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Global warming: an attempt to reconstruct earthworm paleohistory with eDNA

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“Worms have played a more important part in the history of the world than most persons would at first suppose”.

— *The formation of vegetable mould through the action of worms*, Charles Darwin —

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List of papers

This thesis is comprised of four papers, which are referred to by Roman numerals.

- I. *The topological nature of tag jumping in environmental DNA metabarcoding studies.*
Rodriguez-Martinez, S., Klaminder, J., Morlock, M. A., Dalén, L., & Huang, D. Y. T. (2023). *Molecular Ecology Resources*, 23(3), 621-631.
- II. *Tag jumping produces major distortion on metabarcoding-based reconstructions of past and present environments.*
Rodriguez-Martinez, S., Morlock, M. A., Huang, D. Y.-T., Klaminder, J. Manuscript.
- III. *Erosion regime controls sediment environmental DNA-based community reconstruction.* Morlock, M. A.,
Rodriguez-Martinez, S., Huang, D. Y. T., & Klaminder, J. (2023). *Environmental DNA*, 5(6), 1393-1404.
- IV. *Paradoxical views on earthworm natural history leads to contrasting interpretation of environmental data within Fennoscandia.*
Rodriguez-Martinez, S., Polvi Sjöberg, L., Jonsson H., Klaminder, J. Manuscript

Authors contributions

Chapter I:

SRM and JK designed the study. **SRM**, MAM and DYTH collected the data. **SRM** analysed the data and wrote the manuscript with the collaboration of all co-authors (**SRM**, MAM, DYTH, LD and JK).

Chapter II:

SRM and MAM designed the study in collaboration with JK. **SRM**, and MAM. DYTH collected the data and performed the analysis. **SRM** wrote the manuscript in collaboration with all co-authors (**SRM**, MAM, DYTH and JK).

Chapter III:

MAM and JK designed the study. MAM, **SRM**, DYTH and JK contributed to the data collection, analysis, and interpretation. All authors contributed to the writing of the manuscript.

Chapter IV:

SRM and JK outlined the theory of the study. **SRM** and **LP** collected the data, and **SRM** performed the data analysis and wrote the manuscript with collaboration of all co-authors (**SRM**, **LP** and **JK**).

Author abbreviations:

SRM: Saúl Rodríguez-Martínez; **MAM**: Marina Alexandra Morlock; **DYTH**: Doreen Yu-Tuan Huang; **LD**: Love Dalén; **LP**: Lina Polvi; **HJ**: Hanna Jonsson.

Abstract

Earthworms are soft tissue organisms that rarely leave fossils that can be used to identify species. Absence of fossils makes the natural history of earthworm species in post-glacial landscapes of Fennoscandia largely unknown. Analyses of environmental DNA (eDNA) preserved in natural archives such as lake sediments and buried soil layers (paleosols) may offer an opportunity to assess the composition of past earthworm communities. In this thesis, I explore the use of metabarcoding as an analytical method to detect DNA from earthworms that lived in past European environments. I aimed at extracting DNA from various forms of paleosols in Europe and lake sediments, but earthworm DNA is rare in these deposits and amplifying DNA from this group of soil fauna was largely unsuccessful. However, during the scientific progression of my work, I discovered that metabarcoding-based studies are sensitive to ‘tag jumping’, which is a process where sample specific labels (tags) added to sequences for identification of individual samples ‘jump’, resulting in cross-talk between samples. My results suggest that tag jumping i) is mediated by the formation of heteroduplexes (DNA with two strands from different samples), ii) affects interpretations of eDNA studies by adding species to samples where they were not originally present, and iii) makes eDNA assemblages more similar. Importantly, my results also highlight that metabarcoding can generate powerful and trustworthy reconstructions of past environments if conducted with protocols that remove the influence of tag jumps. Reconstructions of terrestrial organisms from eDNA in sediments are also enhanced by erosion events that amplify DNA signals of land-living organisms. I conclude that earthworm DNA is difficult to detect in natural archives using current metabarcoding techniques and that tag jumping, a problem rarely discussed in metabarcoding studies, constitutes a concern in parity with direct sample contamination.

Introduction

A big leap for a small animal

“Any environment, any single life is in a continuous state of change. This is just more obvious when you pay attention to earthworms. Their work may seem unspectacular at first. They don't chirp or sing, they don't gallop or soar, they don't hunt or make tools or write books. But they do something just as powerful: they consume, they transform, they change the earth”.

