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Ms.
Kevine Phalone Ngoufack Djoumessi

Assessment of COI and 16S for Insect Species Identification to Determine the Diet of City Bats

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Faculty of Applied Computer and Life Sciences

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Author:
Ms.

Kevine Phalone Ngoufack Djoumessi

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First Referee:
Prof. Dr. rer. nat. habil. Röbbe Wünschiers

Second Referee:
Dr. Carolin Scholz

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Abstract

Despite the numerous benefits of urbanization to human living conditions, urbanization has also negatively affected humans, their environment, and other organisms that share urban habitats with humans. Undoubtedly adverse while some wild animals avoid living in urban areas, others are more tolerant or prefer life in urban habitats. There are more than 1,400 species of bats in the world. Therefore, they have the potential to contribute significantly to the mammalian biodiversity in urban areas. Insectivorous bats species play a key role in agriculture by improving yields and reducing chemical pesticide costs. Using metabarcoding, it is possible to determine the prey consumed by these noctule mammals based on the DNA fragments in their fecal pellets. This study aimed to evaluate COI and 16S metabarcodes for insect species identification to determine the diet of metropolitan bats. For this purpose, COI and 16S metabarcodes were extracted, amplified, and sequenced from 65 bat feces collected in the Berlin metropolitan areas. Following a taxonomic annotation, I found that 73% of all identified insects could only be detected using the COI method, while 15% could be recovered using the 16S approach. Just 12% of all detected insects were identified simultaneously by both markers. According to this result, COI is more suitable for the taxonomic identification of insects from bat feces. However, given the bias of COI primers, it is recommended to use both markers for a more precise estimation of species diversity. Additionally, based on the insect species identified, I noticed that urban bats fed mainly on Diptera, Coleoptera, and Lepidoptera. The bat species *Nyctalus noctula* was most abundant in the samples. His diet analysis revealed that 91% of the samples contained the insect species *Chironomus plumosus*. 14 pest insect species were also found in his diet.

Zusammenfassung:

Trotz der zahlreichen Vorteile, das Stadtleben für die Lebensbedingungen der Menschen mit sich bringt, gibt es auch negative Auswirkungen auf die Menschen, ihre Umwelt und andere Organismen in städtischen Lebensraum. Während einige Wildtiere das Leben in städtischen Gebieten zweifellos meiden, sind andere toleranter oder bevorzugen das Leben in städtischen Lebensräumen. Es gibt mehr als 1 400 Fledermausarten auf der Welt. Sie haben daher das Potenzial, erheblich zur biologischen Vielfalt der Säugetiere in städtischen Gebieten beizutragen. Insektenfressende Fledermausarten spielen eine Schlüsselrolle in der Landwirtschaft, da sie die Erträge verbessern und die Kosten für chemische Pestizide senken können. Mit Hilfe des Metabarcodings ist es möglich, die von diesen nachtaktiven Säugetieren verzehrte Beute anhand der DNA-Fragmente in ihren Kotpellets zu bestimmen. Ziel dieser Studie war es, COI- und 16S-Metabarcodes zur Identifizierung von Insektenarten zu vergleichen, um schlussendlich die Ernährung von Fledermäusen in urban Gebieten zu bestimmen. Zu diesem Zweck wurden mittels COI- und 16S-Metabarcodes aus 65 Fledermauskotproben DNA extrahiert, amplifiziert und sequenziert, die in Berliner Großstädten gesammelt wurden. Nach einer taxonomischen Annotation stellte ich fest, dass 73 % aller identifizierten Insektenarten nur mit der COI-Methode nachgewiesen werden konnten, während 15 % mit dem 16S-Ansatz wiedergefunden werden konnten. Lediglich 12 % aller nachgewiesenen Insektenarten wurden gleichzeitig mit beiden Methoden identifiziert. Diesem Ergebnis zufolge ist COI für die taxonomische Identifizierung von Insekten aus Fledermauskot besser geeignet. In Anbetracht der Verzerrung der COI-Primer wird jedoch empfohlen, beide Marker zu verwenden, um die Artenvielfalt genauer einschätzen zu können. Anhand der identifizierten Insektenarten konnte ich außerdem feststellen, dass sich Fledermäuse in Städten hauptsächlich von Diptera, Coleoptera und Lepidoptera ernähren. Die Fledermausart *Nyctalus noctula* war in den Proben am häufigsten vertreten. Seine Diet-Analyse ergab, dass 91% der Proben die Insectenart *Chironomus plumosus* enthielten. 14 Insektenpestarten wurden ebenfalls in seiner Ernährung gefunden.

Acknowledgement

I would like to express my gratitude to my thesis advisors for their guidance and encouragement throughout the preparation of this work. Special gratitude to Dr. Carolin Scholz for his availability whenever I ran into trouble or had a question about my research or writing. Many dearest thanks to Fabienne Pritsch for her technical support in the metabarcoding laboratory of the Leibniz-IZW. I would also like to acknowledge PD Dr. Christian Voigt as the second reader of this thesis, and I am gratefully indebted for his very valuable comments on this thesis. Finally, I must express my profound gratitude to my family for their unfailing support and continuous encouragement during my years of study and the writing of this thesis.

Kevine Phalone Ngoufack Djoumessi

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Abbreviations

COI:	Cytochrom C Oxydase 1
DNA:	Deoxyribonucleic Acid
Bp:	Base pair
PCR:	Polymerase Chain Reaction
HTS:	High-Throughput Sequencing
BOLD:	Barcode of Life Data System
rRNA:	Ribosomal Ribonucleic Acid
eDNA:	Environmental DNA
DADA:	Divisive Amplicon Denoising Algorithm
ASV:	Amplicon Sequence Variant
IZW:	Institute for Zoo and Wildlife Research
BeGenDiv:	Berlin Centre for Genomics in Biodiversity Research
IPM:	Integrated Pest Management

1. Introduction

Despite the numerous benefits of urbanization to human living conditions, urbanization has also negatively affected humans, their environment, and other organisms that share urban habitats with humans. The expansion of the cities destroys natural habitats, constraining wild animals in small spaces. Over the recent decades, pollution, flooding, global warming, and other climate change impacts have significantly increased (Patella et al. 2018). Urbanization goes along with many additional physical and chemical changes, such as eutrophication, increased waste generation, altered hydrology, increased anthropogenic noise and artificial light at night (Voigt und Kingston 2016, S. 15). Unplanned development of urban areas leads to the loss of natural habitats which consequently impacts the wild animals. According to the (World Bank 2022), the destruction of natural habitat is expected to increase by 1.2 million km² worldwide by 2030. Furthermore, urban expansion decreases the total amount of available natural habitats and increases the isolation of remnant non-urban habitat patches within the urban matrix (Grimm et al. 2008). The loss or fragmentation of natural habitats caused by urbanization adversely impacts biodiversity and ecological processes. According to (McDonald et al. 2019), land use changes are responsible for the most significant biodiversity losses. In some urban areas, species richness decreased by more than 50% compared to intact natural habitats (McDonald et al. 2019). One of the main consequences of the change in habitat configuration and connectivity is the disappearance of vulnerable species. While some wild animals tend to decline in urban environments, others are more tolerant to urban habitats.

