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Pinning down the role of common luminal intestinal parasitic protists in human health and disease – status and challenges

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Review

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Abstract

While some single-celled intestinal parasites are direct causes of diarrhoea and other types of intestinal pathology, the impact of other gut micro-eukaryotes on human health remains elusive. The fact that some common luminal intestinal parasitic protists (CLIPPs) have lately been found more often in healthy than in diseased individuals has fuelled the hypothesis that some parasites might in fact be protective against disease. To this end, the use of new DNA technologies has helped us investigate trans-kingdom relationships in the gut. However, research into these relationships is currently hampered by the limited data available on the genetic diversity within the CLIPPs genera, which results in limited efficacy of publicly available DNA sequence databases for taxonomic annotation of sequences belonging to the eukaryotic component of the gut microbiota. In this paper, I give a brief overview of the status on CLIPPs in human health and disease and challenges related to the mapping of intestinal eukaryotic diversity of the human gut.

Introduction

Recent research into the human gut microbiome has vastly increased our understanding of the relationship between gut microbes and human health and disease. For instance, we now know that in adults, a low-diversity microbiota with increases in proportions of facultative anaerobes is linked to acute diarrhoea, inflammatory bowel disease, *Clostridium difficile* infection, metabolic syndrome and liver disease, just to mention a couple of conditions (Cani, 2018; Kriss *et al*., 2018). Although most gut microbiome research has focussed on prokaryotic diversity, we have also gained significant insight into the micro-eukaryotic diversity of the human gut. DNA-based methods have been instrumental to this advancement. Three important points have emerged: (1) For some common luminal intestinal parasitic protists (CLIPPs), genetic diversity is surprisingly high; still, DNA sequence data available in publicly available databases such as the NCBI database is rudimentary, hence not reflecting this amount of diversity (Stensvold *et al*., 2011*c*, 2012, 2018; Royer *et al*., 2012; Poulsen and Stensvold, 2016). (2) We have come to realize that some CLIPPs are very common and often more common in gut-healthy individuals than in those with functional and inflammatory bowel diseases, contrary to previous general belief (Petersen *et al*., 2013; Andersen *et al*., 2015; Krogsgaard *et al*., 2015, 2018; Rossen *et al*., 2015; Beghini *et al*., 2017; Jokelainen *et al*., 2017; Mirjalali *et al*., 2017). (3) Robust links between CLIPPs and gut bacteria have been identified by several research teams (Stensvold and van der Giezen, 2018).

These three points currently stimulate interdisciplinary research across the fields of parasitology, clinical microbiology, gastroenterology and ecology. Nevertheless, compared with advances within e.g. bacteriology and virology, progress in the research into CLIPPs and their role in human health and disease is still reflected mostly in simple stool microscopy-based surveys of parasites in selected populations, and is therefore still facing some major challenges. In the following, I will try to detail the status of the three above-mentioned points, and highlight some of the limitations and challenges to the work ahead that aims to identify the significance of CLIPPs in human health and disease.

Which are the most common luminal intestinal parasitic protists?

Intestinal eukaryotes that need a host to complete their life cycles (i.e. organisms that are referred to as 'parasites') include both helminths and protists. More typically, the distinction is made between 'helminths' and 'protozoa', but from a taxonomical point of view, the group of organisms referred to as protozoa does not include one of the most common micro-eukaryotes, namely *Blastocystis*, and so, the term 'protists' appears more relevant and applicable than 'protozoa' in this context. Moreover, while some parasitic intestinal protists are invasive (e.g. sporozoa) or adhere to the mucosal lining (e.g. *Giardia*), quite a few genera appear to be confined mainly to the gut lumen. These include *Blastocystis*, *Dientamoeba*, *Endolimax*, *Iodamoeba* and most species of *Entamoeba*, and given their prevalence, they could be referred to as CLIPPs.

