Advances and prospects of environmental DNA in neotropical rainforests

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Running title: eDNA in Neotropical rainforests

Advances and prospects of environmental DNA in Neotropical rainforests

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Abstract: The rainforests of the Neotropics shelter a vast diversity of plant, animal and microscopic species that provide critical ecosystem goods and services for both local and worldwide populations. These environments face a major crisis due to increased deforestation, pollution, and climate change, emphasizing the need for more effective conservation efforts. The adequate monitoring of these ecosystems has proven a complex and time consuming endeavour, which depends on ever dwindling taxonomic expertise. To date, many species remain undiscovered, let alone described, with otherwise limited information regarding known species population distributions and densities. Overcoming these knowledge shortfalls and practical limitations is becoming increasingly possible through techniques based on environmental DNA (eDNA), i.e. DNA that can be obtained from environmental samples (e.g. tissues, soil, sediment, water, etc.). When coupled with high-throughput sequencing, these techniques now enable realistic, cost-effective, and standardisable biodiversity assessments. This opens up enormous opportunities for advancing our understanding of complex and species-rich tropical communities, but also in facilitating large-scale biomonitoring programs in the neotropics. In this review, we provide a brief introduction to eDNA methods, and an overview of their current and potential uses in both terrestrial and aquatic ecosystems of neotropical rainforests. We also discuss the limits and challenges of these methods for our understanding and monitoring of biodiversity, as well as future research and applied perspectives of these techniques in neotropical rainforests, and beyond.

Keywords: eDNA, DNA metabarcoding, environmental genomics, conservation biology, biomonitoring, neotropics, rainforests.

1. Introduction

Faced with the current environmental crisis, there is an ever growing need to accurately assess existing policy and legislation which aims to protect ecosystems, such as the Paris Climate Agreement, the REDD+ framework, and the Aichi targets (Marques et al. 2014), as exemplified by the IPBES framework (Díaz et al. 2019). This is particularly true for the neotropical moist broadleaf forests, i.e. those occurring from southern Mexico and Florida to Argentina (Olson et al. 2001; Morrone 2014). Of these forests, the rainforests occurring across Amazonia are the most substantial, covering 40% of the region, and representing the primary source of biodiversity across most taxa (Olson et al. 2001; Jenkins et al. 2013; Antonelli, Zizka, et al. 2018).

The biodiversity of neotropical rainforests provides critical ecosystem goods and services for both local and worldwide populations (Rice et al. 2018; Chaplin-Kramer et al. 2019), but these are threatened by increasing human pressures. The region has experienced a 10-fold increase in population densities over the past few decades (Tritsch & Le Tourneau 2016), coupled with a drastic increase in human activities such as deforestation, agricultural expansion, mining and infrastructure construction (e.g. roads, damns; Castello et al. 2013; Rice et al. 2018). These unsustainable land transformations considerably modify abiotic conditions across habitats, and lead to species extinctions, resulting in altered ecosystem functioning and service provision (Rice et al. 2018; FAO 2019). In addition, current predictions for the Amazon basin suggest that climate change will translate to increased droughts, forest-to-savanna transitions, carbon stock losses, and an alteration of the hydrologic and biogeochemical cycles which currently structure this ecosystem

(Nepstad et al. 2008; Davidson et al. 2012).

Assessing the fate of biodiversity with global change and the efficiency of management policies relies largely on the measurement of biological variation at genetic, population, community and ecosystem levels. Such measures, termed “Essential Biodiversity Variables” (EBV, Pereira et al. 2013; Table 1) are most effective when they can be measured in a standardized way that can be employed at varying scales. Currently, these measurements are based on sampling and direct observation of individuals and their description as species by taxonomists. However, obtaining EBVs for neotropical forests is not straightforward. The majority of species occurring in the Neotropics are rare and often exhibit a high level of cryptic diversity (ter Steege et al. 2013; Antonelli, Ariza, et al. 2018; Zizka et al. 2018), making them difficult to describe. The description of such hyperdiverse ecosystems thus relies on considerable taxonomic expertise, yet these skills are in decline (Paknia et al. 2015).

Such a shortfall inherently affects our understanding of species spatial distribution, abundance, evolutionary history, feeding and habitat preferences, as well as functional properties (Hortal et al. 2015). Even when species are identifiable, uncertainties surrounding their spatial distribution remain considerable for neotropical rainforests, since biodiversity assessments are often spatially restricted and biased towards a limited number of accessible areas. These issues pose major limitations to characterising these ecosystems, to better anticipating their responses to global change, and ultimately to implementing effective policies of biodiversity conservation across the region.

Environmental DNA (eDNA) based methods (Figure 1) are now considered as key tools to overcome the aforementioned challenges (Taberlet, Coissac, Hajibabaei, et al. 2012; Deiner et al. 2017; Taberlet et al. 2018; Table 1), providing numerous advantages over classical inventory approaches. Firstly, DNA for taxonomic identification allows an objective analysis of sequence composition, as opposed to more subjective determination using specimen morphology. Secondly, the sampling of the DNA released in the environment by organisms, or environmental DNA (eDNA) is straightforward, due to its prevalence almost everywhere.

In its narrowest sense, eDNA corresponds to the mixture of DNA that can be found in any environmental matrix, whether consisting of soil, sediment or water. This DNA can belong to organisms that are present within the sample in an active or dormant stage (e.g. microbes, spores, pupae, or seeds). Alternatively, it can belong to organisms living in the sample vicinity, since organisms continuously expel DNA into the environment through excretion, secretion, decomposition, or sloughing of tissues. An environmental sample therefore contains a “metagenome”, i.e. a pool of complete or partial genomes from many different species. This metagenome is made up of DNA that can be intracellular or extracellular, dissolved or adsorbed on organic or mineral particles (Nagler et al. 2018).

In its broadest sense, eDNA also corresponds to the DNA that can be extracted from any biological material collected in natural systems, whether it corresponds to a single specimen or a whole community (e.g. bulk samples made of a mass trapping of arthropods or fish larvae). In both cases, the DNA recovered from such a sample does not only contain that of the specimens, but also encompasses the genes/genomes of the specimens symbionts, parasites, or more generally of their microbiota, as well as of their prey (Taberlet et al. 2018; Hacquard et al. 2015).

Thus, the biodiversity retrieved from an eDNA sample is trans-kingdom and multitrophic. Combined with high-throughput sequencing (HTS), environmental DNA-based methods (section 2, Figure 1) now make large-scale and multi-taxa surveys possible from material that is easy to collect, requiring minimal taxonomic expertise. So far mostly used in temperate environments, such surveys could considerably speed up the acquisition of EBVs in general (Jetz et al. 2019), and in species rich and challenging ecosystems such as neotropical rainforests (Table 1).

First, eDNA can provide information on the occurrence of invasive species (Takahara et al. 2013; Valentin et al. 2018), human and agricultural pathogens or pests (Lievens et al. 2006; Harwood et al. 2014; Bass et al. 2015), endangered species or populations (Harper et al. 2018; Tessler et al. 2018) and of wild species in general (Kirshtein et al. 2007; Scibetta et al. 2012). Likewise, it can be used to monitor species that indicate the health of ecosystems (i.e. bioindicators), in particular when these are microbes or invertebrates, of which identification requires advanced and often rare taxonomic skills (Mächler et al. 2014; Pawlowski et al. 2014), especially in tropical ecosystems (Rousseau et al. 2013; Bowles & Courtney 2018 and references herein).

Second it can provide reliable information on the diversity and community composition of soil or aquatic microbes (e.g. Lauber et al. 2009; Zinger et al. 2011; Gilbert et al. 2012), as well as of invertebrates (Pansu et al. 2015; Bista et al. 2017; Zinger, Taberlet, et al. 2019), fish, amphibian, and mammalian communities (Boussarie et al. 2018; Schnell et al. 2018). eDNA can be further used as a standard impact assessment tool in both aquatic (Chariton et al. 2010; Li et al. 2018) and terrestrial ecosystems (e.g. Drenovsky et al. 2010), or as an evaluation tool for the success of restoration and conservation strategies (Bohmann et al. 2014; Perring et al. 2015). Finally, eDNA can provide information for multiple taxon at the same time (e.g. Li et al. 2018; Zinger, Taberlet, et al. 2019), and thus on biological interactions (Vacher et al. 2016). For example, using the eDNA retrieved from the faeces or gut content of a given species can reveal feeding habits (Pompanon et al. 2012), as well as host microbiota and the occurrence of potential pathogens/parasites (Bass et al. 2015). This enables the study of full ecological networks across environmental or land disturbance gradients.

The objectives of this review are therefore (i) to provide a brief overview of eDNA-based methods, (ii) to assess their implementation to describe biodiversity in both terrestrial and aquatic ecosystems of neotropical rainforests, (iii) to highlight the limits and challenges of these methods for providing reliable assessments of EBVs in these environments, and (iv) to propose several avenues for future research in this field.

[INSERT Table 1 here]

2. Overview of eDNA methods

The study of eDNA is made possible through the extraction of DNA from its environmental/biological matrix and its separation from any chemicals that can affect DNA amplification or sequencing reactions (e.g. humic substances, polyphenols, etc.). Once the DNA extract is obtained, four main methods are now routinely applied depending on the final objective (Figure 1). They rely either on the amplification or enrichment of a target genomic region of the metagenome (i.e. species detection, DNA metabarcoding, or capture/enrichment), or on the direct - or “shotgun” - sequencing of the metagenome (i.e. metagenome skimming or metagenomics). We briefly describe each of these approaches below and in particular emphasize DNA metabarcoding throughout this review, as this method is currently the most widely used in the field, in particular in neotropical rainforests. For more detail regarding the molecular and bioinformatics procedures involved, we refer the reader to dedicated literature (e.g. de Bruijn 2011; Bálint et al. 2016; Deiner et al. 2017; Taberlet et al. 2018).

The “species detection” approach consists of detecting/quantifying the amount of a DNA marker that is specific to a single or a small set of species. This approach is most relevant when one aims to detect a species with a high level of sensitivity, including low density populations or dormant/juvenile life forms. The DNA markers used for this approach must correspond to a highly polymorphic locus, enabling the design of primers which are highly species-specific. The approach currently preferred is a direct quantification of the number of copies of the target DNA marker through quantitative PCR (i.e. qPCR, sometimes referred to as real time PCR; Rees et al. 2014) or digital droplet PCR (ddPCR; Doi et al. 2015). These two quantitative methods can help to assess species population density or biomass in the studied area (e.g. Pilliod et al. 2013). This approach is relatively cheap, since it does not rely on sequencing, and is therefore more suitable for large-scale or temporal studies, although is limited to focusing on only one or a reduced set of species.

“DNA metabarcoding” (Taberlet, Coissac, Pompanon, et al. 2012) is the most popular approach to study eDNA (see Figure 1 for more detailed information). This approach has also been referred to in the literature as "amplicon sequencing", "ecometagenetics", "metataxogenomics", but should not be confused with "metagenomics", which we define below. As with species detection, DNA metabarcoding relies on the amplification of a target DNA region by PCR. However in this case, the DNA region targeted is used as a *“barcode”* to discriminate the species comprising the metagenome under study. A relatively large number of samples processed with DNA metabarcoding can be sequenced in a single HTS run (Figure 1). The obtained sequencing reads are then processed bioinformatically to retrieve a list of species (or Operational Taxonomic Units, OTUs).

Enrichment capture on eDNA is very similar to DNA metabarcoding in that it consists of sequencing the same targeted regions. However, it differs in that the target DNA to be sequenced is not enriched through PCR amplification, but instead by capturing it with multiple, taxon-specific DNA probes bound to magnetic beads. This approach is often used for the analysis of ancient DNA of single species or simple species assemblages (e.g. Carpenter et al. 2013) and is increasingly used for the analysis of modern eDNA and complex communities (e.g. Shokralla et al. 2016; Wilcox et al. 2018), although the sensitivity and limitations of this approach are yet to be evaluated.

The last alternative relies on shotgun sequencing, i.e. random sequencing of DNA molecules from the environmental sample. “Metagenomics” is the most direct and comprehensive DNA-based technique, and consists of sequencing as much of the metagenome as possible so as to retrieve organisms taxonomic identity, their phylogenetic relationships, as well as to their metabolic properties. However, it is also the most challenging approach. First, much of the information contained within metagenomes remains undescribed. Second, a metagenome contains a huge diversity of genes and noncoding regions, of which a tiny fraction are highly repeated (e.g. ribosomal RNA genes), and a majority of which are rare. Fully describing this complexity therefore requires substantial sampling, in this case sequencing effort, which today remains costly. Finally, most environmental samples are dominated by microbial DNA, which reduces the probability of detecting larger organisms. Consequently, metagenomics is for now mostly used in environmental microbiology (e.g. de Bruijn 2011) or for ancient DNA analyses (Thomsen & Willerslev 2015).

