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Trends in Parasitology

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Review

Diverse Applications of Environmental DNA Methods in Parasitology

David Bass,[1,2,*] Grant D. Stentiford,[1] D.T.J. Littlewood,[2] and Hanna Hartikainen[3]

Nucleic acid extraction and sequencing of genes from organisms within environmental samples encompasses a variety of techniques collectively referred to as environmental DNA or 'eDNA'. The key advantages of eDNA analysis include the detection of cryptic or otherwise elusive organisms, large-scale sampling with fewer biases than specimen-based methods, and generation of data for molecular systematics. These are particularly relevant for parasitology because parasites can be difficult to locate and are morphologically intractable and genetically divergent. However, parasites have rarely been the focus of eDNA studies. Focusing on eukaryote parasites, we review the increasing diversity of the 'eDNA toolbox'. Combining eDNA methods with complementary tools offers much potential to understand parasite communities, disease risk, and parasite roles in broader ecosystem processes such as food web structuring and community assembly.

Trends

The rapidly developing field of environmental DNA (eDNA) provides modern tools to reveal and quantify novel and known diversity. The relevance to parasitological research is clear because parasitology requires the identification of some of the smallest and most cryptic eukaryotes known.

Many parasite taxa are genetically divergent, but the use of lineage-specific primers in many cases can reveal substantial previously undetected diversity. An additional advantage of eDNA analysis is the possible quantification of both parasites and hosts, which can resolve complex interactions and give novel insights into host-associated microbiomes, pathology, and etiology.

eDNA approaches offer non-invasive and comprehensive methods for assessing parasite diversities and abundances. However, translating this information into assessment of disease risk, or its use as diagnostic evidence, remains challenging and requires extensive validation before its use in notification procedures or detection programs.

A Brief History of eDNA

eDNA (see Glossary) is a conceptual term referring to an increasingly broad repertoire of research using molecular biology and sequencing technologies to investigate the diversity and distribution of organisms. The approach originated with the use of small subunit ribosomal RNA genes (rDNA) for bacterial evolution [1], and developed to reveal hitherto unknown genetic diversity in diverse and extreme habitats (e.g., [2]). Later, these methods were adapted for similar studies of microbial eukaryotes, again revealing unexpectedly large diversity in many environments, and catalyzing a revolution of research on eukaryotic evolutionary relationships, diversity, and ecology (e.g., [3,4]). As the component techniques and technologies became easier to manipulate and cheaper to execute, eDNA approaches were co-opted much more widely and with great effect into other areas of biology, encompassing a broader range of molecular markers and techniques [5,6].

What Do We Mean by 'eDNA'?

We define eDNA in parasitology as studies starting with DNA (or RNA) being extracted from environmental or organismal matrices, in other words from the environment or the host organism. In contrast to a recent review [6], our definition of eDNA includes extracellular DNA, but most importantly targets DNA from organisms present in the sample.

After nucleic acid extraction the DNA (or RNA, reverse transcribed to **cDNA**) may be subject to general or specific amplification steps, sequenced directly (Box 1), and/or used in other sequence-based formats such as fingerprinting and microarrays. We see a continuum of such methods applied to 'traditional' eDNA template matrices – nucleic acids extracted from soil,

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Box 1. Extraction and Sequencing Strategies

(i) The question of DNA versus RNA as starting material is an important one. DNA may be present in both active and dormant cells, cysts and other persistence forms, eggs, dead organisms, or embedded in material shed from organisms (e.g., mucus), or as free extracellular DNA. By contrast, RNA is indicative of active gene transcription, and will be proportionally less evident in dormant stages than in metabolically active cells [112]. The instability of RNA means that it is less able to persist extracellularly and degrades quickly in dead or sloughed-off cells. Therefore DNA can be used as a rough proxy for biomass, whereas RNA better indicates activity [4,113,114].

(ii) Amplicon-based methods involve PCR-amplifying a specific region of a specific gene. This has the benefit of focusing all analytical effort on a required informative fragment, and is extremely sensitive when using group-specific primers targeting a short amplicon. However, interpretation is limited to the information contained in that fragment and PCR biases may lead to preferential amplification of some template sequences over others. Therefore, the resulting diversity and abundance profile of the sequence reads does not necessarily reflect that of the community in the sample. The chosen sequencing method for amplicons depends on the specificity of the primers used. Broad, eukaryote-wide primers amplify very high diversities, for which sufficient sequencing coverage can only be achieved by high-throughput techniques (e.g., Illumina platforms). Lineage or group-specific primers amplifying lower diversity levels may also be amenable to traditional cloning approaches followed by Sanger sequencing.

(iii) Metagenetic analyses require HTS technologies and sample randomly across all of the genomes or transcriptomes present in the eDNA sample. Metagenomes include non-coding as well as coding regions of the genome, and therefore provide access to non-transcribed and/or excised loci such as microsatellites and ribosomal RNA gene internal transcribed spacers I and II. If necessary, a whole-genome amplification step can be used to generate enough material for sequencing, but may introduce amplification biases. Metatranscriptomes comprise only expressed regions of the genomes present in the eDNA sample, and can be used for gene expression, microRNA, and RNAi analyses. Sequencing RNA directly (without conversion to cDNA) avoids biases associated with reverse transcription of RNA [115,116] but has not yet been used in an eDNA context because obtaining sufficient quantities of RNA from many eDNA sample types may be prohibitive.

sediments, filtered water, etc., through fecal and gut samples, to whole organisms or individual tissues from a particular host (Box 2). At one end of this scale the aim might be to generate billions of sequences in a **metagenomic** survey to capture as much diversity in a sample as possible. At the other end are studies focusing on specific lineages, sometimes below the level of species, in one or more host species, in epidemiological or ecological contexts. Although some would consider it a stretch to describe the latter as 'eDNA' studies, the conceptual link with larger-scale, more general work, especially when considering parasites, is unbroken and logical. This review focuses on eukaryote parasites, mostly microbial, but eDNA methods can be applied to all groups of parasites and pathogens, and we consider its potential to provide new insight into metazoan parasites such as helminths and myxozoans (Box 3).

