Science of the Total Environment 895 (2023) 165087

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Amazonian earthworm biodiversity is heavily impacted by ancient and recent human disturbance

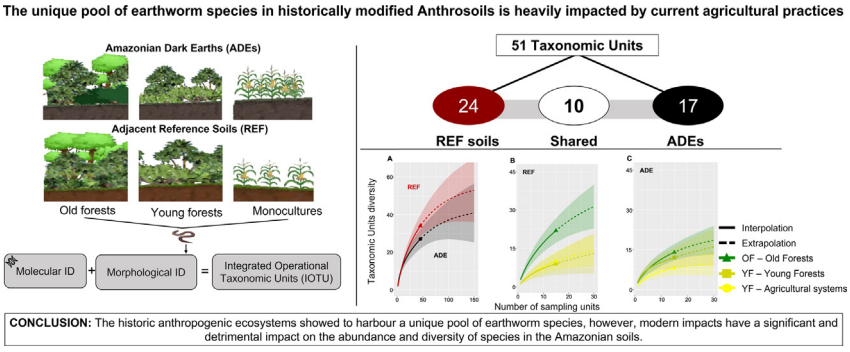
Ana C. Conrado [a], Wilian C. Demetrio [b], David W.G. Stanton [c], Marie L.C. Bartz [d], Samuel W. James [e], Alessandra Santos [b], Elodie da Silva [f], Talita Ferreira [b], Agno N.S. Acioli [g], Alexandre C. Ferreira [h], Lilianne S. Maia [b], Telma A.C. Silva [i], Patrick Lavelle [j], Elena Velasquez [k], Sandra C. Tapia-Coral [l], Aleksander W. Muniz [h,m], Rodrigo F. Segalla [b], Thibaud Decaëns [n], Herlon S. Nadolny [b], Clara P. Peña-Venegas [o], Amarildo Pasini [p], Raimundo C. de Oliveira Júnior [q], TPI Network [1], Peter Kille [c], George G. Brown [b,f], Luís Cunha [d,r,*]

[a] Biochemistry Department, Federal University of Paraná, Curitiba, PR 81531-980, Brazil
[b] Department of Soil Science, Federal University of Paraná, Curitiba, PR 80035-050, Brazil
[c] Cardiff University, Cardiff CF103AT, United Kingdom
[d] Centre for Functional Ecology, Associate Laboratory TERRA, Department of Life Sciences, University of Coimbra, 3000-456 Coimbra, Portugal
[e] Maharishi International University, Fairfield, IA 52557, United States
[f] Embrapa Florestas, Colombo, PR 83411-000, Brazil
[g] Federal University of Amazonas, Manaus, AM 69067-005, Brazil
[h] Entomology Department, Federal University of Paraná, 81530-900 Curitiba, PR, Brazil
[i] Instituto Nacional de Pesquisas da Amazônia, Manaus, AM 69067-375, Brazil
[j] Institut de Recherche pour le Développement, Cali 763537, Colombia
[k] Universidad Nacional de Colombia, Palmira 32 #12-00, Colombia
[l] Servicio Nacional de Aprendizaje, SENA Regional Amazonas, Leticia, Colombia
[m] Embrapa Amazônia Ocidental, Manaus, AM 69010-970, Brazil
[n] CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France
[o] Instituto Amazónico de Investigaciones Científicas SINCHI, Leticia, Colombia
[p] Universidade Estadual de Londrina, Londrina, PR 86057-970, Brazil
[q] Embrapa Amazônia Oriental, Santarém, PA 68020-640, Brazil
[r] School of Applied Sciences, University of South Wales, Pontypridd CF374BD, United Kingdom

HIGHLIGHTS

- Amazonian Dark Earths show an exclusive pool of earthworm species.
- Integrated Operational Taxonomic Units (IOTUs) robustly assess earthworm diversity.
- IOTUs allow the use of data based on immature individuals and cocoons.
- Current land use affects earthworm abundance and diversity.
- The historical land use explains the species pool.

GRAPHICAL ABSTRACT



* Corresponding author at: Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, 3000-456 Coimbra, Portugal.
E-mail address: luis.cunha@uc.pt (L. Cunha).
[1] For a complete list of authors included in this network, please refer to the list of participants in the Web: bit.ly/3O5gQLt.

http://dx.doi.org/10.1016/j.scitotenv.2023.165087
Received 21 February 2023; Received in revised form 20 June 2023; Accepted 21 June 2023
Available online 26 June 2023

ARTICLE INFO

Editor: Manuel Esteban Lucas-Borja

Keywords:
Crassiclitellata
Amazonian Dark Earths
Agriculture
Land-use change
DNA barcoding

ABSTRACT

Despite the importance of earthworms for soil formation, more is needed to know about how Pre-Columbian modifications to soils and the landscape. Gaining a deeper understanding is essential for comprehending the historical drivers of earthworm communities and the development of effective conservation strategies in the Amazon rainforest. Human disturbance can significantly impact earthworm diversity, especially in rainforest soils, and in the particular case of the Amazonian rainforest, both recent and ancient anthropic practices may be important. Amazonian Dark Earths (ADEs) are fertile soils found throughout the Amazon Basin, created by sedentary habits and intensification patterns of pre-Colombian societies primarily developed in the second part of the Holocene period. We have sampled earthworm communities in three Brazilian Amazonian (ADEs) and adjacent reference soils (REF) under old and young forests and monocultures.

To better assess taxonomic richness, we used morphology and the barcode region of the COI gene to identify juveniles and cocoons and delimit Molecular Operational Taxonomic Units (MOTUs). Here we suggest using Integrated Operational Taxonomical units (IOTUs) which combine both morphological and molecular data and provide a more comprehensive assessment of diversity, while MOTUs only rely on molecular data. A total of 970 individuals were collected, resulting in 51 taxonomic units (IOTUs, MOTUs, and morphospecies combined). From this total, 24 taxonomic units were unique to REF soils, 17 to ADEs, and ten were shared between both soils. The highest richness was found in old forest sites for ADEs (12 taxonomic units) and REFs (21 taxonomic units). The beta-diversity calculations reveal a high species turnover between ADEs and REF soils, providing evidence that ADEs and REFs possess distinct soil biota. Furthermore, results suggest that ADE sites, formed by Pre-Columbian human activities, conserve a high number of native species in the landscape and maintain a high abundance, despite their long-term nature.

1. Introduction

Earthworms represent 40 to 90 % of the total macrofauna biomass of tropical soils depending on the land use system (Fragoso et al., 1999). Through their feeding and bioturbation activities, earthworms mix organic materials and minerals in the soil, which can affect nutrient cycling (Lavelle et al., 1997). In addition to these well-known mechanisms, recent studies have suggested that earthworms may also interact with soil bacteria to enhance soil health and plant growth (Jacquiod et al., 2020; Medina-Sauza et al., 2019). Over time, these processes can lead to profound changes in the soil, making earthworms a critical component of the tropical ecosystem.

There are 5738 valid species/subspecies of earthworms described in the literature (Mısırlıoğlu et al., 2023), but the total for the globe is estimated to be around 30.000 species (Orgiazzi et al., 2016). The diversity of the earthworm population in a particular location is affected by climate, soil, vegetation and anthropic practices (Brown and Domínguez, 2010; Fründ et al., 2010; Lavelle, 1996; Pulleman et al., 2012; Rutgers et al., 2016), and human disturbance can have major impacts on earthworm diversity, especially in rainforest soils (Fragoso et al., 1997). The conversion of rainforest to pastures and polyculture agroforestry systems often increases earthworm populations due to invasion by exotic earthworms (Chauvel et al., 1999; Marichal et al., 2014, 2010; Römbke et al., 1999; Römbke and Verhaagh, 1992), but the conversion of rainforest to annual crop systems often drastically reduces both earthworm diversity and abundance (Fragoso et al., 1995; Lavelle and Pashanasi, 1989).