"Metagenome skimming" is a cheap version of metagenomics (Linard et al. 2015; Papadopoulou et al. 2015), albeit more expensive than methods targeting a particular DNA region. In this case, the metagenome is sampled at a shallow sequencing depth so as to sequence only highly repeated DNA regions, i.e. the ribosomal RNA gene regions and the organelle genomes for eukaryotes. These regions can then be partially or fully reconstructed, and thus used to identify the species present but also their phylogenetic relationships.

[INSERT Figure 1 here, full width]

3. Current use and challenges of eDNA applications in neotropical rainforests

Current studies of biodiversity in neotropical rainforests that rely on eDNA based methods mainly describe community composition and diversity changes along environmental or disturbance gradients in order to identify patterns in diversity and their drivers. These studies are reviewed below across ecosystems and focal organisms, and examined to determine what eDNA from different sources can reveal regarding ecological communities from neotropical rainforests and how sampling can be tailored to suit the ecological question. We will restrict our review to contemporary environments, as - to our knowledge - eDNA approaches *per se* have not been used yet in the neotropical rainforests for palaeoecological purposes. We refer interested readers to dedicated reviews on this particular application (Rawlence et al. 2014; Thomsen & Willerslev 2015; Taberlet et al. 2018).

3.1. In terrestrial ecosystems

Microbial communities. These have been mostly analysed in the soil environment, with the study of eDNA from soil samples having a relatively long history in soil microbial ecology (Tiedje et al. 1999 for an early review). Available studies for neotropical rainforests have shown that soil prokaryotic and microeukaryotic communities vary across altitudes (Nottingham et al. 2018), soil conditions, forest types and tree species composition (Ritter et al. 2019; Vasco-Palacios et al. 2019). Numerous studies also report steep changes in composition with increased drought (Waring & Hawkes 2015; Kivlin & Hawkes 2016a; Pajares et al. 2018), deforestation and reconversion to different types of sylviculture (Carney et al. 2004; Ndaw et al. 2009; Kivlin & Hawkes 2016a; Kivlin & Hawkes 2016b), arable farming (Rodrigues et al. 2013; Paula et al. 2014; Mendes et al. 2015; e.g. Franco et al. 2019), and even as a result of pre-columbian activities (Kim et al. 2007; Grossman et al. 2010; Navarrete et al. 2010). Likewise, soil microbial diversity differs between old-growth and secondary forests (Araújo et al. 2014; McGee et al. 2019). All these studies exemplify the utility of soil eDNA for providing microbial-derived EBVs that are meaningful for monitoring the impact of climate change and land use practices.

Invertebrates. Soil micro- and macro-invertebrates (i.e. nematodes, earthworms, insects and springtails) have seldom been studied with eDNA from neotropical rainforest soil samples (Wu et al. 2011; Zinger et al. 2016; Ritter et al. 2019), and in such cases, rather as part of the whole soil eukaryote diversity, through the use of universal primers. A global-scale analysis suggests that neotropical rainforests are dominated by arthropods and enriched in soil annelids (Wu et al. 2011). Locally, soil micro- and mesofauna communities exhibit primarily random spatial patterns that are more pronounced for the mesofauna as compared to microscopic organisms, as shown at a forest site in French Guiana (Zinger, Taberlet, et al. 2019).

The large majority of studies of soil or above-ground invertebrates have so far rather relied on eDNA extracted from bulk samples and analysed through DNA metabarcoding, which is a fast alternative to time consuming sorting and identification of hundreds to thousands of specimens that are difficult to identify. Using this approach, Porazinska *et al.* (2012) were able to observe strong variation in soil nematodes communities across sites and habitats of Costa Rican rainforests. This approach has also enabled the description of above-ground terrestrial arthropods, such as sandflies occurring at several sites in French Guiana (Kocher, Gantier, et al. 2017), or arthropods from a forest canopy in Honduras (Creedy et al. 2019). The latter study also tested the effect of animal size on species detection, with results suggesting such effects are not visible when sequencing depth is sufficient. Enrichment capture has also been used to analyse bulk samples of arthropods sampled with malaise traps in a forest of Costa Rica (Shokralla et al. 2016). This method was found to be more accurate in describing biodiversity than DNA metabarcoding on the same samples and classical observations.

Mammals. Using eDNA from bulk samples of faeces or hematophagous arthropods also seems particularly promising for sampling terrestrial vertebrate diversity as well. For example, DNA extracted from owl pellets in central Brazil provided meaningful information regarding the diversity of small mammals (Rocha et al. 2015). Likewise, vertebrate communities are better described by the DNA contained in blood feeding arthropods collected with Malaise traps and pitfall traps than with classical or camera trap-based inventories, as shown for forests in Panama and Brazil (Rodgers et al. 2017; Lynggaard et al. 2019). This approach further revealed variation in vertebrate community composition, consistent with a gradient of anthropogenic pressures in French Guiana, with a decline of diversity in the areas experiencing the highest pressures (Kocher, de Thoisy, François Catzeflis, et al. 2017). Alternatively, water samples could also be used to study terrestrial mammals, since water bodies should accumulate and transport material from the whole catchment areas through erosion (Naiara Guimarães Sales et al. 2019).

Plants. Initial attempts to describe plant diversity with eDNA used bulk samples of dried, fine roots isolated by hand from soil cores that were collected following a grid or regular sampling scheme in the Barro Colorado Island in Panama (Jones et al. 2011; Barberán et al. 2015). With this approach, one soil core exhibited an average diversity of *ca*. 4 plant species and the DNA imprint of each tree individual was detectable from 1 to *ca*. 20m from the stem. Similar figures can be retrieved by directly using soil as starting material, as shown in a lowland rainforest in French Guiana (Fig. 2A, see also Taberlet et al. 2018, (Yoccoz et al. 2012). Thus, root and soil eDNA can offer new insights into plant root distribution in the soil and their functional implications. The aboveground plant community might be better assessed by targeting plant DNA markers on bulk samples of herbivorous arthropods, but to our knowledge, this approach has not been tested yet.

Constraints and limits. The above shows that organisms from terrestrial environments are either studied using environmental DNA extracted from soils, which are noticeable reservoirs of both intra- and extracellular DNA and mostly contain the signature of soil organisms, or using bulk samples of invertebrates. The former material is probably the easiest to sample from a practical point of view, and less biased/variable than different arthropod sampling techniques (Missa et al. 2009). However, one critical aspect when studying diversity using eDNA extracted from soil is the heterogeneous and complex nature of soil substrates themselves, in terms of physical, chemical and biological properties (Bardgett & van der Putten 2014). This can be an issue when comparing contrasting environments. Typically, the amount of available extracellular DNA, useful for detecting non-microbial organisms, is strongly constrained by soil chemical properties. For example, DNA has a much stronger affinity to clay than sand (Levy-Booth et al. 2007), which thus could introduce bias to comparisons between white-sand vs. *terra firme* forest soils. This heterogeneity not only applies horizontally in space but also vertically, with clear differences in prokaryotic and micro-eukaryotic communities between the organo-mineral horizon and the litter layer from a taxonomic point of view (Figure 2B), and most likely from a functional one (Fragoso & Lavelle 1992; Basset et al. 2015; Ritter et al. 2019). This raises the question of if and how one should integrate this vertical dimension.

The same applies for how much soil samples should be collected across space, whatever the taxon targeted. For example, *terra firme* soils/litter and white sand litter may require higher sampling effort than white sand soils to estimate the plot-scale diversity, due to higher spatial heterogeneity (Figure 2C). Alternatively, sampling effort could be reduced when comparing diversity or community turnover across conditions by for example building composite samples at different sampling points to capture local diversity while keeping down experimental costs. At the sample scale, extracting eDNA from volumes of material that are larger than those from most commercial soil DNA extraction kits (typically 250 µg) should best capture local diversity, which is now possible for ≥10 grams of starting material, as shown for neotropical rainforest soils (e.g. Zinger et al. 2016). Thus the required sampling effort is likely to be highly system dependent, and further analyses across habitats will help better define sampling standards for neotropical rainforests when using soil as a starting material.

In any case, soil samples are unlikely to be the most relevant material for sampling the diversity of plants or aboveground animals at the plot scale because eDNA is poorly transported in soils and thus highly patchily distributed (Levy-Booth et al. 2007; Nagler et al. 2018). This patchiness most likely also results from the reduced DNA persistence in tropical rainforest soils due to high demands of the living biomass for phosphorus, which is otherwise highly limitating (Dalling et al. 2016), thus reducing the probability of detecting large organisms. Accordingly, experiments show that dead root DNA is almost totally degraded after 15 days (Bithell et al. 2014). Likewise, microbes and soil fauna communities exhibit marked seasonal and yearly dynamics (Fragoso & Lavelle 1992; Kivlin & Hawkes 2016b; Kivlin & Hawkes 2016a; Pajares et al. 2018), and so most likely does their DNA as compared to that of rooted plants, which continuously release DNA in soils (Figure 2D).

[INSERT Figure 2 here, full width]

3.2. In aquatic ecosystems

Microbial communities. Several studies using water eDNA have been conducted across different systems to study microbial communities. For example, Tessler *et al.* (2017) showed that bacterial communities from Brazilian floodplain lakes were highly distinct from other areas of the globe, while within Brazilian sites, the composition was overall fairly similar. Other studies suggest the opposite for micro-eukaryotic plankton: Brazilian rivers seem to exhibit marked spatial patterns with relatively high community turnover, even within the same location (Lentendu et al. 2019). These discrepancies raise the question as to whether they arise from biological differences between microeukaryotes and bacteria or from methodological inconsistencies, which emphasize the need for increased efforts in studying aquatic microbial communities in these ecosystems.

Tank bromeliads (Bromeliaceae) accumulate rainwater at the base of each leaf axil and thus represent freshwater islands in a terrestrial matrix. They harbour various aquatic organisms ranging from prokaryotes to macroinvertebrates (Benzing 2000; Leroy et al. 2016). eDNA methods have provided insights into their community structure through either metagenomics (Rodriguez-Nuñez et al. 2018) or DNA metabarcoding (Louca et al. 2016; Louca et al. 2017), revealing bacterial communities that are substantially different from freshwater lake sediments and soil, but remarkably similar in functional structure due to an adaptation to oxygen-limited conditions.

Invertebrates. The use of water/sediment eDNA for targeting aquatic invertebrates (aquatic insects, crustaceans) has, to our knowledge, not yet been applied to neotropical rainforest ecosystems. A recent study has shown its usefulness for assessing macroinvertebrates community composition in the tropical freshwaters of Singapore (Lim et al. 2016), suggesting that such an approach could be relevant to neotropical rainforest ecosystems. As for terrestrial environments, the use of bulk samples for aquatic systems is emerging, such as with the study of Talaga et al. (2017), which details the development of DNA reference libraries for Guianese mosquito larvae to distinguish species from bulk samples of freshwater invertebrates. Still, eDNA studies of freshwater invertebrates in neotropical rainforests are currently limited by knowledge deficits related to their taxonomy and ecology and a lack of previously implemented studies . Although several macroinvertebrate indices enabling the biological evaluation of freshwater ecosystems are available (e.g. Couceiro et al. 2012; Dedieu et al. 2016), these are seldom used because to our knowledge, there is currently no environmental law or regulation relying on these in this ecoregion. One exception in that respect is French Guiana, which must comply with the European Water Framework Directive.

Fishes. The potential of water/sediment eDNA has received comparatively much more attention for studying fish communities. This has been particularly stimulated by the strong limitations of traditional sampling methods, which provide biased estimates and/or cause substantial fish mortalities. Indeed, gill nets provide only partial inventories, and ichthyocides such as rotenone, which were widely employed in the past, are increasingly banned. Electric fishing, which is often a good sampling alternative in other environments can be inefficient in neotropical streams because of the very low conductivity of the water (Allard et al. 2014). Hence, fish eDNA has rapidly emerged as the most promising non invasive alternative to traditional sampling for small streams, rivers, lakes and the sea. Cilleros *et al.* (2019) compared eDNA and traditional sampling (nets and ichthyocides) both in small streams and rivers across French Guiana. Not only did they find that species assemblages were congruent between eDNA and traditional records, but also that eDNA results were more efficient in distinguishing the fauna from different river drainages. eDNA also enables the study of fish communities at cryptic life stages, i.e. the ichthyoplankton. Nobile *et al.* (2019) used DNA metabarcoding on mock communities built from fish eggs and larvae in the Grande River in Brazil, and obtained an average detection rate higher than 95%, and a relatively good estimate of larvae abundances. Likewise, capture enrichment on bulk samples for catfish larvae from the Peruvian Amazon provided a good description of the community in terms of both species and abundance (Maggia et al. 2017; Mariac et al. 2018).

