Global Dispersal and Ancient Cryptic Species in the Smallest Marine Eukaryotes

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Small eukaryotic species (<1 mm) are thought to behave as prokaryotes in that, lacking geographical barriers to their dispersal due to their tiny size, they are ubiquitous. Accordingly, the absence of geographical insulation would imply the existence of a relatively small number of microeukaryotic species. To test these ideas, we sequenced and compared several nuclear, mitochondrial, and chloroplast genes from the isolates of a marine picoeukaryotic alga (~2 μm), *Micromonas pusilla,* collected worldwide. Independent and combined phylogenetic analyses demonstrate that this traditional single morphospecies actually comprises several independent lineages, some of which are shown to be ubiquitous in oceans. However, while some lineages group closely related strains, others form distant clusters, revealing the existence of cryptic species. Moreover, molecular dating using a relaxed clock suggests that their first diversification may have started as early as during the Late Cretaceous (~65 MYA), implying that "*M. pusilla*" is the oldest group of cryptic species known to date. Our results illustrate that global dispersal of a picoeukaryote is possible in oceans, but this does not imply a reduced species number. On the contrary, we show that the morphospecies concept is untenable because it overlooks a large genetic and species diversity and may lead to incorrect biological assumptions.

Introduction

The smallest eukaryotes are widely represented in oceanic waters, although their diversity, as revealed by molecular surveys, appears to be very large and far from being completely understood (Palumbi 1994; Díez, Pedrós-Alió, and Massana 2001; López-García et al. 2001; Moon-van der Staay, De Wachter, and Vaulot 2001; Finlay 2002). Eukaryotic picoplankton (<2–3 μm) plays an essential role in primary production and accounts for an important biomass fraction of the euphotic zone (Stockner 1988). Among photosynthetic picoplanktonic eukaryotes, members of the Pelagophyceae (Heterokonta), the Prymnesiophyceae (Haptophyta), and the green algal class Prasinophyceae (Chlorophyta) are abundant. Prasinophytes generally display minute cell sizes and simple morphologies, as exemplified by *Ostreococcus tauri* (0.8–1.1 μm diameter), the smallest eukaryote known (Courties et al. 1994). Few prasinophyte species have been described, and they are considered ubiquitous. Among them, *Micromonas pusilla,* the first picoplanktonic species ever described (Butcher 1952), constitutes an exceptional model to test assumed ideas of picoeukaryote limited species number and global dispersal in the ocean. The minute sizes and large populations of most microbial species would greatly facilitate their dispersal because they would not be constrained by effective geographical barriers. Consequently, speciation by geographical isolation would not take place, resulting in both ubiquitous species and low species number (Finlay 2002).

Micromonas pusilla is a pear-shaped, solitary small green alga, usually not exceeding 2 μm, which bears one flagellum and contains one mitochondrion and a single chloroplast. It has been identified worldwide in oceans (Throndsen 1970; Not et al. 2004), and it is well represented in culture collections (table 1). To explore the genetic diversity within the morphospecies *M. pusilla,* we carried out multilocus genotyping of 17 isolates from different oceanic regions (Pacific, Atlantic, Indian, and Mediterranean) available in culture collections (table 1) by sequencing five major loci from the three genomes coexisting in the cell (nuclear, chloroplastic, and mitochondrial). We provide the first genetic evidence of the ubiquity and the oceanic dispersal for *M. pusilla*. However, we also show that *M. pusilla* is a complex of several morphologically indistinguishable cryptic species that diverged very long time ago.

Key words: multilocus genotyping, microbial speciation, biogeography, ubiquity, molecular dating.

