ARTICLE

Multi-marker DNA metabarcoding reflects tardigrade diversity in different habitats[1]

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Abstract: Like meiofauna in general, tardigrades are often neglected in ecological and environmental surveys. Tardigrades occur in all parts of the world, from deep marine sediments to alpine environments, and are present in most ecosystems. They are therefore potentially good candidates for biomonitoring programs. However, sampling of these minute animals is both tedious and time-consuming, impeding their inclusion in large-scale ecological surveys. In this study we argue that using a multi-marker metabarcoding approach on environmental DNA (eDNA) partly can overcome this barrier. Samples of moss, lichens, and leaf litter were investigated both by morphology-based methods and DNA metabarcoding, and the results were compared in terms of tardigrade diversity and community composition of the sampled microhabitats. DNA metabarcoding using three markers detected more species of tardigrades than identification by morphology in most samples. Also, metabarcoding detected the same community differences and microhabitat distribution patterns as morphology-based methods. In general, metabarcoding of litter samples was unreliable, with only one out of three markers consistently amplifying and detecting tardigrades. The low availability of tardigrade reference sequences in public databases restricts the taxonomic resolution in eDNA surveys, but this impediment is partly circumvented by utilizing multiple markers.

Key words: Tardigrada, environmental DNA, eDNA, 18S, COI, DNA barcoding.

Résumé : Comme la méiofaune de manière générale, les tardigrades sont souvent négligés dans les inventaires écologiques et environnementaux. Les tardigrades sont rencontrés dans toutes les parties de monde, des sédiments dans les profondeurs marines jusqu'aux environnements alpins, et sont présents dans la plupart des écosystèmes. Ils constituent ainsi de bons candidats potentiels pour des programmes de biosurveillance. Cependant, l'échantillonnage de ces animaux minuscules est à la fois fastidieux et coûteux en temps, ce qui représente une entrave à leur inclusion dans des inventaires écologiques à grande échelle. Dans cette étude, les auteurs avancent que l'utilisation d'une approche de métacodage à barres à l'aide de multiples marqueurs sur des ADN environnementaux (ADNe) pourrait permettre de partiellement surmonter ces entraves. Des échantillons de mousses, de lichens et de litière de feuilles ont été analysés à la fois par des méthodes fondées sur la morphologie et le métacodage à barres de l'ADN. Les résultats obtenus ont été comparés aux micro-habitats étudiés en matière de diversité des tardigrades et de composition de la communauté. Le métacodage à barres de l'ADN à l'aide de trois marqueurs a permis de détecter plus d'espèces de tardigrades que l'identification morphologique chez la plupart des échantillons. De plus, le métacodage à barres a détecté les mêmes différences en matière de composition des communautés et de distribution au sein des micro-habitats que les méthodes fondées sur la morphologie. En général, le métacodage à barres des échantillons de litière se sont avérés peu fiables, avec un seul des trois marqueurs permettant d'amplifier et de détecter des tardigrades. Le faible nombre de séquences de référence pour les tardigrades au sein des bases de données

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publiques limite la résolution taxonomique dans les analyses d'ADNe, mais cette entrave est partiellement contournée en faisant appel à des marqueurs multiples.

Mots-clés : Tardigrada, ADN environnemental, ADNe, 18S, COI, codage à barres de l'ADN.

Introduction

Tardigrades are hygrophilous, microscopic invertebrates inhabiting nearly all ecosystems on earth. Currently, the phylum holds about 1270 described species (Degma et al. 2019), of which most belong to the classes Heterotardigrada or Eutardigrada. These bilaterally symmetrical bodied micrometazoans resemble small bears in their appearance, often being recognized by the slow and inept motions of their four pairs of limbs when observed in a microscope. In the different trophic levels, various species of tardigrades are found as carnivores, herbivores, or bacterivores, demonstrating their broad range of interactions in the food web. They consume plants, algae, nematodes, bacteria, and other tardigrades (Hohberg and Traunspurger 2005; Schmid-Araya et al. 2002), and have been observed to consume prey amounting up to 43% of their own biomass in less than 4 h (Ramazzotti 1962). As they often occur in high abundances (>1000 individuals per gram of microhabitat), their influence in food web interactions can be far-reaching (Nelson et al. 2015). Whereas the distribution of many tardigrade species has received notable attention, studies investigating habitat specificity and preference of species have been found to lack sufficient sampling effort (e.g., Guidetti et al. 1999; Ito 1999; Nelson and Bartels 2007), even in cases with sample size exceeding 1000 samples (Nelson et al. 2019). Such studies, which are mostly based on morphology, are time-consuming, require a high taxonomic expertise, and often require the presence of both adult animals and eggs for confident identifications (Guidetti and Bertolani 2005). These efforts commonly still fail to identify a notable portion of the specimens to species level.

The use of DNA metabarcoding techniques facilitate rapid, large-scale identification of species based on the presence of their DNA (Deiner et al. 2017), and have the potential to circumvent the aforementioned impediments for species level identification of tardigrades in environmental samples. Recent studies have shown the advantages of metabarcoding in documenting invertebrate diversity in soils (e.g., Boggs et al. 2019; Watts et al. 2019), and DNA metabarcoding has been used to investigate prokaryotic and eukaryotic (including tardigrade) community structure in moss samples (Arakawa 2020). However, whether such an approach is applicable and comparable to morphological identification of tardigrades has not been investigated. Separation of tardigrade specimens from their surroundings is difficult, as their clawed limbs allow solid attachment to any object in their proximity. Consequently, a DNA metabarcoding protocol able to identify tardigrades without prior isolation would complement current sampling techniques and perhaps provide a more comprehensive list of species inhabiting a certain habitat.

This study aimed to investigate whether metabarcoding of environmental DNA (eDNA) successfully captures tardigrade diversity. To do this, samples of common tardigrade habitats such as moss, lichens, and leaf litter were investigated by DNA metabarcoding and conventional, morphology-based methods. The captured diversity from both methods was directly compared, including whether each method captured the community compositional differences among microhabitats.

Materials and methods

Field sampling

Fieldwork was conducted on 11 August 2017 in Skråstadheia Nature reserve (58.19899°N, 7.99329°E; 28 m a.s.l.) in southern Norway. Substrate samples of ~100 cm^2 were collected within a 50 m × 50 m plot by using a metal grid. Mosses, lichens, and leaf litter — five samples for each substrate — were collected in a stratified manner, yielding a total of 15 samples, as described in the supplementary data, File S1[2]. The distances between samples were at least 3 m away from each other to prevent gathering samples with overlapping populations and DNA migration. Each sample was placed in a sterile plastic bag, marked with a unique tag, and stored in a cooler for subsequent transport to the laboratory. All samples were dried in a fume hood and transferred back to their respective bags for storage.