Vertebrates. Several studies have focussed on vertebrates inhabiting aquatic environments for at least a part of their life, such as amphibians. Comparing traditional visual and audio survey techniques with DNA metabarcoding of water samples showed that eDNA accurately reflects the conclusions of the other methods whilst cutting the length of fieldwork required studying for frog communities in freshwater streams in the Brazilian Atlantic forest (Lopes et al. 2017; Sasso et al. 2017). Likewise, a comparison of cost models suggests that eDNA-based surveys are a cost-efficient alternative to traditional surveys in amphibian species rich areas such as in the neotropical forest-savannah ecotones of Bolivia (Bálint et al. 2018). All these studies further show that eDNA-methods circumvent biases of traditional approaches linked with species abundance and life history traits. Indeed, they not only allow for the detection of species closely associated with streams, but also of frog species at cryptic life stages (e.g. tadpoles or eggs). These are often missed by traditional surveys, but detectable with eDNA since they release DNA into the environment irrespective of their life stage. Likewise, eDNA is also able to detect endangered species in a non-destructive way, such as for the bromeliad inhabiting Trinidad golden tree frog (Brozio et al. 2017). Beyond amphibians, Sales et al (2019) also detected eDNA from both aquatic and terrestrial mammals when sampling water in the Amazon’s mainstream and tributaries, in addition to a river of the Brazilian Atlantic forest. Comparing these results with camera trapping data confirmed the congruence between the methods (Naiara Guimarães Sales et al. 2019). Interestingly, some of the species detected using eDNA from water samples belong to strictly terrestrial species such as bats or anteaters, which can be explained by the fact that water conveys DNA from terrestrial to aquatic ecosystems. However, further studies are needed to validate this protocol for capturing terrestrial vertebrate diversity.

Constraints and limits. The above shows that eDNA for studying aquatic ecosystems can be extracted from either water or sediment samples, or bulk samples. For bulk samples, the trapping system is likely to be an important factor, as for traditional observations. For water or sediment samples, the interpretation of eDNA data from these two substrates remains unclear. Apart from microbial communities that highly differ between these two environments due to contrasted oxygen nutrient availability (Zinger et al. 2011; Thompson et al. 2017), the discrepancy between the results obtained from water and sediments when targeting larger organisms has been highlighted by several studies. Some studies have shown that fish eDNA concentration in sediments is higher and detectable over longer timescales than in water (Turner et al. 2015; Naiara G. Sales et al. 2019). However, other studies found that sediments were less effective than water samples, e.g. allowing the recovery of only 10% of the fish species in an oligotrophic lake in Mexico (Valdez-Moreno et al. 2019). Water remains to date the most commonly used substrate for eDNA studies in neotropical rainforests due to its ease of collection. Sampling of eDNA is mainly conducted using filtration that is either directly performed in the field or subsequently in the laboratory (Lopes et al. 2017; Naiara G. Sales et al. 2019).

For both water and sediment samples, the concentration of eDNA in the environmental matrix strongly determines how much material should be collected to appropriately sample freshwater diversity. For example, Cantera et al. (2019) sampled up to 340 L of water in streams and rivers in French Guiana to study the impact of sampling effort on fish detection. They showed that with a total filtration of 68 L, 91% of fish diversity could be detected in streams, and 74% in rivers. These results resonate with those obtained by Lopes et al. (2017), showing that filtering larger quantities of water (from 20L to 60L) increases the detection probability for amphibian species and thus covering local amphibian diversity in the Brazilian Atlantic forest. Nevertheless, according to Cantera et al. (2019), filtering 34 L of water is sufficient for the recovery of 64% of the local fish fauna in Guianese streams and rivers, with a strong redundancy between eDNA replicates. Such a limited sampling effort seems hence sufficient to distinguish fish communities between sites and between ecosystem types (i.e. streams vs rivers).

The concentration of eDNA in freshwaters is a function of the local living biomass, but also of the transport and degradation rate of eDNA in freshwater ecosystems, which depends on environmental conditions (Barnes et al. 2014; Barnes & Turner 2015). These processes require further investigation in both waters and sediments of neotropical rainforest ecosystems, in order to best define the sampling effort required to conduct reliable eDNA studies in these areas. It is now well established that low pH conditions, high oxygen demand and primary production, and high temperatures all accelerate the degradation of aquatic eDNA (Barnes et al. 2014; Strickler et al. 2015), which is likely to strongly vary across neotropical rainforest rivers and streams. As such, a study found an unexpected higher mammal species richness in the Brazilian Atlantic forest compared to the Amazon (Naiara Guimarães Sales et al. 2019), which is suspected to arise from a higher degradation rate of eDNA due to the low pH of the Amazon waters (pH ≤ 4).

Another important point, strongly linked to the degradation rate, is the transportation of eDNA with water flow. Studies are ongoing on this aspect in neotropical rivers, but Cantera et al. (2019) report that fish species detected from a stream site were no longer detected in eDNA samples collected in a river site located 300m downstream from the confluence with the river. This suggests either a rapid degradation and hence a relatively short distance of eDNA transportation in neotropical waters, or more generally a high dilution downstream, which should make eDNA detection more difficult at sites distant from where it has been released. Finally, precipitations and stream size should also define local eDNA concentrations. For example, Sales et al. (2019) reported noticeable compositional differences between samples collected from the same location following a 3-week interval. While this might be due to real variation in species composition, it is also possible that variation in water volume linked to increased precipitation at the time of sampling affected species recovery.

3.3. Common field, wet, and dry lab biases

Besides the clade- and environment-specific considerations mentioned above, the processing of eDNA data typically consists of a series of methodological steps (Figure 1) that are all subject to various biases (Dickie et al. 2018; Zinger, Bonin, et al. 2019). We will briefly outline some of them and their associated solution when crucial for applications in neotropical rainforests ecosystems, as these issues are extensively addressed elsewhere (e.g. Deiner et al. 2017; Taberlet et al. 2018; Alberdi et al. 2019). This discussion will be mostly focused on DNA metabarcoding, as it is the approach the most widely used in eDNA research.

At the sampling step, the extent of the sampling area, sampling point locations, number of biological replicates, sample conditioning and transport, *etc*. are all important points to critically consider to avoid compromising the results (reviewed in Dickie et al. 2018; Taberlet et al. 2018) and will inherently depend on the particularities of the ecosystem and taxon under study (see above). Appropriate sample conditioning is also critical in tropical climates, in which microbial growth and DNA degradation is faster and more likely to occur during sample transport. Sample cooling in ice can considerably slow down DNA degradation and microbial growth, but this is seldom logistically feasible when working in remote and warm sites. To circumvent this limitation, DNA extraction can be done directly in the field with specified protocols requiring minimal infrastructure (e.g. Zinger et al. 2016; see Taberlet et al. 2018 for a detailed protocol). Alternatively, the sample can be dessicated with *silica gel* for soils or sediments, or more generally conserved with preservation buffers. These are typically used for aquatic eDNA samples, either for conserving water filters on which eDNA has been captured (Cilleros et al. 2019) or for direct addition to water samples, although preservation buffers seems less effective than sample cooling for eDNA recovery and taxon detection (Naiara G. Sales et al. 2019).

After collection, the molecular processing of samples also has a variety of biases that can reduce the detection or distort the abundance of the taxa retrieved, an important limit for species population EBVs (Table 1). DNA extraction methods are not equally efficient in extracting and purifying DNA, due to variable success of cell lysis for microbes, and more generally to strong variations in the chemical composition of the starting material, with some being noticeable PCR inhibitors (e.g. humic acids). The methods employed for the extraction of DNA should be tailored to the starting material and question, or it may miss or overrepresent certain taxa. Once DNA is extracted, PCR amplification should be done with primers whose specificity-to- and generality-within the clade of interest should have been verified following a thorough literature review, preliminary tests, or the use of *in silico* PCR softwares (e.g. Ficetola et al. 2010; Elbrecht & Leese 2017). Use of inappropriate primers will both strongly bias the retrieved taxa abundances and in some cases, their detection altogether.

Both PCR amplification and sequencing can also generate artifactual DNA fragments/sequences, especially when the target DNA is rare (reviewed in Taberlet et al., 2018). These artifacts are generally in low abundance and very similar to genuine fragments (e.g. only one or a few different nucleotides). They are hence difficult to identify and can artificially inflate taxonomic diversity estimates, this attribute being a community composition EBV candidate (Table 1). Nevertheless, such errors can be reduced by clustering DNA sequences at a certain sequence identity level using supervised or unsupervised approaches (Figure 1). However, it should be noted that the bioinformatics tools used, as well as their associated parameters (e.g. clustering methods and thresholds, sequences distance indices) are not all equally efficient in reducing this artifactual variability, and can even fail to detect genuine biological variability (Coissac et al. 2012; Bálint et al. 2016; Zinger & Philippe 2016; Deiner et al. 2017; Taberlet et al. 2018). The same applies when using supervised approaches, as the taxonomic assignment quality of a sequence/ OTU inherently relies on the completeness and accuracy of the reference databases. For example, using an incomplete reference database, i.e. without conspecific sequences, can lead to an increase of 20% of erroneous taxonomic assignments as compared to the use of a complete one, as shown for Amazonian mammals (Kocher, de Thoisy, Francois Catzeflis, et al. 2017).

Diversity estimates can also be inflated through the presence of genuine DNA fragments that are not initially present in the sample. The most obvious source of such a problem is exogenous contamination, which can occur not only at the sampling step, but also at the extraction, PCR, and sequencing steps because labs and reagents all contain a number of contaminants (Salter et al. 2014). Beside this problem, the multiplexing of samples within a single sequencing library or sequencing lane also produces apparent cross-sample contamination. The exact underlying mechanisms remain not well understood, but DNA fragments that are multiplexed seem to exchange the small tags used to identify their sample of origin (Figure 1), a bias often referred to as ‘tag-switches’, ‘tag-jumps’, or ‘cross-talks’ (e.g. Schnell et al. 2015; Esling et al. 2015). Although this bias produces contaminants at usually low abundances, it can have strong consequences if downstream analysis relies on presence/absence and occurrences.

Given the different artifacts mentioned above, the reader should now be aware that the inclusion of negative and positive controls at the sampling, extraction, amplification and sequencing steps as well as technical replicates is critically important to ensure not only data reliability but also to optimize the processing and curation procedures of the obtained sequences through bioinformatics pipelines. The problem of false positives can be reduced by using PCR-independent methods, such as metagenomics/metagenome skimming, or capture enrichment. However both approaches still require substantial developments and cost reductions to be applicable in large-scale studies. In addition, these approaches are not error-free. They still include tag-jumps or sequencing errors (Taberlet et al. 2018; Wilcox et al. 2018) that remain difficult to detect and filter out.

Artifactual signals can have dramatic effects on estimates and patterns of alpha, and to a lesser extent beta diversity (Calderón−Sanou et al. 2019), as well as on model parameters inference such as for Hubbell’s neutral model (Sommeria-Klein et al. 2016). Since these artifacts are generally low in frequency, end-users should also be careful when focusing on rare taxa. This corresponds to the majority of species in neotropical rainforest ecosystems (ter Steege et al. 2013; Antonelli, Ariza, et al. 2018; Zizka et al. 2018), which suggests that it is unlikely that current eDNA-based approaches provide reliable estimates of species richness, i.e. the number of species being present in the ecosystem studied. Nevertheless, these approaches can still provide meaningful information on alpha or beta diversity patterns by using diversity indices penalizing low-abundance OTUs or taxa such as those based on Hill numbers, which includes well known indices such as Shannon or Simpson diversity (Chao et al. 2014). These have been shown to provide more reliable ecological inferences (Calderón−Sanou et al. 2019), and should be favored over other indices where singletons (e.g. Chao, ACE, Fisher's alpha indices) or rare species have a strong weight (e.g. inferences based on species abundance distribution or on presence-absence data). Nevertheless, new occupancy models able to detect both false negative and false positives are currently emerging (Ficetola et al. 2016; Guillera-Arroita et al. 2017), and their inclusion in current data curation procedures will certainly allow overcoming the above-mentioned limitations.

3.4. Biological interpretation of eDNA

Beyond the methodological considerations raised above, eDNA has specific intrinsic properties which must be considered when interpreting derived results. Even if eDNA data resembles a traditional species abundance table, the abundances correspond to sequencing read counts and species correspond to species, genera, or to OTUs defined at a given level of sequence similarity. This difference can have strong implications for the type of EBV that eDNA can actually measure (Table 1), as well as on ecological inferences depending on the question addressed and types of inference tools used, in particular when they involve theoretical frameworks and models that rely heavily on species and abundances (e.g. niche or neutral models, species abundance distributions).

