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INSIGHT INTO INVERTEBRATE COMMUNITY IN SOLONCHAK SOIL TYPE USING eDNA METABARCODING – A PILOT STUDY

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CVIJETA LAZIĆ[*1], *MILOMIR STEFANOVIĆ*[1], *NATAŠA KOČIŠ TUBIĆ*[1], *ANA KUZMANOVIĆ*[1], *DRAGAN RADNOVIĆ*[1], *DRAGANA TAMINDŽIJA*[1], *STANKO MILIĆ*[2], *JOVICA VASIN*[2], *JORDANA NINKOV*[2], *MIHAJLA DJAN*[1]

[1]University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Novi Sad, Serbia
[2]Institute of Field and Vegetable Crops, National Institute of the Republic of Serbia, Novi Sad, Serbia
**Corresponding author*: cvijeta.lazic@dbe.uns.ac.rs

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SUMMARY

Solonchaks are soils characterized by high concentrations of soluble salts. These soils form unique ecosystems that provide habitats for organisms adapted to such extreme conditions. Invertebrates are one of the groups of organisms that inhabit these soils and play a significant role in ecosystem resilience contributing to soil health and fertility. However, the diversity of invertebrates inhabiting the solonchaks remains underexplored. The development of eDNA metabarcoding method has facilitated the detection of soil invertebrates, overcoming the limitations of conventional labor-intensive and time-consuming methods. eDNA metabarcoding made it possible to study/monitor changes in soil invertebrate diversity. This pilot study employed eDNA metabarcoding to detect soil invertebrates in solonchak soils at two agricultural fields in Vojvodina, Serbia. Furthermore, we compared two analytical methods of eDNA metabarcoding data – clustering and denoising sequences. eDNA metabarcoding method proved to be effective in detection of invertebrates. Using BOLD database, 0.21% OTUs and 0.11% ESVs were successfully assigned to expected Invertebrate phyla (Arthropoda, Annelida and Nematoda), with two specific species identified: Aporrectodea jassyensis and Sminthurinus elegans. Comparison of the two analytical approaches, denoising and clustering, revealed that these approaches identified the same taxonomic categories. However, given the limited sample size, further studies should compare these two approaches using a more extensive dataset to better estimate their usefulness.

Key words:
solonchak, clustering, denoising, environmental DNA, invertebrates, soil biodiversity

INTRODUCTION

The changing environment and increasing negative anthropogenic influence pose significant threats to biodiversity (Prakash & Verma, 2022). These changes are driven by the rapid growth of human population and expansion of sealed land surfaces, mining activities, agricultural practices, food production, among other factors (Geisen et al., 2019). Therefore, understanding genetic diversity, species diversity and ecosystem diversity is crucial for detecting changes in a timely manner and recognizing the effects of adverse impacts. However, the biodiversity of many critical habitats remains insufficiently explored and knowledge of the communities inhabiting them is scarce. One such understudied habitat is soil.

Soil is a habitat to a large number of complex and diverse communities, from bacteria to large organisms such as moles (Bardgett & van der Putten, 2014). Preserving soil health requires knowledge of the diversity of soil organisms and their roles in the ecosystem (Ferris & Tuomisto, 2015). Also, determination of soil biodiversity can reveal the key processes occurring in a certain soil type, and soil organisms can serve as biomarkers of soil quality (Doran & Zeiss, 2000). An important and diverse group of organisms inhabiting soil are invertebrates. Invertebrates play various roles in soil ecosystems. Small invertebrates, including nematodes, mites and springtails, function as biological regulators by controlling the populations of other invertebrates and microorganisms. Larger invertebrates, such as insects, earthworms, ants and termites, act as ecosystem engineers, modifying and creating habitats for other smaller organisms. Furthermore, they assist in regulating resource availability for other organisms (Turbé et al., 2010). Invertebrates that inhabit soil contribute to its health and fertility. Measuring the diversity of invertebrates is important for assessing soil ecosystem resilience and understanding the impacts of different types of land use on their communities and soil health (Dopheide et al., 2020).

