Czech University of Life Sciences Prague

Faculty of Environmental Sciences

Department of Ecology

Bachelor Thesis

Qualitative comparison of DNA isolation methods from insect specimen

Ana Anđelković

Supervisor: Ing. Pavel Jakubec, Ph. D.

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BACHELOR THESIS ASSIGNMENT

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Thesis title

Qualitative comparison of DNA isolation methods from insects specimen

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Objectives of thesis

Goals of the thesis are:

1. Write literature review dealing with the usage of different extraction methods used in forensic research and praxis, with focus on commercially available DNA extraction kits.
2. To get familiar with common laboratory techniques used for DNA isolation and apply these techniques in a comparative study.
3. Collect qualitative and quantitative data for three different amounts of tissue collected from beetle Thanatophilus sinuatus using three different methods (two commercial DNA extraction kits and Direct PCR method).
4. Compare statistically these methods.

Methodology

DNA will be isolated from specimens of Thanatophilus sinuatus stored in 96% EtOH. Three methods will be used for DNA extraction. Two commercially used kits – DNeasy Blood & Tissue Kit (Qiagen), Nucle-oSpin DNA Insect (Macherey-Nagel) and the direct PCR. For each of the extraction methods 1,2, and 3 legs will be used as a source of genomic DNA. The amount of isolated DNA will be determined by Nan-oDrop 1000 UV Visible Spectrophotometer and by Qubit 4 Fluorometer. The DNA extraction will be performed once for each method and amount of the source DNA. The DNA extraction will be followed by amplification of Cytochrome oxidase subunit I (COI) using the forward and reverse primer sequences. Obtained sequences will be then visualized using the software Chromas 2.6.6. and blasted against GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

The proposed extent of the thesis

30

Keywords

DNA, isolation, entomology,

Recommended information sources

Grevelding, C.G., Kampkötter, A., Hollmann, M., Schäfer, U. & Kunz, W. (1996) Direct PCR on fruitflies and blood flukes without prior DNA isolation. Nucleic Acids Research, 24, 4100–4101.

Kranzfelder, P., Ekrem, T. & Stur, E. (2016) Trace DNA from insect skins: A comparison of five extraction protocols and direct PCR on chironomid pupal exuviae. Molecular Ecology Resources, 16, 353–363.

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Murthy, M.K., Khandayataray, P., Tara, M., Buragohain, P., Giri, A. & Gurusubramanian, G. (2022) Optimisation of DNA isolation and PCR techniques for beetle (Order: Coleoptera) specimens. International Journal of Tropical Insect Science.

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The Bachelor Thesis Supervisor

Ing. Pavel Jakubec, Ph.D.

Supervising department

Department of Ecology

Advisor of thesis

Ing. Karolina Mahlerová

Electronic approval: 23. 3. 2023

prof. Mgr. Bohumil Mandák, Ph.D.

Head of department

Electronic approval: 23. 3. 2023

prof. RNDr. Vladimír Bejček, CSc.

Dean

Prague on 30. 03. 2023

Declaration

I declare that I have worked on my bachelor thesis titled "Qualitative comparison of DNA isolation methods from insect specimen" by myself and I have used the sources mentioned at the end of the thesis. As the author of the bachelor thesis, I declare that the thesis does not break copyrights.

In Prague on 30.03.2023.

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ABSTRACT

Correct identification of the species is one of the most crucial steps in forensic entomology. While morphological identification can be performed, it has a high margin of error, therefore making it a non-reliable source of information in forensic research. Molecular identification based on DNA has proven to be a significantly more accurate method and is the preferred way of identifying insects found on remains. The methods of obtaining the DNA vary in quality of the results, price and ease of handling. In this research, three different methods of extractions were performed and assessed. The DNA was isolated from previously collected and bred specimens of *Thanatophilus sinuatus* (Fabricius, 1775) and the specimens were stored in 96% EtOH. Two of the assessed methods of extraction were commercially available extraction kits: DNeasy Blood & Tissue Kit (Qiagen), NucleoSpin DNA Insect (Macherey-Nagel), and the third method used was direct PCR. Three different tissue amounts were also assessed throughout all three extraction methods: 1 leg, 2 legs and 3 legs. The amounts isolated were then measured using NanoDrop 1000 UV Visible Spectrophotometer and by Qubit 4 Fluorometer. The extracted DNA was followed by the amplification of the Cytochrome oxidase subunit I (COI) using the forward and reverse primer sequences. Obtained sequences were then visualized using the software Chromas 2.6.6. and blasted against GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The results showed that all three methods had a satisfactory performance in terms of obtaining the desired sequence. The NucleoSpin DNA Insect Kit has shown the best performance in all the assessed parameters, but the price per sample and the requirement of owning not so commonly used laboratory equipment had to be taken into account. The direct PCR method had the lowest success rate, although the price and handling time presented as a significant advantage. This research suggests that, in the field of forensic entomology where precision is of the utmost importance, the NucleoSpin DNA Insect Kit would be the best extraction method, while direct PCR method could be used in situations where the amount of samples is high and the available funds are low.

Key words: Coleoptera, Silphinae, *Thanatophilus sinuatus*, Forensic Entomology, DNA extraction, extraction kits, PCR

ABSTRAKT

Správná identifikace druhu je jedním z nejdůležitějších kroků v oblasti forenzní entomologie. Morfologická identifikace může mít vysokou míru chybovosti, což z může činit nespolehlivý zdroj informací v oblasti forenzního výzkumu. Molekulární identifikace založená na DNA se ukázala jako značně přesnější metoda a je preferovaným způsobem identifikace hmyzu nalezeného na pozůstatcích. Metody získávání DNA se liší kvalitou výsledků, cenou a snadností provedení. V této studii byly provedeny a hodnoceny tři různé metody extrakce. DNA byla izolována z dříve sbíraných a odchovaných exemplářů druhu Thanatophilus sinuatus (Fabricius, 1775), které byly uloženy v 96% EtOH. Dvě z hodnocených metod extrakce byly komerčně dostupné extrakční kity: DNeasy Blood & Tissue Kit (Qiagen), NucleoSpin DNA Insect (Macherey-Nagel), třetí použitá metoda byla metoda přímé PCR. Tři různé množství tkáně byly hodnoceny v rámci všech tří metod extrakce: 1 noha, 2 nohy a 3 nohy. Izolované množství bylo poté měřeno pomocí spektrofotometru NanoDrop 1000 UV Visible a fluorometru Qubit 4. Extrahovaná DNA byla následována amplifikací Cytochrome oxidase subunit I (COI) a sekvenováním oblasti COI. Získané sekvence byly poté vizualizovány pomocí softwaru Chromas 2.6.6. a vyhledány v databázi GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Výsledky ukázaly, že všechny tři metody vedly k produkci fragmentů k amplifikaci COI. Kit NucleoSpin DNA Insect prokázal nejlepší výkon ve všech hodnocených parametrech cena na vzorek a požadavek na přístup k někdy ne tak často používaného laboratornímu vybavení). Metoda přímé PCR měla nejnižší úspěšnost, i když cena a doba manipulace představovaly významnou výhodu. Tato studie naznačuje, že v oblasti forenzní entomologie, kde je přesnost nanejvýš důležitá, NucleoSpin DNA Insect Kit byl nejlepší metodou extrakce, zatímco metodu přímého PCR by bylo možné použít v situacích, kdy je vysoký počet vzorků a jsou omezené finanční prostředky.

Klíčová slova: Coleoptera, Silphinae, Thanatophilus sinuatus, soudní entomologie, extrakce DNA, extrakční kity, PCR.

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1. Introduction and objectives

1.1 Introduction

Extraction of DNA is often a crucial step in species identification, especially in cases where the species possesses a multitude of immature forms, making the visual recognition based on species specific morphological characteristics challenging. In forensic entomology, the species recognition plays a significant role in providing information about the time of death and conditions in which the body was stored. When using arthropod specimen in death investigations, the first and most crucial step is accurate species identification (Joseph et al., 2011). This, however, may pose a major problem if entomologists are confronted with large amounts of partial arthropod remains such as exuviae, limbs, and unidentified arthropod biomass however, molecular methods such as polymerase chain reaction (PCR) and consequent sequencing can provide further insight into the species identification (Chimeno et al., 2018).

