The molecular ecology of microbial eukaryotes unveils a hidden world

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In spite of the great success of small-subunit ribosomal RNA (SSU rRNA)-based studies for the analysis of environmental prokaryotic diversity, this molecular approach has seldom been applied to microbial eukaryotes. Recent molecular surveys of the smallest eukaryotic planktonic fractions at different oceanic surface regions and in deep-sea Antarctic samples revealed an astonishing protist diversity. Many of the phylotypes found in the photic region affiliate with photosynthetic groups that are known to contain picoeukaryotic representatives in the range 1–2 µm. Surprisingly, a vast diversity of presumably heterotrophic or mixotrophic lineages is also found. Among these, several novel lineages of heterokonts, and a large diversity of alveolates clustering in two major groups (Groups I and II), are present at all depths in the water column. Many of these new phylotypes appear biogeographically ubiquitous. These initial studies suggest that a wide diversity of small eukaryotes remains to be discovered not only in the ocean but also in other environments. For both ecology and evolutionary studies, it is predicted that environmental molecular identification of eukaryotes will have a profound impact in the immediate future.

The use of molecular markers has had major consequences for the study of prokaryotic diversity and evolution [1]. In just a few years, the analysis of small-subunit ribosomal RNA (SSU rRNA) sequences directly amplified from environmental samples has completely changed our view of the biosphere [2]. An amazing diversity of prokaryotic life has been revealed in many different environments, including the most inhospitable regions of our planet [2–4]. MOLECULAR ECOLOGY (see Glossary) has thus verified an old suspicion of microbiologists dealing with natural environments: most microbial diversity escapes isolation attempts, and the species that we isolate are not necessarily the most abundant. Besides these repercussions for microbial ecology, the evolutionary outcomes of these studies include the discovery of many new microbial lineages even at the taxonomic phylum or division level. Within the Bacteria, at least 13 novel candidate divisions have been catalogued [5,6] (Fig. 1). Major groups of uncultured microorganisms are also recognized in the two archaeal kingdoms Crenarchaeota and Euryarchaeota [3,7,8]. Furthermore, a third candidate kingdom, the ‘Korarchaeota’, has been proposed, grouping several basal hyperthermophilic phylotypes [3,9]. Other basal archaeal phylotypes have subsequently been identified in hyperthermophilic environments [10–12] (Fig. 1).

Despite the transcendence of molecular ecology techniques for prokaryotic microbiology, these techniques have rarely been applied to the study of microbial eukaryotes, or PROTISTS. A few exceptions exist; van Hannen et al. described the eukaryotic diversity in a detritus-derived experimental system using molecular methods, identifying new protist lineages [13]. SSU rRNA-gene-specific amplification has also been used to characterize eukaryotic diversity in fossil glacier ice cores [14]. The scarcity of these studies can be explained by the historical lack of connection between protistiologists and (prokaryotic) microbiologists, and also by the idea that protist diversity could be successfully explored using classical isolation techniques, and morphology and ultrastructure data. Curiously enough, contrary to the prokaryotic case, the application of SSU rRNA-based methods for quantification in the eukaryotic world has preceded diversity studies [15–19].

The diversity of protists described by classical methods is very large [20–22]. According to Patterson [22], in terms of cytological diversity and abundance

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Glossary

- **Alveolate** (Alveolata): a vast protist group comprising ciliates, dinoflagellates and the parasitic apicomplexa and perkinsoza; characterized by the possession of a system of abutting sacs (alveoli) underlying the cell surface.
- **Aphotic zone**: dark region of the water column below the photic zone.
- **Archezoa**: group of early branching eukaryotes, mostly parasites, originally thought to predate the endosymbiotic acquisition of mitochondria, but now known to be secondarily amitochondriate.
- **Heterokont** (Heterokonta): a large and diverse protist group including photosynthetic and heterotrophic organisms; mostly unicellular biflagellates with one short and one long flagellum, but also amoeboid, aflagellated or mycelial, including extremely large organisms (e.g. kelp). Also known as stramenopiles.
- **Mixotroph**: organism capable of performing two different types of energetic metabolism (e.g. heterotrophy and phototrophy).
- **Molecular ecology**: a new discipline arising from the application of molecular (DNA-based) methods to the study of biological diversity.
- **Nanoplankton**: planktonic organisms found in the size fraction 2–20 µm.
- **Photic zone**: upper region of the water column where sunlight penetrates; it varies depending on several parameters (e.g. latitude, season and water conditions), reaching a maximum depth of ~200 m.
- **Phylotype**: an environmental SSU rRNA sequence representing a lineage that has not been cultivated.
- **Picoeukaryote**: eukaryote whose cell diameter is ~< 2 µm.
- **Picooplankton**: planktonic organisms, eukaryotic or prokaryotic, whose cell diameter is ~< 2 µm.
- **Protist**: generally refers to eukaryotic microorganisms, but not all protists are always microbial (e.g. kelp) and not all microbial eukaryotes are considered protists (e.g. yeasts). Several authors include all eukaryotes except fungi, plants and animals.
- **Stramenopile**: see heterokont.
(if not species diversity), they dominate the world of eukaryotes. The recent and nearly simultaneous discovery of a vast hidden diversity of protists, mostly prokaryotic-sized, in the sea [23–25] will open new avenues of exploration and could indeed confirm the existence of an unforeseen richness of eukaryotic species on our planet.