A first uncertainty is on the extent to which sequences or OTUs can be used as a proxy for species. In most eDNA studies, species or OTUs are defined by using a threshold of 97% of sequence similarity. This threshold has been historically defined for full-length barcode genes (e.g. Stackebrandt & Goebel 1994; Hebert et al. 2003; Schoch et al. 2012). However, current eDNA studies target small regions within these barcodes (Figure 1) in order to comply with both the sequencing limits of current HTS instruments and, when applicable, with the fragmented nature of extracellular DNA. This constraint inherently comes with a loss of taxonomic resolution, which may have consequences for subsequent ecological inferences. The "Amplicon-" or "Exact Sequence Variant" concept (ASV or ESV, Callahan et al. 2017) has been recently proposed to, amongst other reasons, circumvent this problem, yet this remains sensitive to some molecular artifacts. Sometimes interpreted as intraspecific variability, which can be a desirable output of eDNA (Table 1), ASVs may also yield ecological signals that differ from what one should expect when considering species. Finally, eDNA markers do not have the same taxonomic resolution across clades. For example, the fungal Internal Transcribed Spacers, or the metazoan cytochrome oxidase subunit I (COI) can exhibit some intraspecific variability for certain groups, and only genus to family level variability for others (Schoch et al. 2012). Phylogenetic-based approaches can to a certain extent deal with these limitations. However, while these can be employed with metagenomics or metagenome skimming data (Andújar et al. 2015; Papadopoulou et al. 2015), the short and hypervariable nature of most classical DNA markers used for DNA metabarcoding do not enable making robust phylogenetic inferences, which limits the use of such data to retrieve co-ancestry relationships (Table 1). For such data, the phylogenetic diversity should be retrieved through phylogenetic placement methods, provided that a robust backbone phylogenetic tree is available (e.g.

Matsen et al. 2010; Czech et al. 2019), which remains challenging for neotropical taxa (see section 4.1.).

The other uncertainty of eDNA data relates to the meaning of sequencing reads counts. As mentioned in section 3.3, a DNA extract is subjected to a suite of molecular manipulations that can distort the original distribution of DNA fragment abundances. Adding spiked DNA of known composition and concentrations in environmental samples could allow for the retrieval of absolute values of eDNA molecules (e.g. Smets et al. 2015; Thomas et al. 2016). However, while the abundance (relative or not) of eDNA molecules has been found to correlate with organism biomass in simple experimental set ups (e.g. Nobile et al. 2019) or when quantifying single species *in natura* with qPCR (reviewed in Taberlet et al. 2018), several factors can alter this relationship, and hence, assessment of population abundance (Table 1). First, eDNA persistence and transport in the environment makes it difficult to know whether this biomass is local and contemporary. This is likely to be especially true for soils or sediments as compared to water, the latter being more exposed to high temperature and UV radiations, which favor DNA degradation (Barnes & Turner 2015; Nagler et al. 2018). Even if this bias is limited, relating eDNA abundance to population abundance *per se* remains challenging. Indeed, the number of DNA marker gene copies depends on the taxon, on the tissues from which eDNA is released, the biomass/size of the organisms, but also its life stage (Maruyama et al. 2014). To our knowledge, there is no tool which can retrieve individual counts from sequencing reads or eDNA molecules at the scale of the biological community. These uncertainties has often led researchers to prefer presence-absence metrics over abundance-based ones. However, unless the representativeness of the data curation procedure can be proven, we advocate against such reasoning due to the high error rate of PCR and sequencing based approaches (see section 3.3.).

Given the above-mentioned differences in the intrinsic nature of eDNA data as compared to traditional species abundance tables, this raises the question of whether one can draw ecological inferences with classical tools. Typically, it remains largely uncertain whether inferring community diversity and related characteristics from eDNA-based species abundance distribution or using process models involving explicitly species and individuals is a correct approach. For example, adaptation of Hubbell's model to account for body size or biomass could be more appropriate (O'Dwyer et al. 2009; Sommeria-Klein et al. 2016). There is hence a need for development of related tools and theories in ecology that would better comply with the nature of eDNA data.

4. Future directions and perspectives

The past decade has seen enormous advances in the development and extension of eDNA-based approaches, as well as a large number of potential applications in various environments, including neotropical rainforests. However, these applications remain largely underused in this part of the world when compared with other far less diverse regions (this paper; Mulatu et al. 2017; Belle et al. 2019). This is because countries harboring lower diversity are in general more developed economically: infrastructure for molecular-based research is accessible, with associated personnel now relatively well trained for eDNA data generation and analysis. On the other hand, the Nagoya Protocol on Access and Benefit Sharing restricts the access of genetic resources to the country where the sample has been collected, protecting local countries, which are often less economically developed, from unethical practices by collectors outside of and within the scientific community. We argue that current efforts to develop eDNA-based research in neotropical countries should be encouraged and strengthened through international collaborations between researchers from Neotropical countries and researchers from countries that have already overcome issues relating to methodological application, technical infrastructure and skill acquisition. Such efforts will enable the acquisition of EBVs related to taxonomic diversity, but also beyond to provide information such as species distributions or biotic interactions (Table 1), as well as associated underlying processes. In this final section of the review, we will explore how eDNA can be better used to improve research methods and their subsequent applications, and in doing so ultimately contribute to improving conservation programs and management strategies for these hyperdiverse ecosystems.

4.1. Making better sense of eDNA data with better reference databases

A key limit to current eDNA studies in neotropical rainforests is the provision of relatively poor taxonomic information. This drawback arises in part from the limitations of eDNA-based methods mentioned above, but is further exacerbated when dealing with neotropical taxa in that they are largely underrepresented in current DNA reference databases, and/or they have an unresolved taxonomy. This is particularly true for micro-eukaryotes, for which a significant proportion of OTU and sequencing reads remain unassigned to a taxon, even at the phylum level (Ritter et al. 2019; Zinger, Taberlet, et al. 2019). The deficit in DNA references also applies to less cryptic organisms. For example, only 58% of the São Paulo tree flora has genetic records in international DNA reference databases (Lima et al. 2018). While eDNA does facilitate the identification of challenging taxa at gross taxonomic levels, it is therefore unlikely to provide a satisfactory solution for resolving the Linnean shortfall and provide on its own information on EBVs related to species evolutionary history and functional traits. We hence argue that the future of eDNA remains inherently intertwined with the continued efforts of taxonomists and naturalists to sample, identify and store physical specimens in order to complement DNA reference databases, but also to describe their morphology, evolutionary history, functional traits, and to solve taxonomic problems (Sheth & Thaker 2017; Dormontt et al. 2018; Pinheiro et al. 2019).

Augmenting the completeness of DNA reference databases is crucial not only to facilitate the assignment of unknown sequences. It is also essential to ensure, or verify the plausibility of the retrieved signal, which can be extremely noisy as discussed above. However, one of the difficulties in improving DNA reference databases is the current lack of consensus when choosing the DNA regions to be used across studies. Indeed, these may differ from the ones used in curated reference databases linked to voucher specimens such as the BOLD system for animals and plants (Ratnasingham & Hebert 2007) or databases dedicated to the ribosomal clusters for microorganisms (e.g. UNITE, Abarenkov et al. 2010; SILVA, Quast et al. 2013), which only contain gold standard barcoding genes (i.e. COI for animals, *rbc*L or *mat*K for plants, and ITS for fungi). This is because gold standard barcodes are not necessarily compatible with all applications of eDNA, which often require DNA primers that target broad taxonomic groups and DNA markers that are short to suit existing sequencing technologies or the degraded state of eDNA. Conserved priming sites across broad taxonomic groups are often absent within these gold standard barcodes, an issue highlighted for animals (Bruce E. Deagle et al. 2014) and plants (Hollingsworth et al. 2011). As a consequence, existing primer sets targeting classical barcode subregions are often biased toward certain taxa or on the contrary lack of specificity because they contain too many degenerate bases (B. E. Deagle et al. 2014; Collins et al. 2019). Alternative DNA markers fulfilling these conditions are often located in mitochondrial or chloroplastic introns or ribosomal genes (Figure 1) which are better conserved. However these regions also often exhibit lower taxonomic resolution and are much less referenced in DNA databases. When choosing a DNA marker, the end-user must hence usually compromise between more precise taxonomic information versus unbiased sampling of biodiversity. These considerations go beyond the scope of this review and we refer interested users to dedicated litterature on the subject (Hollingsworth et al. 2011; Bruce E. Deagle et al. 2014; Taberlet et al. 2018).

As stated above, the choice of a given DNA marker strongly relies on the biological question to be addressed, the starting material used and because current reference databases have large deficits in neotropical organisms. Therefore, we encourage the construction of custom reference databases for the targeted DNA region from local taxa that are likely to be detected with the eDNA analysis, as done for example in studies using the mt 12S rRNA gene of neotropical mammals (Kocher, de Thoisy, Francois Catzeflis, et al. 2017) and of Guianese fishes (Cilleros et al. 2019), or for the ITS1 region for the Basidiomycota of French Guiana (Jaouen et al. 2019). Although often considered as a costly endeavour, it can be achieved at relatively low expense (as low as *ca.* 5 $USD / specimen) by using freshly collected specimens, or herbarium/museum collections (e.g. Dormontt et al. 2018) and by multiplexing thousands specimens in a single HTS run. Another promising alternative that will alleviate the lack of standard DNA markers across studies lies in the building of “marker-free” DNA reference databases. This is now possible with genome skimming (Dodsworth 2015), which is similar to metagenome skimming but relies on a single specimen. This approach produces sequences usable for both gold standard and other barcodes as it generates sequences of the complete organelle genomes and full nuclear ribosomal regions (Coissac et al. 2016). Although this remains relatively expensive (as low as *ca.* 100 $USD / specimen), it is likely to become more affordable with continued decreases in sequencing costs.

In addition to compiling DNA information across species, reference databases could complement taxonomic data with ecophysiological characteristics, such as foliar, root or seed traits for plants, and morphological characteristics such as body size for animals. Such information would be extremely valuable, allowing eDNA studies to go beyond the simple description of taxonomic and phylogenetic diversity of the studied system (Table 1). For example, inferring taxon function or gross ecological traits from eDNA data is now possible for bacteria and fungi through databases that compile both metabolic, life history traits, or broad lifestyle types (e.g. PiCrust, Langille et al. 2013; FUNGuild, Nguyen et al. 2016/4). To our knowledge, such tools are currently not directly available for macro-organisms, although several databases compiling taxonomic and functional information in a number of groups have been developed (e.g. FishBase, Froese & Pauly 2019; TRY, Kattge et al. 2019; Atlantic Bird Traits, Rodrigues et al. 2019; or the Global Ants Database, Gibb et al. 2019). Their coupling with DNA reference databases would certainly help advance the field of eDNA studies to include more process-based approaches.

4.2. Toward eDNA-based occurrence portals for the Neotropics?

The greatest strength of eDNA-based approaches is their relative ease of implementation for both long-term and large-scale monitoring of complex communities. Even if these data are not necessarily well resolved at the species level, they still constitute invaluable occurrence data and thereby provide more information on species distributions, another EBV (Table 1), that is currently largely lacking for neotropical rainforest taxa (Antonelli, Ariza, et al. 2018).

To date, eDNA data and metadata reporting the location, time and exact protocol of the sampling are disseminated individually using study specific web repositories, as in data papers (e.g. Murienne et al. 2019) or more general repositories (e.g. Dryad, datadryad.org , Zenodo, zenodo.org ; or the Short Read Archives from Genbank, www.ncbi.nlm.nih.gov/sra). However, the construction of dedicated portals compiling eDNA-based taxa occurrence can now be envisioned for all neotropical rainforests and beyond following the examples of the occurrence portal GBIF (www.gbif.org), the BOLD system (www.boldsystem.org) which integrates DNA data with occurrence, or the EMP (www.earthmicrobiome.org) which compiles occurrence and diversity of microbial taxa across the globe. The success of such an endeavour depends on the adequate standardization of data, a challenge given that ecological signal from eDNA data is influenced by the technique used, the DNA region targeted, and the protocols of molecular biology and bioinformatics chosen. While defining standards for such purposes will certainly facilitate the integration of data across studies, it is also likely that this will be difficult to apply to all desired situations, which may ultimately undermine scientific advances. Several alternatives have been proposed to circumvent this issue. The first is to adopt sequence taxonomy classification as a standard unit (Ramirez et al. 2018). As highlighted above, such an approach heavily depends on taxonomic expertise and enriched DNA reference databases to make the best use of eDNA data. The second is the implementation of “eDNA biobanking”, i.e. the development of storage facilities for eDNA samples that could be reused with different technologies (Jarman et al. 2018).

Although less precise than traditionally collected occurrence data, which are limited in other ways, sections 3.1-2 demonstrate how eDNA-based studies can unveil the abiotic determinants of neotropical diversity. Increasing eDNA-based taxonomic inventories across environmental gradients will provide insights into taxa environmental/physiological tolerances/preferences (Table 1), information which remains scarce in neotropical rainforests. From a more applied perspective, increasing eDNA sampling across land use gradients will enable the identification of indicator taxa for environmental impacts or umbrella taxa that are specific to this ecoregion. However, this application currently remains limited by the difficulty in retrieving population size information from eDNA as discussed above. Without significant developments for this particular aspect, eDNA-based approaches will likely remain of limited utility when assessing the conservation status of neotropical taxa.

