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Original Paper

Barcoding analysis for identification of insect species on decaying remains

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Identifying necrophagous individuals on a dead body is most often associated with forensic practice and the field of forensic entomology. Insects are found on a dead body depending on climatic conditions, with the seasons naturally accelerating the decomposition of tissues and successive waves gradually reducing the amount of soft tissue remaining. The aim of this work was to compare the composition of the necrophagous invertebrate species occurring on different carcasses (*Gallus gallus* vs *Sus scrofa* f. *domestica*). A second objective was to evaluate which of the identification tools comparing *COI* gene sequences is currently more appropriate for barcoding and whether there are significant differences in the results of the BOLD and BLAST identification tools between the samples examined. Molecular determination was done using Sanger sequencing of the *COI* fragment of mitochondrial DNA. In total, 96 individuals and 13 species were identified in the *Gallus gallus* carcass and 50 individuals and 17 species on the *Sus scrofa* (f. *domestica*) carcass. *Calliphora vomitoria* was determined as the most abundant species in both corpses' remains. The reliability of the BOLD and BLAST identification methods is comparably high.

Keywords: forensic entomology, barcoding, *COI*, BOLD, BLAST

1 Introduction

A specific ecosystem, the necrobiome, is created by the complex and distinctive interactions of related species that occur in and around animal carcasses (Pastorelli et al., 2023). The decomposition of a carcass is understood as an ecosystem that undergoes several successive phases interconnected during the decomposition of all vertebrates (Stack, 2015). The speed of decomposition depends on the dimensions of the carcass and the surrounding environment, which significantly affects the time required for decomposition to complete (Amend et al., 2011; Hodecek & Jakubec, 2022). As this phenomenon has been described for a prolonged time and the successional waves of invertebrates occurring on a body are consistent, a comprehensive field of study known as forensic entomology has emerged to address this fact. The dead body serves as an excellent source of entomological material, attracting numerous species of invertebrates throughout its decomposition. Experimental carcasses are particularly unique as they attract not only primarily necrophagous invertebrate species but also predatory species, both in larval and adult forms, taking advantage of the abundance of food available in one location (Pastorelli et al., 2023). Rapid and limited carrion decomposition fosters intense competition among insect species that must detect, identify, and exploit the substrate. This competition plays a crucial role in shaping the ecological interactions between necrophagous insects (Pastorelli et al., 2023), ultimately influencing the abundance of necrophagous species on carcasses. Forensic entomology falls within the field of applied biology, with its primary application, as the name suggests, being in forensic science to investigate serious criminal cases. One of the most common applications is determining the postmortem interval (Joseph et al., 2011; Aly & Aldeyarbi, 2020; Ubomir et al., 2021). To accurately assess this interval, it is essential to have knowledge of the life cycles of all necrophagous species involved. The basis of forensic entomology lies in the colonization of carcasses by beetles, flies, and other forensic animals at characteristic intervals. Subsequent

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sampling of insects and their larval stages help forensic entomologists determine the approximate time of death based on the actual age of the present insects (Joseph et al., 2011). Forensic entomology also encompasses the study of human and animal parasites, as well as pests in the food industry (Ubomír et al., 2021). Focusing specifically on forensic entomology, it has become inherently linked to molecular biology, particularly barcoding, in the past decade (Aly & Aldeyarbi, 2020; Lutz et al., 2021). Since the publication of the "Universal Primer Sequences" by Folmer et al. in 1994, unique loci on the mitochondrial genome can be identified for each genus and species. This is possible because mitochondrial DNA (mtDNA), inherited exclusively from the mother, is present in significantly higher copy numbers within cells compared to nuclear DNA (nuDNA) (Shmookler & Goldstein, 1983; Willerslev & Cooper, 2005). The "universal" primers (Sharma & Kobayashi, 2014) are designed for the cytochrome c oxidase subunit 1 gene (*COI*), which is currently the most widely used gene for identifying cadaver invertebrates. However, for the identification of certain insects, it is appropriate to combine this gene with another gene, such as a nuclear gene, to ensure the accuracy of the identification. Some studies have identified combinations of two or more genes specific to each group of animals found on a carcass for barcoding, even in cases where the *COI* gene is absent. Nevertheless, the *COI* gene is predominantly utilized for the identification of necrophagous insects on carcasses with the LCO and HCO primers or their variations. Morphological characteristics can also be used for the identification of necrophagous insects present on a carcass. However, this identification is often challenging for juvenile stages, which are prevalent in the early stages of decomposition (Aly & Aldeyarbi, 2020). Additionally, a wide range of invertebrates, including representatives from the orders Diptera, Coleoptera, Lepidoptera, and Hymenoptera (parasitoid wasps), can be found on carcasses (Lutz et al., 2021). Although morphological identification keys exist for the developmental stages of forensically important individuals, such as those written by Szpila (2009) or Grzywacz et al. (2017), it is still challenging to identify juveniles found on a carcass, especially outside the geographic area of Central Europe. Genetic markers offer an additional advantage as there is no requirement to study the entire body of a necrophagous individual for molecular identification. With extensive databases and identification tools, molecular identification is more efficient, accurate, and faster. The main drawback may be the financial aspect. However, despite the numerous advantages that DNA barcoding can bring, complications in identification can arise if the method is not designed well experimentally (Collins & Cruickshank, 2012).

