**CZECH UNIVERSITY OF LIFE SCIENCES – PRAGUE**
Faculty of Environmental Sciences

# BACHELOR THESIS


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**CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE**

Faculty of Environmental Sciences

Bachelor

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**BACHELOR THESIS**

# Qualitative comparison of DNA isolation methods from insects specimen

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# CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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# BACHELOR THESIS ASSIGNMENT

Rodrigo Ordoñez

Environmental Engineering

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 nature conservation and biodiversity research with focus on cheap and fast methods that can be used to obtain DNA sequences.
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 (Direct PCR, Hot-Shot, and one commercially available DNA extraction kit).
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. One commercially used kits – DNeasy Blood & Tissue Kits (Geneaid), direct PCR and the HotShot method (Truett et al. 2000). 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 NanoDrop 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/ger

**The proposed extent of the thesis**

30

**Keywords**

DNA, isolation, entomology,

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**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**

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Dean

Prague on 27. 03. 2023

DIPLOMA THESIS AUTHOR'S DECLARATION

I hereby declare that the work presented in this thesis, to the best of my knowledge, is my independent original work, under the supervision of Pavel Jakubec. I have listed all literature and publications from which I acquired information.

21.03.2022

Rodrigo Ordoñez

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## Abstract English

This study compares the performance of different molecular methods, including DNA extraction kits and direct PCR in biodiversity research using the specimen model *Thanatophilus sinuatus*. By determining the efficacy and accuracy of these molecular techniques, we aim to identify the most effective method for enhancing the proper identification of targeted species. The results of this study will provide valuable insights into the strengths and limitations of each method and their future implications in the biodiversity research study field. DNA was extracted from specimens of *Thanatophilus sinuatus* using a commercial DNA extraction kit (Genomic DNA Mini Kit) and also performed direct PCR amplification on the same specimens without prior extraction. The efficacy and accuracy of these two molecular methods was compared in terms of DNA yield, quality, and suitability for downstream analyses, including PCR amplification and sequencing. The DNA was later amplified and sequenced the cytochrome c oxidase subunit I (COI) gene from each sample, and then compared the quality and reliability of the resulting sequences. Also the cost-effectiveness and time-efficiency of the two methods was compared. The results suggest that direct PCR amplification is a cost-effective and time-efficient alternative to commercial DNA extraction kits (Genomic DNA Mini Kit) for biodiversity research. Direct PCR produced PCR and sequencing results that were comparable to those obtained using the DNA extraction kit. Furthermore, direct PCR amplification was significantly cheaper and faster than the DNA extraction kit, making it a more practical and efficient molecular method for biodiversity research. The results of this study shows the reliability of Direct PCR method as a cost-effective and time-efficient alternative to the commercial DNA extraction kit used, for biodiversity research, giving out a valuable contribution to the field of biodiversity research studies.

**Key words:** DNA, isolation, entomology

## Abstract Czech

Tato studie porovnává výkonnost různých molekulárních metod, včetně sady pro extrakci DNA a přímé PCR, při výzkumu biodiverzity na modelu vzorku *Thanatophilus sinuatus*. Cílem je určit účinnost a přesnost těchto molekulárních technik a identifikovat nejúčinnější metodu pro zlepšení správné identifikace cílových druhů. Výsledky této studie poskytnou cenné poznatky o silných stránkách a omezeních každé metody a jejich budoucích důsledcích v oblasti výzkumu biodiverzity. DNA byla extrahována z exemplářů *Thanatophilus sinuatus* pomocí komerční sady na extrakci DNA (Genomic DNA Mini Kit) a také byla provedena přímá PCR amplifikace na těchto exemplářích bez předchozí extrakce. Účinnost a přesnost těchto dvou molekulárních metod byla porovnána z hlediska výtěžnosti DNA, kvality a vhodnosti pro další analýzy, včetně PCR amplifikace a sekvenování. DNA byla následně amplifikována a sekvencována gen cytochromu c oxidázy podjednotka I (COI) z každého vzorku a porovnána kvalita a spolehlivost výsledných sekvencí. Také byla porovnána nákladová efektivita a časová efektivita obou metod. Výsledky naznačují, že přímá PCR amplifikace je cenově efektivní a časově efektivní alternativou k sady pro extrakci DNA (Genomic DNA Mini Kit) pro výzkum biodiverzity. Přímá PCR produkovala výsledky PCR a sekvenování, které byly srovnatelné s těmi získanými pomocí sady pro extrakci DNA. Navíc byla přímá PCR amplifikace výrazně levnější a rychlejší než sada pro extrakci DNA, což z ní činí praktičtější a efektivnější molekulární metodu pro výzkum biodiverzity. Výsledky této studie ukazují spolehlivost metody přímé PCR jako cenově efektivní a časově efektivní alternativy pro použití sady pro extrakci DNA v oblasti výzkumu biodiverzity, a tím přináší cenný přínos oboru studií biodiverzity.



**Klíčová slova**: DNA, izolace, entomologie

Table of Content

# 1. Introduction

Biological diversity or biodiversity refers to the variety of living organisms from all different ecosystems and ecological complexes on the Earth (Gaston & Spicer, 2004). It encompasses a wide range of biological elements including genetic, morphological, and demographic variations within species, as well as interactions between organisms, and ecosystem diversity in the landscape (Fischer et al. 2010).

The biodiversity loss around the world keeps accelerating due to various anthropogenic disturbances, such as land use change, being a major driver for changes on the ecosystem, for example the fragmentation and degradation of land for agricultural or cultural purposes and the general decay of the environmental stability (Farooqi et al. 2022). As a result of this constant and alarming decline in biodiversity, the interest on the topic has also seen an increase on the functional part of biodiversity research which focus and explores the drivers and functional changes on biodiversity itself (Fischer et al. 2010).

Molecular technologies take part into the biodiversity research as tools to help identify species down to their taxonomic level, which provide essential information for its conservation as it can define and distinct specimens phylogenetic position. This allows to follow the specimens history, which information is important to manage and understand the populations dynamics and function on their ecosystem(Karp et al. 1997).

The following study is set to focus on the comparative analysis of different molecular techniques like DNA extraction method and Direct PCR as well as different quantities of the extracted material tissue from the carrion beetle species *Thanatophilus sinuatus* (Fabricius, 1775), for the better DNA evaluation and identification from the specimens, which will be conducted by testing two different molecular extraction methods and three different amounts of material tissue for each method. The outcome of the thesis will significantly improve future biodiversity study protocols by giving out a clear perspective of the quality of DNA yield for each extraction method and quantity of material tissue used.

## 2. Objectives

The thesis deals with the comparison and analysis of different techniques used in nature conservation and biodiversity research for the extraction of material tissue from the carrion beetle *Thanatophilus sinuatus* (Fabricius, 1775), and is set to focus on cheap and fast methods, which results that can later be used to obtain DNA sequences. Furthermore the research aims to determine and show the best reliable option from the tested methods.

## 3. Literary research

### 3.1 DNA extraction

The aspects and conditions of the DNA directly affect the analysis of genome structure and gene expression in the study of specific DNA sequences within complex DNA populations. Therefore, the DNA which compose a small percentage of the cell from which most of it, approximately 90%, is localized at the nucleus, while the rest can reside on different organelles present in the cell, has become a targeted compound, and the DNA extraction  an essential step on molecular biology (Surzycki, 2000).

