Use of massive DNA barcoding to monitor biodiversity: a test on forest soil fauna

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Abstract

Biodiversity monitoring primarily focuses on particular taxonomic groups for which identification expertise is widely available, such as vascular plants or birds. As a result, the response of many taxonomic groups to forest management is much less documented. With the advent of low-cost next generation barcoding approaches, it is now possible to envisage monitoring strategies based on molecular approaches, beyond metabarcoding/eDNA strategies that do not give access to species abundances and integrate species presence over largely unknown spatio-temporal scales. In this contribution, we demonstrate the use of massive DNA barcoding – also referred as megabarcoding – as a promising solution to overcome identification difficulties in demanding taxonomic groups. We performed a proof of concept study in a mountainous beech forest in the Massif Central, France. We sampled soil macrofauna at 25 sampling sites and managed to visually identify at the species level 130 out of the 1413 sampled individuals. Using megabarcoding on the 1283 remaining non-identified individuals, we managed to assign 1124 additional individuals to an operational taxonomic species at a competitive cost. We present the summary statistics of barcoding success in the different taxonomic groups encountered and in larvae versus adult individuals. We demonstrate that larvae individuals, which can hardly be visually identified at the species level, make a substantial contribution to overall macrofauna diversity. We finally showcase how this megabarcoding approach provides a concrete avenue for forest biodiversity monitoring, by assessing the cost and labour intensity of this approach.

1. Introduction

Forest biodiversity monitoring mainly focuses on a limited number of taxonomic groups, among which plants, birds and beetles have attracted much attention (Paillet et al. 2010). Rationale for monitoring such particular taxonomic groups have been abundantly developed: these groups may harbour some ecological and/or conservation importance; they can potentially be used as indicator species; and their monitoring is practical, or at least feasible. Indeed, plants constitute habitats and resources for a wide-range of often specialized phytophagous invertebrates (Jaenike 1990), hence monitoring plants is also a way to indirectly monitor the potential presence of a series of interacting organisms (Basset et al. 2012, Castagneyrol and Jactel 2012). Plants can also be used as indicator species of a wide range of environmental (Tichy et al. 2023) and historic (Hermy et al. 1999) variables. Birds have also been proposed as potential indicator species of forest disturbance (Canterbury et al. 2000), as well as wood-inhabiting beetles (Nilsson et al. 2001). Wood-living beetles in particular are important components of forest biodiversity and play a key role in wood decomposition and nutrient cycling (Stokland et al. 2012). The functional composition of this taxonomic group is further influenced by forest management practices (Gossner et al. 2013) and is thus a group of conservation concern.

Focusing forest biodiversity monitoring on particular taxonomic groups also comes with a number of shortcomings. Indicator species remain surrogates of variables of interest and are also often influenced by the environmental, historical and landscape contexts in which they are applied (Gossner et al. 2014, Lalechère et al. 2017). Besides, several taxonomic groups may respond differently to human management or natural perturbations, rendering the generalization from one group to the others risky (Barlow et al. 2007, Cours et al. 2023). It is thus important to expand forest biodiversity monitoring to a larger number of taxonomic groups than what is currently performed (Nilsson et al. 2001, Burrascano et al. 2023). Among the often-neglected taxa, soil organisms account for ca. 60% of the total number of species on Earth (Anthony et al. 2023) and have an essential role in ecosystem functioning (Bardgett and Van der Putten 2014). Yet, relatively few monitoring programs focus on forest soil biodiversity (Korboulewsky et al. 2016).

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A major obstacle to the study of the diversity of soil organisms is the rarity of suitable
64 taxonomic expertise (Eisenhauer et al. 2017), and the difficulty or impossibility to identify larval stages that can substantially contribute to soil fauna communities (Paquin and Coderre
66 1997). Molecular approaches have been developed for more than two decades for taxonomic identification of a wide range of taxa (Hebert et al. 2003), including soil organisms (Rougerie
68 et al. 2009, Decaens et al. 2013). They consist in sequencing a small region of the genome of the studied organism that is called the DNA barcode. The cost of DNA barcoding was however
70 prohibitive until recently, limiting its widespread use in biodiversity monitoring programs. But this cost has dramatically decreased recently, thanks to the use of nanopore sequencing
72 technologies and of low-cost protocols for DNA extraction and amplification (Srivathsan et al. 2021). In the meantime, reference libraries are continuously completed for a wide range of
74 taxa (e.g., Rougerie et al. 2015, Santos-Perdomo et al. 2024). For instance, the Barcode of Life Database (BOLD) now gathers more than 16 million barcode sequences, and coordinated
76 efforts to fill the remaining gaps are underway (Roslin et al. 2022). The massive barcoding of individual specimens therefore constitutes a promising avenue to speed-up and largely
78 develop biodiversity monitoring, in particular for less studied taxa.