Traditional extraction and morphological identification

We extracted tardigrades from 100 cm^2 substrate samples by homogenizing (crushing) each and subsequently dividing them into four equal parts. The first 25% of the biomass was used for tardigrade extraction for morphological identification, the second for construction of reference sequences using single individual isolation and DNA barcoding, the third for the DNA metabarcoding experiment, while the fourth was kept as backup. For extraction of specimens, the subsamples of moss and lichens were weighed and immersed in 500 mL of dH$_2$O for 30 min to reactivate the tardigrades. The sample was then vigorously shaken for 1 min before being poured through a 500 μm sieve into a 500 mL measuring cylinder. After 45 min, the top 400 mL of water was decanted, whereas the bottom 100 mL with precipitate was transferred into a glass container. For leaf litter samples, the

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[2]Supplementary data are available with the article at https://doi.org/10.1139/gen-2019-0218.

subsamples were washed through a sieve stack with the top sieve (500 μm mesh size) and bottom sieve (45 μm mesh size), to catch all tardigrades and eggs between the sieves. Collected specimens and debris were transferred to glass containers. Using a glass pipette, the extracts were transferred to Petri dishes and examined thoroughly using a stereo microscope (Leica MZ6) to collect tardigrades and eggs.

All specimens were mounted in Hoyer's or Faure's medium and investigated for species identification using phase contrast and differential interference contrast. Using 63× and oil immersed 100× objectives on a Leica DM6000 compound microscope, all individuals and eggs were identified using key taxonomic literature (Bingemer and Hohberg 2017; Fontoura and Pilato 2007; Kaczmarek and Michalczyk 2017; Pilato and Binda 2010; Nelson et al. 2010). Specimens are deposited in the collections of the NTNU University Museum, Trondheim, Norway (NTNU-VM), and in the Bertolani Collection of the University of Modena and Reggio Emilia, Italy.

DNA barcodes from individual specimens

To facilitate higher taxonomic resolution when matching sequences generated by DNA barcoding, DNA barcodes of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) were generated from isolated specimens in the second subsample of the original moss, lichen, and leaf litter samples. Tardigrades were first extracted following the protocol described above, subsequently individually mounted on a slide in a drop of water, photographed, and identified at 63× magnification. Adding more water to the slide allowed for the individuals to be recollected (Cesari et al. 2011). One to 15 individuals of each putative morpho-species were kept, pending their availability, while excess individuals were discarded. Specimens and eggs selected for barcoding were individually washed and transferred to wells on standard 96 well microplates. DNA was extracted by adding 70 μL QuickExtract™ DNA Extraction Solution kit (Lucigen) and incubating the samples for 2 h at room temperature, followed by 15 min at 65 °C and 2 min at 98 °C. Plates were subsequently briefly spun in a centrifuge to ensure that tardigrade exuviae were placed at the bottom of each well. Avoiding the exuviae, 30 μL of each DNA extract was transferred to new wells on a microplate and sent to the Canadian Centre for DNA Barcoding at the Centre for Biodiversity Genomics at the University of Guelph (Canada). DNA barcodes were produced using the primers C_LepFoIF/C_LepFoIR (Hernández-Triana et al. 2014) and LCO1490-Cola_t1/HCO2198_t1 (Folmer et al. 1994; Messing 1983) with subsequent bi-directional Sanger sequencing. In total, 369 individuals were submitted using this protocol. All metadata and sequences are publicly available through the dataset DS-TARNOFOR Tardigrades in Norwegian Forests in BOLD (www.boldsystems.org, Ratnasingham and Hebert 2007); DOI: dx.doi.org/10.5883/ DS-TARNOFOR. Sequences were assigned Barcode Index

Numbers (BINs) by BOLD (Ratnasingham and Hebert 2013). Specimen vouchers were made by recovering the exuviae and mounting them in Hoyer's medium on microscopy slides. These are deposited in the collections of the NTNU University Museum, Trondheim, Norway (NTNU-VM).

DNA extraction for metabarcoding

Extraction of DNA for DNA metabarcoding (on the third subsample) first followed the sample processing protocol for tardigrade extraction described above. Used equipment was sterilized in 5%–10% bleach between samples to avoid cross-contamination. Then, DNA was extracted from three different products of the substrate treatment: the actual substrate left in the sieve (sieve substrate), the water from the top 400 mL of the measuring cylinder (top of water column), and the precipitate (bottom of water column) (Fig. 1). For all subsamples, the substrate from the sieve (the filtered substrate fraction) was scraped into collection tubes and processed with the DNeasy PowerMax Soil Kit (Qiagen) following manufacturer's protocol. From the measuring cylinder, the top 400 mL (the supernatant) and the bottom 100 mL with precipitate were separated and filtered through mixed cellulose ester (CN) filters (0.45 μm) connected to an electrical vacuum pump. The filters were processed with the DNeasy PowerSoil Kit (Qiagen) following manufacturer's protocol. From each subsample, there were therefore three extracts, yielding a total of 45 extracts for the 15 samples. The extracts were used as template to amplify two fragments of COI and one fragment of the 18S gene (V4 region).

Library preparation

The two fragments of the mitochondrial COI gene (hereafter COI-A and COI-B) and the V4 region of the 18S rRNA marker were amplified in triplicate using the primers described in Table 1. All primer pairs and publicly available tardigrade sequences (13 December 2018) were inspected in silico in the AliView software (Larsson 2014) to assess whether the primers matched with tardigrade sequences. The universal eukaryotic 18S V4 primers from Stoeck et al. (2010) matched with tardigrades (along with a multi-tude other eukaryotic taxa) and were not modified. The available tardigrade COI sequences included more variation in the BF2 primer position than the original target freshwater macroinvertebrates (Elbrecht and Leese 2017). Therefore, BF2 primer was modified to include more degenerate bases (see Table 1). Similarly, TardR primer was modified from HCOooTAR tardigrade specific primer (Guil and Giribet 2009) to match all publicly available tardigrade sequences. TardR was used to ensure detection of tardigrades, as the vast majority of the DNA in the samples likely derive from other organisms, and using universal primers might result in only a small capture of tardigrade sequences. The initial PCR amplification was carried out using