Bats include more than 1,400 species worldwide and thus have the potential to constitute a substantial proportion of mammalian biodiversity in urban regions (Simmons und Cirranello 2020). Although most bats avoid urban areas, some bat species may still thrive in cities. Indeed, among vertebrates, bats form the most diverse group of mammals remaining in urban areas (Ree und McCarthy 2005). However, numerous studies indicate that bat activity and species diversity are greatest in more natural areas and decrease as urbanization increases (Kurta und Teramino 1992; Legakis A et al. 2000). The analysis of the environmental parameters showed that these flying mammals are more attracted to sites with abundant vegetation and also to those that lie at the edge of cities in sparsely built areas (Legakis A et al. 2000). In their search for food, bats are also drawn to areas with moderate light levels, which attract insects (Legakis A et al. 2000).

Bats play a key role in maintaining the ecological balance. Over two-thirds of the estimated 1,400 extant bat species are obligate or facultative insectivorous mammals (Kasso und Balakrishnan 2013). According to (Boyles et al. 2011), these insectivorous bats are known as voracious predators of many crop and forest pests. With this ability, insectivorous bats contribute significantly to improving agricultural yields and reducing chemical pesticide costs (Aguiar et al. 2021). Aguiar et al. (2021) posit that bats may contribute to saving US$94 per hectare of cornfields, accounting for an annual savings of US$ 390.6 million per harvest in Brazil (Aguiar et al. 2021). Bats are also considered bioindicators (Jones et al. 2009). While their abundance can indicate changes in populations of arthropod prey species, their diminution can reveal the deterioration of water quality, agricultural intensification, loss and fragmentation of forests, fatalities at wind turbines, disease, pesticide use, and overhunting (Jones et al. 2009). For all these reasons, the protection and conservation of bats are mandatory. To better protect these nocturnal animals, the analysis and understanding of their diet can be an important starting point.

The bats' diets play a crucial role in their health and survival, as well as in the ecosystem dynamics of their habitats. In this thesis, I aim to evaluate COI and 16S metabarcodes for insect species identification from bat faeces. Specifically, I asked: (1) which of these metabarcodes is the most suitable to analyze the diet of bats based on fecal pellet, (2) which insect species are consumed by bats that occur in a major metropolitan area in Central Europe, (3) do these bats also consume insect pests? My hypotheses is that: (1) the 16S marker can be an alternative to the COI markers for the metabarcoding of insects in bat faeces, and (2) some urban bats include pest insects in their diet.

To evaluate the hypothesis, I analysed bat faeces collected in the Berlin metropolitan area. I used COI and 16S minibarcodes to identify prey species in these pellets. Using statistical analyses, I compared the results of the two biomarkers and examined the diversity and composition of the diet of bats observed in the Berlin metropolitan area. Since COI markers have already been developed and used during the past decades, this approach benefits from an extended reference database. If 16S markers are also suitable to assess the diet of bats, I predict that a substantial portion of insect species identified with COI markers will also be identified with 16S markers. Further, if urban bats are consumers of pest insects, I predict that a substantial portion of their diet consists of pest insects.

2. Background

2.1.DNA metabarcoding

DNA metabarcoding(hereafter "metabarcoding") has recently emerged as a method for conducting simultaneous, multi-species identification of complex mixed communities (OUP Academic 2019). The term DNA metabarcoding was established by Taberlet et al (2012), but it has been also referred as "DNA metagenetic" (Creer et al. 2010), „environmental barcoding" (Hajibabaei et al. 2011), metagenomic amplicon sequencing or simply "marker gene surveys" (Bik et al. 2012). Compared to other methods, DNA metabarcoding sequences have a better taxonomic resolution (Huse et al. 2010). This DNA-based method has revolutionized the world of biodiversity research. Indeed, the primer targets short regions (< 300 bp) of mitochondrial or ribosomal DNA allowing DNA amplification in all types of samples. So, this method can be used in scenarios like diet analyses, forensic and ancient DNA studies, because degraded DNA can be amplified. Additionally, metabarcoding-based approaches are cost-effective and produce comprehensive datasets faster than standard monitoring methods (Ji et al. 2013).

2.1.1. Metabarcoding Workflow

DNA metabarcoding combines two powerful technologies for optimal performance: DNA barcoding and high-throughput DNA sequencing (Ji et al. 2013). The first technology involves naming an organism taxonomically using a DNA fragment, called barcode. To be chosen as a barcode, a gene region must satisfy three criteria: (1) contain significant species-level genetic variability and divergence, (2) possess conserved flanking sites for developing universal PCR primers for wide taxonomic application, and (3) have a short sequence length to facilitate current capabilities of DNA extraction and amplification (Kress und Erickson 2008). One of the main challenges of metabarcoding is to develop universal PCR primers. Ideally, these primers should have broad intra-group coverage, non-biased amplification across species, and high taxonomic resolution (Sakata et al. 2022, S. 16). In metabarcoding, the choice of PCR primers has a great influence on the probability of detection of species or specific taxonomic groups (Alberdi et al. 2018).

The second technology, high-throughput sequencing (HTS), allows the sequencing of targeted genes. Before sequencing, the genetic material is extracted and amplified using the polymerase

chain reaction (PCR). After amplification, high-throughput sequencers are used for the sequencing of individual DNA molecules. The result is an extensive list of DNA sequences, which will then be processed using bioinformatic methods.

2.1.2. Metabarcodes for insect species identification

Depending on the species to be identified, the selection of the metabarcode plays an important role in the success of metabarcoding (Deagle et al. 2014, S. 3). Indeed, this selection is not only based on the ability of the metabarcode to differentiate taxa but also on the availability of a reference database for the taxonomy assignment (Liu et al. 2020). In the case of insects, the Cytochrome C Oxidase 1(COI) gene is the standard metabarcode and the Consortium for the Barcode of Life (CBOL) has adopted it. In fact this marker has the required attributes: its variation allows species-level discrimination, it can be PCR amplified from most animals and the associated database (BOLD) now boasts millions of taxonomically verified DNA sequences (PubMed Central (PMC) 2022). This marker permitted the discrimination of closely related species in Lepidopterans, a group of insects with modest rates of molecular evolution and high species diversity (Hebert et al. 2003). Unfortunately, being a protein-coding gene, COI is highly variable in the third position of most codons due to genetic code redundancy, making it difficult to design primers for metabarcoding with good taxonomic coverage (Marquina et al. 2022). Due to the amplification bias of COI primers, alternative metabarcodes have been explored. One of these alternatives is the 16S ribosomal marker.

The 16S rRNA is a mitochondrial gene with a length of 1500bp. The sequencing of the entire gene is difficult. It requires using of multiple primer combination and different partial fragments that vary in length and region (Chan et al. 2022). However, only short fragments of 16S rRNA are used for species identification, because they are easy to sequence, and their performance has already been proven (Chan et al. 2022; Elbrecht et al. 2016). Several studies have recognized the mitochondrial large subunit rRNA gene (16S) as a marker with the potential for species-level resolution and more conserved regions (Elberecht et al. 2022). According to Li et al. (2010) the sequence of 16S rRNA accumulates mutations more rapidly than the nuclear rDNA genes and can infer relationships beneath the family level within insects. The performance of this new marker has been tested in insect metabarcoding with promising results (Marquina et al. 2022). In fact, by comparing the performance in silico and the efficiency in vitro of the two markers, Clarke et al.

(2022) showed that COI primers amplified in silico less than 75% of insect species with complete mitochondrial genomes available, whereas new primers targeting 16S provided more than 90% coverage. Additionally, the utilization of 16S primers in the amplification of freshwater invertebrate mock communities confirmed the previous results by showing a very good efficiency for species identification (Elbrecht et al. 2016). Marquina Hernández (2020) has also shown that 16S is better than COI for metabarcoding of environmental DNA(eDNA) samples because the less degenerate 16S primers do not amplify as many off-target organisms. However, the performance of 16S for identifying insects present in fecal pellets of insectivorous bats has not been extensively evaluated.