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Contrary to the situation in developing countries, the number of carriers of helminth infestations other than those attributable to pinworm (*Enterobius vermicularis*) appears to be rapidly plummeting in human populations in the Western world (Verweij and van Lieshout, 2011; Verweij, 2014), and also in some parts of the developing world, which would probably reflect improved hygienic standards. Still, and for incompletely known reasons, a substantial proportion of the population is colonized by CLIPPs, especially *Blastocystis* and *Dientamoeba* (Verweij and van Lieshout, 2011; Roser *et al*., 2013; Krogsgaard *et al*., 2015, 2018; Jokelainen *et al*., 2017) and, to a lesser extent, by one or more of the Amoebozoa, e.g. *Entamoeba coli* (Bruijnesteijn van Coppenraet *et al*., 2009; Krogsgaard *et al*., 2018; Stensvold and Nielsen, 2012; ten Hove *et al*., 2007); these organisms will be introduced briefly below.

Blastocystis

Blastocystis is a genus comprising a perplexing variety of ribosomal lineages that are arguably separate species, judging from the amount of genetic diversity across complete nuclear ribosomal genes. So far, at least 17 ribosomal lineages, the so-called 'subtypes', have been acknowledged in humans, non-human primates, other mammals and birds (Alfellani *et al*., 2013*b*; Clark *et al*., 2013; Stensvold and Clark, 2016). Also reptiles, amphibia and insects have been identified as hosts for various species of *Blastocystis* (Yoshikawa *et al*., 2016). While this parasite appears to be a rare or at least not so common finding in strict or moderately strict carnivores such as cats, dogs and hyenas (Ruaux and Stang, 2014; Wang *et al*., 2014; Heitlinger *et al*., 2017; Cociancic *et al*., 2018; Moura *et al*., 2018; Udonsom *et al*., 2018), it may be more common in omni- and herbivores, including pigs, cows and sheep (Pakandl, 1991; Navarro *et al*., 2008; Ramirez *et al*., 2014; Masuda *et al*., 2018; Moura *et al*., 2018; Udonsom *et al*., 2018). Nine distinct ribosomal lineages, the so-called 'subtypes', have been isolated from humans, with subtypes 1–4 predominating. Some subtypes even exhibit extensive within-subtype diversity that to some degree is host-specific; e.g. ST3 (Alfellani *et al*., 2013*a*). Colonization is common in older children and adults than in infants and young children (El Safadi *et al*., 2014; Scanlan *et al*., 2014; Poulsen *et al*., 2016; Salehi *et al*., 2017; Scanlan *et al*., 2018), with prevalence rates reaching 100% in developing countries (El Safadi *et al*., 2014). Moreover, *Blastocystis* may colonize the human gut for several years (Scanlan *et al*., 2014).

Dientamoeba fragilis

DNA-based methods helped overcome the diagnostic challenges related to the detection of *Dientamoeba fragilis*, a non-flagellated flagellate for which a cyst stage was reported only very recently (Munasinghe *et al*., 2013; Stark *et al*., 2014). *Dientamoeba fragilis* is the only known species in the genus. The first DNA-based detection methods for *D. fragilis* appeared in the mid-00s (Peek *et al*., 2004; Stark *et al*., 2005*a*, 2005*b*, 2006; Verweij *et al*., 2007). Since then, such methods have helped us to realize that this parasite is very common in some populations, especially in Northern Europe (Röser *et al*., 2013; de Jong *et al*., 2014; Ögren *et al*., 2015; Holtman *et al*., 2017; Jokelainen *et al*., 2017). In Denmark, *D. fragilis* is almost an obligate finding in children (Röser *et al*., 2013; Jokelainen *et al*., 2017). In other regions where methods of high sensitivity are also used, such as Australia, the parasite appears to be a lot less common (Stark *et al*., 2016); however, studies involving screening of asymptomatic individuals for *D. fragilis* are very rare, and so the prevalence of the parasite in individuals without symptoms in most parts of the world remains largely unknown. Apart from humans, *D. fragilis* has been found in non-human primates and pigs (Stark *et al*., 2008; Cacciò *et al*., 2012). The diversity within the species appears very limited, and most cases of *D. fragilis* colonization are attributable to one of only two acknowledged genotypes (Genotype 1), no matter where sampling is performed (Stark *et al*., 2005*a*, 2005*b*; Stensvold *et al*., 2013; Cacciò *et al*., 2016; Greigert *et al*., 2018).