Fig. 1. Illustration of the DNA extraction protocol used in this study. From each sample, DNA was extracted from the sieved substrate, the water column (top of column), and the precipitate (bottom of column). Each extract was subjected to three PCR replicates for each marker.
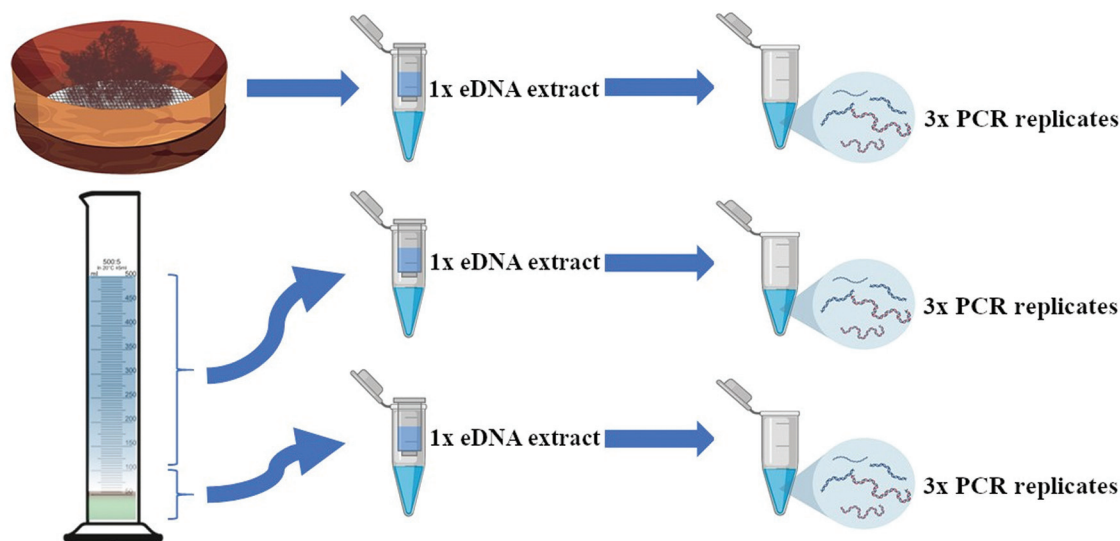

Table 1. Primers used for amplifying the different markers during PCR.

DNA fragment	Amplicon length (bp)	Primer name	Primer direction	Primer sequence (5′−3′)	Primer source
COI-A	422	BF2_TardF	Forward	GC**N**CC**N**GAYATR**KC****N**TTYCC	Elbrecht and Leese 2017 (modified from BF2)
		BR2	Reverse	TC**D**GG**R**TGNCCRAARAAYCA	Elbrecht and Leese 2017 (modified from BF2)
COI-B	443	BF2_TardF	Forward	GC**N**CC**N**GAYATR**KC****N**TTYCC	Elbrecht and Leese 2017
		TardR	Reverse	GG**W**A**R**AAT**HA****R**AATATA**DAC**	Guil and Giribet 2009 (modified from HCOooTAR)
18S rRNA	383–384	18S_TAReuk454FW	Forward	CCAGCASCYGCGGTAATTCC	Stoeck et al. 2010
		18S_TAReukREV3r	Reverse	ACTTTCGTTCTTGATYRA	Stoeck et al. 2010

Note: Bold and underlined nucleotides represent modifications of the source primers. For all primer pairs, the Illumina overhang attached to forward primers were TCGTCGGCAGCGTCAGATGTGTATAAG-AGACAG, while reverse primers were GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAG.

2.5 μL 10× reaction buffer (200 mM Tris HCl, 500 mM KCl, pH 8.4), 0.2 μL Invitrogen's Platinum Taq polymerase (5 U/μL), 1 μL dNTP mix (10 mM), 1 μL MgCl$_2$ (50 mM), and 0.5 μL of each primer (10 mM) with Illumina adapters. Then, 17.3 μL biology-grade H$_2$O and 2 μL template DNA was added to make the final volume 25 μL for each reaction.

The PCR programs used for the COI-A marker involved 3 min initial denaturation at 94 °C, followed by 35 cycles of 94 °C denaturation for 40 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 40 s. Final elongation at 72 °C followed for 2 min. The PCR program for the COI-B marker was identical except using 48 °C annealing temperature. The same program was also used for the 18S marker, except 1 min annealing period at 52 °C, and 25 PCR cycles.

The DNA extracts were amplified in three separately tagged PCR replicates per marker, where each PCR plate had six negative controls. The amplicons of the PCR reactions were examined on a 1.2% agarose gel by electrophoresis, while a subset of samples was quantified using the dsDNA HS Assay kit on a Qubit 2.0, following manufacturer's protocol. The DNA concentration of each marker was normalized by dilution, so that each well contained equal concentrations of all markers from one PCR replicate per sample extract. Likewise, the negative controls from the different marker PCRs were pooled three by three. A second PCR was run with 10 cycles to dual index the Illumina-tailed amplicons, using Nextera XT indices (FC-131-1002, Illumina, San Diego, CA, USA). The indexed amplicons were then transferred to one of two pools. The first pool consisted of 66 sample extracts and five negative controls, and the second of 67 sample extracts and four negative controls — both pools being balanced in regards of sample–substrate composition. Using SPRI magnetic beads, the pools were cleaned following the protocol of Fisher et al. (2011) using a bead:sample ratio of 0.55:1. The purified volume

was quality checked by dsDNA HS Assay kit in Qubit 2.0 and BioAnalyzer and was measured to a DNA concentration of 19.5 ng/μL and 16.4 ng/μL. Finally, each purified pool was used as template for constructing two libraries on standard flow cells using the 600 cycle V3 Illumina MiSeq sequencing kit (MS-102-3003). The raw data are publicly available in the European Nucleotide Archive (ENA) sequence read archive (PRJEB40985).

Data cleaning and filtering

Sequences of the different markers were separated in mothur v.1.41.3 (Schloss et al. 2009) using a *.oligos file including the sequence of each primer. Next, the forward and reverse strands of the sequences were merged in vsearch (Rognes et al. 2016) using the –*fastq_ mergepairs* command. Subsequent filtering of low quality paired-end sequences was done using the commands –*fastq_filter*, –*fastq_maxee*, –*fastq_minlen*, –*fastq_maxlen*, and –*fastq_maxns*. For all markers, max ambiguous nucleotides were set to 0, while max error rate used was estimated for each marker following the protocol of Rognes et al. (2016). Using the *summary.seqs* command in mothur, the lengths of the sequences of each marker, with quantiles, were calculated. This, in addition to inspecting the length of known tardigrade sequences, was used to determine the minimum and maximum sequence length allowed during the filtering. Sequences fulfilling these criteria were relabeled by sample names based on their combination of tags and were kept as separate Fasta files for each sample. These files were then merged in mothur before primer sequences were trimmed. Dereplication to unique sequences was done in vsearch with the command –*derep_fulllength*, followed by de novo chimera detection using the command –*uchime_denovo*, with –*abskew* set to 5. Next, all non-chimera sequences were clustered using the swarm 2.0 software (Mahé et al. 2014), with parameters -d 1, -t 2, -z, -w and using the *fastidious* command. The original quality-filtered sequences, excluding singletons, were then affiliated to the different swarms in preparation for the taxonomic annotation. These swarms were regarded as separate molecular operational taxonomic units (MOTUs). To assign taxonomic labels to these MOTUs, and to assess their validity as true MOTUs, reference sequences were used. For the 18S marker, all tardigrade sequences were downloaded from NCBI, while the COI reference sequences were downloaded from NCBI and BOLD (a neighbour joining tree exemplifying the COI divergence is given in File S2[2]). All marker reference databases were trimmed to reduce computational time, using the cutadapt unix command (Martin 2011). This trimming kept the sequences containing primer anchor sites matching at least two thirds of the primer sequence. Furthermore, this allowed the retainment of the hypervariable regions between the primers, while removing uninformative flanking regions. Taxonomic annotation of sequences of each marker was achieved by searching MOTUs and their nested sequences to the reference databases using a 97% and 98% similarity threshold for COI and 18S, respectively. This was done in vsearch with the commands –*usearch_global*, –*maxaccepts 0*, –*maxrejects 32*, and –*id 0.97* or *0.98*. The COI threshold was chosen based on a Barcode Gap Analysis integrated in BOLD, while the 18S threshold was picked based on literature reports and by exploring how our species data changed with different thresholds. The 98% gave consistent MOTU numbers compared to that of COI and the morphological data. The output was converted to OTU tables, assigned with read numbers of DNA-species/MOTUs in each sample. Negative controls contained raw read sequences, and were included in the bioinformatic pipeline. They contained no tardigrade sequences. To remove inconsistent PCR replicates, and to remove samples that yielded no tardigrade sequences, an iterative R-script was run until no further replicates were removed. This was done by comparing the composition of read numbers and MOTUs between PCR replicates and discarding outlier replicates, as presented in File S3[2]. The script retained 67% of the initial replicates. To avoid the influence of stochastic bias in read numbers among replicates of the same sample, the average number of sequences per MOTU was used when combining (pooling) results from each replicate. This resulted in 12, 12, and 11 quality extracts of moss, lichens, and leaf litter samples in the final OTU table. Only MOTUs with at least 10 reads in a sample were considered as present.

