A user guide to environmental protistology: primers, metabarcoding, sequencing, and analyses

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Abstract

Protists – all eukaryotes besides fungi, animals, and plants - represent a major part of the taxonomic and functional diversity of eukaryotic life on the planet and drive many ecosystem processes. However, knowledge of protist communities and their diversity lags behind that of most other groups of organisms, largely due to methodological constraints. While protist communities differ markedly between habitats and biomes, they can be studied in very similar ways. Here we provide a guide to current molecular approaches used for studying protist diversity, with a particular focus on amplicon-

based high-throughput sequencing (metabarcoding). We highlight that the choice of suitable primers artificially alters community profiles observed in metabarcoding studies. While there are no true 'universal' primers to target all protist taxa as a whole, we identify some primer combinations with a wide taxonomic coverage and provide detailed information on their properties. Although environmental protistan ecological research will probably shift towards PCR-free metagenomics or/and transcriptomic approaches in a near future, metabarcoding will remain the method of choice for in-depth community analyses and taxon inventories in biodiversity surveys and ecological studies, due its great cost-efficiency, sensitivity, and throughput. In this paper we provide a guide for scientists from a broad range of disciplines to implement protists in their ecological analyses.

Keywords: Microbial ecology, protists, high-throughput sequencing, environmental microbiology, primer bias, 18S rRNA gene, metabarcoding

Introduction

The past three decades have seen massive developments in our understanding of microbial diversity in most of the Earth ecosystems. Molecular approaches, particularly the advent of high-throughput sequencing (HTS), have enabled cultivation-independent diversity analyses of microorganisms. PCR-based amplicon HTS (here defined as metabarcoding) data for bacteria and archaea have rapidly accumulated throughout this period, revealing a vast and mostly unknown taxonomic diversity (Gans, Wolinsky, & Dunbar, 2005; Leininger et al., 2006; Louca, Mazel, Doebeli, & Parfrey, 2019; Parks et al., 2017; Sogin et al., 2006). While sequencing errors were shown to have artefactually inflated the inferred diversity in many early studies (Huse, Huber, Morrison, Sogin, & Welch, 2007), increased quality control and refined techniques have subsequently confirmed that the global diversity of prokaryotes likely exceeds a million bacterial species-level lineages (Louca et al., 2019; Parks et al., 2017; Thompson et al., 2017), with many previously unknown clades emerging. Similarly, fungal diversity has been studied extensively using HTS and taxon diversity has been estimated to exceed 100,000 (Buée et al., 2009; Tedersoo et al., 2014), if not an order of magnitude greater (Bass &

55 Richards, 2011; Hawksworth & Lucking, 2017). Protist metabarcoding is lagging behind that of
56 prokaryotes and fungi (Geisen et al., 2017), yet suggesting global species-level diversity estimates in
57 the millions (de Vargas et al., 2015; Mahé et al., 2017).

58 Protists are of key importance for ecosystem functioning and are major drivers in diverse nutrient
59 cycling pathways. Autotrophs (algae) are the counterparts of land plants as main carbon fixers in
60 aquatic environments (Worden et al., 2015). Heterotrophic protists catalyse nutrient cycling in aquatic
61 and terrestrial environments as selective consumers of bacteria and fungi, being critical drivers of the
62 so-called 'microbial loop' (Azam et al., 1983; Bonkowski, 2004). Parasitic protists influence community
63 dynamics of larger eukaryotic hosts, including plants and animals (S. Geisen et al., 2018; Mahé et al.,
64 2017; Worden et al., 2015). Through these abiotic and biotic processes, protists make nutrients
65 available to either smaller or larger members of food webs and enhance eco-system dynamics
66 (Bonkowski, 2004) and/or connectivity (Lima-Mendez et al., 2015). Nevertheless, the diversity of
67 protists is often excluded from microbiome and food-web studies (Lundberg et al., 2012; Mendes et
68 al., 2011). Therefore, we are missing information of an important biotic component that regulates
69 ecosystem functioning.

70 The protist biodiversity knowledge gap at least partly originates from methodological constraints.
71 Standardized PCR-based approaches with a relatively limited number of recommended primer pairs
72 are applied to study bacteria (J. Gregory Caporaso et al., 2010; J. G. Caporaso et al., 2011; Cole et al.,
73 2014; Knight et al., 2018; Walters et al., 2016) and fungi (Ihrmark et al., 2012; Lindahl Björn et al., 2013;
74 Nilsson et al., 2019). However, a consensus on comparable molecular approaches using a standardized
75 PCR-based approach to study protists does not exist. Several primer sets are regularly used (Adl et al.,
76 2019; Adl, Habura, & Eglit, 2014; Hadziavdic et al., 2014), although their benefits and shortcomings are
77 generally poorly known. Ideally, a PCR protocol would amplify all members of the target group with
78 equal likelihood, avoid excessive amplification of non-target lineages, and provide amplicons with high
79 taxonomic resolution. The target sequence should also be well represented in public databases, and

provide enough phylogenetic signal for eco-evolutionary analyses. However, a single such ideal PCR system cannot be developed. In reality, each primer set has some phylogenetic bias, and the use of many different sets therefore limits comparability across studies (Fouhy, Clooney, Stanton, Claesson, & Cotter, 2016; Ramirez et al., 2018; Stoeck et al., 2010). In fact, primer pairs have often been designed to answer specific questions in protist community profiling (see below), based on the experience or preference of individual researchers. A comparative synthesis of existing 'protist primers' for non-experts is still lacking. We therefore review some of the most frequently used primer pairs, evaluate their pros and cons, and provide suggestions for optimal primer choice in different environmental settings. We then introduce guidelines to consider in HTS-based analyses of protist biodiversity. Lastly, we provide an overview of future opportunities and challenges introduced by PCR free HTS approaches.

18S rRNA gene: the universal marker to assess protist biodiversity

The 16S (small subunit) ribosomal RNA gene (Woese, 1987) has been used as a genetic marker for prokaryotic (bacteria and archaea) diversity since the earliest days of amplicon-based diversity studies (Giovannoni & Cary, 1993; Pace, 1997). Its eukaryotic counterpart (18S) was later adopted for studies of micro-eukaryotes (Lopez-Garcia, Rodriguez-Valera, Pedros-Alio, & Moreira, 2001; Moon-van der Staay, De Wachter, & Vaulot, 2001; Moreira & López-García, 2002; van Hannen et al., 1999). The 16S/18S rRNA gene encodes the RNA molecule that forms part of the small subunit of the ribosome. The ribosome is a macro-molecule essential to all organisms to translate mRNA into proteins, its function thus being conserved across the tree of life. While other markers are sometimes preferable for finer-resolution, often group-specific, biodiversity assessment (Fig. 2 in Jan Pawlowski et al. (2012)), the 18S rRNA is the best suited gene for exploring the diversity of protists as a whole. The 18S rRNA gene contains nine relatively variable regions flanked by more conserved regions that together span a range of evolutionary rates, enabling phylogenetic comparisons of both distant and closely related taxa (Neefs, Van de Peer, De Rijk, Chapelle, & De Wachter, 1993). Primers can be designed in more or less

conserved regions to target a wide diversity of taxa. However, there are important considerations to be made when choosing or designing primer sets to amplify 18S rRNA gene regions. Despite being most comprehensive, NCBI's GenBank, is not recommendable to classify 18S rRNA gene sequences due to large amount of errors that include wrongly annotated sequences. At least two general databases are dedicated to the 18S rRNA gene, Silva (Pruesse et al., 2007) and PR^2 (Guillou et al., 2013), while more specialized databases have been designed for specific taxonomic groups, e.g. foraminifera (Morard et al., 2015) or dinoflagellates (Mordret et al., 2018).