Entamoeba

A number of *Entamoeba* species can colonize the human intestine. Infections due to the potentially highly pathogenic *Entamoeba histolytica* are relatively rare compared with colonization by *Entamoeba dispar*, *Entamoeba hartmanni*, and, especially, *Entamoeba coli*, which has been found to colonise between 20 and 30% of individuals in surveyed populations in Brazil (Aguiar *et al*., 2007; Neres-Norberg *et al*., 2014; Higa *et al*., 2017; Jeske *et al*., 2018). Substantial genetic variation has been detected within *E. coli*, with *E. coli* subtype 1 and subtype 2 differing by 13% (Stensvold *et al*., 2011*c*). Overall, the genetic diversity within octonucleated Entamoebas appears vast and still largely unaccounted for (Jacob *et al*., 2016; Elsheikha *et al*., 2018). *Entamoeba polecki* rarely infects humans; nevertheless, four subtypes have been detected with quite varying geographical distribution and host reservoirs (Stensvold *et al*., 2011*c*, 2018); all four subtypes have been found in humans (Verweij *et al*., 2001; Stensvold *et al*., 2018).

It is currently unclear to which extent non-histolytica Entamoebas contribute to the development of intestinal symptoms.

Some other protists show up in stool every now and then (often accompanied by other CLIPPs) and these include parasites belonging to ciliates and the Amoebozoa. Although the amount of documentation is scarce, it is clear that for some of these parasites, especially *Iodamoeba* and *Endolimax*, the intra-generic diversity is vast, with a maximum genetic divergence of at least 31% (Stensvold *et al*., 2012; Constenla *et al*., 2014; Poulsen and Stensvold, 2016). *Endolimax nana* was recently shown to colonize 28.8% of 3245 individuals attending the Evandro Chagas National Institute of Infectious Diseases, Rio de Janeiro, Brazil (Faria *et al*., 2017). To date, no complete, annotated nuclear genome sequences have been published for CLIPPs other than *Blastocystis* and *Entamoeba dispar*.

The extensive genetic diversity documented so far within these CLIPPs has informed the taxonomic terminology, and so, depending on the availability of morphology data and genetic diversity and SSU rDNA sequence coverage, sequences are annotated to species, subtypes, ribosomal lineage, or conditional lineage (Jacob *et al*., 2016). Importantly, it appears that specific taxonomic terminologies are being developed for individual genera; these are based first and foremost on a pragmatic basis.

CLIPPs in a gut ecology setting

In addition to exploring parasite diversity in the gut, it could be important to try and look to gut microbial and ecological relationships in non-human hosts and in the environment, respectively, to better understand the role of CLIPPs in human health and disease. In the field of ecology, protists have been identified as important components of terrestrial and aquatic environments where they are integral constituents of trophic chains and nutrient cycles (Bates *et al*., 2013; Maritz *et al*., 2017). In geothermal springs, protist diversity appears to rely on pH and temperature (Oliverio *et al*., 2018). The introduction of *Acanthamoeba* into the rhizosphere of *Arabidopsis thaliana* leads to rapid changes in associated bacterial communities due to the grazing of the amoeba (Rosenberg *et al*., 2009). Gut flagellates and ciliates assist termites and ruminants in metabolizing/fermenting carbohydrates (Veira, 1986; Ohkuma, 2008; Moon-van der Staay *et al*., 2014); examples are endless. The presence of protists in various

