Marcos*, pp. 39-49.

Lukhtanov, V. A., Sourakov, A., Zakharov, E. V. & Hebert, P. D. (2009) DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. *Molecular Ecology Resources,* **9**, 1302-1310.

Lyneborg, L. (1970) *Taxonomy of European* Fannia *larvae (Diptera, Fanniidae)*. Staatliches Museum für Naturkunde, Karlsruhe.

Mahony, J. B., Luinstra, K. E., Waner, J., McNab, G., Hobranzska, H., Gregson, D., *et al.* (1994) Interlaboratory agreement study of a double set of PCR plasmid primers for detection of Chlamydia trachomatis in a variety of genitourinary specimens. *Journal of Clinical Microbiology,* **32**, 87-91.

Maistrello, L., Lombroso, L., Pedroni, E., Reggiani, A. & Vanin, S. (2006) Summer raids of *Arocatus melanocephalus* (Heteroptera, Lygaeidae) in urban buildings in Northern Italy: Is climate change to blame? *Journal of Thermal Biology,* **31**, 594-598.

Mann, R. W., Bass, W. M. & Meadows, L. (1990) Time since death and decomposition of the human body: Variables and observations in case and experimental field studies. *Journal of Forensic Science,* **35**, 103-111.

Manoj, K., Tushar, B. & Sushama, C. (2007) Isolation and purification of genomic DNA from black plum (*Eugenia jambolana* Lam.) for analytical applications. *International Journal of Biotechnology & Biochemistry,* **3**, 49-56.

Manrique-Saide, P. & Delfín, H. (1997) Importancia de las moscas como vectores potenciales de enferme- dades diarreicas en humanos. *Revista Biomédica* **8**, 163-170.

Marchenko, M.I. (2001) Medicolegal relevance of cadaver entomofauna for the determination of the time of death. *Forensic Science International,* **120**(1-2):89-109.

Marchetti, D., Arena, E., Boschi, I. & Vanin, S. (2013) Human DNA extraction from empty puparia. *Forensic Science International,* **229**, e26-29.

Mardis, E. R. (2008) The impact of next-generation sequencing technology on genetics. *Trends in Genetics,* **24**, 133-141.

Marshall, S., Roháček, J., Dong, H. & Buck, M. (2011) The state of Sphaeroceridae (Diptera: Acalyptratae): A world catalog update covering the years 2000-2010,

with new generic synonymy, new combinations, and new distributions. *Acta Entomologica Musei Nationalis Pragae,* **51**, 217-298.

Marshall, S. A. (2012) *Flies: The Natural History & Diversity of Diptera*. Firefly Books, Richmond Hill.

Marshall, S. A., Hall, S. C. B. & Hodge, S. (2009) A review of the genus *Phthitia* Enderlein (Diptera: Sphaeroceridae: Limosininae) in New Zealand, with the description of two new species. *New Zealand Entomologist,* **32**, 48-54.

Marshall, S. A., Skevington, J. H., Kelso, S. & Zhou, C. (2015) A redefinition and review of the genus *Myrmolimosina* Marshall (Diptera: Sphaeroceridae), with morphological and molecular assessments of new species from Mexico and Guatemala. *The Canadian Entomologist,* **147**, 696-701.

Martín-Vega, D. (2011) Skipping clues: Forensic importance of the family Piophilidae (Diptera). *Forensic Science International,* **212**, 1-5.

Martín-Vega, D., Gómez-Gómez, A. & Baz, A. (2010) The Austrian Piophilidae (Diptera) in the collection of the National Museum of Natural Sciences of Madrid, Spain. *Beiträge zur Entomofaunistik,* **11**, 9-12.

Martín-Vega, D., Hall, M. J. R. & Simonsen, T. J. (2016) Resolving confusion in the use of concepts and terminology in intrapuparial development studies of cyclorrhaphous Diptera. *Journal of Medical Entomology,* **53**, 1249-1251.

Martín-Vega, D., Gómez-Gómez, A. & Baz, A. (2011a) The "Coffin Fly" *Conicera tibialis* (Diptera: Phoridae) breeding on buried human remains after a postmortem interval of 18 years. *Journal of Forensic Sciences,* **56**, 1654-1656.

Martín-Vega, D., Gómez-Gómez, A., Baz, A. & Díaz-Aranda, L. M. (2011b) New piophilid in town: The first Palaearctic record of *Piophila megastigmata* and its coexistence with *Piophila casei* in central Spain. *Medical and Veterinary Entomology,* **25**, 64-69.

Martin, L. D. & West, D. L. (1995) The recognition and use of dermestid (Insecta, Coleoptera) pupation chambers in paleoecology. *Palaeogeography, Palaeoclimatology, Palaeoecology,* **113**, 303-310.

Matuszewski, S., Bajerlein, D., Konwerski, S. & Szpila, K. (2008) An initial study of insect succession and carrion decomposition in various forest habitats of Central Europe. *Forensic Science International,* **180**, 61-69.

Matuszewski, S., Bajerlein, D., Konwerski, S. & Szpila, K. (2010a) Insect succession and carrion decomposition in selected forests of Central Europe. Part 1: Pattern and rate of decomposition. *Forensic Science International,* **194**, 85-93.

Matuszewski, S., Bajerlein, D., Konwerski, S. & Szpila, K. (2010b) Insect succession and carrion decomposition in selected forests of Central Europe. Part 2: Composition and residency patterns of carrion fauna. *Forensic Science International,* **195**, 42-51.

Matuszewski, S., Bajerlein, D., Konwerski, S. & Szpila, K. (2011) Insect succession and carrion decomposition in selected forests of Central Europe. Part 3: Succession of carrion fauna. *Forensic Science International,* **207**, 150-163.

Matuszewski, S. & Szafalowicz, M. (2013) Temperature-dependent appearance of forensically useful beetles on carcasses. *Forensic Science International*, **229**, 92-99.

Matuszewski, S., Szafałowicz, M. & Grzywacz, A. (2014) Temperature-dependent appearance of forensically useful flies on carcasses. *International Journal of Legal Medicine*, **128**, 1013-1020.

Maxam, A. M. & Gilbert, W. (1977) A new method for sequencing DNA. *Proceedings of the National Academy of Sciences*, **74**, 560-564.

Mazzanti, M., Alessandrini, F., Tagliabracci, A., Wells, J. D. & Campobasso, C. P. (2010) DNA degradation and genetic analysis of empty puparia: Genetic identification limits in forensic entomology. *Forensic Science International*, **195**, 99-102.

Mazzarelli, D., Vanin, S., Gibelli, D., Maistrello, L., Porta, D., Rizzi, A., *et al.* (2015) Splitting hairs: Differentiating between entomological activity, taphonomy, and sharp force trauma on hair. *Forensic Science, Medicine and Pathology*, **11**, 104-110.