A comparative overview of 18S rRNA gene primers with large taxonomic spectrum

HTS protist metabarcoding studies have employed dozens of different primers in various combinations, targeting a range of variable 18S rRNA gene regions. Among the most widely targeted regions in early protist-focused HTS metabarcoding studies was the V1-3 region (Euringer & Lueders, 2008), a region with relatively high taxonomic resolution (Hadziavdic et al., 2014; Tanabe Akifumi et al., 2015). However, this region is decreasingly being used to target protists due to insufficient reference sequence coverage of the 5' end of the 18S rRNA gene, its excessive length for most current HTS methods, and unsuitable regions for primer annealing (Hugerth et al., 2014). Instead, the V4 and V9 regions are most commonly used nowadays; they are the focus of this review, as summarised in Table 1.

The V9 region was the most frequently used metabarcoding target in early high throughput studies as its relatively short amplicon length (< 200 bp) was better suited to the sequence length limitations of early HTS technologies. This short length makes the V9 region a suitable target also for contemporary ultra-deep metabarcoding technologies, making it a most cost-effective approach to study protist community diversity, while allowing stringent quality control. The downsides of the V9 region is

associated with its short length (Fig. 1), providing limited phylogenetic information. As such, the 400-500 bp V4 region fragment is increasingly used (Table 1). Furthermore, the V4 region is closest of all HTS-suitable 18S rRNA gene regions to that of the full-length gene and allows the high phylogenetic/taxonomic resolution often to species- or genus-level (Dunthorn, Klier, Bunge, & Stoeck, 2012; Hu Sarah et al., 2015).

The V4 region also enables the most accurate taxonomic placement of unassigned HTS amplicon sequences (Mahé et al., 2017). For these reasons it has been suggested as the main universal protist barcode region (Jan Pawlowski et al., 2012). However, some disadvantages are associated with the V4 region such as its length, which is too long for some short-read optimized sequencing methodologies such as Illumina HiSeq and NovaSeq (Fig. 1, Fig. 2). V4-based metabarcoding surveys also suffer from PCR/sequencing biases due to amplicon length variability (Fig. 1) and variable secondary structures.

Challenges of 18S rRNA gene targeted diversity analyses

The 18S rRNA gene is more complex than the prokaryotic 16S rRNA gene. It is on average three hundred base pairs longer (appr. 1,800 bp) and displays profound evolutionary rate variation between different taxa. This renders the design of truly universal primers impossible (Adl et al., 2014; Hadziavdic et al., 2014). Primers described as ‘universal’ are better thought of as ‘broadly-targeted’. Furthermore, even the most broadly-targeted primer pair will not amplify all protist lineages equally well, such as higher taxonomic ranks (e.g. eukaryotic supergroup; Fig. 3), but differential amplification exists also at lower taxonomic ranks such as at the class level (Fig. 4). Some highly divergent lineages will not amplify at all, as their 18S rRNA gene sequences can differ in multiple nucleotide positions even in the most conserved primer sites. This is the case for many parasitic protists (Bass, Stentiford, Littlewood, & Hartikainen, 2015; Hartikainen et al., 2014), but also diverse free-living lineages (Bass et al., 2016; Bass,

153 Yabuki, Santini, Romac, & Berney, 2012; Fiore-Donno, Weinert, Wubet, & Bonkowski, 2016); at high
154 taxonomic rank this is most obvious for Discoba (within Excavata; Fig. 2). For those groups, alternative
155 approaches are needed to study their communities, such as applying group-specific primers. Even
156 when primers are a perfect sequence match to the template, differences in length and secondary or
157 tertiary structures of the amplified region, due to insertion-deletions (indels) or the presence of
158 introns, which leads to biases, as shorter and structurally simpler fragments are preferentially
159 amplified over longer ones (Figs. 2 and 3). This can influence the sequence representation of protists
160 at all levels, even at supergroups. For instance, Stramenopiles and Alveolates have relatively short V4
161 amplicon lengths leading to preferential PCR-amplification, while the longer V4 regions of Discoba
162 (including Excavata) and Amoebozoa (Figs. 2 and 3) amplify less efficiently (Geisen, Laros, Vizcaíno,
163 Bonkowski, & de Groot, 2015).

164 The ribosomal gene operon is present in multiple copies, and copy numbers vary greatly between
165 taxonomic groups by up to three orders of magnitude (Gong, Dong, Liu, & Massana, 2013; Zhu,
166 Massana, Not, Marie, & Vaulot, 2005). There is evidence in some protist groups that the number of
167 18S rRNA gene reads (suggesting gene copies) positively correlates with relative cell abundance (Giner
168 et al., 2016), biomass (Pitsch et al., 2019) or biovolume (de Vargas et al., 2015), but these correlations
169 do not hold across divergent protist groups (Pitsch et al., 2019). Variable copy numbers between
170 lineages can profoundly alter interpretation of relative abundance or biomass of taxa within protist
171 communities in metabarcoding studies. Inferred lineage diversity can also be biased by intragenomic
172 variation between rRNA gene copies. The degree of intragenomic variation of rRNA gene copies is
173 generally low (0-1%), although in some groups it can reach several percent in some ciliates (Gong et
174 al., 2013), Myxomycetes (García-Martín, Zamora, & Lado, 2019), and Foraminifera (Weber &
175 Pawlowski, 2014). Both of these factors can vary greatly between closely related taxa (Andre et al.,
176 2014; García-Martín et al., 2019; Gong et al., 2013; Nassonova, Smirnov, Fahrni, & Pawlowski, 2010;
177 Weber & Pawlowski, 2014). This can artificially increase diversity estimates as single taxa can then be

178 treated as different species or, if intraspecific sequence diversity is high, as different taxonomic groups
179 (Caron & Hu, 2019).

180 The phylogenetic resolution a barcoding region provides is key for HTS studies. However, because
181 taxonomic marker gene evolution occurs at different rates relative to phenotypic evolution across
182 protist groups, there is no fixed or consensus relationship between marker genes dissimilarity and
183 taxonomy (Boenigk, Ereshefsky, Hoef-Emden, Mallet, & Bass, 2012). Therefore, fixed percentage
184 differences between lineages cannot be used to infer taxonomic distances between groups, and often
185 within them. Different solutions to this can be approached for individual groups, such as targeting a
186 combination of diverse variable regions or using barcoding regions other than the 18S rRNA gene. This,
187 however, is often not feasible in larger-scaled ecological studies due to cost-limitations, and a lower
188 resolution has to be accepted. Another approach is the use of selective targeted bioinformatics
189 analysis for specific groups of interest, for example mapping short reads onto robust phylogenetic
190 trees constructed with longer sequences.

191 Lastly, a metabarcoding approach targeting protists will depict a community of protists that differs
192 from the protist community present *in situ* or *in vivo*, because of the above-mentioned taxon-specific
193 PCR biases and copy number differences. As such, the primer pairs chosen alter the inferred
194 composition of protist communities in a primer-pair specific manner, which is illustrated in studies
195 applying primers targeting both the V4 and V9 regions (Pagenkopp Lohan, Fleischer, Carney, Holzer, &
196 Ruiz, 2016; Stoeck et al., 2010; Tragin, Zingone, & Vaulot, 2018). For example, V9 datasets generally
197 contain relatively more sequences that can only be assigned at the domain level (eukaryotes)
198 compared to V4 assignments (Pagenkopp Lohan et al., 2016).

199 Group-specific primer sets

200 Lineages that do not amplify well with broadly-targeted primers or when non-target sequences are
201 expected to dominate the results can be targeted in metabarcoding approaches with group-specific
202 primers. These target a range of different 18S rRNA gene regions (Supplementary Table 1). For

instance, Cercozoa have specifically been targeted in soils to avoid amplification of fungi (Fiore-Donno Anna et al., 2017; Harder et al., 2016; Lentendu et al., 2014). However even within Cercozoa, amplification of some clades requires even more specific primers, such as the divergent coprophilic *Helkesimastix-Guttulinopsis-Rosculus* clade (Bass et al., 2016). Supplementary Table 1 provides a summary of group-specific primer sets that have so far been used in 18S V4 region rRNA gene protistan metabarcoding efforts. Note that this table only includes primers that have been used and proven successful in HTS metabarcoding efforts. Many other primer sets have been proposed (Adl et al., 2019; Adl et al., 2014) that might be applicable but have not yet been tested in metabarcoding approaches.