The DNA extraction was firstly done by Friedrich Miescher in 1869 when he was trying to isolate lymphoid cells, he discovered a new molecule, the Nuclein, a term he used to call what we know today as the DNA, and determined that it was made up of hydrogen, oxygen, nitrogen and phosphorus. Now the DNA extraction is a DNA purifying method, which consists on using different chemical and/or physical practices, that allow the separation of the DNA from cell membranes, proteins, and other molecular components, so that later on this genetic material extraction can be utilized for future readings including the study of individual genes, sequencing an entire genome, or modifying sections of the DNA (Alberts et al. 2002). Extraction techniques used of the isolation of  DNA are crucial for obtaining remarkable yields of high-quality and pure DNA, as this techniques aim to remove any contaminant such as proteins and RNA that might interfere with downstream applications (Guapta, 2019).

DNA extractions can be done manually or by utilizing commercial kits available for DNA extraction. Numerous tissues can be used for DNA extraction, such as blood, body fluids, frozen tissue section, ethanol preserved tissue, etc. This process the involves destruction of cells, denaturation of proteins and solubilizing the DNA, followed by the removal of the contaminants present in the solution (Guapta, 2019).

The techniques for DNA extraction can vary and do not always follow the same steps, as well as the time that it takes to accomplish an extraction. Every aspect depends on

the technique or equipment utilized. Different techniques can include organic extraction and nonorganic method (Lahiri et al. 1992).

### 3.1.1 Organic extraction

The organic extraction is time consuming and requires a lot of work. This method use organic solvents such as chloroform and phenol, which have a major drawback as they have caustic and toxic properties, for the extraction of nucleic acids. To extract the nucleic acids, the cell must undergo the process of lysing, which is the destruction of the cell so that the molecules inside can be easily separated from the DNA. The residues from the lysis are proteins, RNA and lipids, and cell debris. Cell debris is removed by centrifugation. Lipids are denatured by organic solvents. Proteins are denatured with a protease treatment, then removed by centrifugation. RNA is removed with a RNAse. And the nucleic solution is then precipitated with ice-cold ethanol. The precipitation can be recovered by centrifugation and redissolving the solution in TE buffer (Thomas et al. 1989).

### 3.1.2 Nonorganic extraction

The nonorganic extraction doesn't use organic reagents like phenol or chloroform and requires less time and labor. This method has reported higher quality of DNA yields and a reliable cost effective and safer solution. The nonorganic DNA extraction is non-toxic and works by adding lysis buffer into the sample solution. After the lysis of the tissue cell, the solution is suspended in Proteinase K that is used to denature and remove proteins and SDS (sodium dodecyl sulfate) used to denature proteins. The solution is then incubated overnight at 55-65°C. Temperature helps denature proteins.. A saturated NaCl is then used to decrease protein solubility. In the end the resulting supernatant containing the DNA is extracted into a tube and ethanol can be used to precipitate it (Dairawan & Shetty, 2020).

## 3.2 Comparison of different extraction methods

### 3.2.1 Chelex

Chelex 100 is composed of styrene divinylbenzene copolymers containing paired ions that act as chelating groups in binding polyvalent metal ions such as magnesium. (Samczynski 2006) DNA extractions with Chelex 100 are suitable to use in polymerase chain reaction (PCR) and have been proposed for the rapid extraction of DNA from forensic samples for use with the polymerase chain reaction. The procedure consists of the lysing of cells by heating at 100 C in 5% Chelex suspensions in water, where the chelex works as a protective layer for the DNA, that works against the degradation of the DNA at high temperatures. The DNA can then be amplified by PCR. This method has proved to be relatively fast, and the contamination is minimized as it can be performed in a single tube, reducing the handling of the sample solutions (Casquet et al. 2012).

### 3.2.2 Commercial DNA extraction kits

Obtaining a traceless/clean sample of DNA is the optimal first step in a successful molecular biology experiment. A high-quality purified of DNA sample ensures the success of downstream processes, such as cloning, sequencing and efficient PCR amplification. The quality of the DNA can be easily affected by many factors, like cross contamination when working with multiple samples at the same time, the degradation of the nucleic acid during its preparation, or even the sample size that is being tested (Brevnov et al. 2009).

To ensure a proper extraction Standardized kits for DNA isolation and purification can provide a manual and tools to properly work and extract samples, that if done correctly can result in a high-quality extraction of DNA, they provide a rapid and efficient method for high yield extraction, its reagents are formulated to minimize the contamination and nucleic acid degradation. It also minimizes the user-error ensuring consistent isolation and minimal sample variation (Smith et al. 2003).

### 3.2.3 HotSHot

The rapid simple, inexpensive, and reliable alkaline lysis PCR-quality DNA extraction method of the HotSHot/Hot sodium hydroxide and Tris (buffer) extraction, makes it a reliable option when it comes to extraction from a large variety of tissues, from mammals, to fish, to invertebrates, even to some fungi, plans and bacteria. The lysing of the cell and denaturation of DNA is done by diluting the solution and heating it in a thermocycler. Then this solution is neutralized with a buffer solution that also helps to stabilize the DNA. This is a two-step process of cell lysis and neutralization, a one tube format that minimizes the risk of contamination. This type of alkaline extractions are the best option when it comes to DNA-rich tissues that are low in PCR-inhibiting substances. Some of its advantages are its ease to use, low-cost samples, scalability from single to hundreds of samples. This method is not suitable for high concentrations of PCR-inhibition compounds, total extractions of DNA, unfragmented DNA, or double-stranded DNA (Montero-Pau et al. 2008). Lysing reagents and buffer solutions are not considered irritant or hazardous according to EU regulations and classifications, it also doesn't include ethanol, therefore its safer than many other extraction methods that use reagents such as chloroform or guanidine salts, that may cause damage for prolonged exposure or harmful in case its swallowed (HotShot Extraction Protocol).

## 3.3 Quality of the extraction

Prior to the DNA amplification from the extracted samples, it is an important step to assess their quality. The quality of the DNA can be evaluated by different factors, for example the purity and the yield. Purity refers to the presence of contaminants present in the sample, like proteins or RNA, while the yield refers to the amount of DNA present in the sample. Although it is known what to look for, the size of the sample makes it impossible to determine all the factors by plain sight, therefore specific devices are employed to carry out this job (Boesenberg-Smith et al. 2012).

Two of said devices which are used to conduct the evaluations on this research are NanoDrop 1000 UV Visible Spectrophotometer and by Qubit 4 Fluorometer. NanoDrop uses spectrophotometry to measure the absorbance of nucleic acids at wavelengths of 260 nm and 280 nm, nucleic acids absorb better the 260 nm than the 280 nm, while proteins, in this case contaminants, are opposites as they absorb better 280nm than 260 nm, therefore the procedure for determing the quality of DNA reads the ratio of absorbance from 260 nm to 280 nm and its named A260/A280 ratio, if the sample has an absorbance ratio A260/A280 of 1.8-2.0 ng/ul, the DNA is considered pure. To analyze the purity of the sample, 1 ul of the sample is placed of the spectrometer of the device, which send the information to a computer program that shows the results in real time (Bunu et al. 2020). On the other hand Qubit uses fluorescence analysis to measure the nucleic acid concentrations, by exciting the nucleic acids with specific wavelength, to which they reply by sending light, present on the fluorescent bindings in the nucleic acids, back. This device target only the nucleic acids which gives more accurate information on the yield, although its not capable of determining the purity of the samples (Nakayama et al. 2016).

### 3.4 DNA amplification

Biomolecular experiments that focus on the study detections of individual genes, specific DNA regions or even mutations of interest, are often challenged by the large quantity of nucleic acids that are needed for the study. As it is in the case of genomic sequencing, a detection method used to capture the entire genome of an organism, it requires micrograms of extracted DNA template, which in terms of DNA is considerably high, this need for DNA material has allowed different amplification techniques to emerge and keeps developing new techniques to facilitate the amplification of DNA for further uses (Lasken, 2009).

