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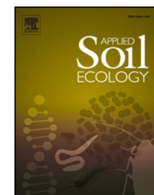

Short communication

Comparing earthworm biodiversity estimated by DNA metabarcoding and morphology-based approaches

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ABSTRACT

Conventional identification based on morphology is today's tool of choice for monitoring earthworm diversity. However, it requires skills to distinguish specific features, which can become the bottleneck in soil ecological studies. DNA-based metabarcoding is increasingly used to monitor diversity of soil organisms. Few studies have compared this method with conventional methods. The aim of the study was to compare metabarcoding based on the mitochondrial 16S rRNA region with conventional morphology-based identification, where metabarcoding captured more species (eight) than identified by conventional method (five). Additionally, we tested the effects of two commercial DNA extraction kits: PowerSoil based on 0.25 g of soil and PowerMax based on 10 g of soil (both from Qiagen), where the PowerMax covered higher richness than PowerSoil.

1. Introduction

Earthworms play vital roles in soil health and ecosystem functioning and are ecosystem engineers (Lavelle et al., 1997; Blouin et al., 2013). Studying distribution and diversity of earthworms is largely based on conventional morphology-based identification (CMI) requiring taxonomic expertise and extended experience. Furthermore, in many cases juvenile earthworms cannot be reliably identified to species level and cryptic species cannot be identified by CMI (e.g., Bartlett et al., 2010; James et al., 2010). Therefore, the collection and identification of earthworms using CMI can become a bottleneck in soil studies.

In recent years, environmental DNA (eDNA) metabarcoding using high throughput sequencing has revolutionized ecological studies and is becoming a tool in community-based studies of soil invertebrates (Capra et al., 2016; Sapkota and Nicolaisen, 2015; Taberlet et al., 2018) and studies for earthworms (King et al., 2008; Porco et al., 2018; Stürzenbaum et al., 2009). DNA metabarcoding workflows reduce time and costs, and no prior knowledge on earthworm taxonomy is required to characterize entire communities. However, comparison of DNA metabarcoding and conventional methods is needed to validate DNA-based methods.

Here we compare the performance of the CMI and eDNA metabarcoding in terms of alpha diversity, community structure, and abundance of the earthworm assemblage in cultivated fields in an experiment with different fertilizer treatments. As DNA can remain stable in soil for days to years (Taberlet et al., 2018), eDNA might detect earthworms that are not present at the time of sampling. Hence, we hypothesize that higher earthworm species richness will be captured by eDNA than by conventional methods. Moreover, we hypothesize that DNA metabarcoding results will differ between soil DNA extractions methods mainly due to soil volume used.

2. Materials and methods

Soil samples were collected from KLIMINI experimental plots (https://projects.au.dk/klimini/) at Foulumgård (clayey sand) and at Højbakkegård (sandy clay) in Denmark. In brief, three treatments (chemical fertilizer, pig slurry and a control without fertilizer) were applied to three plots of randomized blocks designs at both locations, in total 18 plots. Each plot consisted of 3 m × 3 m area and was cultivated with spring barley. Soil sampling was carried out in May–June 2020, four weeks after sowing and fertilization. The soil sampling strategy for

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eDNA consisted of collecting 15 soil cores of 20 cm depth and 2.5 cm in diameter in a Z-pattern in each plot which were pooled and mixed. Subsamples of 40 g were collected into Falcon tubes, frozen in liquid nitrogen and stored at −80 °C. Additional subsamples of 200 g were collected into plastic bags which were stored at −20 °C before DNA extractions. For CMI, a spade was used to dig two soil blocks (l × w × d: 20 × 20 × 30 cm) within each plot, and the soil was carefully sorted by hand to fetch earthworms. The earthworms were later identified and weighed in the lab. Earthworms with uncertain identification by CMI were Sanger sequenced (see Supplementary material 1).