Genes other than the 18S rRNA can be targeted to increase the taxonomic resolution of specific protist lineages(Jan Pawlowski et al., 2012). For instance, ITS sequences have been used to assess the distribution of uncultivated heterotrophic marine protists (Rodríguez-Martínez, Rocap, Logares, Romac, & Massana, 2011), often providing species-level resolution(Stern et al., 2012). The first subunit of the mitochondrial cytochrome oxidase as the universal barcoding region in animals (Hebert, Ratnasingham, & deWaard, 2003) also allows high-taxonomic discrimination of many protist taxa (Heger et al., 2011; Singer et al., 2018). The binding sites of these primers are so conserved that the same protocols as for animals have been used for several divergent Amoebozoa (Kosakyan et al., 2012; Nassonova et al., 2010). For specific groups of photosynthetic protists such as diatoms the *rbcL* gene can be used (Rimet et al., 2019).

An alternative to group-specific primers that target specific groups of protists is the use of primer combinations that amplify broadly but exclude certain groups of protists and other eukaryotes. As such, fungal sequences can be avoided in soils (Fiore-Donno et al., 2016; Geisen, 2016; Lentendu et al., 2014), and in holobionts for the exclusion of plant or metazoan host-associated sequences.

Assessing host-associated protist diversity

The highly parallel throughput of currently available sequencing techniques means that protist diversity can be effectively sampled and analysed even in samples dominated by non-protist taxa.

However, when targeting host-associated protists, co-amplification of host DNA can overwhelm the protist signal. In these cases, group-specific primer sets can be used to focus on a particular group. This strategy is typically used for high-resolution diversity analyses of fungi or Peronosporomycetes (formerly Oomycetes) inside plant roots (Ramirez et al., 2019; Sapkota & Nicolaisen, 2015). However, the disadvantage of this approach is that multiple groups that might be of ecological importance as symbionts cannot be simultaneously targeted. To fully assess the eukaryotic microbiome ('eukaryome' (Javier del Campo, Bass, & Keeling, 2019)) in animal hosts, broadly-targeted eukaryotic 18S rRNA gene V4 region primers that avoid amplification of most animal sequences are available (Bower et al., 2004; Javier del Campo et al., 2019). Alternatively, blocking primers can also be applied that reduce amplification of selected, non-target DNA and thereby increase the amount of target DNA (Tan & Liu, 2018; Vestheim & Jarman, 2008). To the best of our knowledge, plant-blocking primers have not yet been developed to enable amplification of protists within or on plant tissue.

Downstream sequence analyses

Downstream analyses of raw sequence reads are needed process the data and to ensure high data quality. The key steps are common to analyses of all metabarcoding datasets: merging paired-end sequence reads, and quality control steps such as removing low quality, short-read and chimeric sequences (J. Gregory Caporaso et al., 2010). For the subsequent clustering step of processed reads into sequence types representing taxonomic units, an increasingly wide choice of methods is available that significantly impacts measures of diversity and subsequent interpretation. This step aims to eliminate erroneous sequences created during PCR and sequencing, along with sequences originating from intragenomic and intraspecific polymorphisms (Cédric Berney, Fahrni, & Pawlowski, 2004; Richards & Bass, 2005) that can artificially inflate diversity estimates.

Operational Taxonomic Unit (OTU) clustering commonly groups sequences by a user-defined percentage similarity – typically 97 to 99% - using software such as mothur, qiime, usearch, vsearch, etc. (Edgar, 2010; Rognes, Flouri, Nichols, Quince, & Mahé, 2016), using more flexible high taxonomic

resolution approaches such as the clustering method SWARM (Mahe, Rognes, Quince, de Vargas, & Dunthorn, 2014, 2015) or the denoising method implemented in dada2 to produce amplicon sequence variants (ASVs). This last approach is now widely used for bacterial communities across the globe (Callahan, McMurdie, & Holmes, 2017; Delgado-Baquerizo et al., 2018; Thompson et al., 2017), and is increasingly used to analyse protist diversity (Chénard et al., 2019). It is possible that the application of ASVs might lead to an over-estimate of protistan diversity as intragenomic gene copy variants or and artefactual sequences may be interpreted as separate taxonomic units (Caron & Hu, 2019; Xiong et al., 2019).

Several methods exist for taxonomic annotation of OTUs/swarms/ASVs: assignment by the Uclust consensus taxonomy assigner**,** blast, GGSearch (Pearson, 2014), SortMeRNA, RTAX, Kraken or naïve Bayes classification (RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007)). Two main curated reference databases are available for annotation of 18S rRNA gene reads: SILVA (Pruesse et al., 2007) and PR^2 (Guillou et al., 2013) (https://github.com/pr2database/pr2database). SILVA incorporates all existing 18S rRNA sequences and relies on automatic annotation, while PR^2 focuses on the coherence of a taxonomy framework, for example using resources such as Algaebase (Guiry & Guiry, 2008) for photosynthetic protists, and on taxonomic re-annotations performed by supervised pipelines such as EukRef (Boscaro et al., 2018; Javier del Campo et al., 2018). Taxonomic annotation of the same dataset to different databases can result in discrepancies (Dupont, Griffiths, Bell, & Bass, 2016). A community based and expert driven, universal taxonomic framework for protist is underway (UniEuk (C. Berney et al., 2017)) and aims at providing a unified reference taxonomy for protists that will be implemented through the European Bioinformatics Institute (EBI).

Beyond currently applied metabarcoding approaches

Some new sequencing technologies can generate far longer reads than previously possible (Clarke et al., 2009). Amplicons of thousands of base pairs can be generated with platforms such as Pacific Biosciences and Oxford Nanopore. The potential of these long-read platforms to increase phylogenetic

inference and obtain species-level resolution has been shown for fungi and bacteria (Benítez-Páez, Portune, & Sanz, 2016; Tedersoo, Tooming-Klunderud, & Anslan, 2017), and recently for protists (Jamy et al., 2019). Direct RNA sequencing without a cDNA intermediate is also now possible and could allow untargeted protist and general microbiome community profiling (Graham et al., 2019; Marinov, 2017).

In comparison to sequencing platforms focusing on increased read lengths, other platforms (especially Illumina) focus on increased sequencing depth. These are particularly suitable for PCR-free "omics" strategies, including whole community profiling via metagenomics and metatranscriptomics (Carradec et al., 2018; Prosser, 2015). When PCR amplification to increase product yield is avoided, -omics methods circumvent PCR/global-amplification biases that distort diversity analyses and provide community profiles of all three domains of life plus viruses, as well as functional gene information (Flues, Bass, & Bonkowski, 2017; Karsenti et al., 2011; Pesant et al., 2015; Turner et al., 2013; Urich et al., 2008). The systematic collection of multi-omics data (metagenomic, metatranscriptomic, single-cell omics) in aquatic (Carradec et al., 2018; Cuvelier et al., 2010; Seeleuthner et al., 2018) and soil (Geisen, Tveit, et al., 2015; Jacquiod et al., 2016; Turner et al., 2013) systems are unveiling a more true picture of diversity and community composition of protists. A drawback of omics approaches is the high cost per sample since a much deeper sequencing of 10-100 million reads per sample is required. Coverage of any particular target group is also limited, as metagenomic datasets may be dominated by bacterial sequences or host sequences from animal/plant samples (Tedersoo et al., 2015; Urich et al., 2008). The key opportunity for protists afforded by -omics approaches, i.e. the elucidation of patterns of gene expression and interactions, is so far constrained by the deficiency of reference databases compared to those available for prokaryotes (Caron et al., 2017). As this information gap is increasingly filled, however, functional -omics information will help us to better understand ecosystem processes beyond taxonomy-based inferences of protist functions that are currently largely limited to nutrient uptake modes (Stefan Geisen et al., 2018). The first studies linking protist taxa to biological functions using -omics data have revealed the immense potential of these approaches (Hu et al., 2018; Ottesen et al., 2013). An alternative -omics approach that focuses more on taxonomic diversity

analyses and as such reducing sequencing costs compared with full metagenomic and metatranscriptomic analyses is mitochondrial enrichment (mitogenomics) (Andújar et al., 2015; Liu et al., 2016) – an approach yet to be implemented for studying any microbial group including protists.

