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Quick guide

Diplonemids

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What are diplonemids and where do they belong? Diplonemids have been classically described as heterotrophic biflagellated unicellular eukaryotes (protists) from the kingdom Euglenozoa (part of the supergroup Excavata), which also contains important pathogens of humans, livestock and plants called kinetoplastids (with *Trypanosoma*, *Leishmania* and *Phytomonas* as the most notorious representatives) and mostly photosynthetic euglenids (represented for example by ubiquitous *Euglena*). Compared to these widespread, diverse and important kin, diplonemids were until very recently only rarely found in marine or freshwater environments and only half a dozen species of two genera had been described. Diplonemids are generally considered to be predatory eukaryovores, although parasitic and possibly also symbiotic life strategies are described for some species. The flagship species, *Diplonema papillatum*, is a sack-shaped cell equipped with two short, thin flagella and, together with a few other diplonemid members, is available from American Type Culture Collection.

Honestly, if we were to pick candidates for exciting protists just a few months ago, diplonemids would be at the bottom of our list. Indeed, even specialized protistological textbooks usually devote just a paragraph or two to these obscure flagellates, which have consistently been studied by a single lab, the group of Gertraud Burger in Montreal. But diplonemids recently emerged as one of the most diverse and abundant eukaryotes. And the amazing thing is that we barely know what they look like or what they do. How could such an apparently important group remain totally overlooked for such a long time? The answer lies in the environment they occupy, which is primarily the depths of the ocean.

Are there any molecular features unique to diplonemids? Like their sister group the kinetoplastids, diplonemids harbor a huge mitochondrial genome, composed of thousands of circular DNA molecules, which are either relaxed and interlocked into a single network, or free and supercoiled. We know a lot about mitochondrial RNA editing and processing in the pathogenic *Trypanosoma brucei*, and it seemed likely that similar mechanisms would be in place in related diplonemids. However, diplonemids developed another unique way of dealing with their mitochondrial transcripts. While in *T. brucei* mitochondrial mRNAs are heavily edited by multiple post-transcriptional insertions and/or deletions of uridines, pretty much the same handful of transcripts is processed in a dramatically different manner in *D. papillatum* and *Rhynchopus* spp. No intact full-size gene has ever been found in their mitochondrial genomes, with each circular DNA molecule encoding just a single gene fragment. In a puzzling mechanism, the individual fragments are transcribed and spliced together by an extensive, yet totally uncharacterized *trans*-splicing machinery. By gradual addition of fragments, a mature and translatable molecule is generated. The machinery must be extremely precise, able to pick among dozens of different gene fragments, splicing the neighbors together in an exact manner. This is already a very twisted and unprecedented way of generating transcripts of just about a dozen mitochondrial-encoded genes, yet it is further complicated by limited RNA editing. It can be safely said that so far this is the most baroque example of maturation of any organellar transcript.