In the particular case of the Amazonian rainforest, both recent and ancient anthropic practices are important (Heckenberger et al., 2007a, 2007b), however, the main differences between both is the scale and intensity of human impact. Recent changes include the conversion of large areas to agriculture, reducing the quality and quantity of organic matter and soil moisture, impairing earthworm survival (Barros et al., 2003; Mathieu et al., 2009). Modern agricultural practices often involve larger areas of land and higher levels of mechanization and are based on external knowledge and technologies (with the use of hybrid seeds, fertilisers, and pesticides) that may not be well-suited to the local environment and can have more severe environmental impacts on ecosystem health, than traditional slash-and-burn agriculture (Foley et al., 2005; Gomiero et al., 2011; Pretty, 2008). Deforestation rates have also increased significantly in recent decades, leading to large-scale biodiversity loss and carbon sequestration capacity (e.g., FAO and UNEP, 2020; Gibbs et al., 2015). Ancient anthropic changes include the modification of landscape structure and vegetation through the slash and burn practices, selection and dissemination of useful plants, and by Pre-Columbian human colonization of many areas in the Amazonian basin (Clement et al., 2015; Kawa et al., 2011; Levis et al., 2018; Maezumi et al., 2018). In fact, indigenous peoples in the Amazon rainforest have developed sophisticated knowledge systems and traditional practices that have allowed them to manage their environment for centuries (Heckenberger et al., 2007a, 2007b; Posey, 1985; Schmidt et al., 2022). However, one of the most impressive ancient anthropic changes is the generation of Amazonian Dark Earths (ADEs, also known locally as “Terra Preta de Índio” in Portuguese) during centuries of Amerindian occupation over a period of several thousand years, from approximately 2500 years ago to the arrival of Europeans in the region in the 16th century (Iriarte et al., 2020; Lombardo et al., 2022; Smith, 1980). ADEs cover from 0.1 to 3 % of the total area of Amazon rainforest (McMichael et al., 2014; Sombroek et al., 2003), are characterized by their dark color and high levels of carbon, available calcium and phosphorus, and pH (J. Lehmann et al., 2003; Macedo et al., 2017), and have attracted interest for the development of agriculture due to their high fertility (Glaser, 2007). Because of their different physical and chemical properties, their biological diversity also differs from that of typical adjacent soils in the region. For example, bacterial populations in ADEs differ from those of adjacent soils (Grossman et al., 2010; O'Neill et al., 2009). However, so far only one study has been performed on soil macrofauna diversity in ADEs compared to other soils in the region (Demetrio et al., 2021a), and little is known about how they contribute to the formation and fertility of these soils (Cunha et al., 2016).

Traditionally, earthworms are identified only with morphological characters, but an expected large taxonomic deficit, the lack of adequate local keys, and problems with identification of juveniles, has encouraged the use of molecular methods (Decaëns et al., 2016; Maggia et al., 2021). Hence, in this study we used DNA barcodes to identify molecular operational taxonomic units (MOTUs) within the earthworm communities in the study areas and integrated these with morphological data. This approach better represents the diversity of the community because it not only identifies adults but also juveniles and cocoons (Decaëns et al., 2016).

In the present study, we compared earthworm communities in ADEs and reference soils under different vegetation types: medium-old forest, young forest and agriculture. The reference soils (REFs) in this study are found in the adjacent areas to the ADEs showing similar background history (vegetation growth, land use and crops) within an area of the same soil type but in this case with no evidence of ADE (e.g., no darkened horizon A, no artifacts) formation by pre-Columbian societies, and therefore used for

comparison purposes. By doing this, we aimed to clarify whether both ancient (pre-Columbian soil modifications) and modern human impacts affected earthworm abundance and diversity in the Amazon basin.

2. Material and methods

2.1. Study sites

Earthworm communities were sampled in three Brazilian Amazonian regions: Central (Iranduba), Southwestern (Porto Velho) and Lower (Belterra) (Sup. Fig. 1A). In each municipality, paired ADE and non-anthropic reference soils (REF) were selected under three different land use systems (LUS): medium to old secondary forest (MOF), i.e., forests older than 20 yr without recent human disturbance; young secondary forest (YF), i.e., forests with more recent (<20 yrs) disturbance by slash and burn; and agricultural systems (AS), cultivated with maize (Iranduba), soybean (Belterra), or under perennial pastures (Porto Velho). This forest age classification was used since secondary tropical forests have normally recovered most (around 80 %) of the original vegetation (Rozendaal et al., 2019; Smith et al., 2023), and soil macrofauna community (Serra et al., 2021) diversity after around 20 years of regeneration (MOFs). Sites were selected envisaging similar background history (age, structure and disturbance level) within an area having the same geochemical background.

The study sites included MOFs (ADE and REF) in Belterra, located at the Embrapa Amazônia Oriental Belterra Experiment Station (ADE forest around 20 years old, REF >80 years old) and at the Tapajós National Forest (both ADE and REF >80 years old), which had been previously studied for ADEs (Maezumi et al., 2018). The other MOFs (ADE and REF, both >40 years old) were located at the Caldeirão - Experimental Station of Embrapa Amazônia Ocidental in Iranduba, and have been extensively studied in the past for soil fertility, pedogenesis, and soil microbial diversity (Alho et al., 2019; Germano et al., 2012; Grossman et al., 2010; Lima et al., 2014; Macedo et al., 2017; O'Neill et al., 2009; Taketani et al., 2013). The YFs (ADE and REF) in Porto Velho were approximately 4 and 10 (ADE) and 15 and 10 years old (REF) in Teotônio and Santa Paula districts, respectively (each on opposite sides of the Madeira River), and around 13 years old (ADE and REF) in Iranduba. A major part of the tree composition in our medium-old forest sites was notably old, with some assumed to be older than 300 years (e.g., Belterra site in Tapajos National Forest; Vieira et al., 2005), reflecting the long history of the targeted forested areas. Although 20 years might be considered relatively young for a forest, research by several authors, including Rozendaal et al. (2019), Serra et al. (2021), and Smith et al. (2023), argues that forests older than 20 years can already possess a well-established community of fauna, flora, and soil macroinvertebrates. Moreover, within the context of slash-and-burn agriculture in the Amazon, a period of over 20 years can represent a significant duration for forest regeneration. It is noted that forests can regenerate relatively rapidly in certain cases, particularly in areas with ample rainfall and fertile soils, as highlighted by Barlow and Peres (2004).

ADE formation in Iranduba was estimated to have begun approximately 1050–950 yr before present (bp) (Neves et al., 2004), while in Belterra it began around Late Pre-Columbian period 2000 yr bp (Maezumi et al., 2018). At Porto Velho, ADE formation began much earlier, around 6000 years bp (Watling et al., 2018).

The annual crops in Iranduba and Belterra, had been continuously grown for a minimum of 4 (maize) and 7 (soybean) years, respectively. In Porto Velho pastures were dominated by *Urochloa* (REF) and *Paspalum* (ADE) grasses and were approximately 9 and 12 years old, respectively. The soil types in REF sites were classified as dystrophic Ferralsols and Acrisols based on the World Reference Base for Soil Resources (IUSS, 2015) (Table 1). These soil types are the most common in the Amazon region (Gardi et al., 2015). At one young regeneration forest site in Porto Velho, both ADE and REF soil horizons were overlying a plinthic horizon, and the REF soil was classified as a Plinthosol. All ADEs were classified as Pretic Clayic Anthrosols, with dark organic-matter-rich surface soil horizons that were usually over 20 cm thick. Some of the main characteristics of the sites are provided in Table 1, and additional information on the sites can be found in (Demetrio et al., 2021b). A pre-Columbian footprint was assumed at all sites with ADEs because of the overarching impact of the soil and environmental modifications promoted during the creation of ADEs by the Pre-Columbian societies. As shown by several authors (e.g., Barbosa et al., 2020; Kern et al., 2017; Macedo et al., 2019; Schmidt et al., 2014) these modifications have an impact on soil chemical properties and botanical composition of the site (with a higher proportion of useful plant species), thereby impacting the soil biotic (including macrofauna) community present, determining the species assemblage. Furthermore, recent human use and management at these sites can also have an impact, which is why we divided the land uses in the present study into the medium-old forest, young forest and agricultural systems. The last two would show a more “modern” human footprint, whereas, in the medium-old forests, we assumed that the pre-Columbian footprint would dominate, with little “modern” human footprint, as these would not have been subjected to pesticides, inorganic fertilisers, machine-driven tillage and other “modern” inputs used in present-day agriculture.

Table 1
Land use, soil type (IUSS, 2015), vegetation cover and geographic coordinates (Geographic Coordinate Systems -GSM) of the earthworm sampling sites in three regions of the Brazilian Amazon. YF = Young forests (<20 yrs regeneration); MOF = Medium-Old forests (>20 yrs regeneration); AS = Areas currently being used for agricultural production.

