

Response of forest soil euglyphid testate amoebae (Rhizaria: Cercozoa) to pig cadavers assessed by high-throughput sequencing

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Abstract Decomposing cadavers modify the soil environment, but the effect on soil organisms and especially on soil protists is still poorly documented. We conducted a 35-month experiment in a deciduous forest where soil samples were taken under pig cadavers, control plots and fake pigs (bags of similar volume as the pigs). We extracted total soil DNA, amplified the SSU ribosomal RNA (rRNA) gene V9 region and sequenced it by Illumina technology and analysed the data for euglyphid testate amoebae (Rhizaria: Euglyphida), a common group of protozoa known to respond to micro-environmental changes. We found 51 euglyphid operational taxonomic units (OTUs), 45 of which did not match any known sequence. Most OTUs decreased in abundance underneath cadavers between days 0 and 309, but some responded positively after a time lag. We sequenced the full-length SSU rRNA gene of two common OTUs that responded positively to cadavers; a phylogenetic analysis showed that they did not

belong to any known euglyphid family. This study confirmed the existence of an unknown diversity of euglyphids and that they react to cadavers. Results suggest that metabarcoding of soil euglyphids could be used as a forensic tool to estimate the post-mortem interval (PMI) particularly for long-term (>2 months) PMI, for which no reliable tool exists.

Keywords Environmental DNA · Euglyphid testate amoebae · Illumina high-throughput sequencing · Metabarcoding · SSU rRNA gene V9 region · Forensic ecology

Introduction

The estimation of time since death and more generally the ability to detect the presence of cadavers even when the remains are no longer present are the two major objectives in forensic research [1, 2].

Calculation of the post-mortem interval (PMI), an essential element of legal medicine and criminal investigation to establish the timing of events that led to the death of a person, becomes less precise with the advance of the decomposition process. Until now, two main approaches are used to estimate the PMI. The medical method provides information ranging from a few hours to several days after death [3–5]. The second method, forensic entomology, is based on the observation of larval stages of necrophagous flies and beetles and can be used to establish a PMI up to some weeks [4, 5]. Although well established, the accuracy of entomological methods has been questioned after the full development of the first generation of necrophagous insects [5]. Therefore,

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the development of additional indicators for PMI estimates beyond 1 month would constitute a welcome addition to the toolkit of forensic criminal investigators.

According to Payne [6], the decomposition of cadavers can be separated into six stages: fresh, bloated, active decay, advanced decay, dry and remain stages. During the 'bloated' and 'active decay' stages [6, 7], the release of cadaver liquids into the soil changes the chemical parameters drastically [8]. This perturbation of the soil environment has been referred to as 'ephemeral resource patches' [9] leading to the development of 'cadaver decomposition islands' (CDI) [7]. Although most of the decomposition takes place in the first few weeks under optimal conditions, cadaver effects on the soil environment can be long lasting. For example, Towne [10] showed that nitrogen and phosphorus concentration and pH were still significantly enhanced in soil samples taken under cadavers 2 years after laying ungulate cadavers on a prairie, while Melis et al. [11] reported enhanced soil calcium content and pH as late as 7 years post mortem in a CDI. Such environmental changes were shown to have an effect on the soil fauna [12], bacteria [13–15] and fungi [16, 17]. However, knowledge about cadaver effects on soil communities remains very limited, and almost nothing is known about the response of soil protists [18].

In this study, we focused on euglyphid testate amoebae (Rhizaria: Cercozoa), a highly diverse and abundant group of protists that reacts rapidly to environmental changes by shifts in community structure and abundance [19]. Euglyphids include about one quarter of the ca. 300 testate amoeba morphospecies known to occur in soils [19]. These amoeboid unicellular protists range mostly between 20 and 150 μm in length, and their densities typically range between ca. 10^6 and 10^8 individuals per square metre [20]. They build a shell (test) reinforced with ornamented self-secreted siliceous plates, and these shells allow species identification even after the death of the organism [21, 22]. Most euglyphids are heterotrophs and feed mainly on bacteria and fungi [23]. The distribution patterns of soil testate amoebae along environmental gradients and their response to environmental changes have been well studied, including soil humidity [24–26]; temperature [27, 28]; pH [29–32]; and pesticide [33], nitrogen, phosphorus and sulphate concentration [34–37]. They can thus be expected to also respond to the presence of decomposing cadavers. The generation time of euglyphids, which ranges from ca. 2 days to 1 week under natural conditions [38], is considerably longer than that of bacteria or smaller protozoa such as nanoflagellates, and this represents an advantage regarding their use as bioindicators. It is indeed short enough to allow them to (re)colonise rapidly suitable habitats [39, 40] and respond to environmental change over a period of weeks. However, as euglyphids are highly sensitive to environmental conditions, the effects on communities can be expected to be long lasting under continuous environmental stress. So especially for estimating longer PMIs, euglyphids might be a group to consider for forensic applications.

However, a current limitation to the development of euglyphid analysis (or that of other soil protists) as a standard tool for PMI estimates is taxonomy. Sound taxonomy is indeed a prerequisite for the use of a group of organisms as bioindicators. Up to now, all ecological studies on testate amoebae were based on morphology-based species identifications. The morphological identification of testate amoebae requires taxonomic expertise and is time-consuming. Furthermore, recent molecular taxonomy studies on euglyphids have revealed the existence of a substantial higher diversity than estimated based only on morphology [41, 42], and this hidden/unknown diversity may prove to have bioindication value. The molecular approach presented in this study overcomes the current limitation of morphology-based taxonomy and is also faster (i.e. weeks instead of months for the number of samples analysed here).