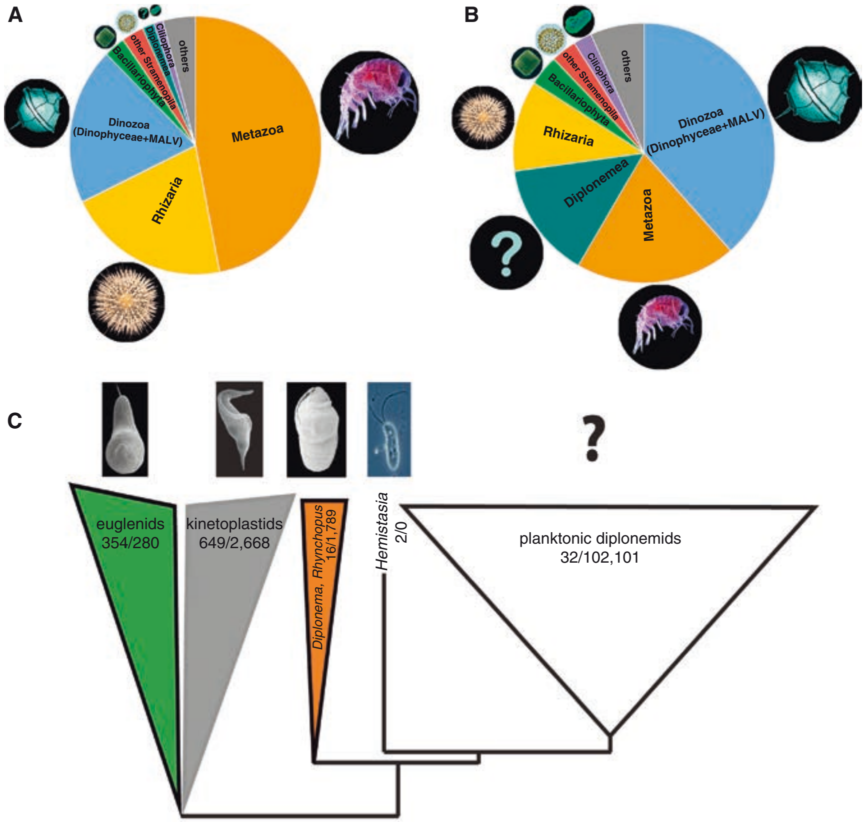

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Figure 1. Diplonemid abundance and diversity. (A) Pie chart showing the 7 most abundant eukaryotic planktonic lineages according to the counts of the V9 sequence, a fragment of the 18S rRNA gene. (B) Pie chart showing the 7 richest eukaryotic planktonic lineages according to operational taxonomic unit (OUT) counts. OTUs are defined with the linkage clustering 'Swarm' approach. (C) Schematized maximum likelihood phylogeny of diplonemid evolution based on V9 sequences. Digits below taxa names show numbers of reference V9 sequences available in public databases/numbers of unique V9 reads as revealed by a global metabarcoding survey of the photic ocean zone.

What is the real diversity of diplonemids? The environmental sequencing revolution at the turn of this century revealed the existence of two previously unknown yet abundant eukaryotic clades. The first comprises important parasites of plankton related to classic dinoflagellates called Marine Alveolate Group I and II (with five lineages being recognized today). The second group is known as Picozoa (originally picobiliphytes), miniscule heterotrophic flagellates of unclear life strategy. Somewhat in the shadow of these important discoveries, the analysis of 18S rRNA sequences from the Drake Passage planktonic samples revealed the existence of an environmental clade related to classic diplonemids. This new lineage gradually started expanding with sequences obtained from the mesopelagic to abyssopelagic layers of the Atlantic Ocean and Mediterranean Sea.

The aim of the international Tara Oceans expedition (2009–2012) and Tara Oceans Polar Circle expedition (2013) was a holistic assessment of eukaryotic diversity from planktonic samples collected across the tropical, temperate and polar worlds. Although novel diversity emerged at all taxonomic levels, diplonemids stood out as they represented one of the most diverse and abundant eukaryotic groups (Figure 1A,B). The analysis is based on ~800 million short V9 barcode sequences, a fragment of the 18S rRNA gene that is phylogenetically informative due to its variability. In this survey, diplonemid barcodes constituted the 6th most abundant eukaryotic group in marine plankton (Figure 1A). They were present in the photic layer of all 45 worldwide-distributed sampling stations, but their abundance clearly grew with oceanic depth. According to a detailed analysis of this V9 dataset, the mesopelagic layer, typically ranging from 200 to 1,000 meters, contributed more than 80% of the global diplonemid abundance, and diplonemids comprised up to 58% of all eukaryotic barcodes of the mesopelagic zone at some stations. Such abundance was certainly unexpected, but what is even more surprising is that, from the perspective of sequence diversity, diplonemids rank 3rd only after the well-studied dinoflagellates and metazoans (Figure 1B).

Based on a rather conservative definition of an operational taxonomic unit, the dataset contains ~12,300 diplonemid species. This is a true bonanza given that we have no idea what any of these organisms look like. Importantly, the phylogenies also show that the classic diplonemids from textbooks, for which at least some molecular and morphological data are available, constitute a branch that is a rather distant sister group to this extremely diverse marine diplonemid-like planktonic clade (Figure 1C).