2.2. Molecular methods for diet analysis

Diet analysis is an important component of animal ecology because it allows us to understand the species' nutritional ecology (Liu et al. 2021a). Traditional diet analysis methods generally rely on preserving remains found in the fecal pellets or stomach contents of studied species to determine their diet, using visual recognition of morphological features by macro- or microscopic methods (Pompanon et al. 2012). These methods are useful, but they are exceedingly difficult to apply in some cases, such as the observation of small invertebrates, nocturnal animals, or species living in the soil or under water. Microscopic examination also has important limitations. In fact, it is not only labor intensive, but the results depend on the knowledge of the person conducting the analysis.

To overcome these difficulties, new methods have been developed. One of these methods is the molecular-based analysis of animal diets. This new approach, known as metabarcoding, has recently become popular, as it provides high resolution and accuracy (Liu et al. 2021b). Through the combination of high-throughput sequencing, the use of “universal” PCR primers to maximize DNA detection from the widest possible range of prey species, and new bioinformatic tools for the selection of appropriate molecular barcodes and data curation, the metabarcoding of mixed samples (fecal pellets, stomach, and gut contents) is widely used. (Lara et al. 2022). One of the advantages of metabarcoding is that it can rapidly characterize the species present in an animal diet. With the capacity to analyze hundreds of samples on a single HTS run, DNA metabarcoding can increase the number of samples analyzed while reducing the time needed for it and the associated cost (Liu et al. 2022).

The diet analysis using metabarcoding is done through the following steps: collection of sample (stools or gut contents); DNA-extraction; PCR amplification of small DNA fragments using specific primers and DNA sequencing with NGS. The obtained DNA sequences are then filtered and analyzed to assign taxonomy using bioinformatic pipelines. Although not exhaustive, here is a selection of the most commonly used pipelines : QIIME-uclust (Callahan et al. 2016), MOTHUR (Schloss et al. 2009), USEARCH-UPARSE (Edgar 2010), Qiime2-Deblur (Amir et al. 2017), DADA2 (Callahan et al. 2016) and USEARCH-UNOISE3 (Edgar 2010). While the first three regroup sequences with typically 97% identity into operational taxonomic units (OTUs), the last three attempt to reconstruct the exact biological sequences present in the sample, called Amplicon Sequence Variants (ASVs) (Prodan et al. 2022).

2.3.Bioinformatic analysis: DADA2 PIPELINE

DADA (Divisive Amplicon Denoising Algorithm) is an algorithm used to denoise sequences from high-throughput sequencing without training the data (Rosen et al. 2012). DADA2 is an open-source R package that extends and improves the DADA algorithm. (Callahan et al. 2016). This package can extract exact amplicon sequence variants (ASVs) from amplicon data. Compared to other methods, the ASVs produced have fewer false positives and maintain high sensitivity. DADA2 also reduces sequence error and sequence de-replication through quality filtering, denoising, read pair merging and PCR chimera removal. The package computes the error model of forward and reverse reads independently. Additionally, DADA2 classifies the ASVs taxonomically using a choice of supplied databases (Ewels 2022).

3. Aim and objectives

Metabarcoding has revolutionized the investigation of the animal world by allowing, among others, rapid analysis of their diet. It has the potential to both inform basic natural history and ecology for many mammals and elucidate key dietary requirements for others of conservation concern (Ingala et al. 2022). Among mammals, bats are one of the groups benefiting from this innovative technology. Despite their small body size, nocturnal foraging activity, and ability to fly, metabarcoding allows detailed analysis of bat diets from feces or stomach contents.(Ingala et al. 2022). The COI metabarcode, because of its established database, is the most used marker for the diet analysis of these nocturnal mammals. However, taxa abundance estimation and species detection are limited due to their primer biases (Elbrecht et al. 2016). This limitation led to the exploration of other markers, such as the 16S rRNA gene, which has been suggested due to its potential for species-level resolution and more conserved regions (Deagle et al. 2014; Clarke et al. 2014). This work aims to evaluate COI and 16S metabarcodes for insect species identification to determine the diet of bat living in and around Berlin metropolitan area. To achieve this goal, we first compared the results obtained by the COI and the 16S metabarcodes to determine which is more suitable for the analysis of the diet of bats using feces. Secondly, we identified the insects consumed by bats living in the metropolitan area of Berlin. Finally, we investigated the role of these bats in maintaining the ecological balance of this city by identifying if they also consume a considerable proportion of insect pests.

4. Material and Methods

4.1. Study area and sampling

In 2019, bat feces were collected at 16 different sites in and around Berlin during March, May, June, and August (Figure 1 and Table 1). Most of the samples were collected in artificial daytime roosts, also called bat boxes. However, five of the samples were found by chance at construction sites. A total of 65 samples were collected and transferred to tubes. The tubes were then transferred to sterile bags and frozen at -20°C before being transported to the Leibniz Institute for Zoo and Wildlife in Berlin for DNA extraction and amplification.

