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REVIEW

Editor's Choice

Environmental DNA and metagenomics of terrestrial mammals as keystone taxa of recent and past ecosystems

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ABSTRACT

1. Terrestrial mammals shape their ecosystems, and mammalian community assemblages can be important indicators of ecosystem functioning and ecosystem changes over time. Numerous taxa of terrestrial mammals are currently threatened by habitat loss and face displacement to new geographical areas or systems to which they are less suited and where they may affect the original communities.
2. Understanding past ecosystem changes is important for predicting future responses of species assemblages to changes in their environments. Thus, ecological and evolutionary history, as well as adaptive capacity, are important predictors of future population viability. Genomic and metagenomic approaches using environmental or ancient DNA offer a wealth of information regarding genome-wide variation of changing communities or of taxonomic groups over time, which may help explain past changes and predict future responses of communities to changes in their environment; however, to date, such studies are relatively scarce.
3. We review studies on environmental DNA and environmental genomics of terrestrial mammals to assess the potential of such approaches regarding past, contemporary, and future terrestrial ecosystems, identify inherent challenges, and discuss potential applications. We elaborate on lessons to be learned from mammal genomics of past ecosystems and compare metabarcoding with general metagenetic and metagenomic techniques. We provide a comprehensive overview of current applications, challenges, and future potential of environmental DNA with regards to terrestrial mammals.
4. As current major challenges regarding mammalian eDNA we identify its scarcity and patchy distribution, along with the persistent necessity of genomic reference data. While the latter are steadily increasing, the former can only be tackled by explicitly mapping the environment to gain understanding of spatial eDNA distribution. Such understanding may facilitate informed choices of sample sites and substrates and, together with new sequencing techniques, this can allow mammalian eDNA to be maximally exploited as a source of biodiversity data.

INTRODUCTION

With approximately 6000 recognised species, mammals are important taxa of almost every known ecosystem and, particularly, of all terrestrial biomes (Jones & Safi 2011). Terrestrial mammals typically exert crucial effects on primary producers through nutrient cycling, energy flow, pollination, seed dispersion, and other bottom-up and

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top-down processes (Lacher et al. 2019). These functions have marked effects on community structures of primary producers, which again affect communities of primary consumers. However, the current rapid decline in abundances as well as extirpation and extinction of numerous mammal species raise conservation concerns, not only with respect to the taxa in question, but also regarding ecosystem functioning (Lacher et al. 2019). Comprehensive assessment of presence, abundance, and ecological functions of terrestrial mammals requires comprehensive monitoring, preferably with data covering long periods of time. The specific mammal community assemblage, that is taxonomic diversity, relative abundance, and genetic diversity, can be an important indicator of ecosystem functioning and ecosystem changes over time (Lundgren et al. 2020). Thus, monitoring present mammal populations and understanding population changes in the past are important components of terrestrial ecosystem assessments (Morellet et al. 2007).

Extant species assemblages and relative abundances are conventionally assessed by direct observation, live trapping, or camera trapping. However, these methods may be problematic due to considerable logistic effort, the need for long temporal and spatial coverage, and labour-intensive data processing. Moreover, taxonomical resolution by visual identification may in some cases be insufficient where closely related (or phenotypically similar) species are concerned. Such problems are amplified in poorly studied ecosystems that are remote from human infrastructure. Terrestrial mammals continuously shed genetic material into their environment through substances such as faeces, mucus, saliva, aerosol, hair, gametes, epithelial cells, and decomposition of tissue. As such, environmental DNA (eDNA; see Box 1) contains a plethora of information which cannot be captured using traditional methods. Currently, the most common application of eDNA is detecting the presence of a particular taxon in a given habitat (Rees et al. 2014, Ruppert et al. 2019); in this regard, eDNA allows accurate taxonomic resolution to the species level, including cryptic intraspecific genetic diversity (Thomsen & Willerslev 2015), which may reveal unknown biodiversity and can even be used for individual identification (Seeber et al. 2017). In some cases, eDNA-based survey methods can outperform traditional survey approaches to determine species presence (Civade et al. 2016, Deiner et al. 2016). At the genomic level, eDNA can deliver a vast amount of information on wildlife populations, which can offer insights in aspects ranging from individual genetics to evolutionary adaption. The function of mammals as pathogen hosts or vectors is of particular interest regarding the spread of diseases between populations and interspecies transmission. Investigating host genomics and eDNA of pathogens can help elucidate disease transmission patterns (Lang & Blanchong 2012, Dayaram et al. 2021), which may be crucial for conclusions and management measures regarding pathogen dispersal in wild mammals and at the interface of wild and domestic animals.

Regarding the distributions of terrestrial mammals in the past, research must traditionally rely on organismal remains, which are analysed visually. These can be bones (van der Plicht et al. 2015) and other macroremains, such as hair (Iacumin et al. 2005) or coprolites (Chin & Kirkland 1998). Such direct evidence of mammal presence is not always readily preserved, the probability of discovering fossil remains of mammals is low, and the fossil record is never complete. This means, for example that the youngest fossil of a species never represents the true last appearance – a principle known as the Signor–Lipps effect (Signor & Lipps 1982). In addition to direct evidence, palaeoecological studies can also utilise specific proxies for the presence of mammals, in particular dung fungi (Davies 2019, Goethals & Verschuren 2019) and parasites such as oribatid mites (Chepstow-Lusty et al. 2019).