80 This approach has been recently coined as “megabarcoding” (Chua et al. 2023) to distinguish it from the “metabarcoding” approach in which mixtures of organisms or their living media
82 (environmental DNA) are sequenced. The potential of metabarcoding, and in particular environmental DNA, for biodiversity monitoring has been abundantly developed in the
84 literature (Rodriguez-Ezpeleta et al. 2021). The great advantage of metabarcoding is its speed of use: an environmental sample is quickly processed and its metabarcoding informs on a list
86 of species that have left DNA traces in the sample. The main limitation of this approach is that the inference of species abundances from metabarcoding results remains challenging
88 (Elbrecht and Leese 2015, Beng and Corlett 2020). Another limitation of the environmental DNA approach is that it integrates the DNA signal over spatio-temporal scales that are still
90 difficult to appraise (Bloor et al. 2021). In contrast, megabarcoding enables to keep track of species abundances and of the spatiotemporal scale of sampling. In particular, it should enable

to track spatial and temporal changes in species relative abundances, which usually prelude species turnovers.

Given the rapid and promising developments of low-cost megabarcoding, comparisons of its efficiency among a full range of taxa composing each ecosystem compartments is now needed. Low-cost megabarcoding has been successfully applied to Dipteran specimens (Srivathsan et al. 2021, Hartop et al. 2024). A recent work using a higher cost protocol extended this application to a wider range of taxa, but mainly epigeal ones (Hebert et al. 2025). Soil macrofauna gathers various taxonomic groups with varied physiological and anatomical attributes that may compromise the ability of low-cost protocols to efficiently extract and amplify DNA, such as the presence of protective shells (in Styllommatophora), of dense exoskeletal cuticles (in Insecta, Isopoda and Diplopoda) or the presence of mucus (in Styllommatophora and Haplotaxida). For instance, mollusc slime has been shown to compromise amplification success (Chakraborty et al. 2020). Our aim in this study was thus to test the applicability of low-cost megabarcoding to monitor soil fauna communities in a temperate forest. We specifically assessed 1) whether the low-cost protocol proposed by Srivathsan et al. (2021) for the extraction, amplification and purification of DNA can be effectively adapted to soil fauna taxa, 2) the completeness of the BOLD database for the taxa encountered in our survey, and 3) the relative ability to monitor larval stages with DNA barcoding, compared to the one obtained for adult individuals.

2. Material and methods

2.1 Soil fauna collection and barcoding protocol

We performed an inventory of soil macrofauna in a beech forest stand located in Laqueuille in the French Massif Central (exact location: 45.6236 N, 2.7393 E, altitude: 1182m) in June 2022. Twenty-five soil samples were gathered within a 50 m square. Each sample consisted in hand sorted individuals larger than 3 mm extracted from soil monoliths of 18 cm x 18 cm and 10 cm deep together with the litter above. These individuals were then immediately stored in

96° ethanol. Individuals were first visually identified using a stereomicroscope (Zeiss Stemi
122 2000-C). This visual identification enabled us to sort the 1413 individuals into morpho-species
that had variable taxonomic resolutions. Among them, 130 individuals were either identified
124 at the species level, or were singletons of a morpho-species and were thus treated as a distinct
species in the following. The remaining 1283 partially identified individuals were barcoded
126 following the protocol detailed in Srivathsan et al. (2021).