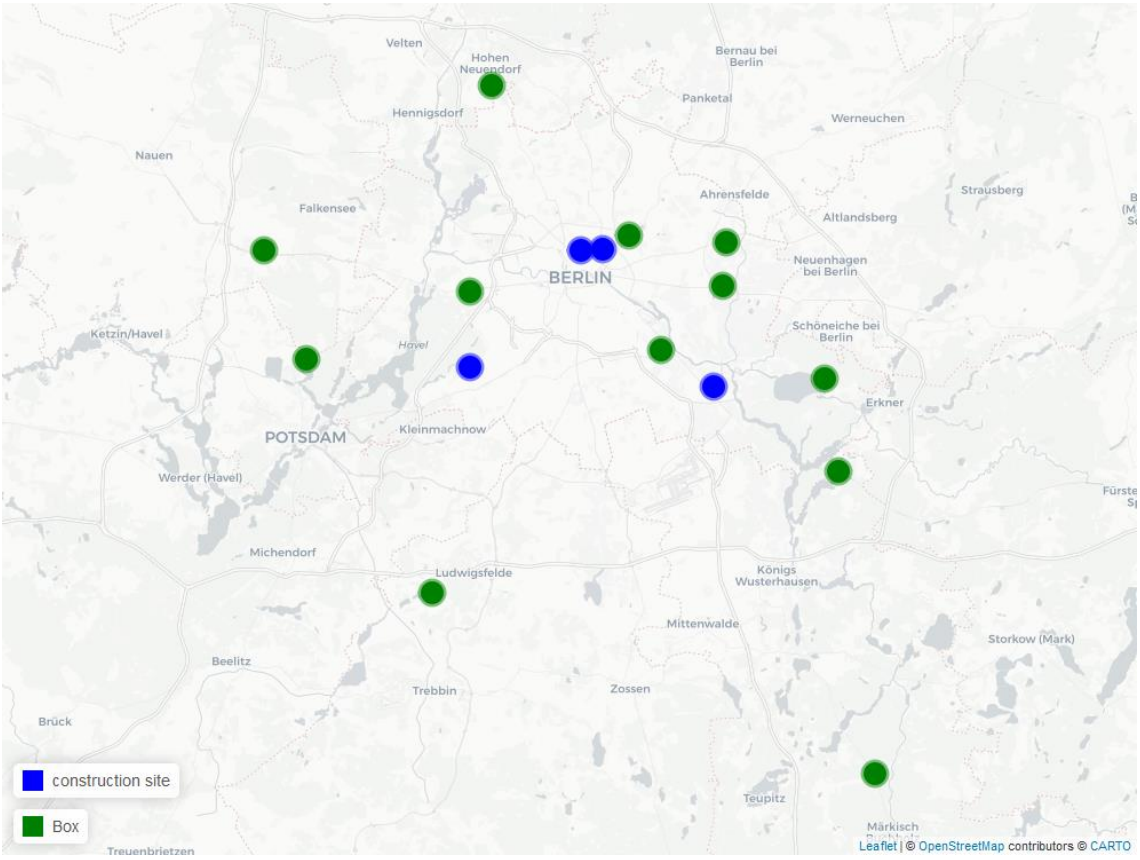

Figure 1: Collection sites of bat stools

Table 1: Sampling details of bat feces used in this study

	Number	Date (2019)	Habitat
Börnestraße 1, 13086 Berlin	1	12.08.	Box
Dörpfeldstraße 37, Berlin Treptow	1	30.07.	Box
ehem. Kaserne Krampnitz bei Potsdam	3	28.06.	Box
Hochsitzweg 121, Berlin. Zehlendorf	1	12.07.	Construction site
Hussitenstraße 7, Berlin Mitte	2	04.03.	Construction site
Invalidensiedlung 30, Berlin Reinickendorf	1	04.06.	Construction site
Knaakstraße 68, Berlin Pankow	1	12.06.	Construction site
Königsheide Bln. Baumschulenweg	12	14.08.	Box
Müggelsee	3	28.08.	Box
Teufelssee	1	28.08.	Box
Oberförsterei Hammer, Nähe Märkisch Buchholz	4	01.07.	Box
Olympisches Dorf, Elstal	1	22.07.	Box
Parkfriedhof Marzahn	11	17.07. 21.08.	Box
Schloßpark Biesdorf	19	23.05.;16.07.; 21.08.	Box
Schmöckwitzwerder, am Gosener Kanal	2	14.08.	Box
Siethen Schloß, Nebengebäude, Potsdamer Chaussee	1	15.07.	Box

4.2.Laboratory procedures

The DNA extraction and the Polymerase Chain Reaction (PCR) were conducted at the Leibniz Institute for Zoo and Wildlife Research in Berlin, while the sequencing was performed at the Berlin Centre for Genomics in Biodiversity Research (BeGenDiv). Laboratory protocols were strictly followed throughout the experiment to prevent contamination by foreign DNA and PCR products. The presence of contaminants was also checked during the whole laboratory procedure via different negatives and positive controls.

4.2.1. DNA extraction

After thawing the bat pellets, DNA was extracted in the IZW laboratory using the NucleoSpin Stool Kit (Macherey-Nagel GmbH & KG, Düren, Germany) according to the manufacturer's instructions. Two DNA extractions were performed for each sample to maximize the amount of extracted DNA. The extracted genetic material was then quantified by determining its concentration using the Qubit Fluorometer (Qubit fluorometric quantification dsDNA High Sensitivity Kit, ThermoFisher Scientific, Walham, USA). To eliminate PCR inhibitors, the DNA extraction was completed purifying some DNA extracts utilizing Zymo-Kit (Zymo Research, 17062 Murphy Ave, Irvine, CA 92614, USA).

4.2.2. PCR amplification and DNA sequencing

We amplified two minibarcodes: COI (~ 133 bp) and 16S (~ 155 bp). For the amplification of the COI markers, we used the primers recommended by Galan et al. (2017), while 16S amplification was done via primers recommended by Taberlet et al. (2018)**(**Table 2). Amplicon libraries were constructed using a two-step PCR strategy combined with the dual-index paired-end sequencing approach. The targeted minibarcodes were amplified during the first PCR via their specific forward and reverse primers. Each of these primers was linked at the 5'-end with Illumina sequencing primers and a heterogeneity spacer to create an artificial nucleotide diversity during the first cycles of the Illumina sequencing (Galan et al. 2017, S. 5). We amplified each target region in separate 25 µl reactions per fecal sample containing 5 µL of FIREPol ReadyMix, 0.5 µL of each primer, 14 µL of water, and 5 µl of DNA extract. PCR conditions for the amplification of COI consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 30 seconds, and then finally an extension step at 72°C for 10 min and hold at 8°C. For the 16S minibarcode, the reactions

were performed under the following thermal profile: initial denaturation at 95°C for 5 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes and a conservation at 8°C.

The products of this first PCR were checked with agarose gels and stronger products were purified with magnetic beads (CleanNGS, GC biotech, Waddinxveen, Niederlande). The concentration of each product was measured using fluorometric quantification (Quant-iT™ dsDNA Assay Kit, high sensitivity, ThermoFisher Scientific, Walham, USA) in a Tecan plate reader (infinite M200, Tecan, Switzerland).

During the second PCR, indexes were added to the target region. This PCR2 was performed in 25 µL reaction volume using 10 µL of PCR1 products as a template. Each 25 µL reaction volume contained 13,75 µL PCR Master-Mix Herculase and 0,625 µL of each indexed primer. The PCR2 started with a first denaturation step of 95°C for 2 minutes, followed by 8 cycles of denaturation at 95°C for 20 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 30 seconds followed by a final extension step at 72°C for 3 minutes (Supplement 1). The index combination was unique for every PCR2 product. Each COI metabarcode was amplified and indexed in two independent PCR reactions. It was impossible to perform technical replicates for the 16S markers because the sample quantity was insufficient. At the end of this second PCR amplification, the products were checked again with an agarose gel and cleaned twice with magnetic beads (CleanNGS, GC biotech, Waddinxveen, Niederlande). Using the fluorometric quantification, the concentration of amplified DNA products was measured in the plate reader (Quant-iT™ dsDNA Assay Kit, high sensitivity, ThermoFisher Scientific, Walham, USA) and pooled in equimolar concentration. The final library was cleaned and concentrated using CleanNGS beads. The Agilent 2200 TapeStation with D1000 ScreenTapes (Agilent Technologies, Santa Clara, California, USA) was used to verify the library's integrity and quality.

At the BeGenDiv, samples were sequenced in three runs on the Illumina MiSeq platform (Illumina, San Diego, California, USA) with v3 chemistry with 600 cycles. FastQC v.0.11.9 and multiqc have been used to check the quality of the reads which have been generated. Cutadapt (version 2.8) was used to remove the remaining adapter. Due to the insufficient quantity of amplified products, technical replicates were done only for the COI but not for the 16S.