McAlpine, J. F. (1977) A revised classification of the Piophilidae, including 'Neottiophilidae' and 'Thyreophoridae' (Diptera: Schizophora). *Memoirs of the Entomological Society of Canada*, **109**, 1-66.

McAlpine, J. F., Peterson, B. V., Shewell, B. V., Teskey, H. J., Vockeroth, J. R. & Wood D. M. (1981) Manual of Nearctic Diptera. Research Branch Agriculture Canada, (Monograph, 27).

Mckenna, D. D., Farrell, B. D., Caterino, M. S., Farnum, C. W., Hawks, D. C., Maddison, D. R., *et al.* (2015) Phylogeny and evolution of Staphyliniformia and Scarabaeiformia: Forest litter as a stepping stone for diversification of nonphytophagous beetles. *Systematic Entomology*, **40**, 35-60.

Mégnin, J.-P. (1894) *La Faune des Cadavres: Application de l'Entomologie à la Médecine Légale*. G. Masson, Paris.

Megyesi, M., Nawrocki, S. & Haskell, N. (2005) Using accumulated degree-days to estimate the postmortem interval from decomposed human remains. *Journal of Forensic Science*, **50**, 618-626.

Mei, M., Whitmore, D., Giudice, G. L. & Cerretti, P. (2013) A neotype designation for the bone-skipper *Centrophlebomyia anthropophaga* (Diptera, Piophilidae, Thyreophorina), with a review of the Palaearctic species of *Centrophlebomyia*. *ZooKeys*, **310**, 7-28.

Meier, R., Kotrba, M. & Ferrar, P. (1999) Ovoviviparity and viviparity in the Diptera. *Biological Reviews*, **74**, 199-258.

Melander, A. L. & Spuler, A. (1917) *The Dipterous Families Sepsidae and Piophilidae*. State College of Washington, Agricultural Experiment Station, Pullman.

Merritt, R. & Wallace, J. (2009) The role of aquatic insects in forensic investigations. In *Forensic Entomology. The Utility of Arthropods in Legal Investigations* (ed. by J. H. Byrd & J. L. Castner), pp. 271-319. CRC Press, Boca Raton.

Metzker, M. L. (2010) Sequencing technologies—the next generation. *Nature Reviews Genetics*, **11**, 31-46.

Meyer, R. (1995) Nachweis gentechnologisch veränderter Lebensmittel mittels Polyerase-Kettenreaktion (PCR). *Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene*, **86**, 648-656.

Miller, M. L., Lord, W. D., Goff, M. L., Donnelly, B., McDonough, E. T. & Alexis, J. C. (1994) Isolation of amitriptyline and nortriptyline from fly puparia (Phoridae) and beetle exuviae (Dermestidae) associated with mummified human remains. *Journal of Forensic Science*, **39**, 1305-1313.

Milos, P. (2008) Helicos BioSciences. *Pharmacogenomics*, **9**, 477-480.

Moemenbellah-Fard, M. D., Keshavarzi, D., Fereidooni, M., Soltani, A., Gholamzadeh, S., Montazeri, M., *et al.* (2015) First case report of *Calliphora vicina* (Diptera: Calliphoridae) on an outdoor human corpse with an estimation of postmortem interval from Iran. *Journal of Entomology and Zoology Studies*, **3**, 400-402.

Moreau, C. S., Wray, B. D., Czekanski-Moir, J. E. & Rubin, B. E. (2013) DNA preservation: A test of commonly used preservatives for insects. *Invertebrate Systematics*, **27**, 81-86.

Morihara, D. & McCafferty, W. (1979) The *Baetis* larvae of North America (Ephemeroptera: Baetidae). *Transactions of the American Entomological Society (1890-)*, **105**, 139-221.

Morrow, J., Taylor, L., Peck, L., Elowsky, C., Stewart Owens, L., Eeckhout, P., *et al.* (2017) Pseudoscorpions of the family Cheiridiidae (Arachnida: Pseudoscorpiones) recovered from burial sediments at Pachacamac (500–1,500CE), Perú. *Journal of Arachnology*, **45**, 370-375.

Morrow, J. J., Baldwin, D. A., Higley, L., Piombino-Mascali, D. & Reinhard, K. J. (2015) Curatorial implications of *Ophyra capensis* (Order Diptera, Family Muscidae) puparia recovered from the body of the Blessed Antonio Patrizi, Monticiano, Italy (Middle Ages). *Journal of Forensic and Legal Medicine*, **36**, 81-83.

Mozayani, A. & Noziglia, C. (2010) *The Forensic Laboratory Handbook Procedures and Practice*. Springer Science & Business Media, Berlin.

Müller, A. (1995) Morphological specializations in Central European bees for the uptake of pollen from flowers with anthers hidden in narrow corolla tubes (Hymenoptera: Apoidea). *Entomologia Generalis*, **20**, 43-57.

Mullis, K., Erlich, H., Arnheim, N., Horn, G., Saiki, R. & Scharf, S. (1987) One of the first Polymerase Chain Reaction (PCR) patents.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986) Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. In *Proceedings of the Cold Spring Harbor Symposia on Quantitative Biology*, pp. 263-273. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Mumcuoglu, K., Ioffe-Uspensky, I., Alkrinawi, S., Sarov, B., Manor, E. & Galun, R. (2001) Prevalence of vectors of the spotted fever group Rickettsiae and murine

typhus in a Bedouin town in Israel. *Journal of Medical Entomology,* **38**, 458-461.

Nabity, P. D., Higley, L. G. & Heng-Moss, T. M. (2006) Effects of temperature on development of *Phormia regina* (Diptera: Calliphoridae) and use of developmental data in determining time intervals in forensic entomology. *Journal of Medical Entomology,* **43**, 1276-1286.

Nagy, Z. T., Sonet, G., Glaw, F. & Vences, M. (2012) First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. *PLoS One,* **7**, e34506.

Negrobov, O. & Naglis, S. (2016) Palaearctic species of the genus *Medetera* (Diptera: Dolichopodidae). *Zoosystematica Rossica,* **25**, 333-379.

Nei, M. (1996) Phylogenetic analysis in molecular evolutionary genetics. *Annual Review of Genetics,* **30**, 371-403.

Nei, M. & Kumar, S. (2000) *Molecular Evolution and Phylogenetics*. Oxford University Press, Oxford

New, T. R. (2008) *A Guide to Australian Moths*. Springer Science & Business Media, Berlin.

Novák, V. (2014) *Beetles of the Family Tenebrionidae of Central Europe*. Academia, Prague.

Nuorteva, P. & Nuorteva, S.-L. (1982) The fate of mercury in sarcosaprophagous flies and in insects eating them. *Ambio,* 34-37.

Nyrén, P., Pettersson, B. & Uhlén, M. (1993) Solid phase DNA minisequencing by an enzymatic luminometric inorganic pyrophosphate detection assay. *Analytical Biochemistry,* **208**, 171-175.