This barcoding protocol consisted in rinsing each individual with purified water (Elga Labwater
128 Purelab Ultra MK2) and placing it in one well of a 96-well PCR microplate. If the individual was
too large to fit into a well, one of its legs (or anus for earthworms) was detached and put into
130 the well. DNA extraction was performed using a HotShot solution (Truett et al. 2000) with a
heating at 65°C for 18 minutes and 98°C for 2 minutes. Extracted DNA was then amplified
132 within a mixture of 3 μL of extracted DNA, 7 μL of 2x Mastermix (CWBio CW0682L), 1 μL of
BSA, 2 μL of tagged primers. Left and right primers were tagged with 9 bp tags developed by
134 Srivathsan et al. (2024) so that each well had a unique combination of left and right tags. We
used the BF3-BR2 primer set (Elbrecht and Leese 2017) to amplify a 418 bp region of the COI
136 barcode for 808 individuals and the LCO1490-HCO2198 primer set (Folmer et al. 1994) to
amplify a 658 bp region of the COI barcode for the remaining 475 individuals. We used these
138 two different sets to compare their efficiency to amplify the COI "Folmer" region of soil fauna.
A previous benchmark on 21 primer sets by Elbrecht et al. (2019) revealed that the BF3-BR2
140 primer set performed better than the classical LCO1490-HCO2198 set when analysing a
Malaise trap sample. We wanted to test whether this enhanced performance was also
142 recovered on soil taxa. We used the following PCR settings: 15 min at 95°C followed by 25
cycles of 30 s at 94°C, 1 min 30 s at 50°C and 1 min 30 s at 72 °C, followed by 10 min at 72°C.
144 Amplified DNA was then pooled and purified using Ampure XP beads at 0.5 X. Library
preparation for sequencing on a flongle followed the ONT protocol (with the modifications
146 explained in Srivathsan et al. 2021). Srivathsan et al. (2021) recommended to pool up to 3 PCR
plates on a flongle, while Hebert et al. (2025) demonstrated that up to 20 PCR plates could be
148 pooled on the same device. We thus used an intermediate number and pooled between 6 and
10 PCR plates on each flongle.

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2.2 Bioinformatic processing

152 Raw sequences were then processed with the software ONTbarcoder 2.0 (Srivathsan et al. 2024) and only quality-checked consensus barcodes (i.e., no ambiguities nor
154 insertion/deletion) for each well were retained. The individuals for which the first sequencing round failed were re-processed from scratch (extraction, amplification, purification and library
156 preparation) for a second round of sequencing, and those still in failure were processed a third time. The barcodes obtained were compared to the BOLD database (Ratnasingham and
158 Hebert 2007) using the animal library (public and private) and the exhaustive search options. This step was performed on the sixth of January 2025. We then clustered barcodes that could
160 not be matched to a species on the BOLD database into distinct OTUs (operational taxonomic units) using a UPGMA method and a similarity threshold of 99% (Buchner and Leese 2020).

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2.3 Data analysis

164 We computed the barcoding efficiency as the percentage of consensus barcodes obtained (the number of obtained barcodes divided by the number of sequenced individuals). We repeated
166 this computation for each of the three rounds of sequencing and separately for this list of taxonomic groups: Arachnida, Chilopoda, Diplopoda, Diplura, Haplotaxida, Insecta, Isopoda
168 and Stylommatophora.

We also computed the barcoding efficiency for each set of primers (BF3/BR2 and
170 LCO1490/HC02198) separately. To test whether the BF3-BR2 primer set lead to a larger barcoding efficiency than the LCO1490-HCO2198 set, we first computed the barcoding
172 efficiency for each PCR microplate separately and treated these microplate-level barcoding efficiencies as statistical replicates, since a single primer set was used on each microplate. We
174 then used a one-sided Wilcoxon test to compare the barcoding efficiencies of microplates that involved the first primer set (BF3-BR2) with those of microplates involving the second primer
176 set (LCO1490-HCO2198).

We finally computed species accumulation curves to assess the completeness of our species
178 inventory, distinguishing the adult-only and total assemblages of soil fauna individuals. This was performed with the function “specaccum” of the vegan R package (Oksanen et al. 2007).

180 Datasets to replicate the analyses are included in the Supplementary Information (Tables S1 and S2) as well as the corresponding R script (Text S3). This material has also been deposited
182 on public repositories, the datasets can be found on:

https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=doi:10.57745/LPYOC1;
184 and the R script on: https://forgemia.inra.fr/franck.jabot/barcoding_laqueuille_forest.

186 ## 3. Results

Using a low-cost DNA barcoding protocol, we obtained an overall barcoding efficiency of 88
188 %: we recovered 1124 DNA barcodes out of the 1283 individuals processed (Fig. 1A). These barcodes clustered into 124 distinct Operational Taxonomic Units (OTUs) among which 55 (44
190 %) had a species name in the BOLD database (Fig. 1B). Interestingly, OTUs without species names in the BOLD database were not solely the rarest taxa in our samples (Fig. 1C). We
192 obtained a larger barcoding efficiency on larvae (96 % of the 234 larvae processed) than on adult individuals (86 % of the 1049 adults processed). Barcodes of larvae had moreover a
194 larger percentage of species name presence in the BOLD database (59 % versus 35 %).

