Chapter 19

Environmental and invertebrate-derived DNA: a powerful approach for surveying and monitoring biodiversity

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Abstract

To know the organisms that surround us has always seemed a fascinating idea and a necessity to characterize an area and estimate its natural values. However, performing complete surveys of the organisms is not always an easy task, because some of them inhabit difficult-to-access areas, and some are rare or difficult to be visualized. The analysis of the DNA retrieved from environmental samples (for example, water, soil and air) (eDNA) is a promising tool to study rapidly and efficiently the species that inhabit any place. This approach seems to show a lot of advantages over traditional biodiversity sampling due to its higher sensitivity and reduced cost and time needed. Similarly, the use of DNA derived from invertebrates (iDNA), such as blood-feed mosquitoes and carrion flies, can also be helpful for surveying animal communities, and it has been demonstrated to be an important tool to answer other questions, such as distribution, range, as well as interactions among coexisting species. In this chapter, we will demonstrate how eDNA and iDNA samples combined with molecular tools have been used to survey

vertebrate and plant species in the neotropics, highlighting the potential of this approach for conservation strategies.

19.1 Introduction - eDNA and iDNA for monitoring biodiversity

Biodiversity is facing a worldwide crisis (Brooks et al. 2002; Bellard et al. 2012; Dirzo et al. 2014; Haddad et al. 2015). Thus, species monitoring is currently a top priority for biodiversity protection. However, owing to various limitations, traditional biodiversity monitoring methods may not always achieve the given monitoring purpose, raising the urge for additional methods capable of reliably monitoring species on wider spatio-temporal scales. Aiming to fill this gap, the advances in high-throughput sequencing paved the way for a new era in the biodiversity monitoring field, with recent advancements now opening new opportunities for studying biodiversity by sequencing DNA retrieved from a plethora of sampling media. Some of the main emerging techniques rely on the collection of traces of DNA present in the environment, the so-called "environmental DNA" (eDNA). Since all organisms release DNA continuously into the surrounding environment (e.g., shed skin, excretes, gametes, saliva, hair, feathers, scats), eDNA surveys aim to obtain such DNA remnants from environmental samples (e.g., water, sediments, soil, air, lake and ice cores) and identify the taxa through the use of specific molecular markers (Fig. 19.1).

Also, as in a science fiction movie, invertebrates that feed on vertebrates or use them to fulfill vital functions of their cycle (e.g., oviposition) have been used as a source of vertebrate DNA. Ingested DNA or invertebrate-derived DNA (iDNA) studies (Calvignac-Spencer et al. 2013; Schnell et al. 2015; Drinkwater et al. 2021) have previously investigated leeches, mosquitoes, flies and beetles to successfully survey wildlife and in some situations to conduct ecological and biodiversity assessments (Fig. 19.1).

Species detection studies can be focused on targeting a single species or the whole community within an area. Single-taxon approaches use specific narrow-target molecular markers and PCR, ddPCR, qPCR amplification (Ficetola et al. 2008; Takahara et al. 2013; Piaggio et al. 2014), while community-level approaches use universal markers and parallel sequencing to detect a broad range of taxa (i.e., "metabarcoding") (Wilcox et al. 2013; Rees et al. 2014; Thomsen and Willerslev 2015; Turner et al. 2015). Species-specific essays have been proven as the most suitable method when targeting one or few species, especially when considering rare and difficult-to-detect taxa (e.g., Hernandez et al. 2020), whilst the metabarcoding approach has been shown as a more efficient and cost-effective option for a

broad characterization of the ecosystem, also allowing the detection of unexpected species or hidden diversity (Gillet et al. 2018).