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Materials and Methods

Algal Strains

Strains of *M. pusilla* (Butcher 1952) from Pacific (3 strains), Atlantic (10 strains), Indian (2 strains), and Mediterranean (2 strains) regions were obtained from the following algal culture collections (table 1): Commonwealth Scientific and Industrial Research Organization, Australia (http://www.marine.csiro.au/algaedb/default.htm); The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), United States (http://ccmp.bigelow.org/); The North East Pacific Culture Collection (NEPCC)–The Canadian Center for the Culture of Microorganisms, Canada (http://www.botany.ubc.ca/cccm/); and Marine Biotechnology Institute Culture (MBIC), Japan (http://seasquirt.mbio.co.jp/mbic/).

DNA Purification, Polymerase Chain Reaction Amplification, Cloning, and Sequencing

Cells were lysed in the presence of 80 μg ml^{-1} proteinase K, 1% sodium dodecyl sulfate, 1.4 M NaCl, 0.2% β-mercaptoethanol, and 2% cetyltrimethylammonium bromide (final concentrations) at 55°C. DNA was then extracted twice with phenol-chloroform-isoamylalcohol and once with chloroform-isoamylalcohol. Nucleic acids were concentrated by ethanol precipitation. The nuclear small-subunit ribosomal DNA (SSU rDNA) and internal transcribed spacer ribosomal DNA (ITS rDNA), comprising a first internal transcribed spacer (ITS1), the 5.8S rDNA, and a second internal transcribed spacer (ITS2) region immediately upstream the large subunit 28S rDNA (LSU rDNA), were amplified using the specific eukaryotic primers

Table 1
***Micromonas pusilla* Strains Used in This Work**

Strain	Collection Site	Coordinates	Collection Date	Cell Size (μm)	Growth Temperature (°C)
CCMP488	Sargasso Sea, Oceanus Cruise 83, Station I (20–25 m depth)	34° N - 65° W	July 7, 1980	1–2 × 1–2	22–26
CCMP489	Sargasso Sea, Endeavor Cruise (120 m depth)	28° 58′ 59.9″ N - 64° 22′ 0.1″ W	May 30, 1988	1.2–2.4 × 1.2–1.6	22–26
CCMP490	Nantucket Sound, Woods Hole, Mass.	41° 31′ 30″ N - 70° 40′ 25″ W	June 18, 1964	1.4–2.7 × 1.3–1.8	22–26
CCMP491[a]	English Channel, near Plymouth, Devon, United Kingdom	50° 21′ 43.2″ N - 4° 10′ 12″ W	January 1, 1950	1–2 × 1–2	17–26
CCMP492	Gulf Stream, Oceanus Cruise 83, Station II, North Atlantic (20–25 m depth)	38° 42′ 7.2″ N - 72° 22′ 0.1″ W	July 11, 1980	1–2 × 1–2	22–26
CCMP493	Gulf of Mexico, North Atlantic	25° N - 90°W	February 15, 1981	1–2 × 1–2	22–26
CCMP494	Gulf of Maine, Eastward Cruise	43° N - 69°W	1980	1–2 × 1–2	22–26
CCMP1195	Bigelow Lab Dock, West Boothbay Harbor, Gulf of Maine, Maine	43° 50′ 38.8″ N - 69° 38′ 28.7″ W	November 19, 1986	1–2 × 1–2	11–21
CCMP1545[a]	English Channel, near Plymouth, Devon, United Kingdom	50° 21′43.2″ N - 4° 10′ 12″ W	January 1, 1950	2–3 × 2–4	18–22
CCMP1646	Gulf of Naples, Italy	40° 45′ N - 14° 19′ 48″ E	April 8, 1993	2–3 × 2–3	18–22
CCMP1723	Sicily Channel, open Mediterranean Sea	37° 12′ N - 11° 24′ E	NA	2–3 × 2–3	17–22
CCMP1764	Gulf of Panama, 1/2 mile off Samba River, Darien, Panama	8° 30′ N - 78° 13′ 12″ W	March 1, 1997	1–2 × 1–2	18–22
CCMP2099	Baffin Bay, Canadian Coast Guard Ice Breaker Pierre Radisson (55 m depth)	76° 16′59.9″ N - 74° 45′ W	April 7, 1998	2–3 × 1–3	0–6
CS170	North West Shelf, Soela Voyage SO6/82, Western Australia, Australia	18°–21°S - 116°–119° 30′ E	January 12, 1982	NA	25
CS222	Corio Bay (near Melbourne), Victoria, Australia	38° 6′ S - 144° 36′ E	January 1, 1988	NA	18
MBIC10095	International waters, Pacific Ocean	NA - NA	NA	NA	22
NEPCC29	Jericho Beach, Vancouver, BC, Canada	48° 18′ N - 123° 18′ W	NA	NA	NA