Nystrom, K. C., Goff, A. & Goff, M. L. (2005) Mortuary behaviour reconstruction through palaeoentomology: A case study from Chachapoya, Perú. *International Journal of Osteoarchaeology,* **15**, 175-185.

Oliveira, J. S., Baia, T. C., Gama, R. A., & Lima, K. M. (2014) Development of a novel non-destructive method based on spectral fingerprint for determination of abused drug in insects: An alternative entomotoxicology approach. *Microchemical Journal*, **115**, 39-46.

Opitz, W. (2010) Classification, natural history, phylogeny and subfamily composition of the Cleridae and generic content of the subfamilies (Coleoptera, Cleridae). *Entomologica Basiliensia et Collectionis Frey,* **32**, 31-128.

Østbye, E. & Lauritzen, S.-E. (2013) A checklist of invertebrates from Norwegian caves and mines. *Fauna Norvegica,* **33**, 35-51.

Otranto, D., Huchet, J. B., Giannelli, A., Callou, C. & Dantas-Torres, F. (2014) The enigma of the dog mummy from Ancient Egypt and the origin of '*Rhipicephalus sanguineus*'. *Parasites & Vectors,* **7**, 2.

Owens, L. S., Eeckhout, P., Arguelles, P., Higley, L. & Reinhard, K. J. (2015) Pachacamac: Prehistoric peruvian burial customs revealed by application of

forensic entomology techniques. In *Proceedings of the 1st International Conference in Funerary Archaeoentomology*, pp. 11.

Ozerov, A. (2004) On the classification of the family Piophilidae (Diptera). *Zoologicheskii zhurnal,* **83**, 600-608.

Panagiotakopulu, E. (2001) New records for ancient pests: Archaeoentomology in Egypt. *Journal of Archaeological Science,* **28**, 1235-1246.

Panagiotakopulu, E. (2004) Dipterous remains and archaeological interpretation. *Journal of Archaeological Science,* **31**, 1675-1684.

Panagiotakopulu, E. & Buckland, P. C. (2012) Forensic archaeoentomology - An insect fauna from a burial in York Minster. *Forensic Science International,* **221**, 125-130.

Pape, T. (1996) Catalogue of the Sarcophagidae of the world (Insecta: Diptera). In *Memoirs on Entomology, International*, pp. 1-558 American Entomological Institute, Logan.

Pape, T., Blagoderov, V. & Mostovski, M. B. (2011) Order Diptera Linnaeus, 1758. *Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa,* **3148**, 222-229.

Pape, T., Dahlem, G., Mello Patiu, C. d. & Giroux, M. (2006) The world of flesh flies (Diptera: Sarcophagidae). http://www.zmuc.dk.

Papp, L. (1984) Family Milichiidae. In *Catalogue of Palaearctic Diptera* (ed. by A. Soós & L. Papp), pp. 110-118. Akadémiai Kaidó, Budapest.

Papp, L. (1998) Families of Heleomyzoidea. *Contributions to a Manual of Palaearctic Diptera,* **3**, 425-455.

Papp, L. & Wheeler, T. A. (1998) Family Milichiidae. In *Contributions to a Manual of Palaearctic Diptera* (ed. by L. Papp & B. Darvas), pp. 315–324. Science Herald, Budapest.

Parry, S., Linton, S.M., Francis, P.S., O'Donnell, M.J. & Toop, T. (2011) Accumulation and excretion of morphine by *Calliphora stygia,* an Australian blow fly species of forensic importance. *Journal of Insect Physiology,* **57**(1):62-73.

Pastula, E. & Merritt, R. (2013) Insect arrival pattern and succession on buried carrion in Michigan. *Journal of Medical Entomology*, **50**, 432-439.

Payne, J. A. (1965) A summer carrion study of the baby pig *Sus scrofa* Linnaeus. *Ecology*, **46**, 592-602.

Payne, J. A. (1972) Insect succession and decomposition of pig carcasses in water. *Journal of the Georgia Entomological Society,* **7**, 153-162.

Payne, J. A. & King, E. W. (1969) Lepidoptera associated with pig carrion. *Journal of the Lepidopterists' Society,* **23**, 191-195.

Payne, J. A. & King, E. W. (1970) Coleóptera associated with pig carrion. *Entomologist's Monthly Magazine*, **105**, 224-232 pp.

Payne, J. A., King, E. W. & Beinhart, G. (1968) Arthropod succession and decomposition of buried pigs. *Nature,* **219**, 1180-1181.

Peel, M. C., Finlayson, B. L. & McMahon, T. A. (2007) Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences,* **11**, 1633-1644.

Perotti, M. A., Braig, H. R. and Goff, L. M. (2010) Phoretic mites and carcasses: Acari transported by organisms associated with animal and human decomposition. In *Current concepts in forensic entomology* (ed. by J. Amendt, C. P. Campobasso, M. Grassberger & M.L.Goff.), pp. 69-92. Springer, New York.

Philips, J. R. & Dindal, D. L. (1990) Invertebrate populations in the nests of a screech owl (*Otus asio*) and an American Kestrel (*Falco sparverius*) in Central New York. *Entomological News,* **101**, 170-192.

Phillips, A. J. & Simon, C. (1995) Simple, efficient, and nondestructive DNA extraction protocol for arthropods. *Annals of the Entomological Society of America*, **88**, 281-283.

Picard, C. J. & Wells, J. D. (2009) Survey of the genetic diversity of *Phormia regina* (Diptera: Calliphoridae) using amplified fragment length polymorphisms. *Journal of Medical Entomology,* **46**, 664-670.

Pinheiro, J. (2006) Decay process of a cadaver. In *Forensic Anthropology and Medicine: Complementary Sciences from Recovery to Cause of Death* (ed. by A. Schmitt, E. Cunha & J. Pinheiro), pp. 85-116. Humana Press, Totowa.

Pinna, M. (1954) *Il clima della Sardegna*. Goliardica, Pisa.

Pitkin, B. R. (1988) *Lesser Dung Flies: Diptera: Sphaeroceridae*. Royal Entomological Society of London, London.

Pless, J. E., Worrell, M. B. & Clark, M. A. (1997) Chapter 9. Postmortem changes in soft tissues [in] forensic taphonomy: the postmortem fate of human remains. In *Forensic Taphonomy: The Postmortem Fate of Human Remains* (ed. by W. D. Haglund & M. H. Sorg), pp. 151-164. CRC Press, Boca Raton.

Pont, A. C. (1980) Calliphoridae. In *Catalog of the Diptera of the Afrotropical Region* (ed. by R. W. Crosskey), pp. 779-800. British Museum (Natural History), London.

Pont, A. C. (2012) Muscidae. In *Fauna Europaea: Diptera. Fauna Europaea version 2.5.* http://www.faunaeur.org (ed. by T. Pape & P. Beuk).

