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Landscape-scale distribution patterns of earthworms inferred from soil DNA

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ABSTRACT

Assessing land-use effect on the diversity of soil biota has long been hampered by difficulties in collecting and identifying soil organisms over large areas. Recently, environmental DNA-based approaches coupled with next-generation sequencing were developed to study soil biodiversity. Here, we optimized a protocol based on soil DNA to examine the effects of land-use on earthworm communities in a mountain landscape. This approach allowed an efficient detection of earthworm diversity and highlighted a significant land-use effect on the distribution patterns of earthworms that was not revealed by a classical survey. Our results show that the soil DNA-based earthworm survey at the landscape-scale improves over previous approaches, and opens a way towards large-scale assessment of soil biodiversity and its drivers.

1. Introduction

Earthworm communities play an important role in terrestrial ecosystems as ecosystem engineers (Lavelle et al., 1997): they regulate aeration, water infiltration, and nutrient cycling in soils. Their distribution is mainly influenced by soil properties and vegetation type (Curry, 2004; Salomé et al., 2011). At the landscape-scale, human land-use induces a strong spatial heterogeneity of earthworm communities by altering their biological, physical and chemical habitat and food supply (Grossi et al., 1995; Grossi and Brun, 1997; Curry, 2004; Stauffer et al., 2014). As the heterogeneity of earthworm communities translates to some extent into the spatial patterns of ecosystem functions (Ettema and Wardle, 2002; Blouin et al., 2013; Hedde et al., 2013), earthworms are increasingly used as bioindicators of soil quality (Römbke et al., 2005; Pérès et al., 2011).

Assessment of land-use effect on the diversity of soil biota is difficult to implement over large areas due to technical constraints, bias linked with current methods of extraction, and the general lack of taxonomic skills. Current International Standard for earthworm sampling is based on handsorting and/or chemical expellant (NF EN ISO 23611-1, 2011). This method is time consuming (Bartlett et al., 2006) and its efficiency depends on soil parameters, season, species characteristics and life stages (Lawrence and Bowers, 2002; Coja et al., 2008). Moreover, taxonomic assignment is often difficult, especially for juveniles, and does not account for cryptic species. During the past decade, DNA barcoding was successfully used for earthworm identification (Rougerie et al., 2009; James et al., 2010; Decaëns et al., 2013). This approach provides a more accurate estimation of taxonomic richness by accounting for both juveniles and cryptic diversity (King et al., 2008; Richard et al., 2010; Klarica et al., 2012). Despite these progresses in the taxonomic identification of specimens, problems inherent to earthworm sampling methodologies, particularly their variable efficiency and difficulties

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to implement them over large areas, limit our understanding of earthworm diversity.

Recently, Bienert et al. (2012) demonstrated the potentiality of a more integrative metabarcoding approach based on soil extracellular DNA and next-generation sequencing to identify earthworm species (Lumbricidae). Persistence of DNA in soils allows overcoming constraints linked to earthworm sampling methodologies, while benefiting from the taxonomic precision of DNA barcoding. The pioneer study of Bienert and colleagues constituted a proof of concept but also pointed out several directions to improve earthworm detection. Among them, authors identified soil sampling, more particularly the coverage of the studied area and the depth of soil cores, as the critical step towards an exhaustive inventory of earthworm diversity using environmental DNA metabarcoding (Bienert et al., 2012).

Here, we optimized the sampling step of this non-invasive soil DNA approach for assessing its potential to characterize earthworm communities at the landscape-scale, in order to propose a robust methodology complementary to classical earthworm surveys. For this purpose, (i) we compared the results obtained by a classical earthworm survey across a mountain range to those deriving from DNA extracted from a mixture of soil plus litter, collected following the same spatial sampling scheme. Then, (ii) we assessed an alternative spatial soil sampling procedure for soil DNA covering the entire surface of the studied plots. Finally, (iii) we compared factors influencing the observed distribution patterns, in relation with land-use temporal trajectories.

2. Material and methods

2.1. Study area

The study was conducted in "Quatre Montagnes" area, a 255 km^2 landscape in the Vercors mountain range (Northern French Alps, elevations range between 1200 and 1600m a.s.l.). Following previous works (FORGECO project, https://forgeco.cemagref.fr/), we selected 18 homogeneous 1 ha plots, corresponding to the three dominant land covers of the studied area (n = 6): old beech coppice, young spruce plantation and pasture (Table 1 and Supplementary Table S1). Land-use trajectories of these three types of plant communities are as follows: old beech (*Fagus sylvatica*) coppices are ancient forests (at least > 200 years-old) where some firs (*Abies alba*) can be found; young spruce (*Picea abies*) plantations are ca. 50–60 years old and were previously managed as temporary meadows or croplands; pastures are grazed by cattle during summer and their surface is currently decreasing in this mountain area due to abandonment and recolonization by shrubs and trees (Redon et al., 2014).