Conventional morphological approaches commonly used for describing diversity of soil invertebrates had certain limitations. These include the problems in detecting organisms in non-adult life stages and potential habitat disturbances during the research. Additionally, these approaches are labor-intensive, time-consuming, and costly. Development of Next-Generation Sequencing (NGS) has enabled simultaneous sequencing of DNA from different species isolated from environmental samples i.e. environmental DNA (eDNA) (Taberlet et al., 2012a). Analysis of short, taxonomically informative DNA fragments obtained from eDNA allows for taxonomic identification of organisms present within an environment (Guerrieri et al., 2021). This approach, known as eDNA metabarcoding (Taberlet et al., 2012b), has proven to be highly effective for biodiversity studies in different environments, including freshwater (Macher et al., 2018), sea (McClenaghan et al., 2020), air (Clare et al., 2022) and soil (Rota et al., 2020). Applying eDNA metabarcoding in the analysis of soil invertebrate diversity offers rapid and efficient community profiling, with higher taxonomic resolution, enabling detection of a wider spectrum of diversity compared to traditional soil sampling and analysis. Additionally, eDNA metabarcoding identifies taxa present as individuals in the sample as well as those represented solely by biological traces.

After field and laboratory work, a major task involves analyzing sequences of DNA regions which are taxonomically informative in a rapid, user-friendly and efficient way. For bioinformatic processing of metabarcoding data, pipelines such as OBITools (Boyer et al., 2016), DADA2 (Callahan et al., 2016), QIIME 2 (Bolyen et al., 2019), APSCALE (Buchner et al., 2022) are frequently used. Regardless of the pipeline used, one of the challenges in data processing is choosing an adequate approach for sequence clustering. In metabarcoding studies of invertebrates, sequences can be clustered by the percentage of similarity into Operational Taxonomic Units – OTU (Kirse et al., 2021) or the unique sequences can be retained, i.e. Exact Sequence Variants – ESV (Hermans et al., 2022). Although opinions vary regarding the most suitable approach (Callahan et al., 2017; Antich et al., 2021), the choice largely depends on the study objectives, as each approach has its own advantages. OTUs more accurately reflect interspecies diversity, while ESVs better describe genetic diversity (including intraspecies diversity) (Brandt et al., 2021). Another challenge in metabarcoding studies is taxonomic identification of OTUs and ESVs, as a large number of OTUs and ESVs are not taxonomically assigned due to incomplete reference databases (Zepeda Mendoza et al., 2015) or are assigned only at higher taxonomic categories (Hermans et al., 2022). Regardless of taxonomic categories that can be detected using metabarcoding approach, pilot studies are important as they indicate groups of organisms specific to certain habitats, and therefore can direct further research to specific target groups.

One of soil types in which invertebrate diversity has not been sufficiently investigated is solonchak. According to domestic soil classification (Škorić et al., 1985) solonchaks are halomorphic soils containing at least 1% salt (chloride and sulfate salinization) or more than 0.7% salt (sodic salinization) in any horizon down to 125 cm of depth. In Serbia, solonchaks are widespread across the Vojvodina region, occupying 15.000-20.000 ha (Vasin et al., 2013; Zeremski et al., 2021). Due to their unfavorable physical characteristics, solonchaks are rarely used as agricultural land. However, with appropriate ameliorative measures, they can be used for agriculture, particularly for growing salt-tolerant cultures rather than salt-sensitive species (Pavlović et al., 2017). Regardless of the land use, solonchaks form unique ecosystems, making it important to expand our understanding of the diversity of organisms that inhabit and are characteristic of these soils.

The goals of our study were to detect invertebrates inhabiting this relatively understudied soil type, solonchak, and to compare different analytical methods of eDNA metabarcoding data obtained from soil samples.

MATERIAL AND METHODS

Soil sampling was conducted in July 2022 in Rančevo, Autonomous Province of Vojvodina, Serbia (45°52'2.03"N, 19° 7'19.15"E), on solonchak soil type (as classified in the soil map by Nejgebauer et al., 1971) from two agricultural

fields (soybean field and maize field). Soil sampling followed the "circular reference sample" method (Bogdanović, 2014). The central point was georeferenced with a GPS device and subsamples were collected in accordance with the layout of circles and points relative to this central position, as illustrated in Figure 1. From each sampling site, 20-25 individual subsamples were collected to make an average sample of approximately 250 g of soil representing an area of 0.5 to 3 ha. Five average samples, i.e. five replicates, were taken from each investigated area, yielding a total of 10 samples from two fields. Sampling was carried out with an agrochemical probe to a depth of 30 cm under sterile conditions. Between each sampling, the probe was cleaned with distilled water, sprayed with ethanol and flame-sterilized. Soil samples were placed in sterile plastic bags, stored in a portable thermal box, and transported to the laboratory, where DNA extraction was performed on the same day.

