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## Ecological and evolutionary significance of novel protist lineages

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### Abstract

Environmental molecular surveys targeting protist diversity have unveiled novel and uncultured lineages in a variety of ecosystems, ranging from completely new high-rank lineages, to new taxa moderately related to previously described organisms. The ecological roles of some of these novel taxa have been studied, showing that in certain habitats they may be responsible for critical environmental processes. Moreover, from an evolutionary perspective they still need to be included in a more accurate and wider understanding of the eukaryotic tree of life. These seminal discoveries promoted the development and use of a wide range of more in-depth culture-independent approaches to access this diversity, from metabarcoding and metagenomics to single cell genomics and FISH. Nonetheless, culturing using classical or innovative approaches is also essential to better characterize this new diversity. Ecologists and evolutionary biologists now face the challenge of apprehending the significance of this new diversity within the eukaryotic tree of life.

### Introduction

During the last 15 years, the discovery of multiple microbial eukaryotic lineages has strongly modified general concepts in ecology, revealing unknown groups, some of which appear to be extremely abundant in the environment (Moreira and López-García, 2002). Due to their abundance and key functional role, they are often critical for biogeochemical processes in a given ecosystem (Caron et al., 2012). This new diversity also challenges our understanding of eukaryotic evolutionary relationships. Important changes in the global taxonomy of eukaryotes happened partly because of the discovery of divergent protist lineages occupying pivotal positions in the phylogenetic tree (Burki, 2014). Studying the life

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history traits of such novel groups may bring up shared features among lineages that will help us to better understand the evolutionary history of certain groups and, ultimately, the whole eukaryotic tree. The discovery of this new diversity within protists began with the use of culturing-independent molecular methods. From the first diversity studies based on the amplification, cloning and Sanger sequencing of 18S rRNA genes to more recent high-throughput metabarcoding, metagenomics or single cell genomics, these techniques have allowed us to move forward faster, freeing us of the constraints and limitations of culturing (del Campo et al., 2013a), although they have their own biases and pitfalls (Amend et al., 2010; Berney et al., 2004). The use of molecular tools does not mean that culturing became obsolete, but rather asks for innovative culturing strategies targeting the new diversity. The combined use of culture-independent techniques and renovated culturing efforts during the last years has pushed forward our knowledge of the eukaryotes and is filling the gaps on complex ecological puzzles and the still incomplete eukaryotic tree of life.

## Approaches to Learn the Biology of Novel Protists

### Environmental surveys

Almost 15 years ago three seminal papers revealed that the picture we had of the microbial eukaryotic diversity in marine systems deviated from reality (Diez et al., 2001; López-García et al., 2001; Moon-van der Staay et al., 2001). Using clone libraries of environmental 18S rRNA genes these studies revealed that protist diversity at the picoplanktonic level was dominated by two novel groups of organisms that were named MALV (Marine Alveolates) (Grosillier et al., 2006; Guillou et al., 2008) and MAST (Marine Stramenopiles) (Massana et al., 2004). The MALV include five independent lineages that branch at the base of dinoflagellates in phylogenetic trees. Known representatives of these environmental clusters belong to Syndiniales (Syndinids, Dinophyta) and are all parasites. As such, they play a key regulating role for high-biomass populations like those forming toxic harmful blooms (Chambouvet et al., 2008; Coats et al., 1996; Kim and Park, 2014). They can also impact higher trophic levels and fisheries by infecting radiolarians (Cachon, 1964), cope-pods (Skovgaard et al., 2005), fish larvae (Meneses et al., 2003) and crustaceans such as lobsters and crabs (Shields, 1994). MAST, on the other hand, include a diverse collection of polyphyletic groups of small (2–5  $\mu$ m) bacterivorous flagellates branching at the base of the Stramenopiles, each lineage potentially having slightly different ecological niches (Massana et al., 2014). The large clonal abundance of these new clades (both groups combined represent on average nearly half of the clones, Massana and Pedrós-Alió, 2008), suggest that they may also have a strong impact in the food web and in the biogeochemical cycles. The fact that both MALV and MAST cells are heterotrophic protists seems to reflect the higher difficulty of culturing heterotrophs than phototrophs, and therefore the former are better candidates to hide novel diversity. Apart from these two groups a myriad of branches sprouted in different parts of the eukaryotic tree, corresponding to additional novel, potentially uncultured, lineages.