— *The Earth Moved: On the Remarkable Achievements of Earthworms*, Amy Stewart —

Human activities have increased the rate and scale of biological invasions to extraordinary levels (Pauchard et al., 2016; Ricciardi, 2007). Belowground invasions are particularly difficult to detect, which often results in lack of early information about the spatial and temporal dynamics of the invasion, lowering the potential for later countermeasures and management strategies (Abbott, 2006). This is the case of European earthworms in North America. Despite being introduced by early colonizers and dispersed in forests for centuries, their ongoing invasion of the North American continent was only realized in late 20th century (Frelich et al., 2006; Hendrix et al., 2008; Langmaid, 1964). It is now well established that invasive earthworms have altered the soils for centuries over large areas in the USA and Canada (Mathieu et al., 2024).

Earthworms as invasive species may appear harmless or even beneficial given their positive impacts in agriculture and gardening. The positive effects from earthworm activities in these settings include improved nutrient availability (Van Groenigen et al., 2019), increased crop production (Van Groenigen et al., 2014) as well as increased in-mixing of soil organic matter (Caravaca and Roldán, 2003; Zhang et al., 2013) and formation of aggregates (Lavelle, 1997), conditions that improve soil aeration (Bertrand et al., 2015). Nevertheless, earthworm impacts on ecosystems may not always be as positive from an ecological standpoint. Some earthworm species dry out soils (Ferlian et al., 2020), compact the soil (Blouin et al., 2013), disturb nutrient cycles (Ferlian et al., 2020; Resner et al., 2015), modify plant communities (Craven et al., 2017; Hale et al., 2006), and alter plant palatability with intensified grazing as a result (Jonsson et al., 2023). The numerous ways invasive earthworms can disturb ecosystems at various trophic levels has been highlighted as a significant environmental problem (Frelich et al., 2019). Given the potential ecological effects from earthworms, their natural history in Fennoscandia becomes

crucial for understanding how they affected past conditions, influence present conditions, and will change future environments.

In North America, earthworms were eradicated during the last glaciation (Gates, 1982; Tiunov et al., 2006). Although northern Europe has a similar glacial history as North America, it is rarely discussed to what extent earthworms can be invasive in Fennoscandia and how and when they arrived in the post-glacial landscape. Nevertheless, a recent theory proposes that at least some of the earthworm species present in Northern Fennoscandia were introduced through anthropochory (Blume-Werry et al., 2023) – i.e., the distribution of cultivated plants and/or seeds by humans. This latter view suggests that the land-use practices of humans are key vectors for the dispersal of earthworms and highlights that some of the species in contemporary Fennoscandian environments could have been introduced relatively recently. This view that earthworms may have been introduced by humans relatively recently opposes the more accepted view that earthworm populations are controlled by the intrinsic properties of ecosystems, including soil texture, climate, and vegetation (Lundmark, 1988, 1986). In this latter theory, earthworms inhabit soils that are suitable for them, so earthworm species in Fennoscandian forests are considered to be part of natural processes rather than possibly of anthropogenic origin. Determining which earthworm species have been introduced recently by humans in the Fennoscandian environments is difficult as little is known about the post-glacial migrations of earthworms. Earthworm history in the post-glacial landscape of northern Europe has only been studied in one paleoecological study, where DNA from a litter dwelling earthworm (*Lumbricus variegatus*) was detected in sediments deposited 10,800 years ago (Lammers et al., 2019). However, Lammers et al. (2019) conclude that *L. variegatus* could possibly be another undescribed species, making it somewhat unclear if a terrestrial earthworm species was indeed present shortly after deglaciation in Norway.

These conflicting views of the history of Fennoscandian post-glacial earthworm species, which have been shown to cause negative impacts in North American forests (mainly mineral soil dwelling earthworm species), emphasize how little we know about the origin of earthworm species in northern Europe. In fact, two contrasting post-glacial migration theories are present in published literature. One theory largely mimics that outlined for North America: soil macrofauna in Fennoscandia was eradicated during the last glacial period (Julin, 1949), a presumption that has been implicit in studies claiming that mineral soil earthworms in Fennoscandian ecosystems were likely brought there by the first human settlers or through modern land-use practices (Blume-Werry et al., 2023; Wackett et al., 2018). A contrasting theory suggests that earthworms survived in glacial refugia in the Scandes and that

some species listed as non-native and potentially invasive such as *Lumbricus rubellus* might have a native origin that predates deglaciation of Fennoscandia (Stop-Bowitz, 1969). So when did different earthworm species come to Fennoscandia? Did they arrive as the result of human activities? These questions need to be answered before we can judge the extent earthworms that are currently re-shaping soils in Fennoscandia are truly native. Considering that earthworm DNA has been detected in ancient sediments (Lammers et al., 2019), paleoecological studies based on metabarcoding approaches seem to be promising avenues for reconstructing the presence of earthworms in past environments.