Table 2: Primer pairs used for the amplification of COI and 16S

Targeted minibarcode	Length	Primer combination (Direction 5'-3')	References
COI	~133 bp	Forward: ATTCHACDAAYCAYAARGAYATYGG	Galan et al. 2017
		Reverse: ACTATAAARAARATYATDAYRAADGCRTG	
16S	~155 bp	Forward: RGACGAGAAGACCCTATARA	Taberlet et al. 2018
		Reverse: ACGCTGTTATCCCTAARGTA	

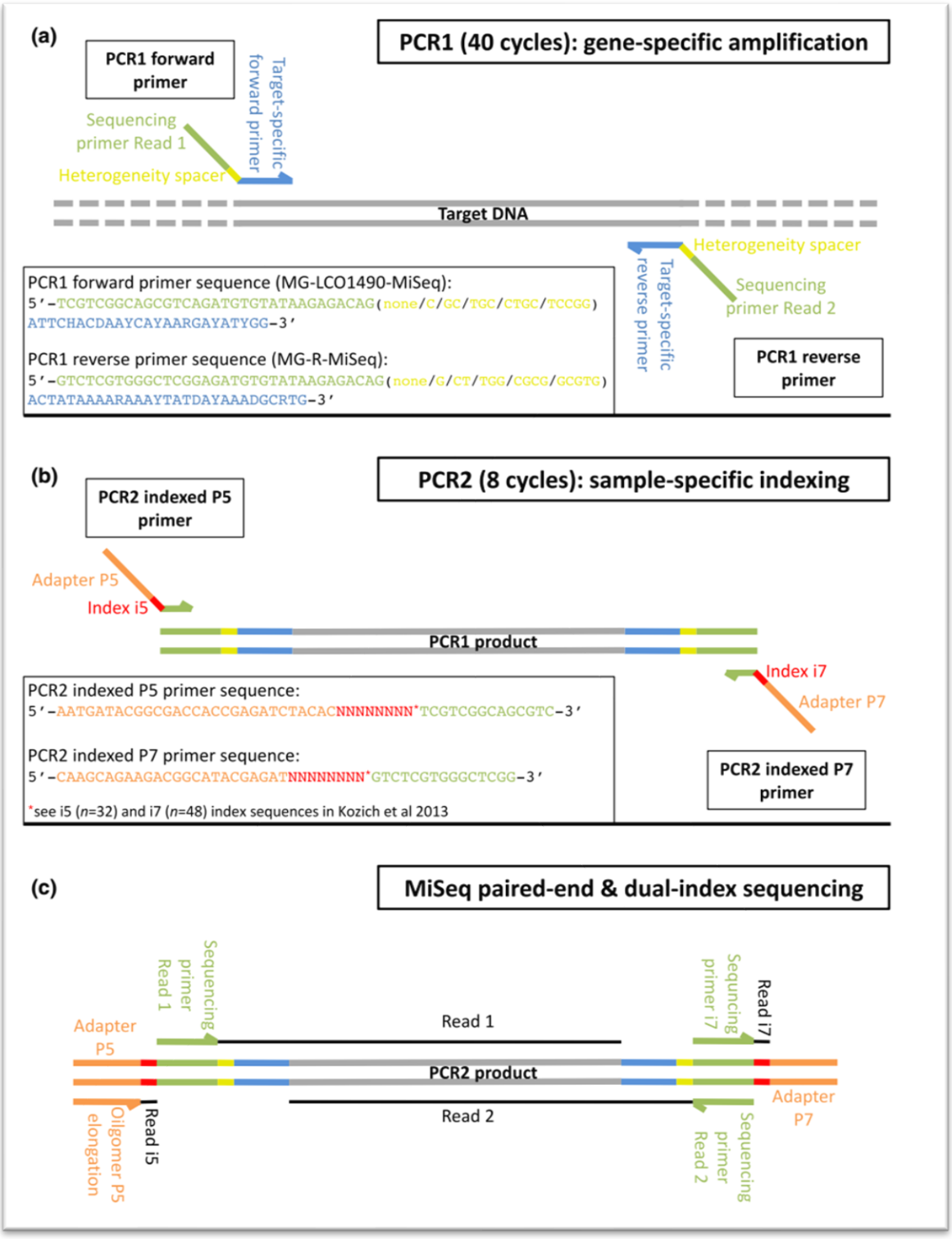

Figure 2: Schematic description of the library construction using two-step PCR and MiSeq sequencing for COI (Galan et al. (2017)

4.3.Bioinformatic processing of data

The bioinformatic analysis was done in R version 4.2.0 (R Core Team 2022) and R Studio 2022.02.1. The R package "dada2" (Callahan et al. 2016) was used to process sequencing reads from quality check to taxonomic assignment. After importing the data to R, the quality of some forward and reverse reads was checked using the function "plotQualityProfile*".* Then, based on the obtained quality plots, I used the function "*filterAndTrim*" to truncate the forward and reverse reads where the quality distribution had been crushed. For the COI marker, forward and reverse reads were truncated at position 160. For the 16S minibarcodes, the quality plot for the forward reads was good, while it was worse for the reverse reads. For this reason, I have cut the forward reads at position 180 and the reverse reads at position 80. Forward and reverse primers were removed based on their respective length.

Furthermore, the functions "*learnErrors"* and "*derepFastq"* were used for error estimation and dereplication of forward and reverse reads. Using these dereplicated reads and the estimated error model, we applied the core sequence-variant inference algorithm to calculate abundance p-values for each unique sequence. This step is particularly important because it allows the identification and elimination of reads caused by errors. After removing erroneous reads, the function "*mergePair*" was utilized to merge the denoised forward and reverse reads only if they overlapped exactly 20 nucleotides minimum. Afterwards, the amplicon sequence variants (ASV) table was constructed, and the chimeras were checked and removed using the functions "*makeSequenceTable*" and "*removeBimeraDenovo*". Finally, we assigned taxonomy to the inferred Amplified Sequence Variants up to the species level with the function "*assignTaxonomy*". This taxonomy assignment is based on the single best hit or a last common ancestor (in case of multiple best hits) with 50 out of 100 bootstrap replicates as minimum bootstrap confidence. I used the reference database provided by Heller and colleagues for the annotation of COI sequences (Heller et al. 2018). 16S sequences were annotated using a database containing insect 16S minibarcodes, which I implemented in my previous works.

For further analysis, I used the R package "*phyloseq"* (McMurdie und Holmes 2013) to compile the assigned ASVs by sample ID and species annotation into a single occurrence matrix. I also removed the contamination using the package "*microDecon*" (McKnight et al. 2019). In fact, with its function named "*decon*", contaminant reads can be systematically identified and removed from

metabarcoding data by using the proportions of ASVs present in blank samples (negative controls). For statistical analysis, I created a dataset combining the number of ASVs found in each sample and their taxonomic annotation. Then I removed all species not belonging to insects and bats from the COI-file. In addition, I kept only the ASVs that were annotated to the species level

4.4.Statistical analysis

The statistical analysis was done in R version 4.2.0 (R Core Team 2022) and R Studio 2022.02.1. Before analysis, I calculated the average COI reads of each sample using the number of reads present in the technical replicates.To evaluate the capacity of both primers to be used for insect identification, I first used the Jaccard similarity index to assess the similarity of both minibarcodes at species level. Furthermore, I used double bar charts to visualize the difference between the number of orders generated using the COI and 16S markers for insect identification. To determine the composition of the bat diet, I merged the datasets containing the annotated metabarcodes and removed all duplicates. Using the R package "*vegan*", I analyzed the bat diet diversity by determining Shannon's and Simpson's diversity indices. Then, I used a pie chart to represent the proportion of each insect order consumed by bats. Finally, thanks to the bat DNA present in the feces, I identified the bat species that producted the fecal pellets. Then I performed dietary analysis of the most represented bat, *Nyctalus noctula*, using the samples where it was detected alone. Using a list of pest insect species collected in public databases such as InsectBase (Mei et al. 2022) and Forest Pest Europe (LOS 2023), I also identified and classified pest insects present in diet of *Nyctalus noctula*.

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5. Results

5.1. Sequencing results and data filtering

Across all 65 samples, I successfully amplified 59 samples using 16S primers and 63 samples using COI primers. MiSeq sequencing generated 411933 reads belonging to the 16S minibarcode, while 3226999 reads were generated for COI minibarcode. After filtration, denoising, and merging, 387391 sequences of 16S minibarcodes against 3076969 sequences of COI minibarcode were recovered. Chimera removal and taxonomic annotaion allowed the identification 3496 ASVs with the COI and 371 ASVs with 16S. After decontamination and selection of identified hexapods at the species level, the final datasets contained 326 COI metabarcodes against 105 16s´S metabarcodes (**T*a*ble 3**).