The Phylogenetic Diversity of Micro-Eukaryotic Parasites

Micro-eukaryotic parasites are phylogenetically and functionally extremely diverse, but our understanding of them is very uneven. By far the best known are those associated with human and livestock health, for example *Plasmodium falciparum* (malaria), *Trypanosoma* (sleeping sickness, Chagas disease), *Toxoplasma*, *Entamoeba*, *Leishmania*, *Enterocytozoon bieneusi* (microsporidiosis), *Giardia*, and *Blastocystis*. Even this small number of pathogens includes representatives of four eukaryotic supergroups [7] (Figure 1). However, parasitism is extremely common and widespread across the eukaryotic tree, and is not necessarily associated with disease in the conventional sense; parasites should also be considered as biological moderators and regulating factors contributing significantly to ecological equilibria (e.g., [8,9,10]). Most major groups are either partly, or exclusively, parasitic (Figure 1). Within metazoans, Myxozoa, long considered a group of protists, were recently confirmed to represent a diverse radiation of cnidarians [11,12]. Nevertheless, these morphologically reduced cnidarians readily lend themselves to the eDNA methods used for micro-eukaryotic parasites in general.

Challenges and Solutions for Micro-Eukaryotic Parasitology

Micro-eukaryotic parasites present particular challenges for detection and characterization. Many are very small (some less than 1 μm), intracellular or intra-organellar, and often occur at low densities

Glossary

Amplicon: gene region amplified during PCR. Placement of oligonucleotide primers determine the gene region and length of the amplicon generated. Primers may be designed to be phylogenetically broad, for example targeting all bacteria or all eukaryotes, or group-specific (e.g., fungi, haplosporidians, *Cryptosporidium*), or species/strain-specific.

Barcoding: the use of (usually) short standardized gene regions for non-phylogenetic species recognition. Regions of the mitochondrial gene encoding cytochrome *c* oxidase I (COI) is frequently used for animals, the large subunit of the chloroplast ribulose-1,5-biphosphate carboxylase-oxygenase gene (RuBisCo/rbcL) for plants, ITS rDNA for fungi, and the V4/V9 regions of 18S rDNA as a 'pre-barcode' for protists.

Complementary DNA (cDNA): RNA reverse transcribed by means of a reverse transcriptase primed by random hexamer primers or group/taxon-specific primers.

Denaturing gradient gel electrophoresis (DGGE): a molecular fingerprinting method which separates PCR-generated amplicons on denaturing polyacrylamide gel based on sequence differences rather than amplicon length.

Environmental DNA (eDNA): nucleic acids extracted from 'true' environmental samples (soil, sediment, filtered water, etc.), organism-derived material (feces, tissue, sloughed cells, etc.), and organisms themselves (as hosts of parasites, pathogens, and microbiomes). Thus eDNA comprises organismal material and whole (micro-)organisms in addition to extracellular DNA, which can be isolated separately through precipitation. eDNA can be sequenced directly as metagenetic libraries or after PCR amplification of specific target gene regions.

High-throughput sequencing (HTS): highly-parallel sequencing technologies including Illumina platforms, 454 Sequencing, Solexa, Ion Torrent, Pacific Biosciences, Nanopore.

Metabarcoding: the use of HTS sequencing platforms or Sanger sequencing for generating large

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Box 2. eDNA Sources, Sampling, and Sample Processing

Awareness of the many ways in which parasite DNA (and RNA, Box 1) may be represented in a given eDNA sample is an important element in the design of an eDNA experiment. The presence of target DNA may represent active forms of the parasite (autonomous dispersal, infectious stages), inactive forms (cysts, spores, eggs) in water columns, sediments, or soils, or extracellular DNA disassociated from any organism [6].

Individual hosts may be sampled directly and molecular analyses applied to whole organisms, tissues, or excreta. Larger host organisms may be incubated in sterile media, followed by analysis of the incubation water to detect newly released parasite stages [47] (Figure IA). Where hosts are small (zoo- or phytoplankton, meiofauna, etc.) they can be sampled together with their parasites, offering the possibility of directly linking the organisms involved and further studying their interactions.

An informative approach for pinpointing planktonic hosts is serial filtration and eDNA extraction (Figure IB). Follow-up using fluorescent *in situ* hybridization or *in situ* hybridization staining has been used to great effect to visualize the host and parasites [10]. Isolation of infected hosts by sample subdivision [117], coupled with eDNA testing to reveal positive size fractions, can be used to associate individual hosts with parasite signal (Figure IC). Environmental matrices, whole organisms, or isolated tissues can be screened using both amplicon-based and metagenetic methods (Figure ID). Extracellular DNA can sometimes be extracted separately from intracellular DNA from the same sample [118].

All these approaches provide access to different sources of parasite (and host) DNA, and offer different data resolution regarding hosts, parasites, and their interactions. Some are purely descriptive and useful for parasite discovery, others are more appropriate for quantitative analysis and therefore can be used for predictive (e.g., disease risk, ecosystem function and connectivity) assessments. The choices of sample type and processing, and DNA amplification and/or sequencing (Box 1), together provide a multi-faceted and highly-adaptable set of methods for eDNA analyses. Note that although the diagram in this box shows an aquatic example, exactly the same principles apply to terrestrial ecosystems, although detection in the atmosphere or in dense substrates may be more challenging than in aquatic environments.