DNA metabarcoding

During the last decade, sequencing techniques have greatly improved, and their applications have increased in variety and reliability. Environmental DNA (eDNA) extracted from environmental matrices (sediments, soils, water, and air) has been used as a source of information to describe both contemporary and past environments (Fernández et al., 2018; Haile et al., 2009; Kjær et al., 2022; Reich et al., 2023). Metabarcoding – i.e., multispecies identification using sequenced eDNA extracted from soil, water, or sediments (Taberlet et al., 2012) – has been shown to be a powerful and accurate way to retrieve eDNA data and reconstruct plant and mammal communities inhabiting past and present environments. For example, metabarcoding has been successfully used to reconstruct environments in the Holocene and beyond (Alsos et al., 2021; Clarke et al., 2019; Lammers et al., 2019; Zale et al., 2018). However, the technique is still being perfected (Zaiko et al., 2021).

Metabarcoding is an automated way to identify small fragments of DNA from multiple species present in a sample (Taberlet et al., 2012). These small fragments are first amplified in a polymerase chain reaction (PCR) and then sequenced. The resulting sequences are then compared to a database to match the DNA to specific species. This process allows for the reconstruction of whole communities of target groups with a relatively inexpensive and non-invasive sampling technique. In the pioneer works finding earthworm DNA in lake sediments, a general mammal (16S) primer was used (Lammers et al., 2019). However, primers specifically targeting earthworms already exist (Bienert et al., 2012), indicating the use of a more specific primer could increase the likelihood of retrieving earthworm DNA from natural sedimentary archives. Thus, metabarcoding has the potential to reconstruct past earthworm communities and bring light to their nativity in Fennoscandia. In addition, metabarcoding could potentially link these earthworms to events caused by the arrival of humans.

As mentioned before, there are still some errors affecting metabarcoding that need to be purged. One source of error is caused by a process referred to as tag jumping (Schnell et al., 2015). High throughput sequencing can handle many samples at the same time and tag jumping may affect the integrity of ‘labels’ used to identify individual samples in pooled datasets. In pooled datasets, each DNA amplicon is labelled (tagged) by adding a pair of short but unique oligonucleotide sequences (hereafter referred to as tags) to the 5’ end of both primers in a PCR reaction (Binladen et al., 2007). Tagging pipelines giving each sample a unique set of tags make it possible to identify each sample during the posterior separation processes. Here, tags leave specific marks in the amplicons that can be identified during the later bioinformatics (demultiplexing) step and used to backtrack the sequence to its original sample. This tagging technique is a well established approach that has been applied on high throughput screening platforms (e.g., Quéméré et al., 2013; Shehzad et al., 2012); however, these tags can be changed during the tag jumping process, resulting in sequence translocations and cross contamination of the data.

Tag jumps are not the only source of ‘errors’ that affect metabarcoding studies. In the context of eDNA-based ecosystem reconstructions, lake sediments are probably the most used natural archive (e.g., Alsos et al., 2021; Clarke et al., 2019; Ficetola et al., 2018), but the reliability of the sedimentary eDNA record has also been questioned (Birks and Birks, 2016). The sediment-derived errors listed by Birks and Birks (2016) can be grouped into four main causes of uncertainties: i) strength of the DNA source; ii) variable transport vector of DNA to the sediment record; iii) impacts from sediment diagenesis (decomposition and post-depositional mobility); and iv) analytical uncertainties. Noteworthy, the latter source of uncertainty was believed to be caused primarily by methodological steps (e.g., sampling and DNA extraction), and Birks and Birks (2016) did not specifically mention tag jumps as a cause of analytical uncertainties. Similar sources of errors have also been highlighted as a weakness of biodiversity assessments based on sedimentary eDNA (Edwards, 2020; Giguet-Covex et al., 2019). Nevertheless, lake sediment records usually show good fit between DNA in the sediment and known historical changes in the landscape, and plants near lake shores seem to be a dominant source of plant DNA (Alsos et al., 2018; Niemeyer et al., 2017). Studies using other proxies (e.g., pollen) also seem to support the idea the main contributor of plant material to the sediments are the communities near the shore line (Jørgensen et al., 2012; Parducci et al., 2019; Pedersen et al., 2013; Sjögren et al., 2017).