Porta, A. (1923) *Fauna Coleopteroruum Italica*. Stabilimento Tipografico Piacentino, Piacenza.

Post, R. J., Flook, P. K. & Millest, A. L. (1993) Methods for the preservation of insects for DNA studies. *Biochemical Systematics and Ecology,* **21**, 85-92.

Povolny, D. & Verves, Y. (1997) The flesh-flies of Central Europe (Insecta, Diptera, Sarcophagidae). *Spixiana Supplement,* **24**, 1-262.

Prado e Castro, C. & García, M. (2010) Additions to the Piophilidae (Diptera) fauna from Portugal, with new records. *Graellsia,* **66**, 101-105.

Prado e Castro, C., Serrano, A., Martins da Silva, P. & García, M. (2012) Carrion flies of forensic interest: A study of seasonal community composition and succession in Lisbon, Portugal. *Medical and Veterinary Entomology,* **26**, 417-431.

Prieto, G., Goepfert, N., Valladares, K. & Vilela, J. (2014) Sacrificios de niños, adolescentesy camélidos jóvenes durante el intermedio tardío en la periferia de Chan Chan, Valle de Moche, costa norte del Perú. *Arqueología y Sociedad,* **27**, 255-296.

Raoult, D., Dutour, O., Houhamdi, L., Jankauskas, R., Fournier, P. E., Ardagna, Y., *et al.* (2006) Evidence for louse-transmitted diseases in soldiers of Napoleon's Grand Army in Vilnius. *Journal of Infectious Diseases,* **193**, 112-120.

Rasnttsyn, A. P. & Haichun, Z. (2010) Early evolution of Apocrita (Insecta, Hymenoptera) as indicated by new findings in the Middle Jurassic of Daohugou, Northeast China. *Acta Geologica Sinica-English Edition,* **84**, 834-873.

Raspi, A., Pisciotta, S. & Sajeva, M. (2009) *Milichiella lacteipennis*: New record for Lampedusa Island (Italy). *Bulletin of Insectology,* **62**, 133-135.

Reibe-Pal, S., Strehler, M., Mayer, F., Althaus, L., Madea, B. & Benecke, M. (2008) Dumping of corpses in compost bins - Two forensic entomological case reports. *Archiv für Kriminologie,* **222**, 195-201.

Reilly, E. (2003) The contribution of insect remains to an understanding of the environment of Viking-age and medieval Dublin. In *Medieval Dublin IV* (ed. by S. Duffy). Four Courts Press, Dublin.

Reinhard, K. J. & Buikstra, J. (2003) Louse infestation of the Chiribaya culture, Southern Peru: Variation in prevalence by age and sex. *Memorias do Instituto Oswaldo Cruz,* **98 Suppl 1**, 173-179.

Reiss, R. A. (2006) Ancient DNA from ice age insects: Proceed with caution. *Quaternary Science Reviews,* **25**, 1877-1893.

Rice, P., Longden, I. & Bleasby, A. (2000) EMBOSS: The European Molecular Biology Open Software Suite. *Trends in Genetics,* **16**, 276-277.

Richards, O. W. (1930) *The British Species of Sphaeroceridae (Borboridae: Diptera)*. Zoological Society of London, London.

Rick, F. M., Rocha, G. C., Dittmar, K., Coimbra, C. E., Jr., Reinhard, K., Bouchet, F., *et al.* (2002) Crab louse infestation in pre-Columbian America. *Journal of Parasitology,* **88**, 1266-1267.

Rivers, D.B. & Dahlem, G.A. (2014) *The Science of Forensic Entomology*. John Wiley & Sons, Chichester.

Roberts, R. B. & Vallespir, S. R. (1978) Specialization of hairs bearing pollen and oil on the legs of bees (Apoidea: Hymenoptera). *Annals of the Entomological Society of America,* **71**, 619-627.

Robinson, G. S. (2009) *Biology, Distribution and Diversity of Tineid Moths*. Southdene Sdn Bhd, Kuala Lumpur.

Rochefort, S., Giroux, M., Savage, J. & A. Wheeler, T. (2015) Key to forensically important Piophilidae (Diptera) in the Nearctic Region. *Canadian Journal of Arthropod Identification*, **27**, 1-37.

Rodriguez Loredo, C. (2001) Las ofrendas de camélidos en un cementerio del Formativo Superior, costa central, Perú. In *El Uso de los Camélidos a Través del Tiempo* (ed. by G. Mengoni Goñalons, D. Olivera & H. Yacobaccio), pp. 221-240. Ediciones del tridente, Buenos Aires.

Roeterdink, E. M., Dadour, I. R. & Watling, R. J. (2004) Extraction of gunshot residues from the larvae of the forensically important blowfly *Calliphora dubia* (Macquart)(Diptera: Calliphoridae). *International Journal of Legal Medicine*, **118**, 63-70.

Rofes, J. (2003) Prehispanic guinea pig sacrifices in Southern Perú, the case of El Yaral. In *Behaviour Behind Bones: The Zooarchaeology of Ritual, Religion, Status and Identity* (ed. by S. J. O'Day, W. Van Neer & A. Ervynck), pp. 95. Oxbow Books, Oxford.

Rognes, K. (1986) A check-list of Norwegian Muscidae (Diptera). *Fauna Norvegica / Serie B*, **33**, 77-85.

Rognes, K. (1991) *Blowflies (Diptera, Calliphoridae) of Fennoscandia and Denmark*. E.J. Brill/Scandinavian Science Press Ltd., Leiden.

Rognes, K. (2005) The Calliphoridae (Blowflies) (Diptera: Oestroidea) are not a monophyletic group. *Cladistics*, **13**, 27-66.

Rognes, K. (2011) Short notes 12. Diptera, Calliphoridae. In *Biodiversity of Marganai and Montimannu (Sardinia).* (ed. by G. Nardi, D. Whitmore, M. Bardiani, D. Birtele, F. Mason, L. Spada & P. Cerretti), pp. 852−857. Cierre Edizioni, Sommacampagna, Verona.

Rohacek, J. (1983) Succession of adults of Sphaeroceridae (Diptera) on bear excrement in central Slovakia (Czechoslovakia). *Biologia (Bratislava)*, **38**, 591-598.

Roháček, J. & Marshall, S. (1998) Revision of Homalomitrinae subfam. n. (Diptera: Sphaeroceridae), with the description of a new genus and three new species. *European Journal of Entomology*, **95**, 455-491.

Ronaghi, M. (2001) Pyrosequencing sheds light on DNA sequencing. *Genome Research*, **11**, 3-11.

Ronaghi, M., Uhlén, M. & Nyrén, P. (1998) A sequencing method based on real-time pyrophosphate. *Science*, **281**, 363-365.

Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., *et al.* (2011) An integrated semiconductor device enabling non-optical genome sequencing. *Nature*, **475**, 348–352.