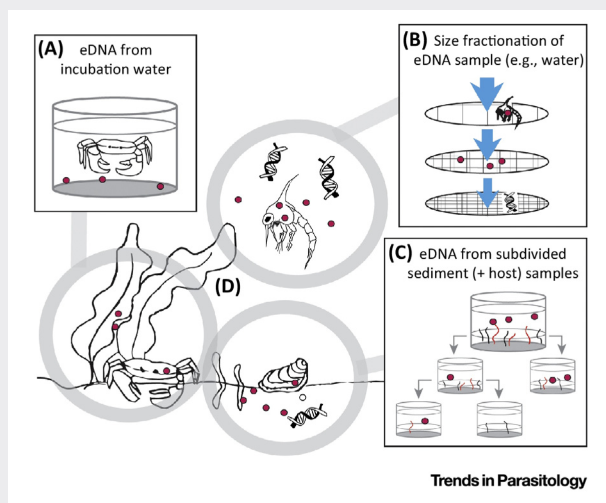

Figure I. Selected Methods for Targeted eDNA Sampling of Parasites. (A) Incubation of larger organisms for parasite expression/release, (B) Serial filtration for size fractionation, (C) Subdivision of samples and consecutive molecular screening, (D) direct sampling of whole organisms, tissues, and environmental matrices (water/sediment/biofilm, etc.).

numbers of barcode or barcode-like amplicons from clone libraries or HTS library preparations.
Metagenetic: collective term for metagenomic and metatranscriptomic.
Metagenome/-ic: DNA molecules or sequences representing the total DNA complement of a sample, whether derived from the environment or a particular organism (e.g., gut, tissue). Note that metagenome/-ic is sometimes used to refer to small subunit rDNA amplicon studies. We strongly advise against this usage because it risks confusing two completely different approaches.
Metatranscriptome/-ic: RNA molecules or sequences representing the total RNA complement of a sample, whether derived from the environment or a particular organism (e.g., gut, tissue). Usually reverse transcribed to cDNA.
Quantitative real-time PCR (RT-qPCR): PCR used to simultaneously amplify and quantify the amplicon during each cycle of the PCR reaction. Quantification relies on fluorescent reporter dyes and, depending on instrumentation, may include additional reporter probes which increase specificity of the reaction.
Sequence library: set of DNA fragments or sequences generated from a single or set of samples by PCR amplification, cloning, or (meta) genomic/(meta)transcriptomic preparation.

and/or asymptomatically. Although often genetically highly divergent, microbial parasites are frequently morphologically indistinct, with high levels of morphological conservation and convergence. They can therefore be difficult to locate and/or to distinguish in histopathology preparations. Molecular detection methods can circumvent or alleviate many of these barriers, but face challenges of their own. One class of these relates to the nature of the matrix from which nucleic acids are extracted: both host tissues and environmental samples can be high in non-target DNA or in compounds that cause problems for DNA extraction and/or inhibit PCR [13]. In addition, accessing parasite DNA may require physical disruption of robust cysts or egg cases. This is particularly an issue for helminth eggs, for which stringent extractions protocols have been developed [14–16]. However, it is unknown how physically-resistant many micro-eukaryotic parasite stages are, and there is likely to be negative bias against resistant stages when using standard extraction protocols.

Consideration of the nature of the environmental sample itself is crucial – is it expected to include the host, or cells from the host, or indeed a possibly unknown alternative host/vector? Are

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Box 3. eDNA and Metazoan Parasites

The detection of metazoan parasite infections, particularly helminths, within definitive hosts often relies on associated pathology, serology, or the detection of eggs shed in feces or urine. Although DNA studies have been developed to identify and genotype many human and veterinary parasites from such samples, the techniques are rarely applied to environmental samples, although this can be equally important [119]. Widespread water or soil surveys specifically for metazoan parasites have been lacking. Low parasite prevalence, particularly when detecting eggs in heterogeneous soil samples, or within intermediate hosts, may make such a prospect costly. However, the ability to diagnose environmental samples may influence the effectiveness of control measures when parasite viability in the environment can persist for months or even years, as is often the case for nematodes, and in particular those causing soil-transmitted helminthiasis (e.g., [120]). Contaminated water or soil requires analysis for effective management and disease eradication [121]. For example, although the clinical conditions of Guinea worm infection (dracunculiasis) in humans are obvious [122], the persistence of this nematode, despite concerted control efforts [123], would surely benefit from an understanding of its prevalence as eggs in freshwater and as larvae in freshwater copepods to understand transmission to humans as well as non-human (dog, other primate) hosts.

The ability to screen environments for the presence of potential (intermediate or definitive) hosts as well as the parasites themselves has implications for human health, animal welfare, freshwater fisheries, coastal aquaculture, conservation, and ecosystem health.

dispersal or persistence stages known or possible? What scale of sampling is necessary to have a reasonable likelihood of detecting a particular parasite? If little is known of the ecology of the parasite then temporal variation in transmission and proliferation must be taken into account. Box 4 provides a generalized parasite life cycle, which illustrates some of the options for eDNA intervention to aid resolving complex parasite life cycles.