Region	Soil type	Vegetation	Land use	Geographic coordinates
Amazonian Dark Earths				
Iranduba	Pretic Clayic Anthrosol	Rainforest	YF	−3.253069, −60.229175
	Pretic Clayic Anthrosol	Rainforest	MOF	−3.230342, −60.268731
	Pretic Clayic Anthrosol	Maize	AS	−3.229481, −60.268700
Belterra	Pretic Clayic Anthrosol	Rainforest	MOF	−2.784236, −54.999936
	Pretic Clayic Anthrosol	Rainforest	MOF	−2.685328, −54.918642
	Pretic Clayic Anthrosol	Soybean	AS	−2.684386, −54.918861
Porto Velho	Pretic Clayic Anthrosol	Rainforest	YF	−8.864422, −64.063342
	Pretic Clayic Anthrosol	Rainforest	YF	−8.850328, −64.067519
	Pretic Clayic Anthrosol	Pasture	AS	−8.865703, −64.061297
Reference soils				
Iranduba	Xanthic Dystric Acrisol	Rainforest	YF	−3.246944, −60.225197
	Xanthic Dystric Acrisol	Rainforest	MOF	−3.226242, −60.273222
	Xanthic Dystric Acrisol	Maize	AS	−3.225364, −60.274772
Belterra	Xanthic Dystric Ferralsol	Rainforest	MOF	−2.784608, −54.998133
	Xanthic Dystric Acrisol	Rainforest	MOF	−2.687194, −54.917583
	Xanthic Dystric Acrisol	Soybean	AS	−2.684322, −54.920208
Porto Velho	Xanthic Dystric Plinthosol	Rainforest	YF	−8.869861, −64.055044
	Xanthic Dystric Ferralsol	Rainforest	YF	−8.847089, −64.066444
	Xanthic Dystric Ferralsol	Pasture	AS	−8.876472, −64.066272

2.2. Earthworm sampling

For each site, earthworm samples were collected by hand-sorting soil monoliths (25 × 25 cm up to 30 cm depth) following the methodology of the Tropical Soil Biology and Fertility (TSBF) Programme (Anderson and Ingram, 1993) proposed as the standard method by the International Organization for Standardization-ISO (ISO 23611-5, 2017). At each sampling site, we defined a 1 ha plot in size, and the five sampling points were located at the corners and center of a square plot measuring 60 × 60 m with 20 m distance from the edge of the plot (Sup. Fig. 1B). The soil monoliths were then collected at these five points, resulting in a total of five samples per site. Each monolith was subdivided into surface litter (if present) and three 10 cm-thick soil layers at depths of 0–10 cm, 10–20 cm, and 20–30 cm. The earthworms, including cocoons (when spotted by eye) were then hand-sorted from the soil samples and fixed in 92 % ethanol, which helps to preserve the specimens for later analysis. This methodology allowed for a thorough and standardized collection of earthworm samples from multiple depths and locations within each sampling site. Later, weighing, counting and taxonomic classification were done at the levels of family, genus and species. Density (number of individuals) and biomass (weight in grams) were extrapolated per square meter.

2.3. Traditional morphological taxonomy

Earthworms were identified to family, genus and morphospecies using the available taxonomic keys (Blakemore, 2002; Michaelsen, 1900; Righi, 1995, 1990). If the earthworms showed differences in more than one character traditionally considered as a key to allocate to a different species, then it was assigned to different taxonomic units, referred to as morphospecies.

2.4. DNA barcoding

After the morphological identification, an individual of each morphospecies from each depth of each monolith was selected for DNA extraction. Of the total specimens collected, 299 were selected (142 adults, 34 cocoons, 123 juveniles and fragmented specimens) for DNA analysis. A small piece of body wall or piece of cocoon tissue (≅0,4 cm^2) was collected from each individual and the extraction performed using a Qiagen® extraction kit (DNeasy 96 Blood and Tissue kit) following the manufacturer's instructions. We sequenced a region of the cytochrome oxidase-1 (COI) gene following standard of the International Barcode of Life project (http://ibol.org/), using the primer pairs LCO1490/HCO2198 (Folmer et al., 1994). Failed samples after this first pass were amplified using the internal primers 2Fall and 2Rall (Sup. Table 1). All sequences are available on BOLD database (dataset: DS-EWTPI Earthworms from Amazonian Dark Earth (http://dx.doi.org/10.5883/DS-EWTPI)).

2.5. Classification of molecular operational taxonomic units (MOTUs)

The nucleotide sequences obtained were edited in the MEGA7 program and aligned using the ClustalW tool (Thompson et al., 1994). For the distance analyses, we used a Neighbor-Joining (Saitou and Nei, 1987) algorithm with the Kimura-2 parameter model (Kimura, 1980) to estimate genetic distances. Robustness of nodes was evaluated through bootstrap re-analysis of 1000 pseudo-replicates. The tree was re-plotted using the online utility iTOL v4 (Letunic and Bork, 2019) and the MOTUs were defined with the ABGD algorithm (Puillandre et al., 2012), which is recognized as an efficient and reliable method for earthworm MOTU delineation on relatively small datasets (Goulpeau et al., 2022). The “barcode gap” was identified between intra- and interspecific distances. In this gap, it was assumed that MOTUs delineated could be considered valid biological entities (Plaisance et al., 2009). A conservative cutoff limit defined at 16 % for interspecific divergence was used to delimit the MOTUs.

2.6. Delimitation of morphospecies and integration of molecular data

We developed an integrative approach where both morphological data and molecular data were used for delimitation of an Integrated Operational Taxonomic Unit (IOTU) using the following consecutive criteria:

1. Morphologically identified morphospecies for which no DNA barcoding data was available or/and data was concordant with the genetic similarity (0–6 % of homology) were assumed to be a unique taxonomic unit, and allocated to a distinct IOTU;
2. In situations where the morphological identification was unable to resolve the species, only the genetic similarity was used;
3. Cocoon, tissue pieces and juvenile specimens were allocated to already generated IOTUs or MOTUs using exclusively the genetic similarity, or in case of a unique sequence then allocated to a new MOTU.

Data were combined to create a concatenated list of delimited morphospecies, IOTUs and MOTUs. This integrated dataset was used to generate the diversity metrics.

2.7. Statistical analysis

The distributions of earthworm data (density, total and mean individual earthworm biomass and diversity indices) were analyzed by Shapiro-Wilks to evaluate their fit to a normal distribution. In cases of deviation from normality, we found that the best fit models for data adjustment were Poisson for density and richness, and Gamma for biomass. Generalized Linear Models (GLiM) were used considering soil category (ADE and REF) and LUS (OF, YF and AS) as factors. Ancient human impacts were assessed by comparing the soil category, while modern human impacts were assessed by comparing the LUS (different levels of disturbance). When differences were significant ($P < 0.05$), Tukey's test was used to determine differences among treatments using multcomp package in R software (Hothorn et al., 2008).

2.8. Biodiversity indicators

Mean earthworm species richness (mean number of species found), species richness within samples (number of species sample $^{-1}$) and Shannon diversity index were calculated using standard formulae (Magurran, 2004).

Beta-diversity (β) indices were calculated using species occurrence and disregarding singletons (species represented by single individuals) to assess the species turnover components. Using Betapart package (Baselga and Orme, 2012) we calculated β Sørensen (βSør) dissimilarity index (max. diversity) and β Simpson (βSim) dissimilarity index (turnover) and Nestedness (βSør – βSim). β diversity values were partitioned according to the following factors: LUS (mean of beta-diversity indices obtained within a region in the same soil category); regional/spatial (obtained comparing the same LUS within each soil category); and soil category effect (result from comparisons between ADEs and REF soils in the same LUS within each region). The Venn diagram was made using InteractiVenn (Heberle et al., 2015) to show the distribution and overlap (shared) of the taxonomic units among LUS categories, LUS categories within the ADE and REF, and among the three regions (Central: Iranduba, Southwestern: Porto Velho, and Lower Brazilian Amazonia: Belterra) where the soil samples were collected. Species richness was compared by plotting rarefaction curves showing how species/morphospecies cumulated according to sampling intensity (as a function of the number of sampling units, i.e. the number soil monoliths). Rarefaction curves were adjusted globally for ADE and REF and for each LUS in each soil category using the iNEXT package (Hsieh et al., 2018).