Data processing and analysis

The tardigrade species inventories recorded in the different extracts from each sample (sieve substrate, top of water column, bottom of water column) were compared using Bray–Curtis (Bloom 1981) and Jaccard (Jaccard 1901) similarity indices on pairwise comparisons of recorded species proportions, as shown in File S4[2]. The mean of the three extracts was used to represent a sample. Sample similarity and community differences were analyzed independently for morphological identification and each metabarcoding marker, and were visualized using Multidimensional scaling (MDS) by Principal Coordinate Analysis (PCoA). The pairwise comparison of all moss, lichen, and leaf litter samples were analyzed for sample variability internal to each habitat versus the between-habitat variability. This was done by running a dispersion test to evaluate the degree of homogeneity of variance between samples of each habitat type, using the betadisper() and permute() commands from the vegan package (Oksanen et al. 2010) in R (R Core team 2018). Further, the among habitat sample variability was evaluated by PermANOVA using the adonis() command. To investigate how congruous metabarcoding and morphologically based methods are at identifying tardigrade community differences between moss, lichen, and leaf litter samples, a mantle test was run (Sokal 1995). This model was run on the similarity matrices to

compare the methods' estimates of sample similarity, and was done using the mantle.rtest() command of the ade4 package (Dray et al. 2015) in R (R Core team 2018), using 9999 permutations. Sample 240 was not investigated by morphological identification, but was metabarcoded to balance the design among substrates. For comparisons across metabarcoding and morphological identification, sample 240 was excluded.

Results

Using the conventional, morphology-based identification method, 3788 tardigrades were recorded and identified to 39 morpho-species, spanning 2 classes, 5 orders, 7 families, and 19 genera. The average number of species and specimens observed in samples of each habitat were highly variable among samples and are reported for each substrate in Table 2. Barcodes were successfully retrieved for 24 of the species, yielding 32 BINs.

Coalescing the samples of each substrate type resulted in large areas of overlap in species presence between the different substrates (Fig. 2), based on traditional morphology data. Only three species were found exclusively in leaf litter, while the numbers for moss and lichen were six and nine, respectively.

For the DNA metabarcoding data, the sequencing of each library resulted in 9 667 862 and 6 302 056 raw reads, yielding on average 144 297 and 94 060 reads per sample (divided among the three PCR replicates). The merging and filtering reduced this number to 5 396 300 sequences. For COI-A and COI-B, 31 and 32 MOTUs, respectively, were matched to tardigrade species when using a >97% identity threshold. The 18S V4 marker matched 39 MOTUs to tardigrade reference sequences using a >98% similarity threshold. All three markers successfully detected a wide range of tardigrade species, spanning many of the major terrestrial families and genera. Of the 24 obtained morpho-species sequenced for COI barcode construction (32 BINs), all DNA-species and BINs except *Pseudechiniscus suillus* and one cryptic variant of the *Mesobiotus harmsworthi* group were retrieved by both COI markers. These two species were identified by morphology in quite high abundances in several of the investigated samples, yet remained undetected by both COI markers. The 18S marker did, however, detect *Pseudechiniscus* sp. and *Pseudechiniscus facettalis*, the latter belonging to the same species complex group as *P. suillus* (Cesari et al. 2020). Marker 18S also identified two *Mesobiotus harmsworthi* group species. For the *Macrobiotus hufelandi* species complex, identified as two morphotypes by morphological identification, DNA barcodes revealed six distinct genetic clusters (BINs). All of these were recovered by both COI markers.

A grand total of 48 different DNA-species were recorded by one or more metabarcoding markers. These included 81.5% of the species identified by morphology, and detected seven additional species not recorded by traditional methods (File S2[2]), in addition to genetic

Table 2. Average number of species and abundances in moss, lichen, and leaf litter samples, reported with the variability between samples of each substrate type.

	Moss	Lichen	Litter
Mean no. of species	12.3, SD = 3.5	8.0, SD = 3.2	10.8, SD = 2.0
Mean no. of individuals	272, SD = 135.3	176, SD = 131.3	61.5, SD = 19.3

Note: All samples contained tardigrades, ranging from 35 to 480 individuals. SD, standard deviation.

Fig. 2. Venn diagram of the number of tardigrade species recorded exclusively in and shared between the sampled substrates using morphological identification.
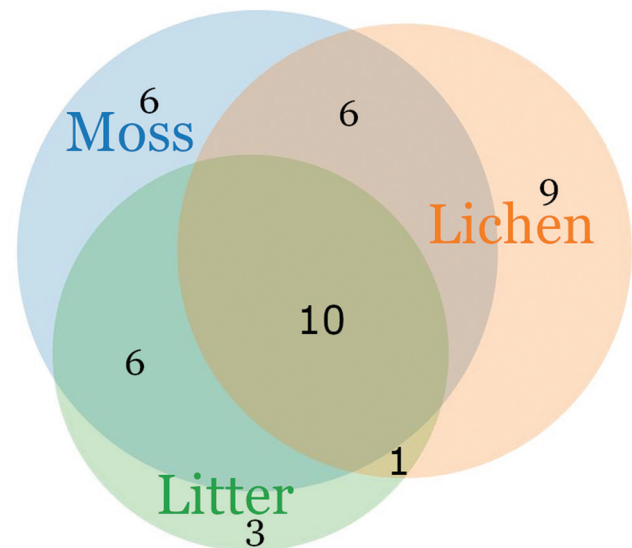
clusters inseparable by morphology (potentially cryptic species). In addition, nine species were detected by 18S and morphological identification, but not by any of the COI markers. Nine other species were detected by COI and morphological identification, but not by 18S (Fig. 3). Among the DNA-only species, three have previously been recorded from Norway, while four are new records. One was a match to *Acutuncus antarcticus*, a species endemic to Antarctica. Of the species exclusively identified by morphology, five out of seven species lacked reference sequences in probed databases. Furthermore, of the two species with available reference sequences, one was only found as a single individual in two samples, while the other was an uncertain identification at the species level (*Echiniscus* cf. *testudo*).