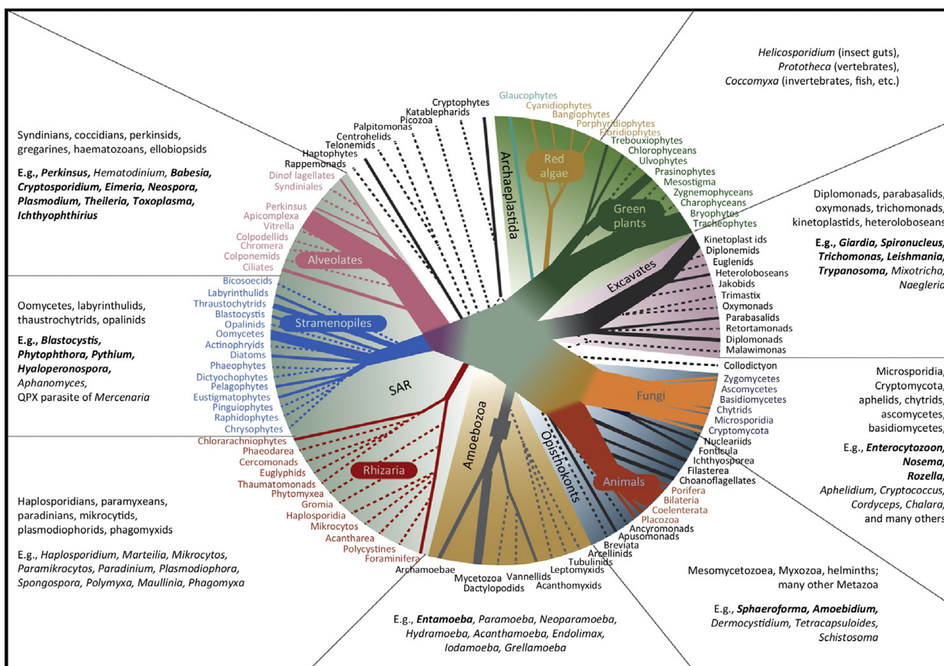

Figure 1. Schematic Eukaryotic Tree of Life. The scheme shows how representative parasitic lineages are distributed across the supergroups SAR (stramenopiles, alveolates, Rhizaria), Amoebozoa, Opisthokonta, Excavates, and Archaeplastida. In each segment, key parasitic groups are listed, followed by example genera. Genera in bold indicate those for which genome sequences are available (adapted with permission from [124]).

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Box 4. eDNA Insight into Parasite Life Cycles

Parasite life-cycle forms and stages outside of their main host(s) of human concern are in many cases unknown. Infective forms (which may be free-living), alternative hosts, mechanical vectors, and persistent or dormant stages can be key to understanding how a parasite endures and disperses in the environment, where and when infection pressure on hosts varies, and the means by which hosts can become infected. A generalized life-cycle diagram in Figure I illustrates the various points at which eDNA interventions may be useful. An unappreciated element in the life cycle can unlock the way to predictive understanding and the development of preventative measures. eDNA methods offer a multitude of insights into both known and unknown elements of the life cycle, and can be applied at various stages of the suspected parasite life cycle.

Where life cycles are known, eDNA tools can be used for monitoring spatial and temporal distribution of transmissive stages in the environment, parasite strain diversity within hosts (multiple infections and within host interactions of parasite strains), and quantitative measures of parasite abundance. In cases where life cycles are poorly known, novel vectors, hosts, and occurrence can be detected by sampling various environmental matrices that may contain parasite dispersal and resting stages or vectors and alternative hosts [101,125]. eDNA can detect dormant stages in environmental parasite reservoirs, or indicate trophic or strictly vertical transmission routes when no free parasite stages are detected.

Appropriate experimental designs (Box 2) allow interrogation of the life cycle to confirm, for example alternative hosts, shedding of infective stages, or the presence of vertical transmission. Relevant case studies include: (i) increased detection of *Hematodinium* spp. in eDNA samples before infections in the known host (blue crab) led to a previously unknown stage in the parasite life cycle being suggested [126,127]. (ii) The role of the copepod *Paracartia* sp. in the transmission of the paramyxean *Marteilia refringens* was clarified by temporal detection patterns of *M. refringens* in zooplankton samples, supporting a possible transmission route via copepod eggs [128]. (iii) Recognition of the polychaete *Nephtys australiensis* as an alternative host of *Marteilia sydneyi*, the causative agent of QX disease in Sydney rock oysters [129].

eDNA surveys of parasites can be complemented by those targeting their (potential) hosts, to develop more ecologically integrated and predictive models. For example, extracellular eDNA surveys of fish communities and movements [130,131] could suggest putative hosts for parasites detected in the same samples, and be used to monitor and predict parasite migration and range shifts.

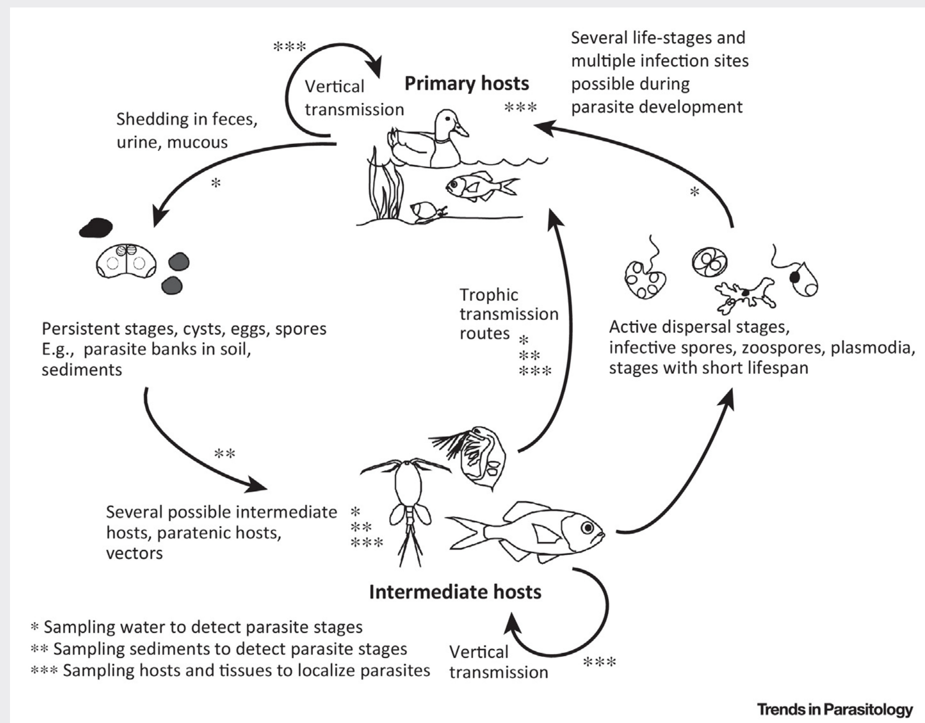

Figure I. Possible eDNA Intervention Points in a Generalized Parasite Life Cycle. The general sample categories are indicated with asterisks.

