

RESOURCE ARTICLE

Establishing arthropod community composition using metabarcoding: Surprising inconsistencies between soil samples and preservative ethanol and homogenate from Malaise trap catches

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Abstract

DNA metabarcoding allows the analysis of insect communities faster and more efficiently than ever before. However, metabarcoding can be conducted through several approaches, and the consistency of results across methods has rarely been studied. We compare the results obtained by DNA metabarcoding of the same communities using two different markers – COI and 16S – and three different sampling methods: (a) homogenized Malaise trap samples (homogenate), (b) preservative ethanol from the same samples, and (c) soil samples. Our results indicate that COI and 16S offer partly complementary information on Malaise trap samples, with each marker detecting a significant number of species not detected by the other. Different sampling methods offer highly divergent estimates of community composition. The community recovered from preservative ethanol of Malaise trap samples is significantly different from that recovered from homogenate. Small and weakly sclerotized insects tend to be overrepresented in ethanol while strong and large taxa are overrepresented in homogenate. For soil samples, highly degenerate COI primers pick up large amounts of nontarget DNA and only 16S provides adequate analyses of insect diversity. However, even with 16S, very little overlap in molecular operational taxonomic unit (MOTU) content was found between the trap and the soil samples. Our results demonstrate that none of the tested sampling approaches is satisfactory on its own. For instance, DNA extraction from preservative ethanol is not a valid replacement for destructive bulk extraction but a complement. In future metabarcoding studies, both should ideally be used together to achieve comprehensive representation of the target community.

KEY WORDS

environmental DNA, insects, Malaise traps, metabarcoding, nondestructive extraction, preservative ethanol

1 | INTRODUCTION

The spectacular diversity of insects, coupled with the shortage of adequate expertise and resources for taxonomic identification, has long hindered studies of the structure and functioning of the insect component of ecosystems. However, DNA metabarcoding are making it feasible to analyze the composition and structure of entire insect communities in detail, opening up research questions in community ecology that have simply not been possible to be addressed before (Gibson et al., 2015). As exciting as these prospects may be, the methodology of DNA metabarcoding of insect communities is still immature. Several different approaches have been tried but it is still unclear how consistent results are across methods and what protocol is optimal for a particular study (see Majaneva, Diserud, Eagle, Hajibabaei, & Ekrem, 2018). As an example, there is still debate over the optimal marker for DNA metabarcoding of insect communities, and to what extent combinations of markers can be used to improve the resolution and accuracy of the analyses (Andújar, Arribas, Yu, Vogler, & Emerson, 2018; Clarke, Soubrier, Weyrich, & Cooper, 2014; Gibson et al., 2014). The efficiency and biases of different sampling and analysis protocols are also poorly understood: insect communities could be studied using metabarcoding of the preservative ethanol from Malaise trap samples, from homogenized tissue from such samples, or from environmental DNA (eDNA) in soil samples, but we lack an understanding of consistency of results across these methods. This paper is devoted to exploring such methodological issues associated with metabarcoding studies of insect communities.

Multi-marker metabarcoding approaches are used to obtain biodiversity information from several domains of life in a particular habitat, combining the most used markers for each of the taxa: 16S for prokaryotes, 18S for eukaryotes, COI for animals and *rcbl* or *matK* for plants (Drummond et al., 2015; Gibson et al., 2014; Krehenwinkel et al., 2018). In other cases, two markers are used to provide complementary data on the same taxonomic fraction of the sample. In such cases, it is common to use a conserved marker with high taxonomic coverage (such as 18S) to ensure the detection of all the main groups of the target taxon, and a more variable marker (such as COI) providing information at higher taxonomic resolution but at the cost of some degree of amplification bias (Coward et al., 2015; Tang et al., 2012). This approach has the advantage that the two markers complement each other, with one marker ameliorating potential biases in amplification of a specific taxon by the other marker (Freeland, 2017; Wangenstein, Palacín, Guardiola, & Turon, 2018). Yet, the lack of resolution of the conserved marker decreases its effectiveness (e.g. Clarke, Beard, Swadling, & Deagle, 2017; Tang et al., 2012).

In response, some authors have advocated the use of an alternative marker of intermediate variability: more conserved than the

high-resolution marker (typically COI), but more variable than the nuclear rRNA genes. The mitochondrial rRNA gene 16S has been offered as such a marker (Clarke et al., 2014; Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014; Marquina, Andersson, & Ronquist, 2018). To our knowledge, only a few studies have tested the potential of using 16S as a replacement for COI in insects *in vitro* (Clarke et al., 2014; Elbrecht et al., 2016; Epp et al., 2012), or as a complement (Alberdi, Aizpuru, Gilbert, & Bohmann, 2018), and only one study made use of both markers simultaneously in resolving ecological rather than methodological questions (Kaunisto, Roslin, Sääksjärvi, & Vesterinen, 2017). These studies have reported that the performance of 16S is considerably poorer compared to COI (Alberdi et al., 2018; Elbrecht et al., 2016), but this discrepancy may be attributed to the fragment of the 16S gene amplified being very short (160–190 bp approximately), and to the fact that the reference library of the 16S gene is substantially smaller than that of COI (Andújar et al., 2018; Deagle et al., 2014). Using longer fragments of the 16S gene, the taxonomic resolution increases to levels comparable to COI (Clarke et al., 2014; Marquina et al., 2018).

Malaise traps have been widely used in morphology-based biodiversity surveys (e.g. Global Malaise Trap Program [GMP, <http://biodiversitygenomics.net/projects/gmp/>]). More recently, catches from such traps have been among the first types of bulk samples subjected to DNA metabarcoding analysis (Gibson et al., 2014; Ji et al., 2013; Shokralla et al., 2015; Yu et al., 2012), offering promising vistas for automated, large-scale biomonitoring of complex communities (see Porter & Hajibabaei, 2018). Yet, the optimization of protocols for metabarcoding of Malaise trap samples and other terrestrial arthropod samples (e.g. canopy fogging) has lagged behind the methods for other types of mixed samples, such as freshwater or marine benthos, or soil. Only a few studies have focused exclusively on methodological improvements of samples of this type, although the numbers are increasing in recent times (Creedy, Ng, & Vogler, 2019; Krehenwinkel et al., 2017, 2018; Morinière et al., 2016; Wilson, Brandon-Mong, Gan, & Sing, 2018). Much of the work done on metabarcoding of freshwater invertebrates can be applied to terrestrial bulk samples. Such transfer of methods may include, for instance, the use of the preservative ethanol to extract DNA without destroying the morphology of the organisms. Nevertheless, insect communities found in freshwater benthos and Malaise traps have different characteristics, with freshwater samples dominated by larval stages and often characterized by low diversity and abundances (see Baloğlu, Clews, and Meier (2018) for exceptionally diverse kick net samples), while Malaise trap catches are dominated by adult insects and generally characterized by high diversity and abundances. As a consequence, the transfer of techniques between these types of samples demands specific testing and optimizing.

An alternative to analyzing DNA from trap catches is to use traces of DNA present in the environment. When successful, such environmental DNA (eDNA) samples e.g. soil or water may reveal a large fraction of taxa present in the region, and serve as an efficient indicator of local biodiversity (Bohmann et al., 2014; Creer et al., 2016; Deiner et al., 2017). However, most of the template DNA in environmental samples consists of highly degraded extracellular DNA from decomposed tissues, cells and excreta of organisms. The low quality of the template DNA makes extraction and amplification extra challenging, and primers that are successful for metabarcoding of trap samples may not work for environmental samples. Importantly, eDNA analysis is focused on recovering traces of total biodiversity of the medium the sample was taken from. This can result in a case where the PCR or extraction step are saturated with off-target DNA, thus yielding few sequences of the intended taxon. Highly degenerate primers worsen this situation, especially when such high degeneracy is needed to amplify the full diversity of the target taxon (Horton, Kershner, & Blackwood, 2017; Macher et al., 2018). Even when less degenerate primers are used, like those for 18S, DNA amplified from undesired organisms can still form half of the sequencing output (Yang et al., 2014). Studies comparing the results of metabarcoding of eDNA and trap samples have been scarce, and have mainly focused on freshwater samples (Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016; Macher et al., 2018), or soil (Horton et al., 2017; Watts et al., 2019). To our knowledge, only one study has combined both types of samples with an ecological aim, rather than a methodological one (Yang et al., 2014).