2.9. Soil physical and chemical attributes

Demetrio et al. (2021b) provides data on soil physical and chemical properties (Sup. Table 2), which were determined by analyzing 2–3 kg

samples collected from depths of 0–10, 10–20, and 20–30 cm after hand-sorting the earthworms from each TSBF monolith. Mean values were calculated over the 0–30 cm depth range using standard methodologies (Teixeira et al., 2017) for various soil properties, including pH ($CaCl_2$), Ca^{2+}, Mg^{2+}, and Al^{3+} (KCl 1 mol L^{-1}), K^+, available P (Mehlich $^{-1}$), total nitrogen (TN), and carbon (TC) through combustion (CNHS). Base saturation, sum of bases (SB), and cation exchange capacity (CEC) were calculated using standard formulas, while soil texture (% sand, silt, and clay) was determined using the FAO soil texture triangle. The soil physical and chemical properties were correlated with earthworm parameters using R software and the 'ggcorrplot' package (Kassambara, 2019). Furthermore, a Principal Component Analysis (PCA) with a Monte-Carlo test was performed using the earthworm data (density, biomass, and diversity indices) and soil chemical and particle size fractions with the ADE-4 package (Dray and Dufour, 2007) in R software.

3. Results

3.1. Earthworm density and biomass

In the 18 sampled sites, a total of 970 earthworm individuals were collected, including 596 adults, 37 cocoons, and 305 juveniles and fragmented specimens. The density of earthworms was significantly lower in AS than in YF and MOF on REF soils, while no difference was observed in density between the different LUS on ADEs (Fig. 1A and Sup. Table 3). Earthworms were mostly found within the first 10 cm of the soil profile in both soil categories (ADE, REF), although they tended to be more superficial in ADEs than in REF soils (Fig. 1C). In AS on REF soils, the two topmost soil layers (0–10, 10–20 cm) had >90 % of all individuals collected. Few earthworms were found in the litter layer and most of them were observed only in MOF sites (ADE and REF).

Earthworm biomass in the soil profile (Fig. 1B and Sup. Table 3) was distributed in a similar way to density. In REF soils, highest earthworm biomass was found in the MOF, followed by YF and lowest in AS (Fig. 1D). On the other hand, no significant differences were found in biomass in the ADE sites.

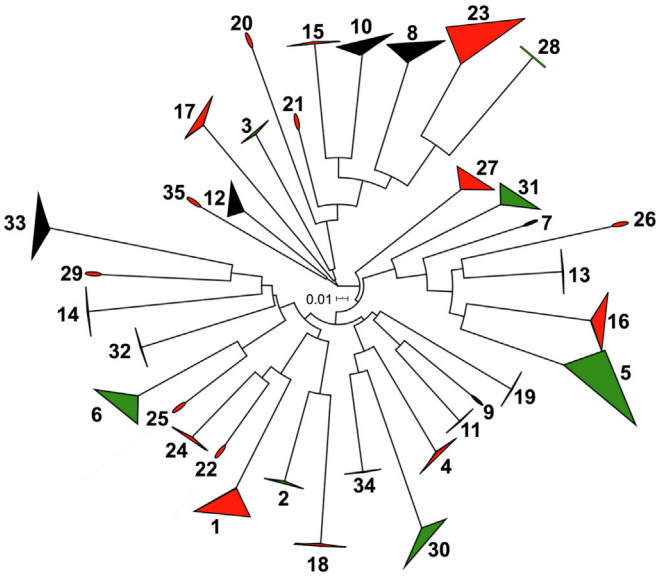


Fig. 2. Neighbor-joining tree of 257 COI sequences obtained from specimens collected in 18 different Amazonian sites. Each triangle represents a molecular operational taxonomic unit (MOTU) identified based on a genetic distance threshold of 16 %, and the numbers correspond to the assigned taxonomic unit number. The longer and shorter lateral edges of each triangle indicate the maximum and minimum intra-divergence values, respectively, within each MOTU. Green triangles indicate MOTUs found in both Amazonian Dark Earths (ADEs) and reference soils (REFs), red triangles represent MOTUs present only in REFs, and black triangles represent MOTUs present only in ADEs.

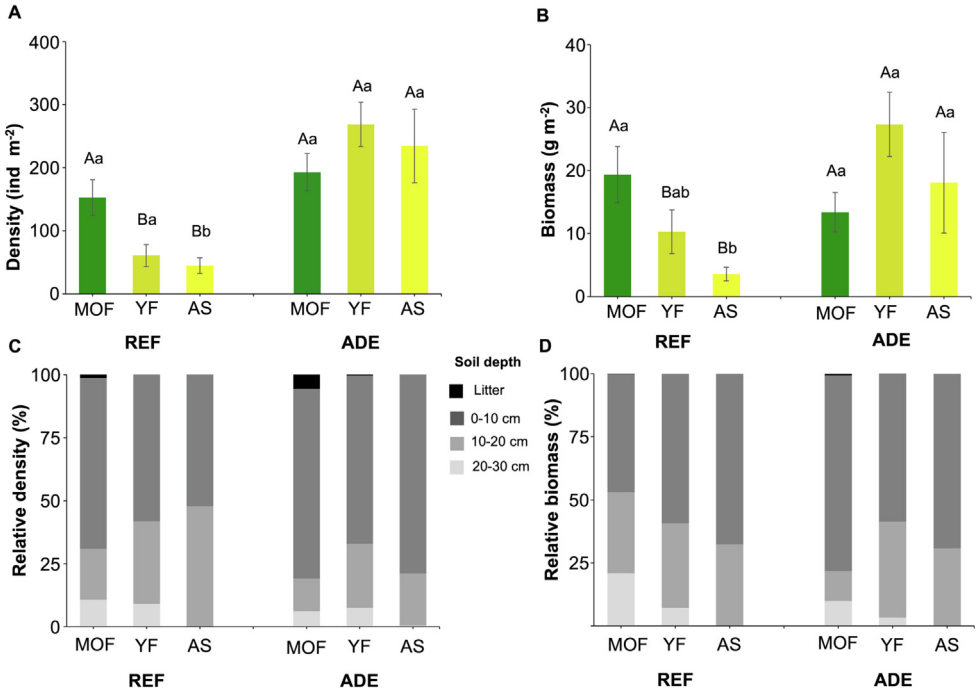


Fig. 1. Earthworm communities in Amazonian Dark Earths (ADE) and reference soils (REF): A) mean earthworm density (ind. m^{-2}) in different land-use systems: Medium-old forests (MOF, green bars), young forest (YF, dark yellow bars), and agricultural/pastoral system (AS, yellow bars); B) Biomass (g. m^{-2}) in land-use systems (LUS); C) relative density of earthworms (%) showing the proportion of earthworms found at each soil depth; D) relative biomass of earthworms (%), showing the proportion of earthworm biomass found at each soil depth. *different letters indicate significant differences ($p < 0.05$) between soil categories within each land-use system (capital letters) and between land use systems within the same soil category (small letters). Bars indicate standard errors ($n = 15$).

3.2. Classification of the taxonomic units

We obtained 257 COI sequences (i.e. 89 % sequencing success) which clustered into a total of 35 MOTUs (Fig. 2 and Sup. Table 4). The intra-MOTU divergence ranged from 0 to 4 % and inter-MOTU divergence ranged from 16 to 39 %, considering a genetic distance of 16 % as a threshold for separating interspecific MOTUs (Sup. Fig. 2). Using sequence data, we were able to delimit 19 MOTUs concordant with the morphological identification (IOTUs). Juvenile, tissue fragments and cocoons added a total of 16 MOTUs, representing 54 % of the total MOTUs and 37 % of the total taxonomic units. Additionally, 16 morphospecies were kept as unique taxonomic units because no DNA sequence was obtained from them. In conclusion, the integrative approach with both sequence data and morphological identification resulted in a total of 51 taxonomic units (IOTUs, MOTUs and morphospecies combined) among these, 1 singleton and 3 doubletons found in ADEs, and 11 doubletons found in REF sites.