Subsequently, this bonanza of novel diversity was revealed also in freshwater systems (Amaral-Zettler et al., 2002; Richards et al., 2005), hydrothermal vents (Edgcomb et al., 2002; López-García et al., 2003), and soils (Lawley et al., 2004). Often the relevance of

these novel lineages is not obvious to estimate because they may not necessarily be the most abundant organisms and therefore eclipsed by the sequences of dominant ones. Sometimes, it is even hard to differentiate novel diversity from sequence errors or pseudogenes (Thornhill et al., 2007). A way to make this novelty emerge is through meta-analysis and reanalysis of published data, allowing the report of novel clades within a wider range of groups (Richards and Bass, 2005) and populating specific regions of the eukaryotic tree (del Campo and Massana, 2011; del Campo and Ruiz-Trillo, 2013). An obvious challenge in future investigations is to increase the breadth of habitats to explore, focusing on under sampled, uncommon or remote ecosystems that may harbor additional new diversity.

The environmental clone library approach (Sanger sequencing) has been fundamental to retrieve complete 18S rDNA sequences for phylogenetic reconstructions of the novel diversity. While still useful to explore the diversity of novel divergent lineages, this approach is clearly limited for describing community diversity. Direct massive sequencing using new technologies such as 454 pyrosequencing (now obsolete) (Sogin et al., 2006) and Illumina (Amaral-Zettler et al., 2009) have now replaced clone libraries for the study of protist diversity in the environment. These so called High Throughput Environmental Sequencing (HTES or metabarcoding) techniques are cheaper, faster and retrieve several orders of magnitude more sequences from the analyzed samples than clone libraries; for example a single Illumina MiSeq run can produce up to 25 millions reads [ $2 \times 300$  base pairs] (Logares et al., 2012). The HTES techniques are limited by the size of the retrieved reads, currently about 450 bp, which leads to a decrease in the phylogenetic resolution. To partially overcome this issue, two hyper-variable regions of the 18S are mainly used to characterize communities: V4 and V9 (Stoeck et al., 2010). Complete sets of tools have been developed or adopted to process this big data, such as the suites QIIME (Caporaso et al., 2010) and Mothur (Schloss et al., 2009) as well as R packages like vegan (Dixon, 2003) or phyloseq (McMurdie and Holmes, 2013). Proper reference sequence databases are crucial to work with HTES reads, and nowadays there are two available for eukaryotes: Silva (Quast et al., 2013) and PR2 (Guillou et al., 2013). After the discovery of novel diversity based on clone libraries, HTES has completely changed the picture again, since the increase of the sequencing power gives access to new diversity within the so-called “rare” biosphere encompassing organisms at very low abundances (Logares et al., 2014) and allows sampling a wider range of habitats (Heger et al., 2014).

The use of meta-omic techniques, the retrieval of the genomes and transcriptomes of the whole community, represents an alternative strategy to study novelty. This way has not been as deeply explored as the metabarcoding but has shown its potential (Massana et al., 2008; Not et al., 2009; Piganeau et al., 2008). Furthermore meta-omics are not only used to identify but also to describe the ecology and distribution of novel organisms (Cuvelier et al., 2010; Geisen et al., 2015; Worden et al., 2012). In the next years advances in genome recruiting methods (Albertsen et al., 2013; Luo et al., 2012; Spang et al., 2015) will allow assembling individual genomes (Metagenome-Assembled Genomes or MAGs; Garcia et al., 2015) from unknown organisms present in metagenomic and metatranscriptomic datasets and to infer their putative functions and improve or resolve their phylogenetic position.

## FISH linking identity with morphology and function

Although environmental clone libraries were a great approach to unveil novel diversity in their time, as are HTES methods today, both share a common problem. There is not a lot that can be inferred about the abundance and biology of the new taxa just by analyzing the reported sequences, apart from their phylogenetic position and their putative habitat. Here is where a technique like FISH (Fluorescence in situ hybridization) can help in linking sequence identity and morphology and even function. The fundament of this technique is to design fluorescent oligonucleotidic probes (18–22 bp) targeting particular environmental 18S rDNA sequences that will hybridize to the rRNA located in ribosome. As hundreds of ribosomes are usually present in a cell, the target can be observed by epifluorescence microscopy (Amann et al., 1995). By visualizing the novel cells embedded in a complex natural assemblage, FISH provides rough pictures of the cell size and shape, and of the presence of several cell components (such as flagella or chloroplasts). In addition, it provides true cell counts, allowing to determine their real contribution in terms of abundance and biomass in the ecosystem. Moreover, combined with other tools such as bacterivory experiments or sophisticated microscopy, FISH provides also information about functional roles of new clades.