4.3. Shedding new light on biotic interactions

The increasing use of eDNA will also certainly fill the current gap of knowledge on species interactions (Table 1) by improving the description of complex and multi-trophic communities for both well studied taxa and more elusive organisms. Such assessments are urgently needed at a time where environmental changes already cause direct species loss and cascading extinction via bottom-up or top-down effects, especially in tropical ecosystems, including neotropical rainforests, where biotic interactions are often expected to be highly specific (Barnes et al. 2017).

It is now possible to analyse the diet of a particular species by collecting faeces, gut contents or even the DNA traces herbivores or pollinators leave on plants (Koskinen et al. 2019; Thomsen & Sigsgaard 2019). These applications are routinely used in temperate ecosystems (Bohmann et al. 2014; Taberlet et al. 2018; Alberdi et al. 2019). By contrast, only few diet studies have been performed on neotropical organisms, i.e. on tapirs from French Guiana (Hibert et al. 2013), on white-face capuchins from Costa Rica (Mallott et al. 2018), on neotropical vampire bats (Bohmann et al. 2018) and rodents (Lopes et al. 2015), and on particular arthropods (Paula et al. 2016; Kocher, de Thoisy, François Catzeflis, et al. 2017; Rodgers et al. 2017). New protocols of diet assessment based on faeces or gut contents are now available and optimized to reduce host DNA concentration in DNA extracts (e.g. Krehenwinkel et al. 2017). Such improvements considerably reduce the costs associated with molecular treatments and sequencing and hence allow for the implementation of large-scale studies of full food-webs composed of understudied and hyperdiverse taxa. This will certainly enable improved characterisation of trophic niche and breadth for many neotropical taxa, thereby improving documentation of feeding behaviour in relation to species functional traits and competitive interactions.

Likewise, eDNA can be used to unravel plant-pollinator networks. Pollinators yield substantial amounts of pollen on their bodies, and conversely the surfaces of leaves and flower petals also harbour traces of DNA belonging to visiting pollinators. This material can be used to build reliable plant-pollinator insect interactions, as shown in temperate ecosystems (Pornon et al. 2016; Thomsen & Sigsgaard 2019). The applicability of the methods has, to our knowledge, not yet been tested in neotropical rainforests and remains to be critically assessed due to the particular climatic conditions, much greater richness, and also the greater amount of vertebrate pollinators in these ecosystems, which can be more challenging to sample than arthropods.

Similarly, improved understanding of host-microbiota interactions can have important implications for threatened species conservation (West et al. 2019). This can be done by studying microbial communities occurring at the surface or within larger organisms in a more comprehensive way than before. So far, existing studies have principally aimed to describe microbial communities and, in some cases, their assembly mechanisms. This has been done mostly for leaf or root endophytes in trees (Kembel et al. 2014; Bonfim et al. 2016; Schroeder et al. 2019; Donald et al. 2020), palms (Donald et al. 2019), grasses (Higgins et al. 2014) or fern species (Del Olmo-Ruiz & Elizabeth Arnold 2017) and for the microbiota of frogs to assess its potential role in the resistance to the chytrid fungus *Batrachochytrium dendrobatidis* (Hughey et al. 2017; Catenazzi et al. 2018). To our knowledge the microbiota associated with neotropical mammals as been only assessed for the endangered Andean Bear (Borbón-García et al. 2017), and the same holds true for the microbiota of invertebrates, which has been so far mostly studied on emblematic arthropods such as ants (Sapountzis et al. 2015; Pringle & Moreau 2017). Although few studies have shown experimentally that the plant microbiota can promote the growth and survival of seedlings (Christian et al. 2017; Leroy et al. 2019), much remains to be done to understand the functional contribution of the microbiota to host health, and how this can affect community level distribution or diversity patterns (e.g. Janzen-Connell effects accounting for the whole microbiota (Janzen 1970, Connell 1978)).

The approaches discussed above mostly enable reconstructing bi- or tri-partite networks, but future applications are likely to span the whole ecological network to advance our understanding of the resistance and resilience of biological communities to disturbance. Indeed, eDNA can provide co-occurrence data for multiple taxonomic, functional and trophic groups retrieved from soil, sediments or water. While these co-occurrences do not represent biological interactions *per se*, these can assist in the discovery of a large variety of interactions at larger temporal/spatial scales, provided that these inferences are evaluated with *a priori* knowledge of the system or statistical tools (Vacher et al. 2016).

4.4 Epidemiology and healthcare

Neotropical rainforests ecosystems harbours many emerging infectious diseases, and use of eDNA for monitoring their agents or vectors has enormous implications for human health. Most parasites and pathogens are usually only detected when aggregating on or in their hosts and without eDNA, their detection remains challenging in the environment (Bass et al. 2015). Recent results from Sengupta *et al.* (2019) indicate that free living larval aquatic phases of *Schistosoma* can be detected with eDNA from water samples, opening an avenue to the control of this neglected tropical disease affecting >250 million people worldwide, mainly in Africa, but with human infestations in several regions of South and Central America. Although using eDNA as diagnostic evidence for pathogens or parasites requires extensive validation before it is used in notification procedures or detection programs (Bass et al. 2015), developments of such methods in the region would considerably improve the monitoring and fight against agents of tropical diseases.

A number of human diseases require a vector, typically an insect, to transmit the pathogen and surveillance programs usually rely on monitoring potential vector populations. Such a task can prove daunting given a single night of trapping using a standard CDC trap (Center for Disease and Control) could yield thousands of mosquitoes / sandflies that need to be identified to species level. eDNA-based approaches greatly reduce the time and costs related to these identifications (Kocher, Gantier, et al. 2017; Talaga et al. 2017), and could be used for routine monitoring of vector species and help in the control of vector-borne diseases.

Classical epidemiological monitoring programs largely focused on pathogens or their vectors, yet it is increasingly recognised that the prediction of transmission risk should include a better understanding of the ecosystem as a whole. This is particularly true in a context of biodiversity erosion and habitat degradation, which could be connected to the emergence of diseases as a result of trophic food-web modifications. For example, deforestation has been suggested to lead to the emergence of diseases such as malaria (Vittor et al. 2009) or Buruli ulcer (Morris et al. 2016), through reductions in diversity and modifications to the species composition of aquatic food-webs. Because eDNA-based methods can provide not only rapid information on pathogens and vectors, but also a broad characterisation of the whole ecological network, we believe they will strongly modify our approach to epidemiology and understanding of disease emergence in the next few years.

4.5. Conservation and impact assessments in neotropical rainforests and beyond

Managing ecosystems and biodiversity requires efficient detection of the species of interest, but also standard, cost- and time-effective protocols that can be implemented repeatedly across large spatial scales and through time, with low, or limited impact on organisms. Such protocols are currently not available for monitoring neotropical rainforests and, more generally, neotropical ecosystems. This review shows that eDNA-based methods fulfil these criteria while enabling characterisation of the taxonomic composition of multiple trophic communities, and could even constitute proxies of other EBVs. These methods complement remote sensing tools since eDNA provides information at a much finer taxonomic resolution, thereby better complying with some of the Aichi Targets that focus on endangered and invasive species (Marques et al. 2014; Bush et al. 2017). Their use could hence greatly facilitate the establishment of Rapid Biodiversity Assessment programs.

eDNA-based rapid biodiversity assessments hold great potential for the evaluation of environmental impacts, in particular for the ever increasing unsustainable use of land in neotropical rainforests, as exemplified with soil organisms (e.g. Franco et al. 2019). Likewise, eDNA-based methods will be able to help evaluate the success of different restoration and conservation strategies (Fernandes et al. 2018). However, the use of eDNA for informing management and political decisions will inherently require the development of quick and standardised sampling protocols that work across varying environment types and can be easily applied by practitioners. Beyond standardisation, which we show here to be a challenging issue, such an application implies the development of biotic integrity indices that are easily transferable to stakeholders, resource managers, and policy makers, and eDNA research is still in its infancy on this particular matter (Cordier et al. 2019). Nevertheless, we are confident that these limits can and will be overcome in the near future.

Aside from rainforests, the Neotropics holds large areas of other biomes that face threats that are not necessarily the same as for tropical rainforests but whose diversity remains poorly described with both traditional and eDNA methods (Antonelli, Ariza, et al. 2018). For example, eDNA could be particularly relevant to describe and monitor white-sand ecosystems which harbour a unique flora and fauna (Fine & Baraloto 2016), but which are currently threatened by increases in cattle ranching, deforestation for firewood or mining for sand (Ferreira et al. 2013). Likewise, it could be used for savanna and dry forest conservation, habitats which currently experience greater pressures than other neotropical biomes, typically as deforestation, localised human disturbance and increasing drought frequency and intensity (Strassburg et al. 2017; González-M et al. 2018). For example, the revision of Brazil’s Forest Code in 2012, the Cerrado (Brazilian savanna) indirectly encouraged Brazilian agribusiness to invest in this biome (Soares-Filho et al. 2014; Strassburg et al. 2017). Estuaries, including mangrove forests, also represent neglected and threatened habitats in the Neotropics, whilst harbouring rich communities and serving as a nursery for many fish and crustaceans (Mumby et al. 2004). In these environments, the turbidity and strong water currents make species inventories difficult, a limit that could be circumvented with eDNA (Stoeckle et al. 2017). A last example is the Pantanal biome, a savanna wetland which hosts a unique diversity, supports essential ecosystem services, and is currently under strong human pressure (Alho 2008). Descriptive and monitoring studies using eDNA analysis in these neglected yet important ecosystems would therefore help to better characterize their diversity and how they respond to various pressures.

From a more basic perspective, the possibility to implement comprehensive, large-scale and long-term biodiversity observatories will certainly help to gain insights into the origin and maintenance of neotropical biodiversity, and its singularity in many ecosystems. Reconstructing past ecosystems from ancient DNA (Thomsen & Willerslev 2015) would be extremely valuable in such a case, and would further improve our understanding of the long-term dynamics of neotropical ecosystems, and hence better predict their future. However, it remains unclear whether eDNA can persist in the long term in tropical ecosystems and further studies are required in this area. Nevertheless, long term dynamics can be assessed through monitoring initiatives along transitions between different biomes. For example, savannas and dry forests constitute transitory or alternative stable states of rainforests in response to global changes (Nepstad et al. 2008; Dexter et al. 2018), and monitoring these sites through eDNA should provide useful information on their dynamics, enabling the identification of early warning markers of major ecological transitions. Acquisition of such data will prove valuable for anticipating the status of these environments and prioritizing corresponding conservation or restoration actions to mitigate such transitions.