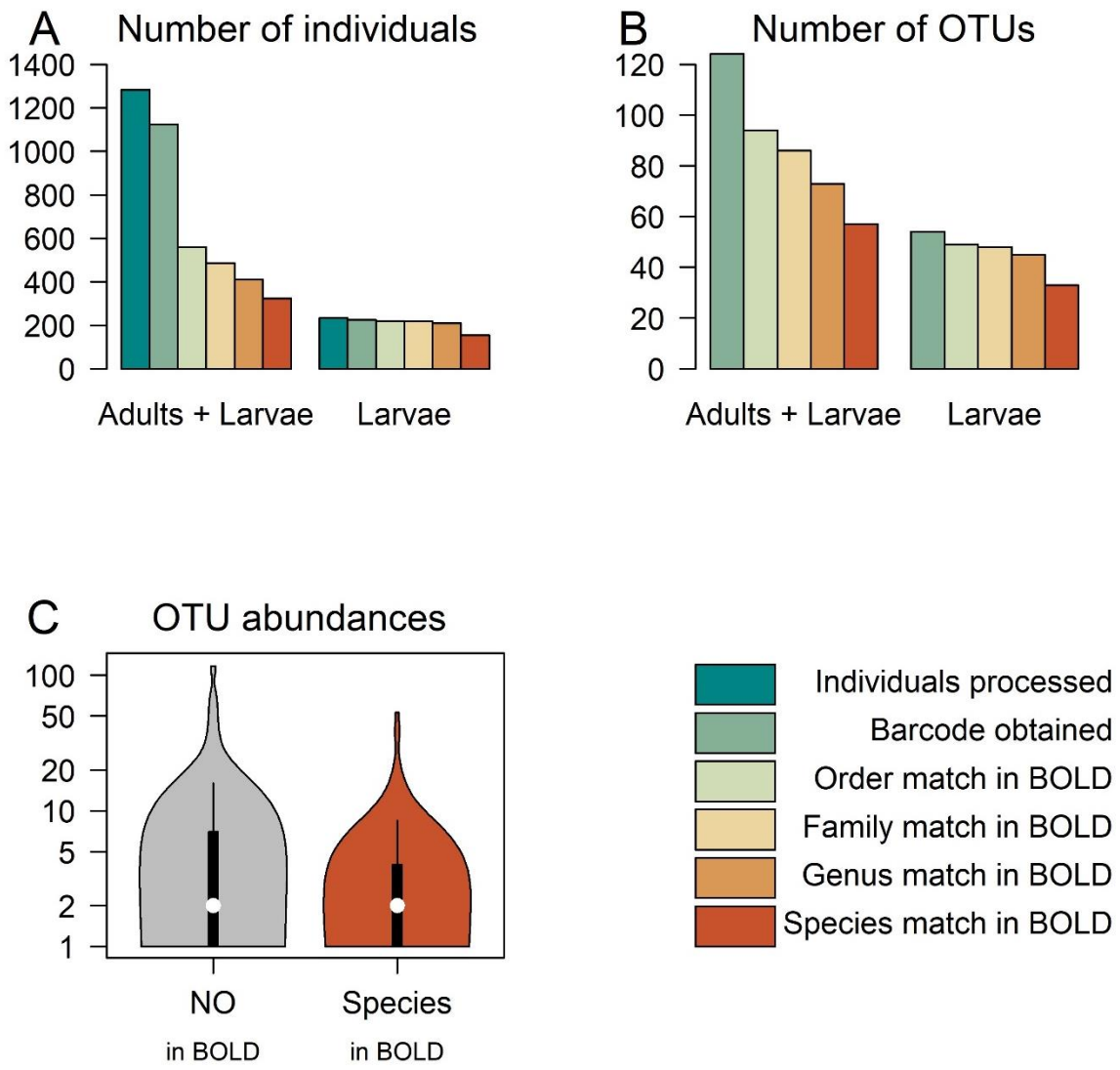

Figure 1: DNA barcoding efficiency of soil macrofauna. Panel A: number of individuals for which DNA barcodes have been obtained and the corresponding matches at various taxonomic levels in the BOLD database. Panel B: number of Operational Taxonomic Units (OTUs) and the corresponding matches at various taxonomic levels in the BOLD database. Panel C: Distribution of OTU abundances among OTU with a species name present or absent in the BOLD database.