Genes COI for animals, and rbcL and matk for plants were previously chosen for species identification by sequencing biological samples of individual specimens in the DNA barcoding approach (Hebert et al. 2003; Hollingsworth 2011). However, for these emerging approaches, the development of new molecular markers was and still is necessary, especially because the DNA obtained from eDNA and iDNA samples is often degraded. The need to choose smaller fragments (mini-barcodes) for amplification of degraded genetic material, which is expected in eDNA and iDNA samples, led to the use of new genes and new primers to be targeting these shorter gene regions. For animals, the use of primers targeting small fragments, about 50-170bp long, from the genes 12SrRNA (targeting vertebrates) (e.g., Riaz et al. 2011; Miya et al. 2015) and 16SrRNA (mammals, e.g., Taylor 1996, and frogs e.g., Bálint et al., 2018) have provided satisfactory results for the identification of the biodiversity when working with mixed DNA samples. For plant identification, the use of the genes matK and rbcL was complemented with ITS (Hollingsworth 2011; Song et al. 2012) and trnL fragments (Riaz et al. 2011; Fanher et al. 2016). The matK has provided better results when used for invasive species identification due to the presence of specific regions that amplify only in target species (Scriver et al. 2015). ITS2 and rbcL were more efficient in general studies with vascular plants, when not using customized reference databases or local surveys (Fahner et al. 2016). However, the chloroplast trnL (UAA) intron is proposed as the most suitable marker for plant eDNA metabarcoding (Riaz et al. 2011; Coissac et al. 2012), although the reference database must be supplemented (Taberlet et al. 2007).

Since its onset, the number of studies that have addressed different aspects of the application of environmental DNA towards the detection of species has increased almost exponentially. This has led to a recent and broad knowledge gathered in this field, mainly in temperate and already well-studied ecosystems where eDNA and iDNA have been used especially for species detection. However, the combination of eDNA and iDNA samples with NGS technologies has great potential for population genetic studies, as showed in Adams et al. (2019). Still, eDNA and iDNA-based surveys in the neotropical realm, a high biodiversity region that is of great conservation concern, are still incipient, and a boost of studies is foreseen for the next few years. In the following sections, we will focus on demonstrating how eDNA/iDNA approaches have been used to study vertebrate and plant species, with a greater focus on the neotropical region.

19.2 Environmental samples

In biodiversity and conservation surveys, a myriad of biological samples can be obtained in the environment, including scats or gut content to assess the microbiota, understand the species' food preferences or ultimately, conduct biodiversity assessment by analyzing the species which the organisms have fed upon through the identification of gut content, and build interaction networks. Samples collected from the environment without the requirement of handling and/or seeing the animal or its traces are collectively categorized as environmental samples (Lacoursière-Roussel and Deiner, 2021). In eDNA studies, some examples of sampling media include soil and sediments to study both micro and macro-organisms (e.g., from bacteria to large mammals) (Kestel et al. 2022); permafrost to detect ancient DNA (aDNA) and investigate past biodiversity history (Willerslev et al. 2003); air for pathogen detection or even in detecting the presence of terrestrial vertebrates such as mammals (Klepke et al. 2022); and water, that is widely used to study micro and macro-organisms, allowing the detection of both aquatic and terrestrial species.

19.2.1 Water samples

One of the most popular media used in eDNA studies is water. Taking a few millimeters of water from an aquatic environment has already proven to be sufficient to reveal the occurrence of aquatic and terrestrial species present in a given area and its surroundings (Deiner et al. 2017). In this context, water eDNA has been recognized as providing a more complete picture of biodiversity composition when compared to traditional surveys, as well as allowing for multi-trophic analysis in metabarcoding studies (Blackman et al., 2022).

So far, the majority of eDNA studies conducted in neotropical realms used water as the main sampling media. Water eDNA samples have been used to detect the presence of invasive species such as the freshwater dinoflagellate *Ceratium furcoides* in Argentina (Accattatis et al. 2020), the North American crayfish *Procambarus clarkii* in Ecuador (Riascos et al. 2018) and the golden mussel *Limnoperna fortunei* in Brazil (Pie et al. 2017); to study the fish diversity in Argentina (Chalde et al. 2019; Nardi et al. 2020), Brazil (Sales et al. 2019, 2021; Dal Pont et al. 2021; Jackman et al. 2021) and French Guiana (Cantera et al. 2019; Cilleros et al. 2019); and also, to detect the presence of terrestrial vertebrates in Brazil (Sales et al. 2020) and Colombia (Mojica and Caballero 2021; Polanco et al. 2021).

Considering that approximately 71 percent of the Earth's surface is covered by water and thousands of taxa are expected to inhabit the aquatic ecosystem, retrieving genetic data from water samples opens up more opportunities to better detect and monitor species, mainly the rare, elusive and often neglected ones, enabling researchers to better understand the relationship between species and their habitat. As an example, ecological indices gathered from an eDNA study conducted in Curaçao revealed the contrasting anthropogenic pressure on functional diversity and species richness of reef fish (Polanco et al. 2022). Moreover, with the new and future improvements in the field, eDNA surveys are expected to move forward from the biodiversity inventories (e.g., species list) to provide more in-depth ecological data, even being able to provide additional population genetics information.

