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Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity

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Picoplankton—cells with a diameter of less than 3 μm—are the dominant contributors to both primary production and biomass in open oceanic regions[1,2]. However, compared with the prokaryotes[3], the eukaryotic component of picoplankton is still poorly known. Recent discoveries of new eukaryotic algal taxa based on picoplankton cultures[4,5] suggest the existence of many undiscovered taxa. Conventional approaches based on phenotypic criteria have limitations in depicting picoplankton composition due to their tiny size and lack of distinctive taxonomic characters[6]. Here we analyse, using an approach that has been very successful for prokaryotes[7] but has so far seldom been applied to eukaryotes[8], 35 full sequences of the small-subunit (18S) ribosomal RNA gene derived from a picoplanktonic assemblage collected at a depth of 75 m in the equatorial Pacific Ocean, and show that there is a high diversity of picoeukaryotes. Most of the sequences were previously unknown but could still be assigned to important marine phyla including prasinophytes, haptophytes, dinoflagellates, stramenopiles, choanoflagellates and acantharians. We also found a novel lineage, closely related to dinoflagellates and not previously described.

A sequence search with the EMBL gene databank showed that only 2 of the 35 18S rDNA sequences (OLI11030 and OLI11015) from the Pacific Ocean had significant identity (more than 99%) to known sequences: the ubiquitous picoplanktonic species *Pelagomonas calceolata* and a recently sequenced acantharian[9], respectively. The maximum sequence identities of the other environmental sequences to known eukaryote 18S rDNAs ranged from 82% to 97%. The global phylogenetic tree (Fig. 1) obtained with both environmental clones and available sequences is largely congruent with those found previously[10–12], although many evolutionary relationships between the eukaryotic crown taxa are not clear, and the bootstrap values at the nodes are low, as indicated in earlier studies. The phylogenetic positions of the environmental clones are also supported by detailed, separate phylogenetic analyses of subgroups, using more 18S rDNA sequences available (data not shown). The presence of sequences from lineages that are known to harbour picoplanktonic representatives, such as the prasinophytes or the pelagophytes (Fig. 1), and the converse absence of sequences from larger cells such as diatoms despite their ubiquity in Pacific waters[13], confirms that the approach taken specifically targeted picoplankton.

Most of the work on oceanic eukaryotic picoplankton has focused on its photosynthetic component because chlorophyll fluorescence makes it easy to detect by flow cytometry[1] and pigment signatures permit inferences to be made about its taxonomic composition. Among autotrophs, haptophytes constitute one of the major picoplanktonic lineages, as suggested by the dominance of the diagnostic carotenoid 19′-hexanoyloxyfucoxanthin in most oceanic waters[14]. Indeed, four haptophyte clones (OLI11056, OLI11019, OLI11072 and OLI11007) were observed in the Pacific sample. Separate phylogenetic analyses of these environmental clones, adding more haptophyte sequences, suggest that clones OLI11072 and OLI11019 are more specifically related to *Chrysochromulina leadbeateri*. Clone OLI11007 belongs to a recently revealed environmental lineage that is related either to coccolithophorids or to *Phaeocystis*[14]. Clone OLI11056 forms a somewhat independent clade as a sister to the *Chrysochromulina* clade and that uniting the *Prymnesium*, *Imantonia* and part of the *Chrysochromulina* species (data not shown).

Another key group in the picoplanktonic autotrophs is the prasinophytes, primitive green algae that have been repeatedly isolated from marine waters[6]. Indeed, three clones (OLI11059, OLI11305 and OLI11345) were assigned to this class (Fig. 1). Phylogenetic analyses strongly support the affinity between OLI11059 and the unidentified coccoid prasinophyte CCMP 1205, whereas the other two sequences seem to form a new clade, not yet represented in culture. The exact branching order of the lineage leading to these clones and CCMP 1205 in other early diverging prasinophyte lineages is still not clear (bootstrap value $<$50%).