DNA barcoding efficiency was highly heterogeneous among taxonomic groups (Figure 2), varying between 22 % (for Stylommatophora) and 100 % (for Arachnida). The percentage of

206 species name presence in the BOLD database was also highly heterogeneous among these groups, varying between 0.3 % (for Diplura) and 91 % (for Diplopoda).

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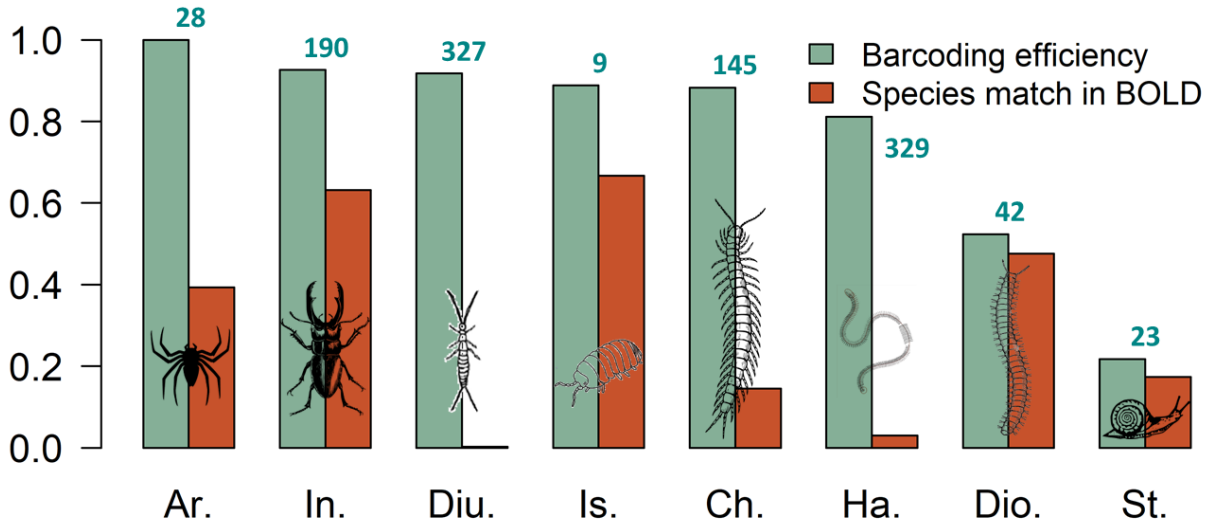

210 **Figure 2**: Heterogeneity of DNA barcoding efficiency among taxonomic groups. Barcoding efficiency is equal to the proportion of individuals in each taxonomic group for which a DNA 212 barcode could be obtained. Species match is the proportion of individuals for which a species match was obtained in the BOLD database. Ar: Arachnida, In: Insecta, Diu: Diplura, Is: Isopoda, 214 Ch: Chilopoda, Ha: Haplotaxida, Dio: Diplopoda, St: Stylommatophora. The numbers above the bars correspond to the number of individuals processed in each taxonomic group.

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Our study also revealed that DNA barcoding failure could largely be reduced by re-processing 218 failed individuals (Figure 3). Using this approach, barcoding efficiency sequentially improved from 59 % (using a single barcoding round for all individuals), to 81 % (using a second 220 barcoding round for individuals in failure) up to 88 % (using a third barcoding round for individuals in failure after the first two rounds). The barcoding efficiency of the first two 222 rounds was close (59 % and 56 % for round 1 and 2 respectively), while the one of the third round was lower (36 %). Finally, we observed an apparently large difference in barcoding 224 efficiency among the two pairs of primers used (68 % and 43 % for BF3/BR2 and LCO1490/HC02198 respectively), but this difference was not significant (one-sided Wilcoxon 226 test's p=0.06).