To some extent, eDNA is expected to be more widespread in aquatic environments and easier to capture species' presence when compared to soil samples. For instance, an integrative biodiversity assessment can be obtained across the land-water interface through the analysis of eDNA transported in river networks (Deiner et al. 2016). The persistence of eDNA molecules can vary greatly depending on sampling media. Water samples are often used to retrieve eDNA signals representing a shorter time span (e.g., days), in comparison to other media (e.g., soil – days to years, permafrost – thousands of years). The effect of eDNA ecology in distinct environments is noteworthy, and the short persistence of eDNA in water is linked to several factors that are usually intertwined, such as eDNA origin, state, transport, and fate, with the latter being impacted by UV exposure, temperature and other environmental factors (Barnes and Turner 2016). However, UV exposure, temperature, and other environmental factors' effects on eDNA persistence remain a puzzle for neotropical areas.

The lack of knowledge regarding the effect of these synergistic environmental factors on DNA degradation rates, coupled with largely restricted understanding of eDNA transport in neotropical water bodies (e.g., streams, rivers) represents one of the key challenges in disentangling signals associated with eDNA *vs* species ecology. For instance, Sales et al. (2021) demonstrated the potential of using water samples to infer spatio-temporal changes in fish assemblages, nevertheless, the effect of eDNA transport and degradation on species detection could not be assessed. Studies aiming to investigate the eDNA ecology in neotropical realms are expected to increase in the forthcoming years, aiming to address these aforementioned limitations in providing a fine-scale spatio-temporal resolution of species detection.

In order to decrease its degradation and optimize eDNA yield, samples must be collected, stored and processed properly (Fig. 19.2). After collection, water eDNA samples

require the shed DNA to be captured and/or concentrated, a process often conducted via centrifugation, precipitation (Fig. 19.2B) or filtration (Fig. 19.2C). Precipitation refers to a chemical process using ethanol to precipitate and isolate the nucleic acids, whereas, filtration is employed to retain DNA molecules using a filter of fine mesh, whilst allowing the passage of water (Jerde et al. 2011; Eichmiller et al. 2016). Filtration has been widely used and considered a better option when retrieving eDNA from water samples. Still, a broad range of filters of different compositions and mesh sizes is available and so far, no consensus has been reached regarding the best filter pore size and material to be used in eDNA surveys. In neotropical areas, it might be even more challenging, as the ideal total volume of water to be filtered has not been analyzed across different ecosystems yet. As an example, Lopes et al. (2021a) filtered 2 to 30 L of water per sample to investigate the presence of frogs, Cilleros et al. (2019) collected approximately 50 L of water in each sample to survey fishes, and Sales et al. (2020, 2021) used 500 mL to 1L to recover eDNA from both fish and mammals. Considering the vast distinctiveness of habitats to be sampled in the neotropics, it is important to conduct pilot tests to evaluate the best filter pore size and composition to be employed. As an example, in very turbid and sediment-rich water bodies, the filtration of large volumes of water might be impossible if using a fine mesh because the filters can get easily clogged preventing the water to go through the filter.

19.2.2 Soil samples

Soil is also a promising source of DNA to study vertebrate, invertebrate, plant and microorganism biodiversity and the interactions of these organisms with the environment (e.g., Oliverio et al. 2018; Zinger et al. 2019; Andersen et al. 2021; Nuñez et al. 2021; Ariza et al. 2022). Unlike in water, DNA can persist in the soil for thousands of years (Haile et al. 2007; Barnes and Turner 2016) as it can bind to environmental compounds, such as clay minerals or organic compounds, that protects DNA from total degradation (Blum et al. 1997; Crecchio and Stotzky 1998). Soil samples can be collected from the surface or from more deep soil profiles, if recent or ancient biodiversity is to be assessed, respectively. It is also generally assumed that the DNA recovered is the same age as the soil in which it was collected. Although this is a reasonable assumption, leaching of DNA to lower strata of soil must be taken into account in non-frozen areas, especially in neotropical areas where rain is more frequent and abundant (Andersen et al. 2012; Haile et al. 2007).