DNA metabarcoding detected a variable average number of species depending on both marker and sample type, but recorded a higher total number of species per sample than morphological identification methods for all samples except 234 and 236. For 10 of the 15 samples, 18S retrieved more species compared to COI-A, COI-B, COI combined, and morphological identification. In

Fig. 3. The overlap in recorded species between the traditional morphological identification and the three markers used for DNA metabarcoding. MOTUs assigned to Tardigrada below the 97% and 98% thresholds are not included in the figure.
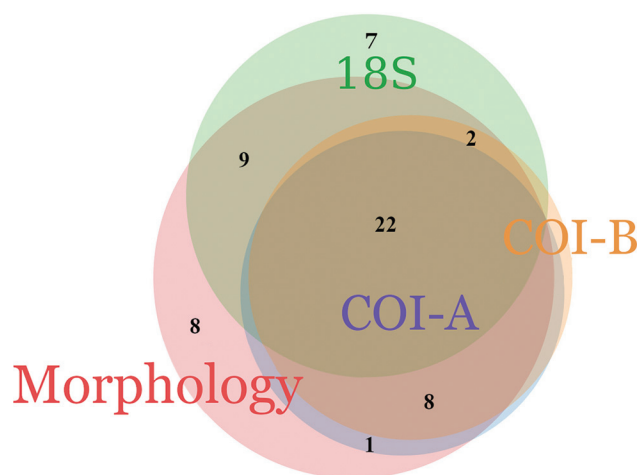
samples 213, 216, and 237, three, two, and three species more were observed by morphology than by 18S. For the mitochondrial primers, COI-B, being the more tardigrade specific, performed better than the more universal COI-A by detecting on average two DNA-species more per sample. Individually, both COI markers recorded on average the same number (COI-B) or two species less (COI-A) than morphological identification, but with large variation among samples. Both COI primers were unsuccessful in retrieving consistent PCR replicates for all extracts of the leaf litter samples 233, 234, 236, and 237. Interestingly, the 18S marker was consistent for all these samples except 236 (which was measured to have a DNA concentration of ~0 ng/µL for at least two of the extracts) (Table 3).

Among the taxa identified by morphology, four were missing from both COI and 18S reference libraries, seven from the COI library alone, and five from the 18S library alone (Fig. 4). Taxa recorded with relatively high abundance by morphology mostly had a high number of reads for one or the other marker in the metabarcoding analysis, although discordances exist (e.g., *Echiniscus spiniger*, Fig. 4).

Based on Bray–Curtis similarity, both morphological identification and metabarcoding recorded moss, lichens, and leaf litter to host statistically significant dissimilar tardigrade communities (Fig. 5). However, while in the communities based on morphological identification there was no difference in the variance between the clusters of samples of moss, lichen, and leaf litter ($p = 0.25$), the communities identified by DNA metabarcoding were significantly dispersed ($p = 0.04$). In both datasets, leaf litter samples contained the most

distinct and consistent composition of tardigrade species. The clusters of moss and lichens showed an area of overlap by both methods. This overlap was in both PCoAs observed by a single lichen sample (226) taken from a rock surface on the ground containing very similar composition of species as the moss samples. Subsequent examination of the sample revealed some mixed-in moss among the lichens in sample 226. Lichen samples contained the highest variability in species composition by both methods, followed by moss samples. Overall, less of the variance in sample distribution was explained in the DNA metabarcoding PCoA (54% by both axes) than in the PCoA based on morphological identification (71% and 74% for axis 1 and 2). This variance represents how well the PCoA maintains the relationship between samples when going from n dimension to two dimensions, and thereby indicates that more information was lost during dimensional compression for the DNA metabarcoding data than for the data based on morphology. Although there were slight differences in the distribution of samples between the methods, the major patterns were similar (Fig. 5).

Sample similarity recorded by metabarcoding and traditional, morphology-based sampling was highly correlated for the 18S marker and moderately correlated for the COI-A and COI-B markers, using both similarity indices. The 18S marker showed high correlation to the morphological data with a Mantel statistic R of 0.76 ($p = 1e-04$) and 0.69 ($p = 1e-04$) for the Bray–Curtis and Jaccard indices, respectively. (Fig. 6). The COI-A and COI-B metabarcoding data fit less well to the morphological data (Table 4), but still had a positive and statistically significant relationship (Table 4).

Discussion

DNA reference barcodes were retrieved from 24 out of the 39 species identified by morphology. While some of this discrepancy might be explained by primer mismatch, it is probably more affected by the difficulties in identifying correct species before DNA extraction and subsequent slide mounting, as well as the size and armouring of the specimens. For instance, although we experienced lower success rate in sequencing the generally smaller and better armoured heterotardigrades (32%) compared to eutardigrades (61%), there was no generic or specific bias in the groups. Moreover, some species were rare in the samples and were undetected when sorting out individual specimens for DNA extraction. For instance, we were unable to sample the species *Hebesuncus conjugens*, *Itaquascon placophorum*, *Mesobiotus montanus*, *Pilatobius oculatus*, and several species of *Echiniscus* for our reference library building.

DNA metabarcoding using COI will in many cases detect higher species richness than morphology-based identification due to its ability to identify cryptic species and species elusive to the sorting methods used to retrieve

Table 3. Number of DNA-species and morpho-species detected in the investigated samples.