Analyses of complex community samples has often been based on extraction from the homogenized tissue present in the sample (Aylagas, Mendibil, Borja, & Rodríguez-Ezpeleta, 2016; Emilson et al., 2017; Kocher et al., 2017). As an alternative approach, Hajibabaei, Spall, Shokralla, and van Konynenburg (2012) and others (e.g. Erdozain et al., 2019; Zizka, Leese, Peinert, & Geiger, 2018)

have suggested extraction from the preservative medium i.e. the ethanol in which the samples are stored. If successful, such an approach would reduce the time of processing and handling, which would decrease the risk of cross-contamination. It would also leave the insects intact. This would not only allow further taxonomic work, but might also allow investigations of the microbiome, parasites/parasitoids or the diet of individual specimens already metabarcoded. However, metabarcoding of preservative fluid may well differ from metabarcoding of homogenized tissue. For instance, small insects with high surface/volume ratio might be expected to be represented by proportionately more DNA in the preservative fluid than in the homogenized tissue, since the leakage of DNA to the preservative ethanol is dependent on body surface. When bulk samples of tissue are homogenized, large-sized organisms will contribute much more DNA to the pool than small ones, biasing the proportion of reads per specimen in the opposite direction (Bista et al., 2018; Elbrecht, Peinert, & Leese, 2017; Krehenwinkel et al., 2017). Other traits can affect the recovery of DNA in the preservative ethanol, such as the degree of sclerotization, or the presence of structures that seal off the body to the entrance of the preservative liquid (Zizka et al., 2018).

In this study, we extend a previous *in silico* study of markers for insect metabarcoding (Marquina et al., 2018) by testing the optimal 16S and COI markers identified there in three different empirical settings. Targeting Malaise trap catches from three different sites at four different time points, we analysed homogenized insects and preservative ethanol from the same samples, and environmental DNA from soil samples taken at the same sites. This allowed us to examine to what extent COI and 16S results overlap or complement each other, and to what extent the sample type affected the performance of the two markers for different insect groups. It also allowed us to explore the degree of consistency in metabarcoding results across two different markers and three different protocols of sampling and to use these results in analyzing the composition of the focal insect communities.

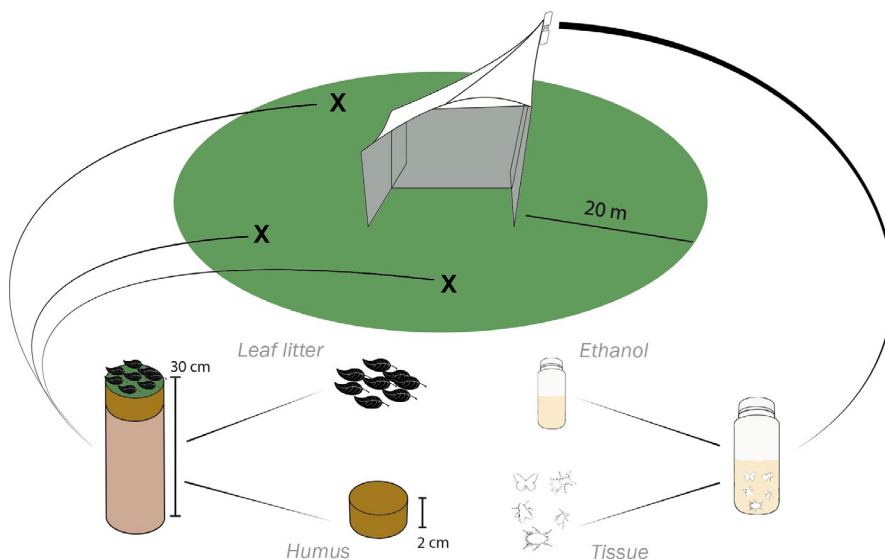


FIGURE 1 Overview of the sampling process and sample typification. At each time point one Malaise trap and three soil cores were taken. The preservative ethanol of the catch was separated and filtered (Ethanol), and the insects were homogenized (Tissue). Each of the three soil cores were separated in leaf litter (Leaf litter) and the two first centimetres of soil (Humus). The three samples for each time point (3 Humus and 3 Leaf litter) were ground and pooled together [Colour figure can be viewed at wileyonlinelibrary.com]

2 | MATERIALS AND METHODS

2.1 | Study area and sampling

To obtain complex environmental samples of insect communities, we targeted the Nacka Nature Reserve at the edge of the metropolitan area of Stockholm (Sweden). Three sites (detailed in Table S1) were chosen to maximize diversity of habitats. The insect community was sampled at four different time points (T1, T2, T3 and T4, corresponding to 4 July, 1 August, 29 August and 26 September 2016) using a Malaise trap run for a duration of 1 week and a soil sample (Figure 1). Each Malaise trap was fitted with a collecting jar filled with 95% p.a. ethanol (no additives). Loss of ethanol volume was not apparent at the moment of collection. Each soil sample consisted of three subsamples taken at random points within a 20 m radius centred at the Malaise trap. Each subsample was taken using a cylindrical core extractor, 30 cm long \times 10 cm diameter, dug down to the mineral soil or to the extractor's maximum length (30 cm deep). Between 2 hr and 3 hr after collection, Malaise trap and soil samples were frozen at -25°C and -80°C , respectively, following standard practices in the laboratory, and were stored for approximately 6 months before analysis. Unique bottles fresh from the manufacturer were used for each Malaise trap sample. The core extractor was subjected to a bleach bath to avoid cross-contamination between sites, and soil samples were stored in individual ziplock bags.

2.2 | DNA extraction

Once in the laboratory, the leaf litter (L hereafter) was isolated from each soil subsample, as well as the first two centimetres of soil (humus or H hereafter). Then, 50 ml of each of the subsamples of leaf litter from the same site and time point were pooled together. The humus subsamples were pooled in the same way. Each pooled sample was thoroughly mixed, manually grinded in a mortar after freezing with liquid N_2 and mixed again, resulting in a total of 24 samples: two layers (humus and leaf litter) \times three sites \times four time points. Each utensil used to manipulate the soil samples (mortar, shovel, etc) was washed with soap, bathed in bleach, thoroughly rinsed and UV-irradiated between samples. Gloves and filter paper were discarded after handling each sample and the bench was cleaned with bleach. Subsequently, 0.4 g of each sample were used for DNA extraction using Nucleospin Soil Kit (Macherey-Nagel) following the manufacturer's protocol.

The Malaise trap samples were handled in a special room of the laboratory dedicated to environmental DNA. This space undergoes frequent cleaning and decontamination (surfaces are bleached and all utensils bleached and irradiated with UV light) and all personnel are required to wear special laboratory coats reserved for this laboratory. The ethanol of the Malaise trap samples was poured through a sieve of 0.6 mm pore size to retain small individuals and body parts. The ethanol was then filtered using Durapore membrane filters (Merk) of 0.45 μm pore size (chosen based on previous positive results in the laboratory

[data not shown]), and discarded. The Durapore filters with the DNA in the preservative ethanol were immediately stored in a 2 ml tube with 400 μl of lysis buffer and stored at -20°C until extraction, while the insects were left to dry on filter paper. The dried insects were homogenized following the same protocol for homogenization and cleaning as with the soil and leaf litter samples, and stored in tubes with 400, 800 or 1,200 μl of lysis buffer depending on the resulting volume of powder after homogenization. All nondisposable utensils and working benches were washed, bleached and UV irradiated between samples using the same procedures as for the soil samples. Both the filter membranes (henceforth referred to as 'ethanol-DNA' or E) and the homogenized insects ('tissue-DNA' or T hereafter) were incubated with proteinase K at 56°C for 24 hr, resulting in a total of 24 samples: two extracts (ethanol and tissue) \times three traps \times four time points. For each sample, 225 μl were used for DNA extraction with a KingFisher Cell & Tissue DNA kit (Thermo Fisher Scientific) on a KingFisher Duo instrument (Thermo Fisher Scientific).