3.3. Earthworm diversity

For diversity analyses we used the combination of DNA barcoding and morphological data. From this total, 24 taxonomic units were unique to REF soils, 17 to ADEs and 10 shared between both soils (Fig. 3). Highest earthworm richness was found in MOF sites, with 25 and 7 taxonomic units (unique + shared taxonomic units) in REF and ADE soils, respectively (Figs. 3, 4A). Ninety percent of the taxonomic units were found in only one region of the Amazon, and only two taxonomic units were present in all

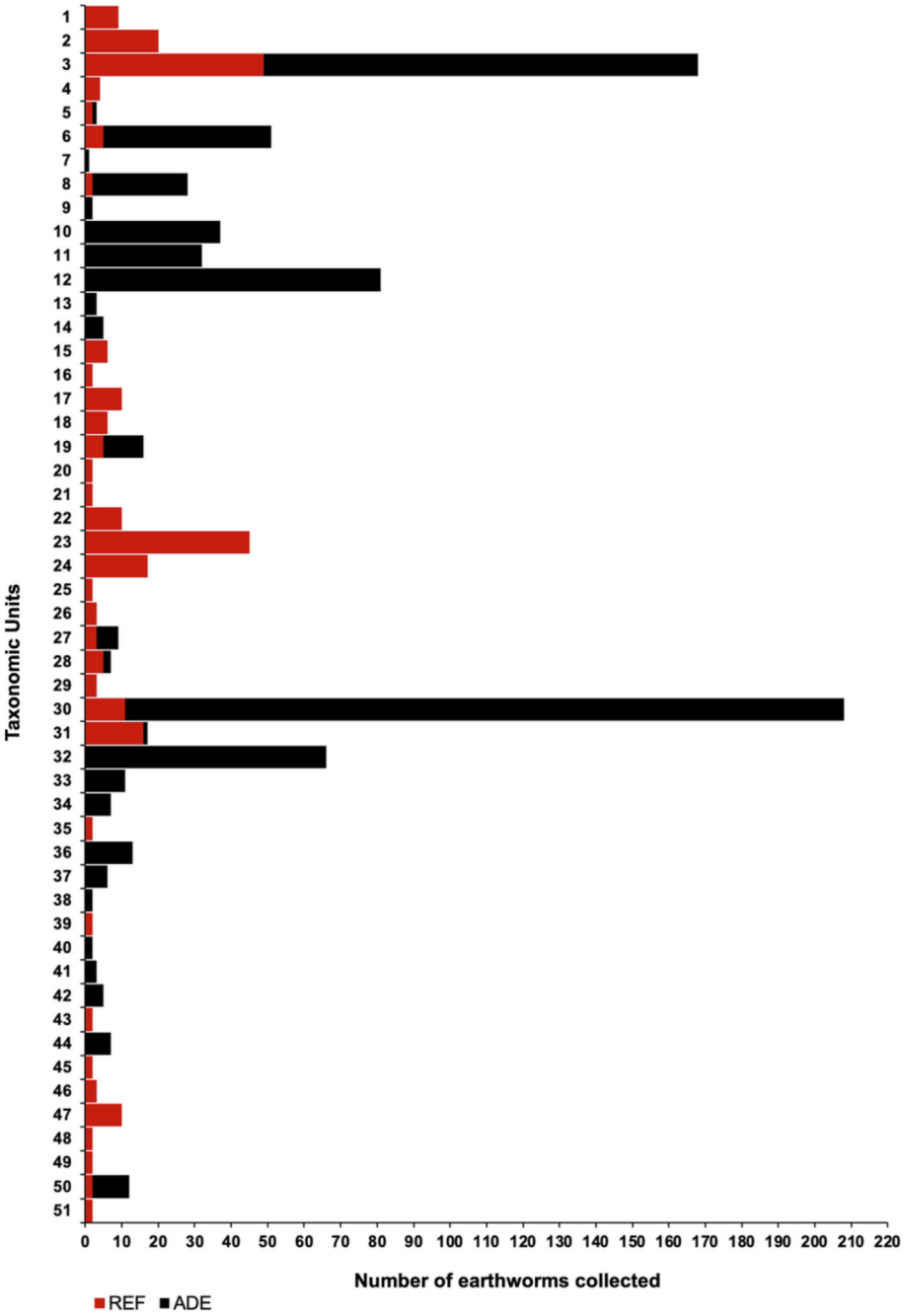


Fig. 3. Total number of individuals collected per taxonomic unit identified through the integrated approach. The red part of each bar represents the number of individuals collected from REF sites, while the black one represents the number of individuals collected from ADE sites. When the two colors are present in the same bar, both start at zero and the most abundant one extends beyond the less abundant.

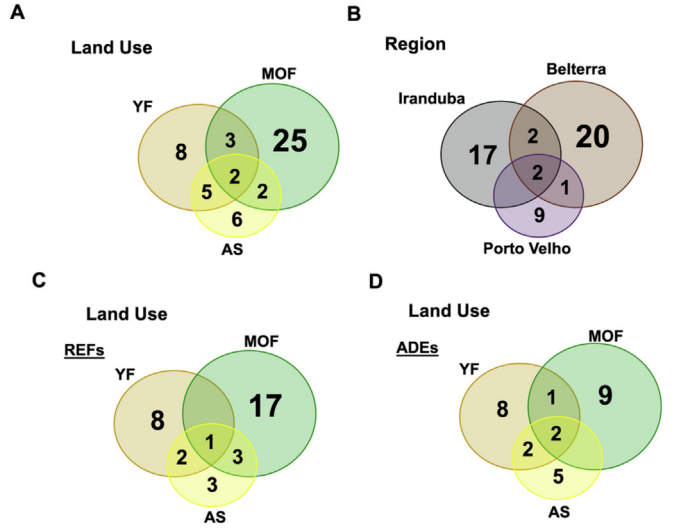


Fig. 4. Venn diagrams showing the distribution: A) of the 51 taxonomic units among the three different land use systems: MOF = medium-old forest; YF = young forest; AS = agricultural/pastoral system; B) of the 51 taxonomic units among the three regions: Central (Iranduba), Southwestern (Porto Velho) and Lower (Belterra) Brazilian Amazonia; C) of the 34 taxonomic units collected in reference soils (REFs) in the three land use systems; and D) of the 27 taxonomic units collected in Amazonian Dark Earths (ADEs) in the three land use systems.

three regions (Fig. 4B), indicating that a significant proportion of the species collected may have a narrow distribution range. Additionally, 64 tissue fragments in ADE and 47 in REF could not be used for morphological or DNA based methods.

Interestingly, ADEs had lower overall taxonomic unit richness than in REF soils (27 vs 34 taxonomic units, respectively), mainly impacted by the lower number in MOFs on ADEs. However, at a lower spatial scale, the average number of taxonomic units per sample was higher in ADEs, indicating that communities tend to be more spatially homogeneous in ADEs than in REFs. The abundance of taxonomic units in MOF on ADEs was similar to that in REF soils, indicating that ADE formation did not have a significant impact on abundance.

Taxonomic assignments at the family to genus levels were possible for 1/3 of the taxonomic units (Sup. Table 4). Only two taxonomic units were identified to species level: *Pontoscolex corethrurus* (Müller, 1857), a well-known peregrine species originating from the Guyana Shield (Dupont et al., 2012; Righi, 1984a) and *Dichogaster modiglianii* (Rosa, 1896) an invasive species originating from Africa (Mainoo et al., 2008). We estimated that at least 31 taxonomic units may be new to science.

The rarefaction curves showed that ADE and REF soils had a rather similar taxonomic unit richness (Fig. 5A). In REF soils (Fig. 5B) MOFs had a higher taxonomic unit richness than YF and AS. Conversely, in ADE soils (Fig. 5C) taxonomic unit richness was similar in all land uses. In the three types of LUS on REF soils and in the OF and YF areas of the ADE soils a sampling effort three or four times greater would be necessary to reach completeness in the sampling of taxonomic unit richness.

Earthworm communities were affected by soil category, with mean richness higher in ADEs (2.6 taxonomic unit sample^{-1}) than in REF soils (2 taxonomic unit sample^{-1}). The effect of LUS showed the same trends as before; in REF soils, mean richness was greater in MOF (2.7 taxonomic units sample^{-1}) than YF (1.2 taxonomic units. sample^{-1}) and AS (1.3 taxonomic unit sample^{-1}), while in ADEs both MOF (2.4 taxonomic units sample^{-1}) and YF (2.7 taxonomic units. sample^{-1}) had higher mean richness than AS (1.4 taxonomic unit sample^{-1}). Total richness in MOF was much higher in REF soils (21 taxonomic units) than in ADEs (12 taxonomic units.), but in YF more taxonomic units were found overall in ADEs (13 taxonomic units) than in REF soils (11 taxonomic units). In the AS, earthworm richness was similar (9 taxonomic units) in both soil categories, and lower than in the other land uses (Fig. 4C).