The first study identifying macroorganisms from eDNA targeted ancient DNA (aDNA) of several terrestrial

Box 1. Glossary

Environmental DNA (eDNA)	DNA isolated from environmental samples (such as water or sediment), that was shed from the original live organism, either as extracellular molecule or within cellular compartments
Ancient DNA (aDNA)	DNA isolated from ancient specimens, including bulk samples
Bulk sample	Samples of environmental material which may include live organisms and eDNA
Barcoding	Identifying species using a short, defined genomic region, such as a fragment of the cytochrome c oxidase subunit 1 for animals
Metabarcoding	Identifying taxa in samples containing DNA from more than one taxon using the sequence of a genomic region that is suitable for this purpose
Metagenetics	Any genetic analyses of samples containing DNA from more than one taxon, using one or few genetic markers
Metagenomics	Producing comprehensive genomic data (or data of numerous markers across the genome) from samples containing more than one taxon, that is on a community scale
Palaeogenomics	Using ancient DNA to produce genomic data

mammals and plants, which was successfully isolated from Siberian permafrost samples (Willerslev et al. 2003). Since this seminal study was published almost two decades ago, considerable progress has been made regarding the use of eDNA of numerous aquatic animals and terrestrial plants, and studies have offered valuable insights into structure and changes in past and current ecosystems (e.g. Deiner et al. 2017, Parducci et al. 2019). By contrast, comparably few studies have focused on terrestrial mammals, despite the vast numbers of potential applications and research questions waiting to be answered, including on biotic interactions in ecosystems, community changes over time, migration patterns, pathogen pressure, and evolutionary adaption to changing environments.

Here, we review studies using DNA from environmental samples and focussed on terrestrial mammals as keystone taxa of many terrestrial environments. We synthesise specific, inherent challenges in adopting eDNA techniques for the study of terrestrial mammals, which explain the relative scarcity of eDNA studies of mammals. We briefly introduce current methodological approaches and elaborate on three aspects of the potential and remaining challenges for eDNA-based studies of terrestrial mammals: 1) obtaining eDNA of terrestrial mammals from different sites and substrates; 2) developing strategies for taxonomic identification of mammalian species assemblages and estimating relative abundances; and 3) deducing information on ecophysiology and behaviour of mammals, as well as on the roles of pathogens, from eDNA. Further, we outline the future of genomic approaches for mammal eDNA to study evolutionary adaptation and aid in species conservation, and we highlight specific potential future applications of (meta)genomic eDNA approaches for mammals.

BRIEF OVERVIEW OF CURRENT METHODOLOGY

Two technical approaches are used for detecting mammal DNA in environmental samples, that is specific polymerase chain reaction (PCR) amplification of a DNA marker of a particular taxon of interest (including metabarcoding of a large taxonomic group), and indiscriminate direct sequencing of a DNA extract (also referred to as 'shotgun sequencing'). Most eDNA studies currently rely on PCR-based approaches; however, inherent biases of PCR such as primer binding and polymerase biases and methodological issues such as under- or over-amplification may confound the results (Nichols et al. 2018). Moreover, fragmentation of eDNA may also render PCR-based approaches such as metabarcoding inadequate or biased, as PCR depends on the integrity of the targeted stretch of DNA between the two primer-binding sites. By contrast, direct sequencing of all DNA in a given environmental sample may help circumvent PCR-inherent biases as eDNA fragmentation is less of a concern. This method, however, typically produces an enormous proportion of off-target sequences, compared to the amount of on-target sequence reads. Hybridisation capture enrichment can substantially reduce the amount of off-target DNA in a sequencing library as target DNA is enriched through selective hybridisation with complementary oligonucleotides. eDNA and aDNA of multiple terrestrial mammals have been successfully enriched from water and sediment samples (Seeber et al. 2019, Murchie et al. 2020), and hybridisation capture enrichment is particularly attractive for highly fragmented aDNA as it does not rely on PCR amplification. It would go beyond the scope of this review to elaborate further on generally applicable technical aspects, thus for details on particular methods and their respective advantages and caveats, see Taberlet et al. (2018).

POTENTIAL AND CHALLENGES OF MAMMAL ENVIRONMENTAL DNA WORK

Obtaining mammal eDNA from different sampling sites and substrates

The major limitation of working with eDNA from terrestrial mammals is its patchy distribution on land. This has been shown in early investigations on mammals (Andersen et al. 2012) and with eDNA of other taxa in terrestrial deposits such as plants (Yoccoz et al. 2012). The sources of mammalian eDNA include body fluids, such as saliva, sweat, urine, and faeces, as well as breath aerosols, hairs, and skin flakes, and these are not uniformly deposited. Efficient tracing of terrestrial mammals from a diversity of substrates (Appendix S1) thus strongly depends on choosing adequate sampling sites, that is 'hotspots' of aggregation or frequentation, such as drinking water sources, migration routes, or confined areas containing other limited resources, which may offer suitable substrates for isolating eDNA of recent terrestrial organisms. Below we review studies targeting different types of substrates; important examples of mammal eDNA work are summarised by substrate type and technical approach in Appendix S1.