In the case of vertebrates, the identification of species presence through eDNA in soil was first applied in areas of permafrost, where DNA is better preserved due to the very low temperatures and leaching is expected not to occur (Willerslev et al. 2003; Pedersen et al. 2015). The quantity and deepness of DNA in the environment can be influenced by a number of biological (animal movement, defecation and other behaviors, demography, rate of cell shedding, etc.), edaphic (pH, particle size, organic matter content, etc.) and climatic factors (precipitation, temperature, UV exposure, etc.) (Levy-Booth et al. 2007; Andersen et al. 2012; Leempoel et al. 2020; Ryan et al. 2022). When these factors are accounted for, eDNA has been pointed out to reflect vertebrate abundance and richness from only a few grams of soil (Andersen et al. 2012). Soil has already been successfully applied to study a variety of vertebrate species (e.g., Kucherenko et al. 2018; Leempoel et al. 2020; Ryan et al. 2022). Although microorganisms have been studied through soil DNA in the neotropical region (Câmara et al. 2022), the potential of this DNA source for neotropical vertebrates has yet to be explored (Carvalho et al. 2022). To our knowledge, only the studies of Ritter et al. (2019) and Lopes et al. (2020) have applied this method in soils from the neotropics.

Ritter et al. (2019) used soil, litter (the organic portion above the mineral soil) and insects to test if the east-to-west biodiversity gradient known to occur in the Amazon forest for birds and trees could be recovered from eDNA and iDNA data. The authors collected 40 soil and 40 litter samples from each of the 39 plots and analyzed the total DNA extracted from the samples. A metabarcoding approach was used by amplifying portions of the genes 16SrRNA for prokaryotes, and 18SrRNA and COI for eukaryotes. There was no relationship between the operational taxonomic units retrieved and the richness of both birds and trees from previous field studies. Furthermore, the west-to-east biodiversity gradient was only partially reflected in the metabarcoding data, due to the effect of outliers in the dataset, which was pointed to be a result of the particularities of each studied area.

Lopes et al. (2020) analyzed litter as a source of eDNA to study the biodiversity of vertebrates, with a special focus on anurans, in an area of the Atlantic Forest in Brazil. Authors collected 32 samples of litter that were later combined into two bulks of 1 kg each. Litter was washed with a mixture of water and buffer and filtered in a fine cellulose membrane. Total and extracellular DNA was extracted from the membrane. A portion of the genes 18SrRNA for eukaryotes and 12SrRNA for vertebrates and anurans was analyzed. The authors were able to retrieve a large eukaryotic diversity with the 18SrRNA gene, but only two sequences corresponding to anuran species with the 12SrRNA gene. They considered the analysis of

eDNA obtained from litter a successful method to characterize the eukaryotic community. Low rate of shedding by amphibians, low number of sampling replicates, low volume of litter collected may have affected the detection of anurans in the area, since these species were observed by researchers in the litter during sampling.

As seen, soil is still beginning to be used and understood as a source of environmental DNA from vertebrates, especially in the neotropics. A few peculiarities of each study area must be taken into account when planning future eDNA studies from soil samples. For example, soils that are acidic or go through a process of acidification could reduce DNA absorption by the soil, as well as higher precipitation can contribute to a higher leaching of DNA (Allemand et al. 2007). Furthermore, the presence of DNA traces in the soil depends heavily on species abundance and soil use (Leempoel et al. 2020; Lopes et al. 2021b; Ryan et al. 2022). Unlike microorganisms that are spread across the soil, vertebrates move, defecate, urinate, shed cells and perform other behaviors not uniformly on the soil, depending on complex biotic and abiotic interactions. Thus, the amount of soil needed to detect biodiversity also varies, from a few grams in a controlled environment (Andersen et al. 2012) to several liters or kilograms in a natural environment (Leempoel et al. 2020; Lopes et al. 2020). This method can also benefit from a more targeted sampling in trails of frequent use or with the use of fences that direct species movement to where soil will be collected (Ryan et al. 2019; Burns et al. 2020). The advantages of using soil to sample vertebrate DNA are that (i) due to the role of soil as a source of DNA for microorganism studies, there are highly efficient commercial kits available that are specially designed to extract DNA from soil, that can be applied to studies focusing on higher taxonomic groups, and (ii) soil samples require low maintenance and processing in the field.