The aim of this work was to compare the composition of the necrophagous invertebrate species occurring on different carcasses (*Gallus gallus* vs *Sus scrofa* f. *domestica*). A second objective was to evaluate which of the identification tools comparing *COI* gene sequences is currently more appropriate for barcoding and whether there are significant differences in the results of the BOLD and BLAST identification tools between the samples examined.

2 Material and methods

2.1 Sampling

One *Sus scrofa* (f. *domestica*) carcass and one *Gallus gallus* carcass were used for this experiment. Invertebrate samples were taken at one month intervals according to the degree of decomposition of the carcass. The two experimental sites were located approximately 40 km apart on extensively farmed land in southern Moravia in similar ecological and climatic conditions and met all hygiene and veterinary regulations. Invertebrates were collected in ethanol and stored at -20 °C. Samples were taken directly from the cadaver, the cadaver bed, and the immediate surroundings. In the period from the spring of 2017 to the autumn of 2018, 160 individuals were collected and analyzed in our laboratory.

2.2 DNA Extraction

Each individual was cleaned with PBS buffer before DNA isolation to avoid contamination of the sample with foreign DNA. Genomic DNA for analysis was extracted from the whole body of each individual using the MACHEREY-NAGEL Genomic DNA Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). All steps were performed according to the standard instructions provided in the protocol.

2.3 PCR Amplification and Electrophoresis

The locus of the mitochondrial cytochrome oxidase subunit I gene (*COI*) was amplified with two sets of primer pairs: Dip_F + Dip_R1 and LCO + HCO (Table 1). They were subsequently accustomed to amplifying a 658 bp fragment of the *COI* gene. Each PCR contained 5 µl of PPP MasterMix (TopBio, Ltd., Vestec, Czech Republic); 0.2 µl of every primer (10 pmol/µl); 0.4 µl $MgCl_2$ (25 mM,TopBio, Ltd., Vestec, Czech Republic); 3.7 µl of ddH_2O (TopBio, Ltd., Vestec, Czech Republic) and 0.5 µl of DNA template. The PCR thermal regime consisted of 1 cycle of 3 min at 95 °C: 40 cycles of 45 s at 95 °C, 40 s at 49 °C and 1 min at 72 °C, and a final cycle of 5 min at 72 °C. These products were purified by MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany), per the manufacturer's protocol, then the PCR products were separated on 3% agarose

Table 1 PCR Primers

Primer	The sequence of primer 5´-3´	Author
LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
HCO	TAAACTTCAGGGTGACCAAAAAATC	Folmer et al., 1994
Dip_F1	GTATAGTAGAAAACGGAGCTG	Horecky et al., 2015
Dip_R1	AATCAACTAAAAATCTTAATTCC	Horecky et al., 2015

gels using 1×TBE (Tris-Borate-EDTA) running buffer at 5 V/cm and visualized by GoodView™ stain (Amplia s.r.o., Bratislava, Slovak Republic). Determination of PCR product concentration was performed using NanoDrop 2000 spectrometer from Thermo Fisher Scientific Inc. (Waltham, USA).

2.4 Sequencing and data analysis

The required amount of PCR product for the sequencing reaction was determined based on the concentration and size. Sequencing was performed at Mendel University in Brno, utilizing the BigDye Terminator Cycle Sequencing Kit v3.1 from Life Technologies Corp. (Carlsbad, USA) and following the manufacturer's guidelines. The final volume of the sequencing reaction mixture was 10 µl. The sequencing products were purified using the BigDye XTerminator™ Purification Kit (Applied Biosystems™, Carlsbad, CA, USA) according to the manufacturer´s recommendations, and ABI 3500 8-capillary sequencer was used for the sequencing process.