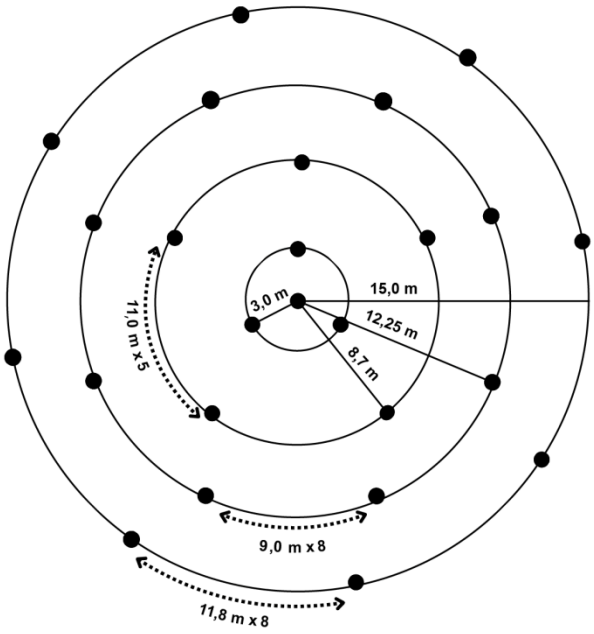

Figure 1. Soil sampling using "circular reference sample" method (Bogdanović 2014; modified)

DNA extraction was performed using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with a few modifications. A 30-minute incubation at 70 °C was added following the addition of Solution CD1 which allows cell lysis. For sample homogenization, BeadBug Homogenizer (Benchmark Scientific, NJ, USA) was used instead of Vortex in Step 2. The samples were homogenized twice for 20 s with a 20-second interval between the runs. DNA was successfully extracted from each of 10 soil samples (0.25 g soil per sample). Concentrations of DNA were measured fluorometrically using DeNovix-QFX Fluorometer (Denovix Inc., DE, USA) and samples were normalized to a concentration of 10 ng/µL.

PCR, library preparation and sequencing were carried out at the Institute for Molecular Medicine Finland (FIMM), Genomics unit, supported by HiLIFE and Biocenter Finland. PCR amplification of 421 bp region of cytochrome c oxidase subunit I (COI) gene was done using the primers BF2 (5' GCHCCHGAYATRGCHTTYCC 3') and BR2 (5' TCDGGRTGNCCRAARAAYCA 3') (Elbrecht & Leese, 2017). Sequencing was conducted on the Illumina MiSeq (2 x 300 bp) sequencing platform.

Demultiplexed reads were delivered from a sequencing company. The quality of the raw reads was assessed using FastQC (Andrews, 2010). After quality control, the fastq files were processed using APSCALE pipeline v1.6.3 (Buchner et al., 2022), which combines different programs for metabarcoding processing: vsearch (Rognes et al., 2016) for merging paired-end reads, quality filtering, OTU clustering and denoising; cutadapt (Martin, 2011) for primer trimming; and lulu (Frøslev et al., 2017) for additional post-clustering curation.

For processing of our dataset, several default parameters were adjusted. For merging paired-end reads, parameters were set as follows: the maximum difference percentage = 25, the number of maximum differences = 180 and the minimum overlap length = 10. Primer sequences were then trimmed and sequences without primers were discarded from the dataset. Merged sequences with length of 421 ± 10 bp were retained and filtered with maximum expected error threshold (maxEE=1). After quality filtering, samples were dereplicated, retaining only reads with an