19.2.3 Alternative sources of DNA to assess the biodiversity and species ecology

Scat samples, especially those from species that have a generalist feeding habit, are also a powerful source of genetic material. Scats can provide data to assess biodiversity through the identification of the species that deposited the scats and the species that were fed on, thus allowing researchers to obtain information on species presence, distribution and diet. From these data, it is also possible to make inferences about species' ecological aspects such as networks, and spatial and temporal interactions. Recent studies with rodents (Lopes et al. 2020), bats (Ingala et al. 2021; Martínez-Fonseca et al. 2022) and large carnivores (Quéméré et al. 2021) have made it possible to corroborate or question the knowledge we currently have about the diet of these species in neotropical environments.

Using an indirect and combined approach, Lopes et al. (2020) were able to study the niche overlap of seven rodent species of the *Ctenomys* genus, that live in South America. Through the metabarcoding approach, the authors used rodent scats and soil to investigate plant consumption and plant availability, respectively. Scats were obtained from captured rodents and in burrows used by the species. Authors amplified regions of the P6 loop of the chloroplast trnL (UAA) intron and the first internal transcribed spacer (ITS1) of nuclear ribosomal genes to detect plant species in scat and soil samples. They found that the rodent species consumed 60% of the plant species detected in the soil samples, indicating that these species present a generalist feeding habitat. This result not only revealed the feeding habitat, but also that the allopatric distribution of the rodents reduces interspecific competition for the same resources.

Elusive species such as bats, which are characterized by their small size and nocturnal and volant behavior, are another good example of a group that can benefit from the use of molecular methods to study diet, because it is difficult to observe their feeding habits. In the study of Martínez-Fonseca et al. (2022), the metabarcoding approach was used to investigate the diet of the *Vampyrum spectrum*, a carnivorous bat, in Nicaragua. Scat samples from this species were collected directly under bats in roots and fragments of the COI, 12SrRNA and 18SrRNA genes targeting vertebrate and arthropod DNA were sequenced using the metabarcoding approach. This study revealed a total of 27 different vertebrate species in the bat diet, including birds, rodents and other bat species, besides arthropods, indicating that *V. spectrum* forages opportunistically. In another study, Ingala et al. (2021) investigated the diet of 25 bat species that co-occur in Belize, using DNA metabarcoding for detection of vertebrates, invertebrates and plants. Bats were captured and placed into cloth bags, where they defecated, and scats were collected. This effort allowed the authors to document bat diet at a multi-trophic level and fine-scale association between bat species and dietary items, showing that most of the studied species do not have restricted diets and that their habits are rather opportunistic. Although Ingala et al. (2021) did not propose to provide a full dietary niche breadth for the 25 species, because it would require many replicates, this study paved the way for future studies that aim to understand the coexistence and niche partition in bat assemblages.

Carnivores are also known to be generally elusive, rare (occur at low densities either because of natural or anthropogenic causes) and difficult to capture. This group plays an important role in ecosystems by regulating the population of other trophic levels, therefore, having a better comprehension of carnivore species diet is a pivotal concern that also supports the verification of ecosystems health. For example, the diet of the Endangered riverine

Pteronura brasiliensis, the giant otter, was assessed by collecting scats deposited in communal latrines along river banks or on small islands, in French Guiana (Quéméré et al. 2021). Authors also used a metabarcoding approach based on portions of the 12S and COI genes targeting vertebrate and invertebrate species, respectively. In this study, scat DNA-based metabarcoding was more efficient than conventional methods to study otter diet. It revealed the presence of species from several groups in the giant otter diet, including fishes, amphibians, snakes, birds and earthworms, and provided a basis to better understand possible human-otter conflict due to predation on species that are valuable as resources for human populations.

Some of the more elusive species are difficult to study. For example, little is known about the plant-animal interactions of the lowland tapir *Tapirus terrestris* despite their known role as engineers of the ecosystem. Hibert et al. (2013) studying the scats of the lowland tapir using metabarcoding succeeded in establishing the diet of this large mammal with great taxonomic resolution, increasing in two new families and eight genera the list of plants consumed by the species.