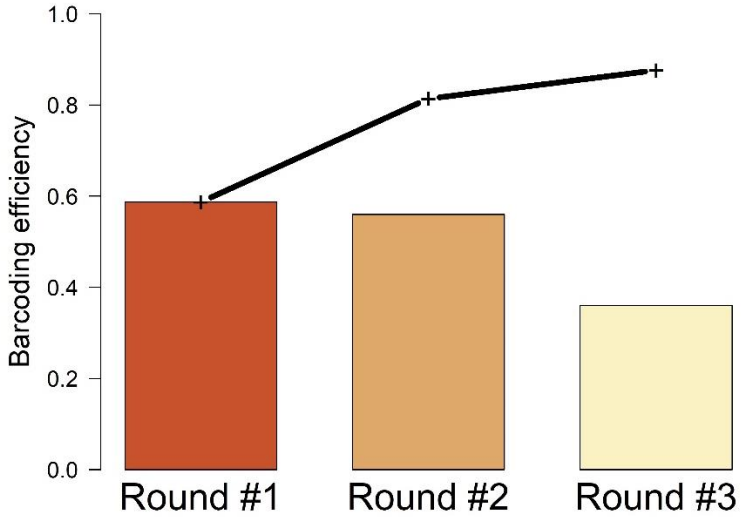
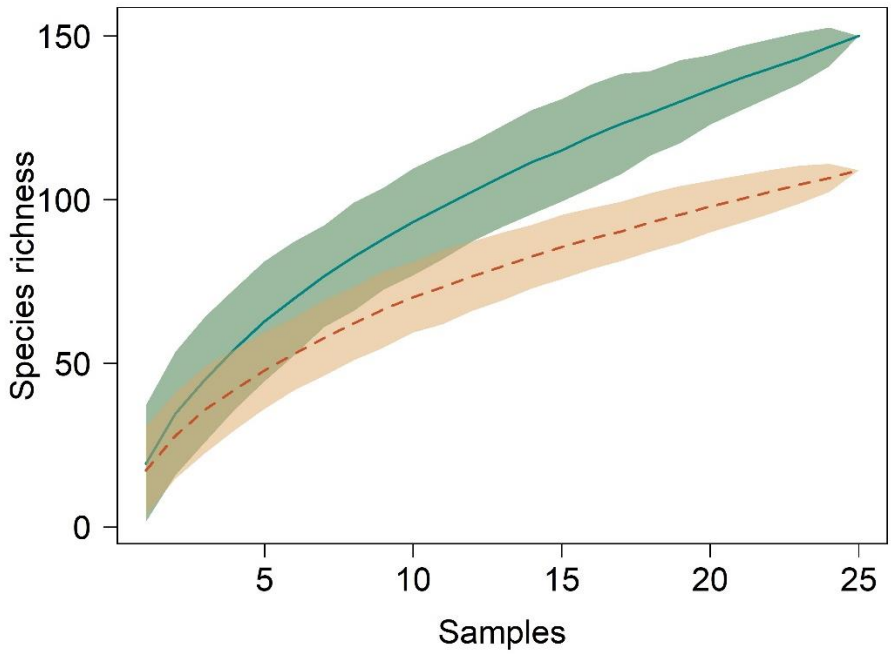


Figure 3: Improvement in DNA barcode efficiency brought by multiple rounds of barcoding. Each bar height stands for the barcoding efficiency of the barcoding round. The broken line represents the cumulative barcoding efficiency after a given number of barcoding rounds.

Our study further revealed that 25 samples of soil monoliths of 18 cm x 18 cm x 10 cm were not sufficient to reach an asymptote in the species accumulation curve (Figure 4). Interestingly, species accumulated faster when including larvae individuals, although larvae only accounted for 18 % of the total number of individuals. This was linked to the fact that 41 of the 149 species (28 %) were only found among larvae individuals.



240 **Figure 4**: Species accumulation curves as a function of the number of samples considered. The plain blue curve represents the species (OTUs) accumulation curve for the total community
242 (adult and larvae individuals) while the dashed orange curve represents the species accumulation curve for the adult individuals only. Light colour areas depict confidence
244 intervals.

246 Our analysis finally revealed that the barcoding-based species inventory strongly modified the initial morphospecies-based inventory by more than doubling the number of species detected
248 (from 74 to 149 species, Figure 5). The barcoding-based species abundance distribution was consequently strongly altered by being much more even than the original morphospecies-
250 based abundance distribution (Figure 5). The taxonomic resolution of the barcoding-based inventory was also sharply improved with the number of individuals with a species-level
252 identification multiplied by more than three, from 130 individuals to 444 individuals.

