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# Picobiliphytes: A Marine Picoplanktonic Algal Group with Unknown Affinities to Other Eukaryotes

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Environmental sequencing has revealed unimagined diversity among eukaryotic picoplankton. A distinct picoplanktonic algal group, initially detected from 18S ribosomal DNA (rDNA) sequences, was hybridized with rRNA  $\lambda$ -targeted (rRNA-targeted) probes, detected by tyramide signal amplification–fluorescent in situ hybridization, and showed an organelle-like body with orange fluorescence indicative of phycobilins. Using this fluorescence signal, cells were sorted by flow cytometry and probed. Hybridized cells contained a 4',6'-diamidino-2-phenylindole–stained organelle resembling a plastid with a nucleomorph. This suggests that they may be secondary endosymbiotic algae. Pending the isolation of living cells and their formal description, these algae have been termed picobiliphytes.

Molecular tools applied to DNA retrieved from marine microorganisms have revealed considerable diversity among the smallest eukaryotic cells (1–3), paralleling that found among marine prokaryotes. Together with a high taxonomic diversity, the finding of many sequences unrelated to those of known organisms was an additional striking feature of these first studies. Clone libraries for the eukaryotic 18S ribosomal RNA (rRNA) gene were constructed at different times from fractionated water samples (using a filter pore size of 3  $\mu$ m) from three coastal sites (4–6), and additional libraries were established from three more open-water sites (7, 8) (table S2). A particular group of sequences was recovered irregularly throughout the year (8) (table S2) and referred to as the “Rosko II” group from partial 18S sequence phylogenies from these sites (4–6). Analyses of full-length sequences (8) reveal that they form an independent phylogenetic group among major eukaryotic taxa (Fig. 1), (9, 10), which we have tentatively called picobiliphytes. Our complex iterative Bayesian analyses (8) indicate that the picobiliphytes are an independent lineage, possibly having a weak sister relationship with the cryptophyte/katablepharid clade, although its true sister group is difficult to assign using a single gene phylogeny. The inability to assign an affinity of the picobiliphytes to any other major eukaryotic group (table S1) in the

eukaryotic 18S rDNA tree was confirmed with the Kashino-Hasagawa test (8) (table S3). Their deep branching suggests that they probably deserve a taxonomic rank of division or phylum.

Picobiliphytes consist of at least three different clades (Fig. 1), for which we were able to identify two signature sequences: PICOBIO1 (5'-GCGTGATGCCAAAATCCG-3') and PICOBIO2 (5'-ATATGCCCGTCAAACCGT-3'), which target most picobiliphytes (tables S4 and S5). They have two or more mismatches with all available GenBank sequences from cultivated protists (tables S4 and S5) and do not display any fluorescence when hybridized to a variety of algal strains from the Roscoff Culture Collection (8, 11) (table S6). In addition, they match a set of five additional environmental 18S rDNA partial sequences: four from the western North Atlantic (12) and one from a mid-Atlantic estuary (Barnegat Bay, New Jersey), extending the possible distribution of the picobiliphytes. These probes enabled us to determine, by microscopy after tyramide signal amplification–fluorescent in situ hybridization (TSA-FISH) (13), the gross morphology of fixed cells from the Roscoff coastal site (Fig. 1 and fig. S1). The morphology of other unknown marine protist groups was also determined by Massana *et al.* (14), using probe methods.

Picobiliphytes are unicellular, slightly oblong, and approximately 2  $\times$  6  $\mu$ m ( $n = 9$  cells) and were recovered in the picoplankton size fraction of our water samples because they probably passed through the 3- $\mu$ m pores in the filter by way of their smallest dimension. Thus, we have referred to them as picoplankton. One remarkable feature is the presence of an organelle-like structure having orange autofluorescence when excited with blue light under epifluorescence microscopy (Fig. 1), a structure similar to that of phycobiliprotein-containing rhodophytes and cryptomonads (fig. S1). These pigments, in contrast to chlorophylls, are water-soluble (15) and thus not removed by the TSA-FISH alcohol dehydration steps. Moreover, any

chlorophyll remaining after alcohol dehydration fluoresces yellow, not orange, under blue light (fig. S1). Thus, picobiliphytes probably have a phycobiliprotein-containing organelle, most probably a plastid. Another distinctive feature is a small body that is stainable with the nucleic acid–specific dye DAPI (4', 6'-diamidino-2-phenylindole), distinct from the main nucleus and consistently seen in close proximity to the presumed plastid (Fig. 1, fig. S1).