All these examples highlight the potential of scat DNA-based metabarcoding to investigate species diet with high accuracy, also supporting the exploration of ecological implications from that. Moreover, the information obtained from diet studies can be interpreted as a biodiversity assessment, especially when studying the diet of generalist species. Thus, diet metabarcoding can be an effective, noninvasive, and economically viable method for biodiversity monitoring, supporting management decisions (Noggard et al. 2021; Shao et al. 2021).

19.3 Invertebrate-derived DNA (iDNA)

Another complementary and more recent approach to biodiversity inventories is the detection of vertebrate species from DNA obtained through gut content of invertebrates that feed on vertebrates or from the insects that use the vertebrates to fulfill vital functions of their cycle (ingested-derived DNA or invertebrate-derived DNA, iDNA) (Calvignac-Spencer et al. 2013; Rodgers et al. 2017). Using invertebrates, such as carrion flies and mosquitoes for sampling DNA of vertebrates is advantageous because these insects are cosmopolites, can be easily sampled using commercial and handmade traps (Fig. 19.3), and can feed on all terrestrial vertebrates (Norris, 1965; Lynggard et al. 2019). Other invertebrates that feed on vertebrates and can be used as samplers include dung beetles (Drinkwater et al. 2021) (Fig. 19.4), leeches (Schnell et al. 2015), sandflies (Massey et al. 2021). The iDNA approach can aid the

biomonitoring of vertebrates that are elusive, rare or present in low population density, and in regions of high biodiversity, such as the neotropics, that are notably areas where a large portion of the local biodiversity remains unknown. However, the use of this approach in the neotropics has been narrowly explored (Carvalho et al. 2022). To date, only six studies used iDNA focused to obtain information about vertebrate communities in the neotropical region. Rodgers et al. (2017) compared the effectiveness of iDNA obtained from carrion flies and traditional methods to survey a well-documented mammal community in Barro Colorado Island, Panama. Although the authors focused on detecting mammal species, other vertebrate species were also recorded, because a mammal-specific (16SrRNA, Taylor 1996) and a broader vertebrate-specific mini-barcodes (12SrRNA, Riaz et al. 2011) were used. A total of 20 mammal species, four birds and one lizard were detected by carrion fly iDNA, a larger number of species than that obtained by the traditional methods (transect court = 13 species; camera-trap = 17 species; iDNA = 25 species) (Rodgers et al. 2017).

Lynggard et al. (2019) asked if it would be possible to detect vertebrate DNA without targeting a specific vertebrate-feeding invertebrate but using arthropod bulk samples. The authors investigated this question by collecting bulk arthropod samples in two regions, including a neotropical area in Brazil and amplifying the same mini-barcodes as Rodgers et al. (2017). Fourteen vertebrate species were recovered in the neotropical region, including anurans, carnivores, chiropterans, primates, artiodactyls and other mammals. This method was efficient in recovering vertebrate biodiversity, as it does not require prior taxonomic knowledge of the collected arthropod taxa and reduces laboratory procedures because arthropods do not need to be processed individually (e.g., preparation and DNA extraction). Authors pointed to the need for a detailed assessment of the number of replicates required to comprehensively assess vertebrate diversity, but also indicated that iDNA from arthropod bulks paired with metabarcoding could serve as a supplementary method to vertebrate monitoring.

In the study of Massey et al. (2021), authors compared the effectiveness of three invertebrates (carrion flies, sandflies and mosquitoes) as iDNA samplers to survey vertebrates in the Amazon region, Brazil. In this area, carrion fly DNA was the best method for landscape scale biodiversity surveillance as it retrieved higher vertebrate richness than mosquitoes and sandflies (gamma diversity). Also, mosquitoes and sandflies showed a feeding preference for humans and armadillos (Dasypodidae family), respectively. In the same study, iDNA results were compared to camera trapping surveys and, although camera trapping showed the highest mean species richness at site-level, it also showed a detection bias towards carnivore and

ungulate species (alpha diversity). Much of the biodiversity detected by the iDNA method was not evidenced by the camera-traps, as this latter method was biased towards large-bodied mammals. These results highlight that the combination of different iDNA samplers can provide better representativeness of the biodiversity.