Analysis of environmental samples (e.g. soil, water, faeces) targeting a specific DNA barcode gene and aiming at characterising the entire community is referred to as metabarcoding [43]. The V9 region of the 18S rRNA gene has sufficient variability for obtaining reasonably high taxonomic resolution [44, 45] and two main advantages for biodiversity surveys as follows: (1) it is short and thus likely to be well preserved in environmental DNA samples and (2) it contains highly conserved sites allowing to designing primers for virtually all eukaryotes [46]. The advent of high-throughput sequencing (HTS) now allows using the metabarcoding approach in ecological studies with high sample numbers (e.g. spatial and/or temporal sampling).

In this study, we used a DNA metabarcoding approach applied to the V9 region of the 18S rRNA gene to assess the temporal response of soil euglyphid testate amoebae to decomposing cadavers over a period of 35 months (1051 days). Given the sensitivity of the technique and the well-documented response of testate amoebae to ecological gradients, changes and disturbances, we expected to find (1) a higher diversity of soil Euglyphids than generally inferred from microscopic analyses and (2) a strong generally negative response of communities to decomposing cadavers with i) rapid disappearance of the majority of taxa following the massive release of cadaver fluids in the soil and ii) slow recovery after the end of the active decay phase, ca. 1–2 months after the peak of cadaver fluid input in the soil. If such patterns were indeed found, this may lead to the development of new PMI indicators in the future.

Materials and methods

Sampling site

The experimental site is situated in a beech- (*Fagus sylvatica*) and oak- (*Quercus robur*, *Q. petraea*) dominated forest near Neuchâtel (Switzerland 47° 00' N; 06° 56' E, elevation 478 m). The overall average temperature measured over the course of the experiment was 10.4 °C (SD 6.09 °C). The mean annual

precipitation of the nearest meteorological station (Neuchâtel) for 1993 to 2013 was 974 mm per year (MétéoSuisse).

Experimental setup

The experiment included three treatments: control (plots of forest soil left under natural conditions), fake pig (plastic bags filled with a volume of soil similar to that of the pigs placed in a cotton cloth) and pig (*Sus scrofa*). The fake pig treatment was used to differentiate the chemical effect of pig cadaveric liquids from the physical effects (i.e. humidity, soil compaction) due to the presence of a carcass on the soil. The bag volume was kept approximately similar to that of the pigs by removing soil from the bag to mirror the volume loss of the pig cadavers over time. The pigs (20 kg±1 kg) were killed on the farm with captive bolt stunning and the cadavers immediately brought to the experimental site. The cadavers were placed in strong metal wire cages (90×100×50 cm) to protect them from scavengers. The cages also allowed moving the cadavers for sampling. Pigs are commonly used in comparable forensic studies due to the similarities with humans, comparable thoracic cage size and almost naked skin [47]. Each treatment was run in triplicate. The sampling plots were organised into three randomised blocks (15–34 m apart). Within each block, the plots were at least 4 m apart.

Sampling and chemical analyses

Eight sets of samples were collected from the onset of the study (August 5th, 2009=D0) until June 21st, 2012 (Table 1). At the onset of the experiment (D0, before the pigs and fake pigs were placed), initial control samples were collected from all sampling plots and pooled for each block (i.e. three pooled samples in total). Sampling days were scheduled according to decomposition stages (Table 1) [6]. On each sampling day, ca. 25 g of soil was taken to a depth of 10 cm in each plot and stored at –80 °C.

Soil subsamples (3 g) were dehydrated (40 °C, 12 h), ground to powder and analysed for total organic carbon (Soil_C) and total nitrogen (Soil_N) using combustion

infrared spectroscopy (CHNEA1108-Elemental analyser, Carlo Erba Instrument) after decarbonation with HCl [48].

Molecular analyses

DNA was extracted from soil samples using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA) following the manufacturer instructions. The SSU rRNA V9 region was amplified by PCR using the specific eukaryotic primers 1380f/1510r (CCCTGCCHTTTGTACACAC/CCTT CYGCAGGTTACCTAC) [44]. Forward primers were tagged on the 5' end with a 10 nucleotides strand, specific to each sample. PCR reactions were run in triplicate with a PTC-200 Peltier Thermo Cycler (BioConcept, Allswill, Switzerland) with 1 ng of environmental DNA, 6 µL of 10x PCR buffer, 0.6 µl of each primer, 0.6 µl of each dNTP 400 µM (Promega) and 0.2 µl of 0.05 U/µl Go Taq (Promega). The volume was adjusted to 30 µL with ultra-pure water. Amplification was conducted with the following conditions: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 57 °C for 60 s and 72 °C for 90 s and final extension at 72 °C for 10 min [44]. PCR products were purified through QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and pooled together with a 4-ng DNA of each sample. A DNA library was prepared using the New England Biolabs's kit NEBNext DNA Sample Prep Master Mix Set 1 (<http://www.neb.com/nebecomm/ManualFiles/manualE6040.pdf>) except for the size selection step. Sequencing was done by the Genomics Core Facility at Brown University (Providence, USA) with an Illumina® HiSeq 2000 sequencer to obtain paired-end reads covering the full length of the V9 region.