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Tables

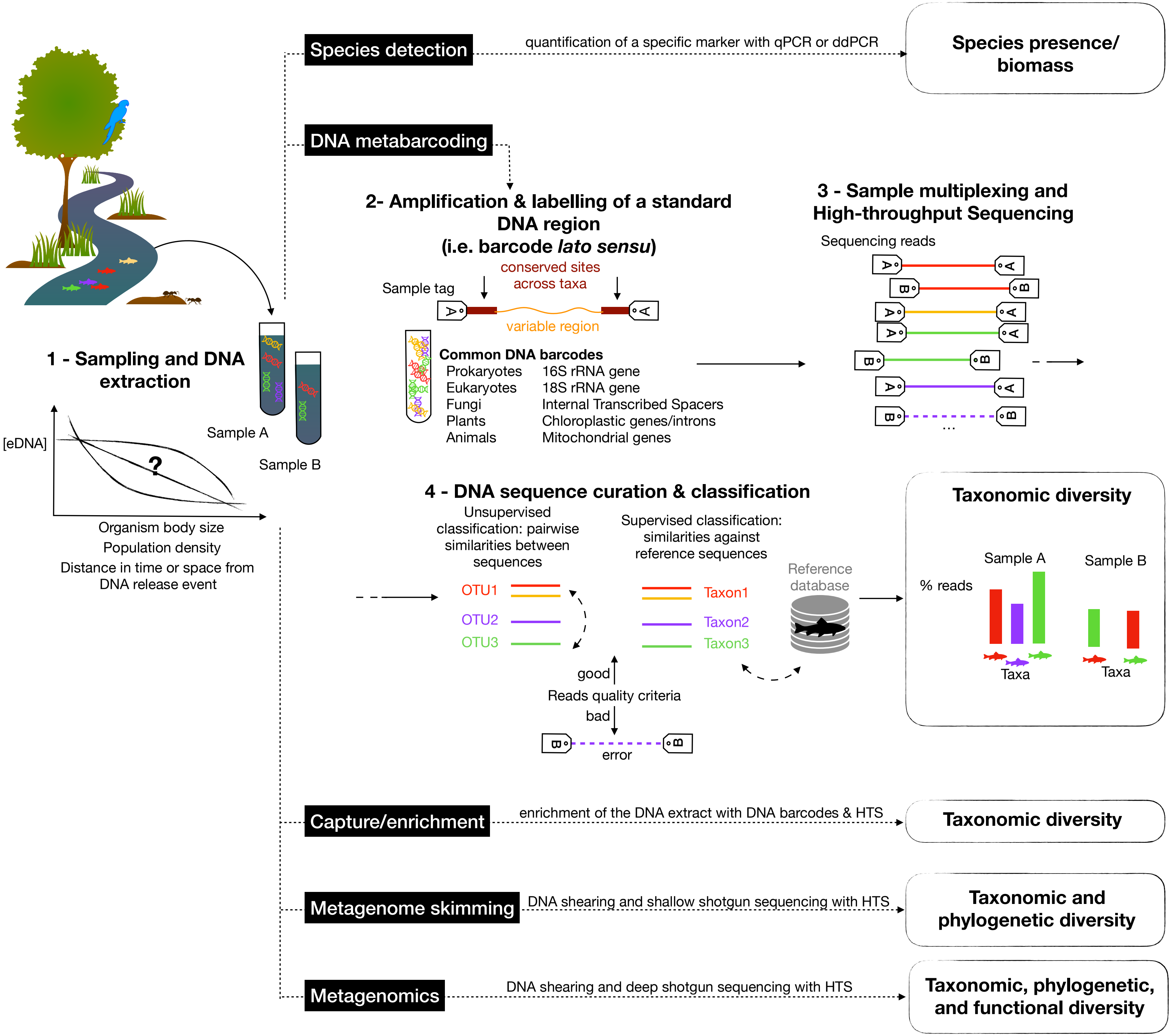
Table 1: Essential Biodiversity Variables (EBVs) and potential utility of eDNA-based methods to measure them in neotropical rainforests. EBVs are as defined by Pereira et al. (2013). Sections of this review or reference paper discussing such applications, or associated limitations are also indicated. NA: no documentation available yet. Usefulness levels are attributed depending on the biases of eDNA for each EBV candidate, the potential costs, as well as the extent to which eDNA information has to be complemented by other sources (e.g. species functional traits).

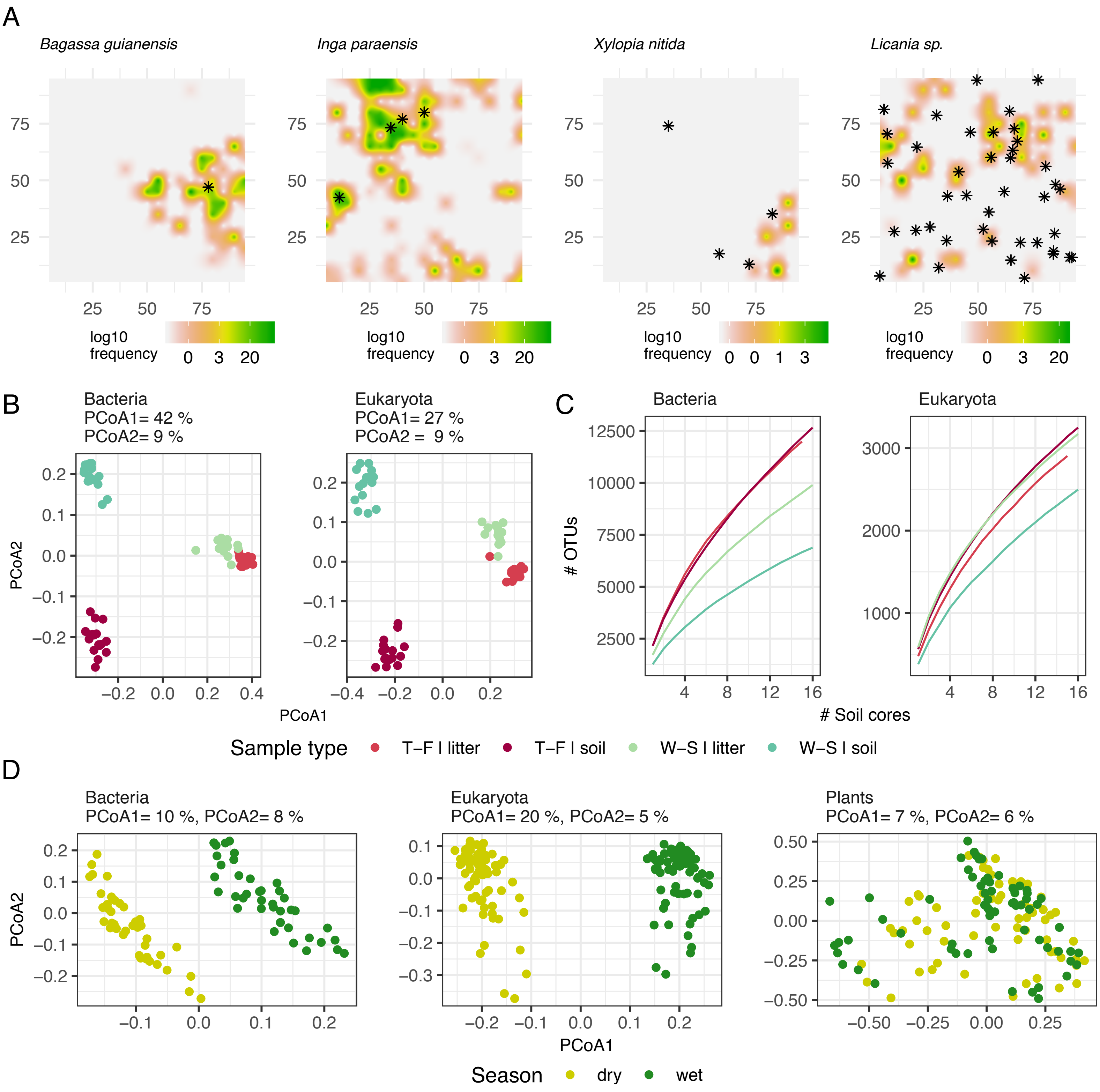
EBV Class	**EBV Candidate**	**Utility of eDNA**	**Sections or references**
Genetic composition	Co-ancestry	Fairly useful	2, 3.4
	Allelic diversity and population genetic differentiation	Fairly useful	(Sigsgaard et al. 2016)
	Breed and variety diversity	Unknown	NA
Species populations	Species distribution	Very useful	1, 2, 3.1, 3.2, 4.3, 4.4
	Population abundance	Poorly useful	2, 3.3., 3.4
	Population structure by age/size class	Useless	NA
Species traits	Phenology	Fairly useful	3.1, 4.1
	Morphology and Reproduction	Useless	NA
	Physiology and movement	Fairly useful	4.1, 4.2
Community composition	Taxonomic diversity	Very useful	1, 2, 3
	Species interactions	Very useful	1, 4.3
Ecosystem function	Net Iary or IIary productivity	Poorly useful	NA
	Nutrient retention	Useless	NA
	Disturbance regime	Fairly useful	3.1, 3.2, 4.5
Ecosystem structure	Habitat structure	Fairly useful	3.1, 3.2, 4.5
	Ecosystem extent and fragmentation	Fairly useful	3.1, 3.2, 4.5
	Ecosystem composition by functional type	Useful	4.1, 4.3

Figures caption

Figure 1: Overview of the main eDNA-based methods with a focus on DNA metabarcoding applied to fish diversity assessment. The broad information that can be retrieved through each of these methods is depicted in white boxes. Step 1 corresponds to DNA sampling and extraction, which is common to all eDNA-based methods (black boxes). Each step of DNA metabarcoding is then described: Step 2 depicts the DNA amplification step and which DNA regions are generally used. It also shows how multiple samples can be sequenced in parallel: by adding a small sample-specific nucleotidic label in the 5' region of each primer (here corresponding to sample A) prior to or after DNA amplification. Step 3 illustrates a multiplex of samples that has been sequenced in a single sequencing run. Between *ca*. 500-1000 samples can be multiplexed on Illumina sequencers depending on the sample diversity. The sequencing step can be seen as a sampling process; the more diverse the pool of amplicons (i.e. containing different barcodes), the more sequencing reads are required to appropriately describe the sample diversity and composition. The dashed sequence in sample B illustrates a tag-jump event. Step 4 broadly summarizes the bioinformatic procedures used to curate/annotate the sequencing data and ultimately retrieve a site by OTU/species table.

Figure 2: Examples of soil/litter eDNA signals in 1 ha forest plots of French Guiana. A) Comparison of the eDNA imprints of different tree species in soil samples collected every 5 m across a 1 ha plot in the Nourague Reserve, and in the top 10 cm of the soil layer. The colour gradient represents the log 10 relative abundance of sequencing reads from each species. Black stars correspond to the location of tree stems with diameter at breast height ≥10 cm. The two left panels show signatures that are consistent with the locations of conspecific stems. The two right panels show inconsistent trends, where "false absences" (i.e. absence of DNA when a stem is present) is likely due to deep rooting systems and "false presences" to roots of small trees not included in the botanical inventory. It is unlikely that they correspond to pollen, seeds or litter, because such material should be present around the other conspecific stems. **B-C)** DNA metabarcoding based analyses of bacterial and eukaryotic communities from soils and litter samples (*ca*. 10 g and 0.5 m3 each respectively) collected in 1 ha plots of a t*erra firme* forest (Nouragues Reserve) and white-sand forest (Mana). The plots show differences **B)** in community composition as measured with the Bray-Curtis index on hellinger-transformed data, summarized with a principal coordinate analysis and **C)** in plot-scale diversity and spatial heterogeneity, as depicted with species accumulation curves. **D)** Seasonal variations in bacterial, eukaryotic and plant community composition in the same plot as in A) and retrieved with soil eDNA. The figure has been produced using the same indices and techniques as in B).


1 - Sampling and DNA extraction
Sample A
Sample B
[eDNA]
?
Organism body size
Population density
Distance in time or space from DNA release event
Species detection
quantification of a specific marker with qPCR or ddPCR
Species presence/ biomass
DNA metabarcoding
2- Amplification & labelling of a standard DNA region (i.e. barcode *lato sensu*)
conserved sites across taxa
Sample tag
A
variable region
Common DNA barcodes
Prokaryotes 16S rRNA gene
Eukaryotes 18S rRNA gene
Fungi Internal Transcribed Spacers
Plants Chloroplastic genes/introns
Animals Mitochondrial genes
3 - Sample multiplexing and High-throughput Sequencing
Sequencing reads
A
B
...
4 - DNA sequence curation & classification
Unsupervised classification: pairwise similarities between sequences
Supervised classification: similarities against reference sequences
OTU1
OTU2
OTU3
Taxon1
Taxon2
Taxon3
Reference database
good
Reads quality criteria
bad
error
Taxonomic diversity
% reads
Sample A
Sample B
Taxa
Capture/enrichment
enrichment of the DNA extract with DNA barcodes & HTS
Taxonomic diversity
Metagenome skimming
DNA shearing and shallow shotgun sequencing with HTS
Taxonomic and phylogenetic diversity
Metagenomics
DNA shearing and deep shotgun sequencing with HTS
Taxonomic, phylogenetic, and functional diversity


A
Bagassa guianensis
Inga paraensis
Xylopia nitida
Licania sp.
25
50
75
log10 frequency
0 3 20
0 0 1 3
B
Bacteria
PCoA1= 42 %
PCoA2= 9 %
Eukaryota
PCoA1= 27 %
PCoA2 = 9 %
PCoA2
PCoA1
0.2
0.1
0.0
−0.1
−0.2
−0.4
0.4
C
Bacteria
Eukaryota
OTUs
12500
10000
7500
5000
2500
3000
2000
1000
4
8
12
16
Soil cores
Sample type
T−F l litter
T−F l soil
W−S l litter
W−S l soil
D
Bacteria
PCoA1= 10 %, PCoA2= 8 %
Eukaryota
PCoA1= 20 %, PCoA2= 5 %
Plants
PCoA1= 7 %, PCoA2= 6 %
PCoA2
PCoA1
−0.3
0.50
0.25
0.00
−0.25
−0.50
Season
dry
wet

Dear Alex,

Thank you very much to you Alex for these recommendations as well as to the referees for their comments. We have followed most of these suggestions, and explain below why when we did not. Overall, we took care to reduce the length and restructured the whole manuscript to make it more fluid and accessible. We also provide a roadmap paragraph at the end of the introduction to facilitate the reading. We believe that all these comments greatly helped us to improve the manuscript, and we hope that this new version will be satisfying and of help for the readers.

Best,

Lucie, on behalf of co-authors.