Controlled and, in particular, captive settings have been a common starting point to establish and validate eDNA assays for a variety of substrates, such as drinking water, soils within enclosures, and artificial surfaces. For example, an early study of mammalian eDNA used soil samples collected in long-term wildlife enclosures to test whether eDNA would reflect biodiversity (Andersen et al. 2012), showing that eDNA generally reflected overall taxonomic composition and relative biomass of individual species. Moreover, the study suggested that animal physiology

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(particularly the digestive system) and animal behaviour affect eDNA deposition. Differences in detection efficiency were observed between soils of different physicochemical properties, such as organic content, which is thus an important factor to consider when planning field experiments and interpreting data.

Other studies conducted in captive settings include the analysis of eDNA on artificial surfaces, such as enrichment toys and feed troughs, which enabled eDNA-based individual identification and the tracking of pathogen shedding within populations of three zebra species (*Equus* sp.; Seeber et al. 2017, 2018), and thus delivered technically simple solutions for obtaining DNA-based data. Water from captive enclosures has also served to trace animals, such as captive coyotes *Canis latrans*, from drinking water (Rodgers & Mock 2015), and has been used to investigate eDNA persistence and behaviour. For example, as part of a pioneering eDNA study to survey multiple waterbodies for freshwater biodiversity, including Eurasian otters *Lutra lutra*, Thomsen et al. (2012) validated eDNA persistence in aquatic environments using the DNA of two amphibian species in mesocosms. For this, the authors examined how long the DNA of the source organisms could be traced after removal of the animals and determined that this DNA degraded within two weeks. Two later studies compared the performance of PCR-based approaches on zoo animal eDNA isolated from water samples to that of similar approaches with eDNA from pond samples from natural settings (Ushio et al. 2017, Harper et al. 2019); as expected, both studies found substantially higher detection rates in zoo enclosures than under natural conditions, owing to the higher density of organisms in enclosures.

Most recently, sampling the environment for mammalian DNA in captive settings has been expanded to include air filtration. So far, this has been accomplished in three studies performed in captive settings: in one study, naked mole-rat *Heterocephalus glaber* eDNA was retrieved from air filters operated in a room in which the animals were kept (Clare et al. 2021), and in the other two studies, indoor and outdoor air filters operated in a zoo were shown to contain eDNA of numerous terrestrial vertebrates, including various mammals (Clare et al. 2022, Lynggaard et al. 2022). However, the efficiency of tracing airborne eDNA of terrestrial mammals in natural settings, where the biomass per air volume is much lower, remains to be evaluated.

Under natural conditions, the detection probability of terrestrial mammals in a sample collected from a given drinking water source depends on general water availability, water requirements, and animal behaviour. Water sources used by terrestrial mammals are furthermore often not clear but turbid, which should be considered in the experimental planning. Large numbers of suspended particles may prevent water filtration to some extent, which thus requires adaption of sampling protocols. Williams et al. (2017, 2018) tested how small water bodies and natural wallows could be used to trace invasive feral pigs *Sus scrofa*. They found that eDNA shed by feral pigs can be detected in water after only 15 min of usage by a single pig, even when the individual's contact with the water source was considered 'minimal'; feral pig eDNA was detectable in water for at least two weeks after removal of the animals, although the study site experienced a humid Subtropical climate, which accelerates DNA degradation. Similarly, Sales et al. (2020) successfully isolated eDNA from Tropical water samples for mammal metabarcoding, which reflected an array of mammalian biodiversity; high-throughput sequencing (HTS) reads were assigned to terrestrial and aquatic mammalian species of 14 taxonomic families, several of which are currently threatened by extinction. These highly diverse data were recovered from Tropical environments which are typically acidic, and low pH causes increased degradation of DNA via chemical hydrolysis (Seymour et al. 2018). In a Temperate climate, Sales et al. (2020) compared camera trapping with metabarcoding of terrestrial mammals from eDNA isolated from water and sediment, and detection rates of terrestrial mammals were similar between the two techniques. This study reported higher detection rates from water than from sedimentary eDNA (Sales et al. 2020), which is contrary to some findings on fish eDNA (Turner et al. 2015).

Natural salt licks were used as hotspots for obtaining terrestrial mammal eDNA in a Tropical ecosystem (Ishige et al. 2017), and eDNA of several Endangered and Critically Endangered large terrestrial mammal species was observed in water samples from salt licks. High salinity may help prevent eDNA degradation to some extent, thus such findings may not exclusively reflect current frequentation but may be biased by past species presence. DNA from saliva can also be recovered as eDNA from specific twigs to allow the identification of browsing species (Nichols et al. 2012, 2015, van Beeck Calkoen et al. 2019) and has been used in a single nucleotide polymorphism assay to assess both species and sex ratios of wild ungulates (Nichols & Spong 2017). This approach may be particularly helpful when conventional methods such as camera trapping deliver insufficient information, for example regarding sex ratios of non-dimorphic species.