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Gene sequencing itself is not an essential component of eDNA workflows [17]. Molecular methods that are built upon previously generated sequence data to provide a quantitative or presence assay greatly reduce data generation and analysis burdens. **Quantitative real-time PCR** (RT-qPCR) and digital droplet PCR [18] are increasingly proven as methods for quantification of target organisms in the environment, and are particularly useful when the targets themselves are rare or difficult to observe directly. The extreme sensitivity of some qPCR applications (by virtue of highly-specific primers and probes defining very short **amplicons** and fluorescence-based reporting) provides the most powerful means of quantifying tightly defined lineages in terms of relative and absolute biomass (DNA) and relative activity (RNA) [19]. Increasingly, isothermal PCR methods such as recombinase polymerase amplification (RPA) [20] offer simple, portable, but sensitive alternatives to qPCR itself.

Although superseded in many ways by the contemporary ease of generating massive sequence datasets, microarrays remain useful for screening samples for a pre-defined set of taxonomic and/or metabolic markers [21], for example screening bacterial diversity in the human gut microbiome [22]. One can envisage microarrays being designed for rapid screening of genes shown (e.g., by environmental metagenomic studies) to be informative sentinels of particular patterns or changes in parasitological systems. After the initial effort and expense of creating the microarray, this approach offers much higher throughput than generating and analyzing massive sequence datasets.

General Eukaryotic eDNA Diversity Studies

eDNA studies using PCR primers to amplify a broad phylogenetic range of eukaryotes from environmental samples suggest that a large proportion of uncultured micro-eukaryotic diversity is represented by parasites. Whether particular sequence types represent parasitic taxa can be initially assessed phylogenetically; sequences which group robustly with known parasitic radiations are also likely to be parasites. eDNA studies continue to reveal a high diversity of parasitic and putatively parasitic phylogenetic lineages across the tree of life in freshwater [23–25], marine water [26–29], and soils [30,31] (Table 1, Key Table). In these cases the eDNA data can provide valuable additional information about diversity and geographical and ecological distributions, depending on the metadata associated with sample provenance and treatment. In some cases, phylogenies including eDNA sequence data have proposed a parasitic lifestyle subsequently borne out by cell isolation or other means of parasite visualization, for example syndineans, cryptomycota, and gregarines [32–35]. Broadly targeted eDNA studies have revealed significant novel diversity in many micro-eukaryotic groups. Some gregarines (apicomplexan alveolates; Figure 1) can be highly represented in broad eukaryote surveys (e.g., [30,36]), as can syndinians and perkinsids (also alveolates, related to dinoflagellates) [3,26,29,37]. However, other apicomplexans, (e.g., coccidians, piroplasmids, *Cryptosporidium*, blood parasites such as *Plasmodium* and *Leucocytozoon*) are largely missing from broadly-targeted surveys, as are the diverse and ubiquitous microsporidia. BLASTn searches [38] against NCBI GenBank of 18S rDNA sequences representing the highly diverse and well-studied genera *Plasmodium*, *Trypanosoma*, *Toxoplasma*, *Cryptosporidium*, and *Eimeria* (the apicomplexan genus containing the highest number of described species, >1700) also produce no broadly targeted environmental sequences until sequence similarities of the BLAST matches are <90% similar to the target sequence, at which point the sequences retrieved relate to different taxa. The same is true of the rhizarian *Haplosporidium*, a diverse genus of mollusk and crustacean parasites, while a related genus parasitizing copepods and prawns, *Paradinium*, has many closely related sequences in marine environmental datasets. Other parasites are intermediate between these extremes, returning smaller numbers of closely related BLAST hits, for example plasmodiophorids [39] and the mesomycetozoean *Sphaerothecum destruens*. Absences of divergent parasitic lineages from broad-scale surveys highlight the concern that many parasitic groups are not captured by general primers. This underlines the importance of designing group-specific primers to assess

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Key Table

Table 1. Examples of Different Types of eDNA Studies with Relevance to Parasitology

Type of Study	Organisms and eDNA Type	Refs
Pan-eukaryote 18S rDNA sequence amplicons	Studies encompassing many parasitic micro-eukaryotes, including alveolates (Apicomplexa, Syndiniales, Perkinsea, gregarines), stramenopiles, fungi and relatives, holozoans, cercozoans, kinetoplastids, etc., in marine and freshwater, soils and sediments	[3,4,9,23,24,27,30,37]
Group-specific amplicons	Use of 18S rDNA, cytochrome oxidase I and other genetic markers for biotic indices, identification and classification, in various matrices including other organisms (hosts), blood, water, soils/sediments, feces	[31,39,46–48,50,60,62, 64,69,71,72,76,86,89, 127,132,136,137]
DNA versus RNA-derived 18S amplicons	Comparisons of biomass (DNA) versus relative activity (RNA) of micro-eukaryotes	[4,113,114]
Micro-eukaryote group-specific studies derived from pan-eukaryote 18S datasets	Syndinians, perkinsids, gregarines, other alveolates, stramenopiles, fungi, cryptomycota, trematodes	[9,16,24–26,28,29,32, 34,41,43]
qPCR/species or lineage-specific quantification	*Ichthyobodo* and *Ichthyophonus* associated with fish, *Aphanomyces* (crayfish plague), *Cryptosporidium*, other parasites and invasive species in water, sediments, tissues, feces, and other matrices	[18,19,55–59,61,63, 98,119,126,138]
Metagenomics	Use of metagenomics for pathogen discovery/inventory/ survey	[80–82,89,91]
Metatranscriptomics	Transcriptomics in environmental assessment in marine water, soils, microbiome genomics in human gut, food production mammals, invertebrates	[36,84–86,107]
Complementary approaches	Combination of *in situ* hybridization microscopy with amplicon sequences to show occurrence and visualize parasites in plankton samples	[10,72,99,112,125, 128,139]
Methodological assessment and development	Primer design, sequencing methods, PCR issues, DNA extraction, phylogenetic analyses, database and barcode development, microarrays, RPA, single-cell PCR	[13,17,20,21,33,40, 51–53,118,133–135]
Microbiome analyses	Microbiomes of humans, bees, food animals, *Sphagnum*	[22,85,88,90,91,94]

the true diversity of certain parasitic lineages; other possible reasons for such absences are discussed further below.