2.3 | PCR and sequencing

Two mitochondrial markers were selected for PCR amplification based on the findings of Marquina et al. (2018). The COI marker was amplified using the primer pair BF2-BR1 (Elbrecht & Leese, 2017; see also Marquina et al., 2018), resulting in an amplicon of approximately 320 bp. The 16S marker was amplified using the primer pair Chiar16SF-Chiar16SR (Marquina et al., 2018), resulting in an amplicon of approximately 350 bp. Primers were tagged with a unique sequence of eight nucleotides at the 5' end (Binladen et al., 2007), and used in different combination of forward and reverse tags for multiplexing samples in a single sequencing run. The PCR mix consisted of one Illustra Hot Start Mix RTG bead (GE Healthcare Life Sciences), 10 pmoles of each primer, 2 μl of DNA template and 21 μl of biology-grade water (final volume: 25 μl). The temperature protocol consisted of an initial phase of denaturation and Taq-activation for 5 min at 95°C , followed by 40 cycles of 95°C for 30 s, annealing phase at $48^{\circ}\text{C}/50^{\circ}\text{C}$ (for COI and 16S respectively) for 45 s and extension phase at 68°C for 45 s, and a final extension phase at 72°C for 10 min. Blanks were prepared with the same volumes, but substituting the DNA with water; the blanks were discarded after checking there was no amplification product in an agarose gel. All PCR runs were duplicated and sample replicates were pooled before library preparation. The concentration of the final PCR products was measured using the high sensitivity reagents in a Qubit Fluorometer (Thermo Fisher Scientific) and they were then pooled to equimolar concentrations and purified. Libraries were prepared with TrueSeq PCR-free kit (Illumina). Desired DNA fragments with adapters were cut off from an agarose gel, purified using QIAquick gel extraction kit (Qiagen), pooled again to equimolar concentrations and sequenced on an Illumina MiSeq, using v3 chemistry and a 2 \times 300 bp paired-end run, at SciLifeLab.

2.4 | Bioinformatic analysis

Bioinformatic analysis was conducted using a combination of the OBITools package (Boyer et al., 2016), other programs are detailed below, custom scripts (available at https://github.com/metagusano/metabarcoding_scripts) and scripts from https://github.com/metabarpark/R_scripts_metabarpark. Read quality was checked using FastQC (Andrews, 2010), and the reads were trimmed after the position in which the average phred score was lower than 28. Paired-end reads with a phred score lower than 30 were discarded. The remaining reads were demultiplexed using the unique sample tags and the primers removed with ngsfilter, and selected based on length. For COI, only reads with a length in the interval 310–330 bp and with no ambiguous bases were retained, while the 16S reads were separated in two categories: 16S 'long' (315–375 bp and with no ambiguous bases) and 16S 'short' (255–305 bp and with no ambiguous bases). These lengths were selected based both on previous results (Marquina et al., 2018, and in silico PCR of arachnid sequences [data not shown]) and on the observed length distribution of the reads, suggesting different amplicon lengths for insects and arachnids. The length constraint used for 16S needs to be relaxed because of the increased frequency of indels in rRNA compared to protein-coding genes. From this point on, the pipeline was conducted in parallel for the three markers (COI, 16S 'long' and 16S 'short'). Reads were dereplicated with obiuniq and chimera removal was conducted using VSEARCH v2.7.1 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016; <https://github.com/torognes/vsearch>), and the remaining sequences were clustered into molecular operational taxonomic units (MOTUs) using SWARM v2.1.13 (Mahé, Rognes, Quince, de Vargas, & Dunthorn, 2015; <https://github.com/torognes/swarm>). Different distance thresholds were tested for the three markers based on previous knowledge of the barcoding gap for each one (Marquina et al., 2018), and the optimal threshold distance, d , was taken to be the value at which the decrease in number of MOTUs reached a stable phase when increasing distance thresholds. The optimal thresholds found using this procedure were: $d = 4$ for 16S 'short', $d = 5$ for 16S 'long' and $d = 9$ for COI (Figure S1). Resulting MOTU tables were curated with LULU v0.1.0 (Frøslev et al., 2017, <https://github.com/tobiasgf/lulu>) to collapse aberrant and presumably erroneous MOTUs. The centroid sequences of each MOTU were compared using ecotag to obtain taxonomic assignment against a custom reference database built with all sequences from Arachnida and Hexapoda from the BOLD database (Ratnasingham & Hebert, 2007; downloaded in June 2018) and the invertebrate and fungi files from release 133 of the nucleotide sequence database from the EMBL repository (Kulikova et al., 2004). At this point, 16S 'long' and 16S 'short' output files were merged. The script *owi_add_taxonomy* was used to complete the taxonomic information (from kingdom to order level) of the annotated MOTUs. In a parallel MiSeq run, a library was sequenced that was prepared with only one of the samples of this study and four controlled tag combinations. The proportion of reads that did not match any of the four combinations was 0.4%. Therefore, the final data set was denoised by deleting from each sample those MOTUs with a relative

abundance of reads lower than 0.4% of the total reads of the sample, and by deleting those with equal or less than 10 reads in total. The detailed bioinformatics pipeline with all programs and scripts used can be found in Supporting Information.

2.5 | Statistical analysis

All analyses of community composition were conducted using the package 'VEGAN' v2.4.4 (Oksanen et al., 2013) and visualized with the package 'GGPLOT2' (Wickham, 2016) in R v3.3.3 (R Development Core Team, 2017). Unless stated otherwise, only MOTUs assigned to Arthropoda were considered. Visualization of the recovered community structure from the different Malaise trap sample types (ethanol or homogenized tissue) was based on a nonmetric multi-dimensional scaling (NMDS) analysis of a Jaccard or a Bray-Curtis dissimilarity matrix with the 16S and COI matrices rarefied to the depth of the sample with least number of reads (function 'rrarefy'). As the results were similar, we only show the results based on the Jaccard index here. To test for differences between the communities recovered from ethanol and tissue with both 16S and COI, we used a Permutation Analysis of Variance (PERMANOVA) with the function 'adonis' for analysis of dissimilarity. Rarefaction curves were obtained with the function 'rarecurve'.

To examine how the morphological traits of individual taxa affected their detectability in the preservative ethanol versus insect homogenate, we scored two sets of characters for each arthropod family: size (large, medium, small) and degree of sclerotization of the cuticle (strong, medium, weak). We then asked two questions: First, how do the traits of the taxon (size, sclerotization) affect its probability of detection (presence/absence) in the two types of samples? Second, how do the traits of the taxon affect its relative read count in the two types of samples?

To answer the first question, we used a generalized linear mixed effects model of presence as a function of fixed effects Sample type (ethanol or tissue), size and sclerotization, along with all two- and three-way interactions. To account for the paired design (where both types of samples were derived from the same Malaise trap catches), we defined Sample ID as a random effect. Since the response was binomial, we assumed a logit-link and binomially distributed error.

To answer the second question, we focused on the log-ratio between the read counts of the same taxon from the same sample, as recorded from ethanol versus tissue homogenate. In other words, we used the formula $R = \log((n_E + 1)/(n_T + 1))$ as our response, where n_E is the taxon- and sample-specific read count from the preservative ethanol, and n_T is the taxon- and sample-specific read count from the tissue homogenate of the same sample. We then fit a generalized linear mixed-effects model of the variable R as a function of size, sclerotization and their interaction, assuming an identity link and normally distributed errors. Following the principle of model reduction, we dropped the nonsignificant interaction term before fitting the final model (Size: $F_{2,74} = 7.54$, $p = .001$; sclerotization: $F_{2,74} = 6.68$, $p = .002$; interaction size \times sclerotization:

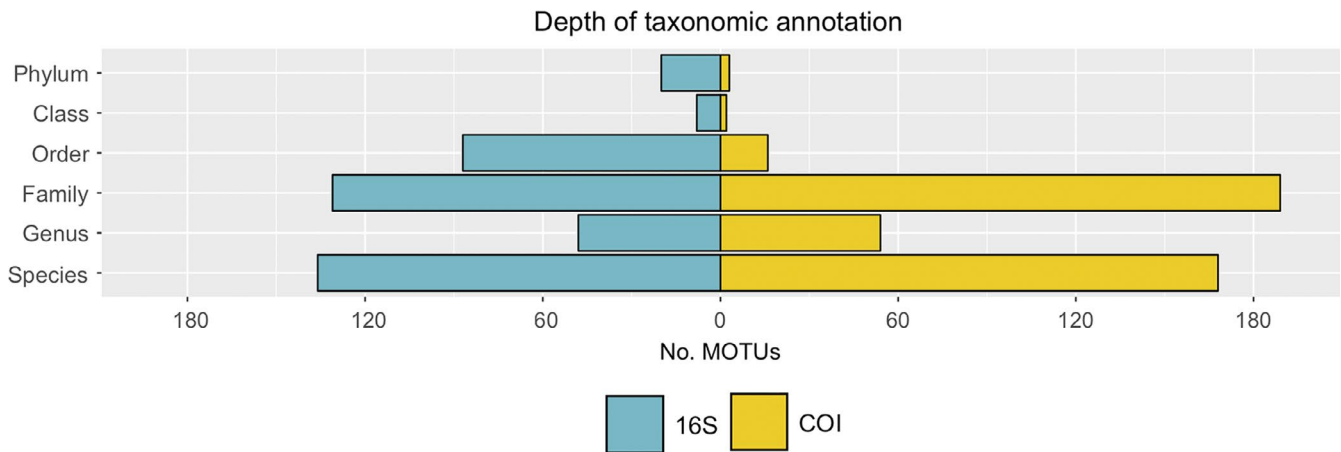


FIGURE 2 Number of MOTUs identified at each taxonomic level for 16S and COI when only Arthropoda OTUs are considered. The total number of arthropod MOTUs for 16S is 430 while for COI is 432 [Colour figure can be viewed at wileyonlinelibrary.com]

$F_{4,70} = 0.22, p = .93$). To visualize the estimated effects, we derived least-squares means from the fitted model. All models were fitted with SAS for WINDOWS version 9.4 (SAS Institute Inc.), procedure GLIMMIX, treating size and sclerotization as categorical class-level variables.

3 | RESULTS

3.1 | Summary of raw reads and MOTUs results

The MiSeq run produced a total of 17.4M reads plus approximately 1.97M control reads of PhiX, of which 13.8M passed the quality filtering and could be assigned to a sample. The average number of reads per sample was $1.73M \pm 732,702$ (SD), with ethanol-16S yielding the most reads (2.64M) and leaf litter-COI the least reads (797,856). Clustering with SWARM produced 60,460 and 34,296 MOTUs for 16S and COI respectively, but after curation with LULU and denoising, this was reduced to 481 and 522 MOTUs, respectively, in the final data set. From these, 430 and 432 MOTUs, respectively, could be assigned to Arthropoda.

The proportion of Arthropoda/non-Arthropoda reads was highly variable depending on the sample and genetic marker (Figure S2). For 16S, the average proportion of Arthropoda reads was 92.1%, ranging from 59.7% to 100% and with no clear pattern among different types of samples. For COI, the value ranged from 0% to 100%, with the leaf litter samples almost entirely comprised by non-Arthropoda reads. Only for three humus samples did we detect any substantial proportion of arthropod reads (20%), as compared to an average of $71.5 \pm 12.0\%$ and $97.5 \pm 7.5\%$ arthropod reads in ethanol and tissue samples, respectively. In all but one sample (16S-H2T2: humus sample from site 2, second time point, amplified with 16S) the rarefaction curves reached the stationary phase well before reaching the maximum number of reads (Figures S3 and S4), suggesting that sequencing depth was enough for recovering all unique PCR products.

3.2 | Comparison between markers

The different taxonomic depth at which the Arthropoda MOTUs were identified reflects differences in the completeness of the reference databases used (Figure 2). The 16S reference database consisted of only 63,325 unique sequences, while the COI database was an order of magnitude larger, consisting of 666,795 unique sequences. As a consequence, the number of MOTUs identified to lower taxonomic levels (species, genus and family) was considerably higher in the COI data set (411 out of 432) than in the 16S data set (315 out of 430).

For most taxa, COI recovered more MOTUs than 16S (Table S2). The number of MOTUs was equal for Hemiptera, Neuroptera, Ephemeroptera and Opiliones, while 16S recovered more MOTUs than COI for Diptera and Psocoptera. Representatives of Trichoptera, Symphypleona, Araneae, Mesostigmata and Sarcoptiformes were only recovered by COI, while MOTUs belonging to Orthoptera (family Acrididae) and Blattodea were only recovered by 16S.

The soil samples present a very different picture (Table S3). Most COI sequences belong to Fungi and other phyla and kingdoms (the unidentified and the non-Arthropoda sequences sum up to 96.37% of the COI reads). Nevertheless, representatives of Orthoptera (family Tettigoniidae) and Araneae were recovered only by this marker in the soil samples. The rest of the orders except Symphypleona, Trombidiformes and Julida were exclusively or predominantly recovered by 16S.

At both species and genus level, most taxa were recovered exclusively by COI, with a lower proportion exclusively recovered by 16S, and even less taxa recovered by both markers simultaneously (Table 1). At the family and order levels, the majority of taxa were recovered by both markers, with a lower proportion detected exclusively by COI or 16S. These patterns are largely due to the incompleteness of the reference libraries. When those taxa recovered by one marker but not present in the reference database of the other marker are eliminated, the situation changes: most species are

TABLE 1 Taxa recovered exclusively by 16S or COI markers from the trap samples (i.e. bulk tissue or preservative fluid), or detected by both markers

Level	Without correction			With correction		
	16S	COI	Common	16S	COI	Common
Species	84	136	29	59	14	29
Genus	53	131	49	53	33	49
Family	10	35	54	10	23	54
Order	3	5	11	3	5	11

Note: The correction refers to eliminating from the reference database of one marker those taxa that were not present in the database of the other marker, with the intent of making detection failures comparable. From this table, we have explicitly excluded the soil samples, which were completely dominated by nontarget DNA amplified by COI (see Figure S2).

recovered exclusively by 16S, and the overlap is higher for the genera, while the numbers for families and orders barely change.

In relation to the individual trap samples, at family level the overlap between 16S and COI was very close to 50%, varying from 40% to 64% depending on the sample (Table S4). Families recovered exclusively by COI represented around 20%–40% of the detected families (with some exceptions). That is, depending on the sample, between 10% to 30% of the total family diversity detected for the target community was not recovered by COI.

3.3 | Trap samples versus soil samples

For both 16S and COI, the homogenized tissue samples were composed almost entirely of reads corresponding to Arthropoda (mean 97.49% and 98.67%, respectively). In the ethanol samples, the proportion of 16S reads corresponding to Arthropoda only decreased slightly (mean 93.45%), while with COI it fell to a mean of 71.54%. In the soil samples, the differences between COI and 16S were even more striking: for 16S, leaf litter and humus samples did not differ significantly from the ethanol in proportion of arthropod reads (mean 88.21%), while COI leaf litter and humus were dominated by non-Arthropoda reads (mean 3.83% Arthropoda reads).

With respect to the number of MOTUs, again COI and 16S present more similar results for the trap samples than for the soil samples. The total number of MOTUs detected in the trap catches (Figure S5) was comparable between COI and 16S. The numbers decreased over time, presumably reflecting the temporal change in the insect communities. The MOTUs detected in preservative ethanol with COI were clearly fewer than those detected with 16S, due to a lower proportion of reads from arthropods. In the eDNA samples (both humus and leaf litter samples; Figure S6), 16S detected varying levels of MOTU richness depending on the sample, ranging from nine to 31 MOTUs. COI only detected a small fraction of the arthropod diversity present in the samples; instead, the COI results were dominated by nontarget DNA sequences (Table S3).