Rowe, J. H. (1946) Inca culture at the time of the Spanish conquest. In *Handbook of South American Indians* (ed. by J. H. Steward), pp. 183-330. Bureau of American Ethnology, Bulletin 143, Washington.

Rowley, D. L., Coddington, J. A., Gates, M. W., Norrbom, A. L., Ochoa, R. A., Vandenberg, N. J., *et al.* (2007) Vouchering DNA-barcoded specimens: Test

of a nondestructive extraction protocol for terrestrial arthropods. *Molecular Ecology Notes,* **7**, 915-924.

Rubink, W. L., Murray, K. D., Baum, K. A. & Pinto, M. A. (2003) Long term preservation of DNA from honey bees (*Apis mellifera*) collected in aerial pitfall traps. *Texas Journal of Science*, **55**, 159-168.

Ruiz, A. (2001) Plague in the Americas. *Emerging Infectious Diseases,* **7**, 539.

Rzhetsky, A. & Nei, M. (1993) Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Molecular Biology and Evolution,* **10**, 1073-1095.

Saccà, G. (1964) Nota sulla presenza in Europa di *Ophyra aenescens* Wied. (Diptera, Muscidae). *Rivista di Parassitologia,* **25**, 295-296.

Sadler, D., Robertson, L., Brown, G., Fuke, C. & Pounder, D. (1997) Barbiturates and analgesics in *Calliphora vicina* larvae. *Journal of Forensic Science,* **42**, 481-485.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* **4**, 406-425.

Salvetti, M., Corbellini, C., Aggiusti, C., Agabiti Rosei, E. & Muiesan, M. L. (2012) *Calliphora vicina* human myiasis: A case report. *Internal and Emergency Medicine,* **7 Suppl 2**, S135-137.

Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., *et al.* (1977a) Nucleotide sequence of bacteriophage φX174 DNA. *Nature,* **265**, 687–695.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977b) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences,* **74**, 5463-5467.

Santos, D., Ribeiro, G. C., Cabral, A. D. & Sperança, M. A. (2018) A non-destructive enzymatic method to extract DNA from arthropod specimens: Implications for morphological and molecular studies. *PloS One,* **13**, e0192200.

Saukko, P. & Knight, B. (2015) *Knight's Forensic Pathology Fourth Edition*. CRC Press, Boca Raton.

Savage, J. & Wheeler, T. (2004) Phylogeny of the Azeliini (Diptera: Muscidae). *Studia Dipterologica,* **11**, 259–300.

Savolainen, V., Cowan, R. S., Vogler, A. P., Roderick, G. K. & Lane, R. (2005) Towards writing the encyclopedia of life: An introduction to DNA barcoding. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences,* **360**, 1805-1811.

Scheffrahn, R. H., Mangold, J. R. & Su, N.-Y. (1988) A survey of structure-infesting termites of peninsular Florida. *Florida Entomologist,* **71**, 615-630.

Schroeder, H., Klotzbach, H. & Puschel, K. (2003) Insects' colonization of human corpses in warm and cold season. *Legal Medicine,* **5 Suppl 1**, S372-374.

Schuehli, G. S. e., Barros de Carvalho, C. J. & Wiegmann, B. M. (2004) Regarding the taxonomic status of *Ophyra* Robineau-Desvoidy (Diptera: Muscidae): A molecular approach. *Zootaxa,* **712**, 1-12.

Schuehli, G. S. e., Barros de Carvalho, C. J. & Wiegmann, B. M. (2007) Molecular phylogenetics of the Muscidae (Diptera: Calyptratae): New ideas in a congruence context. *Invertebrate Systematics,* **21**, 263-278.

Schumann, v. H. (1982) Zur Bedeutung des *Musca domestica*-Antagonisten *Ophyra aenescens* (Diptera: Muscidae). II. Morphologie der Entwicklungsstadien. *Angewandte Parasitologie,* **23**, 86-92.

Séguy, E. (1937) Diptera, family Muscidae. In *Genera Insectorum* (ed. by P. Wystmann), pp. 205-604. P. Wystmann, Bruxelles.

Sevastopulo, D. (1974) Lepidoptera feeding at puddle-margins, dung and carrion. *Journal of the Lepidopterists' Society,* **28**, 167-168.

Shamim, G., Ranjan, S. K., Pandey, D. m. & Ramani, R. (2014) Biochemistry and biosynthesis of insect pigments. *European Journal of Entomology,* **111**, 149-164.

Sharanowski, B. J., Walker, E. G. & Anderson, G. S. (2008) Insect succession and decomposition patterns on shaded and sunlit carrion in Saskatchewan in three different seasons. *Forensic Science International,* **179**, 219-240.

Sharma, M., Singh, D. & Sharma, A. K. (2015) Mitochondrial DNA based identification of forensically important Indian flesh flies (Diptera: Sarcophagidae). *Forensic Science International,* **247**, 1-6.

Shewell, G. (1987) Sarcophagidae. *Manual of nearctic Diptera,* **2**, 1159-1186.

Shin, S. E., Lee, H. J., Park, J. H., Ko, K. S., Kim, Y.-H., Kim, K. R., *et al.* (2015) The first survey of forensically important entomofauna collected from medicolegal autopsies in South Korea. *BioMed Research International,* **2015**, 1-6.

Shinonaga, S. (2003) *A Monograph of the Muscidae of Japan.* Tokai University Press, Tokyo.

Shinonaga, S. & Kano, R. (1983) Four new species and four newly recorded species of the genus *Hydrotaea* Robineau-Desvoidy from Japan with a key to Japanese species (Diptera, Muscidae). *Japanese Journal of Sanitary Zoology,* **34**, 279-287.

Siebert, D. (1992) Tree statistics; trees and 'confidence'; consensus trees; alternatives to parsimony; character weighting; character conflict and its resolution. In *Cladistics* (ed. by P. Forey, C. Humphries, I. Kitching, R. Scotland, D. Siebert & D. Williams), pp. 72–88. Oxford University Press, Oxford.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., *et al.* (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology,* **7**, 539-539.

Sikes, D. S. (2005) Silphidae Latreille, 1807. *Handbook of Zoology,* **4**, 288-296.

Sikes, D. S. (2008) Carrion beetles (Coleoptera: Silphidae). In *Encyclopedia of Entomology* (ed. by J. L. Capinera), pp. 749-758. Springer, Dordrecht.

Singh, B. & Wells, J. D. (2013) Molecular systematics of the Calliphoridae (Diptera: Oestroidea): Evidence from one mitochondrial and three nuclear genes. *Journal of Medical Entomology,* **50**, 15-23.

Singh, D. & Bala, M. (2010) Studies on larval dispersal in two species of blow flies (Diptera: Calliphoridae). *Journal of Forensic Research,* **1**, 1-3.

Skidmore, P. (1962) Notes on the Helomyzidae of Lancashire and Cheshire, including records from other parts of North West England. *Entomologist,* **95**, 226-236.