Picobiliphyte sequences have been found in a variety of marine systems, including the European coast (8), the North Atlantic (from GenBank Blast searches), and the Arctic Ocean (7). A detailed look at their abundance, applying TSA-FISH in size-fractionated (<3  $\mu$ m) seawater samples from the English Channel, revealed that picobiliphytes occurred mostly in fall and winter and were not detected by FISH in summer, although their sequences were occasionally detected in summer clone libraries (tables S2 and S8). Their concentration, up to 80 cells ml<sup>-1</sup>, accounted for about 1.6% of the total picoeukaryote cell counts at one coastal station in the English Channel and corresponded to a major proportion (33 to 81%) of orange-fluorescing picoeukaryotic cells detected by blue laser flow cytometry (tables S7 and S8). In one particular sample, cells exhibiting this fluorescence were sorted by flow cytometry and subsequently hybridized by TSA-FISH with our two probes (8) (table S7). We found that 48 to 61% of the sorted cells were labeled with probes PICOBIO1 and PICOBIO2, suggesting that the picobiliphytes may constitute a substantial proportion of the orange-fluorescing eukaryotic picoplankton previously thought to be cryptophytes (16). The fact that our cells could have been sorted and enriched with a phycobilin pigment signature detected with flow cytometry further supports the contention that they actually possess such pigments (15, 16).

The inferred presence of a phycobiliprotein-containing plastid in picobiliphytes is in good agreement with their putative sister relationship to cryptophytes and katablepharids, the first of which contain phycobiliproteins. Whereas cryptophytes are common in the marine nanophytoplankton, pico-sized cryptophytes are not as abundant, as judged by their relative frequency in clone libraries; and where found, their 18S rDNA sequence places them as an independent lineage within the nano-sized cryptomonads (5, 6). There are also small cell forms among the red algae, such as the marine Porphyridiales, but our group does not belong to the rhodophytes, based on our phylogenetic analysis. Cryptophytes are a well-known example of a secondary endosymbiosis of a rhodophyte, which brings phycobilin pigments to the new host cell. Because picobiliphytes are sister to the cryptophyte/katablepharid clade in most of our complex Bayesian analyses (8) (Fig. 1), it would be most parsimonious to assume that our group is a secondary endosymbiotic alga. The small

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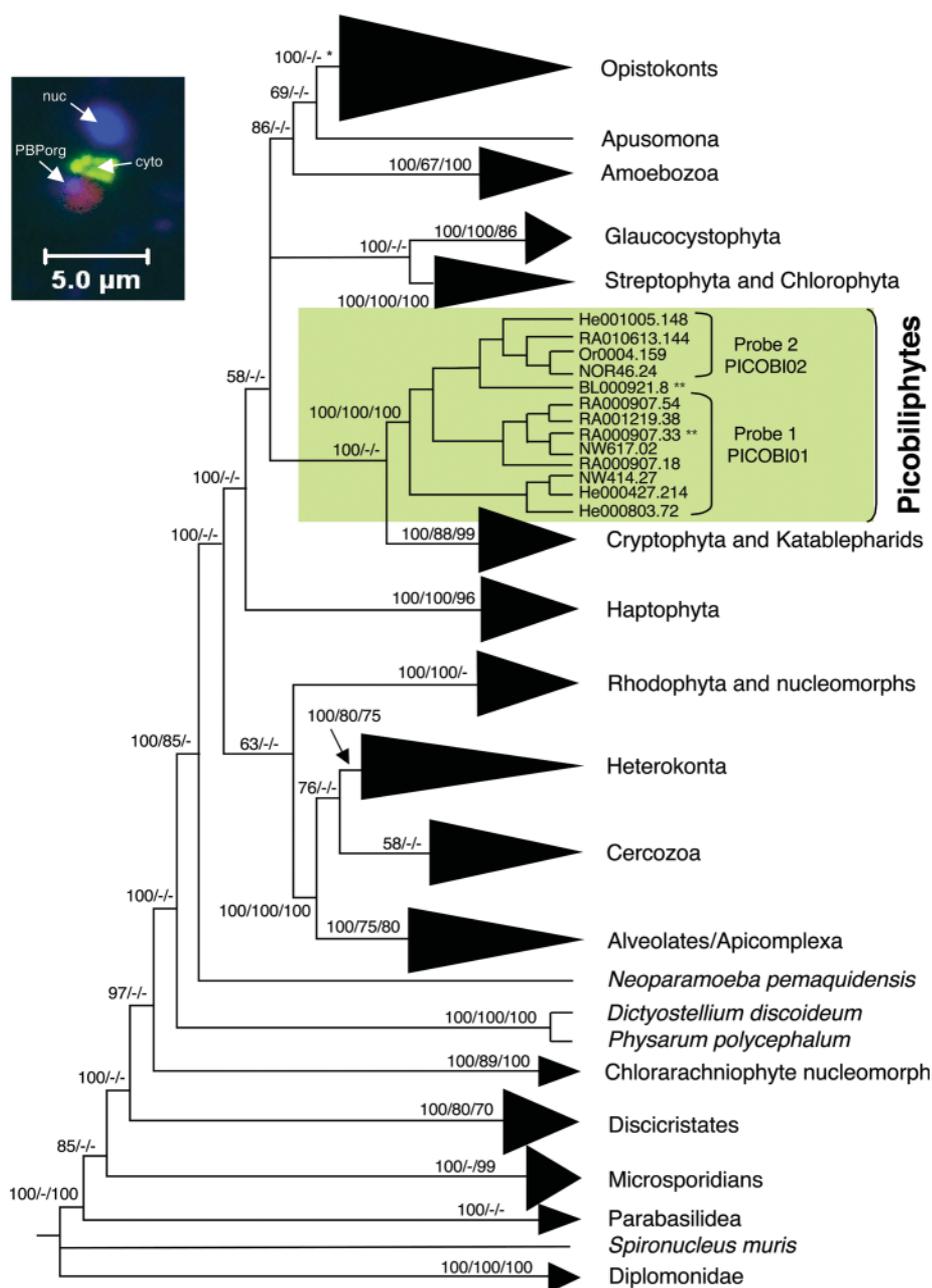
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body stainable with the nucleic acid-specific dye DAPI (Fig. 1) may be a DNA-containing nucleomorph, similar to that found in crypto-