Stramenopiles or heterokonta contain key oceanic algal classes, in particular the ubiquitous diatoms, but also heterotrophic groups such as the bicosoecids. Clone OLI11030 shows 99.6% sequence identity with *P. calceolata* a widespread species, whose discovery in 1993 led to the creation of the class Pelagophyceae[4]. Clone OLI11025 is related to the dictyochophytes, which contain phototrophic, phagotrophic and mixotrophic species. Five other clones are affiliated to two highly diverging heterotrophic stramenopile lineages. One group (OLI11026 and OLI11008) clusters with the oomycetes (*Lagenidium*, *Phytopthora* and *Achlya*), whereas the other group (OLI11066, OLI11150 and OLI11006) apparently represents an early heterotrophic divergence. Clone OLI11066 clusters with clone OLI11150, whereas the affinity of clone OLI11006 with the two former clones is not strongly supported by bootstrapping (54%). The relative branching order between early diverging heterotrophic stramenopiles, for example bicosoecids (*Syluania*, *Cafeteria*), labyrinthulids (*Labyrinthuloides minuta*) and thraustochytrids (*Thraustochytrium kinnei*), is still obscure, as shown in previous studies based on 18S rRNA[15,16].

Dinoflagellates, like the stramenopiles, contain both autotrophic and heterotrophic taxa. Three clones (OLI11255, OLI11027 and OLI11005) can be included in various dinoflagellate clades, although their exact phylogenetic positions are not clear (bootstrap values $<$50%). Seven clones (OLI11115, OLI11261, OLI11055, OLI11023, OLI11010, OLI11009 and OLI11012) form a monophyletic lineage (Fig. 1) that includes the parasitic syndiniophycean *Amoebophrya* sp.[17]. Additional sequences of syndiniophyceans are probably needed for a more detailed characterization of these clones.

The most intriguing discovery from this work is that of an environmental lineage consisting of six clones (OLI11038,

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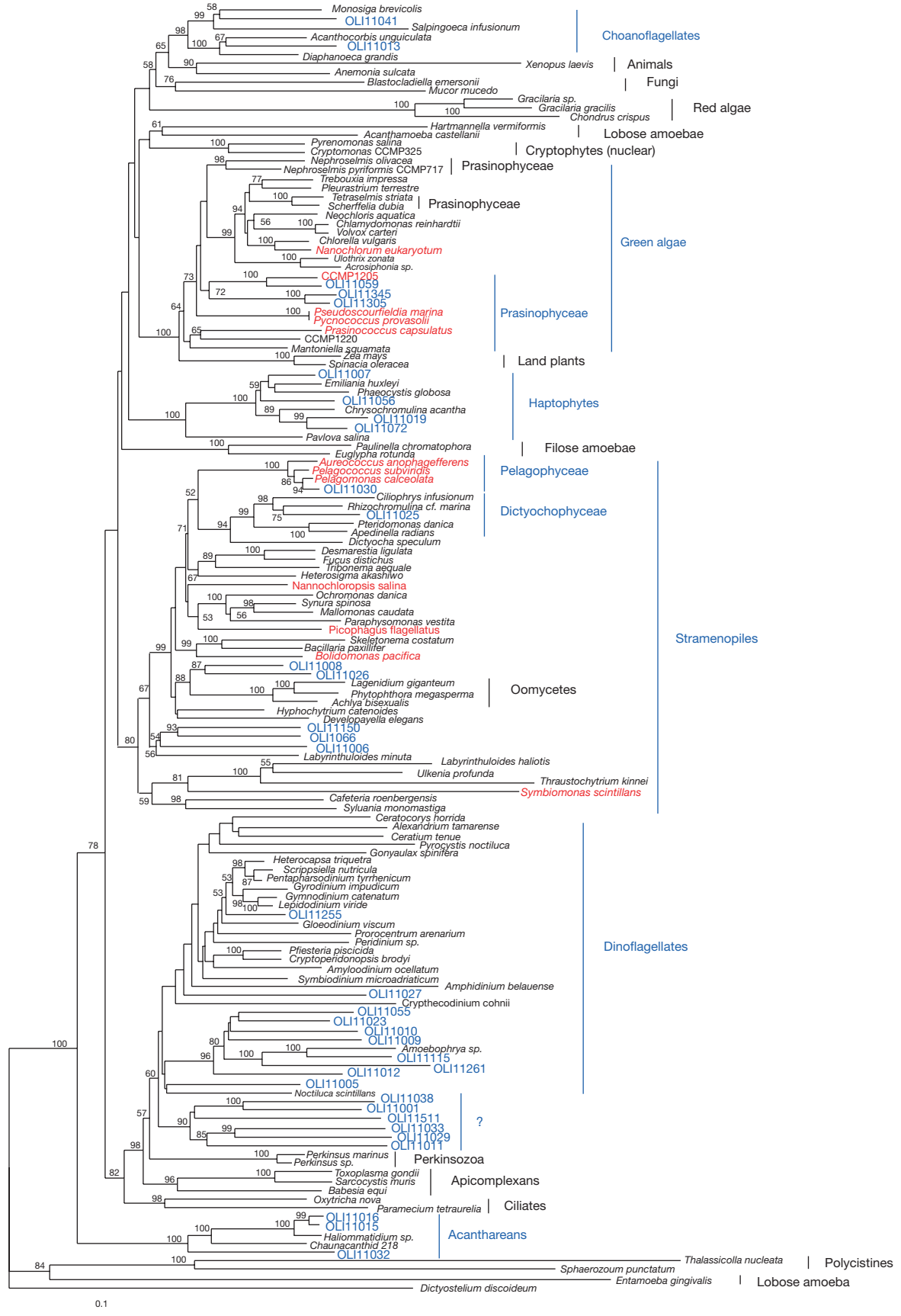