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niches therefore appears to be driven by a variety of host- and environment-derived factors and may in turn have a number of vital or less vital consequences for the associated microbiome, be it the host-associated gut microbiome, plant rhizosphere or terrestrial and aquatic biomes. This understanding has to a large extent failed to resonate with professionals in clinical microbiology and related medical fields, where CLIPPs are generally seen as 'intruders' and (potential) pathogens, despite the fact that most of these are most probably non-invasive and may have unknown functions of potential benefit (Parfrey *et al.*, 2011; Lukeš *et al.*, 2015; Andersen and Stensvold, 2016).

Nevertheless, the concept of certain gut parasitic protists as 'ecosystem engineers' also in humans is sinking in, and studies on trans-kingdom relationships are emerging. For instance, Laforest-Lapointe and Arriet (2018) recently proposed a model for the ecological role of *Blastocystis* in the human gut microbiota. They suggested that *Blastocystis* by predation on abundant bacterial taxa lowers the competition for nutrients and space, leading to an increase in bacterial richness and community evenness. And indeed, carriers of *Blastocysts* and other CLIPPs have been shown to have gut bacterial microbiomes that differ significantly from those who do not carry these parasites in several recent studies, the findings of which were recently summarized by Stensvold and van der Giezen (2018). In fact, higher diversity and higher richness are typically observed in CLIPPs-positive individuals than in those who are negative. What is more is the fact that observations from a recent meta-analysis of metagenomics data indicated that *Blastocystis* carriage is linked to low body mass index (Andersen *et al.*, 2015; Beghini *et al.*, 2017), which again lends support to specific links to gut bacterial diversity. However, it remains to be identified, to which extent *Blastocystis* is actively driving this difference as proposed by Laforest-Lapointe and Arrieta, or whether *Blastocystis* is merely an indicator or specific bacterial community patterns. Stensvold and van der Giezen (2018) recently hypothesized that the increased intestinal oxygen concentrations observed during gut dysbiosis may prevent *Blastocystis* from establishing in the gut, which would suggest a role for *Blastocystis* as an indicator organism.

Experimental models, such as that recently proposed by Pomajbikova and colleagues (Růžková *et al.*, 2018), could be used to develop longitudinal studies on bacterial community changes after the establishment of *Blastocystis* colonization. *Blastocystis* is one the few parasites that is readily established in culture (Clark and Stensvold, 2016), and cysts induced in cultures or obtained from donor material, isolated from stool by gradient centrifugation, can be used for inoculation in order not to co-introduce bacteria that would lead to experimental bias (Rene *et al.*, 2009). Here, the use of both eubiotic and dysbiotic animals could be used to study potential differences in colonization success rate.

The fact that some hosts (e.g. cats and dogs) are not so prone to harbouring a parasite such like *Blastocystis* while others (e.g. humans and artiodactyls) in the same habitat are much more likely hosts, should also be explored in detail, to identify whether this boils down to diet, behaviour (exposure), and/or other factors. If all the subtypes of the parasite are globally pervasive and the overall colonization pressure of *Blastocystis* strong, differences in intestinal colonization between hosts may rely – at least in part – on differences in gut microbiota composition.

It is intriguing that not only *Blastocystis*, but also other CLIPPs have been shown to be linked to specific microbiota patterns (Stensvold and van der Giezen, 2018). Studying gut microbiomes of rural Africans, Morton *et al.* (2015) could predict the presence/absence of *Entamoeba* by 79% accuracy, based on the composition of any individual's gut microbiota. To this end, Xiong *et al.* (2018) identified that shrimp health status could be predicted with 92.4% accuracy based on eukaryotic taxon profiling.