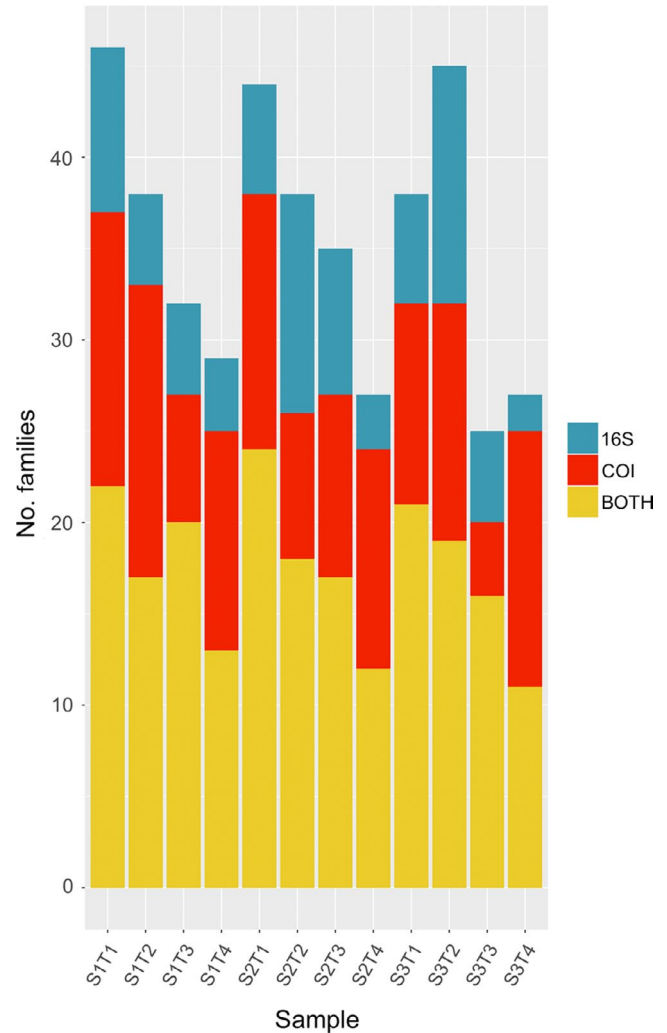


FIGURE 3 Families recovered exclusively by 16S or COI markers from each of the trap samples (i.e. bulk tissue and preservative fluid), or detected by both markers [Colour figure can be viewed at wileyonlinelibrary.com]

From the 430 MOTUs detected by 16S, 337 were found in the trap samples (summing ethanol and tissue) and 120 were detected in the soil (humus and leaf litter). Only 27 of these 430 MOTUs were detected both in the traps and the soil, with Diptera being the order with most MOTUs present in both sample types (18), followed by Coleoptera (three), and other orders with only one MOTU (Hemiptera, Psocoptera, Entomobryomorpha, unidentified Collembola and two unidentified Arthropoda). On each individual site, the overlap between soil and traps consisted of only a few MOTUs (Figure S7). COI detected only 14 MOTUs in the soil samples, out of the 432 found in total, and only two (one Coleoptera and one Symphypleona) appeared in both traps and soil. For COI, the total cumulative number of MOTUs for each site across the four time points (Figure 3) run very close to the curve of the traps, while with 16S, the total cumulative curve is separated from the curve of the traps, and reflects both the increase in both sample types. The increase of the total curve is the result of the sum of the trap and soil curves, indicating no transfer of MOTUs with time from

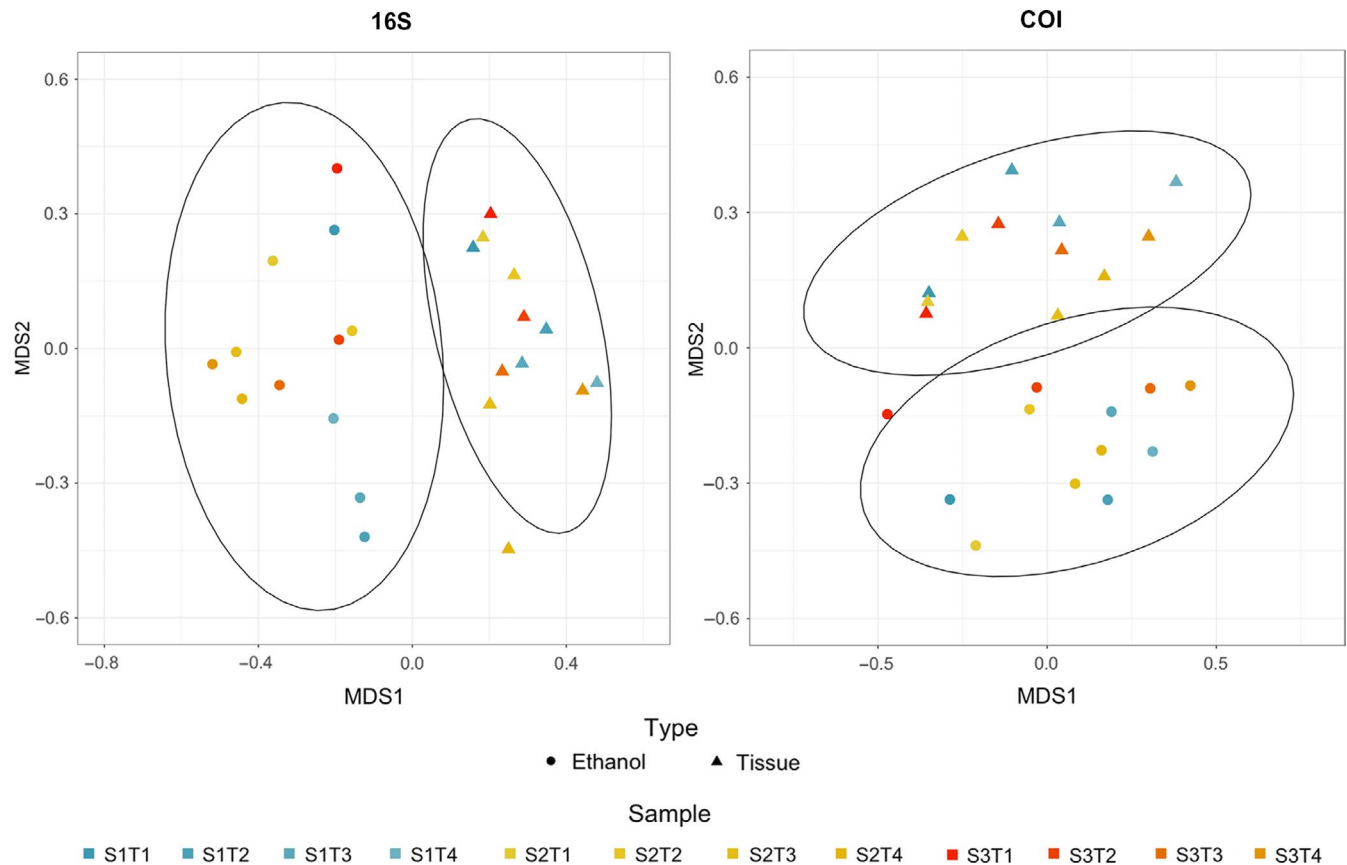


FIGURE 4 NMDS analysis of the recovered communities from ethanol and tissue for both 16S and COI markers, based on a Jaccard dissimilarity matrix [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 The effect of sample type, size and sclerotization on the probability of detecting an insect family

Effect	F-ratio	Num <i>df</i>	Den <i>df</i>	p-value
Sample type	12.33	1	425	.0005
Size	0.18	2	425	.8328
Sclerotization	0.66	2	425	.5175
Sclerotization * sample type	11.86	2	425	<.0001
Size * sample type	12.17	2	425	<.0001
Size * sclerotization	2.77	4	425	.0272

Note: Shown are type III tests of fixed effects of the generalized linear mixed-effects model described in the main text.

Abbreviations: Num *df*, Numerator degrees of freedom; Den *df*, Denominator degrees of freedom; *, interaction.

the soil to the traps, which would be the case if the emergence of flying species from their ground-dwelling larval stages resulted in soil taxa appearing in the Malaise traps after some time lag.