Figure 1 Phylogenetic tree based on nearly complete sequences of the 18S rRNA gene. Sequences in blue beginning with 'OLI11' correspond to those retrieved from the Pacific Ocean (150° W, 11.5° S, 75 m depth). Known marine picoplankton species are indicated in red. Numbers at nodes represent the bootstrap percentages from 100 replicates. Values below 50% are not shown. The scale bar corresponds to a distance of 10 substitutions per 100 nucleotide positions.

OLI11001, OLI11511, OLI11011, OLI11029 and OLI11033) that cannot be assigned to any known eukaryotic taxonomic group (Fig. 1). It seems to be a close relative of both the extant dinoflagellate lineage and the Perkinsozoa[18], a newly established phylum regrouping the marine parasites *Perkinsus* and *Parvilucifera*, of which the latter infects marine dinoflagellates. The monophyly of the clade combining this environmental lineage and the dinoflagellates was not strongly supported by the bootstrap value (60%). This clade could be related to the heterotrophic species *Oxyrrhis*, currently viewed as a "pre-dinoflagellate"[19]. However, because no 18S rDNA sequence is yet available for this taxon, it is not possible to verify this hypothesis.

Among purely heterotrophic protists, acantharians are typical blue-water organisms abundant in temperate and subtropical regions of the world oceans that are important in the global oceanic budget of elements such as strontium and barium[20]. Clones OLI11015, OLI11016 and OLI11032 cluster with known acantharian sequences (Fig. 1). Clone OLI11015 matches very well (99.2% identity) an arthracanthid sequence from the Atlantic Ocean[9] and groups together with clones OLI11016 and *Haliommatidium*. Both clone sequences match perfectly the two acantharian probes designed in a previous study[11], whereas clone OLI11032 matches only one of these acantharian probes. However, there is a strong bootstrap support for the monophyly of the clade including the clone OLI11032 and the acantharians (100%). The vegetative cells of acantharians are usually much larger than picoplankton. However, the sequences are probably from small swarmers that fall in the size range 3–6 μm (ref. 21).

The last set of sequences (OLI11041 and OLI11013) cluster with the heterotrophic choanoflagellates, which are the closest known relatives to sponges. Clone OLI11041 is related to the naked Codosigidae (*Monosiga*) and OLI11013 to the marine Acanthocidae (*Acanthocorbis*), which display delicate silicified lorica.

Although the diversity of prokaryotes has been the focus of much work with molecular methods during the past decade[7], quite surprisingly there has been no investigation of eukaryotic diversity in a similar manner[8]. The sequences, which were retrieved from a single sample, collected at the bottom euphotic layer of the Pacific Ocean, suggest that many new eukaryotic lineages remain to be uncovered from the marine environment, especially within the smallest size class. The genuineness of the new lineages found in this study, such as that related to both dinoflagellates and Perkinsozoa, is demonstrated by the fact that they are represented by several different sequences. Nevertheless, this diversity probably constitutes only the tip of the iceberg for three reasons. First, the primers that we used do not target all protists (for example, Chrysophytes or some Apicomplexa). Second, we restricted ourselves to picoplankton (cells smaller than 3 μm), and diversity is probably greater for larger cells[22]. Last, all sequences originated from a single sample taken at depth in a relatively oligotrophic area: surface waters or more mesotrophic regions probably harbour different organisms. Some species, such as *P. calceolata*, might constitute ubiquitous oceanic 'weeds' in much the same way as the photosynthetic prokaryote *Prochlorococcus*[3], whereas others might be restricted to very well defined niches. This might be true of potentially parasitic species, as suggested by the wide diversity of sequences within the clades found between dinoflagellates and Perkinsozoa (Fig. 1).