What is the lifestyle of planktonic diplonemids? The stunning extent of diversity and abundance is based mostly on the V9 barcode of the 18S rRNA gene, which for interspecific comparison appears to be as suitable for diplonemids as for other protists. Hence, we are facing an unusual challenge. Based on sequences, there is a well-defined group of protists in the world's oceans that we know very little about, in particular whether they are free-living, commensals or parasites. Out of the possible life strategies, we could obviously exclude only phototrophy (both from phylogenetic and ecological reasons). A few studies hint to their parasitic lifestyle but if most diplonemids are indeed parasites they would have to infect the majority of marine eukaryotes, likely other protists for the most part. Alternatively, multiple diplonemid species could infect a single host species, but this would contradict the evolutionary trends seen in other parasitic groups, where exploring new hosts is a driving force of speciation.

There are some clues speaking against the parasitic lifestyle of diplonemids. Firstly, preliminary data indicate that the abundance of diplonemids increases with depth, and is still significant in very deep layers of the ocean, down to 5,000 meters, which supports an even less diverse palette of putative hosts. Secondly, an *in silico* analysis of the same global dataset of V9 barcodes from sunlit oceans offers an insight into possible interactions of planktonic species based on mutual exclusion/co-occurrence of their barcodes. It reveals a plethora of interactions for major marine protist parasites such as syndinians and apicomplexans. In both cases numerous connections tie these parasitic protists with the expected host spectrum. However, even though diplonemids ranked as the 6th most abundant eukaryotic group, they show very little putative interactions with both eukaryotic and prokaryotic components of the plankton community. Thus, the issue of diplonemid lifestyle can only be resolved by obtaining new data, isolating marine diplonemids and analyzing them in the lab.

Is any representative of planktonic diplonemids available in culture? A search for the identity of the planktonic diplonemids and their role in the ocean ecosystem recently yielded an unexpected result with the establishment in culture and concurrent redescription of *Hemistasia phaeocysticola*. Due to the lack of molecular data for the last two decades this euglenozoan ended up with the orphaned *incertae sedis* status. In the currently available extensive 18S rRNA dataset, *Hemistasia* emerged within the robust monophyletic clade of planktonic diplonemids, which constitutes a sister group to the classic diplonemids of the genera *Diplonema* and *Rhynchopus*. Interestingly, *Hemistasia* is a widely distributed although virtually ignored predator or parasite of diatoms, dinoflagellates and haptophytes, as well as metazoans, in particular the copepods. Although there is a considerable genetic distance between *Hemistasia* and most planktonic diplonemids (Figure 1C), it is the only available representative of one of the most abundant and diverse marine eukaryotes.

Have diplonemids been sequenced? More than a dozen genomes of pathogenic kinetoplastids (*Trypanosoma*, *Leishmania*, and *Phytomonas* spp.) have been sequenced, but no genomes are published for diplonemids and euglenozoans. We only have estimates that these flagellates carry genomes several times larger than those of the above-mentioned parasites, which range from 20 to 35 Mbp. It will be interesting to find out whether this huge difference is reflected in higher gene number, as it is somewhat counterintuitive that a free-living protist would need fewer genes than its parasitic relative. From the fragmentary information currently available it seems that the common feature of all euglenozoans, namely the addition of a small RNA molecule called spliced leader RNA onto every nuclear transcript, is conserved also in diplonemids.

What should we do next? The study of diplonemids faces two major challenges. While at least one member of the genera *Diplonema* and *Rhynchopus* is available in culture, an easy-to-grow strain representing the hyper-diverse marine clade is much needed. Next, in order to obtain deeper insight into the vagaries of diplonemids, a genetically tractable strain amenable to methods of reverse and forward genetics will have to be generated. The realization of these goals, together with the recent revelations from the TARA expedition, will help rescue the diplonemids from obscurity and bring them into the spotlight.

Where can I learn more?

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