T*a*ble 3: Number of reads after each data filtering step

	COI	16S
Input	3226999	411933
Filtered	3156755	408571
DenoisedF	3137444	406196
DenoisedR	31388120	407107
Merged	3076969	387391
without chimeras	3064254	386298
Taxonomic annotation	3496	371
Final dataset (only insects' species)	326	144

The taxonomic annotation of the COI metabarcodes allowed not only the identification of insects, but also other organisms. Based on the abundance of reads and the number of samples in which each species where found, I noticed that one of the most represented organisms besides insects was bats. Knowing that these bats could be the ones that produced the fecal pellets, I aimed to identify

the bat species. For this purpose, we retained only the bats that could be identified up to the species level and those with more than one hundred reads. Over the 65 fecal pellets used in this study, bat species were identified in 62. All the detected bats belonging to the family *Vespertilionidae*. Based on the number of samples in which each bat species was found, I noticed that most of the fecal pellets produced were from the species *Nyctalus noctula* (***Table 4***).

Table 4: Identified bat species living in and around Berlin

Species	Total number of reads belonging to each bat species	Number of samples in which each bat species was detected
Nyctalus noctula	229275	57
Plecotus auritus	36200	21
Myotis myotis	22366	14
Pipistrellus pipistrellus	26381	14
Eptesicus nilssonii	20090	10
Vespertilio murinus	631	3
Myotis nattereri	167	2

5.2. COI versus 16S for insect metabarcoding

After analyzing the final datasets, I found that the COI dataset contained 11 insect orders, consisting of 91 families, and 249 genera. Compared to COI dataset, 16S dataset contained 9 insect orders, 51 families and 94 genera (**Table *5***).

Table 5: Summary of the number of insects detected by both markers at each level

	COI	16S
Order	11	9
Family	91	51
Genus	249	94
Species	326	105

Most of the insect orders (9) have been amplified by both markers. With the COI approach, two more insect orders were identified, namely Odonata and Trichoptera. The most represented insect orders for both markers were Coleoptera, Diptera, Lepidoptera, and Hemiptera **()**. Additionally, the

Jaccard similarity index showed that only 12% of the insects were amplified simultaneously by both primers. 15% and 73% of insect species were detected with either the 16S or the COI method.

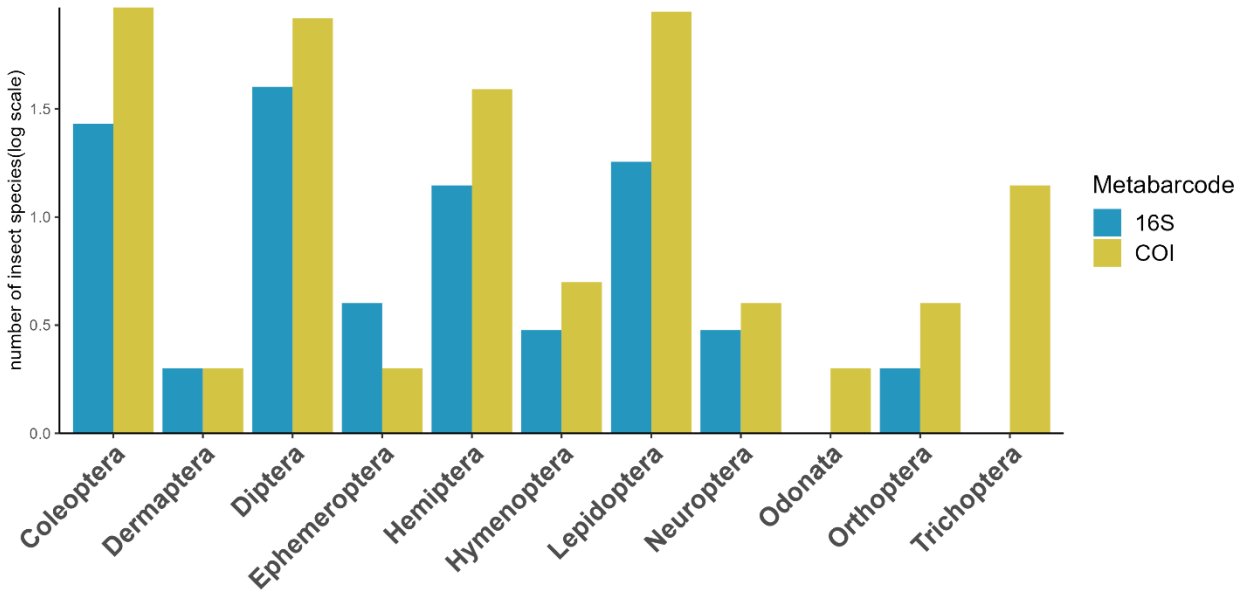

Figure 3: Comparison of the number of detected species per order (log scale) using COI and 16S approachs

5.3.Diet analysis of insectivorous bats from Berlin metropolitan area

I calculated the alpha diversity of prey insects according to the orders detected by both markers. The Shannon and Simpson diversity indices were 1.61 and 0.77 respectively. The low value of Shannon diversity index suggest that the diet of city bat is constituted of few insect orders. The high value of Simpson diversity index not only confirms the low diversity of bat diet, but it also shows that this diet is dominated by few insect orders. Among all the insect detected by both markers, I found that the bat's diet is composed of 11 insect orders. After merging both datasets and deleting the duplicates, I noticed that the most represented insect orders were: Diptera (28% of ASVs), Coleoptera (26% of ASVs), and Lepidoptera (26% of ASVs) (**Figure 4**). Some orders such as Neuroptera (1%), Odonata (0.2%), Orthoptera (0.7%), Ephemeroptera (0.7%), and Dermaptera (0.5%) were represented with low amount of ASVs.

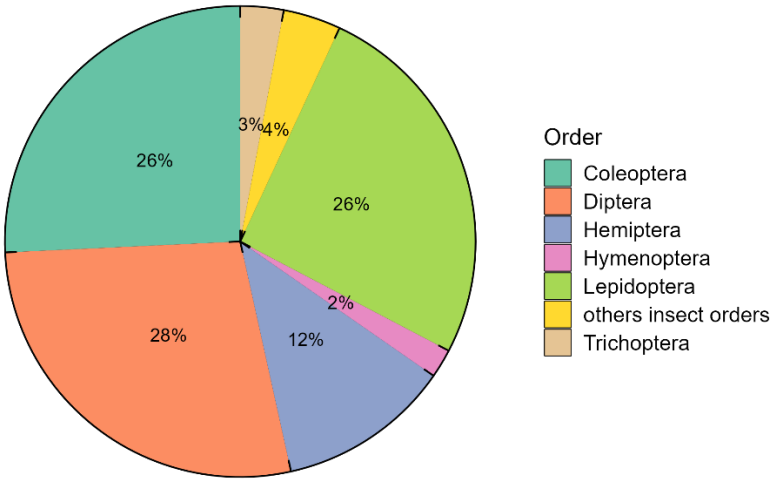

Figure 4:Diet composition of insectivorous bats from the Berlin metropolitan area.