By generating large volumes of sequence data of high lineage richness, eDNA studies can also help to demonstrate the cohesion of groups and resolve phylogenetic relationships [40]. A robust phylogenetic framework is necessary for correct biological interpretation of major new parasitic taxa, recent examples being some gregarines ([32] and references therein), Cryptomycota/LKM clade [34,41], aphelids [42–44], and basal lineages such as *Mitosporidium daphniae*, which branches at the root of Microsporidia [45]. Basally-branching lineages can help resolve phylogenetic placement of long-branched (highly divergent) parasites, particularly when combined with eDNA-derived sequences.

Insights from Group-Specific Primers

Although the large and increasing volume of broadly targeted eukaryotic environmental sequences in public databases is a useful source of micro-eukaryotic parasite data, it provides a very skewed perspective of their diversity, abundance, and importance, as illustrated above. There are several reasons for this, which apply differently to various parasite groups. Many parasite taxa are

genetically divergent, indicated by their long branches on phylogenetic trees, which means that even primers designed to conserved regions of marker genes fail to amplify from divergent lineages, or do so relatively poorly. This is demonstrably the case for haplosporidians, mikrocytids, plasmodiophorids, trypanosomatids, myxozoans, many alveolates, and microsporidians, among others, because PCR primers designed to amplify those groups specifically or preferentially have revealed significant previously unknown diversity, often in environmental samples [39,46–50]. Primers can also be designed to exclude unwanted targets, for example general eukaryote primers with an anti-metazoan bias to reduce host and other 'contaminating' metazoan sequences [51]. Another way to reduce reads originating from the host/non-targets is to use blocking primers, thus focusing a larger proportion of the sequencing effort on the target organisms [52,53].

Other parasites may be relatively rare or patchily distributed, for example X-cell parasites of fish [54], or have physically-resistant stages that are difficult to disrupt during nucleic acid extraction. Lineage-specific probing for blood parasites, or those otherwise tightly host-associated, often reveals novel distribution and diversity from organismal samples, for example multi-primer surveys of avian blood parasites (*Plasmodium*, *Haemoproteus*, *Leucocytozoon*, and *Trypanosoma*) across altitudinal and latitudinal gradients [55,56]. These blood parasites cycle tightly between hosts and therefore may not be found in other matrices (e.g., soil, water). However, a targeted eDNA approach has the potential to detect such host-associated parasites and unknown life-cycle stages.

Targeted Molecular Approaches, eDNA, and Insights into Life Cycles

The most highly targeted eDNA assays are those designed to individual genera, species, or subspecific strains, and are highly-specific, sensitive, and often quantitative. Many RT-qPCR assays have been developed, for example for the fish parasites *Ichthyobodo* and *Ichthyophonus* [57,58], the myxozoan parasite *Ceratonova* (previously *Ceratomyxa*) *shasta* in rivers [59], screening marsupials for *Toxoplasma gondii* [60], and monitoring of intranuclear coccidian parasites in endangered turtles [61]. For very well studied groups or systems, such as *Cryptosporidium*, *Giardia*, and *Enterocytozoon*, the molecular tools have become diverse and refined; for example, allowing identification of differently distributed genetic subtypes in wastewater samples [62] and using quantification to estimate transmission risk [63]. The sensitivity and specificity of these approaches makes them ideal tools for researching life-cycle diversity, host ranges, pathogen reservoirs, etc. Box 4 shows a generalized parasite life cycle, indicating points at which eDNA studies can intervene to illuminate environmental occurrence of different life-cycle stages, potential transmission zones, parasite reservoirs, and identification of areas to prioritize for control or eradication attempts. Even these specific approaches often reveal genetic diversity within their narrow phylogenetic ranges, which may be biologically significant in terms of virulence, distribution, or other characteristics.

eDNA Facilitates Indirect Detection of Both Parasites and Hosts

Despite all safeguards, introductions of hosts, either legitimate or accidental, may be accompanied by non-endemic parasitic hitch-hikers that then have the potential to spread (e.g., *Echinococcus multilocularis* in beaver reintroduction schemes [64] and the spread of *Eimeria* in cottontails introduced for hunting [65]). Sampling of individual hosts to monitor such potential spread is often not feasible (particularly in the cases of migratory birds, mammals, and insects), but eDNA analyses of fecal material has much potential as a host-independent and non-invasive method of parasite monitoring; for example, helminths in rat feces [66], fungal community characteristics associated with the spread of Bat White Nose Syndrome [67], *Blastocystis* in dog feces [68], and *Cryptosporidium* species in cattle feces [69]. Hitch-hiking is a particular concern with micro-eukaryotic parasites, which are at least in some cases more easily dispersed than larger metazoans [70,71] and more difficult to find and/or identify in pathology screens of hosts.

Fecal eDNA analyses can identify hosts along with their parasites, for example the simultaneous detection of crane host species (via mitochondrial 16S gene), individual birds and their sex (via microsatellites and sex-linked markers), and their coccidian parasites (internal transcribed spacer ITS1 rDNA) [72].