Contemporary best practices for protist community analyses

Despite some drawbacks, metabarcoding of the 18S rRNA gene V4 and V9 region represents by far the most cost-efficient way to assess protist environmental diversity across large spatio-temporal scales (i.e. large number of samples). We emphasise that no true eukaryote-wide universal primer pair without biases can be designed for protists due to both the paraphyletic nature of protists and the extreme phylogenetic diversity they encompass. A cumulative overview and information of primer sets used to date that should give an overview for researchers interested in studying protists is shown in Table 1 that together with the other figures provide an overview of advantages and disadvantages. These show, for instance, that the by far most commonly used primer pair 8 (Stoeck et al., 2010) to date, has clear disadvantages and only matches about 67% of all protists (Table 1), especially prevalent in the most common soil protists: Amoebozoa and Rhizaria. Many other primer pairs that can be applied using the highest throughput Illumina sequencing approaches face similar biases and perform only slightly better (Table 1). Therefore, an unbiased comparison between environments and using data obtained so far is difficult and we cannot suggest an ideal primer pair for exhaustive protist community profiling across systems.

Ideally, long-read sequencing using PacBio or Nanopore sequencing is needed to cover most protistan diversity in a sample using primer pair 6 as it covers about 93% of all protists (Table 1). An alternative is accepting shorter read lengths and as such lower phylogenetic resolution using partial single-ended amplicon analyses (Pauvert et al., 2019) with primer pair 6. Pairing sequence reads reduces the possibilities of primers to select from as their amplicon sizes are longer than the most commonly used Illumina platforms can amplify. This means, a lower diversity of protists can be targeted using 2x300bp, 2x250bp or 2x150bp sequencing with the best primers amplifying 88% (primer pair 6), 75% (34) or 63%

(29) of protistan diversity, respectively. If whole microbiome analyses including protists, bacteria, archaea and fungi are envisioned PCR-free metagenomics and metatranscriptomics are suggested, but their costs currently still commonly are preventing large-scaled analyses. A more cost-efficient alternative to get a cumulative microbiome overview is using primer pairs such as 33 that amplify both prokaryotes and eukaryotes.

We summarize the primer selection for a given study system in a user-friendly diagram in Fig. 5. This overview together with additional information provided in this manuscript should help researchers, particularly ecologists, to choose a primer pair that best suits the research question, system studied and sequence platform available. This should facilitate analyses of protistan diversity within mainstream ecological and microbiome studies, and also applied research such as biomonitoring and bioindication (Stefan Geisen et al., 2018; J. Pawlowski, Lejzerowicz, Apotheloz-Perret-Gentil, Visco, & Esling, 2016) . Following this guide, protist community analyses will also become more comparable between studies leading to a greater capacity for, and more meaningful, comparisons of protist communities across eco-systems, similar to that possible for bacteria (Knight et al., 2018) and fungi (Nilsson et al., 2019). Our intention is that this guide develops over time, therefore we intend to regularly update the available metabarcoding primers to study protists (https://github.com/pr2database/pr2-primers). We ask the community for their active input in keeping this work up-to-date, in order to fully establish protist community profiling as a standard tool in future microbiome studies (https://github.com/pr2database/pr2-primers/issues).

Methodology

18S rRNA gene primer sets used in metabarcoding studies were collected from literature. Primer sequences and primer sets (knowing that several primer sets may share at least one primer) were stored in a custom MySQL database (full list available at https://github.com/pr2database/pr2-primers/wiki/18S-rRNA-primer-sets and https://github.com/pr2database/pr2-primers/wiki/18S-rRNA-primers). Primer sets targeting the V4 and V9 region of the 18S rRNA gene were selected to determine in silico amplification of sequences stored in version 4.12.0 of the PR^2 database (Guillou et al. 2013, https://github.com/pr2database/pr2database/releases/tag/v4.12.0). Sequences with ambiguities were discarded. For V4 and V9, sequences with length shorter than 1200 and 1650 bp, respectively, were not considered. Moreover, for V9, since many 18S rRNA do not cover the full V9 region, we only kept sequences that contains the canonical sequence GGATC[AT] which is located at the end of the V9 region, just before the start of the internal transcribed spacer 1. A R script was used to compute the % of sequences matching the forward, reverse and both primers using the Biostrings package function vmatchPattern() with the following parameters: max.mismatch=0, min.mismatch=0, with.indels=FALSE, fixed=FALSE, algorithm="auto". The data were tabulated using the dplyr package and plotted using the ggplot2 package. All scripts are available at https://github.com/pr2database/pr2-primers .

Acknowledgements

This study was funded by the Gordon and Betty Moore Foundation through grant GBMF5257 (UniEuk) and the International Society of Protistologists. SG was supported by an NWO-VENI grant (016.Veni.181.078) from the Netherlands Organisation for Scientific Research. This work was also supported by NERC grants NE/H009426/1 (DB, CB) and NE/H000887/1 (DB), and by funding from the UK Department of Environment, Food and Rural Affairs (Defra) under contract FC1214 (to DB). EL was supported by a grant “Atracción de Talento Investigador” by the Community of Madrid (2017-

T1/AMB-5210). CdV was supported by the French Government “Investissements d’Avenir” program OCEANOMICS (ANR-11-BTBR- 0008).

We thank Linda Amaral-Zettler, Sophie Arnaud-Haond, Sandra Baldauf, Sergio Balzano, Cedric Berney Jens Boenigk, Jane Carlton, Simon Creer, Didier Debroas, Micah Dunthorn, Javier del Campo, Isabelle Domaizon, Virginia Edgcomb, Bente Edwardsen, Noah Fierer, Laure Guillou, Laura Katz, Patrick Keeling, Rob Knight, Franck Lejzerowicz, Purificacion Lopez-Garcia, Connie Lovejoy, Ramon Massana, Sebastian Metz, Edward Mitchell, Angela Oliverio, Xavier Pochon, Chris Seppey, David Singer, Alexey Smirnov, Thorsten Stoeck, Alexandra Worden, Adriana Zingone for initial discussions on primer choices for eukaryotes.

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838

839 Data Accessibility

840

841 All data used in this work are included in this manuscript. All scripts used are available at

842 https://github.com/pr2database/pr2-primers. All data and primer information will

843 continuously be updated at https://github.com/pr2database/pr2-primers/issues).

844

845 Author Contribution

846 SG and DB designed the study; SG, DV, FM and EL drafted the tables with help from all authors; DV and

847 SG created the figures; SG and DB wrote the first draft of the manuscript, supplemented with

848 comments from all authors.

Tables

Table 1. Primer sets targeting the V4 and the V9 region that were used in metabarcoding high-throughput sequencing efforts to study protists; id: primer pair identifier; #: forward or reverse primer name; Fwd: forward primer; Rev: reverse primer; fwd and rev start/end: positions on *Saccharomyces cerevisiae* sequence FU970071; note: id33 also targets prokaryotes; id35 selects against metazoan sequences.