5.4. Diet analysis of *Nyctalus noctula*

For the analysis of the diet of *Nyctalus noctula*, I used 24 of the 65 samples used in this study, because it contained only this species of bat. By combining the results of the two metabarcodes, 182 insect species were identified using the DNA fragments present in the fecal pellets of these bats. Among these 182 insect species, the most dominant prey species were *Chironomus plumosus* (91% of the 24 selected samples), followed by *Cloeon dipterum* (79%), *Spondylis buprestoides* (66%), *Aedes vexans* and *Delia platura* (62% each).

Additionally, I noticed that among the 182 insects detected present in diet of *Nyctalus noctula*, 14 species were pest insect. Based on the number of samples in which each pest insect was found, the most represented pest insects were: *Spondylis buprestoides* (66% of the 24 selested samples), *Plutella xylostelle* (58%), *Amphimallon solstitiale* (54%), *Tipula oleracea* (50%) and *Culex quinquefasciatu*s (45%) (***Figure 5***). Six of the observed pest species are known to be silvicultural pests. I observed three nuisance species. Five insect species were agricultural pests (***Figure 5***).

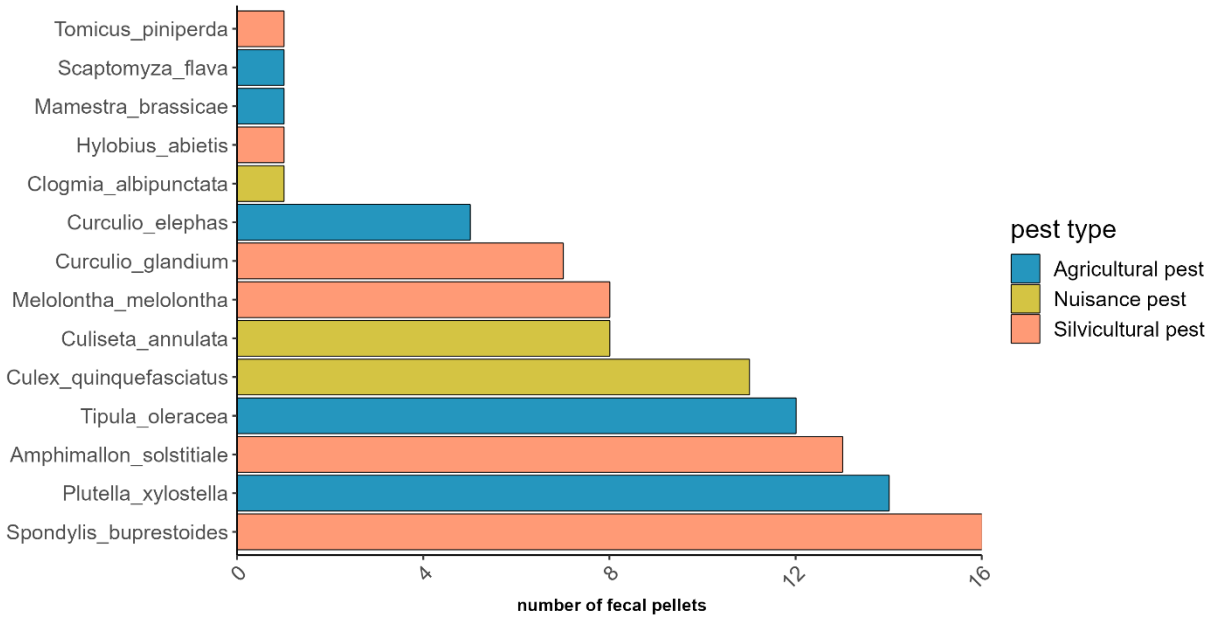

Figure 5: Number of fecal pellets in which each pest species was found

6. Discussion

This work aimed to evaluate COI and 16S markers for insect metabarcoding of bat faeces. More specifically, I wanted to know which of the two markers is best suited for analyzing bat diet. My study was based on fecal samples collected in the Berlin metropolitan area, Germany. This city is recognized in Central Europa as the capital of bats with the presence of more than 31 bat hibernacula (Oberste Naturschutzbehörde des Landes Berlin 2015). Using the COI minibarcodes, I found that the collected pellets exclusively originate from bats of the family *Vespertilionidae*, which are known to be insectivorous. A total of seven bat species were recorded. From the bat pellets, I identified 105 insect species with the 16S minibarcodes and 326 insect species with the COI approach. Based on all the insect species recognized by the two markers, I found that the diet of bats from Berlin is dominated by three insect orders, namely Diptera, Lepidoptera and Coleoptera.

6.1. Comparison between markers

Overall, I found considerable differences between the results obtained by both markers. Compared to COI, fewer insect species were detected using the 16S method. This difference can be explained by the fact that technical replicates were not performed for the 16S minibarcode, whereas they were for the COI approach. The absence of replicates led to the production of fewer ASVs, thus reducing the number of detectable insects. According to van den Bulcke et al. (2021, S. 243), PCR replicates, at least three, not only allows the detection of several species also contribute to reduce the errors generated by PCR amplification. This difference in results is not only observed after the taxonomy assignment. In fact, I also noted a big disparity between the number of reads generated after the sequencing and after the data filtering steps (**T*a*ble 3**). Whereas COI reads amounted to several millions, 16S reads amounted only to a few thousands. This divergence in the number of produced reads may so be related to the fact that COI marker is universal and therefore amplifies all kind of taxa, while 16S is particular designed for insects.

Furthermore, I noticed that 73% of all identified insects were only recognized with the COI minibarcode, indicating that the COI database contains more annotated insect sequences than the 16S database. The number of insects in the COI database equaled 49592 against 33599 for the 16S

database. Since the scientific community recognizes COI as the barcoding marker of choice for animals, particularly for invertebrates, it has a relatively large reference database, which has been actively developed and extended over many years(Ratnasingham und Hebert 2007). The recovery of more annotated COI metabarcodes can also be justified by the fact that the German insect fauna was further studied using COI markers, which allowed the enrichment of its database with the hexapods of this region. This is not the case for the ribosomal database, as this marker is a new minibarcode with limited availability of reference sequences and yet not fully explored taxonomic resolution on the species level for insects (Elbrecht et al. 2016).

Certainly, the COI minibarcode allowed the identification of more insect species, but using the 16S metabarcodes, some species that the COI could not identify were recovered. This illustrates that both markers must be used to maximize insect identification and to generate a comprehensive picture of the biodiversity of a site or sample. My results falsify the hypothesis that 16S metabarcodes can be an alternative to COI metabarcodes, yet they are consistent with other studies that recommend complementary multi-marker approaches in the metabarcoding of insect communities (Clarke et al. 2022; Deagle et al. 2014; Clarke et al. 2014; Marquina et al. 2020).