Parasitic Dark Matter – Uncharacterized Relatives

Interpretation of currently-available sequence data must consider that, for most micro-eukaryotic groups, only a small fraction of their diversity is known. Closely related sequence types may represent biologically distinct parasites. Therefore, interpretation of eDNA sequence data of poorly known parasites is limited by lack of knowledge of the group as a whole [73]. eDNA methods are particularly powerful when linked to complementary studies. For example, phylogenetic analysis of the 18S rDNA sequence of the amphipod parasite *Haplosporidium diporeae* [74] shows that it belongs to haplosporidian clade C [48], a diverse clade of seasonal benthic and planktonic environmental sequences with no known host association when first detected. Other ways in which eDNA approaches can inform parasitological studies include the use of group-specific primers to reveal diversity associated with novel and emerging parasites, for example the newly discovered parasites of *Cancer pagurus* and *Crassostrea gigas* in the UK (*Paramikrocytos canceri* and *Mikrocytos mimicus* respectively) [47].

The high levels of often closely related, 'micro'-diversity accessible via eDNA work raise an important point regarding systematics. This genetic micro-diversity is unlikely to be functionally redundant, as illustrated by both protistan (*Cryptosporidium* [62,75], *Blastocystis* [76], haplosporidians [48], *Amoebophrya* [9]), and metazoan parasites [77,78]. However, taxonomic rigor in the literature is generally decreasing, losing resolution, and retreating up through the taxonomic ranks [78]. The warning of Poulin and Leung [78] that this trend should be reversed to cope with parasite diversity in a changing environment should be sounded even louder in the face of large volumes of specimen-independent eDNA sequence data, and the growing awareness of micro-eukaryotic parasites. A synthesis is needed between morphology- and sequence-based taxonomic methods such that newly generated eDNA data relating to (emerging) diseases caused by biological agents already present in the environment, and to range and host shifts, human impositions, etc., can be interpreted in a robust and adaptable taxonomic framework. Longer sequence reads now possible on **high-throughput sequencing** (HTS) platforms, particularly Illumina and Pacific Biosciences platforms [79], can facilitate taxonomic annotation and phylogenetic analyses of eDNA data.

The Power of Metagenetics

The unique advantage of **metagenetics** is the ability to access the genome/transcriptome-wide gene complement of all organisms within the sample while minimizing or eliminating PCR amplification biases (Box 2). So far, these methods have most frequently been used for describing diversity of viral pathogens [80], but their potential is much wider. One such is pathogen discovery, for example the extremely genetically-divergent ascetosporean *Paramikrocytos canceri*, for which the first (multi-gene) dataset was generated by metagenomic sequencing of DNA extracted from infected tissue [47], where PCR-based approaches had failed. Metagenetics has also revolutionized our understanding of how microbiomes interact with their hosts and the organisms they surround. These methods are particularly appropriate for associating different organisms – hosts, parasites, vectors, and food organisms – to develop multi-context models for disease transmission and etiology [81], for multi-lineage analyses of bacterial and other infections [82], and would be well suited to capturing the wide range of pathogen signatures in fecal samples, for example to track zoonotics [83]. More broadly, metagenetic studies of environmental matrices (soil, water, etc.) are revealing a surprising

diversity and high level of activity of micro-eukaryotic parasites (e.g., [36,84]) that, when better understood, might form the basis of predictive models of disease risk and ecosystem function. eDNA methods (particularly metagenetics) applied to individual hosts can easily elucidate coinfections and hyperparasitism, whose synergistic and antagonistic effects are likely more important than previously recognized.

Potential for Development of eDNA Methods in Parasitology

Once the potential for discriminating between active and inactive parasite material in environmental and organismal matrices at different levels of resolution is realized, more strategic and applied developments of these approaches are possible, and eDNA methods can be better marshaled to identify causative agents of disease. An example of this is the combination of custom microarrays, qPCR, and HTS metagenetics to investigate the range of viral, bacterial, protistan, fungal, and metazoan pathogens associated with colony collapse disorder (CCD) in honey bees [85,86]. Complex infections and host-associated microbiota represent a strongly emerging, process-driven field of study because symbioses are increasingly recognized as drivers of physiological, ecological, and disease-related processes in many organisms, reviewed for example in insects [87]. Human bacterial microbiomes are by far the best-studied host-associated microbiota. HTS provides powerful insight into the role of the highly-complex and abundant bacterial community in the immune system and more general health [88]. The eukaryotic component of gut microbiomes has received less attention, but metagenetic and targeted eDNA methods provide easy access to these by, for example, extracting taxonomically informative markers from HTS datasets [89]. eDNA methods are more recently being applied to other hosts for example mammals [90], plants [91], and invertebrates. Examples of the latter include the use of 16S rDNA **denaturing gradient gel electrophoresis** (DGGE) and clone sequencing to suggest that vector gut microbiota may influence transmission and virulence of *Trypanosoma cruzi* [92], similar methods used for understanding gut microbiota assembly in the western corn rootworm (Coleoptera) and their potential as pathogen vectors [93], variation in bumble bee gut bacteriome between related species and how this varies with pathogen infections [94], and the suggestion via **metatranscriptome** sequencing that shifts in honey bee gut bacteria/community may be a marker of CCD [86]. Pathogens in the microbiotic environment in which organisms live are also important for health and as selective agents: **metabarcoding** of bacterial communities local to European minnow populations has shown that host–parasite interactions drive local adaptation of the major histocompatibility complex (MHC) class IIB loci, in relation to local pathogen diversities [95].

If eDNA signal in an environmental phase (e.g., benthic or planktonic) is to be used as a specimen-independent and non-invasive way of monitoring local infection of or disease risk to larger hosts, calibration studies will be necessary to correlate eDNA signal with directly measured infection. Simply detecting the pathogen in the environment is not sufficient; the requirement is to relate a quantifiable eDNA signal to measured disease or parasite load in local hosts. Generating this signal from RNA rather than DNA samples would identify active infective stages in preference to dormant spores, cysts, or extracellular DNA (Box 1). Where such matches are possible, a temporal calibration effort is then necessary to develop predictive tools. The sensitivity demanded by these applications can be provided by adopting eDNA techniques from other fields: for example invasion biology [96] and conservation work [6,97]. Complementary tools enabling a more integrated understanding of a system are increasingly practicable. For example, a specific *Aphanomyces astaci* (crayfish plague) qPCR assay on both water and crayfish tissue samples allowed correlation of histological evaluation of disease status with parasite load in the environment, and can form a basis for disease surveillance on appropriate spatial scales [98]. Microscopy can also be used for example, *in situ* hybridization (ISH) to localize lineage-specific parasite signal in host tissue in histopathology preparations [47,99].