Usually, a very limited amount of DNA is available from the extractions, so rather than isolating a single copy of the targeted DNA section, it is more useful to generate multiple copies from said target section, or as it is known, to amplify a section from the specific DNA sequence. Therefore, DNA amplification has been proven to be a

key step in many nucleic acid detection protocols. The amplification generates a large number of target copies greatly enhancing its sensitivity. Based on a polymerase activity for primer-directed target amplification, the polymerase chain reaction or PCR, has been the most widely adopted amplification method, due to its simplicity and cost-effectiveness. And thanks to its high exponential process, it has the potential for a single targeted DNA molecule amplification (Zanoli & Spoto, 2013).

Despite all the advantages that the PCR offers, it faces some drawbacks, which has led to the development of alternative amplification methods. On of the disadvantages of the PCR is its sensitiveness to contamination or its inefficiency to amplify unknown targets (Chuang et al. 2013). Some of the new alternatives for the DNA amplification are the Isothermal DNA amplification and the Microfluidic DNA amplification, that as well as the PCR offer some great quality of sequence amplification (Fakruddin, et al. 2013 ; Gorgannezhad et al. 2019).

### 3.4.1 PCR

PCR (polymerase chain reaction) amplification is one of the most widely used amplification techniques, which relies on thermal cycling, despite it being time consuming, as Kim & Easley (2011) state, the repeated heating and cooling can reach a maximum twofold amplification by alternating the denaturation of duplex DNA and the extension of primers that are hybridized to a denatured single-stranded DNA.

This method is capable of detecting and copying as little as one specific sequence, by targeting the DNA template and replicating it exponentially. This replication is done in several steps, denaturation, annealing, extension, and cycling. The denaturation process involves the heating of the targeted DNA template at 95 degrees Celsius to break the double-stranded DNA into two separate strands. The annealing of the strands is then done by lowering the temperature to 50-60 degrees Celsius which allows the DNA primers to anneal on each side of the single stranded template. Once annealed a DNA polymerase enzyme along with nucleotides is added into the mixture and temperature is raised again to 72 degrees Celsius which allows the enzyme to extend the primers and build up the new strands of the desired DNA. Cycling is about

repeating the process for multiple cycles with the purpose of exponentially amplifying the DNA segment. Enough copies are made after 25-30 cycles which estimate that each step takes the maximum amount of time it would take 6 hours to complete the amplification (Joshi & Deshpande, 2010).

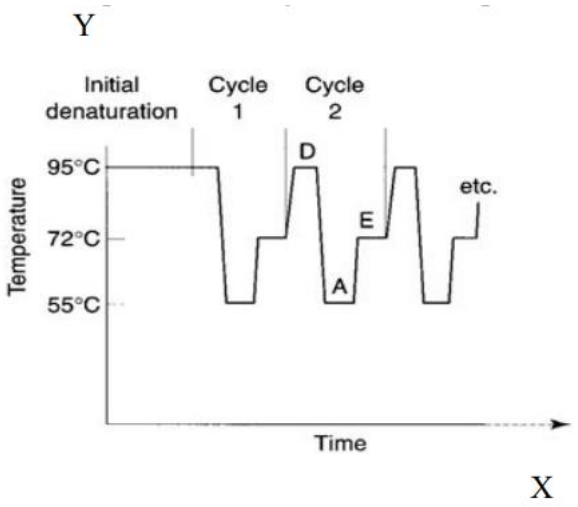


Figure 1. By (Joshi & Deshpande, 2010), shows the process of the repeating cycle of PCR, where D= denaturation, A= annealing, E= extension.

For the proper amplification of the DNA a set of reagents must be utilized in order to ensure the success of the reaction. Starting with the DNA template that is targeted to be amplified. This DNA template can be obtained by different extraction methods. Following with the primers, which will anneal to the specific region of the desired DNA, this set of primers will take place on opposite sides of the DNA strands, one will sit on the positive strand oriented on the 5' to 3' direction while the other set will sit on the negative strand oriented on the 3' to 5' direction. Another important reagent is the DNA polymerase, the most commonly used polymerase for PCR is the Taq (thermus aquaticus) polymerase is resistant to high temperatures, which gives it the advantage on this method. To create and replicate the new strands dNTPs or deoxynucleotides are added to be used as bases for the amplification (Lorenz, 2012).

Day by day the continued development of thermal cycling has improved, and time for the PCR reactions has been shortened by introducing near instantaneous temperature changes and rapid heat exchange within the sample. Although the improvements have shortened the estimated time consumption, an optimized experimental setup and a thermal cycler is required, which makes it difficult to execute. (Kim & Easley, 2011).

### 3.4.2 Isothermal DNA amplification

As stated previously, DNA amplification techniques are an essential tool, not only for basic research, but also for different applications like clinical diagnosis, epidemiology, forensic sciences, among others. In contrast to the PCR, the isothermal amplification does not require thermal cycling, which considerably facilitates the reaction conditions and reduces the equipment required for the reaction. In most cases it is cost effective, easy to use, and in comparison to PCR, is more tolerant to inhibitory components from crude samples. It also shows an equivalent sensitivity and reliability in clinical diagnosis. Additionally, to DNA, the isothermal amplification is capable of using different molecules as input signals, for example mRNA, which have been tested and amplified after converting them into complementary DNA, and proteins. Usually, micrograms of the amplified genomic segments can be obtained from nanograms of the initial DNA within a few hours. Overall, isothermal amplification methods offer a simpler, faster, and more accessible alternative to traditional PCR for DNA amplification (Kim & Easley, 2011).

### 3.4.3 Microfluidic amplification

Microfluidics for nucleic acids amplification has drawn attention since its creation, as it offers the capability of minimizing the usual laboratory operations, by using a fraction of the reagents volume in a much shorter time spam. Microfluidics devices are capable of processing small amounts of nucleic acids by manipulating fluids at a microscopic level. Microfluidic devices are mostly used to improve and facilitate different amplification processes like PCR or Isothermal amplification. Essentially microfluidic devices the principles of said DNA amplification methods and perform

them in an automated programmed way, one example is the droplet-based microfluidics, that provides fully automated programmed PCR assays, which allows the detection and amplification of DNA in thousands and even millions of droplets, resulting in an extremely high throughput. Although utilizing this type of devices could facilitate the detection and amplification of DNA, the use is still limited by trained professionals and expensive laboratory equipment, which limits the use of this technology (Gorgannezhad et al. 2019).

### 3.5 Electrophoresis

Electrophoresis, discovered by Arne Tiselius on 1930, is a laboratory technique used for the separation and analysis of particles. Generally, describes the movement and separation of ions inside an electric field's system called electrophoretic system. This system consists of two opposite charged electrodes connected by an electrolyte, a non-metallic conducting medium, that allows the movement of ions throughout the system. The mobility of an ion particle during separation is influenced by several factors including its size, shape, charge, and temperature (Fritsch & Krause, 2003). Other electrophoresis practices and techniques arose from the creation of electrophoresis, one of this being electrophoresis gel. Electrophoresis gel, which follows the same concept and principle of electrophoresis, is commonly used for the separation of proteins and nucleic acids through a medium of agarose or polyacrylamide gel. Other electrophoresis practices and techniques arose from the creation of electrophoresis, one of this being electrophoresis gel. Electrophoresis gel, which follows the same concept and principle of electrophoresis, is mostly commonly used for the separation of proteins and nucleic acids through a medium of agarose or polyacrylamide gel (Isbir et al. 2013).