Employing FISH, it was shown that the different MAST lineages include cells of different size, abundance, and bacterial grazing behavior, while they collectively account for a very important fraction of heterotrophic flagellates in marine planktonic euphotic regions (Massana et al., 2006, 2009). In the case of MALV protists, the combination of FISH with confocal microscopy has allowed co-localizing MALV cells within putative hosts to confirm their life cycle. Thus, MALV-II cells initiate their life cycle as free-living infective spores (called dinospores) that infect the dinoflagellate host, form large growing endocellular trophonts that leave the host after sporulation as a multicellular vermiform stage which finally disaggregates into new dinospores (Chambouvet et al., 2008). A third example of the use of FISH has been the investigation of the cellular properties of the Cryptomycota or rozellids, an intriguing lineage located at the base of the fungal clade (Lara et al., 2010). Applying FISH on environmental samples it was shown that Cryptomycota cells have flagella in some stages of their life cycle, and that they always lack chitin, once thought to be the primary cell-wall component characterizing fungi (Jones et al., 2011).

## Single cell techniques

Single-cell-omic techniques (Stepanuskas, 2012) represent another culture independent approach to access the novel diversity at the genomic level, sometimes even preserving information on cellular identity, morphology and function. There are three main approaches to obtain single cells: (1) manual picking of single cells under the microscope, a slow method largely dependent on the expertise of the manipulator and the size of the targeted organisms; (2) single cell sorting by Flow Cytometry, a high-throughput method that sorts thousands of single cells in less than a minute (Rose et al., 2004) and is currently the most used; (3) single cell sorting using sophisticated microfluidic devices, an emerging approach that does not only sort the cells but eventually allows to experiment with them at a microscale (Leung et al., 2012; Streets et al., 2014). After sorting, three critical steps are required, each one experiencing current technological improvements: whole genome

amplification, genome sequencing, and sequence analysis (assembly, gene prediction, and annotation).

Single Cell Genomics (SCG) are changing the eukaryotic genomic panorama that was clearly dominated, and still is, by the multicellular groups and their parasites (del Campo et al., 2014). SCG gave access to the first genomic data on two novel eukaryotic groups like Picozoa (previously picobiliphytes) (Yoon et al., 2011) and MAST-4 (Roy et al., 2014). SCG can also help us to better understand the ecology and reveal novel diversity en masse, but instead of analyzing the whole community it is done one cell at a time (Heywood et al., 2011; Lepere et al., 2011; Martinez-Garcia et al., 2012; Woyke et al., 2009). Single Cell Transcriptomics (SCT) is a good complement to SCG that has been already tested with large protists (Kolisko et al., 2014) and not only works fine but ends up being relatively cheap and easy to implement in any lab. Advantages of SCT are that it allows doing comparative transcriptomics (Sebé-Pedrós et al., 2013), provides a more direct view of functional genes, and is a necessary alternative of SCG for organisms with large genomes. Single Cell-omics allow accessing the functional gene pool of uncultured organisms widely reported in the environment, thereby contributing to a better understanding of their ecological function. From an evolutionary perspective, this new genome/transcriptome data can be used to improve the eukaryotic phylogenomic framework (Burki, 2014) by enriching the taxon sampling used by researchers working on specific pathways and processes. Single Cell-omics is a game changer like metabarcoding and will boost our knowledge on the genomics of microeukaryotes.

### Culturing the uncultured

The three strategies we have presented are forming the spearhead of the discovery of new protist diversity because they are useful, high-throughput and fast. But we should not forget that culturing has been the most important method not only to describe new diversity but to understand organismal biology (which is still elusive from sequencing data). Nowadays, despite being time consuming, sometimes thankless, and often overshadowed by molecular methods, culturing is still having a strong impact on novel diversity. If we have a look at some discoveries that happened after the molecular revolution, we will notice that via culturing we have discovered organisms that pushed the field forward. Some new isolates have shaken the old-established eukaryotic tree, such as the still orphan (*incertae sedis*) clades *Telonema* (Shalchian-Tabrizi et al., 2006), *Collodictyon* (Zhao et al., 2012) or Picozoa (Seenivasan et al., 2013) as these three lineages might represent new deep branches of the tree that are not yet properly placed. Other cultured species have induced substantial changes within supergroup topology and composition, like *Carpodiomonas* in the Excavates (Kolisko et al., 2010), *Palpitomonas* for Cryptista (Yabuki et al., 2014) or the Colponemids in the Alveolates (Janouskovec et al., 2013). Some of these discoveries have a deeper impact on general biology, such as *Chomera velia*, a novel algal isolate related to the apicomplexan parasites (Moore et al., 2008). *C. velia* is important not only because it represents a brand new algal group but also because its phylogenetic placement may help to understand the mechanisms behind the origin of apicomplexan parasitism (Woo et al., 2015). While most of these strains have been retrieved by standard isolation procedures, novel methods have been recently developed to isolate organisms that are abundant in the environment but have been

reluctant to being cultured. This is the case for *Minorisa minuta*, an heterotrophic flagellate at the base of the Chlorarachniophytes that was first detected by environmental surveys and then cultured using single cell sorting and growth in an oligotrophic medium (del Campo et al., 2013b).