Sequence treatment

A database was constructed by selecting 44 complete euglyphid V9 sequences from the GenBank database, using sequences derived both from identified organisms and from related environmental sequences retrieved from GenBank. Each environmental V9 read was compared to the database using the

Table 1 Sampling dates and corresponding decomposition stages of the pig cadavers in a *Fagus-Quercus* forest above Neuchâtel, Switzerland

Sample code	Sampling date	Decomposition stage
D0	05/08/2009	Fresh
D8	13/08/2009	Active decay
D15	20/08/2009	Dry stage
D22	27/08/2009	Dry stage
D33	07/09/2009	Dry stage
D64	08/10/2009	Dry stage
D309	10/06/2010	Dry stage
D1051	21/06/2012	Dry stage

Table 2 Summary of the sequence filtering of euglyphid testate amoeba from the control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland

Analysis steps	Total reads	Euglyphid sequences	Euglyphid dereplicated reads	Euglyphid OTUs
Raw fastq	247366905	–	–	–
Blast selection	187566	187566	–	–
Reads >= 5 times	57533	57533	2621	–
OTU building	57724	57724	2621	198
OTU selection	52860	52860	–	51
Triplication D0	57640	57640	–	51

Table 3 Summary of total euglyphid testate amoeba OTU abundance in the control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland

Euglyphid OTUs	Total abundance
eugly_59	4234
eugly_13	4205
eugly_2	4149
eugly_12	3873
eugly_666	3161
<i>Euglypha rotunda</i> AJ418783.1	3056
<i>Euglypha filifera</i> AJ418786.1	2583
eugly_66	2530
Uncultured eukaryote EF025028.1	2279
eugly_81	2048
eugly_151	1949
eugly_156	1933
eugly_5	1916
eugly_136	1630
eugly_183	1451
eugly_322	1400
eugly_38	1227
eugly_307	1199
eugly_54	1186
<i>Assulina muscorum</i> AJ418791.1	1162
eugly_113	1086
eugly_234	946
Cercomonadida env sample EF024983.1	858
eugly_79	675
eugly_33	594
eugly_41	514
eugly_60	417
eugly_290	411
eugly_991	402
eugly_98	382
eugly_320	367
eugly_992	349
eugly_16	314
eugly_473	273
eugly_862	253
eugly_82	238
eugly_76	211
eugly_80	203
eugly_973	195
eugly_233	182
eugly_1245	177
eugly_854	176
<i>Tracheleuglypha dentata</i> X77698.1	176
eugly_282	172
eugly_885	170
eugly_371	165
eugly_250	141

Table 3 (continued)

Euglyphid OTUs	Total abundance
eugly_120	134
eugly_1777	105
eugly_1716	96
eugly_1890	87

BLASTn algorithm [49] in order to select euglyphid sequences. Before the BLASTn, each nucleotide with a Phred score below 28 was changed to an unknown nucleotide 'N' in order to avoid unreliable nucleotides. The BLASTn algorithm was setup with a match/mismatch ratio of 1:-1, gap open and extend penalty, respectively, of 0 and 2 and a word size of 32 nucleotides.

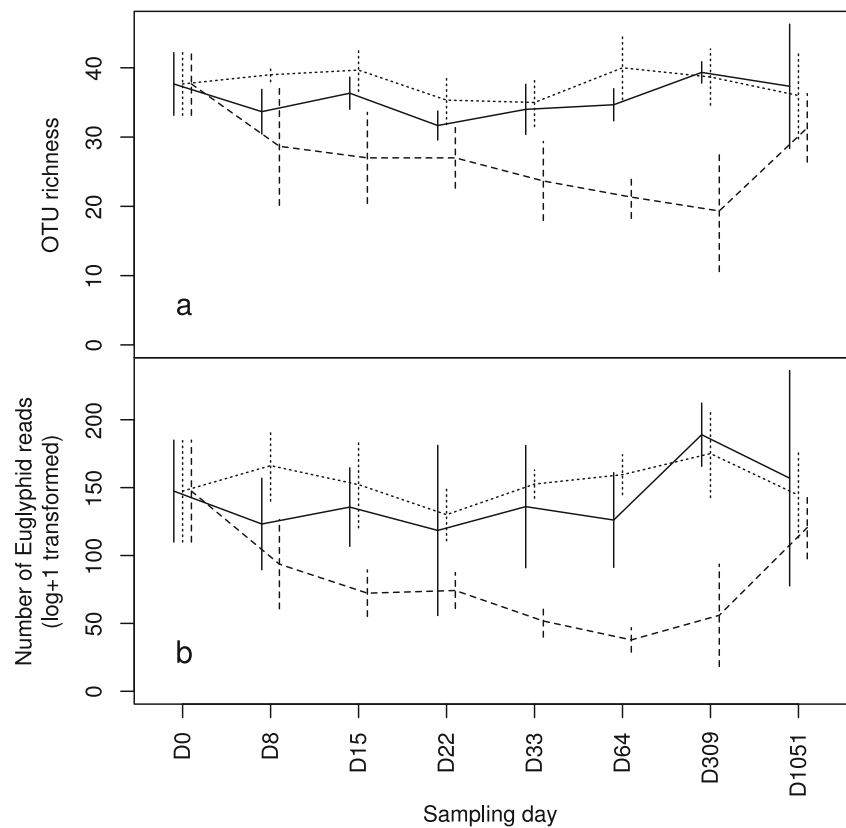
We used an empirically determined e-value threshold as the criterion for classifying a read as belonging to the euglyphids. To determine the appropriate e-value threshold, a subset of eukaryotic V9 sequences (sample D309, block 2, pig treatment) was compared by BLASTn to the previously established euglyphid V9 sequence database with a permissive e-value (i.e. 10). The hit results were sorted by increasing e-value and compared to the GenBank database by BLASTn, using the previous setup, until sequences corresponding to taxa other than euglyphids were found. Once the e-value threshold was found (i.e. $8e^{-29}$), each environmental sequence was compared against the V9 euglyphid database using BLASTn.