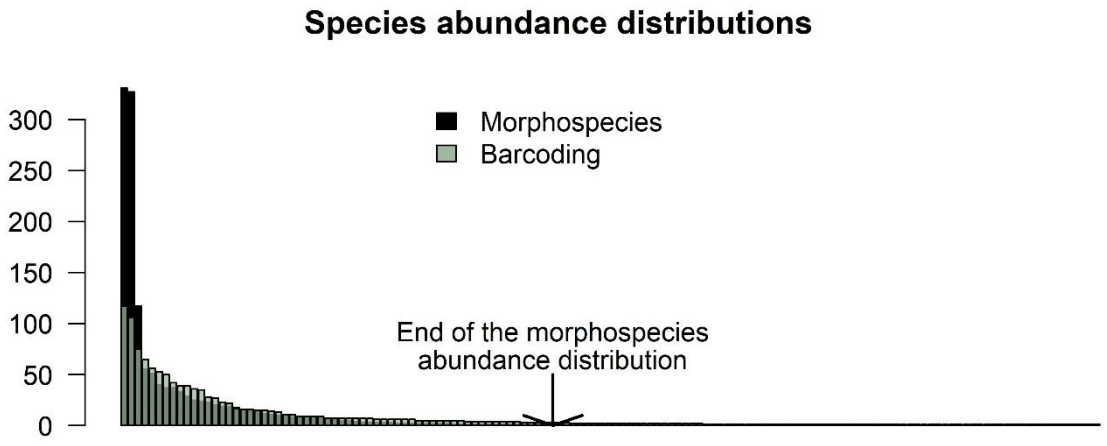


Figure 5: Comparison of the morphospecies-based and the barcoding-based species abundance distributions.

4. Discussion

Our study demonstrates a clear success of megabarcoding to monitor the diversity of forest soil macrofauna. Nearly 90% of the processed individuals were successfully barcoded and we were able to attribute a species name to nearly half of them (Fig. 1). Since taxonomic expertise of soil organisms is particularly rare, megabarcoding of sampled individuals could therefore constitute an operational way to perform soil biodiversity monitoring. Another major advantage of megabarcoding of soil fauna is the identification of larvae. In our study case, we found that larvae accounted for 18% of sampled individuals, and contained a large number of species not found among adult individuals (28% of the total species number). Therefore, the inclusion of soil larvae in biodiversity assessment, enabled by megabarcoding, may profoundly alter our understanding of the rich forest soil fauna diversity (Fig. 4 and 5).

Our study further reveals that barcoding efficiency is variable among taxonomic groups, as previously demonstrated by Hebert et al. (2025). Despite this variation, barcoding efficiency was good to very good (above 80 % or 90 %) for most taxonomic groups, except for Stylommatophora and to a lesser extent Diplopoda (Fig. 2). This may explain why barcoding efficiency was remarkably large (96.1 %) for larvae individuals that mostly belonged to Coleoptera, Araneae and Diptera and thus not to these more problematic taxonomic groups (Table S1). The lower barcoding efficiency for the Stylommatophora and Diplopoda individuals

echoes previous studies that reported barcoding difficulties in these taxonomic groups (Spelda et al. 2011, Chakraborty et al. 2020). It may be due to a variety of reasons (notably, failure of or poor DNA extraction, lack of or low DNA amplification) that would need to be further investigated. Note that Stylommatophora individuals were not extracted from their shells before DNA extraction. This may be part of the explanation for the lower barcoding efficiency of this group, on top of mucus-related amplification difficulties (Chakraborty et al. 2020). The number of processed individuals for these two groups was nevertheless low (23 and 42 respectively, Fig. 2) so that additional tests will be required to confirm or not this lower barcoding efficiency. And solutions to this problem may be sought, for instance by lengthening the heat extraction of DNA in the HotShot solution, by testing alternative primer sets for these groups (e.g., "C_LepFolF- C_LepFolR" as in Spelda et al. 2011), or by sequencing individuals of these groups separately from the other taxonomic groups.

The barcoding efficiency that we reached on the first two rounds was slightly above 50 % (Fig. 3). This is lower than the ones reported by previous studies that were mostly in the range of 80-90 % (Srivathsan et al. 2024, Hebert et al. 2025). Various factors can be put forward to explain the lower barcoding efficiency that we obtained in this study. A first explanation lays in the taxonomic groups studied. Previous megabarcoding studies have notably targeted Dipteran individuals (Srivathsan et al. 2021) that lead to larger barcoding efficiencies than Coleopteran and Arachnid individuals (Hebert et al. 2025). A second explanation linked to the first one is that we pooled individuals of different taxonomic groups in the same flongle. Sorting individuals of different taxonomic groups and sequencing them separately may enable some enhancement in barcoding efficiency, since the DNA sequences of more productive taxonomic groups would not overwhelm the ones of less producing groups. A third explanation lays in the number of microplates that we pooled on each flongle for sequencing. Srivathsan et al. (2024) recommended to pool up to 3 microplates on a flongle, while Hebert et al. (2025) demonstrated that up to 20 microplates could be pooled on a single flongle, but with the use of high-fidelity Taq polymerases for DNA amplification. We chose intermediate numbers and pooled between six and ten microplates on each flongle. Although this strategy led to a smaller barcoding efficiency, we found that it was a good compromise to speed up the treatment of samples, since the library preparation steps are long relative to the extraction and amplification ones (Table S6), and to further decrease barcoding costs (Table S4).