Monitoring larger invertebrates using metabarcoding of soil DNA involves soil sampling, homogenization, DNA extraction, amplicon library preparation, DNA sequencing and bioinformatic analysis. DNA extraction is sensitive to soil processing and the amount of soil used. Thus, we used two commonly utilized soil DNA extraction kits — DNeasy PowerLyzer PowerSoil DNA isolation kit (Qiagen, Denmark) (PS) and the DNeasy PowerMax Soil DNA isolation kit (Qiagen, Denmark) (PM), which use 0.25 g soil and 10 g soil per DNA extraction, respectively. Soil in the Falcon tubes was freeze-dried (Scanlaf Model Coolsafe 55 Lynge, Denmark) and homogenized using a bead beater (Bead Ruptor Elite, Omni International, at 4 m s^{-1} for 30 s in three cycles) prior to DNA extraction of subsamples of 0.25 g soil with the PS kit. DNA was extracted using the PM kit from 10 g of soil without prior freeze drying.

Library preparation was based on ewD/ewE primers targeting the mitochondrial 16S rRNA gene (Bienert et al., 2012) and applying the Nextera XT index kit, using Illumina compatible index primers (Illumina Inc. San Diego, California, USA). Sequences obtained by the Illumina NextSeq 500 platform (San Diego, California, USA) were deposited under SRA accession number PRJNA783879. The DNA reads were analysed after Sapkota et al. (2020), except <120 base pair reads were excluded using VSEARCH ver. 2.6 (Rognes et al., 2016). Data were analysed in R ver. 4.0.3 (R Core Team, 2020), using *vegan* ver. 2.5-7 (Oksanen et al., 2020) and *phyloseq* ver. 1.34 (McMurdie and Holmes, 2013).

Alpha diversity was estimated using observed OTU richness and the significance of differences was evaluated using two-way ANOVA and Tukey's test. For beta diversity-based calculations, the OTU table was transformed to relative abundance and Morisita-Horn index was used for visualization using unconstrained principal coordinates analysis (PCoA). For partitioning of variance, distance matrices were subjected to permutational analysis of variance (PERMANOVA) using the "adonis" test from the "vegan" package.

3. Results and discussion

After quality control, taxonomic assignment, and selecting OTUs belonging to the family *Lumbricidae*, we obtained 1.24 million reads clustered into 1397 OTUs. The maximum number of reads per sample was 181,350, minimum 458 and the median was 24,850. The OTU table was merged at species level using the tax_glom function from *phyloseq* species list. As a result, several OTUs assigned to one earthworm species were consequently considered as one species in the species abundance table (see Supplementary Fig. S1).

Earthworm species richness based on CMI was significantly affected by location ($p < 0.001$) and fertilizer treatment ($p < 0.05$) using two-way ANOVA (Supplementary Table S1). However, location and treatment effects were not found in eDNA datasets based on PS and PM. Pairwise comparison using *t*-test revealed all three methods were different from each other (Fig. 1). PM captured the highest richness followed by PS and CMI methods.

The earthworm community structure differed between the two locations and earthworm identification methods, as shown in the ordination plot (Fig. 2). In the following PERMANOVA of two DNA extraction methods, location explained 23 % ($p = 0.023$) of total variation in PS dataset, and 17 % in PM dataset ($p = 0.05$). The effect of