The smallest cultivated eukaryotes

The existence of eukaryotes of typical prokaryotic size was first discovered almost 50 years ago in marine waters [26]. Later on, microscopy and pigment composition studies confirmed that phytoplankton of bacterial size is composed basically of cyanobacteria (*Prochlorococcus* and *Synechococcus* spp.) and, surprisingly, a complex assemblage of phototrophic eukaryotes of <3 µm [27]. Some of the small eukaryotic photosynthesizers were subsequently described as members of a new order, Parmales [28]. However, the taxonomic composition of this eukaryotic assemblage remained largely unknown, even though the use of flow cytometry techniques suggested that it plays a significant role in oceanic primary production [29]. Quantitative studies using eukaryote-specific probes for fluorescent in situ hybridization (FISH) proved to be a useful method to enumerate small eukaryotic cells in marine samples [18]. FISH experiments resulted in higher counts than those based on DAPI or acridine orange staining, and showed, for example, that Woods Hole coastal eukaryotic plankton was dominated by small protists of ~2 µm in diameter [18].

The first isolated *PICOEUKARYOTES* were indeed photosynthesizers [26]. Many belong to new protist genera and classes, such as the Pelagophyceae [30] and the Bolidophyceae [31]. The smallest eukaryote ever found, *Ostreococcus tauri*, which has a cell size of 0.8–1.1 µm × 0.5–0.7 µm, is a member of the Prasinophyceae [32,33]. It contains the nucleus, one mitochondrion and one chloroplast tightly packed, a compact organization that also appears in other photosynthetic *picoeukaryotes* such as the Bolidophyceae [31]. *O. tauri* has a reduced genome size, 9.5 Mb [34], which is comparable to that of bacteria such as...
as the myxobacteria [35]. Picoeukaryotic members of heterotrophic groups, such as the Bicosoecida, have also been isolated. These also have a compact structure, with only two small mitochondria, one food vacuole and the nucleus [36]. The discovery of these heterotrophs opens the possibility that picoeukaryotes inhabit deep oceanic regions far from the photic zone, something that was demonstrated recently by a study of four deep-sea hydrothermal sites in the Pacific ocean (depths ranging from 2000 to 2550 m). A surprising variety of small protists (most <2 µm in diameter) was isolated from these sites, including members of the Ancyromonadida, Bicosoecida, Cercomonadida, Choanoflagellida, Chrysomonadida and Kinetoplastida [37].

**Small protists in the ocean - the first molecular surveys**

The first molecular survey of eukaryotes in the ocean dates back to only 1998, and indeed was a by-product of bacterial (16S) SSU rRNA studies in Pacific and Atlantic waters [38]. In the course of their bacterial analyses, these authors amplified several plastid genes (chloroplasts derived from cyanobacteria), which allowed them to identify a relatively large number of photosynthetic lineages in the 0.2–10 µm planktonic fraction related to the classes Bacillariophyceae, Cryptophyceae, Prymnesiophyceae, Chrysophyceae and Prasinophyceae [38]. The first specifically eukaryote-directed studies were aimed at determining the abundance and diversity of picoeukaryotic Bolidophyceae [39] and Prymnesiophyceae [40] in different oceanic regions. Despite the fact that they used Bolidomonas-specific primers, Guillou et al. retrieved two divergent sequences, one belonging to a basal heterokont, and another to an alveolate lineage branching off at the base of the dinoflagellates [39]. These were promising hints indicating the existence of an as-yet-unseen protist diversity in the sea, particularly among the smallest eukaryotic sizes.