The process of DNA extraction has been significantly simplified with the use of commercially available DNA extraction kits, although the quality of the kits varies as well as the range of price per sample. In this research, the performances of two different commercially available kits: DNeasy Blood & Tissue Kit (Qiagen) and NucleoSpin DNA Insect (Macherey-Nagel), and direct PCR method were compared in use for species delimitation. Alongside the comparison between the three methods separately, three different tissue amounts were defined and used for each method in order to analyze the impact of the amount of tissue processed on the results of the DNA extraction.

1.2 Objectives

This research primarily focuses on the comparative qualitative analysis of the three different methods of DNA from a carrion beetle *Thanatophilus sinuatus* (Fabricius, 1775), in the context of forensic entomology. Therefore, the analysis of the obtained data is done with not only the price and handling time in mind, but also the quality of the yield from the tested extraction method and the ability to use the material for further testing. The aim of the study is to provide a comprehensive overview of all of the used methods and scale them based on the observed parameters.

2. Literature review

2.1 Species studied

The model species in this research is *Thanatophilus sinuatus* (Fabricius, 1775) (Coleoptera: Silphinae).

Genus *Thanatophilus* (Leach, 1815) belongs to the family Silphinae and contains 23 described valid species (Růžička et al., 2015; Anderson et al., 1985; Navarette-Heredia, 2009). Most of these species occur in the Northern Hemisphere (Holarctic distribution). The members of the genus don't only share the general morphological characteristics, but also have a very similar ecology. Each of the known species are necrophagous in all active stages of development, both as larvae and as adults, and they appear on carrions of large vertebrates, most commonly over the size of 300g, including humans (Midgley & Villet, 2009). They tend to prefer early or mid-stages of decomposition and can begin the breeding process within the first 24 h after death, making them direct competitors with flies (Diptera) for food resources (Midgley & Villet, 2009; Peck, 1990; Sikes, 2005; Payne, 1965; Anderson, 1982). These features make them a very promising group of beetles that could be used as bioindicators in the field of forensic entomology (Jakubec et al., 2019).

Necrophagous beetles are a useful ecological group of insects in the field of forensic entomology (Ridgeway et al., 2014; Charabidze et al., 2016; Midgley et al, 2010). In many cases, they can provide the estimates of the postmortem colonization on remains and the postmortem interval (PMI) as accurate as other groups of insects (e.g., blowflies (Calliphoridae)) (Watson & Carlton, 2005; Midgley et al, 2010). While the list of potentially forensically useful necrophagous beetles is long, only fraction of them can be successfully used as there is a lack of the necessary basic information about their morphology, in particular species and instar identification, and biology, primarily thermal summation models (Jakubec et al., 2019).

T. sinuatus is one of the most abundant and widespread species of the genus *Thanatophilus* (Montoya-Molina et al., 2020). It has a very wide trans-Palearctic distribution, commonly occurring in Europe, Asia, and North Africa (Růžička et al., 2015; Jakubec & Růžička, 2012).

T. sinuatus has a high forensic importance. Both adult and larvae specimens were noted on 13.27% (26 out of 196) of human remains by forensic entomologists in the Czech Republic (Jakubec & Růžička, 2012). It has also been reported that individuals of the species occasionally replace blowflies (Diptera), which is one the crucial groups in forensic entomology, during the colder parts of the year (Bonacci et al., 2011).

Females are semelparous and show no parental care for their offspring. The eggs are typically laid in or on the soil around large vertebrate carcasses (Sikes, 2005; Ikeda et al., 2008). Eggs typically hatch in 4-5 days and larvae feed on carrion remains (Anderson, 1982).

The re-description of the larval stages was done by Jakubec et al. (2019). The need for the thorough re-description came from the fact that *T. sinuatus* often co-occurs with *T. rugosus* (Jakubec & Růžička, 2015; Frątczak-Łagiewska & Matuszewski, 2018). It has proven very difficult to separate these two species morphologically in the earlier larval stages (Von Lengerken, 1929). Only the third instar can be definitively identified to the species level (Novak et al., 2018; Frątczak-Łagiewska & Matuszewski, 2018). Even though the two species possess many similarities, it is generally not safe to assume that the information they provide is interchangeable (Jakubec et al., 2019).
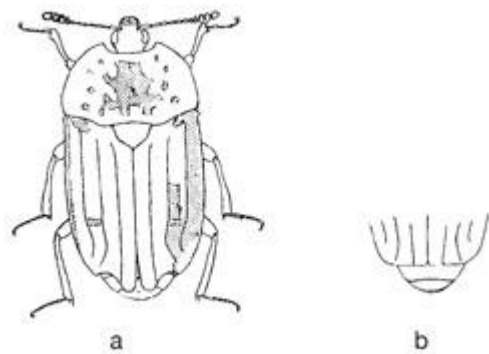

Figure 1. (a) Female *T. sinuatus*. (b) Details of the elytra of male *T. sinuatus.* (Potrevin, 1926)

2.2 Forensic entomology

2.2.1 Definitions and applications of forensic entomology

Forensic entomology is most commonly defined as the analysis of insect evidence for forensic and legal purposes (Amendt et al., 2007). The most important and frequently requested task of this field lies in the estimation of the minimum time since death (also called post-mortem interval (PMI)) (Catts, 1992).

The recently devised techniques allow forensic entomologists to collect evidence that can provide important information in death investigations and answer questions regarding movement and storage of the remains after death, time of injury, trauma site identification, use of drugs and others, connecting suspects to scenes of crimes and their further identification (Campobasso & Introna, 2001). It is also possible to confirm the period of neglect of living humans or animals by analyzing and identifying the insects recovered from infested wounds (miasis) (Amendt et al., 2011).

As mentioned, one of the most important tasks within forensic sciences is estimating the postmortem interval (PMI) of a deceased individual (Alibegović, 2014). PMI is, in the simplest of terms, the period between the occurrence of death and the discovery of the remains (Byrd et al., 2009). In forensic entomology, this term relies on the assumption that insects which are typically associated with decomposing remains appear on the body very shortly after death (Morris et al., 2005).

While numerous medical techniques exist for making this types of estimations, particularly in the field of pathology, those are usually only applicable in the first 72 hours after death, mainly because of the deterioration of the conditions needed for those analysis due to the ongoing decomposition of the body (Amendt et al., 2013). Therefore, when dealing with cases where the body is found in more advanced stages of decomposition, other fields of expertise are necessary for providing clarification, which is where forensic entomology represents a significant source of information, assuming that the colonization is possible and not prevented by limiting factors, such as cold weather. Since the colonization of the remains by arthropod species is assumed to coincide with the beginning of the initial stages of decomposition, which occurs immediately after death, forensic entomologists are able to estimate the PMI, also currently known as time of colonization (ToC) by analyzing the arthropod samples taken from the decomposing remains (Gennard, 2007).

2.2.2 Importance of extracting DNA in forensic entomology

Generally, when using arthropod specimen in death investigations, the first and most crucial step is accurate species identification (Joseph et al., 2011). This, however, may pose a major problem if entomologists are confronted with large amounts of partial arthropod remains such as exuviae, limbs, and unidentified arthropod biomass (Chimeno et al., 2018). Even intact specimens pose a large burden when wanting to apply morphological methods, as eggs, early larval stages, and sometimes even later stages of many different species share similar features making it close to impossible in certain groups, even for a specialized taxonomist, to distinguish between them based on morphology alone (Amendt et al., 2013).

Molecular identification of insects feeding on corpses can be an important technique in species determination, particularly if the indeterminate larval species are recovered at a crime scene. In casework, the life stages collected from a corpse are reared to the adult stage in order to identify the species using morphology (Gennard, 2012). Additionally, the species specific thermal summation models for calculation of PMI can be applied on adult individuals. These models are generally more precise then species specific Isomegalen or Isomorphen diagrams that are used to estimate PMi based on size of the larvae alone(Richards et al., 2008).Identification based on morphology is a slow and often unsuccessful process, so using molecular methods, alongside morphological identification, may, on occasion, be a more rapid and accurate way of providing the basis for determining the PMI. In all instances, specimens for molecular analysis should be killed and stored appropriately and any possibility of contamination minimized (Gennard, 2012).

2.3 DNA extraction

2.3.1 Mechanism of DNA extraction

Deoxyribonucleic acid (DNA) extraction, also known as isolation, is the process by which DNA is separated from proteins, membranes, and other cellular material contained in the cell from which it is recovered (Elkins, 2013). The process of DNA extraction is not a novelty, as the earliest DNA extractions dates back to the 19th century, when Fredrich Miescher performed the first recorded DNA extraction. This extraction was performed in 1869 during his study of cellular function of leucocytes (Dahm, 2005).