Only sequences over 130 nucleotides long and occurring at least five times in the 66 samples were retained, in order to remove possible false-positive sequences. As our database showed that some closely related but nevertheless morphologically and genetically (e.g. COI gene or full SSU) distinct euglyphid morphospecies shared exactly the same V9 sequence (e.g. *Euglypha penardi* (EF456753) and *Euglypha* cf. *ciliata* (EF456754) [50]), we considered each unambiguous difference in the nucleotide sequence as sufficient for discriminating two OTUs. Conversely, when two sequences differed only in ambiguous nucleotides, they were considered as belonging to the same OTU. The resulting OTU sequences were then counted in each sample.

Numerical analyses

We assessed the response of the 51 OTUs found in the 66 samples to the different treatments using partial redundancy analysis (RDA) on Hellinger-transformed data [51] with the blocks used as conditional variable. Rare OTUs (present less than three times in a minimum of ten samples) were removed to reduce noise in the model and optimise the adjusted R^2 [52]. These thresholds were selected after testing several options (presence threshold 1, 3, 5, 7; minimum number of presence 7, 10, 12, 14). The significance of variables (Soil_C, Soil_N, treatment) and ordination axes (first, second and third) were assessed using Monte Carlo tests (999 permutations, p value threshold=0.05).

Fig. 1 Temporal patterns of euglyphid OTU richness (a) and number of reads log +1 transformed (b) in soil samples from control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. Treatments are represented by line type (plain: control, dashed: pig, dotted: fake pig). The vertical lines show the standard deviation of the richness and number of reads for each treatment and sampling date. The lines for the three treatments are slightly offset to improve readability



We assessed the effect of the treatments, relative to control, on the OTU responses over time using a principal response curve (PRC) [53]. The model was also tested using a Monte Carlo procedure (999 permutation, p value threshold=0.05).

All statistical analyses were performed with R-2.13.1 [54] using package 'vegan' [55] for the Hellinger transformation, RDA and PRC analyses.

Retrieval of full-length SSU rRNA gene sequences of selected taxa and phylogenetic analysis

Because sequences of the V9 variable region of the SSU rRNA gene are short (i.e. generally less than 200 bp), they are not suited for inferring the position of OTUs in phylogenetic trees. This is especially problematic if the considered sequences are suspected not to cover a large part of the diversity of the group of interest [56]. In order to place the OTUs of interest (i.e. showing a strong response to cadavers) in a phylogenetic tree, we used the sequence information included in the V9 region to design specific reverse primers and amplified the rest of the SSU rRNA gene. We designed specific primers to amplify specifically two phylotypes that responded positively to the pig treatment: eugly_13R (CACGAACTGAAGGCAAGCCCA) and eugly_666R (TTCACCTCCAATCACAGGAG). The newly designed primers were used in combination with the euglyphid-specific forward primer Eugly1SSUF (GCGTACAGCTCATTATATCA GCA [41]) located at the beginning of the SSU rRNA gene.

DNA extractions, where the OTU was most abundant, were selected for specific amplification of the SSU rRNA gene of interest. Cycling profile was the same as described above (with 40 cycles). PCR products were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and used to transform *E. coli* TOP10' OneShot cells (Invitrogen) according to the manufacturer's instructions. Up to five clones per PCR product were chosen for sequencing. Sequencing was performed with an ABI-3130xl DNA Sequencer (Applied Biosystems). The new sequences obtained were placed into an alignment that comprised all euglyphid sequences retrieved from GenBank, which included both environmental clones and sequences derived from identified organisms. The alignment was performed using MUSCLE [57]. A maximum likelihood tree was built using the RAxML v7.2.8 algorithm [58] as proposed on the portal (<http://phylobench.vital-it.ch/raxml-bb/>) using a general time-reversible model. Rate heterogeneity was estimated using a CAT model.

Results

Diversity and structure of euglyphid OTU assemblages

Of the 247,366,905 raw Eukaryote reads, 187,566 were identified as euglyphids and 57,533 of these were found at least five times overall (Table 2). These 57,533 reads were divided

into 198 OTUs. Of these, 51 OTUs respected the thresholds and were thus retained for further analyses. Six of these OTUs matched exactly with sequences from our database. Total OTU abundance data are summarised in Table 3, and OTU richness and number of euglyphid reads along time for the three treatments are shown in Fig. 1.

The partial redundancy analysis (RDA, Fig. 2) with the blocks used as conditional variable revealed a significant correlation between euglyphid communities and Soil_N and Soil_C (Monte Carlo test, 999 permutations, both $p=0.01$). Axes 1 and 2 were significant ($p=0.005$ for both). The RDA ordination showed that the pig treatment samples diverged from the control and fake pigs along the soil nitrogen content gradient until day 309 after which they converged again with the samples of the other two treatments. The RDA also showed that most OTUs responded negatively to the pig treatment. However, some OTUs responded positively to the pig treatment (e.g. eugly_13, eugly_666).

The principal response curve (Fig. 3) summarises the treatment effects on OTUs over time and shows the average responses of individual OTUs. The first PRC axis explained significantly ($p<0.03$) 42 % of the model variance, while time and treatments explained, respectively, 10 and 27 % of the variance. Qualitatively, the PRC diagram showed an overall negative effect of the pig treatment (D8 to D1051) on the majority of euglyphid OTUs and the positive response of a few OTUs, especially eugly_666 and eugly_13, which were therefore further studied.