Skidmore, P. (1985) *The Biology of the Muscidae of the World*. Springer Netherlands, Dordrecht.

Smith, D. N. (2013) Defining an indicator package to allow identification of 'cesspits' in the archaeological record. *Journal of Archaeological Science,* **40**, 526-543.

Smith, K. E. & Wall, R. (1997a) Asymmetric competition between larvae of the blowflies *Calliphora vicina* and *Lucilia sericata* in carrion. *Ecological Entomology,* **22**, 468-474.

Smith, K. E. & Wall, R. (1997b) The use of carrion as breeding sites by the blowfly *Lucilia sericata* and other Calliphoridae. *Medical and Veterinary Entomology,* **11**, 38-44.

Smith, K. G. V. (1986) *A Manual of Forensic Entomology*. Cornell University Press, Ithaca.

Solis, M. & Mitter, C. (1992) Review and phylogenetic analysis of the Pyralidae (*sensu stricto*) (Lepidoptera: Pyralidae) and placement of the Epipaschiinae. *Systematic Entomology,* **17**, 79-90.

Solomon, M. (1965) Archaeological records of storage pests: *Sitophilus granarius* (L.)(Coleoptera, Curculionidae) from an Egyptian pyramid tomb. *Journal of Stored Products Research,* **1**, 105-107.

Sonet, G., Jordaens, K., Braet, Y., Bourguignon, L., Dupont, E., Backeljau, T., *et al.* (2013) Utility of GenBank and the Barcode of Life Data Systems (BOLD) for the identification of forensically important Diptera from Belgium and France. *Zookeys,* **365**, 307-328.

Sorg, M. H., Dearborn, J. H., Monahan, E. I., Ryan, H. F., Sweeney, K. G. & David, E. (1997) Forensic taphonomy in marine contexts. In *Forensic Taphonomy: The Postmortem Fate of Human Remains* (ed. by W. D. Haglund & M. H. Sorg), pp. 567–604. CRC Press, Boca Raton.

Soszynska-Maj, A. & Woznica, A. J. (2016) A case study of Heleomyzidae (Diptera) recorded on snow in Poland with a review of their winter activity in Europe. *European Journal of Entomology,* **113**, 279-294.

Soszyńska-Maj, A. & Woźnica, A. J. (2006) Dipteron. *Bulletin of the Dipterological Section of the Polish Entomological Society,* **28**, 23-28.

Soszynska, A. (2004) The influence of environmental factors on the supranivean activity of flies (Diptera) in Central Poland. *European Journal of Entomology,* **101**, 481-490.

Southwick, E. E. & Southwick Jr, L. (1992) Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States. *Journal of Economic Entomology,* **85**, 621-633.

Stevens, J. & Wall, R. (2001) Genetic relationships between blowflies (Calliphoridae) of forensic importance. *Forensic Science International,* **120**, 116-123.

Stevens, J. R. (2003) The evolution of myiasis in blowflies (Calliphoridae). *International Journal for Parasitology,* **33**, 1105-1113.

Steyskal, G. (1964) Notes on North American Piophilidae II (Diptera). *Proceedings of the Entomological Society of Washington,* **66**, 177-181.

Stork, N. E. (2018) How many species of insects and other terrestrial arthropods are there on Earth? *Annual Review of Entomology,* **63**, 31-45.

Strasser, B. J. (2011) The experimenter's museum: GenBank, natural history, and the moral economies of biomedicine. *Isis*, **102**, 60-96.

Strong, W. D. & Evans, C. (1952) *Cultural Stratigraphy in the Viru Valley, Northern Peru: The Formative and Florescent Epochs*. Columbia University Press, New York.

Sukontason, K., Sukontason, K. L., Piangjai, S., Boonchu, N., Kurahashi, H., Hope, M., *et al.* (2004) Identification of forensically important fly eggs using a potassium permanganate staining technique. *Micron,* **35**, 391-395.

Sukontason, K. L., Ngern-Klun, R., Sripakdee, D. & Sukontason, K. (2007) Identifying fly puparia by clearing technique: Application to forensic entomology. *Parasitology Research,* **101**, 1407-1416.

Swenson, E. R. (2003) Cities of violence: Sacrifice, power and urbanization in the Andes. *Journal of Social Archaeology,* **3**, 256-296.

Szpila, K. (2010) Key for the identification of third instars of European blowflies (Diptera: Calliphoridae) of forensic importance. In *Current Concepts in Forensic Entomology* (ed. by J. Amendt, M. L. Goff, P. C. Campobasso & M. Grassberger), pp. 43-56. Springer Netherlands, Dordrecht.

Szpila, K., Richet, R. & Pape, T. (2015) Third instar larvae of flesh flies (Diptera: Sarcophagidae) of forensic importance—critical review of characters and key for European species. *Parasitology Research,* **114**, 2279-2289.

Tawfik, D. S. & Griffiths, A. D. (1998) Man-made cell-like compartments for molecular evolution. *Nature Biotechnology,* **16**, 652-656.

Teskey, H. J. & Turnbull, C. (1979) Diptera puparia from pre-historic graves. *The Canadian Entomologist,* **111**, 527-528.

Thevan, K., Disney, R. H. & Ahmad, A. H. (2010) First records of two species of Oriental scuttle flies (Diptera: Phoridae) from forensic cases. *Forensic Science International,* **195**, e5-7.

Thomas, T. D. (2008) The role of activated charcoal in plant tissue culture. *Biotechnology Advances,* **26**, 618-631.

Thomsen, P. F., Elias, S., Gilbert, M. T., Haile, J., Munch, K., Kuzmina, S., *et al.* (2009) Non-destructive sampling of ancient insect DNA. *PLoS One,* **4**, e5048.

Tin, M. M., Economo, E. P. & Mikheyev, A. S. (2014) Sequencing degraded DNA from non-destructively sampled museum specimens for RAD-tagging and low-coverage shotgun phylogenetics. *PLoS One,* **9**, e96793.

Tolrá, M. C. (2011) Some dipterans collected on winter cadavers in La Rioja (Spain):(Diptera: Phoridae, Heleomyzidae and Sphaeroceridae). *Boletín de la Sea,* **48**, 147-150.

Tomberlin, J. K. & Adler, P. H. (1998) Seasonal colonization and decomposition of rat carrion in water and on land in an open field in South Carolina. *Journal of Medical Entomology,* **35**, 704-709.

Tomberlin, J. K. & Benbow, M. E. (2015) *Forensic Entomology: International Dimensions and Frontiers*. CRC Press, Boca Raton.

Traub, R. & Wisseman, C. (1978) The ecology of murine typhus-a critical review. *Tropical Diseases Bulletin,* **75**, 237-317.

Tuccia, F., Giordani, G. & Vanin, S. (2016a) A combined protocol for identification of maggots of forensic interest. *Science & Justice,* **56**, 264-268.