Sometimes a more detailed study of well-established cultures produces significant novel biological insights. Such is the case of *Capsaspora owczarzaki* that was originally isolated and deposited at ATCC as *Nuclearia*. After a proper phylogenetic analysis *Capsaspora* revealed itself as a new branch of the holozoans related to choanoflagellates and metazoans (Ruiz-Trillo et al., 2004) and helped to change our view on the origin and mechanisms of metazoan multicellularity (Sebe-Pedros et al., 2011). Another example within the opisthokonts is the basal fungal clade Rozellida or Cryptomycota (Jones et al., 2011; Lara et al., 2010), a widely diverse parasitic group that was defined by combining data retrieved from environmental surveys and from the cultured *Rozella allomyces*. During the last years an even larger group has been defined at the base of the Fungi that also includes the Microsporidians (Capella-Gutiérrez et al., 2012) and the the Aphelids (Karpov et al., 2013). Finally, another interesting example is *Breviata anathema* (Walker et al., 2006), previously known as *Mastigamoeba invertens* (Cavalier-Smith et al., 2004), once considered to belong to the amoebozoans and now placed at the base of the newly defined eukaryotic supergroup Obazoa, which encompasses Opisthokonts, Apusomonads and Breviates (Brown et al., 2013).

## Conclusions and Future Perspectives

The journey on the discovery of novel eukaryotic lineages is far from being over, it is just starting, and we should be conscious of the importance of this venture. Increasing the knowledge of the eukaryotic diversity at any level of the tree, from the root to the leaves, is of paramount ecological and evolutionary importance, having even implications in other fields such as biomedicine or conservation. We need to fill all possible gaps to fully understand eukaryotic evolution, the rise of parasites, the generation of symbioses, the establishment of multicellularity or the origin of the eukaryotic cell. At an ecological level, we need to know who are the main players of the different processes and we need to characterize these actors. We have been doing great advances so far but we are still facing several challenges that need to be overcome:

### Keep improving our culture independent techniques

Retrieving more and longer reads, processing and analyzing more images, getting better genomes from single-cell-omics, etc. The tools we have been using have provided great results but as explained before they also have their own pitfalls and limitations. We should work to polish them and find more efficient ways to get better data.

### Surfing the data tsunami

Current molecular methods to assess microbial diversity allow processing many samples generating an overwhelming amount of sequences that hardly will be fully analyzed. We should make an effort to escalate the analytical tools available to the amount of data

obtained. It is crucial to develop innovative analytical strategies to get the most from the retrieved information.

### **A picture speaks a thousand words**

Imaging was during decades the only way to describe new organisms for protistologists and there is a relevant knowledge corpus. Except for the few studies that combine ultrastructure and sequencing or tools like FISH, most often imaging and molecular data are decoupled. Integrating both approaches, and keeping them integrated in the future, is extremely important. It is advisable to work with images in the same systematic way than with sequences: imaging acquisition by high-throughput methods and creation of comparable and browsable databases. The technology is already available, just needs to be adopted/adapted to protist studies.

### **Data need to be free**

A critical challenge of the tsunami of data is the lack of proper infrastructures for data sharing in a comprehensive way. As a community, we should make an effort to push for the creation of open databases that allow our peers to have an easy access to the results. Furthermore, additional meta-analytical studies can give a new use of the data and bring up some new results.

### **From the bench to the computer and back to the bench**

So far we have been mostly identifying this new diversity but now we need to fully understand their biology and ecological relevance, demanding experimental scenarios. So it is time for isolation and culturing, and to perform original experiments with natural communities. We encourage the use of classical or novel culturing methods to bring to the lab organisms reported by environmental surveys that can be relevant from an ecological or evolutionary point of view. The protist world is vast and hosts most of the eukaryotic diversity, but we are only starting to grasp the extent of such diversity. Exciting years are ahead of us as we deploy all the potential of high-throughput techniques for the study of microbial eukaryotes. The fact of being understudied from an evolutionary and ecological point of view will make the protists, and the protistologists, the main responsible for reshaping the tree of the eukaryotes and for describing novel ecological interactions.

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