Substrate	Sample ID	COI-A	COI-B	COI Total (COI-A + COI-B)	18S	Total DNA-species	Morph.
Moss	212	11	13	15	15	21	10
Moss	213	7	10	17	16	25	19
Moss	215	8	11	13	21	25	13
Moss	216	5	6	7	12	17	14
Moss	219	10	13	13	15	17	8
Lichen	221	—	7	7	13	14	3
Lichen	222	8	9	17	15	24	9
Lichen	224	12	12	13	19	20	13
Lichen	226	10	15	18	18	24	8
Lichen	230	5	7	7	10	12	7
Leaf litter	233	—	—	—	13	13	8
Leaf litter	234	—	—	—	10	10	14
Leaf litter	236	—	—	—	—	—	11
Leaf litter	237	10	11	16	6	16	9
Leaf litter	240	—	8	9	20	21	N/A

Note: Numbers given by DNA metabarcoding markers for each sample are average numbers of MOTUs per PCR matching reference sequences above the 97% (COI-A and COI-B) and 98% (18S) similarity thresholds. Samples with inconsistent PCR replicates were excluded from the calculations. Species identified by morphology (Morph.) are species recorded by at least one individual or egg in the sample. The dash (—) indicates samples with no reliable species number and were excluded from analysis due to inconsistent results among PCR replicates.

specimens (Tang et al. 2012; Elbrecht et al. 2017; Sun et al. 2019). Previous studies that compare morphological identification, DNA barcoding of single specimens, and eDNA metabarcoding of soil and marine meiofauna show a similar trend, although morphological identification produced higher richness than 18S DNA-based methods for some phyla and families (Leasi et al. 2018; Treonis et al. 2018), and 18S has been shown to produce lower diversity estimates than both morphological identification and COI-based estimates (Tang et al. 2012). For tardigrades, the number of DNA-species detected by DNA metabarcoding using three markers exceeds the number of morpho-species detected by conventional, morphology-based identification in most samples (Table 3). The numbers of species reported in this study are likely underestimates of the actual tardigrade diversity because there are numerous MOTUs assigned to Tardigrada that are below the similarity threshold used for species-level identification. However, the tendency of PCR-based DNA metabarcoding to produce spurious MOTUs (Ashelford et al. 2005; Haas et al. 2011; Quince et al. 2011) complicates the interpretation process when using incomplete reference libraries. The proportion of such artifact MOTUs in our data remains unknown, but as most of these unlabeled MOTUs were detected in several samples, there is a high likelihood that they are true tardigrade species that remain unresolved due to missing reference sequences.

The confidence in the recovered species lists is further supported as many species are independently detected by more than one marker. The multi-marker approach facilitated identification of more species as missing reference sequences for one marker often were complemented by the presence of the respective species' reference sequence for another marker (Fig. 4). Assess-

ments of zooplankton communities using multiple markers have given similar results (Stefanni et al. 2018; Zhang et al. 2018). Additionally, the use of more than one marker can facilitate detection of more species as primer mismatch and resulting amplification failure (Zhang et al. 2018), as well as low barcode resolution, are less likely to occur in all markers. However, interpretation of results in multi-marker metabarcoding is complicated as any recorded cryptic variant identified by both COI and 18S often cannot be confidently consolidated as true equivalents. Extending the reference libraries to encompass sequences of more species will make a single marker approach more viable, and thereby eliminate this issue.

The 18S sequence library for tardigrades used in this study does not provide the same taxonomic resolution as the COI markers. The 18S reference library contains more discordant clusters of 18S sequences from different species than the COI reference library (based on p-distances) (Blaxter et al. 2004). For some taxa, two or more species either share identical or have highly similar 18S sequences (e.g., Blaxter et al. 2004; Guidetti et al. 2019). Although identification of tardigrades by 18S sequences is more challenging, the marker still identified the majority of our tardigrade species. Problematic taxa do exist (e.g., *Mesocrista* sp., *Calohypsibius* sp., *Thulinius* sp., *Hypsibius* sp., and *Paramacrobiotus* sp.), genera of which some or all species seem indistinguishable by the selected sequence similarity threshold (Blaxter et al. 2004; Garey et al. 1996; Jørgensen and Kristensen 2004; Guidetti et al. 2019). Consequently, a lower diversity of tardigrades was expected to be found with 18S compared to COI. The findings by Arakawa (2020) supports the observation of low taxonomic resolution in 18S sequences as metabarcoding of moss

Fig. 4. Heatmap of the different methods and their species detection lists. Abundances are quantified as the logarithm of sequences and logarithm of number of individuals. In cases where more than one variant of a species was recorded, identifications were elevated to genus level to allow comparison of results from the 18S and COI markers. Blank cells indicate no detection. Red asterisk (*) marks species lacking reference sequence for that marker. Black asterisk on species names denote species that were detected at the genus level by the 18S marker and were deduced to likely belong to the listed species based on the morphological identification of specimens from the same sample.

samples was able to identify all morphologically identified taxa but only at genus level. The observed higher diversity identified by 18S is likely a result of the 18S reference library containing sequences from far more species than the COI reference library. Furthermore, public databases commonly contain spurious sequences (e.g., contaminations or taxa wrongly labelled) which could be detected by DNA metabarcoding and bias species richness estimates. By searching all 18S sequences tagged as Tardigrada in GenBank, numerous rotifer sequences were included in the downloaded file. We tried to account for this by excluding such

sequences from the reference library, but cannot guarantee their complete removal. Any DNA metabarcoding survey should evaluate the recorded taxa, as it will significantly increase the confidence in the results. Of the species detected by DNA metabarcoding of 18S, *Acutuncus antarcticus*, *Macrobiotus scoticus*, *Minibiotus gumersindoi*, and *Mixibius saracenus* are considered uncertain records due to their current biogeographical distribution. Neither of these taxa were detected during the morphological investigations. The reason for the potential erroneous identification of these species might be several, including wrongly identified sequences in