Tuccia, F., Giordani, G. & Vanin, S. (2016b) A general review of the most common COI primers for Calliphoridae identification in forensic entomology. *Forensic Science International. Genetics,* **24**, e9-e11.

Tuccia, F., Giordani, G. & Vanin, S. (2018) Forensic Entomology: An overview. In *Crime, Security and Society*. University of Huddersfield Press, Huddersfield.

Turchetto, M. & Vanin, S. (2004a) Forensic entomology and climatic change. *Forensic Science International,* **146 Suppl**, S207-209.

Turchetto, M. & Vanin, S. (2004b) Forensic evaluations on a crime case with monospecific necrophagous fly population infected by two parasitoid species. *Anil Aggrawal's Internet Journal of Forensic Medicine and Toxicology,* **5**, 12-18.

Turchetto, M. & Vanin, S. (2010) Climate change and forensic entomology. In *Current Concepts in Forensic Entomology* (ed. by J. Amendt, Goff, M.L., Campobasso, C.P., Grassberger, M.), pp. 327-351. Springer, Berlin.

Uhle, M. (1903) *Pachacamac. Report of the William Pepper, M.D., LL.D. Peruvian expedition of 1896*. The Deptartment of Archaeology of the University of Pennsylvania, Philadelphia.

Umebachi, Y. (1975) Further studies on the dopamine derivative, SN-1 derived from the yellow pigments of *Papilio xuthus*. *Insect Biochemistry,* **5**, 73-92.

Vallisneri, A. (1733) *Opere Fisico-Mediche Stampate e Manoscritte del Kavalier Antonio Vallisneri Raccolte da Antonio Suo Figliuolo, Corredate d'Una Prefazione in Genere Sopra Tutte, e d'Una in Particolare Sopra il Vocabolario della Storia Naturale.... Tomo Primo [-terzo, ed ultimo]*, Padova.

Van Nieukerken, E., Kaila, L., Kitching, I., Kristensen, N., Lees, D., Minet, J., *et al.* (2016) Order Lepidoptera Linnaeus, 1758. In *Animal Biodiversity: An Outline of Higher-Level Classification and Survey of Taxonomic Richness* (ed. by Z.-Q. Zhang), pp. 212-221. Zootaxa.

Vance, G. M., VanDyk, J. K. & Rowley, W. A. (1995) A device for sampling aquatic insects associated with carrion in water. *Journal of Forensic Science,* **40**, 479-482.

Vanin, S. (2008) Utilità e limiti dell'identificazione tramite DNA delle specie di insetti di interesse forense in Italia e Europa. *Rivista Italiana di Medicina Legale,* **30**, 1403–1411.

Vanin, S. (2012) Archeoentomologia funeraria: Risultati e prospettive dallo studio delle mummie di Roccapelago. In *Roccapelago e le Sue Mummie: Studio Integrato della Vita di Una Piccola Comunità dell'Appennino tra XVI e XVIII Secolo*, pp. 225-229. Academy Editions, Modena.

Vanin, S. (2016) Advances in forensic entomology in missing persons investigations. In *Handbook of Missing Persons* (ed. by S. J. Morewitz & C. Sturdy Colls), pp. 309-317. Springer International Publishing, Cham.

Vanin, S., Gherardi, M., Bugelli, V. & Di Paolo, M. (2011) Insects found on a human cadaver in central Italy including the blowfly *Calliphora loewi* (Diptera, Calliphoridae), a new species of forensic interest. *Forensic Science International,* **207**, e30-33.

Vanin, S. & Huchet, J. B. (2017) Forensic Entomology and Funerary Archaeoentomology. In *Taphonomy of Human Remains: Forensic Analysis of the Dead and the Depositional Environment* (ed. by E. M. Schotsmans, N. Márquez-Grant & S. L. Forbes), pp. 167–186. Wiley, Manhattan.

Vanin, S., Tasinato, P., Ducolin, G., Terranova, C., Zancaner, S., Montisci, M., *et al.* (2008) Use of *Lucilia* species for forensic investigations in Southern Europe. *Forensic Science International,* **177**, 37-41.

Vanin, S., Turchetto, M., Galassi, A. & Cattaneo, C. (2009) Forensic Entomology and the Archaeology of War. *Journal of Conflict Archaeology,* **5**, 127-139.

Vanin, S., Zanotti, E., Gibelli, D., Taborelli, A., Andreola, S. & Cattaneo, C. (2013) Decomposition and entomological colonization of charred bodies - a pilot study. *Croatian Medical Journal,* **54**, 387-393.

Vass, A. A. (2001) Beyond the grave - Understanding human decomposition. *Microbiology Today,* **28**, 190-193.

Verano, J. (2014) Warfare and Captive Sacrifice in the Moche Culture: The Battle Continues. In *Embattled Bodies, Embattled Places: War in Pre-Columbian Mesoamerica and the Andes.* (ed. by A. Scherer & J. Verano), pp. 283-309. Harvard University Press, Cambridge.

Verma, S. & Singh, L. (2003) Novel universal primers establish identity of an enormous number of animal species for forensic application. *Molecular Ecology Notes,* **3**, 28-31.

Verves, Y. G. (2005) A catalogue of Oriental Calliphoridae (Diptera). *International Journal of Dipterological Research*, **16**, 233-310.

Vienna, P. (1980) *Coleoptera: Histeridae*. Calderini, Bologna.

Viero, A., Montisci, M., Pelletti, G. & Vanin, S. (2018) Crime scene and body alterations caused by arthropods: Implications in death investigation. *International journal of legal medicine.* In press. DOI:10.1007/s00414-018-1883-8.

Vikhrev, N. (2008) New data on distribution and biology of the invasive species *Hydrotaea aenescens* (Wiedemann, 1830) (Diptera, Muscidae). *ZooKeys*, **4**, 47-53.

Voelkerding, K. V., Dames, S. A. & Durtschi, J. D. (2009) Next-generation sequencing: From basic research to diagnostics. *Clinical Chemistry*, **55**, 641-658.

Volney, W. J. A. & Fleming, R. A. (2000) Climate change and impacts of boreal forest insects. *Agriculture, Ecosystems & Environment*, **82**, 283-294.

Wake, T. A. (2007) Vertebrate faunal remains. In *Moche Tombs at Dos Cabezas* (ed. by C. B. Donnan), pp. 211–230. Cotsen Institute of Archaeology, University of California, Los Angeles.

Wakeley, J. (1993) Substitution rate variation among sites in hypervariable region 1 of human mitochondrial DNA. *Journal of Molecular Evolution*, **37**, 613-623.

Wallman, J. (2004) The application of entomology to criminal investigations. In *Practice of Crime Scene Investigation* (ed. by J. Horswell), pp. 347-359. CRC Press, Boca Raton.