Extraction is frequently one of the most labor-intensive steps of DNA analysis. Extraction methods sometimes require an overnight incubation (in the second step Fig. xy), although protocols that can be completed in minutes or a couple of hours do exist, alongside some recent procedures that use reagents which allow this step to be skipped completely. (Elkins, 2013).

Because of the lipid structure of the cell and nuclear membranes, presence of proteases and magnesium, and coiling of DNA around histones, many of the available DNA extraction procedures have common elements. The extraction of DNA generally consists of three basic steps:

1. Lysis of the cell membrane to release DNA into the lysis solution.

2. Enzymatic digestion and/or denaturation of proteins to unsequester DNA from nucleoproteins.

3. Isolation of the DNA - separation of DNA from other cellular components and inhibitors (Elkins, 2013, Chong et al., 2021).

The disruption of cell membrane can be done using any of the several possible methods such as detergents, like sodium dodecyl sulfate (SDS), dithiothreitol (DTT) for reducing the disulfide bonds or using heat to increase the fluidity of the cell membrane. The inactivation of proteins, including nucleases, can be done by heat denaturation or by using digestive enzymes, most commonly proteinase K. In situations where nondegraded DNA of high molecular weight is required, the temperature should be maintained below 60°C and the time period should be kept short, anywhere from 15 to 20 minutes. The solid phase immobilizes the magnesium,

without which the activity of the DNA would not be possible. This is followed by the elution of the immobilized material by buffer or salt. Centrifugation is done if the DNA remains in the aqueous phase. This is done to separate the DNA from other cellular materials, such as proteins and lipids. Another way to achieve this is by partitioning them in organic solvents (Elkins, 2013).

2.3.2 Extraction kits
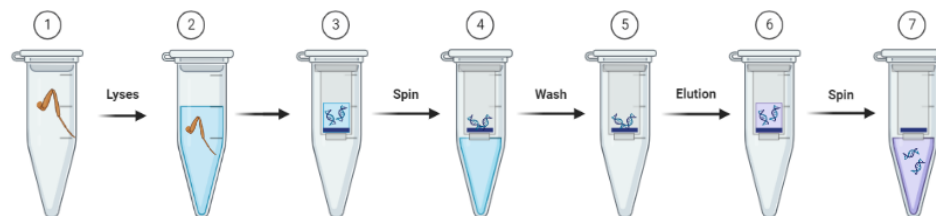
Figure 2. Step-by-step description of the DNA extraction procedure by commercially available extraction kits (BioRender.com)

DNA extraction kits are a simple way of rapidly obtaining high-quality DNA samples from different sources, for example saliva, blood, and animal or plant tissues. The extraction kits include pre-packaged chemical reagents and often include additional supplies, usually those that are kit-specific and aren't usually found a regular laboratory setup. Most commonly, these include spin columns, bead-beating tubes and magnetic stands (DNeasy Blood and Tissue Kit User Handbook, 2021; QIAamp DNA Mini Kit Handbook, 2016).

While there are variations, most of the commercially available DNA extraction kits include the following components:

1. Lysis buffer – solution containing detergents, salts and other chemicals used for dissolving the membranes and release of the DNA into the solution.
2. Proteinase K – enzyme that breaks down proteins in the sample, like the ones that may inhibit DNA extraction or further extraction steps;

3. Wash buffers – main purpose is to remove impurities (salts, proteins and similar contaminants);

4. Elution buffer – a solution that dissolves the DNA-bound matrix and releases the purified DNA into the solution (PureLink Genomic DNA Mini Kit User Guide, 2021; DNeasy Blood and Tissue Handbook, 2021; QIAamp DNA Mini Kit Handbook, 2016).

A vast variety of commercially available DNA extraction kits is available and each offers a specific protocol. Based on some common elements and procedures, the kits can be grouped into several categories, such as:

1. Spin-column kits, which use spin columns filled with DNA binding materials, usually silica, which are subsequently eluted from the column in wash and dilution steps. These include GeneJET Genomic DNA Purification Kit and PureLink Genomic DNA Mini Kit by Thermo Fisher Scientific, as well as QIAamp DNA Mini Kit by Quiagen (PureLink Genomic DNA Mini Kit User Guide, 2021; QIAamp DNA Mini Kit Handbook, 2016; GeneJET Genomic DNA Purification Kit Handbook, 2021).

2. Magnetic bead kits, in which the beads have a covering from a specific DNA binding ligand. Once the binding is complete, a magnetic field is used to separate the beads from the sample and a buffer solution is applied in order to elute the DNA from the beans. Some of the examples of such kits include MagMAX DNA Multi-Sample Kit by Thermo Fisher Scientific and PowerMag DNA Isolation Kit by Mo Bio Laboratories (MagMAX DNA Multi-Sample Kit Manual, 2017; PowerMag DNA Isolation Kit User Manual, 2020).

3. Organic extraction kits, which use organic solvents in order to perform the extraction of the DNA from the samples. Organic solvents most often include phenol or chloroform, amongst others. This step is usually followed by DNA precipitation, typically done by using ethanol, after which the DNA is resuspended in a buffer solution. The extraction kits used in this research, DNeasy Blood and Tissue Kit by Quiagen and NucleoSpin DNA Insect by Macherey-Nagel, fall into the category of organic extraction kits (DNeasy Blood and Tissue Kit User Handbook, 2021; NucleoSpin DNA Insect Kit User Manual, 2016)
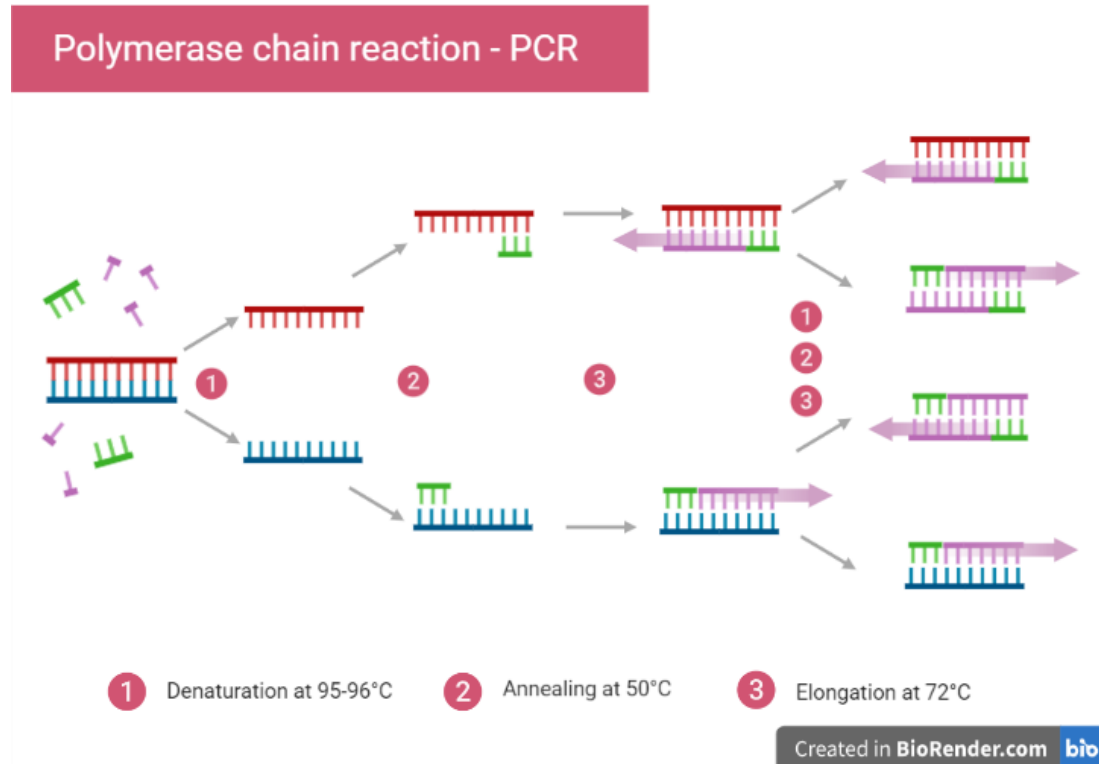
2.4 Polymerase Chain Reaction (PCR)

Figure 3. Visual description of the polymerase chain reaction (PCR) procedure (BioRender.com)

The polymerase chain reaction, or PCR, is a fast and inexpensive way of producing a large quantity of DNA from an often very small quantity of template DNA (Reed et al., 2016). The result of PCR is a significantly amplified sample of a chosen region from the original DNA sample. This chosen region can be any region of the DNA molecule, as long as those region's bordering sequences are known. This is due to the fact that two short oligonucleotides, one for each strand of the double helix, have to hybridize to the DNA molecule. The size of the target sequence doesn't have to be large, since PCR is extremely sensitive and is able to successfully amplify the sample even with just one starting molecule.