Considering that iDNA is still narrowly explored in the neotropical region for biodiversity assessment, Saranholi et al. (2022) aimed to assess the effectiveness of the iDNA approach for surveying terrestrial mammals in a semi-controlled area, a zoo that houses several mammal species. The effectiveness of mosquitoes and flies as iDNA samplers were compared by the number of mammal species detected and by the distance between the trap where an insect was captured and the enclosure of the mammal whose DNA was ingested by the insect. To achieve this, differently from the previous studies, each insect captured was analyzed individually. A total of 45 OTUs were recovered. There was no difference between the number of mammal species recovered per individual insect, but the number of flies captured was higher than that of ingurgitated female mosquitoes, resulting in more mammal species recovered by flies. Eight and twenty mammals were recorded exclusively by mosquitoes and flies, respectively, suggesting that the use of both samplers allowed a more comprehensive screening of the biodiversity. The maximum distance recorded between an insect and the enclosure of the mammal that were fed upon was 337 m for flies and 289 m for mosquitoes, not differing significantly between groups. These results are helpful to raise insights to guide further sampling design and calibrate efforts for surveying mammals in high biodiversity areas, such as the neotropical region.

Besides the examples of iDNA applications cited above that have focused on surveying vertebrate communities and used a metabarcoding approach, iDNA can also be used to study host-vector interactions. Araujo-Pereira et al. (2020) and Rodrigues et al. (2021) investigated the mammal DNA presents in the blood meal of sandflies from the Psychodidae family, a Diptera that can transmit the protozoa that causes leishmaniasis. The identification of blood meal sources of the sandflies and other insects that serve as disease vectors is an important step for vertebrate host identification, supporting the control of vector borne diseases. Both studies captured sandflies in the Amazonian region, Brazil, and identified the vertebrate species from the insect blood meal by sequencing (Sanger) a mini-barcode of the Cytb mitochondrial gene. Humans, the nine-banded armadillo (*Dasypus novemcinctus*) and the lesser anteater (*Tamandua tetradactyla*) were the most frequent mammal species detected in the study of Araujo-Pereira et al. (2020), while the nine-banded armadillo was the most common species

detected by Rodrigues et al. (2021) in the blood meals. Of note, both studies reported many sequences lacking sufficient quality for species identification. This could be explained by the use of the Sanger-based sequencing that does not allow the precise analysis of mixed sources of DNA. In this sense, the use of metabarcoding sequencing must be preferred in future studies that aim to investigate host-vector interactions in the neotropical region.

19.4 Challenges and Perspectives

19.4.1 Methodological concerns

The incompleteness of the reference databases is often assumed as one of the main limitations to eDNA-based surveys reaching their full potential in megadiverse areas (Jackman et al. 2021). Metabarcoding analyses are usually heavily impacted by the quality of databases as it relies on the taxonomic assignment obtained through a comparison between the DNA data retrieved metabarcoding sequencing and the reference sequences available. In the absence of such reference data, a massive amount of data might be lost, consequently leading to an underestimation of the total biodiversity recovered. Particularly in the hyper-diverse neotropics, the lack of reference sequences in public databases has been reported as a critical aspect that limits the use of metabarcoding (Kocher et al. 2017; Rodgers et al. 2017; Banerjee et al. 2022) and efforts to produce such sequences are still needed, as already pointed by Carvalho et al. (2022).

The impact of reference databases is not limited to metabarcoding applications and can also affect single-species essays (e.g., qPCR, ddPCR studies). The establishment of sound eDNA detection through qPCR essays relies on the quality and suitability of genes, primers and probes specificity used. To ensure the robustness of essays, previous analyses should be conducted on the search for potential confounding taxa, including information about closely related taxa (Langlois et al. 2020). The urge for increasing the reference data for a broader range of species is therefore shared across all aforementioned eDNA/iDNA methods. Many researchers agree with this need and therefore there are initiatives to identify the major gaps in the genetic basis (e.g., Marques et al. 2021).

Completing the databases and having genes that complement the identification of all species will not be enough to perform reliable surveys, since all species are not sampled with the same ease, probability or sampling technique (e.g., Massey et al. 2021, Saranholi et al. 2022). Thus, the use of traditional methodologies combined with eDNA and metabarcoding can reach more reliable and faster results, improving cost-benefit ratios (Carvalho et al. 2022).

Also, techniques such as capture enrichment are being developed to increase DNA yield and reduce bias in species detection (Wilcox et al. 2018).