Compared to camera trapping, isolating eDNA of mammals from soil has proven very efficient, in particular for small species, which do not trigger camera traps (Leempoel et al. 2020). The total number of terrestrial mammal species detected from eDNA isolated from soil in a wildlife reserve surpassed that obtained from long-term camera trapping (Leempoel et al. 2020). However, a comparison of data from mammal eDNA with available long-term

data from other methods (Sales et al. 2020) suggests that currently, the most comprehensive data can be compiled by pairing eDNA with conventional methods such as camera trapping. As a different approach, live trapping is particularly relevant for small mammals (e.g. rodents). However, eDNA methods can outperform live trapping with respect to costs and labour (Ferreira et al. 2018) and are not harmful to the study animals.

Identifying animals from their footprints is an established and simple approach which, however, may be error-prone regarding species that leave similar tracks. Several recent studies have shown that eDNA isolated from footprints in snow can be used to identify species reliably. A proof of concept was produced by Dalén et al. (2007), who successfully isolated eDNA from snow footprints which were then assigned to Arctic fox *Alopex lagopus*. Subsequently, eDNA isolated from snow footprints has been used to identify the tracks of rare carnivoran species including Canada lynx *Lynx canadensis*, fisher *Pekania pennanti*, and wolverine *Gulo gulo* in Canada, suggesting that eDNA approaches may be a powerful tool to supplement winter surveys (Franklin et al. 2019). Similarly, eDNA from snow footprints in a Japanese rural area were used to identify three carnivoran species (*Martes melampus*, *Vulpes vulpes*, and *Canis lupus familiaris*) and one ungulate (*Cervus nippon*; Kinoshita et al. 2019). Scandura et al. (2006) used multiple non-invasive eDNA sources, including snow tracks, faeces, blood spots in the snow, and shed hairs, for single- and multi-locus microsatellite genotyping of a wolf *Canis lupus* population. Such comprehensive usage of various sources of eDNA allows researchers to produce a plethora of data which can be used to answer genetic and genomic questions at the individual and population levels (Barnes & Turner 2016).

Mammalian eDNA can also be collected from parasitic invertebrates such as leeches (Hirudinea; Schnell et al. 2012) and arthropods, typically termed invertebrate-derived DNA (iDNA). Carrion flies (Calliphoridae and Sarcophagidae) that were experimentally exposed to mammal cadavers, as well as randomly collected carrion flies in a Tropical environment, were shown to contain iDNA of various large and small terrestrial mammals (Calvignac-Spencer et al. 2013), which provided useful supplementary information for surveying the presence of (cryptic) species. Various species of leeches feed on terrestrial mammals, after which they digest their blood meal for extended periods of time, which makes them potential sources of mammalian iDNA (Schnell et al. 2015). To compare the efficacy of sampling biodiversity through parasitic leeches with camera trapping, Weiskopf et al. (2018) collected 200 leeches in a forest in Bangladesh and identified recent vertebrate hosts by Sanger sequencing iDNA from blood meals; 42% of the leeches produced iDNA of 12 mammalian species. In a similar study in Southeast Asia, Abrams et al. (2019) compared iDNA from leeches with camera-trap data. They observed a larger proportion of ungulate taxa detected by iDNA than through camera traps, and, more importantly, iDNA confirmed the presence of the elusive binturong *Arctictis binturong* which was not detected by camera trapping; however, they noted a bias towards larger host taxa which may limit the use of this approach for smaller mammals. By contrast, Gogarten et al. (2020) found that fly-derived iDNA was biased towards smaller species, when compared to camera traps. Several studies using arthropod and leech samples collected in Brazil, Tanzania, Côte d'Ivoire, and southern and Southeast Asia successfully used iDNA and eDNA for barcoding and metabarcoding of terrestrial mammals (Schubert et al. 2015, Tessler et al. 2018, Drinkwater et al. 2019, Lynggaard et al. 2019, Drinkwater et al. 2021). iDNA may thus facilitate biomonitoring in terrestrial ecosystems over broad spatial and temporal scales and when a lack of hotspots or adverse environmental conditions impede eDNA collection.

The retrieval of aDNA of terrestrial mammals from natural palaeoecological archives was carried out in one of the first demonstrations of the use of eDNA: a seminal paper (Willerslev et al. 2003) on permafrost samples aged 400000–10000 years yielded megafaunal aDNA of several taxa including woolly mammoth *Mammuthus primigenius*, bison *Bison* sp., musk ox *Ovibos moschatus*, and horse *Equus caballus*. Numerous studies had previously been conducted on aDNA isolated from individual macrofossils such as bones or other preserved tissues (Higuchi et al. 1984, Hagelberg et al. 1994, Höss et al. 1994, Greenwood et al. 1999, Franz et al. 2017); however, such studies depend on suitable macrofossils. Assessing the temporal and spatial distribution of animals independently from fossil findings can substantially expand our understanding of range shifts, extinctions, and faunal turnovers. For example, the presence of woolly mammoth and horse was determined in exposures from Alaska as young as 10500 years before present, suggesting that these species persisted several thousands of years longer than presumed from macrofossil findings (Haile et al. 2009). Some sites and exposures yield particularly high numbers of permafrost samples containing mammal DNA, such as permafrost samples analysed by Willerslev et al. (2014), where 18 of the 25 analysed samples contained megafaunal DNA. The use of blocking primers preventing amplification of human DNA in such samples can increase the diversity of retrieved mammalian aDNA sequences (Boessenkool et al. 2012).