region	id	fwd name	fwd seq	fwd start	fwd end	rev name	rev seq	rev start	rev end	length	Reference
V4	1	F-566	CAGCAGCCGCGGTAATTCC	565	583	R-1200	CCCGTGTTGAGTCAAATTAAGC	1178	1199	635	(Hadziavdic et al., 2014)
V4	2	A-528F	GCGGTAATTCCAGCTCCAA	573	591	R-952	TTGGCAAATGCTTTCGC	935	951	379	(Hadziavdic et al., 2014)
V4	3	574*f	CGGTAAYTCCAGCTCYV	574	590	1132r	CCGTCAATTHCTTYAART	1132	1149	576	(Hugerth et al., 2014
V4	4	563f	GCCAGCAVCYGCGGTAAY	563	580	1132r	CCGTCAATTHCTTYAART	1132	1149	587	(Hugerth et al., 2014
V4	5	616f	TTAAAAVGYTCGTAGTYG	616	633	1132r	CCGTCAATTHCTTYAART	1132	1149	534	(Hugerth et al., 2014
V4	6	616*f	TTAAARVGYTCGTAGTYG	616	633	1132r	CCGTCAATTHCTTYAART	1132	1149	534	(Hugerth et al., 2014
V4	7	V4_1f	CCAGCASCYGCGGTAATWCC	564	583	TAReukREV3	ACTTTCGTTCTTGATYRA	963	980	417	(Bass et al., 2016)
V4	8	TAReuk454FWD1	CCAGCASCYGCGGTAATTCC	564	583	TAReukREV3	ACTTTCGTTCTTGATYRA	963	980	417	(Stoeck et al., 2010)
V4	13	3NDf	GGCAAGTCTGGTGCCAG	551	567	V4_euk_R1	GACTACGACGGTATCTRATCRTCTTCG	989	1015	465	(Bråte et al., 2010)
V4	14	3NDf	GGCAAGTCTGGTGCCAG	551	567	V4_euk_R2	ACGGTATCTRATCRTCTTCG	989	1008	458	(Bråte et al., 2010)
V4	15	EUKAF	GCCGCGGTAATTCCAGCTC	570	588	EUKAR	CYTTCGYYCTTGATTRA	963	979	410	(Moreno et al., 2018
V4	16	TAReuk454FWD1	CCAGCASCYGCGGTAATTCC	564	583	V4 18S Next.Rev	ACTTTCGTTCTTGATYRATGA	960	980	417	(Piredda et al., 2017
V4	17	E572F	CYGCGGTAATTCCAGCTC	571	588	E1009R	AYGGTATCTRATCRTCTTYG	989	1008	438	(Comeau, Li, Trembl Carmack, & Lovejoy, 2011)
V4	18	515F	GTGCCAGCMGCCGCGGTAA	561	579	1119r	GGTGCCCTTCCGTCA	1144	1158	598	(Parfrey et al., 2014)

V4	23	590F	CGGTAATTCCAGCTCCAATAGC	574	595	1300R	CACCAACTAAGAACGGCCATGC	1272	1293	720	(Venter, Nitsche, Domonell, Heger, & Arndt, 2017)
V4	24	EK-565F-NGS	GCAGTTAAAAAGCTCGTAGT	612	631	EUK1134-R	TTTAAGTTTCAGCCTTGCG	1120	1138	527	(Simon et al., 2015)
V4	25	NSF563	CGCGGTAATTCCAGCTCCA	572	590	NSR951	TTGGYRAATGCTTTCGC	935	951	380	(Mangot et al., 2013
V9	27	1391F	GTACACACCGCCCGTC	1628	1643	EukB	TGATCCTTCTGCAGGTTCACCTAC	1773	1796	169	(Stoeck et al., 2010)
V9	28	1380F	CCCTGCCHTTTGTACACAC	1617	1635	1510R	CCTTCYGCAGGTTCACCTAC	1773	1792	176	(Amaral-Zettler, McCliment, Ducklow Huse, 2009)
V9	29	1389F	TTGTACACACCGCCC	1626	1640	1510R	CCTTCYGCAGGTTCACCTAC	1773	1792	167	(Amaral-Zettler et al 2009)
V9	31	1388F	TTGTACACACCGCCCGTCGC	1626	1645	1510R	CCTTCYGCAGGTTCACCTAC	1773	1792	167	(Piredda et al., 2017
V4	33	515F Univ	GTGYCAGCMGCCGCGGTAA	561	579	926R	CCGYCAATTYMTTTRAGTTT	1130	1149	589	(Needham & Fuhrma 2016)
V4	34	515FY	GTGYCAGCMGCCGCGGTA	561	578	NSR951	TTGGYRAATGCTTTCGC	935	951	391	(Lambert et al., 2019
V4	36	TAReuk454FWD1	CCAGCASCYGCGGTAATTCC	564	583	V4RB	ACTTTCGTTCTTGATYRR	963	980	417	(Balzano et al., 2015
V4	40	Uni18SF	AGGGCAAKYCTGGTGCCAGC	549	568	Uni18SR	GRCGGTATCTRATCGYCTT	991	1009	461	(Zhan et al., 2013)