The PCR premix typically consists of forward and reverse primer, dNTPs, ddH$_2$O, MgCl and polymerase I enzyme, also known as *Taq* polymerase, which has replaced the DNA polymerase from *Escherichia coli* that was previously widely used (Chien

et al., 1976, Brown, 2016). *Taq* polymerase, which is isolated from *Thermophilus aquaticus*, a bacteria living in extreme temperatures, is very thermostable, which makes it resilient to denaturation deriving for heat treatment. This property is one of the pillars of the PCR methodology success (Brown, 2016).

The PCR procedure commences with creating a mixture from *Taq* polymerase and target DNA (Brown, 2016). One cycle of PCR is made up of three distinct steps, which are carried out at different temperatures. These three steps include:

1. Denaturation of double stranded DNA, in which the mixture is heated to the temperatures of 94-98°C. At this temperature, the hydrogen bonds that hold the two polynucleotides of the double helix break, resulting in target DNA becoming denatured into single stranded molecules. (Reed et al., 2016).
2. Annealing of the primers, where the temperature is decreased to anywhere from 37-65°C. This allows for a partial rejoining of single strands and the attachment of the primers to the annealing positions.
3. Extension of the primers is the final step, where the temperature once more gets raised, this time to 72-74°C.This temperature is right below the optimum for the *Taq* polymerase.

In the first stage of PCR, a synthesis of 'long products' is achieved from each of the target DNA strands. The 5' ends of these polynucleotides are the same, but the 3' ends are random. The randomized 3' ends represent the positions where DNA terminates by chance (Brown, 2016).

The three-step cycle of denaturation, annealing and synthesis is then repeated a multitude of times. In the first repetition, the denaturation of the long products happens, and, during the DNA synthesis stage, the four resulting strands are copied, resulting in four double stranded molecules. Two of those double stranded molecules are identical to the long products obtained in the first PCR cycle, and the remaining two are composed of completely new DNA. During the second repetition, or the third cycle, the two double stranded molecules composed of new DNA result in the production of 'short products', which have both 5' and 3' ends set by the primer annealing positions. These 'short products" double in numbers during subsequent cycles, growing exponentially until one of the components of the reaction is fully depleted. In terms of numbers, the amount of 'short products' after 30 cycles is

above 130 million from every starting molecule, meaning that it's possible to produce several micrograms of product from just a couple of nanograms of target DNA (Brown, 2016; Reed et al., 2016).

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is one of the most commonly used methods of DNA product separation and observation. The separation is done by creating an electrical field which then results in movement of the nucleic acid. To achieve this, an appropriate amount of gel is mixed and poured into a mold, making sure that wells for loading the DNA also exist.

After the gelation process is complete and the device is set up, the DNA is loaded into the wells and a current is applied. Molecules of negative charge then migrate towards the positively charged anode, at varying speed, depending on their size. Molecules of larger molecular weight tend to migrate towards the anode slower than the ones with a small molecular weight, creating a separation on the gel. The results of agarose gel electrophoresis are bands which can be visualized under UV light. To achieve visualization, it is necessary to mix a suitable dye into the solution during the initial preparation of the material for electrophoresis (Yilmaz et al., 2012).

Ethidium bromide is a commonly used dye for sample visualization in electrophoresis. It is a fluorescent dye that intercalates between nucleic acid bases, making the detection of the fragments of nucleic acid in the gels significantly easier (Sharp et al. 1973; Boffey, 1984; Lodge et al. 2007; Harrington, 1993; Lane et al., 1992). It is, however, important to note that ethidium bromide is a potentially carcinogenic substance and should be handled with great care. GelRed has appeared as an alternative to ethidium bromide, but the use is still not as widespread (Yilmaz et al., 2012).

Once sufficient time has passed for DNA migration in the gel, the gel is taken out and an ultraviolet lamp is used to illuminate the gel. The most common way of illuminating and visualizing the results is by using a light box, which had an integrated illumination system and can take images of the gel with the presence of UV light. The stained nucleic acid had a color that is between red and orange, but the images presented are typically shown in black and white (Yilmaz et al., 2012).

3. Materials and methods

3.1 Sample collection

The initial specimen used in the research were collected using two baited pitfall traps, placed at the district of Lysolaje, Prague, Czech Republic (50.1289519N, 14.3670008E). Traps were created by digging a hole that was 20 cm deep with the radius of 20 cm. A hard plastic recipient with drainage holes at the bottom was then placed into the hole and any potential gaps between the recipient and the surrounding soil were corrected. A loose layer of soil was then placed at the bottom of the recipient and the slightly aged poultry at the beginning stages of decomposition was put on top of it. The traps were covered with wiring and metal roofing, in order to protect the traps from the elements and the destruction by larger predators, such as foxes, cats and dogs. The collection process was performed from the months of February to June, with the traps being checked in one-week intervals.

The specimens were collected from the traps, identified, and taken to the incubators, where they were carefully monitored and bred. After reaching maturity, individuals were secluded and placed in 96% ethanol-filled tubes for preservation. The tubes were then labeled numerically, with addition of the abbreviated species name and the

date of the labeling.
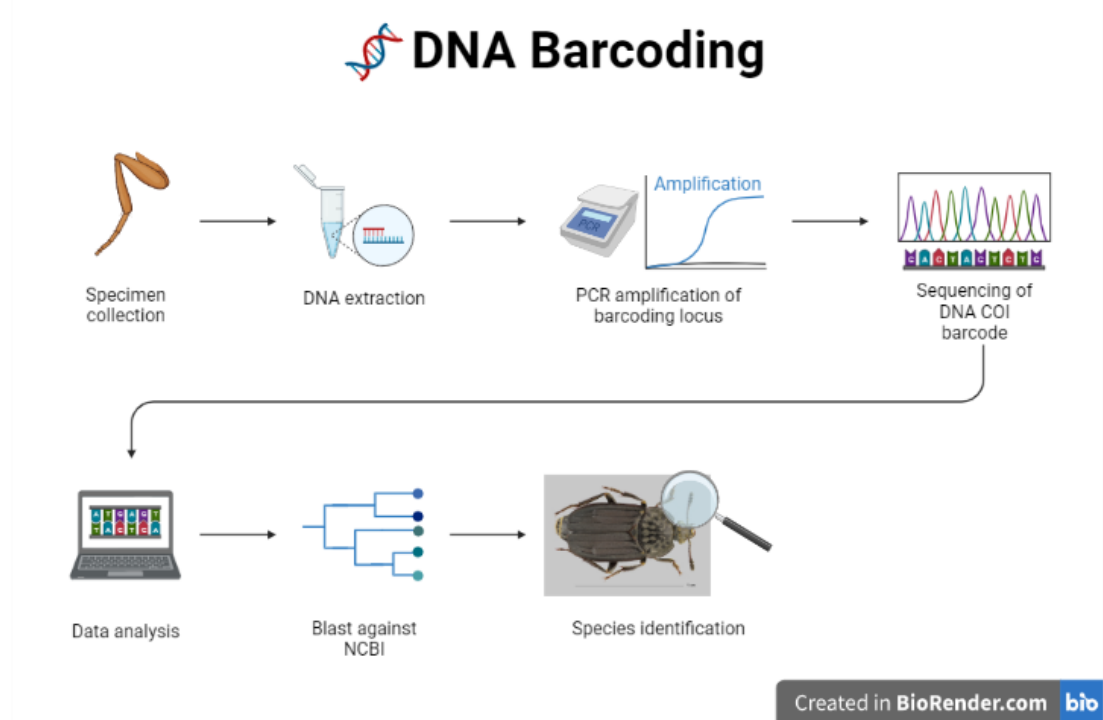
3.2 Design of the DNA extraction

Figure 4. Description of the design of the experiment, starting with the DNA extraction, PCR amplification, sequencing the DNA COI barcode and ending in blasting to provide confirmation of correct identification (BioRender.com)

3.2.1 Sample amount

Apart from comparing different methods of DNA extraction, the comparison between different amounts of tissue was done for each of the methods. Three different amounts of tissue were assessed, expressed through the number of legs extracted from the individuals.