Cave deposits are invaluable hotspots for mammalian aDNA research, as caves frequently have a long-term history of being used by various mammals over numerous generations. Moreover, constant cool temperatures, low

humidity, and protection from ultra-violet radiation are conducive to the preservation of aDNA in caves. Thus, cave sediments were among the very early targets for studies of mammalian ancient eDNA (Hofreiter et al. 2003). Although data from one early study suggested that leaching of highly concentrated recent DNA in non-frozen deposits, such as those from caves, can be a problem (Haile et al. 2007), more recent investigations have not been confounded by this, and a recent paper demonstrated intact microstratigraphic preservation of DNA from cave sediments (Massilani et al. 2022). A current increase in the use of cave deposits was fuelled by the finding that cave sediments can provide data on past hominin presence (Slon et al. 2017, Zhang et al. 2020, Vernot et al. 2021). Cave sediments from different climatic regions have been targeted for non-hominin mammals, revealing, for example 25000-year-old DNA of wolves and bison from a cave in the Caucasus, Georgia (Gelabert et al. 2021), black bears *Ursus americanus* and giant short-faced bears *Arctodus simus* in northern Mexico (Pedersen et al. 2021), extirpated red deer *Cervus elpahus* from a cave in Ireland (Carden et al. 2012), and various mammals from a cave in Australia (Haouchar et al. 2014). Moreover, stalagmites in a cave in Georgia were shown to contain aDNA of various mammals such as bears *Ursus* sp., roe deer *Capreolus* sp., and horseshoe bats *Rhinolophus* sp. (Stahlschmidt et al. 2019).

Lakes serve as catchment basins, preserving in their sediments high-resolution records of ecological changes in the surroundings; therefore, lakes have been the target of many palaeoecological studies, including DNA-based approaches. These have mostly concentrated on lacustrine ecosystems (Domaizon et al. 2017) or plants (Parducci et al. 2017); however, aDNA of terrestrial mammals has also been isolated from lake sediments and provides considerable insights into ecosystem changes (Giguet-Covex et al. 2014, Graham et al. 2016, Murchie et al. 2020). From an Alpine lake sediment core in France, Giguet-Covex et al. (2014) showed that a period of intense erosion during the Late Iron Age and Roman period correlated with the presence of sheep *Ovis* sp. and cattle *Bos* sp., evidenced by mammal DNA, as well as with signals of deforestation, evidenced by plant DNA. Shotgun sequencing of sediment from a lake on St. Paul Island, Alaska, allowed researchers to date the extinction of the local woolly mammoth population and to correlate it with an event of regional climate change and resulting freshwater scarcity (Graham et al. 2016). Pedersen et al. (2016) shotgun-sequenced aDNA from Arctic lake sediments and found significant correlations of large and small mammalian herbivore species assemblages with vegetation structure. However, for many lake sediment cores, attempts to retrieve mammal DNA have failed, exemplified by an investigation by Lammers et al. (2019), who used a mammal-specific assay but found only very few mammal sequences; instead numerous clitellate worm species were detected, likely due to their high biomass in this ecosystem.

Middens may provide archives of past biodiversity and, even in warm climates, seem to favour DNA preservation, probably due to desiccation processes, which prevent eDNA/aDNA degradation. Moore et al. (2020) shotgun-sequenced aDNA from packrat *Neotoma* sp. middens collected in the north-western USA and Mexico, which were more than 30 000 years old, and they identified a wide array of plant taxa. In middens located in hot, arid regions of Australia, and in South Africa's Western Cape province, various mammalian taxa, some of which were locally extinct or endemic, were observed by metabarcoding and HTS (Murray et al. 2012).

In summary, numerous substrates have proven to contain mammalian eDNA, however, its distribution in the environment is not uniform. Therefore, the selection of adequate sampling material from putative hotspots of aggregation is vital for exploiting eDNA and aDNA as valuable sources of information on present ecosystems and palaeoenvironments.

Developing strategies for taxonomic identification and abundance quantification from eDNA

Numerous software tools for taxonomic assignment of HTS reads are available, and it is important to choose an accurate tool so as to interpret the data correctly (Bálint et al. 2016, Ye et al. 2019). The informative value of environmental metagenomic sequence data is at present limited by a lack of comprehensiveness in the available reference databases, and by the varying quality of reference sequences (Breitwieser et al. 2018). While mitogenomes of many extant terrestrial mammal taxa are available and facilitate sufficient taxonomic resolution, genomes and genotype variation of extinct taxa are obviously less well covered. This inevitably reduces the probability of aDNA sequences aligning to those of the available references. Data generated by more selective approaches, such as targeted enrichment or metabarcoding, may also be used sub-optimally, if a lack of reference sequences prohibits data interpretation at the desired taxonomic level. Ongoing endeavours to augment these databases are therefore essential, so that researchers can make full use of environmental genomic data in the future (Alsos et al. 2020, Rhie et al. 2021).