853

854

Figure legends

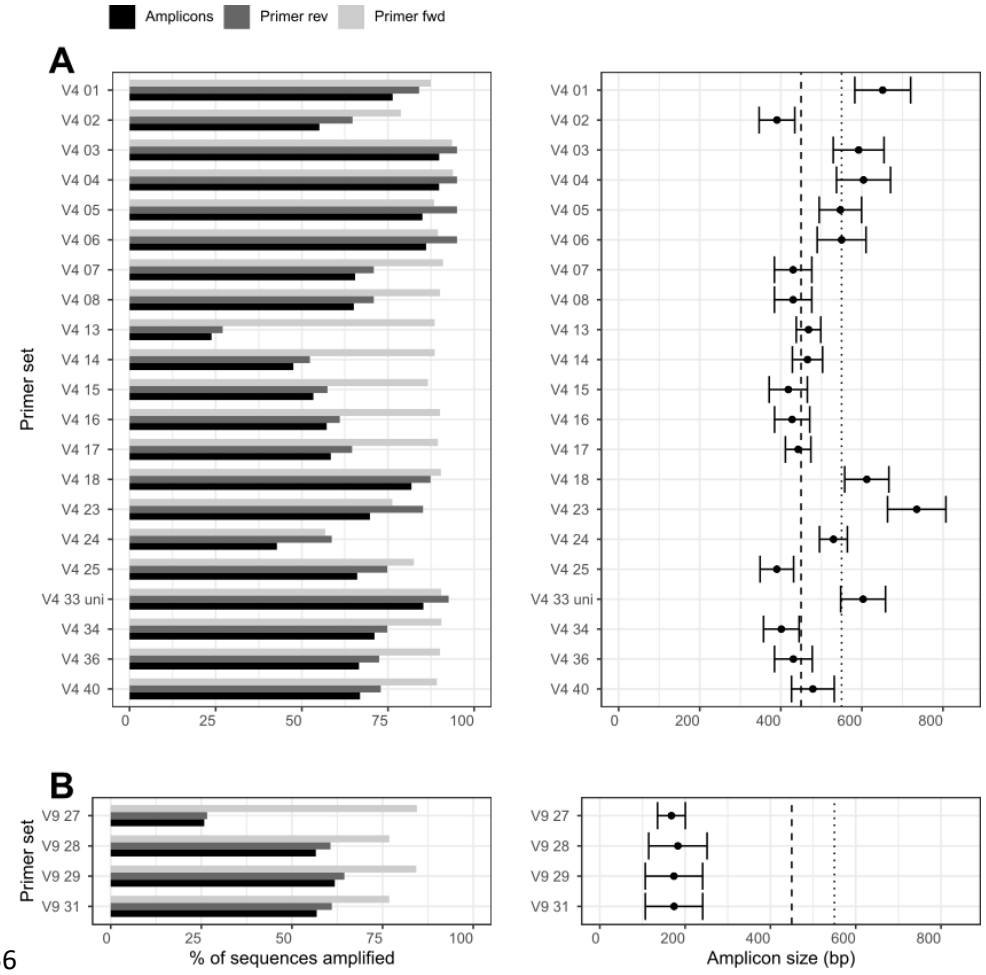
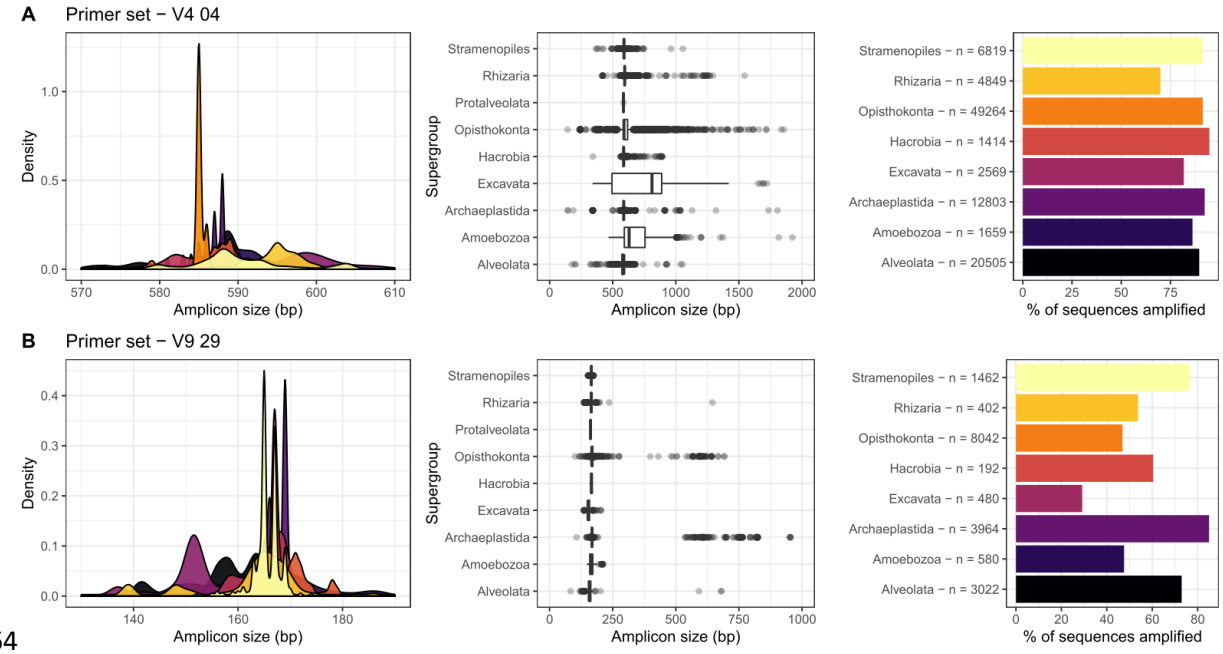
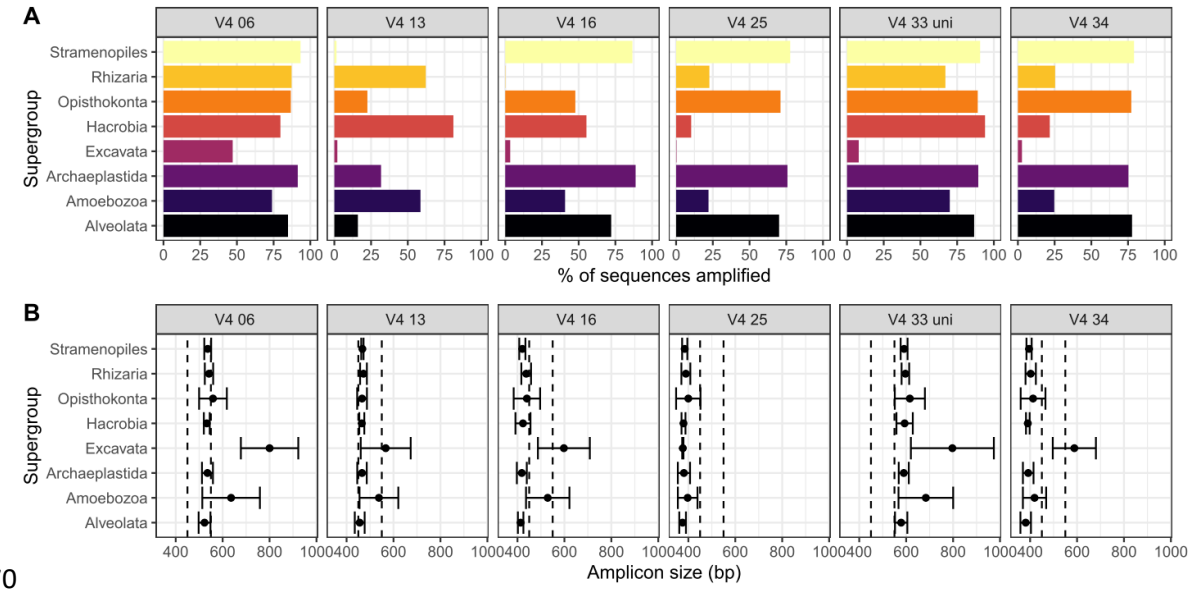


Fig. 1. Left panel. Percentage of reference sequences with a perfect match to both forward and reverse primers individually and the entire (A) V4 and (B) V9 18S rRNA gene region; right panels show amplicon sizes targeted by different primer pairs including lengths that can be covered by the most commonly used Illumina sequencers (dotted line: 2x300 base pairs; dashed line: 2x250 base pairs); error bars represent the standard deviation. PR^2 version 4.11.1 was used the reference 18S rRNA database. Note that this figure provides an overview of all currently used primer sets to target protists; details for each of the primers is given in Table 1.



864

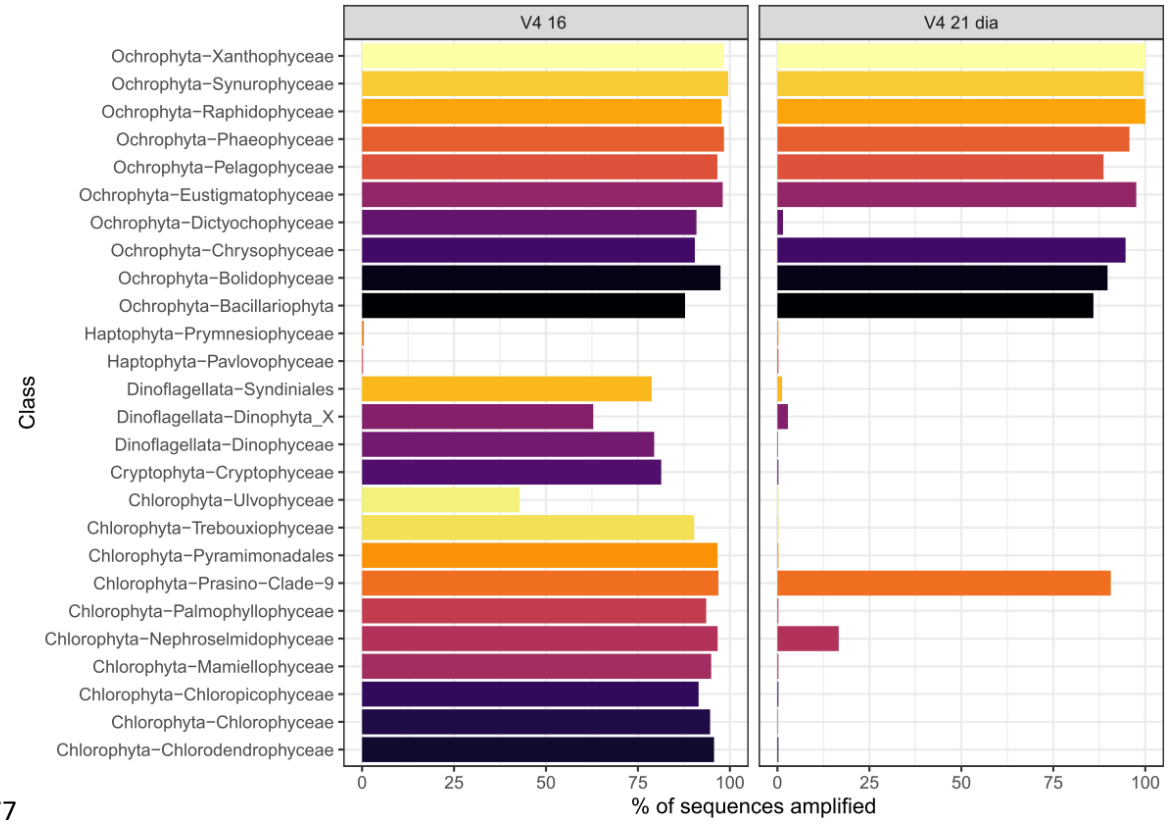
865Fig. 2. Amplicon lengths differences of higher taxonomic level protist lineages exemplified for each of
866a broadly targeted V4 (A) and V9 (B) primer set (Table 1). Lengths differences are prevalent between
867protist groups especially in the V4 region leading to differential amplification efficiency between the
868groups. Note that Hacrobia represents the sum of Haptophytes, Cryptophytes and Centrohelids. n
869represents the number of taxa for a given supergroup present in PR^2.