Retrieval of full-length SSU rRNA gene sequences of eugly_13 and eugly_666

All clone sequences obtained were identical ($n=5$ and 8, respectively). Phylogenetic analyses confirmed the position of the two phylotypes within euglyphid testate amoebae (supported with 100 % bootstrap value) and showed that they did not belong to any barcoded family (Fig. 4) [59]. They were basal to all known euglyphid families. Eugly_13 branched robustly (80 % bootstrap) with an environmental sequence from freshwater sediments (freshwater 13_2.2 AY620297). By contrast, eugly_666 did not branch robustly with any sequence—be it from environmental samples, cultures, or isolated cells.

Discussion

Euglyphid community responses to decomposing pigs

This study showed that the presence of decomposing pig cadavers significantly affected the community structure of euglyphid testate amoebae, showing a drastic decrease in sequence abundance and in OTU richness (Fig. 1). This result is in agreement with our general working hypothesis. The

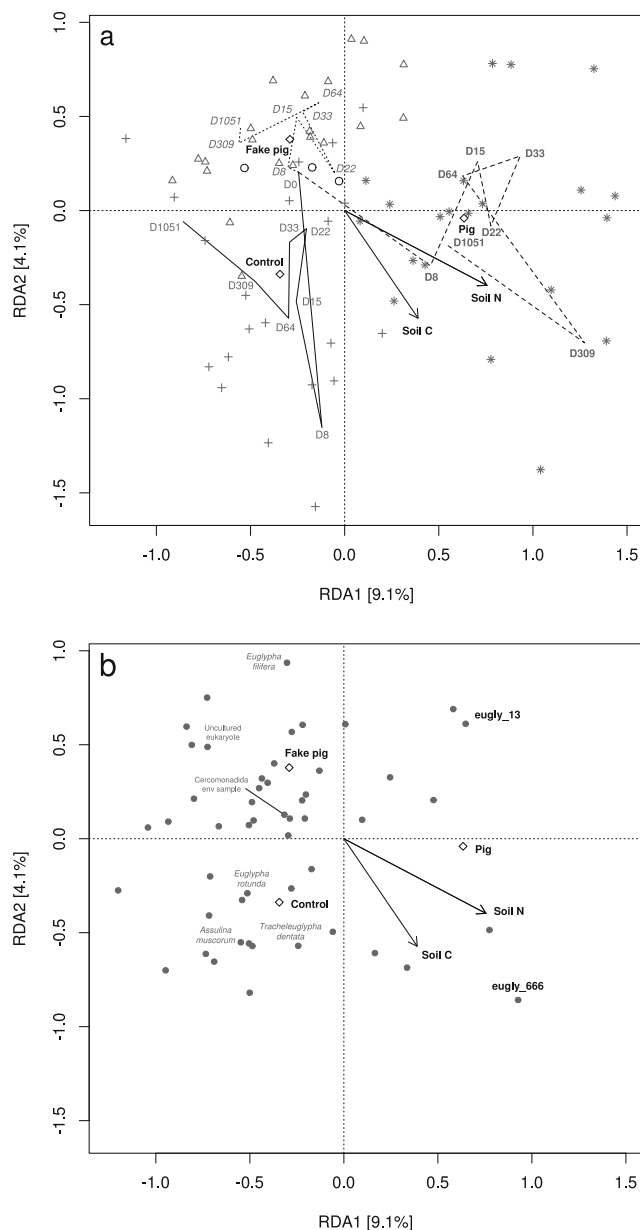


Fig. 2 Partial redundancy analysis (RDA) ordination diagram showing the temporal patterns of soil euglyphid testate amoeba communities (OTUs) in control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. *Diamonds* represent treatment centroids, and *arrows* represent weight percentage of total organic carbon (soil C) and total nitrogen (soil N). Percentages of variance explained by axes are shown in *brackets*. In **a**, successive sampling dates for each treatment (days 0, 8, 15, 22, 33, 64, 309, 1051) are connected by *lines*. The *line corners* correspond to the centroid (average for RDA1 and RDA2 coordinates) of three samples of the same treatment and same sampling day. Treatments are indicated by line type (*plain*: control, *dashed*: pig, *dotted*: fake pig), fonts of the sample labels (*plain*: control, *bold*: pig, *italic*: fake pig) and symbols ('+' : control, 'Δ' : fake pig, 'stars' : pig). *Empty circles* represent day 0 for each block. In **b**, OTU responses are represented by *dots*. The two OTUs for which full SSU sequences were obtained are represented in *bold*. The OTUs with a perfect match with a database sequence are represented with their *names*