Earthworms are terrestrial organisms, and it seems plausible that eDNA from ancient earthworm communities is better preserved in buried soil layers (paleosols)

if these layers functioned as earthworm habitats in the past. However, earthworm DNA has been shown to be preserved over many millennia in lake sediment records (Lammers et al., 2019), findings that suggest transport vectors of earthworm DNA to lakes exist and that their DNA can be preserved for a long time. Lake sediments are the matrix of choice for environmental DNA-based reconstructions as these sediments, in contrast to paleosols, and provide a near-continuous chronology of DNA. I write ‘near-continuous’ here as within-catchment transport vectors of DNA do change in strength over time (Birks and Birks, 2016); hence, temporal changes in transport vectors of DNA and sediment diagenesis may influence the eDNA signal preserved in lake sediments. The probability of detecting the DNA of terrestrial organisms (e.g., mammals) in river water is affected by somewhat different factors than for plants, such as the use of near shore habitats and the slope of the terrain or precipitation intensity (Lyet et al., 2021). This latter study also suggests that sampling during heavy rain or snowmelt could increase the probability of detection of mammal DNA, suggesting that erosion rates could affect the input of DNA into the water. Physical erosion can rapidly transfer soil from distant sources into a lake, potentially altering the DNA pool of the sediment record by bringing in terrestrial DNA from more distant areas. For example, it has been shown that the depth of eroding soil layers determines the abundance of plant DNA in lake sediments (Giguet-Covex et al., 2019). Given that soil erosion may increase both the source area and strength of terrestrial DNA signal, it seems rational to expect that the likelihood of detecting earthworm DNA increase in lakes situated in catchments with high erosion rates.

Aims of the thesis

This thesis uses metabarcoding analysis of lake sediments and paleosols deposits to determine what earthworm species were present in the Fennoscandian environment before the arrival of modern humans. The initial research objective was to determine what earthworm species were present in ecosystems before the known onset of agriculture in northern European ecosystems, which is about 8000 years ago in central Europe, about 1000 years ago in Iceland, and about 200 years ago in the northernmost parts of Sweden. In other words, I aim at providing results that could be used to resolve what species can be considered ‘truly’ native in Northern European environments. However, research does not always go as planned. I realized this the hard way and outline below what I discovered during the processes of trying to extract earthworm DNA from soils and sediments. Specifically, I ended up answering four other research questions when searching for earthworm eDNA in lake sediments and buried soil layers:

1. The main underlying cause to tag jumping through its signature patterns **(I–II)**.
2. The effect of tag jumping on metabarcoding data **(I–II)**.
3. The importance of sediment origin on the eDNA record **(III)**.
4. What is known about the postglacial earthworms migrations Fennoscandia **(IV)**.

Methodology

For these studies, we collected samples from Lake Grossee, Flumserberg, Eastern Switzerland with a percussion piston coring system (Uwitec, Austria) in 2017 (**I-III**) and seven other lakes across the Central Alps in 2020 (**III**), and a sedimentary soil profile developing about 10 km from Abisko Scientific Research Station (**I**) in 2019 and did a literature review about earthworm migrations and effects (**IV**).

Samples collection

To study how sediment origin influences sedimentary DNA results (**III**), we retrieved a sediment sequence spanning the Holocene from Lake Grossee, an alpine lake in Flumserberg, Switzerland (47°04'43.8" N 9°14'47.1" E at 1619 m.a.s.l.). In 2017, we recovered a sediment core from the lake's central basin using a percussion piston coring system (Uwitec, Austria). Cores were split lengthwise and aligned to a composite of 7.73 m as described elsewhere (Glaus, 2018) and sampled at 14, 132, 232, 235, 352, and 529 cm of composite depth (**I-III**). In 2020, we used a gravity corer (Uwitec, Austria) to collect short sediment cores from depocenters of seven lakes in the Central Alps (**III**).

In Lake Grossee, organic-rich sediments are interpreted to represent the background sedimentation, whereas reddish siliciclastic beds are interpreted as flood event deposits (Glaus, 2018). The Holocene record contains more than 100 comparable flood layers, ranging from 0.2-cm to 10-cm thick. Nine of these event layers were sampled together with adjacent background sediments. To assess the influence of sediment origin on the DNA composition, we visually identified event layers in the short cores based on characteristics such as fining-upward sequences, high minerogenic content in contrast to organic-rich surrounding sediment, and the local abundance of plant macrofossils.

The other set of samples originated from a paleosol developing about 10 km from Abisko Scientific Research Station (68°21'17.0" N; 18°48'54" E), where both soils and earthworms have previously been described in detail (Wackett et al., 2018). Soil samples were taken at 30, 90 and 220 cm below the surface (**I**).