[a] CCMP491 and CCMP1545 derive from the same original strain but have been maintained independently since 1992; NA, not available.

18S-42F (5′-CTC AAR GAY TAA GCC ATG CA-3′) and 28S-1R (5′-ATG CTT AAA TTC AGC GGG T-3′) (López-García et al. 2001). β-tubulin genes were amplified with the primers Btub-A (5′-GCA GGN CAR TGY GGN AAY CA-3′) and Btub-B (5′-AGT RAA YTC CAT YTC RTC CAT-3′) (Edgcomb et al. 2001). The chloroplast RuBisco *rbcL* genes were amplified with primers rbcL-F1 (5′-GCT GTA GCA GCT GAG TCA TC-3′) and rbcL-R1 (5′-GGC ATT ACG CCA GGA AGA G-3′). Mitochondrial genes encoding the cytochrome c oxidase subunit I (*coxI*) were amplified with cox-F1 (5′-TTY TTY GGN CAY CCN GAR GTN TA-3′) and CX1BS1 (5′-GGI ACI GGI TGG ACI YTI TAY CCI CC-3′) (Watanabe et al. 1998). Polymerase chain reactions (PCRs) were carried out in 25 μl of reaction buffer containing DNA template (10–100 ng), 1.5 mM $MgCl_2$, deoxynucleoside triphosphates (10 nmol each), and 20 pmol of each primer. Amplification was performed using proofreading *Pfu* DNA polymerase (Stratagene, Amsterdam, the Netherlands) and *Taq* DNA polymerase (Promega, Charbonnieres, France) for 35 cycles with a denaturing step at 94°C (30 s), a primer annealing step at 52°C (60 s), and an extension step at 52°C (60–180 s). PCR products were cloned using the TOPO-TA cloning kit (Invitrogen, Leek, the Netherlands) following the manufacturer's instructions. Inserts of the expected size were sequenced directly using either specific or vector primers by Genome-Express (Meylan, France). For each strain, a minimum of three clones, including multiple PCR and cloning experiments, were sequenced from both directions for cross-checking. Strains CCMP1545 and CCMP491 are derivatives of the reference strain PLY27 isolated in 1950. They exhibit identical nucleotide sequences for all genes despite their separate curation at CCMP, thus serving as an internal control to our experimental approach. The sequences reported in this paper have been deposited in the GenBank database (accession numbers AY954993–AY955063).

Phylogenetic Reconstruction

Nucleotide sequences were automatically aligned using ClustalW 1.83 (Chenna et al. 2003). Hypervariable regions in rDNA and introns were realigned with relaxed gap opening and extensions and manually verified. Individual and concatenated marker trees were reconstructed by minimum evolution (ME), maximum likelihood (ML), and Bayesian methods. ME was based on Kimura-2 parameter distances with 1,000 bootstrap replicates using MEGA3 (Kumar, Tamura, and Nei 2004). ML was based on the GTR + Γ + I nucleotide model with 100 bootstrap replicates using PhyML 2.4.4 (Guindon and Gascuel 2003). Bayesian reconstruction was run on four chains at 0.2 heating with the GTR + Γ + I model using MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003). Parameters of the models were independent over individual noncoding and coding regions in concatenated analyses, including relaxing parameters of first, second, and third codon positions. Bayesian analyses were run repeatedly from random trees and tested for convergence.