Exogenous contamination also represents an important concern in the eDNA/iDNA approach, which can occur at any step (sampling, DNA extraction, PCR, and sequencing). To avoid this, it is important to conduct the studies in an eDNA-dedicated laboratory using UV-sterilized room (Fig. 19.4D) and exclusive equipment for such purposes (centrifuges, thermocycler, pipettes, lab coats, etc.). Since human DNA contamination is almost imminent, the use of blockers during PCR has shown successful results (e.g., Boessenkool et al. 2012) and is highly accepted among the scientific community. However, the use of these tools should be treated with caution, since the identification of some taxonomic groups may be affected by using blockers of phylogenetic related species (for example, humans and primates).

With reduced costs of sequencing, high throughput sequencing is becoming more available. However, the potential of this type of sequencing is still deeply underexplored in the neotropical region, mainly because reagents and equipment are imported, making services being charged in dollars or euros or other currency, which incurs comparatively high costs of this type of sequencing. Besides the decrease in sequencing costs, new possibilities of types of sequencing and applications for eDNA/iDNA are emerging, such as genomic and metagenomic analyses (Seeber and Epp 2022).

Different methods to collect samples that can decrease contamination and improve eDNA sampling, such as automated water collection, are still being developed, tested and improved (Sepulveda et al. 2020, 2021; Wandhekar et al. 2021). One of the approaches that can present a high cost-benefit relationship is what has utilized air samples to collect pollen and survey plant species (i.e., Kraaijeveld et al. 2015). The methodology has proven to be very efficient to identify the species and, to our knowledge, it has not been used to sample high biodiversity regions such as the neotropics. With improved collection, sequencing and analyzing tools for eDNA, we also expect to be able to use environmental DNA data for other applications, such as a source of information for population genetics. Although some studies have been able to study aspects of population genetics from eDNA (Sigsgaard et al. 2017; Baker et al. 2018), this field is still on its onset as the identification of individuals is still an obstacle because it relies mostly on nuclear markers that can be lost more easily to environmental degradation (Adams et al. 2019).

19.4.2 Conservation remarks

The eDNA and iDNA sampling combined with the molecular tools and the advances in the NGS technologies present a great sensitivity for species detection along with reduced costs in comparison to traditional methods, which creates a remarkable opportunity to advance biodiversity monitoring (Sutherland et al. 2013; Cristescu 2014; Kelly et al. 2014b). Also, eDNA and iDNA approaches permit noninvasive monitoring, reducing the risks involved in using methods based on species capture, which is critical especially for threatened species.

The number of environmental DNA surveys has seen an exponential increase in the past decade. However, there is a heavy bias on the proportion of studies towards the global North, with this application still remaining incipient in neotropical countries (Carvalho et al. 2022; Schenekar 2022). Although there are clear advantages with the eDNA and iDNA approaches, it is still little explored in the neotropical region (Carvalho et al. 2022).

Despite the potential highlighted to survey biodiversity through environmental DNA, there are still few official monitoring initiatives using this methodology. It is imperative for eDNA/iDNA to be acknowledged by government agencies as a valid monitoring tool so that standards (e.g., sampling protocols, minimum replicates, data processing) and maximum error values can be defined (Kelly et al. 2014a). The conduction of experiments in controlled situations is expected to generate the necessary data, but this is still deficient in the highly diverse neotropical region where protocols and standards from temperate regions do not apply completely.

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Figure captions

Fig. 19.1 Diagram of environmental DNA (eDNA) and invertebrate-derived DNA (iDNA) applications for surveying and monitoring biodiversity.

Fig. 19.2 Field and laboratory procedures for water eDNA samples. A) collection of the water sample; B) filtration of water samples in a filter using a manual vacuum pump and C) precipitation of the nucleic acids from eDNA water samples.

Fig. 19.3 Traps used to capture insects, modified to study iDNA, preserving the captured insects immediately in alcohol and avoiding the degradation of the genetic material. A) CDC-type trap used to capture mosquitoes; B) Trap made with a plastic bottle baited with a piece of meat to attract flies.

Fig. 19.4 Field and laboratory procedures for iDNA analyses using the dung beetles as samplers. A) Collection of the dung beetles using pitfall traps; B) Dissecting the beetles' gut; C) Digesting the beetles' gut for DNA extraction; D) Performing PCRs in a sterile room for mini-barcodes amplification.