From three subsamples from each depth, we extracted 0.5 g of material using the DNeasy Power Soil kit (QIAGEN). DNA extractions were carried out in a dedicated ancient DNA laboratory at Umeå University, which is isolated from other modern DNA laboratories and has a positive air pressure system accompanied by a HEPA air filter system. We followed protocols to avoid contamination during the

extraction process (Paijmans et al., 2019), including using extensive personal protective gear (facemask, gloves, and clean suit). Multiple extraction blanks and control amplifications were processed alongside all samples to screen for potential contamination (**I–III**).

Molecular methods

Two tagging approaches were used for these studies: a combinatorial tagging approach (**I–II**), where tags on each end are reused to form different combinations, and a twin tagging approach (**II–III**), where tags are not reused, so each sample had the same tag on both ends. Applying these two approaches to the same samples allowed us to compare the performance of both methodologies, to assess the tag jumping mechanism (**I**), and to estimate its effects on metabarcoding datasets (**II**).

DNA from all extracts, including extraction controls, was amplified in three PCR replicates using different primer sets. Amplicons were pooled equivolume (one pool per library) and purified with a QIAQuick PCR purification kit (QIAGEN). Library preparation was done by Novogene following the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB #E7645) without the PCR enrichment step. Samples were sequenced on an Illumina platform.

After sequencing, the quality of the data was assessed with fastQC (Andrews, 2010), and libraries were further processed with the OBITools3 pipeline (Boyer et al., 2016). Here, we put the emphasis on the demultiplexing as it is the key step affected by tag jumping (**I–II**). To avoid confusion of tags due to sequencing errors, we set the demultiplexing to allow no mismatches in the tags. Sequences were then assigned to a 97% similarity to databases derived from the National Centre for Biotechnology Information (NCBI) and filtered by the targeted genomic region. All taxonomic classifications follow the NCBI taxonomy (Schoch et al., 2020).

Results and Discussion

My results show that tag jumping is an important source of bias that likely has been underestimated in the literature (**I–II**). In these two first chapters, I outline the mechanism behind tag jumping and use an alternative tagging system to completely prevent the effects of tag jumping to quantify the distortion created by tag jumping in real datasets (**I–II**). I also present results showing how the origin of sediments affects terrestrial eDNA signals in lake sediments and discuss its importance in the context of paleoreconstructions (**III**). Finally, I discuss two contrasting theories regarding the post-glacial migration history of earthworms in Fennoscandia (**IV**). A more detailed description of my findings follows below.

Tag jumping: cause and effect (I-II)

“Then it doesn't matter which way you walk . . . – so long as I get somewhere.”

— *Through the Looking-Glass and What Alice Found There*, Lewis Carroll —

There is a gap in literature when it comes to tag jumping. Although the process has been previously described (Esling et al., 2015; Schnell et al., 2015), the molecular origin of tag jumps and the magnitude of its consequences have not been sufficiently explored. I believe that this knowledge gap has caused a lack of awareness in the scientific community. To fill this knowledge gap, I first tried to disentangle the molecular origin of tag jumping (**I**).

Previous studies have identified four mechanisms that cause tag jumping: (1) mixed clusters, also called cluster bleeding (Kircher et al., 2012); (2) cross-contamination of primers with different tags, either during synthesis or laboratory handling (Kircher et al., 2012); (3) chimera amplification during library enrichment (Kircher et al., 2012); and (4) DNA polymerase with 3'-5' exonuclease activity during end repair (Schnell et al., 2015; van Orsouw et al., 2007). Only the third and fourth suggested mechanisms are heteroduplex mediated. That is, for tag jumping to happen through chimera amplification or exonuclease activity during end repair, two single stranded DNA molecules of different amplicons must interact. This interaction leaves a particular topological signature on the data as the heteroduplex formation is an interaction that allows one to model and estimate the number of jumps in the data. This topological signature of tag jumping becomes visible only when the sequenced results are arranged following what we call a tagging matrix, an arrangement of samples and blanks based on their tag combinations. In this matrix, the columns represent one of the tags and the rows represent the other tag, leaving the samples at

the intersections. When arranging samples in a matrix like this, a heteroduplex driven process has two characteristics: a) the tag jumps form ‘rook patterns’ locked within a column or a row where one of the tags is shared, never forming diagonal lines, and b) tag jumps positively correlate to the number of interactions within their respective column and row. That is, taking a tile of the matrix, the incoming jumps are the product of the reads on its row times the reads on its column. If the noise in the data presents these two characteristics, we can conclude that the process is driven by a heteroduplex formation. To disentangle which of the two plausible heteroduplex driven processes caused the jumps, we need to look at the applied analytical pipeline. Chimera amplification requires a PCR reaction to happen on the pooled samples, and exonuclease activity needs a polymerase with exonuclease capacity to be used during end repair. If both of these conditions are present in the pipeline, they cannot be disentangled without further experiments. However, because we did not conduct a second PCR, we could attribute any heteroduplex driven tag jump process to the T4 polymerase activity during end repair.