3.4 | Preservative ethanol versus homogenized trap samples

The community recovered from the Malaise trap samples was significantly influenced by sample type (ethanol vs. homogenate)

independent of the marker used (ADONIS 16S ethanol-tissue: $R^2 = .12$, $p = .001$; COI ethanol-tissue: $R^2 = .08$, $p = .001$; Figure 4). A closer look revealed very little overlap between the MOTUs recovered from tissue and ethanol in each sample, and in one case - for primer 16S S3T4 - no overlap at all (Figure S5). For 16S, the number of MOTUs recovered from ethanol was slightly higher than the number of MOTUs recovered from tissue (with some exceptions, e.g. S3T1), while for COI we observed the opposite pattern. This difference may have been caused by the higher proportion of non-Arthropoda sequences of COI detected in the ethanol samples (Figure S2).

Differences in community composition between the two sample types (homogenized tissue vs. preservative ethanol) were correlated with insect traits. The probability with which a family was detected in a sample was significantly affected by sample type (ethanol or tissue), by the two-way interactions between this factor and size and sclerotization of members of the family, and by the interaction between size and sclerotization (Table 2, with family-level patterns shown in Table S5). The effect of size was directly opposite for samples of different type: for ethanol samples, detection probability was highest for insects of small size, whereas for tissue homogenate, detection probability was lowest for small insects and similar for insects of medium and large size (Figure 5, top-left). More highly sclerotized insects were less likely to be detected in both substrates, but these differences were much

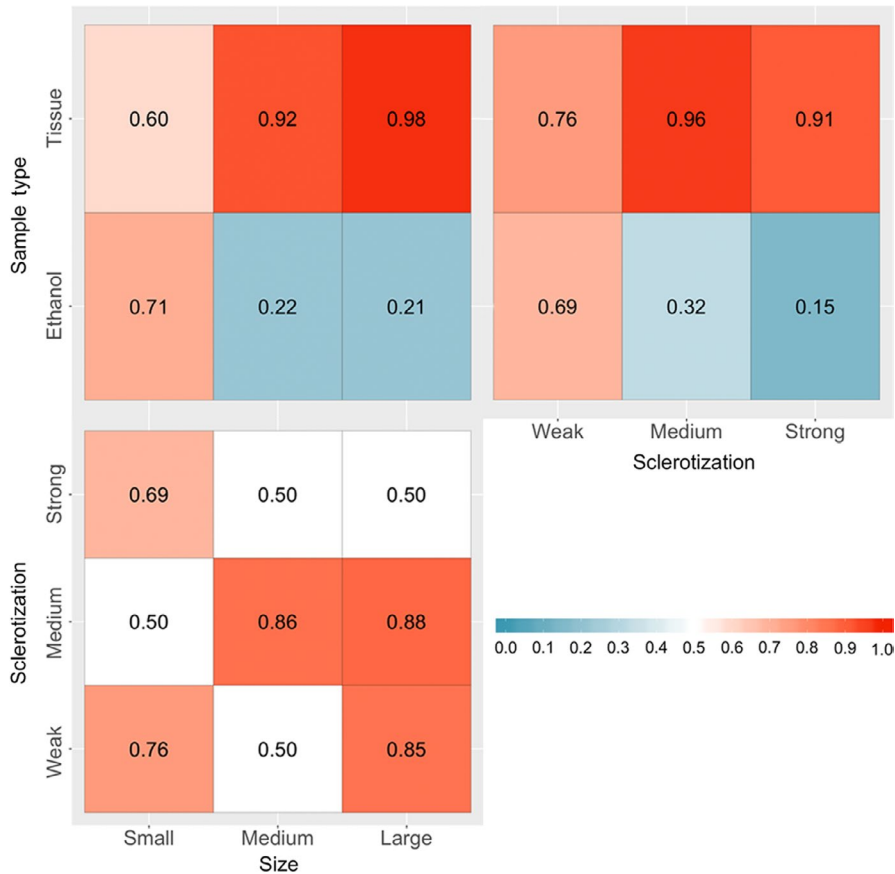


FIGURE 5 Effects of Sample type, size and sclerotization on the probability of detecting an insect family (1: always detected, 0: never detected), as derived from the generalized linear mixed-effects model summarized in Table 2 [Colour figure can be viewed at wileyonlinelibrary.com]

smaller for samples of tissue homogenate than for preservative ethanol (Figure 5, top-right). Insect families of medium sclerotization and medium to large size were the most easily detected, with more complex patterns among other categories (Figure 5, bottom-left).

Likewise, the relative read numbers recovered from samples of different types were strongly affected by insect traits, with both size and sclerotization having a significant effect. As there was no detectable interaction between the two factors (interaction size \times sclerotization $F_{4,70} = 0.22$, $p = .93$), this term was dropped from the final model, in which both Size ($F_{2,74} = 7.54$, $p = .001$) and sclerotization ($F_{2,74} = 6.68$, $p = .002$) retained independent, significant effects. In brief, the log-ratio of counts from ethanol versus tissue declined with increasing size and sclerotization (Figure 6). In other words, the smaller and softer the insect family, the more abundant the reads from ethanol were relative to the reads from tissue.

4 | DISCUSSION

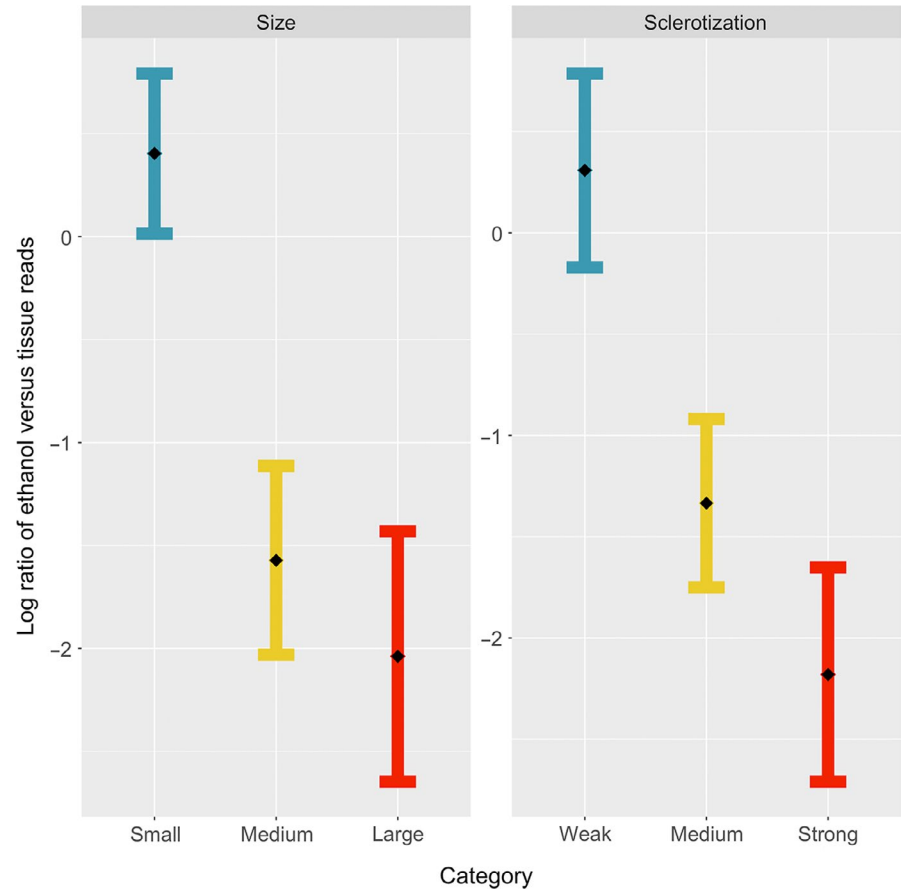
Overall, we found considerable differences between the results obtained with different markers and with different sampling protocols. The most striking result was that analysis of preservative ethanol appeared to preferentially pick out small and soft-bodied insects that presumably comprise a small fraction of the total body mass of the trap sample, but that may comprise a significant fraction of the total species diversity. Our results showed that the combined use of

16S and COI, as well as the metabarcoding of DNA from both the preservative ethanol and homogenized tissue, improved the recovery of arthropod diversity from Malaise trap catches. We also found that the highly degenerate primers required for taxonomically broad COI metabarcoding of insects are problematic in analyses of eDNA samples restricted to this taxon because they pick up so many non-arthropod sequences. The alternative marker 16S recovered more arthropods in soil, but provided little overlap between both bulk and eDNA sample types. Below, we discuss these results in more detail.