870

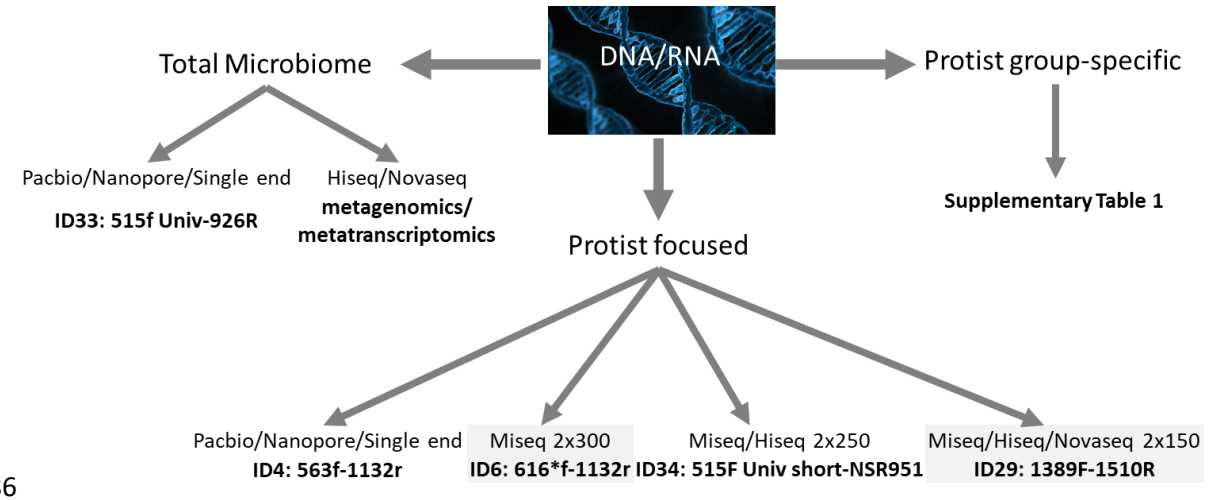
871Fig. 3. Coverage of some of the most broadly targeting primer pairs (Fig. 1, Table 1) as identified with
872perfect matches of both primer pairs to the target sequences for the main protist lineages. This shows
873that (A) primers do not equally amplify higher taxonomic level lineages of protists and (B) amplicon
874lengths differ between supergroups and depending on primer sets. Note that Hacrobia represents the
875sum of Haptophytes, Cryptophytes and Centrohelids. For details on all primer sets see Suppl. Fig. 1.

876



877

878Fig. 4. Coverage differences at higher taxonomic resolution (which corresponds, approximatively, to 879Class level) as identified with perfect matches of both forward and reverse primers illustrated for 880primer sets 16 and 21, showing that amplification success differences between protist groups (see Fig. 8812) can also be present at lower taxonomic levels. The "universal" primer set 16 does not amplify 882Haptophyta *in silico*, but in fact partially amplified them in natural communities *in vivo* likely because 883the mismatch is located in the middle of the primer (Piredda et al., 2017). Primer set 21 which is 884described as specific of diatoms (Zimmermann, Jahn, & Gemeinholzer, 2011) also amplifies other 885Ochrophyta as well as some green algae.



886

887Fig. 5. Decision-making chart to guide molecular approaches for protist community analyses. The ideal 888primer choice depends on the available sequencing platforms and the study question (protist- focused

889or microbiome- that target all prokaryotes and eukaryotes simultaneously). The longer the sequencing
890read, the broader theoretical coverage of protistan diversity. See Table 1 and main Figs. for detailed
891information on the respective primers.

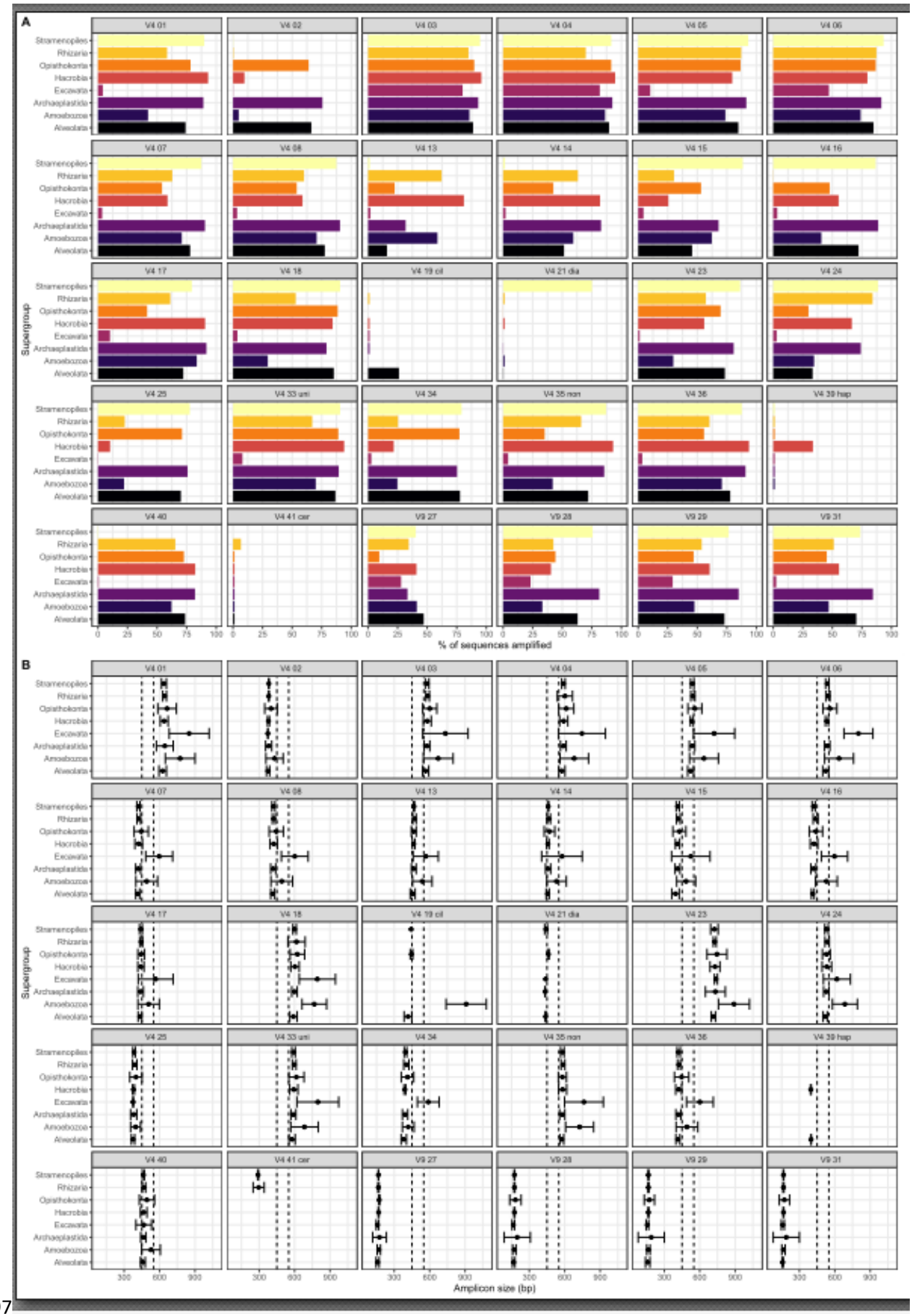
892

893Supporting Information

894Supplementary Table 1. List of protist group-specific primers so far used in high-throughput sequencing
895based metabarcoding studies