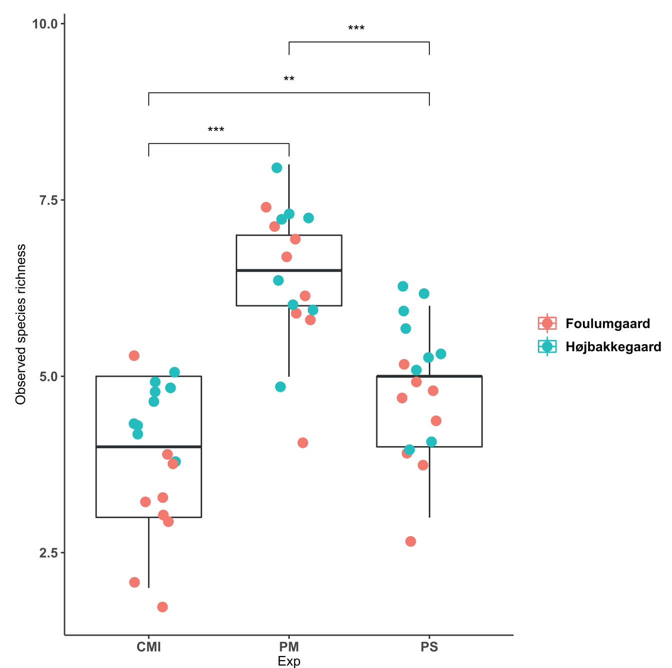

Fig. 1. Box plot showing observed richness of earthworms estimated using metabarcoding of two DNA extraction methods (PowerSoil (PS) and PowerMax (PM)) and Conventional Morphological Identification (CMI). Significance levels show adjusted p-values obtained by Tukey's test. ** = $p < 0.01$, *** = $p < 0.001$.

fertilizer regime and interactions between factors were non-significant. PERMANOVA on the CMI dataset found significant effect of location ($R^2 = 0.52$, $p = 0.002$) and no effect of fertilizer. Despite the difference in richness of earthworm species, we found dominant effect of location on driving community structure in metabarcoding using two DNA extractions methods and CMI dataset.

In all samples, DNA metabarcoding was superior to CMI in detecting earthworm species, as DNA metabarcoding based on both DNA extraction methods showed higher or equal number of occurrences of each species compared to CMI, except unidentified *Lumbricus* sp. This group was probably identified to species by metabarcoding (Fig. 3a). PM showed a higher detection sensitivity than PS. *Aporrectodea tuberculata*, *Lumbricus festivus*, and *Lumbricus terrestris* were not detected by CMI. However, while *L. terrestris* and *Lumricus herculeus* could not be identified morphologically by CMI, subsequent barcoding identified one individual of *L. terrestris* in an adjacent field, confirming that it is found in the Foulum area, while the remaining barcoded specimens were *L. herculeus*. Since *L. herculeus* was collected via CMI and identified using DNA barcoding, this species is included in the CMI dataset.

Earthworm species detected by CMI dominated the relative number of Lumbricidae reads in the PS and PM dataset whereas the species only detected by eDNA were represented with low abundance (Fig. 3b). For instance, *A. tuberculata* in PS and PM samples accounted for only 1 % of total relative abundance, compared to *A. caliginosa* accounting for 37 %.

Metabarcoding based on PS and PM DNA extraction methods both captured significantly higher diversity than CMI. A reasonable explanation is that metabarcoding is likely to pick up DNA of earthworms at all stages, including eggs and juveniles which could explain the higher diversity. Furthermore, sampling method could affect their detection via morphology-based methods, e.g., highly mobile earthworms are detected via eDNA, whereas deep-burrowing species might be missed by CMI. This could be especially relevant for detection of *L. terrestris*, which is sensitive to vibrations and can persist down to one meter depth (Nuutinen and Butt, 2003) and was found in several samples by DNA metabarcoding, but not by CMI. Notably, while *L. terrestris* was present in