The partition of beta-diversity values also showed important effects of LUS on earthworm taxonomic unit turnover in ADEs soils (0.58), though these were slightly lower in REF (0.49) (Fig. 6 and Sup. Fig. 3). Regional effects, which show the diversification of taxonomic units as a result of the

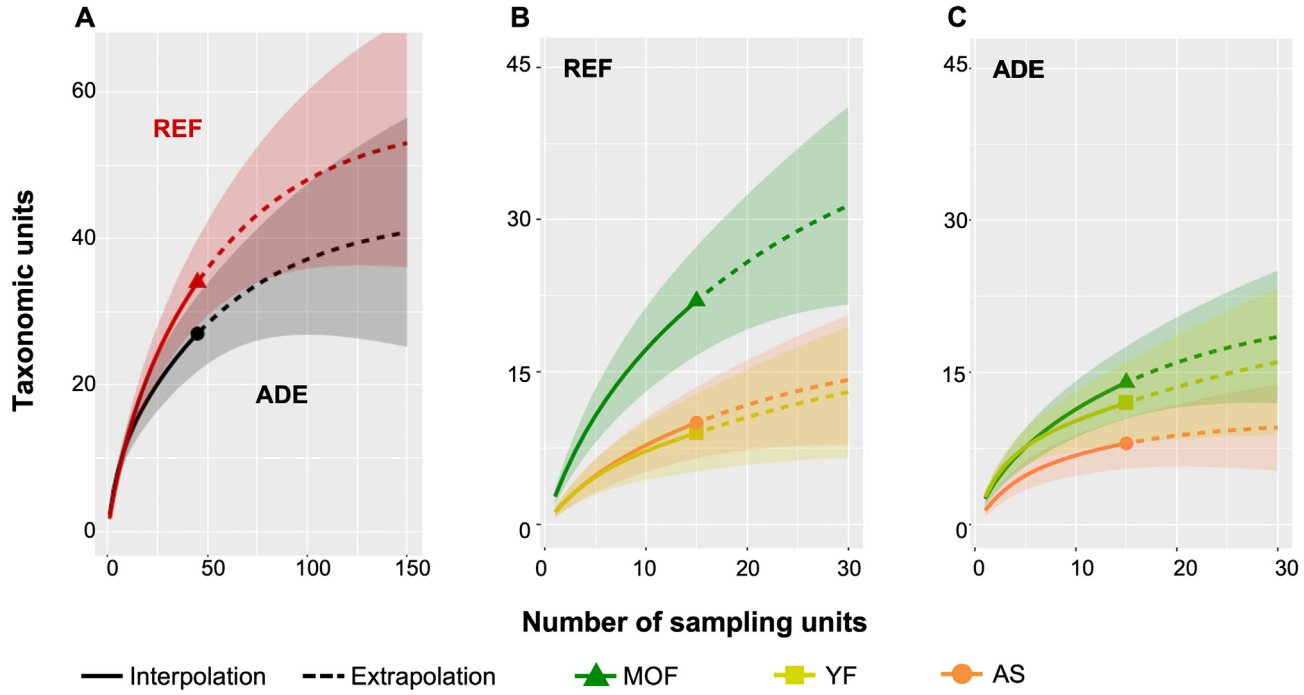


Fig. 5. Earthworm taxonomic unit rarefaction and extrapolation curves. A) Comparison between reference soils (REF: red lines) and Amazonian Dark Earths (ADEs: black lines). B) Comparison of taxonomic unit total numbers in REF under different land-use systems: medium-old forest (MOF, green lines), young forest (YF, yellow lines), and agricultural/pastoral system (AS, orange lines). C) Comparison of taxonomic unit total numbers in ADE under different land-use systems: medium old forest (MOF, green lines), young forest (YF, yellow lines), and agricultural/pastoral system (AS, orange lines). Shaded areas represent 95 % confidence intervals.

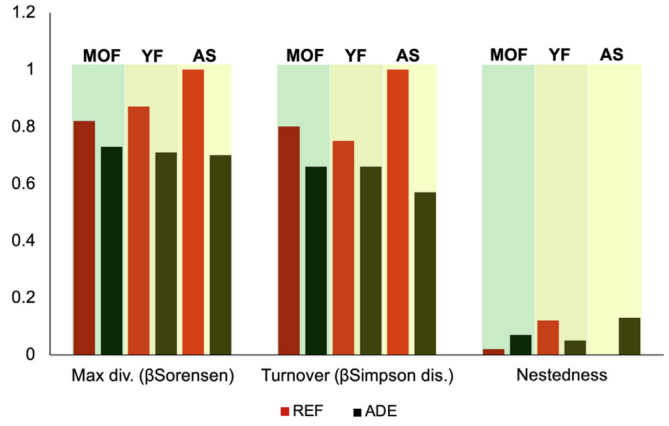


Fig. 6. Partition of beta-diversity (without singletons) of earthworm taxonomic units into β Sørensen (overall diversity), species turnover (β Simpson dissimilarity index) and nestedness among each soil category (REF and ADE) within each land-use systems (MOF = medium-old forest; YF = young forest; AS = agricultural/pastoral system); ADE (Amazonian Dark Earths); REF (reference soils).

spatial/geographical distance, were particularly significant for AS in REF soils (Fig. 6 and Sup. Fig. 3, and Sup. Table 5). The same was observed for the soil effect, where the beta-diversity in AS of the REF soil was higher than in other land uses (Fig. 6 and Sup. Fig. 3, and Sup. Table 5).

3.4. Principal component analysis (PCA)

The PCA analysis showed a clear separation between ADEs and REF soils (Fig. 7A). Axis 1 (PC1) explained 34.6 % of the variance and separated the samples based on soil fertility, with the X-axis (Fig. 7B) related mainly to levels of P, SB (Ca^{2+} + Mg^{2+} + K^{+}), CEC, total carbon and nitrogen and pH. Additionally, Al3+ contributes importantly to PC1 allowing the comparison between ADE and REF soils, mainly due to the higher quantity of Al3+ in REF soils. Axis 2 (PC2) explained 21.2 % of the variance and separated the samples regarding earthworm biomass (total, mean individual biomass) and taxonomic unit richness and soil texture (clay, sand contents).

Earthworm density, total biomass, richness and mean body mass were positively related to soil type and its properties, with higher densities, biomass and richness associated mainly with YF and MOF sites on ADEs. On the other hand, individual biomass (bigger earthworms) was more related

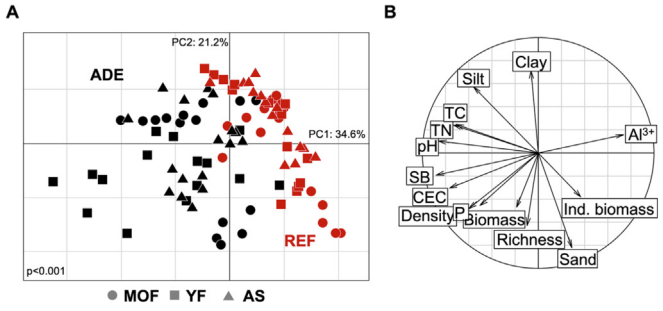


Fig. 7. Principal component analysis of earthworm data (density, biomass, individual biomass and mean taxonomic unit richness) combined with soil chemical and particle size analysis of reference soils (REF: red color) and Amazonian Dark Earths (ADEs: black color) under three land use systems (LUS). A) Factorial map showing sample dispersion according the soil category (ADE, REF) and LUS (MOF = medium-old forest; YF = young forest; AS = agricultural/pastoral system). Significance of the model (soil category or land-use systems) was obtained using Monte-Carlo test (999 permutations). B) Correlation circle showing the links between individual variables and the first two principal components. Meaning of abbreviations in B: TN = Total nitrogen; TC = Total carbon; SB = sum of bases; CEC = cation exchange capacity; P = Phosphorus; (Al^{3+}) = Aluminum.

to REF soils, mainly some MOF and YF samples. Most AS sites on REF soils were inversely associated to all earthworm data.