One important factor in this practice is the addition of ethidium bromide into the running buffer, which is used during the preparation of the gel, this chemical is visible through UV light and has the ability to bind with the DNA molecule, therefore making the DNA visible under the UV light (Sigmon & Larcom, 1996). The preparation of the agarose gel is done in four quick steps and the concentration of the agarose will

depend on sizes of DNA fragments. Most agarose gels have aa agarose concentration of 0.5%-2%. First the proper amount of agarose is deposited into and Erlenmeyer flask, then the running buffer is added into the same flask and swirled to mix both solutions, after this the mixture is heated on the microwave and mixed in short intervals until the agarose is fully dissolved, at last ethidium bromide (EtBr) with a concentration of 0.5 μg/ml is added into the solution. It's good to mention the importance of wearing gloves while handling ethidium bromide, as it is a suspected carcinogen that can be easily absorbed by the skin into the body (Lee et al. 2012).

Using gel for electrophoresis allows the option of running multiple DNA samples, that have been previously amplified by PCR technique, simultaneously, which facilitates its DNA analysis and separation. The electrophoresis gel system consists of two opposite charged electrodes located one on each end and the medium, in this case the gel. The DNA is placed on the negative side of the system, and when the electrical field is applied into the medium, it makes the negative charged molecules like the DNA migrate into the positive side of the system(Rifai et al., 2018). The mobility of the ion particles is also affected by several factors like the concentration of the agarose, electrical parameters, temperature and most importantly the size of the DNA fragments (Isbir et al., 2013).Smaller fragments can move through the gel faster than larger fragments, which allows them to migrate farther away inside the gel. As a result, the different sizes of the fragments will show as distinct bands in the gel after electrophoresis, meaning that the number of fragments at a specific spot determines the luminosity in the gel (Lee et al. 2012).

### 3.6 Sequencing

Several scientific disciplines, such as molecular biology, genetics, and biotechnology, now consider DNA sequencing to be a crucial tool. As I t allows the reading of genetic information stored in DNA molecules by researchers. Sequencing technology detects genetic variants, mutations, and other important aspects that may be used to understand the structure and function of genes, genomes, and whole organisms by identifying the sequence of nucleotides in the DNA. In biodiversity research, DNA sequencing has become a significant tool for studying species interactions and genetic diversity. By sequencing DNA from diverse creatures, scientists may learn about genetic variances and similarities that can reveal the links and evolutionary histories of distinct species. DNA sequencing has a wide range of uses in conservation biology, where it may be used to identify endangered species and monitor their numbers. DNA sequencing is also used to investigate the links between various species in ecosystems in order to gain a better understanding of biodiversity and natural system operation (Sharma et al. 2022). The accuracy and quality of the sequencing data are essential in these applications and are reliant on the quality of the DNA sample. Hence, DNA sequencing is a tool for ensuring the quality and integrity of the DNA sample as well as a way for learning about genetic information. This makes it a crucial stage in many molecular biology, genetics, and biotechnology applications since it is the last assessment of the quality of extracted DNA from any procedure (Guo et al. 2014).

### 3.7 Beetles and biodiversity

Beetles, or Coleoptera, belong to the class Hexapoda, which is the largest class in the phylum Arthropoda, the largest animal phylum. Coleoptera themselves make up almost 40 % of the total insect species, which is about 390,000 described species (Eggleton, 2020). Generally the relevance of insects is usually ignored and overlooked, mostly due to the misinformation and lack of comprehension the general public has on them, especially when it comes to the thought of how such small things can have that much of an impact on the environment, not knowing that in fact their existence is what helps the environment to stay in balance and any major disturbance

might interrupt the ecosystem services that these populations are responsible for. Ecosystem services refer to the benefits that humankind receives from the natural environment and the ecosystem around them, whether it affects their quality of life directly or indirectly, such as food availability, organic decomposition and cycling o nutrients, climate and flood regulation and control, and water (Noriega et al. 2018).

The role of insects in the environment, as previously stated, is critical for the provision of ecosystem services, as all the insects have a different but specific role that they have to achieve for the ecological function. The ecosystem services that insects are mostly responsible for are pollination, although its not exclusive of insects as some mammals are considered pollinators too, pest control, and decomposition(Goutam et al. 2017). In the case of coleoptera, as previously stated, the largest group of insects, can be found performing of many of the different ecosystem services to provide a proper ecological function to the environment (Nichols et al. 2008).

## 3.8 Ecosystem services performed by Coleoptera

### 3.8.1 Nutrient cycling and decomposition

Nutrients are a key factor for the growth of plants, just like humans depend on food, plants depend on nutrients to grow. The more fertile the soil where the plans are developing, the stronger their growth will be. Nutrients such as carbon, nitrogen, phosphorus and sulfur, are essential for a plant to develop properly. And they come into the soil by decomposers which are responsible for degrading organic matter and store it into the soil. As decomposers, beetles can break down dead organic matter, such as leaves, wood or animal carcasses, and tough plant material like lignin or cellulose that other decomposer can not digest, which are then decomposed by microorganisms allowing this nutrients to go back into the soil which then allow plants

to intake this nutrients into their structure and start the cycle all over again (Cheli et al. 2022).

### 3.8.2 Pollination

As most living organisms, the reproduction and the preservation of their species is embedded deep inside their survival instincts, therefore to create the next generation and keep reproducing themselves, while plants might not have the mobility other organisms have, they have developed a method of sexual reproduction called pollination, where the male flowers transfers the pollen into the stigma of a female flower with the help of an external source. As regarding for the coleoptera, flowers are able to attract them by their scent, which catches the attention making them go inside the flowers and pollinate them. In the case of Scarabaeine or dung beeltles, they are restricted to (and often obligate pollinators) of the dung/carrion scented flowers of the families Aracea/Lowiacea. Due to their scent, not many insects are attracted by their scents, which makes coleoptera play an important role on their reproduction (Nichols et al. 2008).

### 3.8.3 Seed dispersal

Mammalian abundance and diversity can be associated with the presence of dung beetles in tropical forests. This beetle species is highly sensitive to most forest disturbances such as deforestation, which makes them function also as a great bioindicator for a non disturbed healthy forest. They are mostly found in the tropical environment, and they excel at several ecosystem services some of them which are the dispersal of seeds and nutrient cycling, this implicates that their population reduction in a forest can have major cascading effects. The dispersion of seeds is and indirect action of the beetles, as the rest of the animals feed on the fruits from the plants, the beetles feed on the excrete from the animals, that contains usually contains large amounts of seed, which are then carried by the dung beetles around the forests into the top layers of the soil. Allowing the plant to sprout in a nutrient rich

environment. A technique that allows plant species to disperse through the forest to avoid competition within plant species (Vulinec, 2000).

### 3.9 Carrion beetles

Silphidae, commonly known as carrion beetles is a small group of Coleoptera that makes up less than 200 species that are spread all around the world. Even though they are few species, they perform a crucial role on the environment which is the decomposition and breakdown , and the recycling of organic matter into the terrestrial ecosystem. Although not all silphids show interest on carcasses, as some of them are phytophagous or feed from the dung or the fungi, but for most of them the carcasses represent a rich source of ephemeral nutrients. The carrion beetles do not feed only on the dead vertebrate carcasses, but also prey on smaller carrion inhabitants, such as maggots, fly eggs, they also show no mercy to smaller species of carrion beetles, and feed on them. The carcass does not only represent a source of food for the carrion beetles, but it also represents a place of growth and colonization for them. As the necrophagous species are attracted with the strong scent of the dead bodies in a predictive sequence called entomofaunal succession, they deposit their eggs, ensuring them a source of food to grow in their larval stages (Mullins et al. 2013)

### 3.10 Taxonomy

The family Silphinae belongs to the superfamily of Staphylinoidea, which is divided by two subfamilies: Nicrophorinae and Silphinae. The Silphids are composed by 183 species divided in 15 genera, distributed in the temperate regions, as the competition for the carrion beetles in the tropical regions is to high due to the larger presence of ants and flies, indicating its presence as being rare to almost nonexistent. They are most abundant in the palearctic region. Compared to nicrophorinae, silphines are more widely distributed, as they are more tolerant to different climates. Silphines also show a larger diversity with 12 genera, meanwhile necrophorinae only 3. Western Europe hosts 28 recorded species of silphidae, 11 which are necrophorinae and 17 silphinae (Dekeirsschieter et al. 2011).