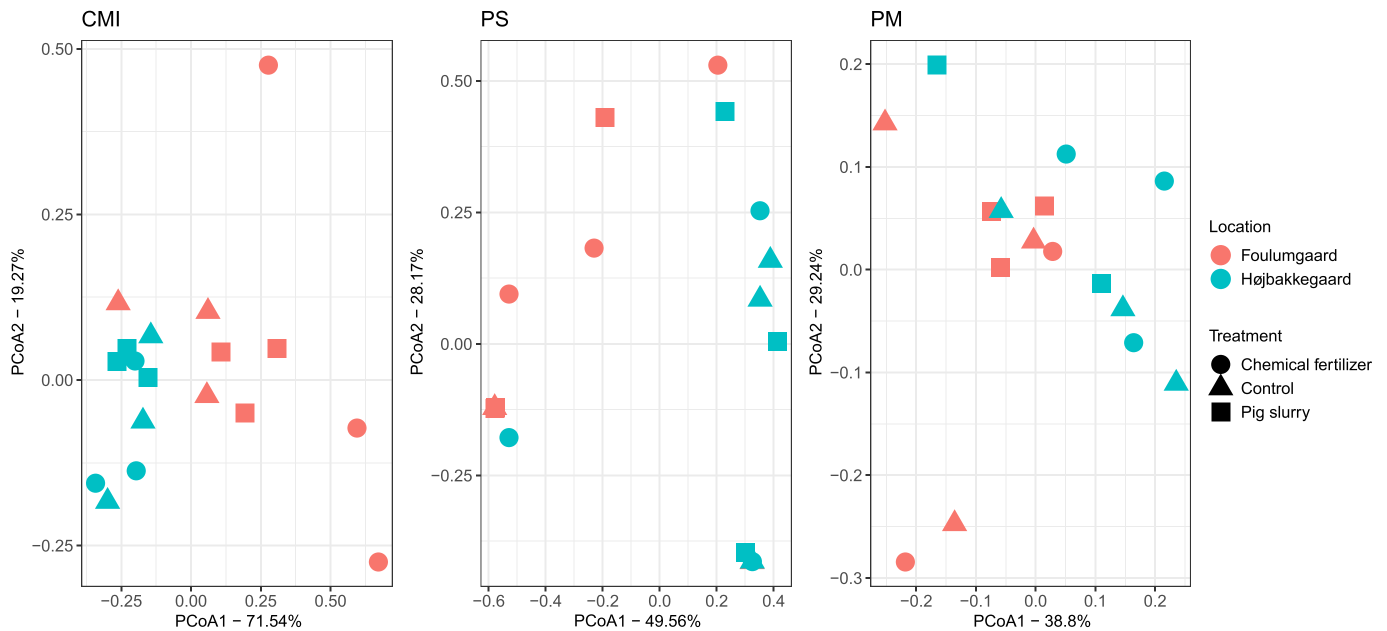

Fig. 2. PCoA plot based on Morisita-Horn index depicting earthworm community structure determined using Conventional Morphological Identification (CMI) and DNA metabarcoding from PowerSoil (PS) and PowerMax (PM) based on OTU tables merged at species level.