Detection of Recombination Events

The recombination events were mapped using the Recombination Detection Program RDP-V2 (Martin, Williamson, and Posada 2005). With a *P* value of 0.05, the RDP, BootScan, SiScan, and GENECONV methods identified multiple potential recombination events within the β-tubulin exons, particularly between residues 340–650 and 337–659 (A and B lineages and CCMP494 + MBIC10095) and 378–816 and 315–687 (C lineage and CCMP494 + MBIC10095).

Molecular Dating

Divergence times were estimated using the program r8s 1.7 (Sanderson 2003), taking into account the penalized likelihood (PL) method and the truncated Newton algorithm. This method is clock independent and estimates divergence times by allowing different branches to have different rates of evolution (Sanderson 2002). The Bayesian consensus tree and the credible set of Bayesian trees were used to estimate the divergence dates. The sequence alignment used consisted of concatenated SSU rDNA, *coxI*, and *rbcL* data sets from Viridiplantae, rooted with the diatom *Rhizosolenia setigera* and the red alga *Cyanidioschyzon merolae*. The alignment with 54 taxa and 3,058 characters was partitioned considering genes and codon positions. The hypervariable third codon position of *coxI* was excluded. Out-group was pruned prior to date estimation. The optimal smoothing parameter determined by a cross-validation was set to 1, permitting the rate variation. To avoid local optima, five random starting values and restarts were permitted. Four nodes were constrained/fixed (Gillespie, Rothwell, and Scheckler 1981; Crane, Friis, and Pedersen 1995; Kenrick and Crane 1997; Bowe, Coat, and dePamphilis 2000; Sanderson et al. 2004) in million years appearance of land plants (432 Myr), seed plant origin (355–370 Myr), split of gymnosperms and a lineage leading to angiosperms (290–320 Myr), and the monocot "versus" eudicot divergence (90 Myr). Using the Bayesian consensus tree, the *Micromonas* divergence was estimated to be 61 Myr. We estimated a time interval of 66 ± 10 Myr from the 95% credible tree interval (300 trees) of the Bayesian analysis. Relaxing the first appearance of land plants to 432–476 Myr and the monocot/eudicot divergence to 90–130 Myr had no significant effect on the first divergence of *Micromonas* estimate yielding 69.5 Myr.

Results and Discussion

To explore the genetic diversity within 17 *M. pusilla* strains collected worldwide, we amplified and sequenced the small-subunit rRNA gene (SSU rDNA), the rDNA ribosomal internal transcribed spacer (ITS including ITS1–5.8S–ITS2 rDNA), and the β-tubulin gene from the nuclear genome; the mitochondrial *coxI* encoding cytochrome c oxidase subunit I; and the chloroplast *rbcL* encoding the large subunit of ribulose 1,5-bisphosphate carboxylase, RuBisco. Sequence analysis revealed that the average genetic divergence varied greatly over each marker, from 1.5% for SSU rDNA to 16.9% for *coxI* (see Supplementary Material Tables S1 and S2). Interestingly, β-tubulin genes contained two introns, which provided additional, less-constrained regions for comparison. Their genetic divergence was sensibly higher (57% and 49%, respectively).