Importantly, by re-sequencing individuals in failure of barcode recovery, we demonstrated
308 that final barcoding efficiency was comparable to the one of previous studies (Fig. 3). For this,
we recommend using two rounds of barcoding, since the third round provides comparatively
310 fewer barcodes (Fig. 3).

As argued before by Srivathsan et al. (2021), the cost of barcode sequencing has dramatically
312 decreased thanks to the use of nanopore technologies. But the total cost of DNA barcoding
also incorporates the costs of reagents involved in the extraction and amplification of DNA.
314 The protocol of Srivathsan et al. (2021) put forward a number of innovations to cut down
these costs, including the use of low-cost HotShot solutions to extract DNA instead of
316 commercial kits and the use of low PCR reaction volumes. We provide in Table S4 the details
of the reagent costs implied in the full protocol and computes that the production of a DNA
318 barcode in this study costed around one euro. Although this cost depends on the commercial
arrangements each lab may get with reagent suppliers, this nevertheless gives the order of
320 magnitude of megabarcoding costs. We also detail the equipment costs to perform
megabarcoding and demonstrate that it amounts to around 20,000 euros for minimal
322 equipment needs (Table S5). Finally, following this protocol, a single lab technician can
barcode around 1000 (2000) individuals per week if the lab is equipped with a single (two)
324 thermocycler(s) and thus obtain around 500 (1000) barcodes (Table S6).

A current limitation of megabarcoding is that reference databases are still lacunar, especially
326 for soil fauna (Recuero et al. 2024). In our study, nearly half of barcodes could not be matched
to a referenced species in the BOLD database. This limitation is however not definitive. Indeed,
328 reference databases are updated at a rapid pace: the number of sequences in the BOLD
database has increased by 67 % in 2024. And coordinated regional and national efforts are
330 under way to barcode missing taxa (e.g., Spelda et al. 2011, Hendrich et al. 2015, Roslin et al.
2022). This implies that currently unmatched sequences could be matched in the future, so
332 that inventories based on megabarcoding can be progressively updated, a feature especially
important for longitudinal and/or comparative studies.

334

5. Conclusion

We demonstrated that the low-cost megabarcoding protocol of Srivathsan et al. (2024) could be fruitfully applied to forest soil macrofauna and that it led to an overall very good barcoding efficiency of nearly 90 % (Fig. 1). Two taxonomic groups, Styllommatophora and Diplopoda, would require further study to reach such high level of barcoding efficiency (Fig. 2). Megabarcoding of soil fauna also enables the identification of larvae individuals. Our study demonstrates that these larvae individuals substantially contribute to macrofauna biodiversity (Fig. 4), with many larvae species not encountered among adult individuals. Finally, megabarcoding enables to keep track of species abundances and therefore provides a strongly renewed assessment of soil macrofauna community structure (Fig. 5). With a cost of ca. one euro per barcode, megabarcoding constitutes a concrete avenue to vastly develop the monitoring of forest soil macrofauna.

CRediT authorship contribution statement

Franck Jabot: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft. **Gwenaëlle Auger**: Data curation, Investigation, Writing – review and editing. **Pauline Bonnal**: Data curation, Methodology, Investigation, Writing – review and editing. **Mathilde Pizaine**: Data curation, Investigation, Writing – review and editing. **Marilyn Roncoroni**: Investigation, Writing – review and editing. **Julien Pottier**: Conceptualization, Supervision, Writing – review and editing.

Declaration of Competing Interest

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

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Supplementary Information

-Table S1: Barcoding-enhanced inventory data. This dataset includes the identification of 1124 forest soil macrofauna individuals by DNA barcoding and the visual identification of 130 additional individuals.

-Table S2: Visual inventory data. This dataset includes the visual identification of 1413 forest soil macrofauna individuals.

-Text S3: R script to replicate the analyses and figures.

-Table S4: Data on reagent costs.

-Table S5: Data on equipment costs.

-Table S6: Duration of each barcoding step.