896

Supergroups	Targeted group	primer name	sense	primer sequence 5'-3'	Marker	region in the	average ampli	Sequencing technology	reference paper	Comments
Alveolata	Tintinnids and related	152+	forward	TTA CAT GGA TAA CCG TGG TAA TTC	18S rRNA	V2-V3	308 bp	MiSeq	Santoferrara, L., et al. (2018) Journal of Plankton Research, 40, 209–221	
		528-	reverse	CCC GGC CCG TTA TTT CTT GT						
	Symbiodinium spp	SYM_VAR_5.8S2	forward	GAATTGCAGAACTCCGTGAACC	ITS 2		250 bp	MiSeq	Hume, B.C.C., et al. (2018) Peerj, 6, 22.	
		SYM_VAR_REV	reverse	CGGGTTCWCTTGTYTGACTTCATGC						
	Dinophyta (=Dinoflagellates)	D1R	forward	ACCCGCTGAATTTAAGCATA	28S rRNA	D1-D2	365 bp	MiSeq	Smith, K.F. et al (2017) New Zealand Journal of Marine and Freshwater Research, 51, 555-576	Original forward primer from a former study, here applied for the first time to metabarcoding
		305R	reverse	TTTAAYTCTCTTTYCAAAGTCC						
Rhizaria	Cercozoa (general)	Cerc479F	forward	TGTTGCAGTTAAAAAGCTCGT	18S rRNA	V4	250-300 bp	Pyrosequencing	Harder, C.B., et al. (2016) ISME J, 10, 2488-97.	
		Cerc750R	reverse	TGAATACTAGCACCCCCAAC						
		Cer2F	forward	ATTTCTGCCCTATCAGCT	18S rRNA	V3-V4	600 bp	Pyrosequencing	Lentendu, G. et al. (2014) Molecular Ecology, 23, 3341–3355	
		Cer1R	reverse	ATACTAGCACCCCCAACT						
		S616F_Cerco	forward	TTAAAAAGCTCGTAGTTG	18S rRNA	V4	350 bp	Illumina MiSeq	Fiore-Donno, A.M. et al. (2018) Mol Ecol Resour, 18, 229-239.	Semi nested protocol: the first two primers (S616F_Cerco and S616F_Eocer) are to be mixed in a proportion 80% to 20% respectively, in combination with the third (S963R_Cerco). The obtained products are to be reamplified using the first two in combination with the fourth (S947R_Cerco)
		S616F_Eocer	forward	TTAAAAAGCGCGTAGTTG						
		S963R_Cerco	reverse	CAACTTTCGTTCTTGATTAAA						
		S947R_Cerco	reverse	AAGAAGACATCCTTGGTG						
	Plasmodiophorida	1301f	forward	GATTGAAGCTCTTTCTTGATCACTTC	18S rRNA	V7-V9	500 bp	Pyrosequencing	Bass, D., et al. (2018) Frontiers in Microbiology, 9	
		1801r	reverse	ACGGAAACCTTGTTACGACTTC						
	Foraminifera	s14F1	forward	AAGGGCACCACAAGAACGC	18S rRNA	Helix 37 and 37	150 bp	Illumina HiSeq	Pawlowski, J., et al., (2014) Molecular Ecology Methods 14, 1129-40	
		s15.3	reverse	CCTATCACATAATCATGAAAG						
Stramenopiles	Chrysophyceae/Synurophyceae	Chryso_240	forward	GGAAACCAATGCGGGGCAAC	18S rRNA	V2-V3	430 bp	Pyrosequencing	Lentendu, G., et al. (2014) Molecular Ecology, 23, 3341-55	Primers first developped for Sanger sequencing, here used for HTS. Semi-nested approach with a generic primer; specific primers are to be used in the second PCR
		Chryso_651	reverse	CTATTTTGCTCACAGTAAATGACGAG						
	Bacillariophyceae (diatoms)	Diat_rbcL_708F	forward	AGGTGAAGTTAAAGGTTCATACTTDAA	rbcL (plastidial)		312 bp	Ion Torrent	Chonova, T. et al. (2019) Frontiers in Microbiology, 10	Primers first designed for Sanger sequencing in a previous study
		R3	reverse	CCTTCTAATTTACCAACAACTG						
		D512for	forward	ATTCCAGCTCCAATAGCG	18S rRNA	V4	390-410 bp	Pyrosequencing	Zimmermann, J. et al. (2015) Molecular Ecology Resources, 15, 526-542.	Primer pair first developed for Sanger sequencing in a previous study
		D978rev	reverse	GACTACGATGGTATCTAATC						
	Peronosporomycetes (=oomycetes)	ITS1oo	forward	GGAAGGATCATTACCACA	ITS	ITS1 and ITS2	650 bp	Illumina MiSeq	Riit, T. et al., Mycokeys, 119-120.	To be used with generic primer ITS4
		ITS3oo	forward	AGTATGYYTGTATCAGTG		ITS2	350 bp			
Discoba	Kinetoplastea	Kineto_80	forward	CATCAGACGYAATCTGCCGC	18S rRNA	V2-V3	700 bp	Pyrosequencing	Lentendu, G., et al. (2014) Molecular Ecology, 23, 3341-55	Primers first developped for Sanger sequencing, here used for HTS. Semi-nested approach with a generic primer; specific primers are to be used in the second PCR
		Kineto_651	reverse	TTGGTCGCRCTTYTTTAGTCACAG						
Metamonadea	Diplomonadida (Fornicata)	DimA	forward	RGGGACRGGTGAAATAGGATG	18S rRNA	V4-V5	280 bp	HiSeq 2500	Cannon, M.V. et al. (2018) Microbiome, 6, 195	shorter 18S rRNA than most eukaryotes
		DimB	reverse	CAAATTGAGCCGCAGACTCC						
Amoebozoa	*Acanthamoeba*	SRAca28	forward	CCAATTACAAGACTCTTRTCGAG	18S rRNA	V2	470-510 bp	Pyrosequencing	Fiore-Donno, A.M. (2016) Scientific Reports, 6, 19068.	Semi-nested protocol: first PCR with general primer S1 (Fiore Donno et al., 2008) and SRAca28 second PCR with S1 and SFAca22
		SFAca22	forward	CGGYGAGACTGCGGATGG						
	Dark-spored Myxomycetes	SR19Dark	forward	GTCCTCTAATTGTTACTCGAD	18S rRNA	V2	550-590 bp	Pyrosequencing	Fiore-Donno, A.M. (2016) Scientific Reports, 6, 19068.	Semi-nested protocol: first PCR with general primer S1 (Fiore Donno et al., 2008) and SR19Dark second PCR with S1 and SF2Dark
		SF2Dark	forward	GTTGATCCTGCCAGTAGTGT						
Haptista	Haptophyta	Lhapto8	forward	CCATCTCATCCCTGCGTGTCTCCGAC	28S rRNA	D1-D2	350 bp	Pyrosequencing	Bittner, L., et al. (2013) Molecular Ecology 22, 87-101	
		Lhapto20R_bis	reverse	TCAGACTCCTTGGTCCGTGTTTCT						
		528Flong	forward	GCGGTAATTCCAGCTCCAA	18S rRNA	V4	450 bp	Pyrosequencing	Egge, E., et al. (2013) Plos one, 8, e74371	
		PRYM01+7	reverse	GATCAGTGAAAACATCCCTGG						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V4 01
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V4 19 cil
V4 21 dia
V4 23
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V4 25
V4 33 uni
V4 34
V4 35 non
V4 36
V4 39 hap
V4 40
V4 41 cer
V9 27
V9 28
V9 29
V9 31
Stramenopiles
Rhizaria
Opisthokonta
Hacrobia
Excavata
Archaeplastida
Amoebozoa
Alveolata
Supergroup
0
25
50
75
100
% of sequences amplified
B
300
600
900
Amplicon size (bp)

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898Suppl. Fig. 1. Coverage of all primer pairs used so far in high-throughput sequencing studies as
899identified with perfect matches of both primer pairs to the target sequences for the main protist
900lineages. This shows that (A) primers do not equally amplify higher taxonomic level lineages of protists
901and (B) amplicon lengths differ between supergroups and depending on primer sets. Note that
902Hacrobia represents the sum of Haptophytes, Cryptophytes and Centrohelids.

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