4.1 | Comparison between markers

In terms of number of arthropod MOTUs, both markers were consistent, recovering 430 (16S) and 432 (COI) arthropods for the total data set, respectively. The evenness in the number of arthropod MOTUs between both markers allowed for an easy comparison of the quality of the taxonomic annotation allowed by each marker. As expected, MOTUs could be identified to a lower taxonomic level for COI than for 16S, although the difference was not as large as the difference in completeness of the reference databases. For COI, 95% of MOTUs could be identified to family, genus or species, while this was true for only 73% of 16S sequences. These results demonstrate that 16S has adequate taxonomic resolution for metabarcoding of insects, but they also confirm the necessity of creating a local reference database to obtain a biodiversity assessment of equal quality to that obtained by COI with this marker (Clarke et al., 2014; Deagle

FIGURE 6 Least-squares means (with standard errors) from the fitted model of log-ratio between the read counts of the same taxon from the same sample, as recorded from ethanol versus tissue homogenate, as a function of the (left) size and (right) sclerotization of insect families. A higher ratio means more sequences obtained from the ethanol than from the tissue fractions and vice versa [Colour figure can be viewed at wileyonlinelibrary.com]



et al., 2014; Elbrecht et al., 2016). However, the lack of reference sequences for 16S was not as problematic as the small size of the 16S reference library would have suggested. This may be because the Swedish insect fauna is fairly well known and might have been subject to more 16S barcoding than the faunas of poorly documented, megadiverse regions of the world, such as the tropics, where COI barcoding may be more dominant. Possible drawbacks of 16S include the creation of spurious MOTUs with rRNA genes (or 'taxonomic inflation') caused by the difficulty in the bioinformatic pipelines of dealing with gaps in the alignments generated by the indels that occur more frequently in rRNA than in protein-coding genes (Clare, Chain, Littlefair, & Cristescu, 2016), and increased chimera formation caused by the higher similarity between the sequences (Wilson et al., 2018). However, after data set curation removing low abundance MOTUs, we obtained very similar numbers for COI and 16S, indicating that these problems can be circumvented by appropriate data cleaning approaches.

Even though 16S recovered fewer MOTUs than COI in most insect orders, 16S marker complemented the results of COI, as has been concluded by several investigators (Clarke et al., 2014; Deagle et al., 2014; Marquina et al., 2018), falsifying the hypothesis that a highly degenerate COI primer pair is enough to detect all biodiversity (Elbrecht & Leese, 2017; Elbrecht et al., 2016). To judge the overlap between the results obtained by the COI and 16S markers, it is necessary to focus on the taxa for which there is sufficient reference material to avoid biases caused by the vastly different sizes of the reference libraries.

When this is done, the overlap is actually relatively high (62% at family level), and it is the 16S marker that recovers more exclusive taxa (59 species, 53 genera and 10 families) and not COI (14 species, 23 genera and 23 families). These numbers should nevertheless be treated with caution, as the incompleteness of the 16S database could have caused unrealistically low values for COI, by removing taxa from the list that might have actually been detected by the latter and not by the former (but this comparison could not be done since they are not present in the 16S database). The COI marker, even one associated with a highly degenerate primer pair like the one we used, is not sufficient to detect all insect biodiversity. Thus, our results are consistent with those of other studies advocating for complementary multi-marker approaches in metabarcoding of insect communities (Clarke et al., 2014; Deagle et al., 2014; Kaunisto et al., 2017; Thomsen & Sigsgaard, 2019) and other taxa (Holman et al., 2018; Wangenstein et al., 2018).

4.2 | Preservative ethanol versus homogenized tissue from Malaise trap samples

Metabarcoding of homogenized insect tissue from trap samples comes at the cost of losing all the morphological information of the collected specimens. This prevents further study of the material, such as description of any potential new species from the material, or reference barcoding or detailed investigations of individual specimens. Therefore, analysis of preservative ethanol would open up many possibilities if it were an adequate replacement for analyses

of tissue homogenate. Shokralla, Singer, and Hajibabaei (2010) were the first to achieve PCR amplification of DNA extracted directly from fixative ethanol from single specimen samples. Later, Hajibabaei et al. (2012) did the same with freshwater benthic bulk samples, reporting high recovery success: 89% of the taxa identified with the tissue extraction were also identified in the ethanol. Since then, and until recent times, studies testing the suitability of DNA metabarcoding of preservative ethanol have been scarce, which is surprising given the significant advantages of this method: in addition to the availability of the material for further sequencing or for taxonomic study, the method also comes with a much lower processing time in the laboratory than other nondestructive protocols, which tend to involve incubation of the sample in a lysis buffer. To our knowledge, there are only four studies on the performance of high-throughput sequencing from preservative ethanol of bulk samples subsequent to the two pioneering papers by Hajibabaei's group. The first focused on genome skimming of mock samples of terrestrial and aquatic Coleoptera (Linard, Arribas, Andújar, Crampton-Platt, & Vogler, 2016), while the other three used metabarcoding of both mock and real samples from freshwater benthic macroinvertebrates (Erdozain et al., 2019; Martins et al., 2019; Zizka et al., 2018). Our study is the first one to present data on DNA extracted from preservative ethanol from Malaise trap samples.

The methodology used to obtain the DNA from the ethanol varies among previous studies. Erdozain et al. (2019) and Hajibabaei et al. (2012) opted for simple evaporation of 1 ml of ethanol at 56°C followed by resuspension in water, while others have followed a more elaborate process: Linard et al. (2016) centrifuged the entire volume of ethanol to precipitate the DNA, let the precipitate dry and extracted the DNA with a commercial kit; Martins et al. (2019) used a vacuum concentrator to evaporate 2 ml aliquots and then extracted the DNA of the precipitate with different commercial kits; and Zizka et al. (2018) passed the ethanol through a filtering membrane, which was later incubated with lysis buffer in a similar way to what is done in eDNA studies of water samples. We chose the latter option to obtain all the free DNA and cells in the ethanol, rather than small aliquots that might have too low DNA concentration and from which some DNA sequences could be absent. Whether the choice of these three methodologies affects the detection of different taxa is unknown, and should be tested in the future.

We found that the insect community recovered from metabarcoding of preservative ethanol was strikingly different from the community recovered from homogenized tissue. Of the taxa we found, 36 families were exclusively found in ethanol, while 23 families were exclusively found in homogenates, and 17 were found in both substrates. The larger and more heavily sclerotized insects were recovered more frequently in the tissue samples, and the smaller and more weakly sclerotized were primarily recovered in the ethanol. Nevertheless, some exceptions to this pattern can be highlighted. For instance, ladybugs (Coccinellidae, Coleoptera) are strongly sclerotized but are nevertheless found exclusively in preservative ethanol in some samples. This could be because they, when stressed, produce defensive excretions of which haemolymph

is a main component, containing large amounts of DNA. Tipulids (Tipulidae, Diptera) are recovered by both the ethanol and the tissue, but their read numbers is almost three times larger in the ethanol. Although being large insects, tipulids have a very high surface to volume ratio, which could facilitate the entry of ethanol into the body and the leakage of DNA into the solvent. Another example is the oribatid mites (Oribatida, Sarcopiformes), exclusively found in the ethanol samples. They have a strong tegument, but they can be so small that they were possibly not retained in the 0.6 mm pore size sieve, and therefore ended up in the ethanol filter rather than the tissue fraction of the sample. Also, some insects are known to vomit or regurgitate the intestinal contents when introduced alive in ethanol, and that can be the explanation of the exclusive presence of some families in preservative ethanol. However, without knowledge of the true taxonomic composition of the samples, it is impossible to say whether the results we observed are caused by any of these taxon-specific features or simply by PCR biases or other biases in DNA recovery during the processing of the sample. The absence of certain families of small insects in the tissue samples can be due to the fact that they represent only a small fraction of the total biomass, and hence of the DNA of the sample, as suggested by Elbrecht et al. (2017). However, the level of sclerotization still appears to play an important role, since most families of small but strongly sclerotized insects are preferentially found in the tissue samples.