The obtained data were analyzed using Seq Scape software v.4.0.

2.5 Sample identifications

We used two different methods for sample identification according to DNA, the BLAST and the BOLD Identification System. Both the BOLD and BLAST identification tools have reference sequences against which they compare the inserted sequence and thus can determine the species. The Barcode of Life Data System (BOLD) was established in 2005 as a comprehensive platform for storing, analyzing, and publishing DNA barcode records. It serves as a repository that encompasses all aspects of the analysis process, from sample collection to the creation of a reliable sequence library. The database, accessible at www.barcodinglife.org (2022), primarily contains *COI* gene sequences, but it also has the potential to accommodate other gene or multigene codes. Each recorded sample in BOLD includes species information, taxonomic classification, details about the primers used in the polymerase chain reaction, and collector records. New records are regularly added to the database, contributing to its exponential growth. Data-sharing partnerships exist between BOLD and organizations such as the National Center for Biotechnology Information (NCBI), the DNA DataBank of Japan (DDBJ), and the European Molecular Biology Laboratory (EMBL). The system uses global alignment, which compares DNA sequences and tries to find the most accurate match in the reference sequences' entire length, thus pointing out possible relatedness. Species sequences are used as the default code search library with at least three records with a maximum consensus deviation of 2%. Other records that have not been fully validated yet are stored in another area of the data because they may carry incorrect information about the identified species. If the identification system finds a match with a difference from the reference sequence of less than 1%, it will provide identification of the individual at the species level. If the identified sequence's difference is less than 3%, the assignment will take place at the genus level. In cases where the sequence difference is more than 3%, the system finds the 100 most similar sequences and taxonomically classifies them (Ratnasingham & Hebert, 2007). The Basic Local Alignment Search Tool (BLAST) was developed in 1985 (www.ncbi.nlm.nih.gov/blast, 2022). BLAST compares all combinations of nucleotide or protein sequences with databases or with each other. Unlike the BOLD, it does not look for a match with a full-length reference sequence but looks for local matches between two sequences (McGinnis & Madden, 2004; Johnson et al., 2008). Sequence similarity searches often provide the first information about a new DNA or protein sequence, allowing scientists to predict their functions from similar sequences (Madden, 2013). The database offers 3 algorithms for nucleotide sequence comparison: megaBLAST, discontiguous megaBLAST and BLASTn. The megaBLAST program compares longer sequences and it is suitable for comparing very similar sequences, mostly within a species where the sequence identity is 95% or more. Discontiguous megaBLAST and BLASTn are more suitable programs for comparisons between species. (McGinnis & Maden, 2004). There are several key differences between the approaches used in BLAST and BOLD. BLAST compares query sequences to database sequences directly, whereas BOLD first verifies whether the query is a functional *COI* gene sequence to avoid pseudogenes or human contaminants. BOLD uses its own sequence databases; this means that the similarity

percentage calculated by BOLD may differ to a certain extent from the similarity percentage calculated by BLAST. However, it is important to note that BOLD uses a different approach for different databases. For example, BOLD includes sequences extracted from the GenBank database, but not all sequences in BOLD are in GenBank. Thus, the percent similarity calculated by BOLD may be more accurate in some cases (Ratnasingham & Hebert, 2007). This fact was also confirmed in our research.

3 Results and discussion

In almost all cases, identification was slightly more accurate when using the BOLD database. Only one exception occurred for the body of *Sus scrofa* (f. *domestica*) and the species *Phormia regina*. The difference in the identification of all individuals studied was no more than 0.6% when comparing the BOLD and BLAST tools. This may be mainly due to the different approaches used in sequence identification and the use of different reference sequence databases.

In all cases, the result of molecular identification was identic when using both databases. However, this may not be the rule, and it may happen that the results may not always be the same for other samples, so we still recommend using both databases for evaluation. A common reason for disagreement is that a species may be missing in one database or is underrepresented. Furthermore, carcasses are also a favourite food source for other invertebrate species, such as the Hymenoptera and wasps (Vespidae). Rarely, leafhoppers (Ichneumonidae) or bumblebees (Bombidae) may also occur near the carcass. All these species contribute to the decomposition of the carcass through their activities (Byrd & Tomberlin, 2019). However, no such problem appeared in our study. The species found on the carcasses of *Gallus gallus* (Table 2) are not surprising as they are routinely reported also in other studies. All individuals can be found in the available literature (Joseph et al., 2011; Byrd & Tomberlin, 2019) as representatives of the necrophagous fauna involved in carcass decomposition. Only one individual of non-necrophagous species was found on an experimental carcass of *Sus scrofa* (f. *domestica*) (Table 3). This species was *Pogonognathellus longicornis* (order Collembola), which bases its feeding strategy more on the ingestion of soil microbiota, such as bacteria, actinomycetes, and algae, with a preference for fungi (Ruess et al., 2007) and it was therefore only a random occurrence on the carcass.