**Table 1.** List of the Western European species of "carrion" beetles (including Mediterranean species), family Silphidae LATREILLE, 1807 — *Liste des espèces de Silphidae de l'Europe de l'ouest (incluant les espèces méditerranéennes), famille Silphidae LATREILLE, 1807.*

| | **Subfamily** | |
|---|---|---|
| | **Nicrophorinae KIRBY, 1837** | **Silphinae LATREILLE, 1807** |
| Genus | *Nicrophorus* FABRICIUS, 1775 | *Ablattaria* REITTER, 1885 1884 |
| | | *Aclypea* REITTER, 1884 |
| | | *Oiceoptoma* LEACH, 1815 |
| | | *Phosphuga* LEACH, 1817 |
| | | *Silpha* LINNAEUS, 1758 |
| | | *Thanatophilus* LEACH, 1815 |
| | | *Necrodes* LEACH, 1815 |
| | | *Dendroxena* MOTSCHULSKY, 1858 |
| Species | * *Nicrophorus germanicus* LINNAEUS, 1758 | **Necrodes littoralis* LINNAEUS, 1758 |
| | * *Nicrophorus humator* GLEDITSCH, 1767 | **Thanatophilus dispar* HERBST, 1793 |
| | * *Nicrophorus investigator* ZETTERSTEDT, 1824 | **Thanatophilus rugosus* LINNAEUS, 1758 |
| | * *Nicrophorus interruptus* STEPHENS, 1830 | **Thanatophilus sinuatus* FABRICIUS, 1775 |
| | * *Nicrophorus sepulchralis* HEER, 1841 | **Oiceoptoma thoracicum* LINNAEUS, 1758 |
| | * *Nicrophorus sepultor* CHARPENTIER, 1825 | **Silpha carinata* HERBST, 1783 |
| | * *Nicrophorus vespillo* LINNAEUS, 1758 | **Silpha obscura obscura* LINNAEUS, 1758 |
| | * *Nicrophorus vespilloides* HERBST, 1783 | **Silpha tristis* ILLIGER, 1798 |
| | * *Nicrophorus vestigator* HERSCHEL, 1807 | **Silpha olivieri* BEDEL, 1887 |
| | * *Nicrophorus nigricornis* FALDERMANN, 1835 | **Silpha puncticollis* LUCAS, 1846 |
| | * *Nicrophorus antennatus* REITER, 1884 | **Silpha tyrolensis* LAICHARTING, 1781 |
| | | *Phosphuga atrata atrata* LINNAEUS, 1758 |
| | | *Dendroxena quadrimaculata* SCOPOLI, 1772 |
| | | *Ablattaria laevigata laevigata* FABRICIUS, 1775 |
| | | *Aclypea opaca* LINNAEUS, 1758 |
| | | *Aclypea undata* MULLER, 1776 |
| | | *Aclypea souverbiei* FAIRMAIRE, 1848 |

* : indicates necrophagous or predaceous species — *indique des espèces nécrophages ou prédatrices* (Heinz, 1971; Hastir et al., 2001; Sikes et al., 2002; Debreuil, 2003a; Debreuil, 2003b; Debreuil, 2004a; Debreuil, 2004b; Debreuil, 2004c; Ružicka et al., 2004; Sikes, 2005)

Figure 2. A closer look of the list of the Western European registered carrion beetle species (Dekeirsschieter et al. 2011).

### 3.11 *Thanatophilus sinuatus*

The necrophagous beetles from the Thanatophilus genus*, Thanatophilus sinuatus*, is the most common species found in Europe. Their potential of use in forensic entomology is remarkably high due to the frequency on which they appear on human remains, whether it is as adults or in their larval stage feeding from the decaying carcass whilst they grow and develop on their initial stages until they become adults (Jakubec et al. 2019).

The ecosystem services this species provides, as previously stated is the decomposition and recycling of nutrients in the environment, by feeding on the vertebrates carcasses they help with the decomposition of the organic matter. The decomposition of the organic matter is done as the beetles feed on the carrion (decaying flesh of a dead animal/human), they organic tissue is broken down into smaller pieces which then is decomposed by fungi or bacteria and returned back into the soil, to be later taken up by other plants or any organism in the ecosystem (Parmenter & MacMahon, 2009).

However the ecological disturbances such as deforestation, habitat destruction and environmental pollution, can have big negative impacts on the forest ecology and biodiversity itself. Around the world forest are being increasingly fragmented, and many of the biological processes critical for the ecosystem function carried out by insects are prone to be disrupted. Cases report the decrease of dung decomposition carried out by dung beetles in fragmented forests as well as species richness of different insect species. In example a study conducted in New York stated that the species richness of the carrion beetle was reduced by two-thirds in fragmented forests, therefore as the land is fragmented, basic ecosystem services that existed before stop functioning, food and water is scarce for animals, and their population start decreasing. As the population of animals decrease, other smaller organism population start decreasing too, such as *Thanatophilus sinuatus*, which feeds and is dependent on the animal carcasses as there is no more food for them, they fly away or simply die because of the lack of resources (Gibbs & Stanton, 2001).

## 4. Methodology

The purpose of the study is to analyze different variabilities of the DNA such as quantity and quality, using three different techniques to extract DNA and do PCR analysis to determine the most viable way to identify the carrion beetle *Thanatophilus sinuatus.* The experiment was conducted by two different researchers simultaneously for the sole purpose of factorial design therefore eliminating the bias, by having more than one independent variable.

The beetles used for the study were collected on the field using pitfall traps. The traps were build by digging a hole on the ground where a plastic recipient, containing rotten chicken meat, was placed an covered with a metal lid with enough space in between for the beetles to get in. The beetles were collected during the months of March and April 2022, and later on put into an incubator where they were taken care of and reproduced to get the amount of beetles necessary to make the study.

Once the beetles reached maturity, they were transferred to a container tube filled with ethanol, which helps preserve the body of the beetle, and later the tubes were numbered and moved to a freezer to be preserved, therefore avoiding the degradation of the body tissue. They were taken out of the freezer once they were ready to be analyzed.

The extraction was done following different methods: extraction protocol kits and Direct PCR extraction, provided by the Czech University of Life Sciences, the extraction kits were Genomic DNA Mini Kit (Geneaid), DNeasy Blood & Tissue Kits (Qiagen) and NucleoSpin DNA Insect. The tissue used for each of the extraction methods are the legs belonging to the previously collected beetles. Each extraction method was repeated 24 times per person having a total of 48 samples per method.. The experiment was separated on the amount of legs used each time, therefore 3 different amounts of tissue was used. The first 8 extractions were done with one leg, the second 8 extractions were done with two legs and the last 8 extractions were done with three legs. So that in the end the amount of tissue used could be compared and then determine the amount of tissue that gives better yields of DNA. A total of 192 extractions were performed and evaluated.

Each extraction kit follows a protocol which allows the user to extract the DNA from insect tissue with detailed step by step instructions. The protocol is done for each extraction and multiple extractions can be done at the same time. Each kit was conducted on separate days.