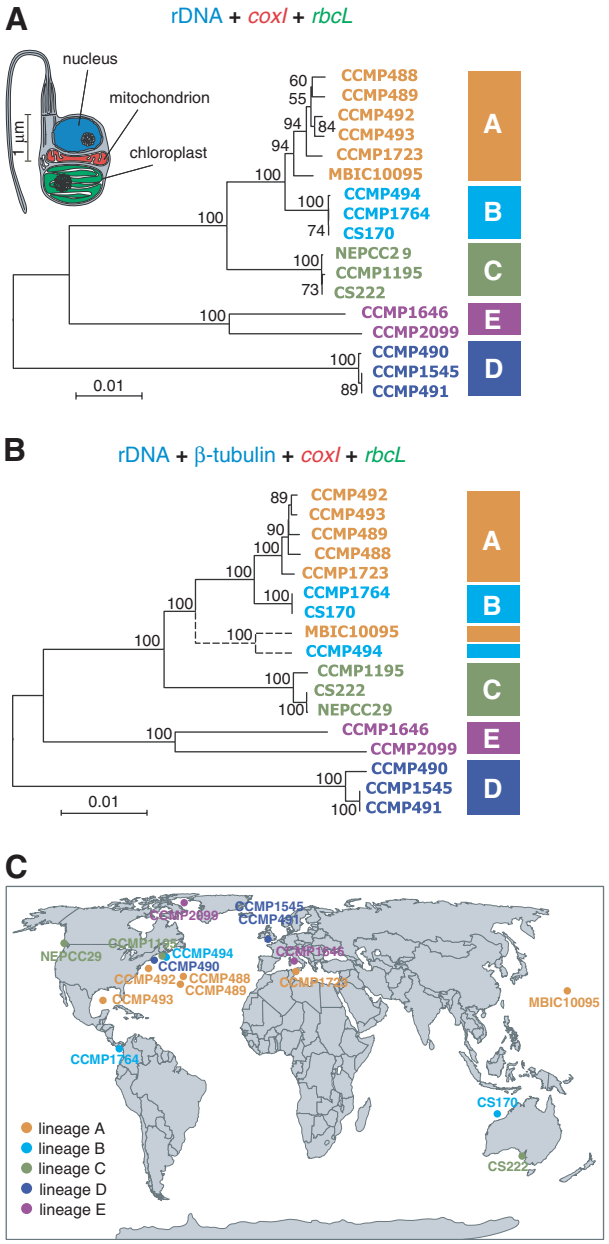


FIG. 1.—ME phylogenetic trees of concatenated markers and geographical origin of 17 *Micromonas pusilla* strains. A schematic drawing of *M. pusilla* is shown in the top left corner. Phylogenetic analysis for the concatenated rDNA + *coxI* + *rbcL* (*A*) and rDNA + β-tubulin + *coxI* + *rbcL* (*B*) sequences. rDNA includes SSU rDNA and ITS1–5.8S–ITS2 rDNA regions. Lineages A–E are indicated by color boxes. Dotted lines indicate putative recombinant strains for the β-tubulin gene (see text). (*C*) Geographical origin of the analyzed strains. Colors correspond to lineages A–E, as in the phylogenetic trees.

Multilocus Genotyping of *M. pusilla* Strains

We carried out phylogenetic analyses of individual as well as concatenated markers. In all cases, the individual phylogenetic trees revealed the existence of several distinct groups or lineages, here arbitrarily named A–E (fig. 1 and

Supplementary Material Fig. S1). All trees were congruent, showing the same five clusters, with a singular exception. It involved two strains that segregated from the lineages A and B to form a new cluster in the β-tubulin tree and also in the tree of the concatenated data set in which this marker was included (fig. 1*B*, and see below).