The first (18S) SSU rRNA-based surveys of small planktonic fractions have confirmed this suspicion. Moon-van der Staay et al. extended the work previously initiated in surface waters (75 m depth) in the equatorial Pacific, using general eukaryotic-specific primers instead of class- or genus-specific primers [24]. They were thus able to identify a wide diversity of lineages, many of them belonging to photosynthetic classes (Prasinophyceae, Haptophyceae, Prymnesiophyceae, Bolidophyceae, and others to typical heterotrophic groups (choanoflagellates and acantharea) or to groups with phototrophic, heterotrophic and mixotrophic representatives (dinoflagellates). Additionally, they retrieved sequences not clearly affiliated to any known organism. Three phyotypes formed a cluster distantly related to labyrinthulids and thraustochytrids within the heterokonts, and several phyotypes appeared at the base of the dinoflagellates within the alveolates [24]. These results are remarkable because of the large diversity found in surface waters. At the same time, and most unexpectedly, we discovered an impressive diversity of protist lineages in Antarctic deep-sea waters ranging from 250 to 3000 m depth, also using a SSU rRNA-based strategy [23].

Little is known about deep-sea planktonic communities and, although some efforts have been directed to study bacterial and archaeal communities [41–43], protist populations have never been characterized. Traditionally, dark, cold, oligotrophic, low-biomass deep-sea waters are considered inhospitable. Despite this, our study uncovered the existence of many new lineages in the 0.2–5 µm fraction (picoplankton and small nanoplankton) at 250, 500, 2000 and 3000 m depth, and also in the nanoplanktonic fraction at 3000 m depth. Many of these lineages affiliated to typical non-photosynthetic groups (dilethae, acantharea, fungi, thraustochytrids and diplomeds), one pseudo-nitzschia-like diatom, several dinoflagellate phyotypes, and also lineages not closely related to any known organism (two early branching phyotypes and two heterokont phyotypes). However, the most striking finding was an assemblage of genetically very diverse sequences, which comprised the vast majority of our clones (from 50% to 76% depending on the depth analyzed), forming two distinct clusters within the alveolates [25].

The results of these initial studies on surface and deep-sea waters point to the existence of a cryptic diversity of tiny protists in the ocean. A recent SSU rRNA survey of picoeukaryote diversity in the surface waters of the Mediterranean, North Atlantic and Antarctic oceanic regions has extended and confirmed the results of Moon-van der Staay et al., with the detection of many photosynthetic (Prasinophyceae, Prymnesiophyceae, Bacillariophyceae, Pelagophyceae, Glaucoctophyceae, Cryptophyceae, Eustigmatophyceae, Bolidophyceae and Chrysophyceae) and heterotrophic (dilethae, cercomonads and fungi) lineages, dinoflagellates and putative new lineages within heterokonts and alveolates [25].

From this small handful of SSU rRNA eukaryotic surveys, it is already clear that a large diversity of protists has escaped detection by classical methods. This could be especially true in the case of picoeukaryotes, whose small size might preclude their recognition under optical microscopy and, when they lack flagella or other conspicuous eukaryotic features, even under electron microscopy. This discovery, still in its early phase, has far-reaching implications for both evolution and ecology studies.

**Evolutionary consequences: major novel groups**

Many of the environmental phyotypes identified in oceanic surface waters correspond to groups of organisms that have already been characterized [24,25]. This is particularly so for the photosynthesizers, as picoalgal algae have been known for a long time [26,27]. Nevertheless, several lineages of heterokonts and alveolates detected in superficial waters are also identified in
Fig. 2. Phylogenetic tree showing the position of new marine alveolate phylotypes based on their small subunit ribosomal RNA (SSU rRNA) sequences. Deep-sea phylotypes detected in Antarctic waters (250–3000 m depth) [23] are in blue; those from the photic region (75 m depth) in the equatorial Pacific [24] are shown in green. The tree was constructed by neighbour-joining with a G–law using nearly complete SSU rRNA sequences (1133 unambiguously aligned positions). The tree is rooted using green. The tree was depth) in the equatorial Mediterranean areas [23–25], most of the alveolate dinoflagellates in the Antarctic, Pacific and Mediterranean and North Atlantic [23,25], and In addition to ciliates detected in the Antarctic, new and diverse marine alveolate Groups I and II (Fig. 2). However, this group is clearly distinct from typical dinoflagellates because of their proximity to the dinoflagellates. Furthermore, quoting P.P. Grassé in his Traité de Zoologie, in which 'the flagellispore is far from resembling a dinospore; it seems also difficult to incorporate Amoebophrya to the dinoflagellates. Here, we do not assign it a precise systematic position; we do not even affirm that it is a flagellate' [45]. Our phylogenetic analysis agrees with this suspicion, and shows that Amoebophrya spp. [44,46]...
Alveolate sequences (in total >50) is identical, indicating a surprising variety that has probably not been fully explored. This, together with low statistical support for many internal branches, led us to propose a radiation early in the alveolate evolution [23]. The incorporation of the sequences determined by Moon-van der Staay et al. further supports this idea. Consequently, the classical tripartite classification of alveolates (ciliates, dinoflagellates and apicomplexa) appears inadequate. Alveolates encompass several other lineages including the novel marine groups and probably those represented by organisms of uncertain phylogenetic position such as the Perkinsozoa, Parvilucifera infectans, Pseudoperkinus tapisis and the environmental phylotype OLI11005 (Fig. 2). Therefore, alveolates are possibly comparable to heterokonts in terms of diversity.