The tissue amounts were defined as follows: one leg (marked as 1L), taken from the first pair; two legs (marked as 2L), taken from the second and third pair; three legs (marked as 3L), taken from the first, second and third pair. The two and three leg samples used one leg from each pair, usually all taken from the same side of the body in order to ensure consistency in the amount of tissue extracted and avoid the aspect of human error.

3.2.2 Sample preparation

At the start of sample preparation, the workspace was prepared. The cleaning of workspace and the tools was done using 75%EtOH. The individual specimens were then taken out of their tubes and placed on a previously disinfected surface. The legs were methodically removed and placed into 1.5 ml microcentrifuge tubes. Between each individual, the tools used for removal of the tissue were cleaned using 75%EtOH and an alcohol burner lamp to prevent contamination.

The tubes containing the tissue were subsequently named using the identifying number of the specimen used, an abbreviation of the species name, the appropriate abbreviation of the amount of tissue and the abbreviated name of the person performing the DNA extraction.

3.2.3. DNA extraction

The DNA extractions were done using two different commercially used extraction kits and direct PCR. All three tissue amounts were tested for each of the methods. For the extraction kits, the legs were removed from the body and processed. For direct PCR, the legs were cut into smaller pieces after removal and then processed.

The extractions done with the extraction kits were performed by protocol, with modifications being made in the lysis time of the DNeasy Blood and Tissue Kit, which was raised from 1 hour to 2 hours. Both kits were completed in two stages, with half of the samples being processed in each stage. The extractions for the NucleoSpin DNA Insect (Macherey-Nagel) Kit were performed on the 15th of November 2022 and 21st of November 2022. The extractions for DNeasy Blood & Tissue Kit (Qiagen) were performed on the 27th of May 2022 and 6th of June 2022.

The protocol for the NucleoSpin DNA Insect (Macherey-Nagel) kit:

1. Place the extracted tissue in NucleoSpin Tubes Type D.
2. Add 100 μl of Elution Buffer BE.
3. Add 40 μl of Buffer MG.
4. Add 10 μl of Proteinase K.

5. Place the closed tubes with the sample into Retsch mixer mill at 30 Hz for 3 minutes.

6. Centrifuge the samples at 11000 xg for 30 seconds.

7. Add 600 µl of Buffer MG.

8. Vortex each sample for 3 seconds.

9. Centrifuge the samples at 11000 xg for 30 seconds.

10. Place the NucleoSpin DNA Insect Columns into 2 ml Collection Tubes.

11. Transfer the 550 µl of Supernatant to NucleoSpin DNA Insect Columns.

12. Centrifuge the samples at 11000 xg for 30 seconds.

13. Discard the Collection Tubes and place the NucleoSpin DNA Insect Columns into new ones.

14. Add 500 µl of Buffer BW.

15. Centrifuge the samples at 11000 xg for 30 seconds.

16. Discard the flowthrough.

17. Add 500 µl of Buffer B5.

18. Centrifuge the samples at 11000 xg for 30 seconds.

19. Discard the flowthrough.

20. Centrifuge the samples at 11000 xg for 30 seconds.

21. Place the NucleoSpin DNA Insect Columns into 1.5 ml tubes.

22. Add 100 µl of Elution Buffer BE.

23. Incubate the samples at room temperature for 1 minute.

24. Centrifuge the samples at 11000 xg for 30 seconds.

25. Discard the NucleoSpin DNA Insect Columns.

26. Close the 1.5 ml tubes and label them.

The protocol for DNeasy Blood & Tissue Kit (Qiagen):

1. Place the extracted tissue into a 1.5 ml tube.

2. Add 180 µl of Buffer ATL.

3. Add 20 µl of Proteinase K.

4. Vortex each sample for 5 seconds.

5. Incubate the samples on the heating block at 56° C with constant mixing for 2 hours.

6. Vortex each sample for 15 seconds.

7. Add 200 µl of Buffer AL.

8. Vortex each sample for 5 seconds.
9. Incubate the samples on the heating block at 56° C for 10 minutes.
10. Add 200 μl of Ethanol.
11. Vortex each sample for 5 seconds.
12. Place Columns into Collection Tubes.
13. Transfer the Supernatant into the Columns.
14. Centrifuge the samples at 6000xg for 1 minute.
15. Discard the Collection Tubes and place Columns into new ones.
16. Add 500 μl of Buffer AW1.
17. Centrifuge the samples at 6000xg for 1 minute.
18. Discard the Collection Tubes and place Columns into new ones.
19. Add 500 μl of Buffer AW2.
20. Centrifuge the samples at 20000xg for 3 minutes.
21. Discard the Collection Tubes and place Columns into 1.5 ml tubes.
22. Add 200 μl of Buffer AE to the center of the Column.
23. Incubate the samples at room temperature for 1 minute.
24. Centrifuge the samples at 6000xg for 1 minute.
25. Discard the Columns.
26. Close the 1.5 ml tubes and label them.

Direct PCR extraction of DNA was done using fresh tissue, without any previous extraction procedures preceding it. There was, however, a difference in the sample handling, as the tissue had to be completely submerged in the chemicals that are used in this method. This means that for direct PCR, unlike for the DNA extraction kits where the legs were used in their entirety without any manipulation, the tissue first had to be cut down and grinded into small pieces that would be able to lie on the bottom of the tube. The subsequent procedure was fairly short, and the protocol after tissue manipulation and placement into tubes went as follows:

1. Add 20 μl of PBS buffer.
2. Incubate the samples on the heating block at 98°C for 2 minutes.
3. Close the 1.5 ml tubes and label them.

A total of 74 samples were extracted with the three extraction methods. For each method, 24 samples were extracted, divided into three tissue amounts, resulting in 8 samples per tissue amount.

3.2.4 Measuring the isolated DNA

The yield of isolated DNA was determined by using two different methods: spectrophotometry and fluorometry. The machines used were NanoDrop 1000 UV Visible Spectrophotometer and Qubit 4 Fluorometer.

NanoDrop 1000 UV Visible Spectrophotometer is used to measure the concentration and purity of nucleic acids in a sample, although it is important to note that it can also be used in measuring other biomolecules. Due to the fact that the system employs short path lengths, a wide range of nucleic acid concentration measurements are able to be performed. Alongside that, the necessary volume used in the analysis is quite low (1-2 μL), allowing for multiple reruns where necessary and minimizing waste. The procedure for usage is relatively uncomplicated and easy to follow, aided by the user friendly interface of the accompanying computer program (Desjardins & Conklin, 2010; NanoDrop 1000 Spectrophotometer User's Manual).

Qubit 4 Fluorometer is a device used for quantification of DNA, RNA, microRNA and protein, amongst other measurements. It functions by using fluorescent dyes, binding to the target molecules. The steps involved in measurement can be categorized into three phases:

1. Sample preparation – depending on the target molecule, the sample preparation is done as per protocol provided;
2. Fluorescence measurement – the Qubit assay tubes are loaded with the prepared sample and are placed into the machine, after which the wavelength of light is emitted, exciting the fluorescent dye and resulting in the emission of a signal proportional to the amount of target molecule in the sample;
3. Data analysis - the signal is received and converted into a concentration value, which is then displayed wither on the screen of the instrument or exported on a connected computer (Qubit™ 4 Fluorometer User Manual).

Compared to the NanoDrop 1000 Spectrophotometer, the sample preparation in for this method is slightly more complex and time consuming, as well as expensive.

For NanoDrop 1000 UV Visible Spectrophotometer, the measurements were performed three times per sample. The measurement process started with the

calibration of the machine with the use of a blank sample, which in this case were the buffers used in each one of the methods of DNA extraction. Each one of the samples was measured three times, and the samples were processed in clusters of three or four, in order to avoid potential loss of data due to a mechanical error. The data was saved after each cluster of measurements in the form of a Microsoft Excel sheet through the "Record" function.

The measurements done by Qubit 4 Fluorometer were performed as per protocol. Two standards were determined before analyzing each of the given methods of extraction. Each sample was measured once. The measurements were transcribed and then manually added to the corresponding Microsoft Excel sheet.

3.3 Data processing

The data obtained with the use of NanoDrop 1000 UV Visible Spectrophotometer and by Qubit 4 Fluorometer was analyzed using RStudio, an integrated development environment for R, which is a programming language primarily used for statistical computing and graphics. The data was compiled together in a Microsoft Excel sheet, combining the data from all processed methods of extraction and measurment. The Microsoft Excel sheet was then saved as a text file and exported into RStudio for further processing.