Apart from biodiversity assessments, eDNA has also been used for attempts to quantify biomass. Effective population size is one of the most crucial factors of population viability, and variations in populations sizes of terrestrial mammals are important indicators of

ecosystem changes relating to selection pressures and species' interactions. eDNA concentration may be positively correlated with biomass, which has so far mostly been examined in (experimental) aquatic systems using organisms such as fish and amphibians (Pilliod et al. 2014, Lacoursière-Roussel et al. 2016); however, the relationship between eDNA and biomass is substantially more complex on land due to the uneven distribution of eDNA in any substrate. Quantification of organisms from eDNA, especially based on HTS read numbers, is disputed due to confounding factors such as differences in DNA shedding between taxa, and methodological and amplification biases (Fonseca 2018, Kelly et al. 2019). Thus, it is crucial to evaluate whether the abundance of a given taxon in the assignment is positively correlated with its biomass or abundance in the biological sample (a process termed 'ground-truthing'). Highly sensitive methods, such as quantitative PCR or emulsion PCR (e.g. droplet digital PCR), may be used to quantify specific template DNA in a given environmental sample, and appear to have higher detection efficiencies for single species than metabarcoding, as tested in aquatic systems (Baker et al. 2018, Wood et al. 2019). So far, quantification of biomass from eDNA has found little application in terrestrial systems. In a simulation, Kelly et al. (2019) found that, within a community sample, biomass was only modestly correlated with eDNA abundance and amplification success. Additionally, due to amplification bias, PCR-based approaches may be less reliable in terms of abundance estimates than other methods such as hybridisation capture (Adams et al. 2019). Taken together, substantial validation effort will be required to establish methods for estimating the abundance of terrestrial mammals from eDNA and aDNA.

Changes in the abundance of large terrestrial mammals during pre-modern eras are even more difficult to assess, from aDNA or from macrofossils, due to the scarce and patchy distribution of both records. A commonly employed alternative is the use of proxy indicators, such as spores of coprophilous fungi, including those belonging to the *Sporormiella-Preussia* species complex, *Sordaria* sp., *Podospora* sp., or *Pilobolus* sp. (reviewed by Perrotti & van Asperen 2019) or oribatid mites (Chepstow-Lusty et al. 2019). Morphological identification of spores of coprophilous fungi is complicated, and results may be error-prone due to insufficient taxonomic resolution of spores and a lack of information on their biology, that is which fungal taxa are dependent solely on dung and therefore are useful indicators of herbivore biomass (Feranec et al. 2011, Johnson et al. 2015). Using fungal eDNA as a proxy was unsuccessful in a fungal sedimentary aDNA metabarcoding dataset from various Siberian lakes (Seeber et al. 2022).

Deducing behaviour, ecophysiology, and the role of pathogens from eDNA

There is potential to use eDNA to study the behaviour of terrestrial mammals, including dietary and dispersion behaviour. eDNA has been successfully used to allocate dietary habits to large herbivores, in particular in the context of restoration of degraded habitats and to increase the capacity of natural areas to sustain higher biodiversity (Nichols et al. 2015, Iacolina et al. 2020, Schure et al. 2020). Space use-related aspects may include changes in the routes of migratory species (Xu et al. 2021) and dispersion of invasive species, for example hippos *Hippopotamus amphibius* in Colombia (Castelblanco-Martínez et al. 2021) and dromedaries *Camelus dromedarius* in Australia (Brim Box et al. 2019); these aspects, however, have not yet been examined through eDNA.

The presence and abundance of large mammals strongly affects the abundance and community structure of small mammals, arthropods, plants, and fungi (Danell et al. 2006, McCauley et al. 2008, van Klink et al. 2015, Olofsson & Post 2018), as well as those of parasites and pathogens (Fauchald et al. 2007, McCauley et al. 2008). Emerging infectious diseases are becoming increasingly relevant for wild mammals due to habitat loss and increase of the wildlife–livestock interface, thus the dynamics of pathogen transmission and associated immune responses in wildlife are of concern (Wiethoelter et al. 2015). Changes in the immune genome of mammals may be an important indicator of disease-associated selection pressures and population viability, but to date the immune genome has been targeted in only a few eDNA or aDNA studies, including that of Pečnerová et al. (2016), who targeted several immune markers of woolly mammoths, and that of Smith et al. (2017) who compared the genomes of woolly mammoths and Asian elephants *Elephas maximus*, and identified genome structural variants concerning immunity-related genes in woolly mammoths.