Novel heterokonts
The recent molecular studies in photic oceanic regions have revealed a large diversity of photosynthetic picocryukaryotic heterokonts (Stramenopiles) including Dictyochophyceae and Pelagophyceae [24] (Fig. 3), as well as diatoms (Bacillariophyceae), Eustigmatophyceae, Bolidophyceae and Chrysophyceae [25]. This observation expands previous studies on picocryukaryotic marine algae [30,39,40], indicating that these small photosynthesizers are widespread and possibly play an important role in oceanic primary production.

However, what is most interesting from an evolutionary perspective is the discovery of several apparently basal phylotypes, some of which are also present in the deep-sea, suggesting that they are heterotrophic (Fig. 3). Thus, a solid cluster (94% bootstrap) is formed by sequences retrieved from surface and the upper part of the aphotic region (250 m depth) from Pacific, Mediterranean and Antarctic waters. This group is related with good support (84% bootstrap) to the oomycetes, which are heterotrophic flagellates [47,48] (Fig. 3).

Among the deep-sea sequences, DH148-5-EKD53 (3000 m depth) occupies an isolated position, although one phylotype found in Mediterranean surface waters (ME 1-17) [25] clusters with it (D. Moreira and P. López-García et al., unpublished). This suggests that this heterokont lineage is ubiquitous in depth and geographical distribution. The 2000 m-deep phylotype DH147-EKD10 appears to be located at the base of labyrinthulids and thraustochytrids, which are typical marine decomposers, although the statistical support is very weak (Fig. 3).

One of the most remarkable observations comes from the combination of novel heterokont phylotypes retrieved from photic regions in different oceanic areas. Moon-van der Staay et al. identified a few sequences that appeared distantly related to the thraustochytrids in a poorly resolved region of the tree [24]. Díez et al. identified 24 sequences of basal lineages without apparently close relatives [25].

**Fig. 3.** Phylogenetic tree showing the position of new marine heterokont phylotypes based on their small subunit ribosomal RNA (SSU rRNA) sequences. Deep-sea phylotypes detected in Antarctic waters (250-3000 m depth) [23] are in blue; those from the photic region (75 m depth) in the equatorial Pacific [24] are in green; those from Mediterranean surface waters are in orange [25]. The tree was constructed by neighbour-joining with a Δlaw using nearly complete SSU rRNA sequences (1134 unambiguously aligned positions). The tree is rooted using 15 alveolate sequences (not shown). Numbers at nodes correspond to bootstrap values (from 1000 replicates). The scale bar corresponds to 15 substitutions per 100 positions for a unit branch length. A novel well-supported group of sequences related to oomycetes is highlighted in yellow. Two putative, although weakly supported, lineages without apparent relatives are in red and purple. These lineages might actually be the first described members of this new group of alveolates. In any case, sequence data from already described species of the order Syndiniidae, including, among others, the parasitic Amoebophryaceae and the Duboscquellaceae, will be required to clarify this issue.

In their study, Díez et al. also identified several alveolate sequences [25] that are not incorporated into the tree in Fig. 2 because, unfortunately, they are only partial sequences. This makes it very difficult to construct a robust phylogenetic tree, which is of prime importance when novel phylogenetic groups are to be defined. However, individual analysis of their sequences indicates that four belong to Group II and one to Group I, further extending the observation of these lineages in other oceanic regions (D. Moreira and P. López-García et al., unpublished).