Wallman, J. F. (2001) Third-instar larvae of common carrion-breeding blowflies of the genus *Calliphora* (Diptera: Calliphoridae) in South Australia. *Invertebrate Systematics*, **15**, 37-51.

Wallman, J. F. & Donnellan, S. C. (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.

Warren McKibben, J. & Micks, D. W. (1956) Report of a case of human intestinal myiasis caused by *Leptocera venalicia*. *The American Journal of Tropical Medicine and Hygiene* **5**, 929-932.

Watson, D. W., Niño, E. L., Rochon, K., Denning, S., Smith, L. & Guy, J. S. (2007) Experimental evaluation of *Musca domestic*a (Diptera: Muscidae) as a vector of Newcastle disease virus. *Journal of Medical Entomology*, **44**, 666-671.

Watt, J. C. (1974) A revised subfamily classification of Tenebrionidae (Coleoptera). *New Zealand Journal of Zoology*, **1**, 381-452.

Wells, J. D., Introna, F., Di Vella, G., Campobasso, C. P., Hayes, J. & Sperling, F. A. (2001) Human and insect mitochondrial DNA analysis from maggots. *Journal of Forensic Science*, **46**, 685-687.

Weston, O. (2002) Flower foraging behavior of the Australian species *Eleale aspera* (Newman) (Coleoptera: Cleridae: Clerinae). *The Coleopterists Bulletin*, **56**, 241-245.

Wheeler, J. C., Russel, A. J. F. & Redden, H. (1995) Llamas and alpacas: Pre-conquest breeds and post-conquest hybrids. *Journal of Archaeological Science*, **22**, 833-840.

Whitaker, A. P. (2007) *Fleas: Siphonaptera*. Field Studies Council, London.

Whitaker, J. O., Clem, P. & Munsee, J. R. (1991) Trophic structure of the community in the guano of the evening bat *Nycticeius humeralis* in Indiana. *The American Midland Naturalist*, **126**, 392-398.

Whitmore, D., Pape, T. & Cerretti, P. (2013) Phylogeny of *Heteronychia*: the largest lineage of *Sarcophaga* (Diptera: Sarcophagidae). *Zoological Journal of the Linnean Society*, **169**, 604-639.

Wigglesworth, V. (1972) Haemocytes and basement membrane formation in *Rhodnius*. *Journal of Insect Physiology*, **19**, 831-844.

Wilson, A. S., Janaway, R. C., Holland, A. D., Dodson, H. I., Baran, E., Pollard, A. M., *et al.* (2007) Modelling the buried human body environment in upland climes using three contrasting field sites. *Forensic Science International*, **169**, 6-18.

Wilson, I. G. (1997) Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, **63**, 3741-3751.

Wolcott, M. J. (1992) Advances in nucleic acid-based detection methods. *Clinical Microbiology Reviews*, **5**, 370-386.

Wood, W. B. (1976) The skeletal material from the Brooloo Range and Rocky Hole Creek burial sites. *Archaeology and Physical Anthropology in Oceania*, **11**, 175-185.

Woodley, N. (1989) Phylogeny and calssification of the" Orthorrhaphous" Brachycera. In *Manual of Nearctic Diptera* (ed. by J. McAlpine & D. Wood), pp. 1371-1395. Agricolture Canada, Ottawa.

Woźnica, A. J. (2006) Three new species of the genus *Suillia* Robineau-Desvoidy, 1830 from the Neotropical region (Diptera: Heleomyzidae). *Annales Zoologici*, **56**, 657-665.

Wylie, F. R., Walsh, G. L. & Yule, R. A. (1987) Insect damage to aboriginal relics at burial and rock-art sites near carnarvon in central Queensland. *Australian Journal of Entomology*, **26**, 335-345.

Wyss, C. & Cherix, D. (2013) *Traité d'entomologie Forensique: Les Insectes sur la Scène de Crime*. Presses Polytechniques et Universitaires Romandes, Lausanne.

Yoshii, T., Tamura, K., Taniguchi, T., Akiyama, K. & Ishiyama, I. (1993) Water-soluble eumelanin as a PCR-inhibitor and a simple method for its removal. *Nihon Hoigaku Zasshi*, **47**, 323-329.

Zahradnik, P. (2013) *Beetles of the Family Ptinidae of Central Europe*. Academia, Prague.

Zaidi, F. H. & Chen, X. x. (2011) A preliminary survey of carrion breeding insects associated with the Eid ul Azha festival in remote Pakistan. *Forensic Science International*, **209**, 186-194.

Zajac, B. K., Martin-Vega, D., Feddern, N., Fremdt, H., e Castro, C. P., Szpila, K., *et al.* (2016) Molecular identification and phylogenetic analysis of the forensically important family Piophilidae (Diptera) from different European locations. *Forensic Science International,* **259**, 77-84.

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.

Zehner, R., Zimmermann, S. & Mebs, D. (1998) RFLP and sequence analysis of the cytochrome b gene of selected animals and man: Methodology and forensic application. *International Journal of Legal Medicine,* **111**, 323-327.

Zhang, S.-Q., Che, L.-H., Li, Y., Dan, L., Pang, H., Ślipiński, A., *et al.* (2018) Evolutionary history of Coleoptera revealed by extensive sampling of genes and species. *Nature Communications,* **9**, 1-9.

Zidani, S., Ferchichi, A. & Chaieb, M. (2005) Genomic DNA extraction method from pearl millet (*Pennisetum glaucum*) leaves. *African Journal of Biotechnology,* **4**, 862-866.

Zuha, R. M., Huong-Wen, S., Disney, R. H. & Omar, B. (2017) Scuttle flies (Diptera: Phoridae) inhabiting rabbit carcasses confined to plastic waste bins in Malaysia include new records and an undescribed species. *Tropical Life Sciences Research,* **28**, 131-143.

Zuha, R. M., See, H. W., Disney, R. H. & Omar, B. (2014) First record of genus *Puliciphora* Dahl (Diptera: Phoridae) associated with rabbit carcasses placed in concealed environments in Malaysia. *Forensic Science International,* **245**, e36-37.

Zumpt, F. (1965) *Myiasis in Man and Animals in the Old World: A Textbook for Physicians, Veterinarians and Zoologists*. Butterworths, London.

Zurgani, E. (2018) Documentation of body transformations during the decomposition process: From the crime scene to the laboratory. University of Huddersfield, Huddersfield. PhD Thesis.

Zuska, J. (1965) A review of the Czechoslovak species of the family Piophilidae with special reference to their imoprtance to food industry (Diptera, Acalyptrata). *Acta Entomologica Bohemoslovaca,* **62**, 141-157.

Zuska, J. & Laštovka, P. (1969) Species-composition of the dipterous fauna in various types of food-processing plants in Czechoslovakia. *Acta Entomologica Bohemoslovaca,* **66**, 201-221.

Appendix A

>D1AA_COI

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GC

>D2AA_COI

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>D4AC_COI

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Appendix B

Appendix C