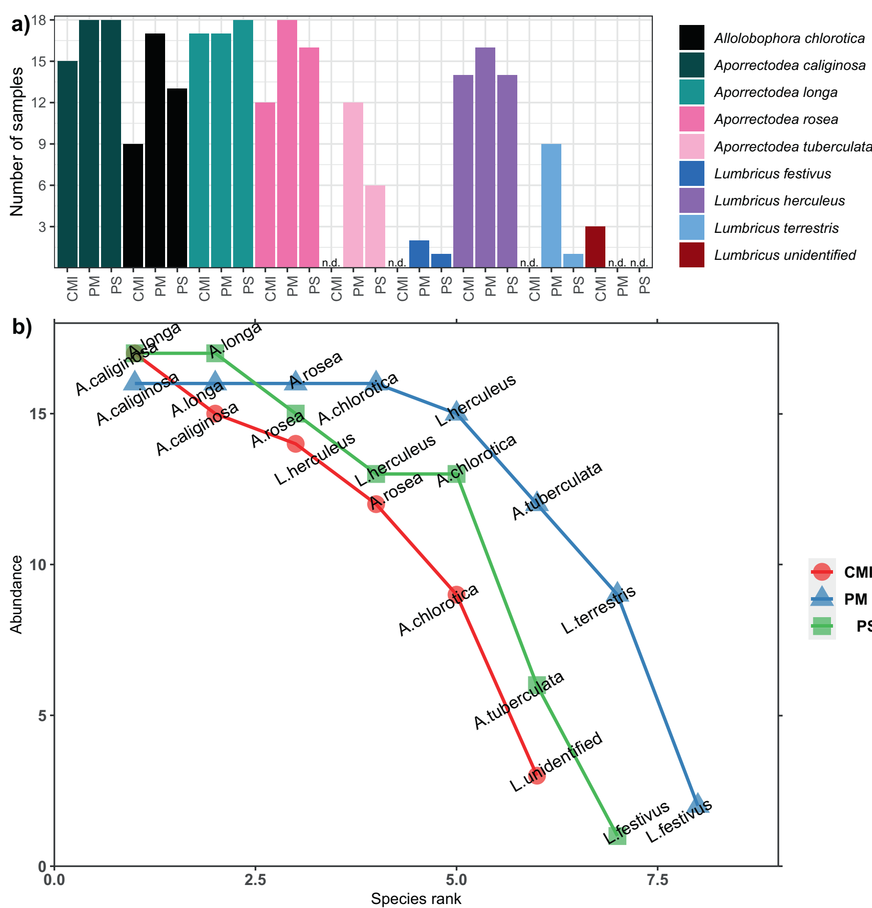

Fig. 3. Comparison of earthworm species from 18 sampling plots found by Conventional Morphological Identification (CMI) and metabarcoding of two DNA extractions methods (PowerSoil (PS) and PowerMax (PM)). a) Barplot showing number of samples with detected earthworm species; b) Rank abundance curves for three methods. X-axis represents the species abundance rank, where most abundant species are ranked as 1 followed by low abundant species. Y-axis shows the occurrence of species per total 18 soil samples. Note, *Lumbricus herculeus* in CMI samples were only identified after subsequent COI barcoding. n.d.: not detected.

nine of the PM samples, it accounted for only 0.08 % of total relative Lumbricidae read abundances across treatments, compared to *L. herculeus* accounting for 5.5 %.

PM and PS are common commercial kits for soil DNA extraction. PS is especially used for microbial communities (Lear et al., 2018). However, little is known about how well they extract DNA from larger, less abundant organisms. Here PM outperformed PS in capturing richness and diversity of earthworms, most likely due to 40× larger amount of

soil for DNA extractions. The main advantages of PS over PM are that its cost is five times lower and the extraction is less time consuming. The elution volume from PS is 100 µl and that of PM is 5 ml, and higher yield could be an advantage if extracts are used in many applications such as multiple qPCR runs.

In conclusion, species richness was significantly higher with DNA metabarcoding compared to CMI, and four earthworm species: *A. tuberculata*, *L. festivus*, *L. herculeus* and *L. terrestris*, were only detected by DNA metabarcoding. Out of the two DNA extraction methods, PM comes with a higher price per sample and time used for DNA extraction, but it captures higher earthworm richness. On the other hand, PS is a cheaper alternative making it a widely used kit for soil microbial studies, and our study shows it captures higher richness than CMI. Effect of location was consistently observed in both DNA metabarcoding methods and CMI. The clear advantages of the eDNA metabarcoding in species detection can pave the way for increased use of eDNA for monitoring earthworms and assessing not only biodiversity but also the ecosystem services provided by the different earthworm species in relation to feeding habitats and burrowing activities. However, CMI also monitors the biomass of earthworms, which is not yet possible by eDNA.

CRediT authorship contribution statement

AW, RS, MAL, ZB, PHK designed and conceived the study. RS, MAL, ZB, AZ conducted the experiment and analysed the data. MAL, ZB and RS drafted the first manuscript. AW and PHK secured the funding. AW, RS, BWH and PHK supervised the study. All authors read, revised and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104798.

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