6.2.The diet of bats in the Berlin metropolitan area

Using the mitochondrial and ribosomal markers a global diet analysis of bats living in Berlin was carried out. Shannon and Simpson diversity indices suggested that the diet of common noctule bats is constituted of few insects. The dietary analysis cconfirmed this result by showing that these bats feed mainly on Diptera, Lepidoptera and Coleoptera. These results were congruent with previous knowledge of the prey consumed by this bat species, e.g morphological and molecular biological analyses(Galan et al. 2017, S. 13; Donatus Waghiiwimbom et al. 2019). Coleopterans are easily found in large quantities in the bat diet because they are the world's largest order of insects comprising about one-third of all insect species (Yom-Tov und Whitaker 2002). The dominance of just three insect orders in the diet of bats supports the hypothesis that the diet of urban bats is dominated by insect orders, which includes a large number of insect species. However, the relative proportions of biomass of the various insect orders were not studied, making it impossible to determine the relationship between prey insects consumed and potential prey insects available, i.

e. whether bats consume Diptera in proportion to their abundance, or whether they actively prefer this insect as their diet. (Yom-Tov und Whitaker 2002, S. 380)

Using metabarcoding, it is possible to identify bat consumed prey at the species level. Indeed, compared to morphological studies that allowed prey taxonomic identification at the order (sometimes family) level, this new DNA-based technology provided more details on dietary composition by increasing prey taxonomic resolution (Galan et al. 2017). With metabarcoding, it is possible to recognize the majority of insects consumed by bats up to the species level, including those present in small quantities, which cannot be identified using morphological analysis. In my case, this approach allows detecting five insect orders that were less present in fecal pellets, namely Neuroptera (1%), Odonata (0.2%), Orthoptera (0.7%), Ephemeroptera (0.7%), and Dermaptera (0.5%).

Additionally, the DNA present in bat fecal pellets allowed the detection of seven insectivorous bat species, all belonging to the family of Vespertilionidae. Based on these number of fecal pellets in which bat species *Nyctalus noctula* were found, I concluded that this bat species could be the most observed bats in Berlin metropolitan area. This species is also known as the common noctule bats. They are very sensitive to light and prefer to live in less-lit areas. In metropolitan areas like Berlin, they are most often found in dark corridors such as urban forests, parks or waterways. (Voigt et al. 2020). The diet analysis of this bat showed that it feed more on certain insect species than others, probably because these insects are the most present in their environment.

Furthermore, this study revealed the presence of 14 pest insect species in the diet of *Nyctalus noctula*, belonging to three groups, namely silvicultural, agricultural pests and nuisance insects. Silvicultural and agricultural pests were the most often consumed, indicating this bat play a potential role in controlling large pest outbreaks in agricultural areas (Monck-Whipp et al. 2018). For this reason, urban farmers wishing to benefit from the insect pest control service provided by bats may incorporate bat-mediated insect suppression into existing IPM strategies by managing a diversity of noncrop habitats and roosting sites to support different bat species foraging over crops (Kolkert et al. 2020, S. 384). Further, Maas et al. (2016) confirmed the importance of bats for insect pest control. The authors highlighted that bats could even be used to suppress arthropod outbreaks at the regional level. Due to the trophic interactions of bats, including their ecosystem services, other predators, such as birds, cannot replace them (Maas et al. 2016). The consumption of nuisance

pests proves that bats can be used to control the proliferation of insect species harmful to humans. However, the ability of bats to regulate insect pests depends on predation risk, light intensity, and life stage.

7. Limits and perspectives

The findings of this study have to be seen in light of some limitations. It is difficult to evaluate the performance of the two markers if the amplification and sequencing were not done under the same conditions. Performing technical replicates for one but not the other prevents identifying the cause of the discrepancy in the results. In this study, with the lack of technical replicates for 16S markers, it is impossible to conclude whether the small number of species identified using this marker is because some metabarcodes were not amplified or because this marker cannot be used to identify certain insect species. After applying the same amplification and sequencing conditions, the databases used for taxonomic annotation must be similar. Thus, depending on the insect species recovered, the performance of each marker can be easily evaluated. These libraries should also contain local insect species, which will increase our ability to assess the robustness of our taxonomic assignments (Ingala et al. 2022).

For the diet analysis, it would have been interesting to determine the diet composition of each bat species living in the Berlin metropolitan area using both metabarcodes. In future studies, capturing and identifying each bat species would be preferable before collecting the feces for molecular analysis. The fecal pellet of each individual should be collected over more than one night (Ingala et al. 2022). Moreover, sample collection should include different seasons at the same sampling site, to allow inferences about differences related to seasonal environmental changes and minimal nutritional requirements during the growth and reproduction stages (Lopes et al. 2015).

8. Conclusion

In this work, I aimed to evaluate COI and 16S metabarcodes for insect species identification from bat feces. The results showed that among all the insects identified, 73% were detected using the COI approach, while only 15% were recovered with the16S approach. This suggests that both markers should be used simultaneous for the metabarcoding of insects in order to maximize the estimation of species richness from environmental samples. area. These bats feed mainly on Diptera, Coleoptera and Lepidoptera. Based on the collected fecal pellets, I discovered that the bat species *Nyctalus noctula* are the most observed bats in Berlin metropolitan. I also noticed the diet of this bat species is dominated by few insect species. More interesting, I found that *Nyctalus noctula* also feed on pest insect species. The most consumed pest insects are agricultural and silvicultural pests, suggesting that common noctule bats can be used to control the proliferation of pest insects. My results provide a foundation of knowledge regarding the bat diet analysis using metabarcoding. But to improve the results and better evaluate the two metabarcodes, it will be preferable to carry out this study on samples containing known insect species. Additionally, the importance of bats in regulating the entomological ecology of urban areas calls for more in-depth studies on the diet of each bat species living in Berlin metropolitan area.

Declaration of authorship

I hereby declare that i have prepared this work independently and only with the use of the literature and aids indicated.

Passages that have been taken verbatim or in spirit from sources are marked as such.This work has not been submitted in the same or similar form to any other examination authority.

Mittweida, 05 February 2023

Kevine Phalone Ngoufack Djoumessi

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Supplementary

SUPPLEMENTARY 1: Adaptors and PCR schedule conditions used for COI and 16S metabarcoding for identification of insects presents in bat.

Adaptaters used for the amplification

	Adapter Name	Sequencing Adapter	Interspacer
Forward	MG-LCO1490-MiSeq_P5_01	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	Y
Reverse	MG-univR_P7_01	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	Y

PCR conditions for first PCR of COI

PCR-Master-Mix FirePol:

Component	final concentration	MM µl each
H_2O	ad 20µl	14,00
FIREPol ReadyMix (5x)	1x	5,00
FP 10uM	0,25 µM	0,50
RP 10uM	0,25 µM	0,50
Template-DNA (~2ng/µl)		5,0µl
Total		25,0µl

PCR program: "PCR barc 45°C"

95°C	5 min	
94°	30s	40 cycles
45°	45s	
72°	30s	
72°	10min	
8°	∞	

PCR conditions for first PCR of 16S

PCR-Master-Mix FirePol:

Component	final concentration	MM µl each
H_2O	ad 20µl	14,00
FIREPol ReadyMix (5x)	1x	5,00
FP 10uM	0,25 µM	0,50
RP 10uM	0,25 µM	0,50
Template-DNA (~2ng/µl)		5,0µl
Total		25,0µl

PCR program: "PCR barc 45°C"

95°C	5 min	
94°	30s	35 cycles
45°	45s	
72°	30s	
72°	10min	
8°	forever	

PCR conditions for second PCR

PCR Master-Mix Herculase:

component	final concentration	MM each (µl)	
H2O	ad 25,0µl	6,875	
Buffer (5x)	1x	5,0	
dNTP-Mix (10mM each)	0,25mM each	0,63	
DMSO	4%	1,0	
Herculase II Fusion (U/µl)	Units	0,25	
			→ dispense á 13,75 µl
Index-Primer P7_xx (10pmol/µl)	0,25 pmol/µl	0,625	
Index-Primer P5_xx (10pmol/µl)	0,25 pmol/µl	0,625	
PCR1-Product:		10,0 µl	

PCR program: "Indexing“

95°C	2 min	
95°C	20 sec	8 cycles
52°C	30 sec	
72°C	30 sec	
72°C	3 min	