The diversity of each of these groups in terms of genetic divergence is similar to that of huge groups such as the dinoflagellates or ciliates. None of these
Two of these sequences were complete, and were therefore included in our analysis (Fig. 3). The rest of the sequences were analyzed individually (D. Moreira and P. López-García et al., unpublished). 13 out of the 24 sequences cluster more or less solidly with the group formed by the phylotypes OLI 11150 and OLI 11066, with the sequence ME 1–24 being more divergent. Another four sequences group with the Pacific phylotype OLI 11006, which might be distant related to the recently described heterotrophic flagellate Woblia lunata [49]. Additionally, four of their basal phylotypes affiliate to the oomycetes-related group, another appears more related to known labyrinthulids, and yet another is closely related to the deep-sea phylotype DH 148-5-EK D53.

In summary, by the diversity retrieved, heterokonts seem to predominate in superficial waters not only by the existence of picoeukaryotic photosynthetic members, but also by the presence of several new lineages of putative heterotrophic flagellates or other kinds of presumable heterotrophs or mixotrophs. Most of these groups tend to appear at the base of the heterokonts. Some authors would see in this confirmation of the idea that the ancestor to the heterokonts was primarily heterotrophic [25,50]. However, there is increasing support for the hypothesis that alveolates and heterokonts are sister groups that had a photosynthetic common ancestor [51,52]. As this is a controversial matter, the precise kind of metabolism performed by these basal phylotypes, especially those found to date only in the photic region (see the groups noted with a question mark in Fig. 3), must be determined unequivocally. Isolation and cultivation of these organisms will hopefully provide an answer in the future. In addition, many of these basal branches are quite unstable, jumping to more apical positions when the taxonomic sampling and the number of compared positions are changed (D. Moreira and P. López-García et al., unpublished). This indicates that the internal phylogeny of the heterokonts is not yet resolved, although the inclusion of environmental lineages should be of great help in this regard.

The impact of small protists on marine ecology
The discovery of a broad diversity of nano- and picoeukaryotic lineages in the sea from these foremost molecular studies anticipates important consequences for marine ecology. Clearly, specific quantitative studies need to be performed. The diversity found from surface to deep-sea waters is remarkable. In fact, all of the sequences presented in these recent studies are different, and are especially divergent in the case of alveolates (Fig. 2). This suggests that their diversity is far from exhausted. Indeed, the analysis of the same SSU rRNA gene libraries in deep-sea Antarctic samples still provides new phylotypes, mostly affiliated to the novel alveolate groups (D. Moreira and P. López-García et al., unpublished). In addition, preliminary in situ hybridization studies with eukaryotic-specific probes indicate that picoeukaryotes also abound in coastal waters (D. Moreira and P. López-García et al., unpublished). Visual inspection of the same environmental samples by scanning electron microscopy allows the detection of some cells with clear eukaryotic traits (Fig. 4). However, these constitute a minute proportion, clearly inferior to counts obtained by several authors for similar environments [18,29]. This suggests that a significant number of picoeukaryotes have morphologies indistinguishable from prokaryotic cells by optical or scanning electron microscopy.

In a recent quantitative study using fluorescent in situ hybridization techniques to estimate bacterial and archaeal cells in picoplankton, Karner et al. reported that prokaryotes account for 63% to 90% of the total cell counts in the deep ocean (Pacific ocean, down to 4500 m depth) [42]. The remaining percentage could be explained, at least partly, by eukaryotes in the picoplanktonic range, in addition to prokaryotic cells containing few ribosomes, as already advanced by these authors [42]. These values might be an indirect indication of the existence of a fairly abundant amount of picoeukaryotes in the deep sea, although quantitative reproducible and varied experimental data are required to validate this idea. If this is the case, the picoeukaryotic contribution to carbon cycling must be important. Until now, most measurements of carbon cycling by prokaryotes have used size-exclusion techniques, which cannot discriminate very small eukaryotes [53]. These measurements should possibly be reassessed to give more accurate information on global biogeochemical cycles.
Biogeographically, and in a similar way to what is known for marine prokaryotes [54,55], picoeukaryotic lineages appear to be ubiquitous in different oceanic regions, as most of the identified phylotypes have close relatives in the equatorial Pacific, Mediterranean, and South Atlantic and Southern oceans. In addition, some of these groups, particularly the novel alveolate Groups I and II, are found at all depths, from surface to 3000 m depth, without any clear stratification.