Using RStudio, the data was processed and visualized by plotting. For higher statistical accuracy, the outliers were eliminated from the data processing procedure. The values processed were taken from the measurements made with NanoDrop 1000 UV Visual Spectrophotometer and Qubit 4 Fluorometer. Both machines provided the values of concentration of the samples and these values were primarily compared between each other. Apart from the concentration, NanoDrop 1000 UV Visual Spectrophotometer also provided other values:

- Abs260, or the absorbance at 260nm, representing the wavelength which is most absorbed by nucleic acids.
- Abs280, or the absorbance at 280nm, representing the wavelength which is most absorbed by proteins in the sample.

- Abs230, or the absorbance at 230nm, representing the wavelength most absorbed by salts and other organic compounds.
- 260/280, which is a ratio that shows if the sample is pure from protein contaminants. For DNA analysis, the optimal value of this ratio is around 1.80.
- 260/230, which is the ratio that shows if the sample is pure from contaminants which absorb the wavelength of 230 nm, such as salts and other organic compounds.

All the results from both machines were collected and evaluated in the context of the extraction kits and the amounts of tissue. The different tissue amounts were also processed individually for each kit.

3.4 PCR and Electrophoresis

After the extractions were completed and the measurements of concentrations of the yield from samples coming from the extraction kits was complete, each sample was used as a template for the amplification of Cytochrome oxidase I (COI) barcoding region, using one primer pair – Jerry and Pat. PPP Master Mix (Top Bio) was used according to manufacturer's instructions, using 12.5 µl of PPP Master Mix, 9.5 µl of H20, 1µl of 10mM Forward primer, 1µl 10mM Reverse primer, and 1 µl of the template DNA. PCR reaction was conducted in the total of 25 µl under following conditions:

1. 98°C 1x 30s
2. 98°C 35x 5s
3. 50°C 35x 5s
4. 72°C 35x 1 min
5. 72°C 1x 1:30 min
6. 4°C hold

The PCR products were visualized by electrophoresis using 1% agarose gel (100V 35 min). The gel was prepared by the following protocol:

1. Add 80 ml of the buffer to the Erlenmeyer flask.
2. Measure 0.8 g of the agarose.

3. Homogenize the solution by lightly mixing.

4. Heat the solution in the microwave for 1 minute and 30 seconds.

5. Cool off the solution by mixing while keeping the Erlenmeyer flask under cool water, making sure that no water enters the flask.

6. Add 2 µl of of ethidium bromide.

7. Mix lightly.

8. Slowly pour the mixture into a previously prepared mold.
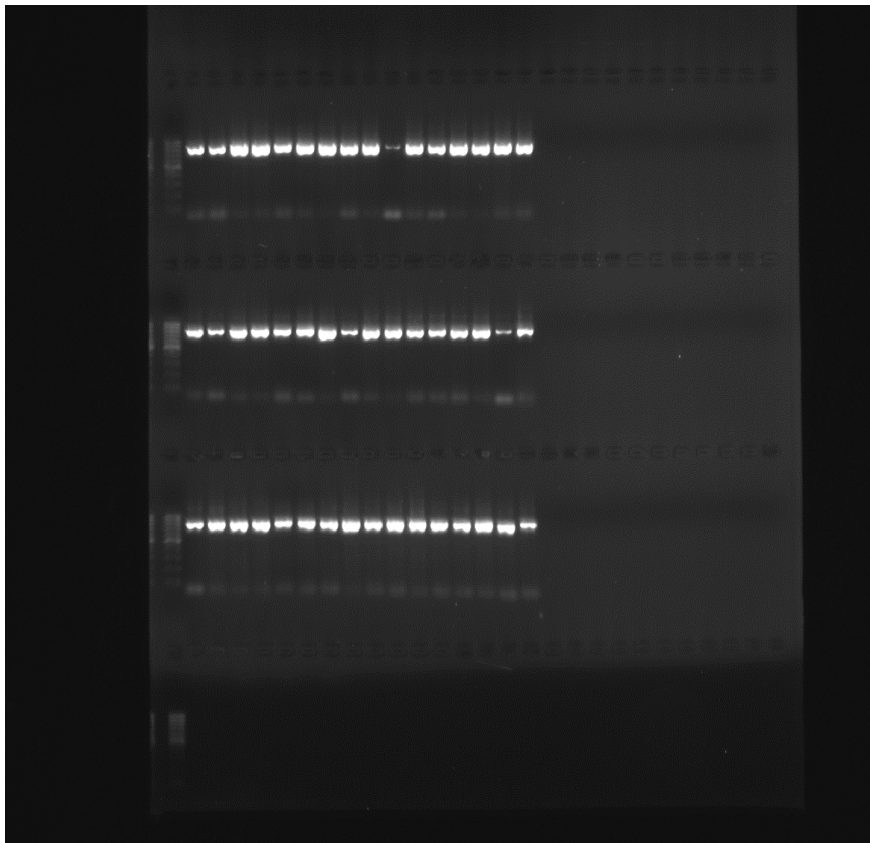
9. Place the combs into the mold.

Figure 5. Example of visualization of the amplified DNA using agarose gel electrophoresis (Mahlerova, 2022)

3.5 Sequencing

Amplified products of the PCR were prepared for unidirectional sanger sequencing. Firstly, each sample was purified using IT Exosap according to the provided protocol and the forward primer was added.

The obtained sequences were visualized using Chromas v2.6.6., trimmed based on the sequence quality. The trimmed sequence was then used as a query for nucleotide BLAST (Basic Local Alignment Search Tool)(https://blast.ncbi.nlm.nih.gov/Blast.cgi). The hit accession, the E value, sequence length, and the percent of probability was noted in order to evaluate the obtained sequence for usage for barcoding of the organism.
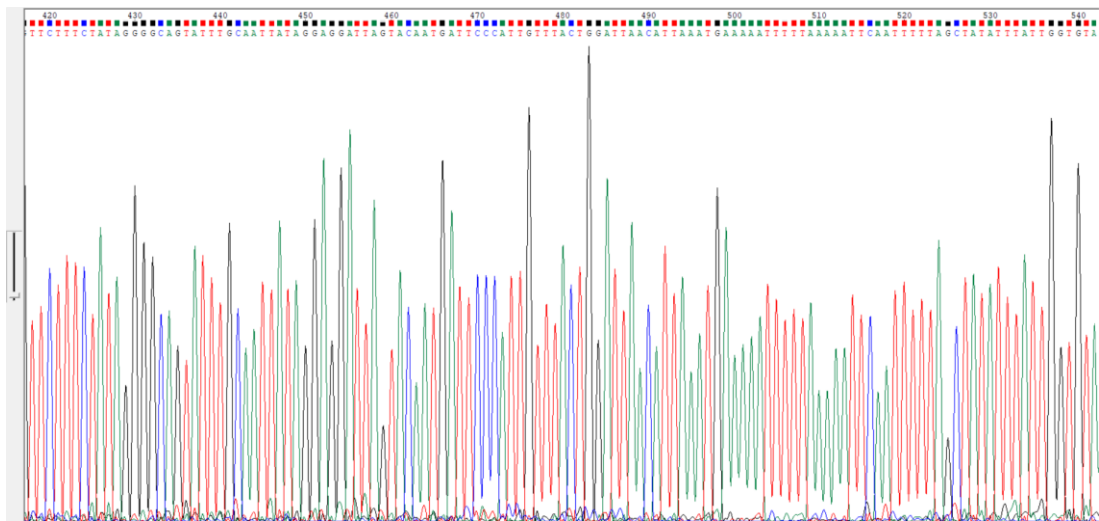

Figure 6. Visualized sample using Chromas v2.6.6.

4. Results

4.1 Methods of extraction

The concentrations obtained from extraction kits were analyzed with NanoDrop 1000 UV Visible Spectrophotometer and Qubit 4 Fluorometer. An important note has to be made when observing the two measuring methods regarding the obtained values, which are noticeably lower for Qubit 4 Fluorometer than NanoDrop 1000 UV Visible Spectrophotometer. Qubit 4 Fluorometer measures only the DNA in the sample, and while NanoDrop 1000 UV Visible Spectrophotometer tries to do the same, it usually also captures the other contaminants present in the sample alongside DNA, resulting in numerically higher readings.