Nucleated life within the human intestine also include fungi. Common genera found in stool include *Candida*, *Saccharomyces*, *Malassezia*, *Pichia* and *Aspergillus* (Laforest-Lapointe and Arrieta, 2018); however, our understanding of the extent to which these genera in fact colonize the human intestinal tract or merely reflect dietary components is incomplete, and recent evidence appears to suggest that fungal colonization of the intestinal tract of healthy individuals is minimal (Auchtung *et al.*, 2018).

The faecal eukaryome – mapping of eukaryotic diversity in vertebrate stool

As observed by e.g. Hamad *et al.* (2018), differences in observed microbiome profiles may reflect differences in DNA extraction protocols, DNA amplification and sequencing technologies, plus queried databases (SILVA, Greengenes, RDP, NCBI, self-curated databases, etc.). So far, mapping of eukaryotic diversity in human and non-human stool samples has used mainly one of two approaches: Shotgun sequencing or amplicon-based sequencing of genomic DNA extracted from stool (Cristescu, 2014). The applicability of shotgun sequencing in terms of detecting and differentiating CLIPPs is hampered by the fact that relatively few CLIPPs genomes are available for reference. Amplicon-based sequencing has typically used nuclear small subunit ribosomal DNA (18S) as the target. However, some variation in amplicon-based approaches is seen, mostly in terms of the choice of target(s) and DNA sequence data processing. The most informative regions of the 18S appear to be the V3, V4, V5 and the V9 regions (Maritz *et al.*, 2017; Krogsgaard *et al.*, 2018). As an example, Krogsgaard and colleagues used three different primer sets for eukaryotic DNA (G3F1/G3R1 and G6F1/G6R1 targeting the V3–V4 region of the 18S rRNA gene and G4F1/G4R1 targeting the V3–V5 region) and one set of primers for prokaryotic DNA [341F/806R (Yu *et al.*, 2005)] (Krogsgaard *et al.*, 2018). Sequences were mapped using BION (http://box.com/bion), a newly developed k-mer-based analytical semi-commercial open-source package which allows annotation to species level. Prokaryotic DNA sequences were mapped against the RDP 11.04 reference database, while eukaryotic DNA sequences were mapped using SILVA version 123 reference database with an improved in-house seven-tier taxonomy for eukaryotes, similar to the tiers defined for prokaryotes (phylum, class, order, family, genus, species and sequence levels).

Published data on differences in the eukaryome across vertebrate populations and links between bactieral and eukaryotic signatures are still scarce.

Krogsgaard *et al.* (2015) found that CLIPPs diversity was higher in healthy individuals compared with patients with irritable bowel syndrome and also observed that individuals colonized by CLIPPs typically had a higher bacterial richness and diversity than those without (Krogsgaard *et al.*, 2018).

Heitlinger *et al.* (2017) used 4 16S and 44 18S primers in a Fluidigm-based approach, followed by taxonomic analysis using dada2 to map eukaryotic diversity in spotted hyenas. While no differences were found in eukaryome richness, diversity, evenness or genus abundance across age groups in a population of spotted hyenas, a more diverse eukaryome was identified in high-ranking than in low-ranking animals (Heitlinger *et al.*, 2017).

Maritz *et al.* (2017) recently developed and evaluated an 18S rRNA assay employing ILLUMINA-based sequencing and annotation of sequence data using locally curated as well as QIIME formatted SILVA databases with a view to detecting and differentiating protists in sewage with special emphasis on trichomonads. The team used vertebrate blocking primers to increase

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protist data yield (Maritz *et al.*, 2017). Choice of primers is critical too as evidenced by the differing outcomes in terms of e.g. Amoebozoan data obtained by Moreno *et al.*, 2010 and Matsunaga *et al.* (2014), who both aimed at mapping eukaryotic diversity in wastewater/sludge.