Using the reasoning outlined above, I constrained the molecular cause of tag jumping in my datasets to the activity of the T4-polymerase used during end-repair **(I)**. In line with the proposed heteroduplex model, I found counts of several species forming horizontal and vertical lines in the tag’s matrix. Using the blanks embedded in the tagging matrix, I could show a strong positive correlation between the incoming tag jumps in a tile and the amount of reads on its row and column. The latter finding in combination with the observed rook patterns proved that tag jumping is a heteroduplex driven process that occurs in absence of a second PCR of the pooled samples. Therefore, the observed tag jumps were driven by the T4 DNA polymerase used for end repair. Moreover, I used the positive correlation between tile position and reads in my blanks to estimate the impacts of tag jumps in my samples and group them into four categories based on the proportion of inferred tag jumps: (1) unreliable detections, (2) uncertain detections, (3) reliable detections, and (4) detections outside the limits of the regression used to classify them. I estimate that between 40–80% of the samples were classified as unreliable due to tag jumping – a mind-blowing high proportion.

Having understood the underlying molecular mechanisms behind tag jumps, it became evident that there was one simple way to solve the issue. In this tagging matrix, molecules move following strict rules, and tag jumps can only move across one row or one column, just like the chess rook. The discovered ‘topological law’ of tag jumping implies that a smart arrangement of samples makes it impossible for incoming jumps to affect the results. If samples are arranged following the main diagonal of the matrix – following the bishop’s trajectory in chess – even when some

sequences turned into rooks and moved, they can never move into another sample and interfere with its results. That is, arranging samples in the main diagonal removes the impacts of tag jumps. Or, in this context, the bishop is mightier than the rook. With that topological law of tag jumping in mind, I proposed twin tagging as the way to completely prevent tag jumping effects (I). Note that some sequences will still jump out of their samples and will be lost, but they will never be mixed with another sample, which is much more detrimental for the results. This new insight also allowed me to produce affected tag jumping and tag jumping free libraries on will.

I produced two sets of libraries – one based on a combinatorial tagging protocol (affected by tag jumps) and one based on a twin tagging protocol (tag jumping insensitive protocol) – to further evaluate the consequences of tag jumping (II). Each dataset was composed of a library for plants and for mammals. It became evident that the twin tagging protocol generated more trustworthy data. All blanks and negatives were clean or showed singular species contamination as opposed to the combinatorial tagged protocol, where all blanks and negatives had virtually all taxa present in their libraries. The magnitude of the effects of tag jumping was also apparent when comparing the results of the two protocols. For example, average detection rates were higher in the combinatorial tagged datasets, which most likely was a byproduct of the ‘extra’ incoming DNA due to tag jumps and the subsequent increase in false positives. In the mammal datasets, the twin-tagged dataset had on average a detection of 7 (range 0–12) taxa per sample compared to 12 (8–15) taxa per sample on the combinatorial-tagged dataset. Similarly, the twin-tagged plants dataset had an average of 20 (14–30) taxa per sample, which was substantially lower than the 35 (13–52) taxa per sample as suggested by the combinatorial-tagged counterpart. This higher detection rate was not linked to higher sequencing depth but to a much higher spread of the same taxa over more samples. For example, taxa such as *Cervus* or *Sus* were constrained to a few of the samples in the twin-tagged dataset but were almost ubiquitous on the combinatorial-tagged dataset.

In contrast to my first study, where I estimated the impact of tag jumping, I could for the first time actually measure the impact of tag jumps on paleoecological and ecological measures. First, I calculated Bray-Curtis dissimilarities between the samples to check if artefactual reads generated by tag jumping affected the statistical differences in-between samples (II). Between sample differences were generally bigger in the twin-tagged datasets, which contained samples with lower intrasample variance and higher between sample differences. The permutational analysis of the variance (PERMANOVA) confirmed that of the 15 possible comparisons all but one couple were significantly different in the twin-tagged datasets (all significant for the plants), but only 8 were still different in the combinatorial-tagged datasets (12

significant for the plants). This reduction of differences was due to the difference in variance of the datasets produced by the tag jumps. In the mammal dataset, the variance using combinatorial tagging was 200% bigger, and in the plants datasets the increase was around 25%, suggesting that the latter dataset was somewhat more resilient to tag jumps. This is, the samples are more different on the twin tagging datasets because they are not influenced by other samples.