The comparison of the obtained concentrations varied within the two measuring methods: NanoDrop showed a better result for the DNeasy Blood and Tissue Kit, while Qubit showed an overall better result for NucleoSpin DNA Insect Kit.
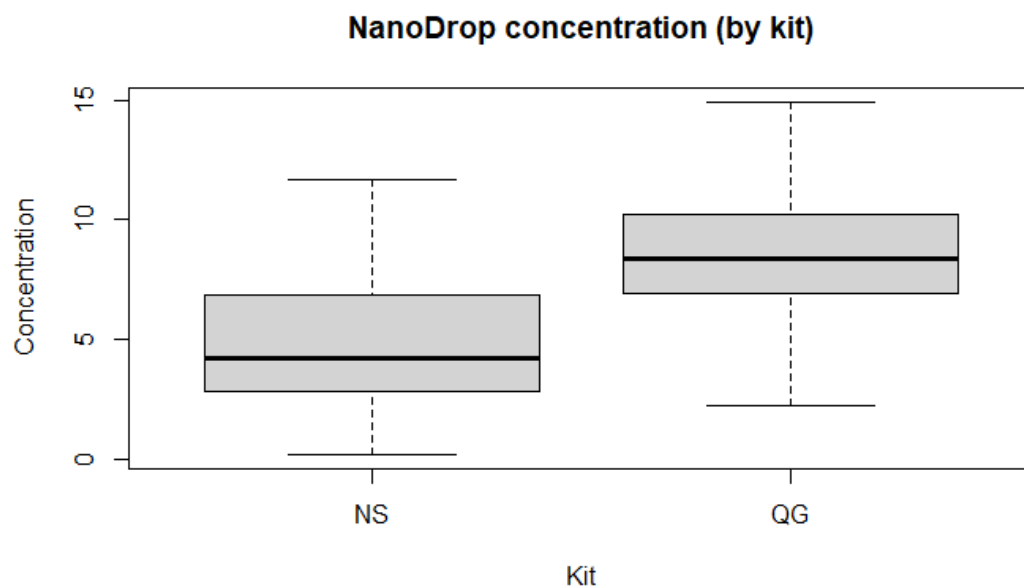

Figure 7. Boxplot representing the difference between concentrations measured by NanoDrop 1000 UV Visual Spectrophotometer. NS represents the NucleoSpin DNA Insect Kit and QG represents the DNeasy Blood and Tissue Kit.
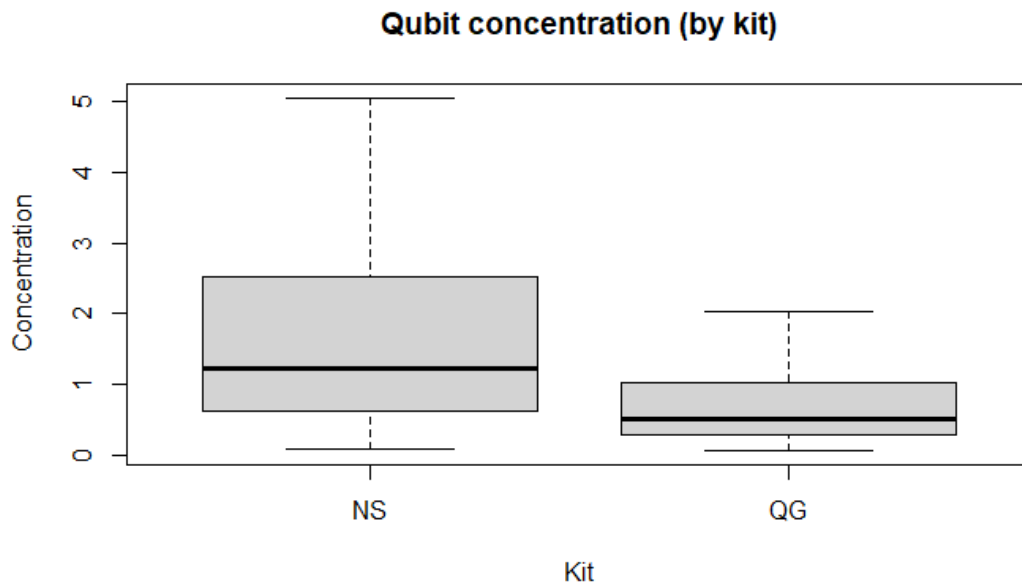
Qubit concentration (by kit)

Figure 8. Boxplot representing the difference between concentrations measured by Qubit 4 Fluorometer. NS represents the NucleoSpin DNA Insect Kit and QG represents the DNeasy Blood and Tissue Kit.

The results obtained using Qubit show a higher correspondence with the results shown by sequencing, where NucleoSpin DNA Insect Kit has shown a better result in terms of both query length and successful sequencing runs. Despite NucleoSpin DNA Insect Kit giving a better result, it is important to note that the difference in the numbers wasn't large and both of the kits gave a yield high enough for successful sequencing.

The number of successful sequencing runs for the direct PCR method was notably smaller than in for the two extraction kits, which was likely caused by human error. In terms of query length, however, the direct PCR method was not far behind DNeasy Blood and Tissue Kit.

Extraction method	Average concentration (NanoDrop) (µg/µl)	Average concentration (Qubit) (µg/µl)	Average query length for sequencing
DNEasy Blood and Tissue Kit	10.98194444	0.710347826	771
NucleoSpin DNA Insect Kit	8.248648649	2.576094595	787
Direct PCR	-	-	740

Figure 9. Comparisons of average numerical differences in concentrations between two commercially available DNA extraction kits, measured by both machines, as well as the comparison of average query length for sequencing including all three methods of extraction.

4.2 Tissue amounts

In analysis of the tissue amounts, all variables were assessed both collectively and individually within different extraction methods. In terms of concentrations of the samples obtained by extraction kits, Measurements of concentration done by NanoDrop for this parameter were dismissed due to inconclusiveness. Instead of concentration measurements, the ratio of 280/260 nm was considered as an alternative measure obtained from NanoDrop, and the results showed the best ratio for the highest tissue amount, which was three legs. The worst results were observed for one leg, but the difference between the best and worst average values was not large.