##############################

REFEREE 1:

R1.1: Authors have reviewed many aspects of the use of eDNA in neotropical rainforest. Based on the structure of the review, reader can easily get lost in all the sections/text and lose track of the reason of why is currently reading this review. Lots of information are repetitions of other reviews that could be removed from the main text and included as table/box or figure, authors even suggest other reviews for specific topics (mainly introduction and current applications). When the reader finally get through those sections, challenges and perspectives are actually providing new information with very good point for better sampling design and how eDNA could be applied in the region.

>>> We understand the referee's point and strongly reduced the length of the manuscript accordingly.

R1.2: In general, the manuscript is rarely interrupt with a figure or table (not present at all) and when present figures do not add additional information than the text (see specific comment on figure 3).

>>> We have included an additional table that we refer to at several instances in the MS. We also removed the former figure 3 and modified figures 1 and 2.

R1.3: The other problem of this review is that not include any information on other techniques that are not sequencing even if one of their main point is that eDNA is not yet a quantitative measure. At the moment all most all the studies that have tried to link eDNA to biomass are based on qPCR results. As the estimation of biomass from eDNA is big problem and authors make it clear in their review it also important adding information on studies that used qPCR in eDNA. eDNA used in combination of qPCR for detecting threated species or invasive is one of the most advance area of research especially for the standardisation of the results across different species/labs (see for example "Reporting the limits of detection and quantification for environmental DNA assays" or "Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate") and have practical application in conservation efforts.

>>> We added such considerations briefly, but did not extend the text on this aspect due to the manuscript length limit and the very limited number of such studies in neotropical ecosystems. In addition, we respectfully disagree that this approach is the most advanced area of research on eDNA, as, to our knowledge, the amount of proof of concept papers equals, or is even lower than for e.g. DNA metabarcoding applications. Therefore, we prefer to refer only to dedicated reviews here.

R1.4: Another point that should be revised is how much importance should be given to microbial ecology studies. Microbial studies are really interesting and are considered the base on which eDNA is developing as the former has a longer tradition as research field. Based on existing literature, studies and reviews have made a clear separation between the microbial ecology studies and eDNA studies, with some overlapping possible within the microeukaryotes communities. In this review, a big part of text is devoted to pure microbial studies (e.g. terrestrial ecosystems and aquatic ecosystems sections) and should be reduced.

>>> We understand the referee's point and took care to strongly reduce the text dealing with microbial ecology. However, we have still let some references to it given the increasing overlap between classical and microbial ecology that are now possible such as with eDNA techniques, as well as with the increasing interest of classical ecologists to host-associated microbes.

R1.5: Keywords: should include one or two words relative to the rainforest or geographical area used for the review

>>> Done

R1.6: 1.2 Environmental DNA definitions: Line 124 Authors use the world "metagenome" to indicate all the environmental DNA that is present in the sample. As the target reader would include non-molecular expert the word "metagenome" can create confusion and been associated with shotgun sequencing. See for example line 260 and following, "Metagenomics is the most direct …". I would suggest using a different word to avoid any confusion.

>>> We disagree with the referee on this point, because metagenome is the exact term that one should use in this case. However, to avoid any confusion, we provide a clear definition of this word l.110ff.

R1.7: Line 130 Authors state that they are going to use the eDNA in the broadest sense including also faeces or gut content in the eDNA starting material. Authors should add a phrase to explain what is included in eDNA in the narrowest term, again as many readers are not familiar with terminology.

>>> We now provide an explicit definition of eDNA in its narrowest and broadest sense l.104ff and 114ff

R1.8: Lines 153-155 and 158-160: Authors suggest that the amount of eDNA is roughly positive correlated with biomass, this is still a matter of debate and an active research area. Authors should point out this uncertainty, the reader can get the wrong notion that biomass can be estimated based on eDNA. The same comment is for the following phrases about spatio-temporal information. The proximity of the source of eDNA is clearly a matter of debate especially in water system in which eDNA can travel up to 200 km from the source.

>>> We have removed this consideration and now discuss more about this particular aspects throughout section 3, in particular section 3.3. and 3.4.

R1.9: *Line 161 "Extracellular DNA cam also persist in the environment from several hours…" this is also true for intracellular DNA and therefore I suggest changing extracellular DNA to eDNA.*

>>> We removed this part of the introduction to comply with the referee's comment R1.1

R1.10: 1.3 Methods for processing eDNA: As said in general comment, this section can easily cut it down or convert in tables/figure. Authors correctly pointed out that several reviews are already present (line 188-189) to cover most aspects of this section and repetition should be avoided.

>>> We now present this in a dedicated section (section 2) and strongly reduced the text to better rely on Figure 1. We still believe that minimum information has to be provided for readers that are not familiar with these technologies (and often use incorrect terminology when talking about eDNA-based methods).

R1.11: Figure 1 In general it is a very clear figure with detailed information for all the aspect that's should cover. Sample B is not too clear, from what I understood is a sample in which an error has occurred, and the wrong specie has been assigned to the wrong fish, is it correct? Authors should explain in the description.

>>> We thank the referee and have now better explained what is new or artifactual in sample B. We also modified the figure to make it more informative for the whole section 2.

R1.12: 2.1 Terrestrial ecosystems: Lines 399-431 Authors dedicate a large section of this section to microbial ecology, which is really interesting, but it is off topic in this context. Authors should consider removing it or reduce it drastically. The following part even if still include microbiology linked with eukaryotes and provide an excellent review on how to sample soil core and what has been done in neotropics bioregion.

>>> We thank the referee for this advice and have amended this section (now section 3.1) accordingly. As mentioned above, we still kept some references on microbial communities, which could be of interest for some of the potential readers of this review.

R1.13: Lines 433-435 Bulk samples and soil eDNA studies are compared but authors have stated in line 130 that they will use eDNA in the broadest sense which include bulk samples therefore they should specify better what they mean with soil eDNA. The use of eDNA in is broadest and narrowest sense through the manuscript is a weak point as this led to inevitable confusion in non-expert reader.

>>> In the revised manuscript, we made sure to clearly specify whether the examples/limitations we discuss apply to soil/sediment/water eDNA, or to eDNA obtained from bulk samples throughout the section 3 when this information is important to mention.

R1.14: Figure 3. Figure is not adding/explaining any particular information that cannot be retrieved by the text. Figure should be removed or changed to add meaningful information compared to the text

>>> We have removed this figure.

R1.15: 2.2 Aquatic ecosystems: Line 636-652 As stated in general comments a good section of this paragraph is about more microbial ecology than eDNA. All microbial ecology part should be shortened.

>>> Done

R1.16: 2.5 Host associated microbiota: This paragraph mostly belongs to microbiology research field. Endophyte communities, root associated microbiota, microbial symbiosis with eukaryotes are all classic area of microbiology research. The gut microbiota could be incorporated with the diet analysis in one section.

>>> We have considerably revised this section and that focusing on diet, which we moved to the perspectives section, as this is probably the application of eDNA that has least been covered in neotropical rainforests. In this new structure, we also considerably reduced the length of the discussion on host-microbiota. l.865ff

R1.17: 3.1 Field and, wet and dry lab biases: In general, the paragraph is well written with several example specific based on neotropical area (lines 831-837) while other parts are mainly repetitioning present in several reviews that authors cite and invite the readers for further reading (lines 838-859). The second part should be cut or presented in a different format and only the relevant point should be present in the text.

>>> We agree and have shortened the text and make better use of Figure 1 here.

R1.18: Lines 874-875 Authors should include a mention to occupancy model that would likely become routinely used to detect false positive/negative.

>>> We now present occupancy models as a promising way of improving eDNA data l.601

R1.19: 3.2 Biological interpretations of eDNA: Line 971-973 eDNA molecules are able to persist in the environment for long period if trapped in soil or sediment, but in water the persistence is reduced, and this should be explicitly said otherwise the reader can get a wrong notion.

>>> We now state this explicitly l.655ff

R1.20: Authors make a clear point in this section on how at the moment eDNA is not quantitative measure for many reasons however in all the manuscript they never mention qPCR techniques even if they include citation as Maruyama et al 2014 (line 991) to explain why eDNA is still a not quantitative measure. Most of the research to make eDNA a quantitative measure is based on qPCR and information on this topic have to be included in the manuscript.

>>> We did not extend the MS on this particular aspect due to length constraints,but now mention this point more explicitly l.649ff.

R1.21: 4.1 Increasing the breath of reference database: Authors are pointing out that at the moment there is a lack of consensus on which marker gene should be considered as gold standard in eDNA studies, I will suggest adding which one they would suggest using to someone new in the field. For example, if one of the readers would like to start an eDNA project in neotropical rainforest authors would suggest using COI or other markers?

>>> While we agree that such discussion is extremely useful for end-users, we do not believe that our review should focus on this aspect, for which guidelines depend on the taxon and ecosystem studied, as well as on the initial question addressed. Providing meaningful advice on this matter would actually require a whole separate review, so we prefer to refer the readers to more specific literature l.746ff

R1.22: I do not completely agree with the sentences in lines 1085-1087 as it will be not so easily achieved. Tropical ecosystems harbour huge biodiversity and as the authors said later in their conclusion usually they lack the facilities to carry out molecular work and the transport of specimen outside the countries are not usually an easy path to follow.

>>> While we certainly agree that improving DNA reference databases can represent a non negligible certain burden (but see our discussions l.760ff), we do believe that this effort has to be mentioned and encouraged through collaboration between northern and southern countries. The same concern (on costs) holds true for use of eDNA in general, which is why we emphasize on the need for international collaborations in the introduction of section 4.

R1.23: Line 1145 and following. Several web repositories for eDNA metadata sharing are available

>>> We could not retrieve to what the referee was referring to, as the line number does not match the referee comment. However, we have added information on existing facilities to share eDNA data and metadata l.793.

R1.24: 4.3 Shedding new light on tropical ecological networks: Line 1236-1254 Authors are highlight how eDNA can be used to test several hypotheses as the Janzen-Connell however then they expand their discussion without any additional information for the reader excluding the final recommendation that should be moved further up in the paragraph.

>>> We totally suppressed this paragraph to shorten the manuscript and in particular the part related to microorganisms, as recommended by both referees.

REFEREE 2:

R2.1: The manuscript "Advances and prospects of environmental DNA in Neotropical rainforests" reviews the existing literature on eDNA use in the neotropics. It introduces eDNA, reviews its current applications in the tropical biomes of the neotropics, discusses challenges of eDNA methods, and looks at future uses of eDNA.

The manuscript presents an extensive review of the literature and could be of relevance to researchers in the neotropics, but has a few serious problems that need to be resolved prior to publication. The main problem is the length of the ms, which is 8,000 words over the suggested word count for this journal. The second major issue is the lack of cohesion throughout the ms, there is no clear focus or goal, and even the exact topic seems to fluctuate between different sections. Lastly, the review would benefit from a more thorough synthesis and interpretation of the literature, rather than mainly providing summaries of previous research.

I recommend major revisions prior to a decision of acceptance. As it stands, the ms is not yet suitable for a high-impact journal such as Advances in Ecological Research. Revisions to the length, cohesiveness, and interpretation of the literature would greatly improve the suitability of this ms for publication in Adv Ecol Res.

>>> We thank the referee for his/her assessment and suggestions. We overall agree with his/her criticisms and in this revision, we took special care to reduce the length of the MS, homogenize the different parts, improve cohesiveness, and clarify the objectives. We hope the revised text will be clearer.

R2.2: General comments: A big issue with the ms is its length. At 22,755 words (16,186 excluding refs), or 77 pages, it is too long (it exceeds the journal's suggested word limit by more than 8,000 words). The considerable effort that undoubtedly went in this ms is drowned out by unnecessary details and repetitiveness, turning this potentially very useful resource into an unwieldy ms that is unlikely to be used by many people. The word count could easily be cut by at least a third, or even half.

>>> We agree and have considerably reduced the length of the MS thanks to both referees comments.

R2.3: Another issue is the lack of a clear cohesive structure in the bulk of the review. The ms reads as if the authors each wrote their section, but there was limited post-writing effort to streamline the manuscript and make it into a cohesive paper. This unfortunately translates in a bulky ms that is in times hard to follow, with considerable repetition and varying quality of English language.

>>> We did our best to improve the manuscript cohesiveness both conceptually and with the language.

R2.4: Maybe this is because no clear goal of the review? The different chapters vary considerably in their focus ("neoptropical rainforests" vs. "tropical biomes of the Neotropics" vs. "Neotropics" vs. "Neotropical ecosystems" vs. "Tropical ecosystems of the Central and South Americas"). Setting up a clear focus and goal of the review in the introduction would help guide readers (and it seems the authors as well).

>>> We agree with the referee and now better make clear that our focus are neotropical rainforests throughout the MS, although we extend the scope of the review in the last section as a perspective (4.5).

R2.5: Sections (particularly Part 2) of the review read as a very traditional literature review (listing summaries of research papers) with limited synthesis of literature. While such reviews can be useful to researchers in a narrow field, it excludes a wider scientific audience and reduces the value for a high impact ecology journal.