The findings described in my first two chapters clearly show that tag jumping is a major concern for metabarcoding-based studies (and possibly all molecular approaches using similar tagging protocols). However, my findings also show that trustworthy data can be collected by using twin tagging approaches, which solves the problems associated with incoming jumps.

Sediment origin and its impact on the eDNA record (III)

The idea that sediment origin can influence DNA composition is not new and has been highlighted as a driver of uncertainty in paleoecological reconstructions based on sedimentary DNA (Birks and Birks, 2016; Edwards, 2020; Giguet-Covex et al., 2019). However, as few studies have specifically tested this theory, the extent of the impact of erosion per se on sedimentary DNA remains uncertain. However, strong erosion events after heavy rainfalls or snowmelt have been suggested to alter the probability of detection of different species through eDNA (Lyet et al., 2021). Therefore, I found it reasonable to think that physical erosion impacts not only the DNA in the water column but also the DNA in the sediment itself. To solve my third main research question, I compared eDNA communities of sediments deposited during erosion events with communities reconstructed using sediment deposited without any morphological signs of erosion (III).

When I analysed the species composition of the sediment deposited during erosion events and the background sediment, I found that the compositions were significantly different even though they were deposited close to one another in the sediment and therefore deposited around the same time, so major changes in catchment communities were unlikely (III). The major differences between communities reconstructed from event layers and background sediments, just millimetres apart, were striking and repeatable for sediment deposited throughout the Holocene. The number of plant genera was higher in the event layers compared to the background sediments for the Holocene core and for the surface cores. There was an overlap of species detected in both kinds of sediments, but the event layers contained more than 30% more genera than the background sediments. However, a few genera were detected only in one of the two sediment types. An indicator species analysis revealed that genera specific to background sediments were mainly aquatic

or semi-aquatic, whereas those specific to event layers were terrestrial plants. Similarly, the results showed separation in the detection of mammals: roughly 50% of the taxa were found in both sediment types, with a small fraction only present in the background sediments and a larger number only in the event layers.

Clearly, sediment origin is important for the DNA signal detected in lake sediments and therefore changes in sediment transport and sources impact the recovered sedimentary eDNA community. The strong link between erosion events and the eDNA community seen in our study adds a new dimension to previously published studies. For example, several studies have linked specific taxa to erosion and used this as an argument for these taxa to be the cause of the erosion (Ficetola et al., 2018b; Giguet-Covex et al., 2014). Although this latter interpretation may be correct, our results also highlight that erosion affects the DNA input to the sediment record, so the relationship between taxa and erosion identified in previous studies could be the reversed. That is, erosion may deposit mammal DNA in the lake sediment only during extreme erosion event, so these animals are only detectable at times when erosion is high, but not necessarily causing it.

What do we know about the post-glacial history of earthworms in Fennoscandia?(IV)

“A bird does not give up flying today because it couldn’t find any worms yesterday.”

— Matshona Dhliwayo —

This project started with a simple question: How did earthworms arrive to the boreal regions of Europe? It did not seem complete without going back to that question at the end. I have been discussing metabarcoding and its caveats for the last few pages, and several caveats prevented me from answering this question, at least in a more orthodox experimental way. The truth is that I could not find a way to solve the case through the use of sedimentary DNA as I originally planned. However, I have compiled some circumstantial evidence from the literature that hints at when earthworms may have arrived in Fennoscandia (IV).

Glacial refugia for earthworms in southern Europe is believed to include areas outside the ice sheet (Julin, 1949), which expanded into Germany. The literature identifies evidence that many earthworm species dispersed very slowly and perhaps by humans. In North America, dispersion rates of 10–20 m/year⁻¹ have been calculated for *Lumbricus* Linnaeus, 1758; (Hendrix et al., 2008; Terhivuo and Saura, 2006). For *Aporrectodea* Orley, 1885, estimated migration rates in northern Fennoscandia are below 10 m/year⁻¹ (Wackett, et al. 2018). With the inferred migration rates of <20 m/year⁻¹ for *Lumbricus* and *Aporrectodea*, natural dispersion