From the phylogenetic point of view, in many cases the new sequences form distinct clades with regard to organisms known from cultures (for example, OLI11345 and OLI11305 within the Prasinophyceae, or OLI11008 and OLI11026 within the stramenopiles). They might therefore shed light on the evolution of these key protist phyla, as well as for oceanic groups, such as the acantharians, that cannot be maintained in culture. From the ecological point of view, these data suggest that a large fraction of eukaryotic picoplankton is heterotrophic, because 24 of the 35 sequences retrieved belong to heterotrophic lineages, some of which are probably parasitic (for example, the dinoflagellate lineage containing *Amoebophrya* and the new lineage between dinoflagellates and the Perkinsozoa). This importance of parasitism might require a revision of our current views on the structure of marine microbial food webs, which consider oceanic heterotrophs mainly as phagotrophic predators. As soon as more eukaryotic sequences from a variety of environment become available, it will become possible to design oligonucleotide probes[23] that will enable us to determine the morphology and ecological role of these unknown protists. □

Methods

Picoplankton collection, genomic DNA extraction and PCR amplification of rDNA

Picoplankton was collected at 75 m depth from nutrient-depleted water of the equatorial Pacific Ocean (150° W 11.5° S) during the OLIPAC cruise in November 1994. The water sample was pre-filtered through Nuclepore PE filters 47 mm in diameter with a pore size of 3 μm; 1 litre of the filtrate was then collected on a glass-fibre filter (Whatman GF/F), which was put into a cryovial filled with lysis buffer (0.75 M sucrose, 400 mM NaCl, 20 mM EDTA, 50 mM Tris–HCl (pH 9.0)), immediately frozen in liquid nitrogen and stored at −80°C. Frozen filters were thawed, then homogenized in cold DNA extraction buffer (10 mM Tris–HCl (pH 8.0); 0.1 M EDTA (pH 8.0); 0.5% SDS). Total genomic DNA was extracted and the 18S rRNA genes were amplified by PCR (30 cycles) with the oligonucleotide primers (5′-ACCTGGTTGATCCTGCCAG-3′, 5′-TGATCCTTCYGC AGGTTCAC-3′) complementary to regions of conserved sequences close to the respective 5′ (*Escherichia coli* position 7) and 3′ (*E. coli* position 1534) termini of the 18S rRNA gene[14]. Although these primers recognize most protists, some of the 18S sequences found in the public databases display one or more mismatches. This is true, in particular, for the following groups: Chrysophyceae, some Chlorarachniophyceae, a few Apicomplexa (*Plasmodium*), some Ciliophora (*Euplotes*), and primitive eukaryotes such as Metamonada or Euglenazoa.

Gene cloning and colony hybridization

PCR products were purified with the Geneclean II kit (Qiagen). The ends of the amplified DNA fragments were modified for blunt-ended ligation with T4 kinase and polymerase. The blunt-ended 18S rRNA genes were purified again and inserted into the calf-intestinal-phosphatase-treated *Hin*cII restriction site of the pGEM3Zf(−) plasmid vector (Promega) and transformed into *E. coli* DH5-α cells. Inserted 18S rRNA genes were screened by colony hybridization with a eukaryote probe (5′-GGGCATCACAGACCTG-3′)[24] labelled with fluorescein-11-dUTP by using the enhanced chemiluminescence (ECL) 3′-oligo-labelling kit (Amersham). Selected recombinants were grown on a nylon membrane disc, Magna Lift (Micron Separation Inc.) placed on Luria–Bertani agar. A clone containing the plasmid vector was applied to the membrane as a negative control. Lysis of cells, DNA denaturation, and removal of bacterial debris were performed as described[14]. The membrane was air-dried and baked at 80 °C for 2 h. Prehybridization was performed at 42 °C for 30 min in hybridization buffer containing 5 × SSC, 0.1% (w/v) hybridization buffer component (from the ECL 3′-oligolabelling and detection kit), 20-fold dilution of liquid block (from the same kit) and 0.02% SDS. Hybridization with the oligonucleotide probes (5–10 ng ml^{-1}) was achieved at 42 °C for 12–17 h. Post-hybridization wash conditions included two subsequent washes (15 min each) with 0.1 × SSC, 0.1% SDS at 42 °C. Filters were exposed to a blue-light-sensitive autoradiography film (Hyperfilm-ECL; Amersham) to detect the bound probes, in accordance with the manufacturer's instructions (Amersham).