Extractions with Genomic DNA mini kit (Genaid) were done on the 26.05.2022 & 14.11.2022

Genomic DNA mini kit (Genaid) Protocol:

Tissue Dissociation

- Transfer the N amount of leg/s to a 1.5 ml microcentrifuge tube.
- Add 200 µl of GT Buffer into the tube.
- Add 20 µl of Proteinase K into the tube.
- Shake for 5 seconds in the vortex.
- Incubate at 60°C for 30 minutes.

Step 1 – Lysis

- Add 200 µl of GBT Buffer.
- Shake for 5 seconds in the vortex.
- Incubate at 60°C for 20 minutes
- Preheat the Elution Buffer (200µl per sample at 60°C)

Step 2 – Binding

- Add absolute ethanol the lysate and shake for 10 seconds in the vortex.
- Place a GS Column in a 2 ml Collection Tube (Prepare 1 per sample)
- Transfer the mixture to the GS Column.
- Centrifugate at 14-16000 x g for 2 minutes.
- Discard the collection tube and fluids.
- Place GS Column into a new Collection Tube.

Step 3 – Wash

- Add 400 µl of W1 Buffer to the GS Column.
- Centrifugate at 14-16000 x g for 30 seconds.

- Discard the flow-through (liquid inside collection tube) and place the GS Column back in the same collection tube.
- Centrifugate at 14-16000 x g for 30 seconds.
- Discard the flow-through (liquid inside collection tube) and place the GS Column back in the same collection tube.
- Centrifugate at 14-16000 x g for 3 minutes to dry the column.

Step 4 – DNA Elution

- Discard collection tube and transfer clean GS Column into a clean 1.5 ml microcentrifuge tube.
- Add 100 µl of the pre-heated Elution Buffer into the center of the column matrix.
- Let it rest for 5 minutes to ensure the absorption of the Elution Buffer.
- Centrifugate at 14-16000 x g for 30 seconds to elute the purified DNA.
- Discard the GS Column and store the microcentrifuge tube for later analysis.

All the reagents of the protocol are properly labeled by the manufacturer to avoid mistakes and confusions.

Before using the kit is important to remark the following:

- The preparation of Proteinase K is done by adding dd$H_2O$ (vortex is recommended to dissolve and spin down). Store Proteinase K at 4°C.
- Add absolute Ethanol to Wash Buffer prior to initial use.

For both reagents its labeled in the bottle labels the necessary amount of dd$H_2O$ or absolute Ethanol to be added.

The cost per sample for this extraction kit is 83,89 czk, ist by far the cheapest kit used. The average time required for this protocol are 3 hrs and 30 min. due to its prolonged incubation times.

Extractions done with NucleoSpin DNA insect were done on the 27.05.2022 & 06.06.2022.

NucleoSpin DNA insect Protocol:

Step 1. Sample preparation

- Place N amount of legs into a NucleoSpin Bead Tube.
- Add 100 µl Elution Buffer (BE).

Step 2. Lysing sample

- Add 40 µl of Buffer MG.
- Add 10 µl of Liquid proteinase K.
- Close and place tube into the tube holder.
- Place the tube holder into the agitator and agitate for 5 minutes.
- Centrifuge tube for 30 seconds at 11,000 x g *No longer nor higher g-force.

Step 3. Adjust DNA conditions

- Add 600 µl Buffer MG and vortex 3 seconds
- Centrifugate at 11,000 x g for 30 seconds

Step 4. DNA Binding

- Place an insect column into a 2ml collection tube.
- Transfer 500-600 µl of supernatant into the insect column.
- Centrifugate at 11,00 x g for 30 seconds.
- Discard collection tube and place column into a new collection tube.

Step 5. Wash silica membrane

- Add 500 µl of Buffer BW.
- Centrifugate at 11,000 x g for 30 seconds.
- Discard only the flowthrough.
- Add 500 µl of Buffer B5.
- Centrifugate at 11,000 xg for 30 seconds.
- Discard only the flowthrough.

Step 6. Dry silica membrane

- Centrifugate at 11,000 x g for 30 seconds.

Step 7. Elute highly pure DNA

- Discard collection tube.
- Place insect column into a 1.5 ml tube.
- Add 100 µl of Elution Buffer BE into the column.
- Incubate for 1 minute.
- Centrigugate at 11,000 x g for 30 seconds.
- Discard insect column and store the 1.5 ml tube for later analysis.

The cost per sample for this extraction kit is 124,38 czk, it's the second most expensive kit. The average time required for this protocol are 2 hrs and 30 min, it is fast and easy to follow.

Extractions done with DNeasy Blood & Tissue Kit (Qigen) were done on the 15.11.2022 & 21.17.2022.

DNeasy Blood & Tissue Kit Protocol:

Step 1.

- Place N amount of tissue into a 1.5 microcentrifuge tube.
- Add 180 µl Buffer ATL.
- Add 20 µl proteinase K.
- Vortex for 5 seconds.
- Incubate at 56°C until lysis of the sample.

Step 2.

- Add 200 µl Buffer Al.
- Vortex for 5 seconds.
- Incubate at 56°C for 10 minutes.

Step 3.

- Add 200 µl ethanol 96%.
- Vortex for 5 seconds.

Step 4.

- Place DNeasy mini spin column into a 2 ml collection tube.
- Transfer mixture into the collection tube.
- Centrifugate at 6000 x g for 1 minute.
- Discard flowthrough and collection tube.

Step 5.

- Place spin column into a new 2 ml collection tube.
- Add 500 µl Buffer AW1.
- Centrifugate at 6000 x g for 1 minute.
- Discard flowthrough and collection tube.

Step 6.

- Place spin column into a new 2 ml collection tube.
- Add 500 µl Buffer AW2.
- Centrifugate at 20,000 x g for 3 minutes.
- Discard flowthrough and collection tube.

Step 7.

- Transfer sin column into a 1.5 ml microcentrifuge tube.

Step 8.

- Elute DNA by adding 200 µl Buffer AE to the center of the spin column.
- Incubate for 1 minute at room temperature.
- Centrifugate at 6000 x g for 1 minute.

Before using the kit is important to remark the following before starting:

- Redissolve any precipitants in Buffer AL and ATL.
- Add ethanol to Buffer AW1 and AW2.

The cost per sample for this extraction kit is 126,08 czk, Its by little the most expensive kit., although the cost difference is not that different form NucleoSpin. The average time required for this protocol are 4 hrs and 30 min and its by little the most expensive kit.

Once the DNA samples were collected, they were read and analyzed using NanoDrop, Qubit. Nanodrop was done using NanoDrop 1000 UV Visible Spectrophotometer. This device is capable of reading 1 µl of the sample at a time through a spectrometer alongside with a computer program. Each measurement was done with a 10 µl pipette, and each sample was measured three different times to avid mistakes generated by the computer, the device or the person in charge. Later the samples where measured by Qubit 4 Fluorometer, the device can detect nucleic acids through a stimulation of wavelength in the sample. Each sample was measured once on Qubit, as the machine is capable of reading the whole sample at once, therefore eliminating the need of doing individual extractions from the sample to read in the device. The data was then put together using RStudio.

Direct PCR

For this method the samples used were the freshly extracted legs without any DNA extraction method. The following procedure is fast and only requires the sample preparation before going to PCR amplification.

Sample preparation:

- Place the leg/s on an Eppendorf tube.
- Pipette 20 µl of PBS into the tube.
- Incubate for 2 minutes at 98°C.

It’s important to remark that PBS buffer must be diluted 10 times. The dilution is done by adding 9 ml dd$H_2O$ to 1 ml PBS 10x to get PBS 1x.

With direct PCR  Phire Hot Start II DNA Polymerase is used as the master mix 17.55 czk per sample.