Fig. 5. PCoA of the Bray–Curtis distances on relative frequencies of species detected by morphology-based identification (upper panel) and DNA metabarcoding of the 18S marker (lower panel). The p-values for tests of the differences between clusters and dispersion within clusters are listed in the corner. GOF abbreviates goodness of fit for the PCoA data to the respective axes.
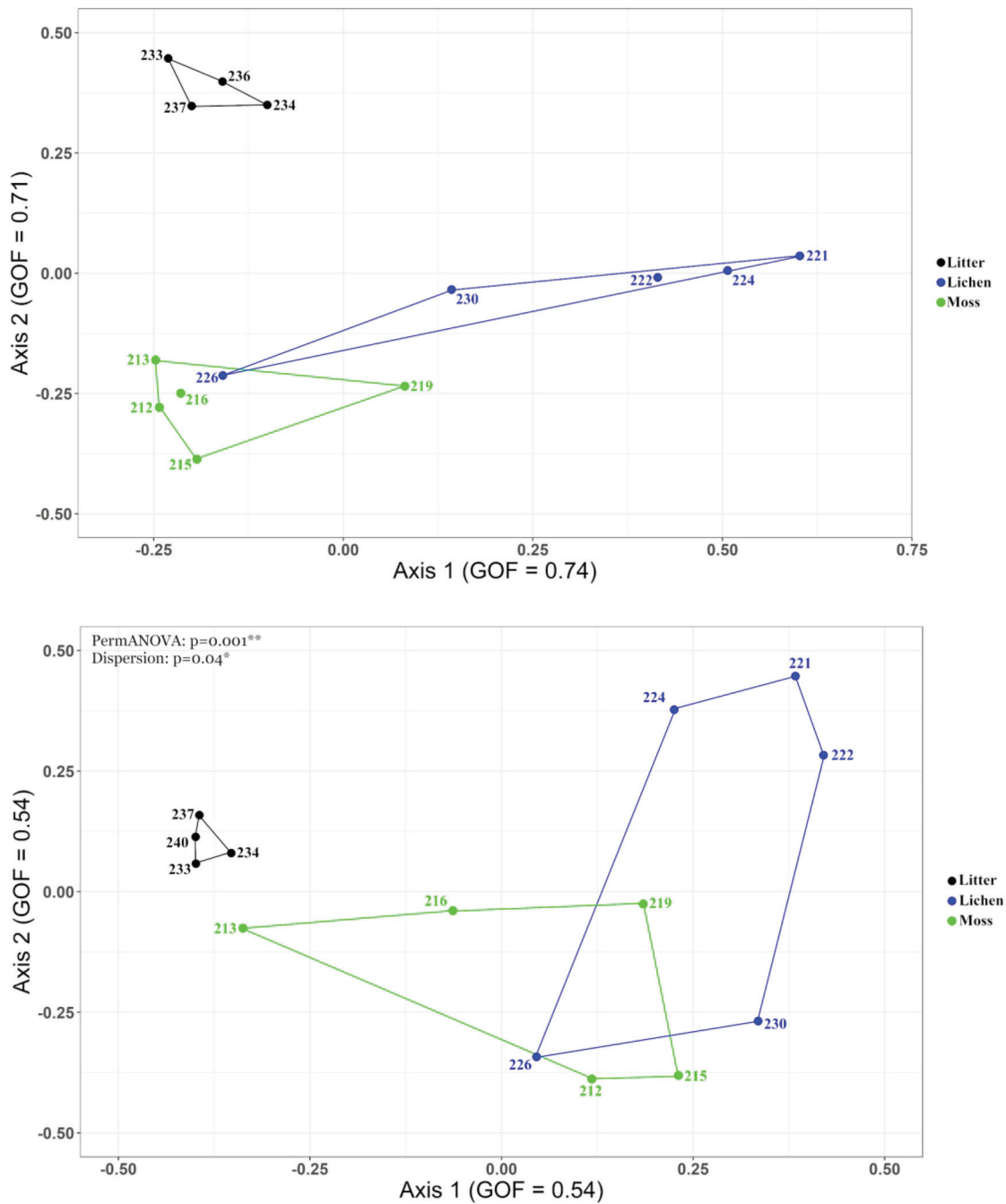
GenBank and our use of a potentially too low threshold for accepting species matches (98%). Nevertheless, their detection highlights the importance of screening DNA metabarcoding data for possible false positives. Furthermore, these species were only recorded when reference libraries included sequences from public databases, and were absent when analyzed using the smaller, local reference library. The presence of *Microhypsibius bertolanii* in the DNA metabarcoding data, but not in the

morphological data are likely due to the elusiveness of the genus. Species of *Microhypsibius* are rarely encountered, and are mostly represented by few individuals when present (e.g., Guidetti et al. 1999; Guidetti and Bertolani 2001; Bertolani et al. 2014; Zawierucha et al. 2016). Individuals belonging to a species of *Microhypsibius* was recorded in samples collected at a locality not far from ours (unpublished data), meaning the genus is likely present also in our samples. Together with several other

Fig. 6. Scatter plot on each marker's Bray–Curtis (upper panel) and Jaccard (lower panel) community similarity matrices in relation to the matrices based on morphological data. Red, dashed line represents the expected 1:1 relationship if the methods were fully congruent.
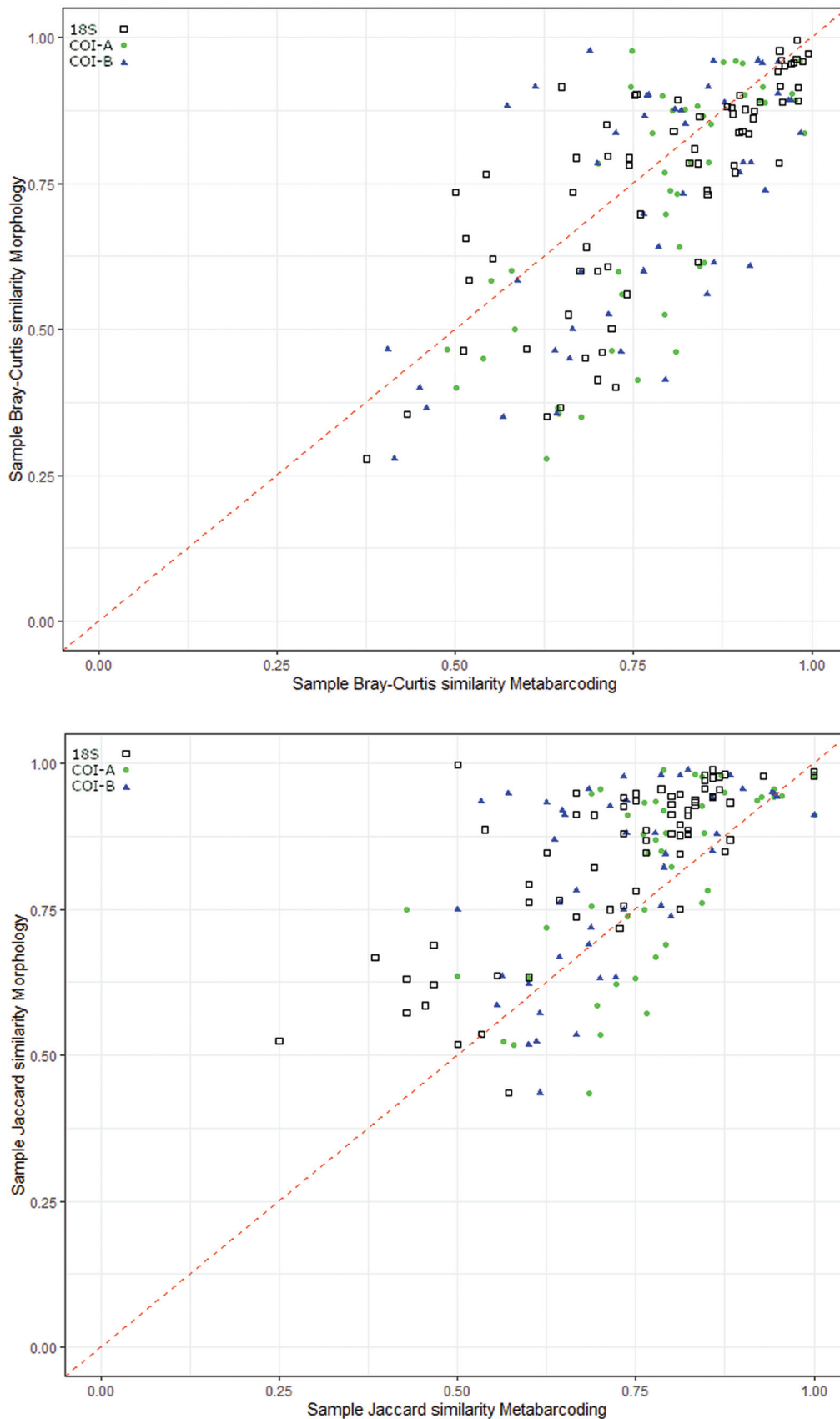
Table 4. Mantel test parameter estimates on pairs of Bray–Curtis and Jaccard similarity matrices of each metabarcoding marker compared to the morphology-based identification method.