Finally, nothing is known about the biology and metabolism of these new lineages. We can reasonably speculate that relatives of known photosynthizers, which are indeed found in surface waters, perform photosynthesis. Similarly, we can presume that close relatives to typical decomposers, such as fungi, or predators, such as diliates and acantharea, display equivalent functions. We can even suppose that deep-sea lineages are probably heterotrophs. However, as many of these new lineages are really divergent from cultivated members, it is not possible to infer anything about their mode of life. Moon-van der Staay et al. propose that many of these lineages are parasitic, at least those belonging to the Group II alveolates, containing Amoebophrya spp. and the Group I, placed between dinoflagellates and Perkinsozoa [24]. However, as these phylotypes are genetically very divergent and diverse, there is no solid ground for this conclusion at the present time. As a matter of fact, among dinoflagellates, which encompass a similar genetic diversity, we find typical parasites and free-living members. Also, the assumption that many basal heterokont lineages are heterotrophic [25] should be regarded cautiously, especially for phylotypes that have only been retrieved from the photic region. Directed cultivation attempts should provide the answer in the future.

Perspectives: what's next?

Protist molecular ecology is just beginning, and will surely have remarkable repercussions in general microbial ecology, and eukaryotic phylogeny and evolution.

The first eukaryotic SSU rRNA-based studies in the ocean have just revealed the existence of many new lineages, possibly abundant, of prokaryote-sized protists. This diversity is probably the tip of the iceberg. A big effort to elaborate a more complete catalogue of marine protist lineages by molecular techniques, and observation by in situ hybridization will be needed to visualize and quantify microbial eukaryotes. This should be combined with classical methodologies to isolate and cultivate representative members of these taxaons, paralleled by electron microscopy techniques providing information about ultrastructure details. This will provide invaluable data to complement sequence phylogenetic information with morphological features. Cultivation and direct observation in nature will help to determine the ecological role of these organisms in the marine food webs. Additionally, these discoveries open up the possibility that picoeukaryotes are diverse and abundant in many other environments that have never been explored from this perspective. These include extreme environments, where life is predominantly microbial and whose eukaryotic component is often disregarded. Earth's biotopes could still hold surprising treasures for protistologist and microbiologist explorers.

The retrieval of new, mainly free-living, eukaryotes could have outstanding significance for phylogenetic studies. The eukaryotic tree based on single molecular markers, including SSU rRNA, is not well resolved, having several unstable regions. The basal part of the SSU rRNA tree, populated with long-branches often corresponding to fast-evolving parasites, is particularly affected by this problem. This generates contradictory phylogenies when different molecular markers are used [56]. At present, there are three ways to overcome this phylogenetic uncertainty. One is retaining good supported relationships retrieved from the analyses of different markers to establish an ideal consensus phylogeny [56]. The other two are analyzing concatenated molecular markers [52,57] and including broader taxonomic sampling comprising novel groups of eukaryotes. In this regard, molecular ecology constitutes a prominent source for novel protist sequences. The addition of free-living protist phylotypes displaying shorter branches could help to alleviate artefactual long-branch attraction effects. Thus, we recently showed that a slow-evolving environmental Diplonema-like sequence had a stabilizing effect in the phylogeny of Euglenozoa [58]. Furthermore, we recently identified two basal, slow-evolving sequences affiliated to the radiolarian classes Polycystinea and Acantharea in a cosmid genomic library constructed with DNA from 500 m depth at the Antarctic Polar Front. Their inclusion in phylogenetic analyses allowed us to retrieve the monophyly of both groups, which constitutes a piece of evidence towards the monophyly of Haeckel's Radiolaria (Polycystinea, Phaeodarea and Acantharea) [59]. This result is at odds with previous work concluding that Polycystinea and Acantharea were not sister groups [60]. That analysis was based on the fast-evolving radiolarian sequences available at the time and likely suffered from a long-branch attraction artefact. Nevertheless, all of these approaches – comparative phylogeny, gene concatenation and molecular ecology – are complementary. With the development of environmental genomics [61], the gene sequence of protein markers can be retrieved from cosmids or bacterial artificial chromosomes ascribed to a given phylotype for subsequent gene fusion and improved comparative phylogenetic analysis.

Finally, combining phylogenetic studies with culture and ultrastructural data will help to place the acquisition of particular structures in eukaryotic evolution, as several of these lineages seem to occupy intermediary positions. Furthermore, molecular ecology of protists in different environments could even provide elements to test current hypotheses on the origin of eukaryotes by allowing the detection of possible missing links.