A total of 96 individuals were identified using the molecular biology methods on *Gallus gallus*, the vast majority of which belonged to the order Diptera (94). One individual from the order Coleoptera was found here (*Aleochara curtula*), and from the order Hymenoptera, *Vespula germanica* was identified. Of the total number of individuals determined in *Gallus gallus* cadaver, 60% of individuals consisted of only 3 species (*Calliphora vomitoria*, *C. vicina* and *Chrysomia albiceps*). Even though there was an attempt to collect as many

Table 2 Results of the molecular identification found in *Gallus gallus* cadaver

N	BLAST determination:	∅ %	BOLD determination	∅ %	Order
76	*Calliphora vomitoria,* Linnaeus, 1758	99.86	*Calliphora vomitoria*	100.00	Diptera
5	*Chrysomya albiceps,* Wiedemann, 1819	99.67	*Chrysomya albiceps*	99.95	Diptera
4	*Hydrotaea ignava,* Harris, 1780	99.62	*Hydrotaea ignava*	100.00	Diptera
2	*Calliphora vicina,* Robineau-Desvoidy, 1830	100.00	*Calliphora vicina*	100.00	Diptera
1	*Lucilia caesar,* Linnaeus, 1758	100.00	*Lucilia caesar*	100.00	Diptera
1	*Hydrotaea aenescens*, Wiedemann, 1819	100.00	*Hydrotaea aenescens*	100.00	Diptera
1	*Aleochara curtula,* Goeze, 1777	99.00	*Aleochara curtula*	100.00	Coleoptera
1	*Scoliocentra brachypterna*, Loew, 1873	99.60	*Scoliocentra brachypterna*	100.00	Diptera
1	*Lucilia ampullacea,* Villeneuve, 1922	100.00	*Lucilia ampullacea*	100.00	Diptera
1	*Sarcophaga argyrostoma*, Robineau-Desvoidy, 1830	99.85	*Sarcophaga* sp.	100.00	Diptera
1	*Vespula germanica,* Fabricius, 1793	99.85	*Vespula germanica*	100.00	Hymenoptera
1	*Suillia bicolor,* Zetterstedt, 1838	99.39	*Suillia bicolor*	100.00	Diptera
1	*Dryomyza anilis,* Fallén, 1820	99.56	*Dryomyza anilis*	99.85	Diptera
96	Total	99.72	Total	99.98	

Table of results of identified individuals on *Gallus gallus* experimental body. ∅% average percent identity (similarity) for selected tools; Total – indicates the total number of examined individuals that could be identified by BOLD or BLAST tool, N-total number of examined individuals, the value of percent similarity of the analyzed sequence

species as possible, the most important decomposers in our climate are mostly representatives of the order Diptera. The three species mentioned belong to the Calliphoridae family, whose representatives are the most abundant in the first stage of carcass decomposition (Byrd & Tomberlin, 2019).

For *Sus scrofa* (f. *domestica*), 50 samples were analyzed using molecular biology methods (Table 3). Four orders (Diptera, Coleoptera, Hymenoptera, and Collembola) were present in this collection. Most specimens belonged to the order Diptera (44) and Coleoptera (5), and one specimen was identified from the order Collembola. Three species, *Calliphora vomitoria* (26%), *Calliphora vicina* (18%), and *Chrysomya albiceps* (16%), were the most frequent, with the others having a minority representation. Given that representatives of the Diptera order are most often found on the dead body in the larval stage of their development, this result is also to be expected.

The results of the study show that the significantly dominant species in both types of cadavers was *Calliphora vomitoria*. It was present on the carcass of *G. gallus* in 79% and on the carcass of *S. scrofa* in 26%. This result may be due to the fact that the larvae usually occur together, which is a consequence of the laying of a certain species, but also the matter of ecological adaptation. In natural settings, aggregations of individuals usually consist of individuals of the same species (i.e., intraspecific aggregates) (Fouche et al., 2018; Aubernon et al., 2019). This could have resulted in the predominant representation of the family Calliphoridae (*Ch. albiceps*; *C. vicina*, *L. caesar*, *L. ampullaceal*) on both examined bodies. On both carcasses, there was also one record of *Aleochara curtula*, which is a member of the family Staphylinidae (order Coleoptera). It is an important and common necrophage in central Europe that occurs on carcasses in the later stages of decomposition (Madra et al., 2014; Byrd & Tomberlin, 2019).