Sequencing and phylogenetic analysis

A total of 103 clones with a rDNA insert from an 18S rDNA clone library were randomly selected. Plasmid DNAs were purified with the FlexiPrep kit (Pharmacia). The rDNA of each clone was partly sequenced (nucleotide positions 1317–1822 on *Artemia salina*). A sequence identity search with the EMBL databank confirmed that all clones were eukaryotic. Because partial sequences did not provide sufficient information for an unequivocal taxonomic identification, 50 representative variants of the clones were screened on the basis of the partial sequence comparison, and the 18S rRNA genes were completely sequenced. Nucleotide sequences were determined for both strands as described previously[14]. Sequence identity searches were conducted with the program FASTA. Secondary structures of environmental sequences fitting the model described in detail elsewhere[25] were determined (data available from the authors) by aligning each sequence with the most similar one already present in a database of 18S rRNA sequences[26], transposing the secondary structure pattern onto the newly aligned sequence, and making slight adjustments where necessary with the nucleotide sequence editor DCSE[27]. On the basis of the evaluation by the CHECK_CHIMERA program of the Ribosomal Database Project[28], analyses of phylogenetic affiliations for separate sequence domains, and predicted secondary structures, 36 of the 50 complete sequences, which showed no evidence for potential chimaeric gene artefacts, were analysed. One clone showed a sequence identity of 99% to a higher plant, *Musa acuminata*. This sequence and the potential chimaeric gene sequences were not considered further. The environmental sequences were aligned with 111 sequences from a range of eukaryotic evolutionary lineages (accession numbers available from the European small subunit ribosomal RNA database[26]) by using the CLUSTALX 1.64 program[29], with manual refinement based on a

consideration of primary and secondary structures. Sequence positions for which putative homology could not be asserted were excluded. Phylogenetic relationships are inferred by the neighbour-joining method with the PHYLIP package, version 3.5c (ref. 30). Evolutionary distances were calculated with the Kimura two-parameter model (transition:-transversion ratio = 2.0). Bootstrap methods provided confidence estimates for tree topology. To avoid potential bias, taxon addition order was randomized.

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Parallel adaptive radiations in two major clades of placental mammals

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Higher level relationships among placental mammals, as well as the historical biogeography and morphological diversification of this group, remain unclear[1–3]. Here we analyse independent molecular data sets, having aligned lengths of DNA of 5,708 and 2,947 base pairs, respectively, for all orders of placental mammals. Phylogenetic analyses resolve placental orders into four groups: Xenarthra, Afrotheria, Laurasiatheria, and Euarchonta plus Glires. The first three groups are consistently monophyletic with different methods of analysis. Euarchonta plus Glires is monophyletic or paraphyletic depending on the phylogenetic method. A unique nine-base-pair deletion in exon 11 of the *BRCA1* gene provides additional support for the monophyly of Afrotheria, which includes proboscideans, sirenians, hyracoids, tubulidentates, macroscelideans, chrysochlorids and tenrecids. Laurasiatheria contains cetartiodactyls, perissodactyls, carnivores, pangolins, bats and eulipotyphlan insectivores. Parallel adaptive radiations have occurred within Laurasiatheria and Afrotheria. In each group, there are aquatic, ungulate and insectivore-like forms.

The combination of fossil and anatomical data has suggested moderately well-resolved phylogenetic trees for the 18 extant orders of placental mammals[1,2]. DNA sequences have provided consistent support for only a few of the proposed superordinal groups, notably for Paenungulata (elephants, sea cows and hyraxes) and Cetartiodactyla (artiodactyls and whales)[3], and have rejected some traditional clades such as Archonta (primates, tree shrews, flying lemurs and bats)[4]. Molecular data have also suggested new sets of relationships; Afrotheria, a group that includes paenungulates, aardvarks, elephant shrews, golden moles and tenrecs, is supported by both mitochondrial ribosomal RNA and nuclear protein-coding genes[5,6]. These same sequences suggest that lipotyphlan insectivores are paraphyletic or polyphyletic.

Increased resolving power may result from concatenations of individual genes[7]. We concatenated DNA sequences for mitochondrial RNA genes and three nuclear genes (A2AB, IRBP, vWF), including 16 new sequences (see Methods). This data set includes 26 placental taxa, representative of all eutherian orders, and two marsupial outgroups (see Fig. 1A). In addition, golden mole and tenrec, belonging to the insectivore families Chrysochloridae and Tenrecidae, respectively, are represented by sequences for all genes