The extent to which primate gut eukaryotic diversity is only rudimentarily reflected in reference databases can be exemplified by the following: In a metabarcoding study of non-human primate gut eukaryomes, only 0.01% of all SSU rDNA reads matched sequences in the Silva 123 database at a 100% threshold (Wilcox and Hollocher, 2018). In that study, *de novo* operational taxonomic unit (OTU) assignment revealed 4293 eukaryotic OTUs at a 97%-identity level, and reference-based taxonomy assignment matched sequences to 2021 unique eukaryotic genera. Investigating the sewage eukaryome of sludge digesters in Japan, Matsubayashi *et al.* (2017) found that 85% of the clones obtained by 18S rRNA gene clone library construction showed less than 97.0% sequence identity to what they termed as 'described eukaryotes', indicating most of the eukaryotes in anaerobic sludge digesters are largely unknown.

Advancing the mapping of intestinal eukaryotic diversity: Wastewater and new sequencing technologies–the way forward?

In summary, the characterization of nuclear small subunit (SSU) ribosomal RNA genes has been the backbone of DNA-mapping the tree of life. In the field of clinical microbiology, taxon-specific genetic variation across nuclear SSU ribosomal RNA genes has been instrumental to the development of a vast variety of targeted DNA-based diagnostic methods over the past few decades (Verweij and Stensvold, 2014); however, the development and use of such diagnostics are limited by the DNA sequence data available in NCBI (Stensvold *et al.*, 2011*b*).

The SSU rRNA gene has proved useful for the detection and differentiation of several species of parasites. For helminths, however, this gene generally appears very conserved, and mitochondrial genes or ITS data are taxonomically more informative. Likewise, ITS data appear more relevant for differentiating between non-parasitic eukaryotic organisms often found in the gut, such as yeasts and molds, and so the genes providing most taxonomic resolution differ and depend on the type of organism.

The presence of large intra-generic diversity in some parasites has spurred hypotheses on differences in pathogenicity being associated with species/subtype/genotype, and so our ability to detect and differentiate not only genera and species but also subtypes, ribosomal lineages, etc., is important. Again, while the 18S has proved particularly useful in differentiating between *Blastocystis* subtypes and even subtype alleles (Stensvold *et al.*, 2011*a*), this marker provides very little resolution within the species of for instance *D. fragilis*. For other parasites, such as a couple of genera belonging to the Amoebozoa, namely *Entamoeba*, *Endolimax* and *Iodamoeba*, we are only beginning to appreciate the vast extent of genetic diversity (Silberman *et al.*, 1999; Clark, 2000; Verweij *et al.*, 2001; Stensvold *et al.*, 2010, 2011*c*; Royer *et al.*, 2012; Jacob *et al.*, 2016; Elsheikha *et al.*, 2018). The work and methodological limitations involved in mapping the intra-generic diversity in these organisms have led to issues related to resolving the phylogeny among this group of organisms and left some 'dark holes' in publicly available databases. Briefly, the largest limitations here are as follows: although hypervariable regions within 18S, ITS or 28S may prove useful for studies into eukaryotic diversity, robust analysis of phylogenetic relationships, including the very delineation of novel ribosomal lineages, and optimal yield of analysis of sequence data from metagenomics or other amplicon-based sequencing studies requires sequencing of complete, or near-complete ribosomal genes. When genomic DNA extracted directly from e.g. stool is used, the application of general primers with a view to amplifying near-complete ribosomal genes often results in preferential amplification of some organisms over other. As an example, individuals colonized by *Iodamoeba* and/or *Endolimax* are typically co-colonised with *Blastocystis*, and because the length of the SSU rRNA gene is only 1.8 kbp in *Blastocystis* while 2.5 kbp or more in *Iodamoeba* and *Endolimax*, *Blastocystis* ribosomal genes are more likely to be amplified from faecal genomic DNA due to the shorter DNA sequence. Another limitation is related to intra-cellular variation (hypervariable regions), which makes Sanger sequencing of polymerase chain reaction (PCR) products of some sequence stretches unsuitable, e.g. due to the presence of sequence variation within homo-polymers. TA cloning of PCR products has been tried with some success, but this is relatively expensive, time-consuming and laborious (Stensvold *et al.*, 2012). Even next-generation sequencing methods such as ILLUMINA do not provide much better solutions to overcoming this issue. Clearly, alternative ways to effectively obtain data are needed.