Table 1
Topsoil (0–5 cm) parameters of the three land-uses (n = 6).

	Land cover		
	Old beech coppice	Young spruce plantation	Pasture
Altitude (m a.s.l.)	1397 (100)	1363 (109)	1573 (56)
Organic C (g kg^{-1})	88.4 (22.8)^a	28.9 (10.2)^b	62.7 (24.2)^a
Total N (g kg^{-1})	5.33 (1.0)^a	1.69 (0.5)^b	5.82 (2.22)^a
C/N ratio	16.7 (3.9)^a	16.8 (2.5)^a	10.8 (0.4)^b
Available P (g kg^{-1})	0.04 (0.02)	0.05 (0.02)	0.05 (0.02)
pH (H_2O)	6.3 (1.0)^a	4.7 (0.4)^b	6.1 (1.2)^a
Na (g kg^{-1})	0.010 (0.002)^a	0.004 (0.002)^b	0.008 (0.006)^{ab}
Ca (g kg^{-1})	6.2 (2.5)^a	1.0 (0.4)^b	5.4 (4.0)^a
Humus index	3.8 (0.4)^a	2.8 (0.8)^b	1.3 (0.5)^c

Values are means (standard deviations). Letters indicate significant differences ($P < 0.05$) between land-uses (one-way ANOVA tests).

2.2. Soil basic characteristics and classical earthworm survey

In each plot, basic characteristics of topsoils (0–5 cm) were determined on composite samples (5 soil cores of 5 cm depth) sieved at 2 mm (Table 1 and Table S1). Soils were Endoleptic to Epileptic Cambisols (Calcaric), (Hypereutric), or (Dystric) depending on soil pH (IUSS Working Group WRB, 2007). The measured properties included organic C and total N contents, C/N ratio, available P (Olsen), pH (H_2O), Ca and Na cation contents, and an assessment of the humus form through the humus index (Ponge and Chevalier, 2006). All reference methods used for measuring basic soil properties are listed in Table S1.

A classical earthworm survey was performed in June 2012 and October 2013 by combining allyl isothiocyanate (AITC) and handsorting in three 0.5 m^2 subplots in a 20 × 20 m square situated in the center of the 1 ha plots, following Zaborski (2003) (Fig. S1a). We irrigated 0.5 m^2 quadrats with two applications of 10 L of AITC solution (10 g l^{-1}) at 10 min intervals. Earthworms emerging at the soil surface were collected during 10 min after each application. The total area of subplots (0.5 m^2) was then dug up to the depth of 20 cm and a manual handsorting was performed. All sampled individuals were stored in alcohol and later morphologically identified according to Bouché (1972).

2.3. Sampling for soil DNA-based earthworm survey

To be properly compared with the classical earthworm survey, composite soil samples for metabarcoding analyses were collected using the same sampling scheme (three 0.5 m^2 subplots in a 20 × 20 m square). Ten soil cores per subplot (*i.e.*, 30 in total per plot), containing both litter and organo-mineral soil (ca. 20 cm depth, comprising O and A soil horizons of these shallow Cambisols), were sampled in October 2013 and pooled together (Fig. S1a). Coring samplers were sterilized between each plot to avoid cross-contamination. Soil samples were stored at 4 °C and the extraction of extracellular DNA was performed in the week following sampling.

2.4. Extraction of soil extracellular DNA

The use of extracellular DNA adsorbed by soil particles was recently proposed to describe soil biodiversity (*e.g.*, Bienert et al., 2012; Taberlet et al., 2012). This DNA can be extracted from large amount and/or volumes of soil samples, enhancing thus species detectability and allowing a wider coverage of studied area in a minimum of time (Taberlet et al., 2012). It further makes possible the detection of elusive organisms that are not necessarily present in the soil sample.

Soil samples were weighted. An equal weight of saturated phosphate buffer (Na_2HPO_4; 0.12 M; pH = 8) was added and the mixture was agitated vigorously for 15 min to release particle-bound DNA (Taberlet et al., 2012). A small fraction of the mixture was centrifuged (10 min at 10000 rpm) and the supernatant was then used as starting material for the following extraction steps. These latters were carried out using a commercial kit (NucleoSpin® Soil; Macherey–Nagel, Düren, Germany), skipping the lysis step and following manufacturer's instructions (Taberlet et al., 2012).

Four extracellular DNA extractions per soil composite sample were performed and four extraction controls were added.

2.5. DNA amplification and high-throughput sequencing

Classical barcodes such as the ca. 650 base pairs (bp) long Cytochrome Oxydase I, (Hebert et al., 2004) are too long to be retrieved entirely in soil extracellular DNA, this latter being