There is much potential to co-opt eDNA methods to better understand how ecological communities influence disease risk from their constituent parasites [100], and to understand and predict disease emergence in an integrated and changeable ecological context [101], as well as the life-cycle interrogation possibilities outlined in Boxes 2 and 3. Molecular genetic techniques are increasingly used in epidemiological studies [102], many of which are obviously or potentially enriched by approaching them from an eDNA perspective as described above, that is, the characterization and systematics of parasite diversity, life cycles, transmission, and coinfection.

Parasites contribute significantly to ecosystem biomass and energy transfer, increasing link density and connectivity relative to food webs constructed using only free-living taxa [103]. Many parasites exist for some time as free-living dispersal stages that can be abundant and important food sources for other organisms [104]. Quantifiable eDNA assays could greatly expand the sample size and phylogenetic resolution of such studies, and identify parasite reservoirs (both environmental and within-host, particularly in the case of covert infections), transmission routes (e.g., via host excreta), and habitat areas in which infective pressure is high or low.

At the largest scale, molecular techniques are increasingly being used to evaluate overall ecosystem and habitat condition/health. Bourlat *et al.* [105] identify many areas in which eDNA-based methods are (and have potential to be) used in monitoring marine health status, including diversity, non-indigenous species, food webs, eutrophication, and seabed integrity [105]. Metabarcoding approaches to marine monitoring have also been proposed [106]. Very recently the use of transcriptomics to integrate eco-toxicogenomics into marine health evaluation has been assessed [107] via contamination-associated gene expression markers. Parasites themselves have been shown to be useful markers of ecosystem status and its response to change [108,109], including response to climate change [110]. The employment of eDNA methods for such studies can be very effective if appropriate sampling and molecular strategies are chosen (Boxes 1 and 2), both reducing sampling effort and maximizing sampling coverage.

Policy Relevance and Application of eDNA

That eDNA approaches have the potential to detect unknown pathogen diversity within ecosystems and can be applied to detect specific taxa of interest is beyond debate. However, it is crucial to consider how data arising from eDNA surveillance approaches may be contextualized for use in a policy setting, for example how the detection of apparently specific genomic material from a politically important (listed) pathogen in an environmental matrix relates to the universally applied principles of 'infection' and 'disease' detection and reporting according to the World Organization for Animal Health (Office International des Epizooties, OIE) (www.oie.int) (for context see [73]). In essence, upon detection of a case (animal infected by the notifiable pathogen) the Competent Authority (CA) within the affected OIE Member Country (MC) is obliged to notify the headquarters of the OIE who subsequently inform other MC CAs of the case [111]. Detection relies on the application of a set of defined and validated diagnostic tests on samples arising from the susceptible (either known or newly identified) host. Within the regularly updated Manual series, the OIE state a requirement for any diagnostic test to be validated to determine its fitness of purpose [111]. Because all tests are currently designed and applied in such a way (i.e., applied to host tissues), detection of apparently the same notifiable pathogen in an environmental context (i.e., outside its host) cannot currently fulfill the criteria required for detection and reporting, according to conditions stipulated by the OIE [111].

In the context of eDNA, although it may be envisaged that metagenetic approaches will be increasingly applied to environmental matrices (or host tissues) to investigate phylogenetic diversity of potential animal pathogens, it is difficult to imagine at present that such a broad approach (and one not applied to infected or diseased host tissues) will have direct utility in the detection and reporting of notifiable pathogens. Rather, a more targeted, amplicon-specific

approach, either directed at detection of a given listed pathogen gene sequence, or at similar sequences from closely related parasites, may provide a more useable test that can be validated against available 'gold standards' [47]. In summary, for future use of eDNA approaches to pathogen detection in a policy context, a new appreciation of the value of 'extra-host' notifiable pathogen detection and, in particular, validation of this environmental signal against other validated tests, is crucially required. Given that detection and reporting of notifiable pathogens initiate a formalized sequence of events that may culminate in trading restrictions of live animals and commodities between nations, we propose that, until such validation is achieved, environmental detection of 'extra-host' pathogen sequence alone is not sufficient diagnostic evidence to initiate notification procedures within OIE MCs.

Concluding Remarks

The application of eDNA in biodiversity and environmental condition indices are rapidly-developing fields. Increasingly sophisticated methods allow eDNA to address questions related specifically to parasites and their ecology. With appropriately designed studies, eDNA can demonstrate parasite diversity and distributions, reveal novel lineages and associations, help to elucidate parasite life cycles and disease etiologies, and investigate how ecological communities influence disease risk from their constituent parasites. Detection of divergent parasitic lineages often requires lineage-specific PCR primers and special considerations regarding the patchiness and seasonality of parasite presence within and outside the host environments. Incorporation of eDNA-based monitoring for pathogen detection in policy contexts has yet to be formalized, and may lead to conflicts where pathogens are detected in eDNA but infection is unconfirmed. The Outstanding Questions Box highlights further methodological and policy challenges ahead but, in general, eDNA-based approaches provide a powerful, developing – and increasingly used – toolbox for parasitological studies.

Outstanding Questions

To what extent can eDNA signals from environmental matrices indicate infection (and health) status of local hosts?

Can eDNA signal be used to predict the risk of disease in local hosts?

What is the parasitic nature of close relatives of known parasite lineages detected in eDNA studies?

How can eDNA data be validated to inform policy and management decisions?

Can quantification of parasite diversity and activity via eDNA methods be used as part of ecosystem assessment and monitoring?

Can a reference dataset be generated and maintained as a taxonomic resource for annotation of parasite data generated by HTS approaches?

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