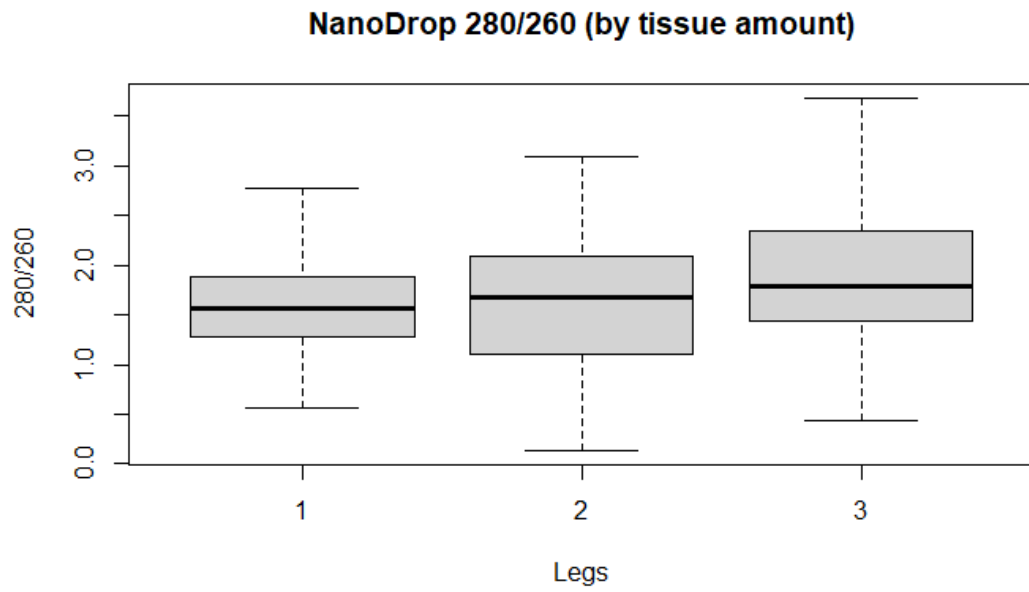**NanoDrop 280/260 (by tissue amount)**

Figure 10. Boxplot representing the 280/260 ratios measured by NanoDrop 1000 UV Visual Spectrophotometer for the three tissue amounts (1, 2 and 3 legs)
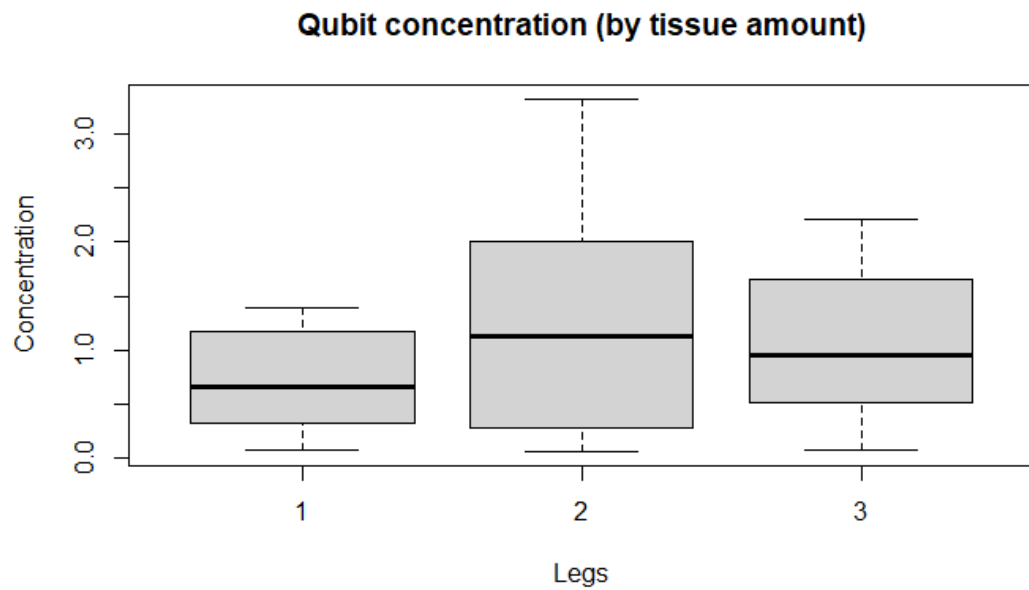

Qubit concentration (by tissue amount)

Figure 11. Boxplot representing the concentrations measured by Qubit 4 Fluorometer for the three tissue amounts (1, 2 and 3 legs).

Measurements for the concentrations based on tissue amounts by Qubit 4 Fluorometer have shown a different result. Samples from one leg results have performed significantly worse than with NanoDrop 1000 UV Visible Spectrophotometer, and the samples with the tissue amount of two legs showed the best results by a great margin.

In terms of sequencing, the results on average coincide with the measurements of concentrations done by Qubit 4 Fluorometer, with the longest average query length coming from the samples where the tissue amount was two legs and the shortest from the one leg samples. Therefore, the results obtained from Qubit 4 Fluorometer were considered as more accurate in terms of assessing the impact of the amount of tissue on the results of the extraction.

Tissue amount	Average concentration (Qubit) ($\mu g/\mu l$)	Average query length for sequencing
1 Leg	0.511	765
2 Legs	2.097625	793
3 Legs	1.49475	772

Figure 12. Average concentration measured by Qubit 4 Fluorometer and average query length for sequencing for the three tissue amounts (1, 2 and 3 legs)

4.3 Handling time and cost

The handling time and cost were taken into consideration in the assessment of the extraction methods.

The cost per sample calculations were done based on the prices for the extractions, without taking into account the additional costs for amplification of the samples, and gave the following results:

- 126.8 CZK/Sample for DNeasy Blood and Tissue Kit.
- 124.38 CZK/Sample for NucleoSpin DNA Insect Kit.

- 17.55 CZK/Sample for direct PCR.

The handling time was divided into two parts: the extraction of tissue and the completion of the experiment. The tissue extraction for DNeasy Blood and Tissue Kit and NucleoSpin DNA Insect was around 1 minute and 30 seconds per sample, while for direct PCR it took around 3 minutes per sample due to tissue manipulation. Opposite to that, the extraction of DNA took the least amount of time for direct PCR – 12 minutes for 12 samples – a minute per sample, whereas NucleoSpin DNA Insect Kit took approximately 10 minutes per sample and DNeasy Blood and Tissue Kit took 20 minutes per sample.

Extraction method	Price per sample (CZK)	Tissue extraction time per sample	DNA extraction time per sample
DNEasy Blood and Tissue Kit	126.8	1 minute and 30 seconds	20 minutes
NucleoSpin DNA Insect Kit	124.38	1 minute and 30 seconds	10 minutes
Direct PCR	17.55	3 minutes	1 minute

Figure 13. Comparison of the average prices per sample and handling times for tissue and DNA extraction, including all the extraction methods.

5. Discussion

The results of this research showed some significant differences between the three extraction methods. In terms of yield concentration and quality of the obtained sequences, NucleoSpin DNA Insect Kit has performed the best out of all the methods.

In forensic entomology, precise identification of the species is of critical importance. For this reason, the quality of the obtained sequences has to be taken into consideration.

Considering speed and cost per sample, direct PCR is the best out of all the analyzed methods. However, the number of unsuccessful samples is notably larger than in either of the extraction kits. Also, the manipulation of the material that is required prior to the procedure makes this method less straightforward and much more susceptible to accidental contamination. This method could be considered in situations where the number of samples is large and speed of processing is important, as well as in situations where there is limited funding.

Extraction kits are certainly more expensive than direct PCR, but there are advantages to their use. The chemicals require little to no preparation prior to first use and the protocols are fairly easy to follow, even for less experienced researchers. The obtained sequences had an almost 100% PCR success rate and sequencing success. In terms of forensic entomology, the extraction kits have the advantage of high yield and high precision. There are, however, some notable disadvantages to the extraction kits. Occasionally, the kits may require laboratories to use and to own very advanced and specific equipment. This, coupled with the significantly higher price per sample, may make the extraction kits unavailable to laboratories with less funding.

The NucleoSpin DNA Insect Kit performed the best, with the highest concentrations measured by Qubit and a100% rate of success in both PCR and sequencing. The cost per sample was smaller for this kit DNeasy Blood and Tissue Kit, although the difference in price was not large. The time of handling per sample, however, was significantly smaller than for DNeasy Blood and Tissue Kit, with a difference of almost 10 minutes, and the protocol was clear and easy to follow. One of the

disadvantages of this particular extraction method is the usage of specific equipment - Retsch mixer mill, which was only needed for this method.

As mentioned earlier, the importance of DNA extractions in forensic entomology lies in the accurate identification of the species collected from the crime scene. In such situations, the use of extraction kits in order to ensure successful extraction and accurate sequencing is justified, especially when the available samples for extraction are limited. Taking that into consideration, as well as the results of this research, the NucleoSpin DNA Insect Kit could be considered the best method for extraction of the DNA.

While not being a primary focus of the research, a significant difference was observed and noted between the two pieces of equipment used for measuring the concentrations of the extracted sample. Although NanoDrop 1000 UV Visible Spectrophotometer provided many different types of results apart from concentration of the sample and was easy to use, the readings were often inaccurate and susceptible to errors that could not be linked to human factor. Qubit 4 Fluorometer has performed significantly more consistently in the measurements of the obtained concentrations and the use of the machine itself was very simple and user friendly, but the preparation for the machine calibration and the sample preparation was time consuming and not as straightforward. The dissimilarity in performance between the two machines is somewhat explained by the price difference, since Qubit 4 Fluorometer is on average 1000$ more expensive than NanoDrop 1000 UV Visible Spectrophotometer.

6. Conclusion

NucleoSpin DNA Insect Kit has overall proven as the best method of extraction for the purpose of forensic research, due to the high success rate in downstream uses of the obtained sample. The price can be a limiting factor that should be taken into account, but the resulting yield from this method justifies the higher cost of sampling. Even though other methods, such as direct PCR, may take less time and have a lower cost, the possibility of unsuccessful results from a valuable and limited samples taken from remains is high. Therefore, when considering the three presented methods and the demands of the field of forensic entomology, NucleoSpin DNA Insect Kit can be recommended as the preferred method of extracting the DNA from the collected insect specimen.

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8. Figure references

Figure 1. Portevin, M.G. (1926). Les grands nécrophages du globe, Silphini-Necrodini-Necrophorini. Encyclopédie Entomologique (A). Vol. 6. *Paul Lechevalier.*

Figures 2., 3. and 4. were created by the author using BioRender.com.

Figure 5. Image taken by Ing. Karolina Mahlerová.

Figure 6. Visualization created by the author using Chromas v2.6.6.