PBS buffer cost of 0.04 czk per sample

PCR

Following the extraction, one primer pair was employed to amplify the cytochrome oxidase I barcode area using each sample as a template- Jerry & Pat (Simons 2000). PPP Master Mix (Top Bio) was used for the reaction in accordance with the manufacturer's instructions, along with 12,5 µl of PPP Master Mix, 9,5 µl of water, 1 µl of 10mM Forward primer, 1 µl 10mM Reverse primer, and 1 µl of the template DNA. The PCR reaction was carried out in 25 µl total under the following circumstances:

- The PCR was visualized using 1% agarose gel electrophoresis (100V 35 min).
- Unidirectional sanger sequencing was prepared using amplified PCR products from the reaction. Before adding the forward primer, each sample was first purified using IT Exosap in accordance with the protocol.
- The resulting sequences were cropped based on the sequence quality and visualized using Chromas v2.6.6. The nucleotide BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) program was then used with the cropped sequence as a query. In order to assess the obtained sequence's suitability for use in the organism's barcoding, the hit accession, E value, sequence length, and probability percentage were evaluated.

PCR cost per sample for the reagent PPP master mix is 10,55 czk.

Cost for the dNTP’s 10 mM is 1.54 czk per sample.

Table 1. Total price per sample including PCR for each method

| Method | Total Price |
|---|---|
| NucleoSpin | 136,47 czk |
| DNeasy Blood & Tissue Kit | 138,17 czk |
| Tissue genomic DNA mini Kit | 111,99 czk |
| Direct PCR | 19,13 czk |

## 5. Results

The comparison of methods was done between the results from the direct PCR and the Genomic DNA mini kit Protocol (Genaid).

Genomic DNA mini kit Protocol results:

Nanodrop

The first readings were done using Nanodrop. The initial comparison done in the kit was the concentration of ng/µl of molecular material per number of legs used for the extraction.

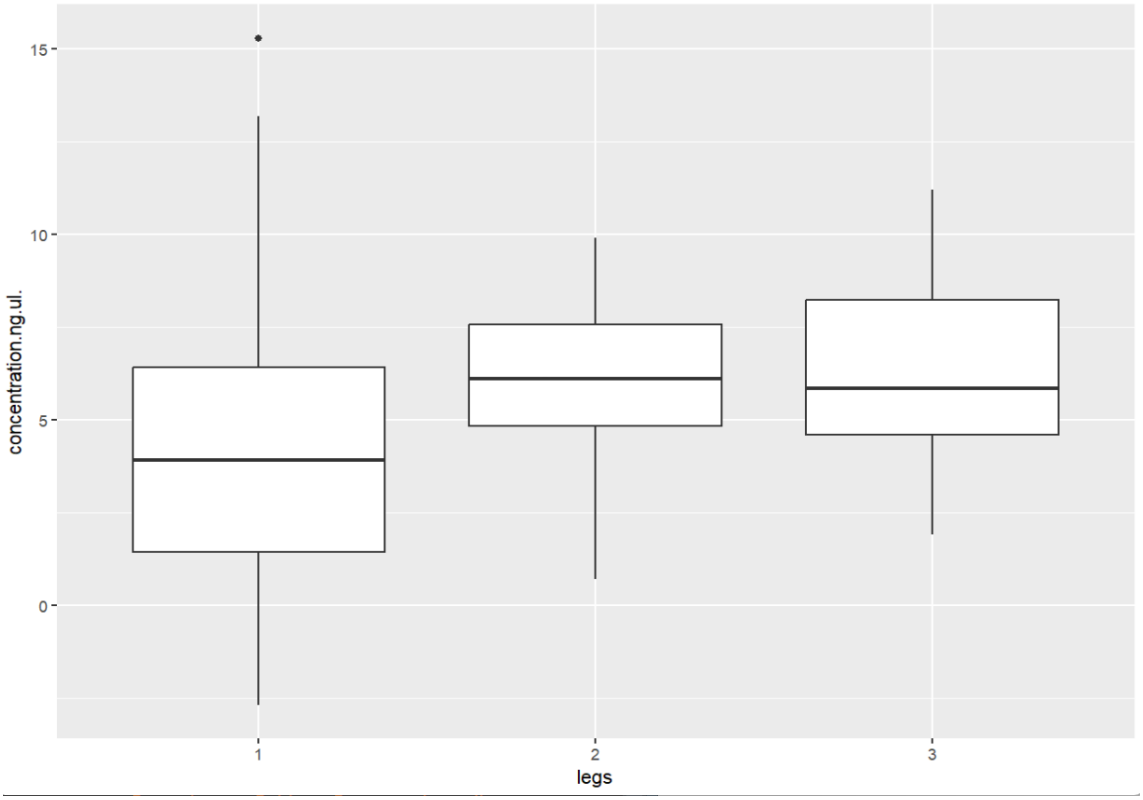

Figure 3. Number on the bottom in the x axis (legs) corresponds to the amount of leg tissue used for the extraction of DNA. Number on the left side in the y axis (concentration ng/µl) corresponds to the concentration of molecular material found in the sample.

The second aspect compared were the readings from the absorbance ratio A260/A280 for each sample.

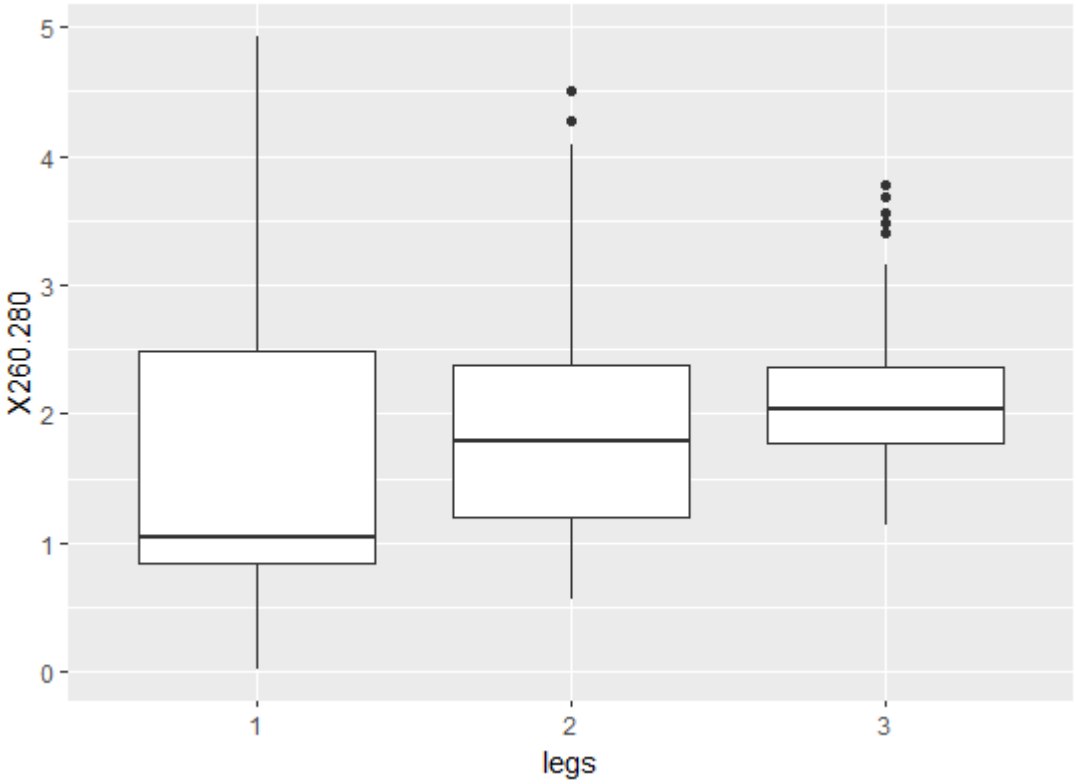


Figure 4. Number on the bottom in the x axis (legs) corresponds to the amount of leg tissue used for the extraction of DNA. Number on the left side in the y axis (X260.280) corresponds to the A260/A280 absorbance ratio.