As stated by Meiklejohn et al., 2019, it is inevitable that any public database will contain some inaccurate data. The generation and submission of incorrect sequences are likely due to incorrect identification of source material, poor isolation techniques (especially in the case of fungi), contamination of cultures, endoparasites in insects (e.g., *Wolbachia*), duplicate entries due to cases of synonymy, and PCR-based errors (e. g.chimeric sequences or inadvertent sequencing of pseudogenes). Therefore, it is crucial to carefully address the problem of inconsistencies in publicly accessible databases to ensure the future trustworthiness of the data for reuse (Cheng et

Table 3 Results of the molecular identification found in *Sus scrofa* (f. *domestica*) cadaver

N	BLAST determination	∅ %	BOLD determination	∅ %	Order
13	*Calliphora vomitoria*	99.82	*Calliphora vomitoria*	100.00	Diptera
9	*Calliphora vicina*	99.50	*Calliphora vicina*	100.00	Diptera
8	*Chrysomya albiceps*	99.79	*Chrysomya albiceps*	99.97	Diptera
3	*Alysiinae* sp.	96.67	Alysia sp.	100.00	Hymenoptera
3	*Sarcophaga caerulescens,* Zetterstedt, 1838	99.76	*Sarcophaga caerulescens*	100.00	Diptera
2	*Lucilia caesar*	99.73	*Lucilia caesar*	99.94	Diptera
2	*Drosophila* sp.	99.86	*Drosophila* sp.	100.00	Diptera
1	*Philonthus succicola* Thomson, 1860	100.00	Philonthus succicola	100.00	Coleoptera
1	*Sarcophaga* cf. *similisaratrix*	99.51	*Sarcophaga* cf. *similisaratrix*	100.00	Diptera
1	*Lucilia illustris*, Meigen, 1826	99.39	*Lucilia illustris*	99.69	Diptera
1	*Lucilia ampullacea*	99.71	*Lucilia ampullacea*	100.00	Diptera
1	*Aleochara curtula*	99.67	*Aleochara curtula*	100.00	Coleoptera
1	*Neoleria ruficeps*, Zetterstedt, 1838	96.65	*Neoleria ruficeps*	99.44	Diptera
1	*Ichneumon inquinatus,* Wesmael, 1844	99.13	Ichneumon inquinatus	99.47	Hymenoptera
1	*Pogonognathellus longicornis,* O.F. Müller, 1776	99.66	*Pogonognathellus longicornis*	99.83	Collembola
1	*Phormia regina,* Meigen, 1826	99.18	*Phormia regina*	99.08	Diptera
1	*Lucilia sericata,* Meigen, 1826	99.49	*Lucilia sericata*	100.00	Diptera
50	Total	99.27	Total	99.85	

Table of results of identified individuals on *Sus scrofa* (f. *domestica*) experimental body. ∅% average percent identity (similarity) for selected tools; Total – indicates the total number of examined individuals that could be identified using the BOLD or BLAST tool, N-total number of analyzed individuals, value of percent similarity of the examined sequence

al., 2023). On the other hand, it is undeniably true that identification tools are constantly improving (Pentinsaari et al., 2020). Nevertheless, it is important to combine all available options as best as possible.

4 Conclusions

Forensic entomology deals with the study of necrophagous insects found at crime scenes and, in conjunction with molecular biology methods, are a valuable tool in elucidating crime scenes. It was found that the necrobiome that formed on the examined cadavers does not fundamentally differ in the context of standard succession conditions for Central Europe and that the 3 most abundant species significantly predominate in the group of founded individuals, predominantly *Calliphora vomitoria*. Thus, from the above results, it can be said that the chosen methodology using the *COI* mitochondrial gene sequence is perfectly suitable for the identification of the whole spectrum of necrophages involved in carcass decomposition, as the sequences of all examined individuals had a percentage of similarity in the given identification tools/databases of more than 99%. This means that it is possible to use BOLD and BLAST, as the identification results are identical in the taxa we examined. The study also confirms the suitability of the proposed methodology for distinguishing taxonomically different individuals.

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