4. Discussion

4.1. Improving earthworm diversity analysis with integrated operational taxonomical units (IOTUs)

Our study on earthworm diversity in the Amazonian soils utilized a combination of traditional morphological taxonomy and molecular data to identify 51 morphospecies, which is a significant improvement compared to other studies in Amazonia that relied solely on morphological identification (Brown et al., 2018; Römbke et al., 1999; Rousseau et al., 2010). The use of Integrated Operational Taxonomical Units (IOTUs) allowed us to analyze earthworm diversity more accurately and effectively, as it enabled us to include juveniles, fragmented specimens, and cocoons that are difficult to identify based solely on morphological characteristics. We found a good agreement between the genetic delineations and morphological information, except for those IOTUs that only contained these challenging-to-identify specimens, which added 19 taxonomic units to our data. The identification of earthworms based on morphological characters is complex and generally only feasible for well-preserved adult specimens (Chang and James, 2011; Decaëns et al., 2013). However, molecular techniques, such as the use of DNA barcodes, were especially useful in identifying juveniles, fragmented specimens, and cocoons (Klarica et al., 2012). We used the DNA barcode to express the earthworm diversity data in our study, which represented 68.6 % (37.2 % for MOTU and 31.4 % IOTU) of the total number of taxonomic units. Our results confirmed previous findings that the use of DNA barcodes allowed integration of juveniles and cocoons in the data, leading to an increase in earthworm diversity (Decaëns et al., 2016; James et al., 2010; Maggia et al., 2021).

The findings of our study provide strong support for the effectiveness of IOTUs in improving earthworm diversity analysis. By using IOTUs, we were able to include a high number of taxonomic units, thereby enhancing our understanding of earthworm diversity. Our results also highlight several benefits of using IOTUs for earthworm diversity analysis. Firstly, IOTUs facilitated the integration of genetic and morphological data, resulting in a more comprehensive assessment of earthworm diversity. Secondly, IOTUs allowed us to include difficult-to-identify specimens, such as juveniles, fragmented specimens, and cocoons, that would have been otherwise excluded, resulting in a more accurate estimation of earthworm diversity. Finally, the use of IOTUs enabled us to accurately represent the diversity of earthworms, with DNA barcodes accounting for 68.6 % of the total number of taxonomic units.

4.2. Overall earthworm diversity

In our investigation, we identified a total of 51 taxonomic units across five families, but only two of these could be classified to the species level. Other studies have also found high frequency of enigmatic or unknown species in collections of tropical earthworms, due to their high diversity and endemism, such as in Amazonia (Decaëns et al., 2016; Lavelle and Lapied, 2003; Maggia et al., 2021). There were only two previous publications on earthworms in ADEs, and these were part of this same study (Cunha et al., 2016; Demetrio et al., 2021a). Nonetheless, Cunha et al. (2016) presented only results from one region (Iranduba) and an additional site in Manaus, while Demetrio et al. (2021a) did not include DNA barcoding results. Furthermore, both studies confirm the unique status of soil fauna communities in ADEs compared with REF soils, which had already been reported for vegetation and microbial richness of these soils (Arroyo-Kalin, 2010; Grossman et al., 2010; O'Neill et al., 2009). Hence, the present study is more complete, and highlights the high species richness of the regional pools and the high number of unique species, some only discovered using molecular techniques.

The two identified species, *Pontoscolex corethrurus* (taxonomic unit 3) and *Dichogaster modiglianii* (taxonomic unit 11), are peregrine

earthworms (Hendrix et al., 2008; Plisko, 2001; Taheri et al., 2018). The first (*P. corethrurus*) is native to the Guyana plateau and has colonized most of the tropical regions of the world via human dispersal activities (Taheri et al., 2018), including extreme environments (Cunha et al., 2014). It is believed to have been dispersed across South America through human-mediated transport associated with the trade of agricultural products (Hendrix et al., 2008; Marichal et al., 2010; Ortiz-Gamino et al., 2016). Today, it is now widely distributed in Brazil (Brown et al., 2006), and appears frequently in samples collected in the Amazon, especially in disturbed environments (Cunha et al., 2016; Lavelle and Lapied, 2003; Marichal et al., 2010). *Dichogaster modiglianii*, on the other hand, is of unknown origin, possibly from central Africa as suggested for some other members of the same genus (Rota and Schmidt, 2006) and probably was also introduced to South America through human-mediated transport (Hendrix et al., 2008). Although it had previously been collected in Brazil, such as in Mato Grosso, Amazonas and Roraima (Righi and Guerra, 1985; Righi et al., 1978; Ayres and Guerra, 1981; Righi, 1984a, 1984b, 1990) this study reports the first finding of *D. modiglianii* in Rondônia. Given the complexity and diversity of the Amazon forest ecosystem, it is justifiable to hypothesize that the reasonable number of unidentified species could represent natives to the area and potentially novel to science. However, it is crucial to conduct additional taxonomic investigations to provide formal descriptions and classifications for these species.

Of the total taxonomic units, one third were from the Central region (Iranduba), 18 % from Southwestern (Porto Velho) and 39 % from the Lower (Belterra) Brazilian Amazonian (Fig. 4B). The lower number of taxonomic units found in the Southwestern Amazon region in the study may be due to the more limited number of land uses, as only agricultural and young forest sites were sampled, with no samples taken from old forests (>20 years). The other taxonomic units that were found in only two regions (i.e., 4 % Iranduba and Belterra; 2 % in Belterra and Porto Velho) probably represent widespread native species, as there are a few of these in Brazil (Brown and James, 2007). The low overlap of taxonomic units and high number of rare and singleton species observed between regions in the present study could confirm the high endemism rate (>90 %) reported previously by Lavelle and Lapied (2003) and Maggia et al. (2021) for the Amazon region, however this must be complemented with a more intense sampling effort. In fact, the high proportion of singletons, the rarefaction curves and richness levels in our results suggests the under sampling of rare species and highlight that more species could occur in the areas of study (Coddington et al., 2009; Lim et al., 2012).

4.3. The unique pool of earthworm species in ADE soils

The limited overlap of only 20 % of taxonomic units between REFs and ADEs, combined with the discovery that 1/3 of the units were exclusively found in ADEs, highlights the importance of these sites as species reservoirs in the Amazonian rainforest. These results could also be explained by a tenacious footprint driven by human-induced landscape changes, which have altered soil fertility and plant communities throughout much of the region (Grossman et al., 2010; Levis et al., 2018). In fact, our study indicates that ADE as an environment explains the variability in the earthworm communities in terms of their species composition, richness, and distribution. Overall, these findings support the notion that human activities have far-reaching consequences on soil biodiversity, including earthworm communities, which play a vital role in soil health and ecosystem functioning. The selection processes of earthworm species in ADEs likely began with habitat interference/disturbance by the Amerindian societies, (including forest clearance, human settlement, slash and burn agriculture, and management of the soil and vegetation cover), followed by the reduction in populations of sensitive native species, population growth and/or introduction of opportunistic species and finally, the colonization of vacant niche spaces by some opportunistic and/or exotic species (González et al., 2006; Hendrix et al., 2006). Interestingly, a relatively high number of native and undescribed taxonomic units were found in ADEs, despite intensive modification of their habitat (slash and burn agriculture, human settlement) and of the soil environment (higher pH, P and Ca contents), over centuries of Amerindian use (Lehmann et al., 2003a, 2003b; Neves et al., 2003; Smith, 1980). Soil characteristics of ADEs are very different from the natural REF soil conditions due to input of animal bones and organic materials, which may impose evolutionary constraints to native soil biota in the Amazon (Demetrio et al., 2021a). The high species turnover observed between ADEs and REF soils, as well as the high turnover associated with land use change for both soil categories, mainly in REF soils, is not surprising. These findings suggest that ADEs and REF soils have distinct soil biota due to differences in soil characteristics and historical land use practices that can also be related to regional differences in soil and land management by human settlement over time (Kern et al., 2017).

The two morphospecies identified at the species level, *D. modiglianii* and *P. corethrurus*, are well-known widespread exotics. While *P. corethrurus* was the most abundant species found in both soil categories, only *D. modiglianii* was exclusively present in the ADEs across all three regions. This indicates that *D. modiglianii* could be considered an indicator species for ADE soils. Cunha et al. (2016) suggested an important role of earthworms, particularly *P. corethrurus*, in soil processes and the genesis and ecosystem functioning of ADEs, in line with previous predictions by Ponge et al. (2006). Moreover, *P. corethrurus* is an earthworm that can ingest, process and transform charcoal, reinforcing a possible functional role in these soils (see Ferreira et al., 2021; Ponge et al., 2006). However, despite the fact that earthworms have been major soil bioturbators and have likely been influencing the soil properties and processes of ADEs since their formation over 6.000 years ago (Watling et al., 2018), there is currently no available information regarding their functional role in these anthropic soils.