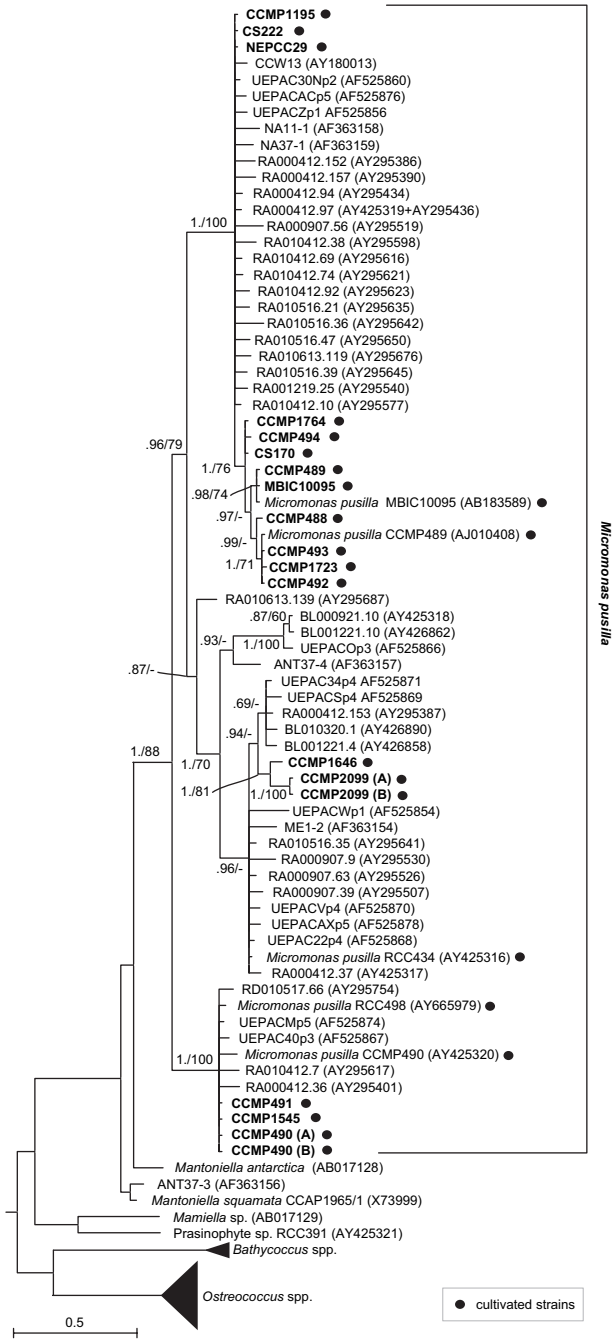
The lineage A appeared rather heterogeneous compared to its sister groups B and C (fig. 1 and Supplementary Material online). While several loci were identical within all strains in lineage A, some polymorphism was detected at other loci, with a maximum of 1.3% and 1.1% sequence divergence for ITS1 rDNA and *coxI*, respectively (Supplementary Material Table S2). An interesting case was that of members of the lineage C (fig. 1), comprising the strains NEPCC29 (Pacific), CS222 (Indian), and CCMP1195 (Atlantic). All three were identical for *coxI* and almost identical for *rbcL*. They exhibited only two nucleotide substitutions at this locus, and the strain CCMP1195 exhibits an AT insertion in the ITS1 rDNA. A few more substitutions were detected in CCMP1195 at the β-tubulin exons (19 substitutions), intron-1 (3 substitutions), and intron-2 (4 substitutions plus 2 deletions) by comparison with the remaining two strains. Even so, this lineage C showed the greatest similarity at the level of all individual loci among all surveyed strains. Therefore, it can be reasonably concluded that this lineage is ubiquitous in oceans.

An even more outstanding situation was that corresponding to isolates forming the lineage B (fig. 1 and Supplementary Material Fig. S1). Indeed, only two isolates, CS170 (Indian) and CCMP1764 (Pacific), both within lineage B, from our 17 strains exhibited identical sequences over all sequenced markers including, remarkably, the two highly variable intron sequences. Similarly, the strain CCMP494 (Atlantic), which clusters with them to form lineage B in all phylogenetic trees except that of β-tubulin and its concatenated derivative, displayed identical sequence for both organellar markers and showed only a single substitution over the entire SSU rDNA with respect to the other B strains. Therefore, the lineage B would represent the best genetic case demonstrating the oceanic ubiquity of a picoeukaryotic species (fig. 1 and Supplementary Material Fig. S1). However, it differed significantly at the nuclear β-tubulin locus, a protein-coding marker with a high conservation degree. The β-tubulin nucleotide tree was identical to that observed for the other markers, except for MBIC10095 and CCMP494. The two isolates which, in the rest of phylogenies, belonged to the clusters A and B, respectively, were placed apart forming an independent group. This pattern was also shown by the phylogenetic tree of the concatenated markers rDNA + *coxI* + *rbcL* + β-tubulin (fig. 1*B* and Supplementary Material Fig. S1). We cannot exclude that they might represent hidden divergent paralogues. However, in all cases only a single PCR product and identical sequence was obtained, despite multiple amplification with *Pfu* and *Taq* DNA polymerases, independent cloning steps, and sequencing for both, CCMP494 and MBIC10095. The alternative, more likely, explanation is that the β-tubulin represents a locus that underwent a recombination event. The application of recombination detection programs showed that CCMP494 and MBIC10095 β-tubulin genes derive from a mix of lineage A and B sequences, which favors the hypothesis that the β-tubulin represents a locus that underwent a recombination event that generated the two divergent sequences. Therefore, with the reserve imposed by the occurrence of natural polymorphism (mostly affecting CCMP1195) and the occurrence of putative recombination events in one locus (β-tubulin), lineages B and C represent up to date the best examples of pan-oceanic picoeukaryotes. At any rate, the detection of this recombination event in one single locus highlights the importance of multilocus studies as the analysis of only one marker might have produced misleading conclusions.