abundance above 2. Two different approaches were tested: clustering (using a 97% similarity threshold for OTU generation) and denoising (using an alpha value of 2 and minimum size of 2 for ESV generation). After processing sequences in APSCALE pipeline, retained OTUs and ESVs were taxonomically assigned against the BOLD database (2024) using BOLDigger (Buchner & Leese, 2020). Following the assignment, additional data was retrieved with the option BOLD API. In order to determine the best fitting hit, the lists with OTU and ESV BOLD results were filtered using the option BOLDigger. Downstream processing of the datasets was performed using TaxonTableTools (TTT) (Macher et al., 2021). Full taxonomy tables (containing information about taxonomic identification, sequences and OTU and ESV frequency) were filtered using the Taxon-based filter option (check marks: none, method: keep) and only against Invertebrate phyla Arthropod, Annelida and Nematoda with a similarity threshold of 84%, as this threshold is generally used to distinguish orders (Cole et al., 2010).

RESULTS AND DISCUSSION

Amplification of COI marker was not successful in 1 out of 10 samples (replicates). In the remaining 9 samples (replicates), the number of reads per sample ranged from 16363 to 78720. From a total 484002 raw reads, we identified 2878 OTUs and 5463 ESVs with only 6 OTUs and 6 ESVs assigned to the expected phyla of Invertebrates (Arthropoda, Annelida and Nematoda) (Tab. 1).

Table 1. Taxonomic categories of invertebrates identified by the eDNA metabarcoding method in soil samples from two agricultural fields of solonchak

Taxonomic category						Number of reads per OTU		Number of reads per ESV	
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Soybean**	**Maize**	**Soybean**	**Maize**
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Aporrectodea	*Aporrectodea jassyensis*	28	0	21	0
Arthropoda	Arachnida	Trombidiformes	Rhagidiidae			0	27	0	19
Arthropoda	Arachnida	Trombidiformes				10	0	8	0
Arthropoda	Collembola	Neelipleona	Neelidae	Megalothorax		0	3	0	2
Arthropoda	Collembola	Symphypleona	Katiannidae	Sminthurinus	*Sminthurinus elegans*	2	0	2	0
Nematoda	Enoplea					3	0	2	0

With both clustering and denoising approaches, we identified the same taxonomic categories, although clustering approach assigned a slightly higher number of reads. From 6 OTUs/ESVs, 1 OTU/ESV was assigned to Annelida class Clitellata, 2 OTUs/ESVs were assigned to Arthropoda class Arachnida, 2 OTUs/ESVs were assigned to Arthropoda class Collembola and 1 OTU/ESV was assigned to Nematoda class Enoplea.

We successfully identified two taxa to the species level: *Aporrectodea jassyensis* (Michaelsen, 1891) and *Sminthurinus elegans* (Fitch, 1863). From the two OTUs/ESVs assigned to the Arthropoda class Arachnida, one could not be assigned to a taxonomic level below order and the other could not be assigned to a taxonomic level below family due to incomplete entries in the BOLD database. OTU/ESV assigned to Nematoda class Enoplea could not be assigned to lower taxonomic levels because the reference database entry was restricted.

Comparing results between the two sampling fields, more taxonomic groups of invertebrates were detected in the soybean field (4 OTUs/ESVs) compared to the maize field (2 OTUs/ESVs).

In order to understand the effects of human activities, such as agriculture, on soil organisms, it is crucial to continuously monitor soil biodiversity and detect anthropogenic pressures. The development of the environmental DNA metabarcoding approach (Taberlet et al., 2012b) has significantly facilitated studying soil organism diversity. Although eDNA metabarcoding has expanded our understanding of the diversity of soil invertebrates (Rota et al., 2020), there are still certain challenges that can be encountered in its application.

Selecting appropriate markers and primers is crucial in detection of the target group of organisms. If the research aims to detect invertebrates, selecting or designing primers that amplify informative barcode regions from all groups is a complex task that requires careful consideration (Dopheide et al., 2019). In our study, we used BF2 and BR2 primers (Elbrecht & Leese, 2017), which have great potential in aquatic ecosystem studies of invertebrate diversity (Elbrecht & Steinke, 2019), but may not be optimal for studying diversity of soil invertebrates. Additionally, these primers amplify a 421 bp region of the COI barcode gene (Hebert et al., 2003), and since the environmental DNA isolated from soil is often degraded, primers that amplify shorter regions may increase amplification yield and, subsequently, improve identification of taxa. Previous studies on soil invertebrate diversity have demonstrated the

efficacy of using primers that amplify slightly shorter region of the COI barcode gene (Watts et al., 2019; Kirse et al., 2021). Also, the results of soil metabarcoding studies can be affected by different land-use practices. It has been shown that agricultural practices can potentially lead to reduced invertebrate biodiversity (Ponge et al., 2013).