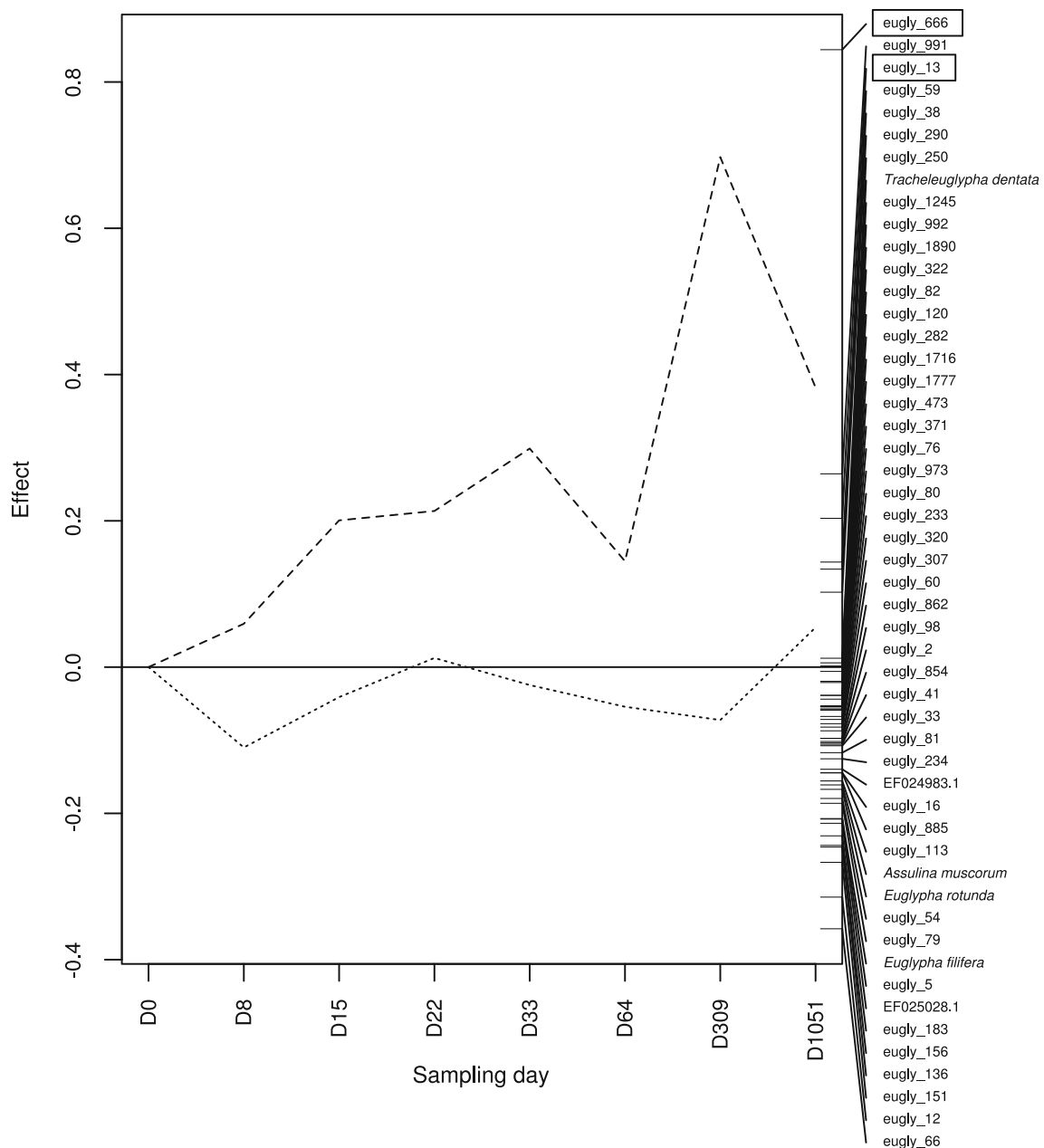


Fig. 3 Principal response curve (PRC) diagram showing the effects of pig (dashed line) and fake pig (dotted line) treatments relative to control treatment over time on soil euglyphid testate amoeba communities in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. The average

response of individual OTUs is shown on the *right axis*. The two OTUs for which full SSU sequences were obtained are framed. GenBank accession numbers represent sequences that matched perfectly with the database

negative effect of a cadaver on euglyphid communities was correlated to the large input of nitrogen and organic carbon in the soil. This result was consistent with previous studies, which show that inputs of nitrogen strongly and negatively influenced testate amoeba communities [34–36]. It is probable that most euglyphids died because of anoxic conditions, but a direct or indirect effect of high nitrogen content is also possible.

However, two well-represented OTUs, namely eugly_13 and eugly_666 (eugly_991 also responded positively but

was less abundant), responded positively to the presence of cadavers, but only in the late decomposition stage (i.e. after 1 month to 1 year). These OTUs were present but rare at the beginning of the decomposition process as well as in the control and fake pig treatment, but their abundance peaked, respectively, at D33 and D309 in the pig treatments only and in the three replicates simultaneously (Fig. 5). This suggests that they did not benefit from the initial perturbation brought by the release of cadaveric fluids but rather found optimal conditions (i.e. abiotic, e.g. soil water chemistry, and biotic, e.g.