>>> We have considerably revised all parts and in particular former sections 2 and 3 (now section 3) in order to better reveal what are the known and unknown for each of these systems from an eDNA perspective.

R2.6: While the conclusion raises very important points, most of the points raised are not mentioned anywhere in the review. This hiatus makes the conclusion seem like an afterthought to rationalise the review, rather than an integral part of the review.

>>> We agree and now consider the problems related to costs, training and Nagoya protocols in the introduction of the perspective section (section 4), as they apply if one is to apply these techniques more routinely in neotropical rainforests and beyond.

R2.7: Throughout the ms there is a tendency for “fuzzy language” to describe quantities (“fairly good”, “some”, “non-negligible”, etc.), which should be avoided in scientific writing. There are some minor language issues (grammar, word order)

>>> In this revised manuscript, we have taken care of avoiding any vague writing and revised the english.

R2.8: I don’t really understand the use of all the specific “shortfalls” (Darwinian, Linnean, Wallacean,…) in the text. As they seem to be explained each time, just leaving the explanation would make more sense than including more unnecessary terminology in an already jargon-heavy manuscript (except for referencing Hortal et al. 2015)

>>> We have excluded this jargon in the revised MS.

R2.9: Detailed comments: Title: The title does not seem to reflect the content of the review, which seems to be about more than only rainforests.

>>> We now better make the point on what ecosystem we focus on throughout the MS (neotropical rainforests).

R2.10: Abstract: l28: “wholly dependant” seems like rather strong statement; l29-30: incorrect use of the term “let alone”, consider switching to: “many species remain undiscovered, let alone described”

>>> Corrected.

R2.11: 40-44: It would be useful to explain this in detail in the ms intro

>>> We now provide a roadmap paragraph in the intro l.150ff.

R2.12: 41: “this ecoregion”: which one? Not introduced in abstract

>>> We have now replaced this by the actual system to which we refer to.

R2.13: Part 1: Intro: Intro does not clearly lay out what the aim of the review is, which makes for a confusing read for the rest of the ms: Is the aim to give an update of knowledge on neotropical rainforests? Explain value of eDNA in neotropical forests? Give a list and summary of each eDNA study in this bioregion? Etc..

>>> We now provide a roadmap paragraph in the intro l.150ff.

R2.14: First two paragraphs in intro are very long, suggest splitting for increased readability (potentially lines 61 and 93)

>>> Thanks for the suggestion. We reduced the whole introduction.

R2.14: l.53: Really? I did not know this, larger than the central Africa, Southeast Asia, etc. combined? Are there references available for this statement?

>>> Corrected.

R2.15:

- l.83: remove “now”
- l.84: not clear what the meaning is of “in general”
- l.98: consider replacing “for” by “even”
- l.109: “reduced” or “limited”?
- l.111: not clear of solving the aforementioned limitations will mitigate global change
- l.116-118: reference would be useful
- l.132-133: not relevant

>>> Done/Clarified/Modified

R2.16: 158-160: I do not agree with this statement, an extremely rare species depositing faeces where a sample is collected would (incorrectly) suggest a very high biomass. Suggest altering statement or providing clear references

>>> We agree and, as indicated for R1.8, the whole paragraph has been revised. These considerations are now only mentioned in section 3.

R2.17: 178: would be useful to refer sooner to figure 1 in this paragraph

>>> Done.

R2.18:

- 321: “extremely” is an exaggeration, consider removing
- 328-332: Repetitive section

>>> We modified the paragraph to avoid repetition.

R2.19: 332-334: The references used here are not risk assessments, but impact assessments. The two are entirely different management tools and cannot be interchanged

>>> We corrected the text accordingly.

R2.20: 340: “bioindication” is jargon and should be defined

>>> We now provide its definition.

R2.21: Part 2: Current applications; The different sections of part 2 are all differently structured. I would expect at least some parallels when comparing how eDNA has been used in different ecosystems / substrates

>>> We thoroughly revised the text in this part to make it more homogeneous and structured.

R2.22: Terrestrial ecosystems: No clear structure + too much detail in describing results of other studies, a review should synthesize more, rather than merely summarise.

Additionally, the paragraphs on different biota in this section seem to have different goals, which is quite confusing

◦ Section (407-440): Microbial DNA: very detailed description of results older studies

◦ Section (441-479): Invertebrates: no detailed info, but synthesis and suggestions for uses

◦ Section (480-502): Larger animals/plants: most detailed info on methods, high level detail on results, interpretation of those results (not done in any other sections)

◦ Section (503-527): what is the topic? Vertical heterogeneity AND microbial and plant seasonal variation AND eDNA persistence in soils?

◦ Section (528-551): This section goes back to animals, but now on other, better sampling methods than soil?

>>> We modified the whole structure of that paragraph and of that on aquatic ecosystems so that to go beyond a simple listing of existing papers and harmonized the structure of both paragraphs. In particular, we provide paragraph headings to better reflect this new structure, which follows discussion on each taxon and then general concerns/challenges particular to each of these environments.

R2.23: Aquatic ecosystems: some language mistakes (grammar, incorrect word use), could do with language edit. Follows different structure (again) from previous section. This section focuses more on method use, as I expected the entire review to do. Would suggest to try to follow format/order of terrestrial section though (e.g. microbial – invert – large species).

>>> See reply to R2.22.

R2.24:

- Diet analyses: interesting section, just some language errors
- Bulk samples: some minor language mistakes

>>> These paragraphs have been integrated either in terrestrial/aquatic ecosystems, or in perspectives (diet, section 4.3)

R2.25: Host associated microbiota: First sentence states this is done using faeces, tissues, or bulk samples, which places it in the previous two sections of Part 2. It is not clear why this is a separate section, especially since parts of this have already been discussed on 699 – 713. Consider integrating this part in the other sections, or re-writing this (and previous) sections so it's clear why this deserves separate section. Too much detail in describing results of other studies.

>>> This section has been strongly reduced and moved in to section 4.5 which discusses the perspective research on biological interactions.

R2.26:

- 370: "most" is too vague, reword
- 372: "certain" is too vague, reword
- 374: not clear what is meant by "achievements": studies, results, conclusions?

>>> Corrected

R2.27: 386-392: repetitive already discussed in intro

>>> We modified the introduction and this paragraph in response to another comment we had, so this comment does not apply anymore.

R2.28: 393: “non-negligible” is too vague, reword

>>> Vague wording has been excluded throughout the MS.

R2.29:

- 394-395: consecutive sentences starting with “however”
- 397: “second”: where was “first”?
- 419-420: irrelevant
- 445-447: references needed
- 490-491: change word order: “by directly using soil as”

>>> Corrected/Clarified.

R2.30: 495-498: Commenting on the methodological details of this single study, but not on any other study seems strangely out of place in this section

>>> We removed this part of the text, and only discuss the results briefly in the Figure 2 caption.

R2.31: 506: “horizontal heterogeneity” was not mentioned above, or anywhere else in the ms

>>> We have clarified this point l.323

R2.32: 513-516: not clear how seasonality fits into a paragraph on vertical heterogeneity

>>> We respectfully disagree with the referee. Both deal with variations in abiotic conditions, either on a temporal axis or on a spatial one. Nevertheless, we separated these two ideas in the revised MS to comply with other comments.

R2.33: 513-528: entire paragraph does not seem to fit into this section

>>> We removed this discussion.

R2.33:

- 529: previous paragraph already started with “finally
- 562: Remove “the”
- 568: Remove “typically”
- 578: Is this sentence about soil or water?
- 579: does “it” refer to water or eDNA?
- 580: what does “particles” refer to? Water, eDNA, other?
- 581: incorrect use of “therefore”
- 607 + 612: suggest using “toxins” or “ichthyocides” instead of “toxicants”
- 691: “some” is too vague, reword
- 692: “their diet”
- 700: “also now” is grammatically incorrect
- 706: change word order to: “by directly targeting”
- 732: “fairly good” is too vague, reword
- 749: remove “indeed
- 754-755: faeces and bulk tissues already addressed in the previous section
- 792: “exploding” is not suitable scientific language

>>> Thanks, all these points are now corrected/modified in the revised text.

R2.34: Part 3: Challenges; This part has very limited linking back to topic of the review (neotropical rainforests), I could find only three small examples in 10 pages. While it might be more difficult in a technical section, clearly relating back to the topic of the review would greatly increase relevance to readers. Word length should be reduced, removing repetitive sections and unnecessary fillers could cut word length by almost half.

>>> We have considerably reduced this section accordingly (now section 3.3 and 3.4). However, we feel that it is important to provide a reminder for these limitations. In our experience as readers, referees and editors ourselves, we still read many manuscripts that poorly account for (or even totally omit) these considerations. We hence believe that they will be useful for naive users/readers.

R2.35: 820 – 834 (intro): the same content could be written in a quarter of the word length

>>> See our reply to R2.34.

R2.36:

- 824: remove “indeed”
- 829: change to “prevent” (grammar: it the particularities that prevent, not the eDNA)

>>> Do not apply anymore.

R2.37:

- 836-845: another intro after the intro?
- 846-935: reduce length, too much circular reasoning and unnecessary filler

>>> See our reply to R2.34.

R2.37:

- 862: Change “beyond” to “After”
- 864: “Usually” or “unusually” low abundances?

>>> Corrected

R2.38: 1025: Not clear what reasoning authors argue against: the use of presence/absence metrics? Or the use of abundance metrics? If PA is considered unreliable, what is favoured instead? Abundance of reads does not reliably relate to real abundance, would be useful to explain more clearly what is meant.

>>> The whole paragraph has been modified and clarified.

R2.38: Part 4: Perspectives Too long, often repetitive, and includes irrelevant information

>>> We have also considerably revised and removed the redundancies in this section. We did our best to remove potential irrelevant information, although the referee did not single out any in particular.

R2.39:

- 1054: “has come of age” is a big statement, which may or may not be entirely correct
- 1061-1066: seems like a rather lengthy way to say “reference databases are poorly resolved”

>>> Corrected

R2.40: 1084-1099:

• It would be useful to introduce/explain what this list is meant to achieve, there is no clear lead to the start of it.

• It is not entirely clear to me how the first and second point differ: they both seem to argue for more complete, high quality reference databases

>>> We have considerably shortened this paragraph to remove potential redundancies

R2.41:

- 1088: “non-negligible” is vague, non-scientific wording
- 1084-1086: Both sentences start with “first” and “firstly”, but there is no “secondly” to follow up

>>> Corrected

R2.42: 1131 – 1134: Trait databases are available for some marine taxa (e.g. FishBase), but not necessarily linked to DNA reference databases

>>> We added examples of such databases l.782.

R2.43:

- 1147: different format of the term “terra-firme” to previous use (402: “terra firme”), same issue with “white sand” (1341), “white sands” (402), “white-sand” (1145)
- 1142: remove “nonetheless”

>>> Corrected.

R2.44: 1136-1172: savannas, tropical dry forest, mangroves, dee sea environments, etc are not rainforests, so not clear why they have been included here.

>>> We excluded deep sea environments to remain focused on terrestrial habitats, but still propose an opening to other ecosystems. Even if the review is focused on Neotropical rainforests, we make clear in section 4.5 (and also in the initial version) that going beyond rainforests in the Neotropics is relevant to better understand the fate of rainforests with global change. More generally, we did not find many studies on other ecosystems, which is also one of the reasons that led us to focus the review on rainforests.

R2.45: 1231-1246: While interesting, the threats to rainforest are well known and have been described in the intro, so most of this section could be removed

>>> These considerations are now partly excluded from the intro and the targeted paragraph has been reformulated.

R2.46:

- 1246: “non-negligible” is vague, reword
- 1262: “a number of empirical evidence”: incorrect grammar
- 1287-1291: needs references
- 1301: what are EBVs?
- 1323: “remains”

>>> Corrected

R2.47: 1310 - 1318: I thought the review was about rainforest and not savannas?

>>> See our reply to R2.44.

R2.48: 1321-1336: this paragraph on sanitation/parasites/healthcare does not belong in a section on “conservation biology and ecological risk assessments”

>>> We created a dedicated section to the topic accordingly l.894

R2.49: Conclusion: 1364: Is this review supposed to be about the Neotropics or neotropical rainforests?

>>> See our reply to R2.44.

R2.50: 1365-1366: This review did not compare research eDNA effort in other, less diverse regions. So this statement should either be removed or backed with references.

>>> Corrected

R2.51: 1365-1376: none of this was discussed in the review, so unclear why it is used as a conclusion

>>> We agreed and discuss these aspects much earlier in the manuscript now l.685ff

R2.52: Figures: Figure 1: needs a more detailed legend to make it easy to interpret

>>> Now provided.

R2.53: Figure 3: not possible to distinguish between the different shades of grey, so cannot interpret this figure

>>> Figure 3 has been removed.