phytes and chlorarachniophytes (17), supporting the idea that picobiliphytes are another secondary endosymbiotic algal group (18).



**Fig. 1.** Phylogenetic trees were reconstructed from full-length 18S rRNA sequence data listed in table S1 and inferred with Bayesian analysis from two parallel runs, each with one million generations with six chains and increased temperature between the chains to facilitate exchange between the chains (8). This tree is the 50% majority-rule tree of the last 100 trees saved from one of the parallel runs. Support for each node was also determined with 100 replicated bootstrap analyses of weighted maximum parsimony and neighbor-joining analyses. Nodes supported by bootstrap or posterior probability values above 50% are labeled for the three methods used (MrBayes/maximum parsimony/neighbor-joining). If a clade was not supported by a method, it is indicated by a dash. The asterisk indicates that internal major clades were supported by 100 posterior probabilities from the MrBayes analysis. PICOBIO1 and PICOBIO2 are specific for the sequences belonging to the three clades as bracketed. (Insert) Picture of a cell targeted by the probe PICOBIO2 (specific for picobiliphyte clade 2) from the Roscoff ASTAN sampling site on 26 September 2001. Arrows point to the DAPI-stained nucleus (nuc) in blue, to the green fluorescence from probe-specific labeling of the small subunit rRNA in the cytoplasm (cyto), and to the red autofluorescence from the phycobiliprotein-containing organelle (PBPorg). Double asterisks indicate sequences not recognized by the probes.

Kleptoplastidy is another possibility, such as in the katablepharids (19, 20), which along with the cryptophytes are the picobiliphytes' purported sister group. However, kleptoplastidy is unlikely in such small organisms. In the absence of living cells to follow through cell division, we screened filtered 3- $\mu$ m-fractionated water for cells that hybridized with our probes, using a ChemScan solid-phase cytometer (8) (fig. S2). We never encountered positive cells without a plastid on the filters scanned by the laser, which implies that the cells are predominately pigmented, so kleptoplastidy does not seem very likely.

Are the picobiliphytes representatives of another red algal secondary endosymbiosis, such as chromo-alveolates, in the broad sense, or do they have kleptoplastids? Without living cells, the status of their endosymbiosis and a formal description will remain unresolved. Nevertheless, picobiliphytes are pigmented and thus contribute to primary production. Molecular analysis confirms that they are a eukaryotic group that should be recognized at the phylum or division level, without any real indication of their sister group. We found that they are well represented in polar and cold temperate coastal marine ecosystems, as judged from their appearance in clone libraries and preliminary FISH data. The putative presence of a DNA-containing body in the purported plastid places them in an intriguing position in the study of plastid reduction to organelles.

Within the past 15 years, four algal classes have been described from the picoplankton [see (5) for details], and picobiliphytes represent another division or phylum. The phylogenetic analysis indicates that they are a highly diverse group, composed of at least three distinct clades. The temporal and spatial scales at which they occur, as inferred from molecular data, indicate that they could make up a substantial picoplankton fraction under certain conditions. The existence of small, sometimes rare, organisms is only now being recognized, and their role in ecosystem function is unknown, but they probably act as reservoirs of genetic capacity that are activated under specific conditions. The discovery of picobiliphytes and their apparent widespread distribution and contribution to marine protist assemblages highlight the imperative of understanding biodiversity before its loss on a global scale.

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discussions about appropriate analytical methods and to S. Frickenhaus for establishing parallel processing and implementing the complex Bayesian analyses. All authors discussed the results and commented on the manuscript. Full-length sequences have been deposited at GenBank with the accession numbers EF050072, AY426835, DQ222872 to DQ222800, DQ060523, and DQ0605236. The authors declare no competing financial interests.

#### Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 and S2

Tables S1 to S8

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