For the more divergent lineages D and E, the strain sampling available was less comprehensive, preventing global comparative analysis. Nevertheless, the situation of the lineage D might be similar to that of lineages B and C. Strains CCMP1545/CCMP491 (English Channel) and CCMP490 (West Atlantic) were also remarkably similar (fig. 1). This points out to the existence of a lineage of high genetic similarity with a broad oceanic distribution. The lineage E was formed by CCMP2099 (Arctic) and CCMP1646 (Mediterranean), which are very divergent for all markers and may be representatives of two different clusters. In any case, the number of independent genetic lineages within *M. pusilla* may be much higher because the inclusion of available prasinophyte environmental SSU rDNA sequences (Stoeck and Epstein 2003; Guillou et al. 2004; Massana et al. 2004; Not et al. 2004; Romari and Vaulot 2004) in phylogenetic trees shows the occurrence of various additional groupings interspersed with the clusters defined in our multigene analyses (fig. 2).

Unexpectedly Ancient Cryptic Species

The detection of *M. pusilla* strain clusters that are ubiquitous, e.g., lineages B and C, implies that there are no barriers to their oceanic global dispersal. Their oceanic circulation may be driven either by surface currents (<400 m depth) or even by the deep-sea currents that redistribute the waters around the globe in times of ~850 years (Broecker, Sutherland, and Peng 1999). Despite its phototrophic lifestyle, it is plausible that *Micromonas* is sometimes drawn to the deep sea, where it travels in a low metabolic state to other oceanic regions where it upwells. However, deep-sea currents assuring the global mixing of contemporary oceans have not always existed or circulated in the same way but have been shut down or changed their direction many times in Earth's history (Broecker 1997; Driscoll and Haug 1998). Shutdowns in global oceanic circulation may have allowed the existence of prolonged periods of geographical isolation ending up in genetic diversification. Notably, molecular clock analysis of SSU rDNA of the polar planktonic foraminifer *Neogloboquadrina pachyderma* "sinistral" suggests that its diversification into Arctic and Antarctic types started with the ice cap expansion after the onset of the Northern Hemisphere glaciation (3.5–2.5 Myr) (Darling et al. 2004). Could oceanic circulation shutdown have had an effect on the genetic differentiation seen within *M. pusilla* strains? To answer, we carried out molecular dating analyses based on available genetic data for the green algal and plant lineage and using several fossil record references available for the plants



Key to the envinronmental phylotypes

CCW	Sippewisset salt march, ME, USA
RA	Coastal Astan station, western English Channel, France
RD	Estuarine Doufur station, western English Channel, France
UEPAC	Pacific ocean
NA11	Within eddy, North Atlantic
NA37	Outside eddy, North Atlantic
BL	Blanes Bay, Mediterranean
ANT37	Ice edge Antarctica, Waddell Sea
ME1	Alboran Sea, Mediterranean

FIG. 2.—SSU rDNA Bayesian phylogenetic tree including all available environmental phylotypes related to *Micromonas pusilla*. The tree was rooted using several prasinophyte and zygnematophyte sequences. Bayesian posterior probabilities >0.5 and ML bootstrap values >50% are shown at nodes. Sequences from cultured *Micromonas* strains are indicated by a black circle. Those determined in this study are in bold. The key to the environmental sequences indicates the geographical origin of the phylotypes.