eDNA metagenomics have been conducted on a few groups of pathogens and parasites relevant to mammals (reviewed by Bass et al. 2015). Such studies can deliver important information in the context of reintroduction for restocking wild populations, as introductions of mammals may also lead to introduction of novel pathogens to naïve systems. Regarding pathogen pressure and monitoring of disease dynamics using eDNA, Alfano et al. (2021) identified a spectrum of mammalian viruses by enriching eDNA isolated from waterholes, and Dayaram et al. (2021) used eDNA-based hybridisation capture, quantitative PCR, and single-molecule real-time sequencing to assemble genomes of pathogens and quantify pathogen abundance in drinking water in water-limited ecosystems. Such

non-invasive methods are of interest for identifying pathogens of mammals and, particularly, for tracing genomic variations in pathogens which may be of epidemiological importance. Tardy et al. (2012) performed whole-genome sequencing on bacterial pathogens isolated from carcasses of Alpine ungulates and identified large integrated conjugative elements and novel prophages in these genomes; such comparative genomics of pathogens may allow conclusions and predictions to be made regarding disease epidemiology.

GENOMIC APPROACHES TO MAMMALIAN ENVIRONMENTAL DNA – CURRENT STATUS AND FUTURE PERSPECTIVES

To date, the vast majority of eDNA studies have been based on PCR-amplification of short regions, which is sufficient for identifying organisms to species or sub-species level; comparably few studies have attempted genomic approaches (Adams et al. 2019). However, environmental samples such as water or sediment allow the retrieval of eDNA from entire communities which can be used for metagenetics or, on a larger scale, for metagenomics (Box 1). The term genomics does not necessarily imply comprehensive sequencing of a genome in its entirety, but is also used for screening of a large number of markers across the genome (Supple & Shapiro 2018). Successful attempts to retrieve genome-scale data from eDNA have mostly targeted organellar DNA, but research is currently moving to the retrieval of low-coverage genomes (Pedersen et al. 2021). This developing field may provide novel insights on evolutionary history, population structure, selection pressures, and adaptive loci in terrestrial mammals in present and past ecosystems.

Adaptation and conservation genomics

Natural populations of terrestrial mammals may respond to habitat unsuitability (due to, e.g. direct anthropogenic effects or climate change) by adaptation, by shifting their geographical distribution, or by reduced population sizes. Accessing eDNA to obtain information on these processes could substantially further our understanding of many processes in mammal populations. Natural selection may result in allele frequency shifts at loci under selective pressure to maximise fitness in a given environment, and, under natural conditions, adaptive capacity is a crucial determinant of population survival (Hoffmann & Sgró 2011). Identifying genetic loci, for example among genes associated with known immunological, metabolic, growth, or reproductive functions, that bear signatures of natural selection is key to understanding adaptation in natural populations (Hoffmann & Sgró 2011). Genome scans have been used successfully to identify signatures of natural or artificial selection in large mammals, albeit predominantly in domesticated animals (e.g. cattle and horses; Yurchenko et al. 2018, Cardoso et al. 2018, Gurgul et al. 2019); less is known about genome-wide adaptive variation in wild mammals.

In many threatened large mammal species, adaptive changes are expected to be insufficient for coping with changes in their environment, given their long generation intervals and the speed and magnitude of current disturbance of natural habitats. As one of many consequences, evasion to less suitable habitats is an increasingly common phenomenon, which, however, alters the composition of local communities and the nature of species' interactions (Kondoh 2003, Parmesan & Yohe 2003). Thus, assessing changes in community structures and community genomics at long temporal scales may produce important information regarding the adaptive capacity of entire ecosystems, which may be of use for predicting future developments and establishing conservation measures (Shafer et al. 2015). Genomics can produce information that is important for efforts to manage potentially threatened wild mammal species by identifying loci that are associated with inbreeding depression and disease susceptibility and for predicting effects of introgression and admixture on Darwinian fitness (reviewed by Steiner et al. 2013, Supple & Shapiro 2018).

Intraspecific genomic diversity can also inform on population size, for example by analysing time-series of population genomic changes. An extensive study on the past distribution and the genetic history of Beringian steppe bison *Bison priscus* showed a dramatic decline in genetic diversity in a population which had been large and diverse until around 37000 years before present, a time which coincided with considerable environmental changes (Shapiro et al. 2004). A study on the evolutionary history of muskox *Ovibos* sp., which at present shows comparably little genetic heterogeneity, suggested that their genetic variability was substantially reduced after the Last Glacial Maximum, indicating that populations of this taxon were brought down to considerably small numbers over much of their original range (MacPhee et al. 2005). Understanding past population dynamics and genomics may be important for predicting responses of populations to future changes. Comparative analyses of collared lemmings *Dicrostonyx torquatus* and narrow-headed voles *Microtus gregalis* using aDNA and ecological niche modelling showed markedly different responses to climatic and environmental changes in these two species which occupy similar but not identical ecological niches (Prost et al. 2013); results suggest

that both species may be threatened by habitat loss and decreased genomic variability.