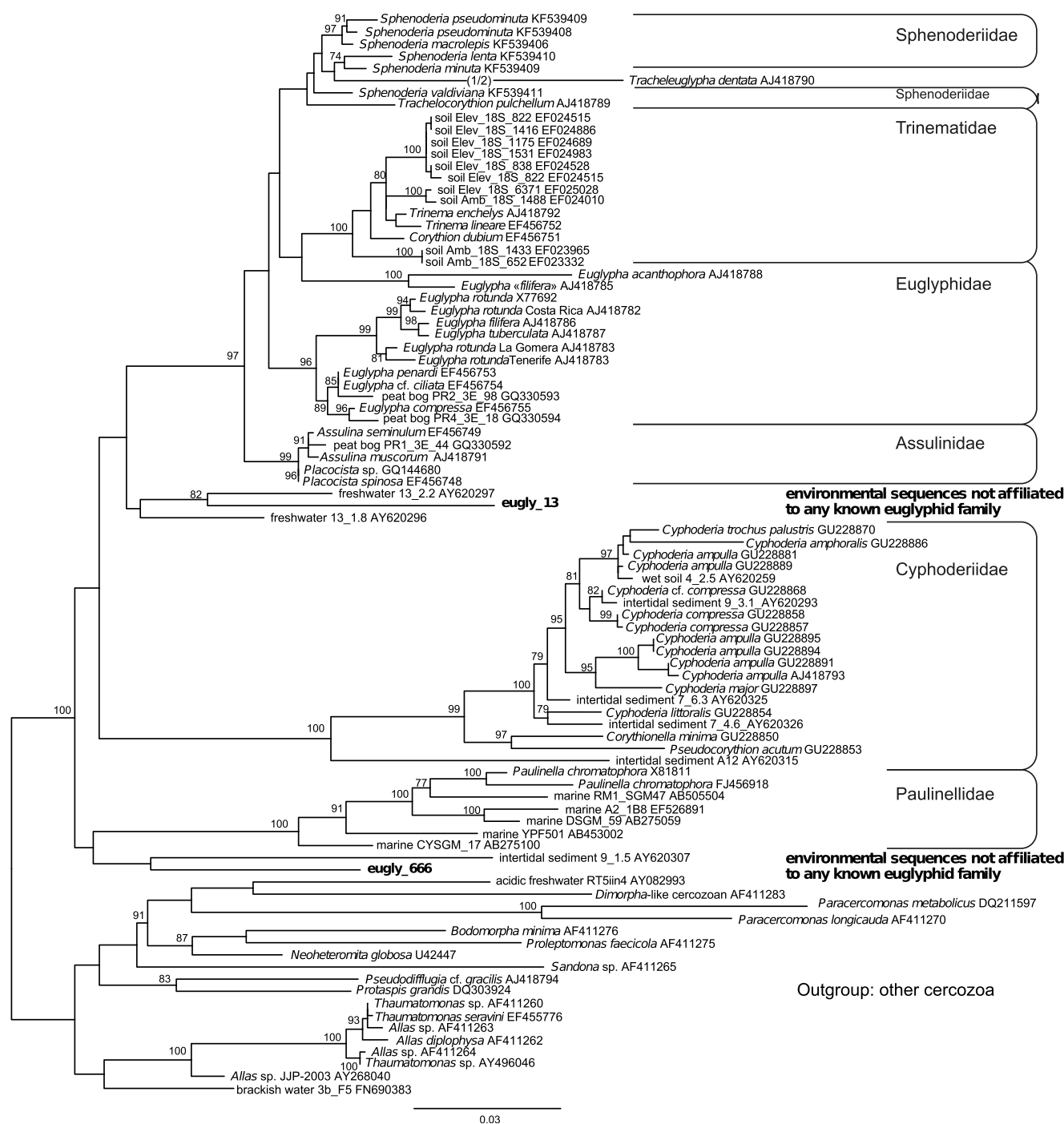


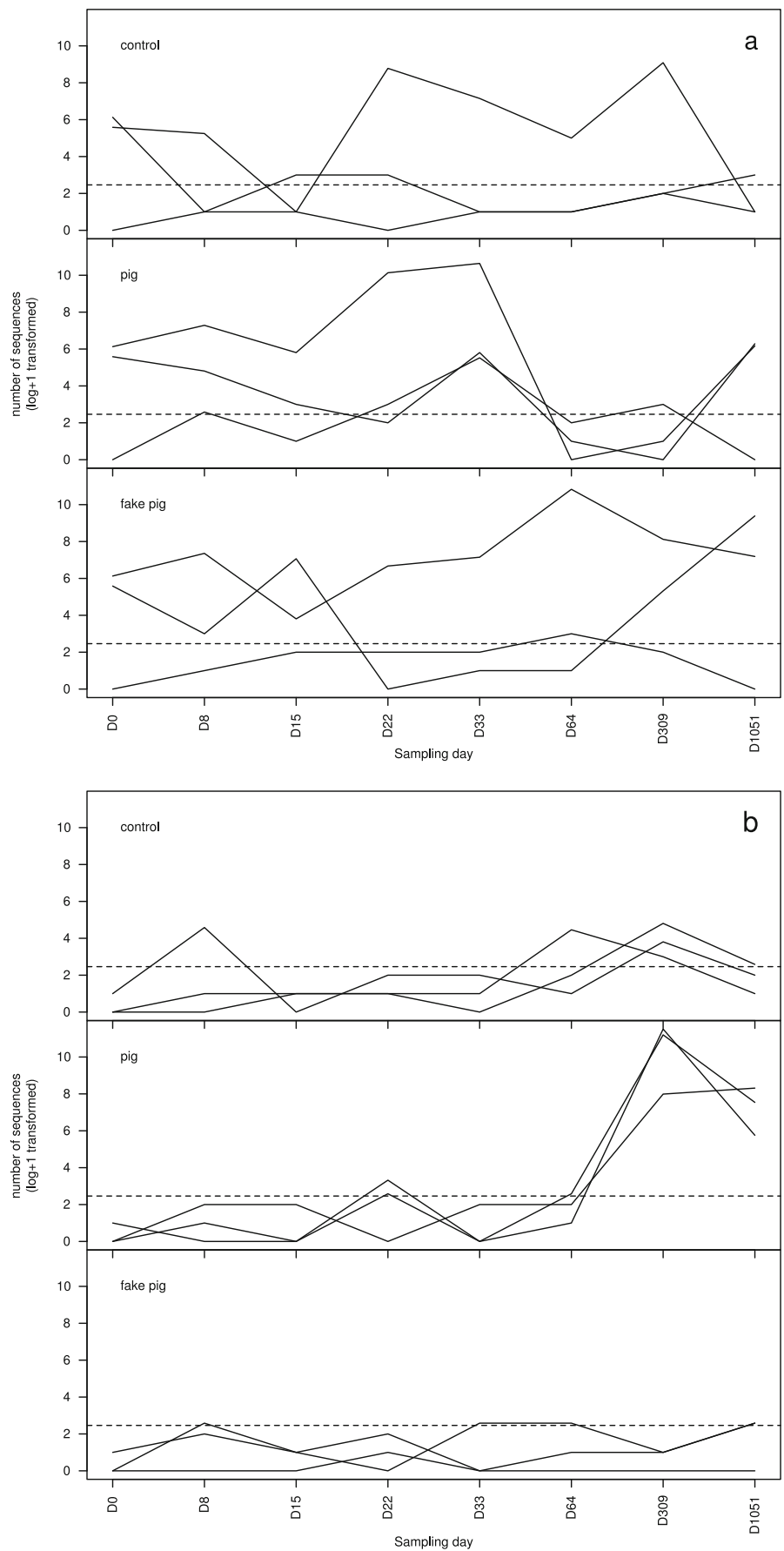
Fig. 4 Maximum likelihood tree built on full SSU rRNA gene sequences of Euglyphida showing the phylogenetic position of **eugly_13** and **eugly_666** full-length sequences (in **bold**) obtained from pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. The tree was built