4.4. Impact of modern and ancient land uses on earthworm communities

Modern agricultural practices, including the use of soil tillage and pesticide application have well-known detrimental effects on both earthworm richness and density (Briones and Schmidt, 2017; Pelosi et al., 2014), which could explain the lower values for both in AS on both REF and ADE sites. Additionally, this confirms previous observations about the negative effects of land use change and intensification on earthworm communities in the region (Barros et al., 2004; Decaens et al., 2018; Fragoso and Lavelle, 1992; Marichal et al., 2014). Deforestation and soil disturbance tend to negatively affect forest earthworms, mainly native epigeic and anecic species (Barros et al., 2004; Decaëns et al., 2004; Fragoso et al., 1997; Paoletti et al., 1991, 1988), due to the decrease in available food and changes in the soil environment (i.e., lower soil moisture and higher temperature due to the absence of a litter layer and no tree cover). In addition, converting forests into agricultural fields cultivated with annual crops (like corn and soybeans) affects earthworms more than permanent pastures, due to the higher level of soil disturbance and the use of pesticides (Decaëns et al., 2004; Fragoso et al., 1997; Lavelle and Pashanasi, 1989).

Earthworm density and biomass from all ADE sites were similar to the MOF areas of REF soils, contrasting with a smaller number of species found in ADE sites. This reinforces the hypothesis that earthworm communities in ADE soils, like recently converted agricultural soils are dominated by colonizer species, probably *r*-strategists, able to quickly populate disturbed environments (Satchell, 1980). This can be exemplified by the case of *P. corethrurus*, a cosmopolitan peregrine earthworm in the tropics which has been successfully introduced worldwide (Taheri et al., 2018). This endogeic earthworm tolerates a wide range of biotic and abiotic environmental conditions (Lavelle et al., 1987), and is an opportunist colonizer after forest clearance (Marichal et al., 2010). Overall, unlike most native species, colonizer earthworms show high ecological plasticity, being able to survive under a wide range of soil and habitat conditions, with variable contents of sand or clay and high or low soil organic matter content (González et al., 2006; Lavelle et al., 1987). Furthermore, species turnover due to soil factor (ADE vs. REF) in MOF was close to 50 %, indicating that even in these old secondary forests, changes could be due to previous Amerindian occupation and more traditional land uses such as slash and burn agriculture, practiced over centuries in ADE sites (Maezumi et al., 2018)

Despite this, soils with ADEs, due to their intrinsic characteristics, managed to maintain high earthworm numbers compared to REF soils. Furthermore, earthworm abundance was similar under all three land uses on ADEs. The higher nutrient resources (particularly soil organic matter) in ADEs, as well as the additional microhabitats created by abundant charcoal and pottery fragments may be important in these phenomena, though the direct relationship between these Amerindian soil additives and earthworms have not yet been tested experimentally (Cunha et al., 2016).

Hence, earthworm populations seem to be more resistant to land use modification in ADEs than REF soils, although nothing is known of the functional consequences of these changes, which deserve further attention. A better description and increasing sampling of the earthworm communities across a broad range of ADEs and reference soils in Amazonia, accompanied by more detailed studies (field, laboratory and greenhouse), on the functional roles of earthworms in these soils is necessary in order to improve the conservation and sustainable management of ADEs throughout Amazonia and to further understand their role as reservoirs for a unique pool of species.

5. Conclusions

This study provides valuable insights into the current earthworm species assemblies in areas impacted by both ancient and modern human activities in the Amazon rainforest. Our findings indicate that ancient human impacts could offer an explanation to changes in diversity and species richness, however not affecting their abundance. ADEs were found to host a unique pool of earthworm species, highlighting the importance of further research into the roles of these communities in this special soil environment and how ADEs might offer a reservoir for species with elevated importance for the conservation of biodiversity in Amazonian forests.

The present study integrated information from conventional taxonomy with DNA barcoding, increasing accuracy and efficiency of our earthworm diversity mapping, and confirming its usefulness as a tool to enhance the reliability of earthworm species assessments in the tropics (with the identification of juveniles and cocoons).

Modern (recent) land use, as already observed in other studies, affected both earthworm abundance and its diversity, though the impacts appeared to be attenuated in ADEs, likely due to the higher organic matter contents.

Nevertheless, we advocate that more research is needed to confirm some of our results and explore the long-term impacts of both ancient and modern human activities on earthworms in the Amazon rainforest. Specifically, future studies should consider landscape factors such as habitat fragmentation, disturbance size and extent, and how these factors interact with earthworm communities and ecosystem functionality. Monitoring these over time would also allow for a better assessment of short-versus long-term impacts of modern anthropogenic activities in both ADE and reference soils. Such research is crucial for developing effective conservation strategies and maintaining the biodiversity and ecosystem functioning of the Amazon rainforest.

CRediT authorship contribution statement

Ana Caroline Conrado Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing: original, review & editing; Willian C. Demetrio Data curation, Formal analysis, Validation, Writing: review & editing; David W.G. Stanton, Validation, Writing: original, review & editing; Marie L.C. Bartz Investigation, Writing: review & editing; Samuel W. James, Investigation, Writing: review & editing; Alessandra Santos, Investigation, Writing: review & editing; Elodie da Silva, Investigation, Writing: review & editing; Talita Ferreira, Investigation, Writing: review & editing; Agno N.S. Acioli, Investigation, Writing: review & editing; Alexandre C. Ferreira, Investigation, Writing; review & editing; Lilianne S. Maia, Investigation, Writing: review & editing; Telma A.C. Silva, Investigation, Writing: review & editing; Patrick Lavelle, Investigation, Writing: original, review & editing; Elena Velasquez, Investigation, Writing: review & editing; Sandra C. Tapia-Cora, Investigation, Writing: review & editing; Aleksander W. Muniz, Investigation, Writing: review & editing; Rodrigo F. Segalla, Investigation, Writing: review & editing; Thibaud Decaëns, Investigation, Writing: original, review & editing; Herlon S. Nadolny, Investigation, Writing: review & editing; Clara P. Peña-Venegas, Investigation, Writing: review & editing; Amarildo Pasini, Investigation, Writing: review & editing; Raimundo C. de Oliveira Júnior Writing: review & editing; TPI Network N/A; Peter Kille, Funding acquisition, Writing: review & editing; George G. Brown, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing: original, review & editing; Luís Cunha Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing: original, review & editing.

Data availability

All sequence data used in this study are available in the BOLD database under the project code DS-EWTPI.

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.

Acknowledgments

The study was supported by the Newton Fund and Fundação Araucária (grant Nos. 45166.460.32093.02022015, NE/N000323/1), Natural Environment Research Council (NERC) UK (grant No. NE/M017656/1), a European Union Horizon 2020 Marie-Curie fellowship to LC (MSCA-IF-2014-GF-660378), and DWGS (No. 796877), by CAPES scholarships to WCD, ACC, TF, RFS, AF, LM, HSN, TS, AM, and RSM (PVE A115/2013), Araucaria Foundation scholarships to LM, AS, ACC, and ES, Post-doctoral fellowships to DWGS (NERC grant NE/M017656/1) and ES (CNPq No. 150748/2014-0, 165702/2015-0), PEER (Partnerships for Enhanced Engagement in Research Science Program) NAS/USAID award number AID-OAA-A-11-0001—project 3-188 to RMF and by CNPq grants, scholarships, and fellowships to ACF, GGB, RF, SWJ, CRC, EGN, and PL (Nos. 140260/2016-1, 307486/2013-3, 302462/2016-3, 401824/2013-6, 310690/2017-0, 303477/2018-0, 307179/2013-3, and 400533/2014-6). We thank INPA, UFOPA, Embrapa Rondônia, Embrapa Amazônia Ocidental, and Embrapa Amazônia Oriental and their staff for logistical support and the farmers for access to and permission to sample on their properties. Sampling permit No. 18131-6 for Tapajós National Forest was granted by ICMBio.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165087.

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