Results from the readings by Qubit

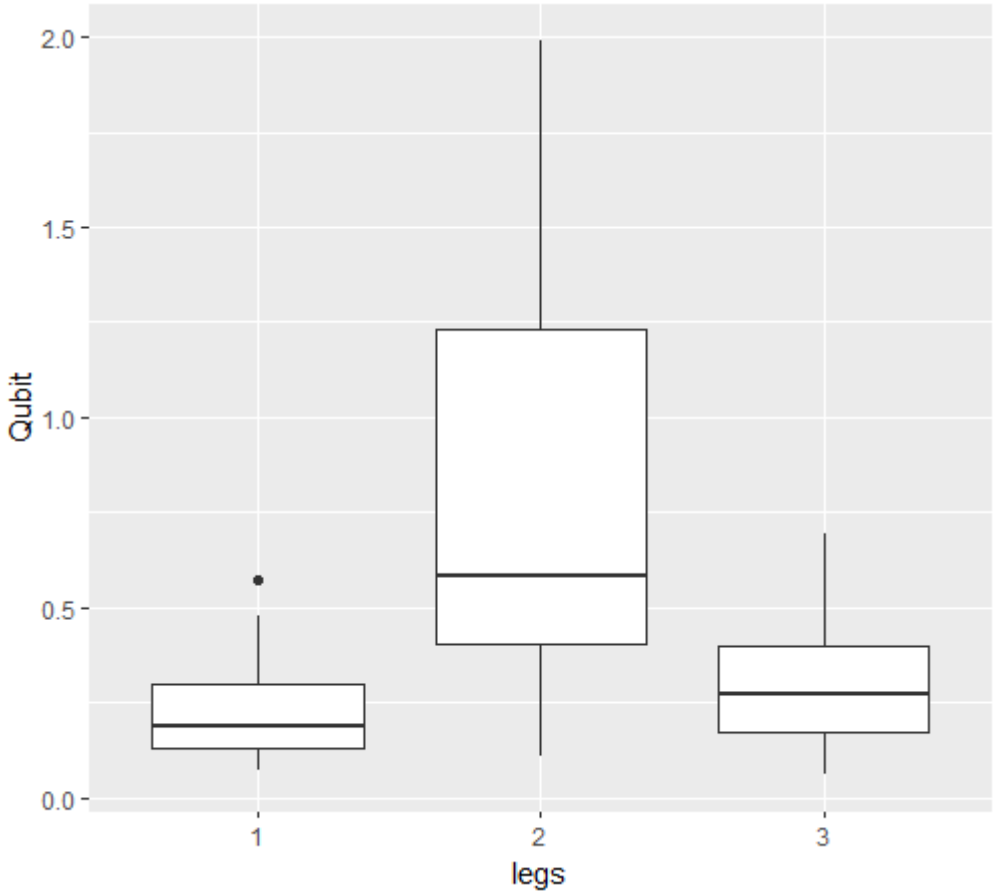


Figure 5. Number on the bottom in the x axis (legs) corresponds to the amount of leg tissue used for the extraction of DNA. Number on the left side in the y axis (Qubit) corresponds to the concentration of nucleic acids.

PCR

All the extraction methods and all quantities of material tissue used for the extractions produced enough amount of DNA for amplification, therefore it was possible to obtain barcodes from the model specimen.

All samples produced valid barcodes for the species *Thanatophilus sinuatus.*

## 6. Discussion

The two methods the study used to compare and determine the best and cheapest method to extract DNA where the extraction kit Genomic DNA Mini Kit (Geneaid) and the direct PCR. This discussion will help analyze each method used and determine which method is the best for biodiversity studies.

The extraction kit determined the quality and quantity of DNA present in the extractions for 3 different amount of tissue levels, meaning DNA extracted from 1 , 2 and 3 legs.

The concentration of molecular material does not specifically tell the amount of DNA present in the samples, it rather tells the amount of DNA and contaminants such as proteins and RNA (Boesenberg et al. 2012). The concentration is more constant in the extraction from the 2 legs, while the extraction form 1 and 3 legs showed more variability in their concentration. Therefore with less variability, more accurate results can be obtained.

The absorbance ratio A260/A280 is used to asses the purity of the DNA in the sample, where 1.6nm-1.8nm absorbance is considered pure DNA, any absorbance lower than 1,6nm is considered contaminated by the presence of proteins or other materials and an absorbance higher than 2nm indicates the presence of pure RNA (Lucena et al. 2016). The results show a better purity of DNA from the samples extracted from 2 legs, as the average lays in between 1.6nm and 1.8nm, while the average from the extracted samples of 1 leg showed closer to 1 nm, which could indicate contamination in the sample and the average from the extracted samples of 3 legs showed higher than 2nm, which indicates the presence of RNA in the sample.

When it comes to the best yield and quality of DNA between the quantity of legs, the kit showed that as a matter of fact utilizing 2 legs for the extraction DNA can provide better quality DNA yields, as it can be seen in both the results of concentration and absorbance ration.

For the Qubit readings, although it is not capable of determining the quality but the yield of DNA, the results showed a higher yield of DNA from the 2 legs, although there's much more variability in the samples compared to 1 and 3 legs, the lowest amount o nucleic acids from the 2 leg extraction was higher than the highest amount of nucleic acids from the 1 and 3 legs extraction.

Overall the extraction from the 2 legs shows to be the best option of quantity of material tissue to be used for DNA extractions using this type of extraction kits.

Even though the other tow amounts of material tissue didn't perform as well in the quality and quantity, when it comes to PCR and sequencing, all of them produced enough DNA to get valid barcodes that determined the model species *Thanatophilus sinuatu.* As well as the direct PCR, despite the fact that no isolation of the DNA was performed with the direct PCR, it also produced the same results when it came to sequencing.

As the focus of the study is to determine the best and cheapest way to extract DNA for the determination of species. PCR is by far the fastest way in comparison to the 3.5 hrs of laborious work from the Genomic DNA Mini Kit, PCR is done in less than 1 hr and it can be a good alternative for DNA extraction kits. As stated by (Videvall et al. 2017), direct PCR provides a reliable, fast and cheap alternative to conventional DNA extraction methods. Especially when it comes to biodiversity studies, direct PCR can help decrease the total cost from the DNA extractions and provide fast and accurate results, which is extremely useful for the biodiversity analysis, as it uses significantly large amount of samples from specific species to assess different analysis for the population, it can significantly reduce the cost and time spent on the specimen analysis.

## 7. Conclusion

Direct PCR can be a reliable alternative to other DNA extraction methods for biodiversity research, as it is low in cost and highly effective to determine the desired specimen through sequencing of DNA. This method doesn't require DNA isolation of any kind, which makes the process even faster. The low cost of this method reduces the amount of money needed for the larger populations, that using other methods could result to be highly expensive, as well as the amount of time sent in the laboratory performing extractions is reduced. Therefore concluding with the findings of the study and determining direct PCR as the best method, between the two compared methods, to be employed on biodiversity research studies.

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## Image references

Figure 1: Joshi, M., & Deshpande, J. (2010). Polymerase chain reaction. International Journal of Biomedical Research 2, 81-97.

Figure 2: Dekeirsschieter, J., Verheggen, F., Lognay, G., & Haubruge, E. (2011). Large carrion beetles (Coleoptera, Silphidae) in Western Europe: a review. Biotechnol. Agron. Soc. Environ. 15, 435-447.