Population genomics and landscape (community) genomics

An important aspect of eDNA is the potential for efficient sampling for genomic data in space without the need to encounter the target organisms directly. This can allow a leap in efficiency and in the size of datasets targeting spatial genomic variation and geographic population differentiation, which can inform on spatial variation in selection pressures and on resulting local adaptation. This aspect is covered by the research field of landscape genomics (Schwartz et al. 2010, Balkenhol et al. 2017). Studies in this field can identify loci of adaptive variation in response to environmental factors such as climate (Joost et al. 2007, Pariset et al. 2009, Lv et al. 2014), or assess the risk of infection with a fatal disease and identify loci associated with putative resistance functions (Vajana et al. 2018). In the face of ongoing wildlife habitat loss and climate change, such information will be increasingly valuable when new pathogens emerge. In contrast to landscape genomics, the concept of landscape community genomics integrates the concept of species interactions by investigating genomes of multiple taxa, to test the effects of biotic and abiotic factors on genomic adaptive variation (Hand et al. 2015). Landscape community genomics can offer a holistic perspective on potential selection pressures and population genetics, and has tremendous potential for understanding effects of environmental heterogeneity on spreading of infectious diseases, parasites, and host adaptive responses (Elbers et al. 2018, Kozakiewicz et al. 2018). At present, little is known about landscape genomics or landscape community genomics of most wild mammals; however, environmental and ancient genomics may be powerful methods to assess dynamic changes in landscape genomics of mammals.

Metagenomics in palaeoecology

The use of metagenomic data in palaeoecology is currently gathering momentum, and recent papers using ancient eDNA have integrated a genomic perspective in the analysis of these data. This is exemplified by the cave sediment studies of Gelabert et al. (2021), who retrieved genomic data of humans, wolves, and bison, and by the study of Pedersen et al. (2021), in which genomic data of black bears and giant short-faced bears were placed in a phylogenetic context. While such studies are challenging due to uncertainties regarding DNA shedding, eDNA distribution, and aDNA taphonomy, they can reveal genome-wide changes in terrestrial mammals. Knowledge of such changes allows researchers to examine responses to various forms of selection pressure which may have affected mammal populations in the past, or which may affect them in the future. Palaeogenomics offers insights regarding evolutionary changes in populations and demographic events over long periods of time (reviewed by Pont et al. 2019), and the possibility to retrieve equivalent data from natural eDNA archives promises the integration of data from a whole range of substrates (Dussex et al. 2021).

CONCLUSION: GENERAL CHALLENGES AND PERSPECTIVES

Despite the considerable potential of eDNA research, tracing terrestrial mammals through eDNA is a challenging endeavour; the predominant issue is the scarcity and patchiness of mammalian eDNA on land. Further complications include the generally limited persistence of eDNA, considerable differences between systems regarding physicochemical conditions which may affect eDNA decay rates and detectability, and the incompleteness of reference databases. Moreover, studies using combined approaches of eDNA and other methods may produce contradicting results, and generalised comparisons of detection efficiency between established survey methods and eDNA between studies are complicated, as non-benchmarked sampling effort and differences in down-stream processing may distort the overall picture.

Tracing and quantifying the abundance of terrestrial mammal populations and predicting adaptive (genomic) responses to ecosystem changes due to climate change, habitat loss, population declines, and emerging diseases are crucial, and such research may be informed by insights into comparable processes and development in historical or palaeoenvironments. Single- and multi-locus microsatellite genotyping has been performed on eDNA to produce large amounts of data which can be used to answer genetic and genomic questions from the individual to the population level (Barnes & Turner 2016). Moreover, eDNA will be of increasing relevance in the future to explore interconnections between taxa, to detect presence of invasive species, and to determine community responses to various factors. So far, most mammal eDNA studies have been focused on detection of particular taxa, and applications to landscape genomics and conservation genetics have received less attention. The use of eDNA for assessing patterns of community diversity and structure is currently being tested and will certainly become an important approach in the future (Broadhurst et al. 2021).

The development of appliances such as hand-held (real-time) PCR machines (e.g. miniPCR; Cambridge, USA) and portable sequencing devices (e.g. MinION; Oxford

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Nanopore Technologies, Oxford, UK) has facilitated acquisition of more (specific) data at lower costs and labour, also for eDNA (Seah et al. 2020, Egeter et al. 2022). So far, these technologies have been used mainly for microbial communities (Urban et al. 2021); however, low-abundance vertebrate DNA can also be targeted. In particular, using targeted real-time sequencing (Kovaka et al. 2021) alongside mapping against reference genomes (Formenti et al. 2022) can offer the possibility to enrich for genomic data of target species immediately in the field. To exploit such novel assays to their maximum potential, we will need explicit benchmarking studies to understand the deposition and occurrence of genomic eDNA of different taxa in a variety of sites and substrates, including abiotic matrices and iDNA. While not all approaches will immediately be successful, rapid methodological progress and the growth of (genomic) reference databases will substantially augment the currently limited informative value of environmental metagenomics in the next few years. This will allow researchers to gather data on genomic and ecosystem changes at unprecedented spatial and temporal scales, and at a very high resolution. Such data may be key in the light of the current biodiversity crisis, as they may provide information for decision-makers to initiate conservation measures at an early stage.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

Appendix S1. Approaches and success of mammalian eDNA studies.