Metabarcoding marker	Similarity index	Mantel statistic (R)	Permutation variance	*P*-value
18S	Bray–Curtis	0.76	0.018	1e–04
COI-A	Bray–Curtis	0.64	0.017	1e–04
COI-B	Bray–Curtis	0.46	0.014	4e–04
18S	Jaccard	0.69	0.016	1e–04
COI-A	Jaccard	0.60	0.018	1e–04
COI-B	Jaccard	0.49	0.020	4e–04

species recorded by few individuals in our morphological data (e.g., *Hebesuncus conjugens*, *Platicrista angustata*, *Milnesium* spp.), which were also detected by DNA metabarcoding, they testify to the power and sensitivity of metabarcoding in detecting rare species.

For ambiguous records in the DNA metabarcoding data, evaluating the ecology and biogeography of probable species can often be used to infer the correct species with high probability. From our study, it seems likely that the recorded *Calohypsibius* sp. and *Astatumen* sp. by 18S are *Calohypsibius ornatus* and *Astatumen triacrinae* as these are common in Norway (Meier 2017; unpublished data) and were found in relatively high number of specimens in the samples. Likewise, the *P. facettalis* detected by 18S is likely to correspond to *P. suillus*, which was observed in high abundances in the samples. Cesari et al. (2020) observed no clear subdivision of these two species based on the COI gene, postulating that the species possibly are synonymous.

The community composition analyses (based on full data for the morphology-based PCoA and data pruned for likely false positives in the DNA metabarcoding PCoA) inferred highly similar patterns of species composition by both methods. However, while the within-cluster (substrate) variability of samples for the morphological data was similar, the large difference in variability in the DNA metabarcoding data breaks the assumption of the PermANOVA regarding equal dispersion of all clusters. This means that the univocal pattern outlined in the PCoA based on morphologically generated data cannot be confidently deduced from the metabarcoding data. Nevertheless, the highly similar clustering and distribution of samples in both PCoAs indicate that DNA metabarcoding does in fact identify the same community composition patterns as morphological methods. On the other hand, for the ecological significance of the inferred community dissimilarity, one cannot conclude a statistical difference. Several studies have investigated community composition of tardigrades in mosses, lichens, soil, and leaf litter, without finding substantial differences (e.g., Guidetti et al. 1999; Ito 1999; Nelson and Bartels 2007; Guidetti and Bertolani 2001; Nelson et al. 2019), although hygrophilous and eurytopic

species seem more common in leaf litter (Guidetti et al. 1999). Despite the biomass volume of samples collected in these studies being smaller than ours, their inferences were based on much higher sample size, indicating that our findings can be an artifact of low sample size. Indeed, the accumulation curves on the recorded (morphology-based) data, described in File S5[2], show that our sampling effort is insufficient for any ecological inference. However, these graphs also indicate that there still remains uncaptured diversity of tardigrades in the sampled habitats, lending support to the higher species richness estimates inferred from the DNA metabarcoding data.

Regardless of the legitimacy of the identified ecological patterns, metabarcoding of tardigrades by the use of eDNA reveals highly similar communities compared to those obtained by morphological identification. Although it is debatable to use sequence numbers to quantify species abundance (e.g., Lamb et al. 2019; Yates et al. 2019; Schenk et al. 2019), using such data as MOTU proportions served informative, given the increased fit and lower estimate uncertainty for the Bray–Curtis data compared to the Jaccard data. For samples hosting close to identical communities, metabarcoding based on the Bray–Curtis index seems to slightly underestimate the similarity. Interestingly, by using the Jaccard Index on the same data, metabarcoding seems to be overestimating the sample similarity. This is, at least in part, a likely effect of the reference library, as rare and potentially spurious MOTUs are weighted more by the Jaccard Index, while being less impactful on the Bray–Curtis index. Consequently, the Jaccard similarity is inflated while the Bray–Curtis similarity is negligibly affected. Such cases can be, at least partly, mitigated through stringent filtering and post-pipeline curation and evaluation of the data, as this can eliminate false positives.

The Mantel test correlations between the distance matrices produced by the metabarcoding and morphological-based method show that the two methods are highly congruent in sampling tardigrade diversity, but that choice of marker matters. Overall, 18S shows the most promise as a metabarcoding marker for community analyses of tardigrades, but obviously suffers from

its inability to distinguish certain genera and families. The COI markers are less affected by this issue, but seem less suitable for describing community differences. Similar findings are reported in numerous studies of other taxa, generally as a result of amplification bias (e.g., Deagle et al. 2014; Elbrecht and Leese 2015; Piñol et al. 2015).

Based on the much less reliable attempts to detect tardigrades in some of the soil and leaf litter samples, it seems that DNA metabarcoding of these microhabitats is more challenging. Leaf litter and soil host similar species richness to, but lower tardigrade abundance than moss and lichens (Bartels and Nelson 2006, 2007; Guidetti et al. 1999; Nelson et al. 2019; this study), indicating lower availability of tardigrade DNA in these environments. Furthermore, the amount of PCR inhibiting components is often high in soil and leaf litter (Miller et al. 1999; Taberlet et al. 2018) exacerbating the PCR potential further. Inconsistency between PCRs is common for eDNA studies (Deiner et al. 2017) and, following Taberlet et al. (2018), up to nine PCR replicates should be included in DNA metabarcoding of soil eDNA. Such a setup would likely improve the reliability of our study. Interestingly, tardigrades were recorded in all PCR replicates of leaf litter in our study, but due to the high inconsistency of intra-sample PCRs, they were not considered trustworthy. By utilizing a higher number of technical replicates, reliable tardigrade species lists will likely be obtainable also for soil and leaf litter samples.

Conclusion

Methods based on morphological identification of individuals are time-consuming and often limited in their taxonomic resolution. They are therefore not always suitable for large-scale ecological studies on tardigrades. DNA metabarcoding of environmental substrates promises an effective and accurate identification of species regardless of morphological constraints and developmental stage. DNA metabarcoding of tardigrades is, however, currently suffering from the incoherence of reference databases. We show that a multi-marker approach partly circumvents this issue, but complicates the interpretation process. This is especially apparent when comparing species sequences captured by more than one marker in the presence of cryptic variants. Nevertheless, metabarcoding of environmental substrates grants superior detection of tardigrade species and allows capture of differences in community composition between substrates. Looking beyond tardigrades, the approach used in this study should be applicable to other micro- and mesofaunal taxa, requiring only minor adjustments to the protocol.

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