using RaxML on 1440 positions and rooted with several cercozoan sequences retrieved from GenBank. Robustness of nodes was evaluated by bootstrapping (1000 replicates)

prey and/or low level of competition or predation) for their growth in later stages. These organisms probably benefited from changes in the bacterial communities, as these are supposed to change deeply and progressively underneath a cadaver [60–62]. Indeed, previous studies have shown that decomposing carcasses cause an increase in soil bacterial biomass [9] but also drastically change bacterial community

structure [14, 15]. As bacteria constitute a large part of euglyphid food regime [22], any change in the abundance or community structure of bacteria is likely to also influence the abundance and community composition of euglyphids. It may also be that these taxa represent nutrient-tolerant organisms that benefit well from high abundance of prey organisms, but with low competitive ability in the normally more

Fig. 5 Temporal pattern of number of sequences (log +1 transformed) over time in control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland, for euglyphid testate amoeba OTUs eugly_13 (a) and eugly_666 (b). Full lines represent the number of sequences in each block. The dashed lines represent the average sequence number in a sample independently of the treatment, block or sampling



oligotrophic conditions. The precise mechanism for this response however remains to be elucidated.

A possible bias could have been due to the import of euglyphids with the cadavers, either from the farm or during transport. However, at D8, cadaver samples were less different from the control than samples from fake cadavers (which could not have been contaminated by the new plastic bags and cotton cloth), and we therefore conclude that such contamination was negligible.

The effect of cadavers on euglyphids peaked at D309 (Fig. 3). This time interval seemed quite long in comparison with the results obtained by Szelezcz et al. [18] from the same field experiment (i.e. complete die-out of testate amoebae 22 days post mortem). However, Szelezcz et al. [18] studied litter and not the underlying mineral soil horizon as done here, and they used a direct observation (microscopy) approach, which most likely underestimated diversity. Indeed, as OTUs eugly_13 and eugly_666 did not belong to any known euglyphid family (Fig. 4), their morphology may differ significantly from known forms, they may be very small and thus overlooked or lost in classical sample preparation protocols using 10–20- μ m filters and/or they may represent naked forms (i.e. without test), as documented in foraminiferans [63].

The RDA and PRC both showed that euglyphid communities had not completely recovered from the influence of cadavers by the end of the experiment (i.e. D1051). This pattern is in line with the observations of Szelezcz et al. [18], who did not observe a full recovery by D309 (end of their experiment). This long resilience time suggests that euglyphid communities (and probably testate amoebae in general) could be used as indicators of cadaver presence over very long periods. The fact that euglyphid communities still indicated an effect of cadavers either shows a lag in return to pre-disturbance community structure or that they still responded to other differences (e.g. soil chemistry, abundance and composition of prey).

In addition—and this is in itself an unexpected result—the positive response of certain euglyphid OTUs at certain time points (eugly_13 at D33, eugly_666 at D309) (Fig. 5) suggests that individual taxa may respond specifically and positively to some decomposition stages. Such patterns suggest the possibility to use soil testate amoebae as bioindicators for estimating the time elapsed since death (post-mortal interval, PMI), a parameter of considerable importance in forensic sciences.

Unknown diversity of soil euglyphid testate amoebae

Even after removing rare OTUs, we still found 51 OTUs, 45 of which did not match any sequence in the database. The V9 region does not allow discrimination between close-related species, and it is unclear to how many morphologically and

genetically different taxa these 51 OTUs correspond. Regardless of the short length of the barcode, these results reveal the existence of a very high overall diversity of euglyphids in forest soils. This technique yields large amounts of data from small sample volumes, requires much less taxonomic expertise than classical morphological analyses and does not depend on the existence of a reliable taxonomy (which is often lacking for protists).

Perspectives and potential future application

Focusing our study on a specific taxonomic group allowed us to define OTUs at high resolution, using a threshold adapted to already barcoded morphospecies. This approach allowed us to use metabarcoding at a taxonomic resolution close to morphological analysis—much more than what is generally achieved in studies using general eukaryotic marker. Indeed in most studies, more sequences are pooled into OTUs, each of which corresponds to broader taxonomic units than what we achieved in this study. The approach we used to study the response of euglyphid testate amoebae to the impact of decomposing cadavers can also be used to study the responses of any other group of soil eukaryotes. It is indeed very likely that many other taxonomic groups will also show comparable responses to those documented here for euglyphids. Our study shows that some of this unknown diversity could be of potential use for applied purposes such as forensic science. If such patterns can be explored in details, we believe that it will be possible to develop accurate and reliable new molecular bioindicator tools for PMI estimations and other applications.

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