would leave most of the post-glacial landscapes in northern Fennoscandia beyond their natural reach even at a Holocene timescale. In addition, natural barriers such as freshwater systems (Ancylus Lake and Baltic Ice lake) and brackish water systems (Yoldia Sea, Littorina Sea, and the Baltic sea), which surrounded the Fennoscandian peninsula for some periods, likely further hindered the northward progress of earthworms from Southern Europe. With the slow migration rate inferred from the North American continent in mind combined with glacial refugia in south central Europe, the presence of earthworms in Scandinavia should be marginal and constrained to the southernmost parts of Fennoscandia. This scenario is far from the actual situation in contemporary Fennoscandia. In the contemporary environment, populations of *Lumbricus terrestris* Hoffmeister, 1843, *Lumbricus rubellus* Linnaeus, 1758, and *Aporrectodea* spp. are all present in the northernmost regions of Finland, Sweden, and Norway. When I consider the slow migration rates of earthworms that would constrain their northern limit to southernmost parts of Sweden in combination with numerous evidence for the importance of anthropochory in contemporary environments (Hendrix et al., 2008), it seems evident that humans have mediated the dispersal of earthworms into northern Fennoscandian ecosystems. In this human centric model, earthworm migrations would be explained by a stratified dispersal (Shigesada et al., 1995), where they arrive to a new territory through long distance dispersion aided by humans and naturally expand their populations from those initial intrusions.

Although I cannot prove my theory of a primarily human centric dispersal models for some of the common earthworm species in Fennoscandia, previously published literature have found indications of human dispersal of earthworms. First, existing spatial gradients where earthworm abundances decrease with increasing distances from human settlements despite limited change in vegetation and climate are difficult to explain without viewing human settlement as the original introduction point (Wackett et al., 2018). Second, Jerand et al. (2023) found that relict soils in northern Sweden were unmixed by earthworms before the arrival of farmers. The fact that bioturbation of soils occurred after the arrival of farmers supports my own observations about the introduction of earthworms in Iceland. My observations of the paleosols of Iceland indicated that tephra layers deposited after the arrival of Viking farmers were bioturbated by earthworms but only in places where the soil was close to settlements. A third circumstance favours the hypothesis that the late arrival of earthworms in Northern Fennoscandia was likely due to human-mediated dispersal, an observation confirmed by local people living in the Scandes. Here, Sami village inhabitants describe that mineral soil dwelling earthworms arrived after the 1970s (Wackett et al., 2018). Similarly, the first farmers arriving in the northern Scandes (Njunjes, N. Sweden) noted that earthworms were not found when they

started farming at a site where *L. terrestris*, *L. rubellus* and *Aporrectodea* spp. are currently very abundant in the sub-alpine birch forest.

With all the above indications, analysis suggests that mineral soil dwelling earthworm species, such as *Lumbricus rubellus* and *Aporrectodea* spp., were likely not present in northern Fennoscandia before the arrival of agriculture and modern land-use (composts, flower beds and lawns). If this condition is enough to consider them ‘alien species’ in northern Fennoscandian ecosystem, despite that these species clearly had European glacial refugia, is up for interpretation. However, I note unwanted effects generated by earthworms in various ecosystems of the world (Hendrix et al., 2008), and advocate the precautionary principle as more research is needed about the impact of earthworms on the Fennoscandian environment.

Concluding remarks

Metabarcoding and other molecular tools still need to be perfected. For that matter, scrutiny of data and error reporting have key value. In recent years, molecular tools have become one of the most relevant set of techniques to understand the past and present, yet they are far from perfection. In this thesis, I contributed to the advance of metabarcoding studies by bringing light to one of its caveats – tag jumping – hoping that avoiding this problem will help others answer the questions that I could not.

Tag jumping, despite being rarely discussed in the literature, possess a major concern for metabarcoding data. My results contribute to the understanding of this issue and its causes and further quantify its impact and consequences for paleoecological datasets. I provide not only the explanations for the processes and a measure of the effects but also outline a simple solution to overcome the issue. In addition, I evaluated the impacts of erosion and lake sediment diagenesis on eDNA preserved in sediments, where I found a strong effect of erosion but not from lake sediment diagenesis. Together, my findings relating to tag jumping and erosion impact on sediment eDNA contribute to improved knowledge that hopefully will lead to more advanced reconstructions of past and present ecosystems.

Finally, I identified several indications that earthworms are present in northern Fennoscandia because of a dispersal history mediated by modern human transport. Although this is not a closed case, literature and observations strongly suggest that human transport vectors have and still do play an important role on earthworm dispersal. If earthworms indeed advanced into Fennoscandia via human transport, a considerable part of the soils in northern Fennoscandia evolved for millennia without earthworms.

“Alles hat ein Ende, nur die Wurst hat zwei.”
(“Allt har ett slut, men korven som har två.”)
(“Everything has an end, but the sausage that has two.”)
(“Todo tiene un final, menos la salchicha que tiene dos.”)

— German proverb —

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