Meanwhile, Pacific Biosciences (PacBio) RS II, considered a third-generation sequencer, uses single-molecule real-time technology and can be used for sequencing of single DNA molecules in real-time without prior amplification steps, enabling direct observation of DNA synthesis by DNA polymerase (Nakano *et al.*, 2017). Importantly, this technology enables the production of long reads (typically >20 kbp with a maximum of 60 kbp) at relatively low costs (Nakano *et al.*, 2017). Orr and colleagues used culturing and targeted PacBio RS II amplicon sequencing to expand on data on the diversity within the class of Diphyllatea, a group of protists that may represent one of the earliest diverging eukaryotic lineages (Orr *et al.*, 2018). By obtaining near full-length 18S rRNA sequences in addition to mining publicly available databases, they were able to resolve the phylogeny within the class and better map the distribution of members of the class. The technology was also recently used for characterizing and quantifying protistan sequences from environmental samples (Jones and Kustka, 2017), and in terms of gut microbial diversity, one of the few studies using it so far is that by Myer *et al.* (2016) to generate data for phylogenetic analysis of rumen bacterial communities.

A limit to this technology is the relatively high rate of sequencing-related introduced errors; however, there are several ways to reduce or completely eliminate these errors using software tools and by decreasing the time the machine is used. Moreover, PacBio appears to be better at overcoming the issues related to the sequencing of hypervariable regions that e.g. ILLUMINA sequencing may have problems with. Critics of PacBio might argue that the use of this technology should rather be seen as an adjunctive, supportive and possibly exploratory tool that may provide a scaffold that could inform and guide more sophisticated and precise analyses. Such analyses could include Illumina-based sequencing of overlapping 300–400-bp amplicons using sequence-specific primers. Nevertheless, complete and accurate *de novo* assemblies of *Escherichia coli* strains could be accomplished using data generated solely from the PacBio RS II (Powers *et al.*, 2013). The team found that addition of other sequencing technology data obtained by Ion Torrent and MiSeq offered no improvements over the use of PacBio data alone (Powers *et al.*, 2013).

Apart from identifying the best possible technological and data processing pipelines, it is also worthwhile considering types of material for studying diversity. For instance, untreated sewage may be particularly useful in terms of detecting and mapping micro-eukaryotic diversity, since this material reflects pooled faecal samples from a large population of humans with some spillover of material from non-human sources.

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Chouari *et al.* (2017) investigated eukaryotic diversity in wastewater using 18S sequencing, and of 1519 analysed sequences, 160 operational taxonomic units were identified. No less than 56.9% of the phylotypes were assigned to novel phylogenetic molecular species, showing <97% sequence similarity with their nearest affiliated representative within public databases. Similarly, Matsunaga *et al.* (2014) observed that 60% of their 18S rRNA gene clones obtained from DNA extracted from municipal wastewater had <97% sequence identity to described eukaryotes. In both studies, data on *Blastocystis* and Amoebozoa were observed. These studies highlight not only the vast DNA data gap in the eukaryotic tree of life, but also the relevance of using sewage as study material for investigations into eukaryotic diversity.

In conclusion, DNA mapping of nucleated life within the intestine and exploring it in ecological contexts are critical to further our understanding of gut microbial diversity and its role in health and disease. Application of more detailed reference data will allow for subtle and robust trans-kingdom analyses of gut microbes and will moreover expand our knowledge on host specificity, transmission patterns and links to clinical phenotypes. The use of genomic DNA from the pooled stool, as e.g. represented by sewage and amplicon-based third-generation sequencing may be a way to ensure the acquisition of quick and robust data to uncover the missing branches of the gut microbial eukaryotic tree.

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