In summary, our results contrast strongly with the findings of Hajibabaei et al. (2012), who reported almost 100% recovery in ethanol of the taxa found in tissue homogenate, and with other results that, although not obtaining a perfect match, are more optimistic than ours: Zizka et al. (2018) found ~65% overlap between ethanol and homogenate; Erdozain et al. (2019) found that metabarcoding of preservative ethanol consistently detected only fewer taxa than morphological identification; and Martins et al. (2019) recovered 70%–95% of the EPTO (Ephemeroptera, Plecoptera, Trichoptera and Odonata) taxa identified through morphology, plus others not identified by morphology. However, our results are in line with those of Linard et al. (2016), who were only able to recover 15 of 40 morphospecies from preservative ethanol versus 37 from the tissue. Neither Erdozain et al. (2019) nor Martins et al. (2019) compared the metabarcoding of ethanol-DNA to metabarcoding of homogenized tissue, only to morphological identification; whether the results of such a direct comparison would still be as positive, or more similar to the results presented here, is unknown. As in the previous cases, detection probabilities were in general affected by the morphology of the insects. This could be the major explanation for the different outcomes, since all studies showing positive results focus on freshwater samples that mostly consisted of larvae and other taxa that are weakly sclerotized in general and therefore should be well represented in preservative ethanol, while the more negative results (current study and Linard et al., 2016) are the only ones we have so far for samples consisting of more heavily sclerotized terrestrial insects. It is possible that minor differences in processing could also have contributed to the different outcomes; this should be studied further. Nevertheless, our results clearly suggest that ethanol-based

DNA cannot be used as a nondestructive replacement of destructive tissue homogenization protocols in metabarcoding of Malaise trap samples. Instead, the two methods complement each other: preservative ethanol appears to retrieve small and weakly sclerotized insects, while homogenized tissue seems to be dominated by insect species with large total body mass. Future studies should address whether a nondestructive extraction protocol based on the incubation of the bulk sample in lysis buffer, a compromise between the two approaches tested here, can more reliably recover the true community composition of a terrestrial arthropod sample.

4.3 | Malaise trap samples versus soil samples

Our results show that COI is problematic in metabarcoding of environmental DNA samples like soil samples when the taxonomic focus is narrow (in environmental samples, a restriction to a single phylum is considered narrow). This is mainly because it is amplified using highly degenerate primers, whose target/off-target amplification rate ratio is greatly affected by the proportion of DNA from nontarget taxa in the sample by binding stochasticity (Polz & Cavanaugh, 1998). In soil samples in particular, the fungal biomass is expected to greatly exceed that of arthropods. This explains the abundance of nonarthropod sequences in COI analyses of soil samples, and the rapid saturation of the rarefaction curves confirms that all amplified templates were sequenced, but suggests that not all present templates were amplified. A similar effect may explain why COI analyses of preservative ethanol tend to pick up more nontarget taxa than 16S analyses. To some extent, ethanol samples can be considered eDNA, since not only the DNA of the collected insects leaks out into the ethanol, but also the DNA of microorganisms (pathogens or symbionts) associated with the insects, and of those organisms that had an interaction with the collected specimen. Another potential explanation for the predominance of fungal over insect sequences in the COI analyses of the soil samples could be that the fungi present in the soil are alive while the insect DNA is primarily degraded DNA from dead specimens. To deal with degradation, primers for shorter COI fragments have been designed (e.g. Vamos, Elbrecht, & Leese, 2017; Zeale, Butlin, Barker, Lees, & Jones, 2011). Nevertheless, this explanation can probably be discarded due to the fact that the 16S amplicon, which is longer than the COI amplicon, was not affected. Thus, it can be concluded that the COI amplification of off-target sequences is caused by saturation of the too highly degenerate primers by nontarget template DNA, and not by the fragmentation of insect DNA.

For surveys focusing on total biodiversity in eDNA samples, highly degenerate primers can provide information from a very wide spectrum of taxa (Deiner et al., 2016; Macher et al., 2018; Majaneva, Diserud, Eagle, Boström, et al., 2018). However, this comes at the expense of lower coverage for any specific taxon, which may result in large parts of the community missing from the resulting reads. Therefore, for eDNA samples, it is often better to use less degenerate primers for more conserved markers, such as rRNA genes (Horton et al., 2017) even when this comes at the expense of reduced

species discrimination capacity (Tang et al., 2012; Wangenstein et al., 2018). COI primers with lower degeneracy can be useful with eDNA samples, picking up fewer nontarget taxa than degenerate primers while still recovering a good portion of the invertebrate diversity, but they are still likely to result in lower coverage of the target taxon than in bulk samples (e.g. Watts et al., 2019). Our results show that the 16S primers chosen work well for analysis of insect community composition in eDNA samples, since the primer-binding site is more conserved than for COI, but the lower degeneracy prevents off-target amplification. At the same time, the fairly long amplicon read (350 bp) gives good taxonomic resolution (Marquina et al., 2018), better than that obtained with other 16S primers tried to date (Alberdi et al., 2018; Clarke et al., 2014; Elbrecht et al., 2016).

We found the limited overlap in MOTUs between soil and trap samples surprising for two reasons: first, we expected to find some soil inhabitant species that had climbed up and into the Malaise traps, and second, we expected to see a time-lagged overlap caused by ground-dwelling larvae detected in the soil samples, which, once developed into adults, would find their way into the traps. There were only a few examples of the first case, involving springtails and beetles found both in the soil and the trap samples. There were more examples of the second case, but still only 18 MOTUs, all belonging to Diptera (some of these cases could actually be explained by dead adults being detected in the soil samples). A possible reason for the absence of a time-lagged overlap between soil and trap catches is that both types of samples were taken late in the season (first samples were collected in early July), when most insects had probably already emerged. However, the minimal overlap between our soil and Malaise trap samples may simply reflect that insect communities found in different biological substrates in the same habitat or location differ significantly in composition (Kozioł et al., 2018). For instance, in a study somewhat similar to ours, Watts et al. (2019) found big differences in the communities retrieved in soil samples and aboveground traps, although in their case the crawling organisms were retrieved in the aboveground traps, whereas they were primarily found in the soil samples (leaf litter fraction) in our study.

In conclusion, we show that a multi-marker approach using COI and 16S markers enhances the recovery of biodiversity from insect bulk samples and eDNA. Our 16S marker does not appear to suffer from significantly lower taxonomic resolution than COI, unlike the nuclear rRNA markers that have been tried previously as complements to COI. This gain in resolution presumably emanates from the fact that our 16S marker is longer. Additionally, we found that the lower degeneracy of the primers amplifying 16S allows for on-target sequencing of the desired taxon in environmental samples, in contrast to the highly degenerate COI primers, which show low specificity and recover many phylogenetically distant nontarget organisms. Thus, COI primers are not suitable for biodiversity surveys based on analysis of eDNA when only a narrow taxonomic focus is desired. Moreover, our results showed that metabarcoding of DNA extracted from the preservative ethanol from Malaise samples is not equivalent to metabarcoding of the homogenized insect tissue from these samples. Thus, the former cannot be used as a nondestructive

replacement for the latter. Nevertheless, metabarcoding of preservative ethanol clearly provides additional and valuable information, especially for small and soft insects, which appear to be underrepresented in the tissue homogenate. Therefore, analysis of preservative ethanol may be used in combination with analysis of tissue homogenate in order to obtain a more complete picture of the insect community than would be possible with only one of the methods alone.

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AUTHOR CONTRIBUTIONS

D.M., T.R., and F.R. conceived and designed the study. D.M. collected the samples, and D.M., and R.E.S. carried out the laboratory work. D.M. performed the bioinformatic and D.M., and T.R. the statistical analyses. D.M. wrote the first draft of the manuscript and prepared the figures and tables. All authors reviewed and contributed to subsequent drafts of the manuscript.

DATA AVAILABILITY STATEMENT

Raw reads for both markers as well as the *ngsfilter* files with multiplexing tags can be accessed at <https://zenodo.org/record/3256244#.XRI7Jq2BOUE>.

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SUPPORTING INFORMATION

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