In metabarcoding studies, a significant challenge in data processing is the selection an appropriate sequence clustering approach that aligns with the study's research objectives. In this study, we compared two different approaches – clustering and denoising, using eDNA metabarcoding data obtained from soil samples. By processing data using the APSCALE pipeline, we obtained 2878 OTUs and 5463 ESVs, and after taxonomic assignment using BOLD database, 6 OTUs and 6 ESVs were assigned to the target group of organisms – Invertebrates. When comparing these two analytical approaches (denoising and clustering), our results showed that both of them yielded the same number of taxonomic units. As this study analyzed a small number of samples, future studies incorporating larger sample sizes are necessary to better assess the usefulness of each approach. Previous research suggests that the clustering approach is sufficient for interspecies diversity studies, while denoising is still recommended for intraspecies diversity analysis (Brandt et al., 2021). However, it should be noted that the COI gene typically shows high intra-species variability and can provide population-level information. Therefore, it is recommended to use clustering and denoising jointly in COI metabarcoding pipelines (Antich et al., 2021).

Another challenge of metabarcoding studies is taxonomic identification of sequences from environmental samples. In our study, only 0.21% OTUs and 0.11% ESVs were successfully assigned to the expected phyla of Invertebrates (Arthropoda, Annelida and Nematoda). One OTU/ ESV assigned to phylum Nematoda could not be assigned to taxonomic rank lower than the class Enoplea, as the reference database entry was restricted, which was previously described in the literature as a gap in using BOLD database (Weigand et al., 2019). Additionally, two OTUs/ESVs assigned to the Arthropoda class Arachnida, could not be assigned to a taxonomic level below order and family due to incomplete entries in the BOLD database. Previous studies have identified incomplete databases as a consistent challenge in studying soil invertebrate diversity (Porter et al., 2019; Watts et al., 2019). Thus, to increase the effectiveness of this method, it is crucial to improve the reference databases. In this regard, integrating metabarcoding with traditional methods in studying soil invertebrates could be helpful. An integrative approach would greatly enhance our understanding and monitoring of soil-inhabiting organisms and effective collaboration with skilled taxonomists is essential for developing a comprehensive reference database.

This study focuses on the solonchak soil type, which is one of the least studied soils in terms of biodiversity in Vojvodina Province, Serbia. Although our results provided only an insight into the diversity of invertebrates living in solonchak, they can indicate which groups of organisms are specific to this soil type, and therefore can direct further studies towards specific target groups.

CONCLUSION

To our knowledge, this is the first study to assess the diversity of soil invertebrates using the environmental DNA (eDNA) metabarcoding approach in Serbia. Our findings demonstrate the potential of eDNA metabarcoding for future soil biodiversity studies. Expanding the geographical coverage, including a broader range of soil types, and potentially using different primers for targeting invertebrates, could enhance our understanding of invertebrate diversity in different soil types in Serbia. Additionally, we propose that this method could contribute to understanding the impacts of agricultural practices on beneficial soil invertebrates, establishing eDNA metabarcoding as a valuable tool in monitoring soil organisms.

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Authors' ORCID iDs:

Cvijeta Lazić (https://orcid.org/0000-0002-9585-6754); Milomir Stefanović (https://orcid.org/0000-0001-6294-3782); Nataša Kočiš Tubić (https://orcid.org/0000-0002-6077-7378); Ana Kuzmanović (https://orcid.org/0000-0002-0684-439X); Dragan Radnović (https://orcid.org/0000-0003-3753-1887); Dragana Tamindžija (https://orcid.org/0000-0001-8179-8285); Stanko Milić (https://orcid.org/0000-0001-7673-9969); Jovica Vasin (https://orcid.org/0000-0002-9407-5470); Jordana Ninkov (https://orcid.org/0000-0003-4148-0453); Mihajla Djan (https://orcid.org/0000-0002-2427-0676)

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