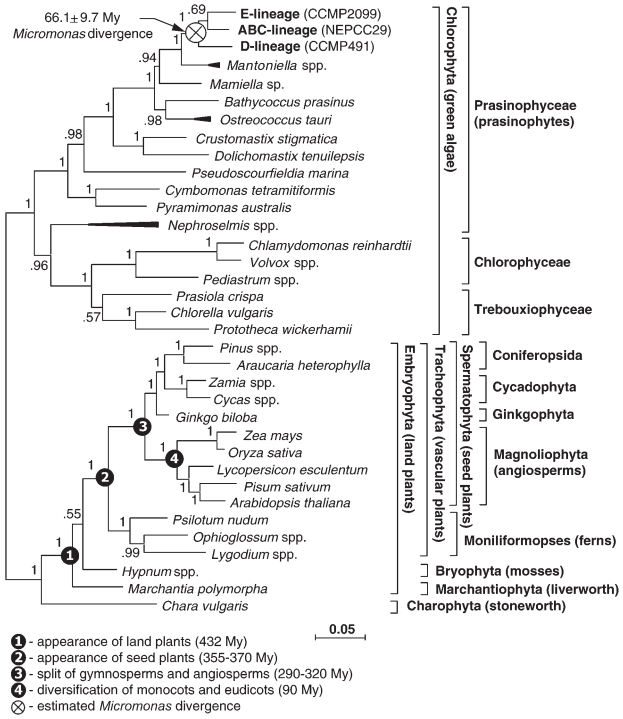


FIG. 3.—Phylogenetic tree of combined SSU rDNA, *rbcL*, and *coxI* nucleotide sequence alignments for *Micromonas pusilla* lineages and representative green algae and plants. Phylogenetic reconstruction was carried out by a Bayesian approach using a GTR + Γ + I model partitioned on genes and taking into account the three different codon positions. Bayesian posterior probabilities are indicated for all nodes. Taxonomic affiliation is given on the right. Constrained nodes for molecular clock analysis (using the PL method) are indicated by black circles 1–4. The divergence time for the deepest node in the *M. pusilla* clade (crossed circle) is shown.

(fig. 3 and *Materials and Methods*). This approach yielded an unexpectedly ancient divergence time for the earliest node in the *Micromonas* cluster of 66 ± 10 Myr, which pushes the diversification of the *Micromonas* lineages back to the Late Cretaceous. This coincides with a period of balmy waters (>15°C) in polar regions (Jenkyns et al. 2004) and, most likely, with oceanic circulation regimes very different from today's (Li and Keller 1999; Erbacher et al. 2001) that might have been accompanied by a reduction in ocean mixing, providing geographical isolation conditions triggering speciation. At any rate, apical nodes in *M. pusilla* phylogenies are much more recent, indicating that genetic diversification and likely speciation are ongoing evolutionary processes. This appears to be occurring in the absence of apparent geographical barriers (sympatric speciation).

Global Dispersal and Speciation in Picoplanktonic Eukaryotes

Our multilocus study of *M. pusilla* strains from various oceanic regions clearly shows that this traditional picoeukaryotic species is in fact an assemblage of diverse lineages. We observe the occurrence of various lineages that are genetically relatively homogeneous (lineage A) to highly homogeneous (lineages B, C, and D), which are indeed ubiquitous in oceans and that, under certain circumstances,

can recombine, as attested by the β-tubulin data set. These recombination events may be at the origin of new genetically divergent lineages, i.e., triggering speciation. In fact, the level of genetic divergence between some *M. pusilla* lineages is so high that they should be considered different cryptic species. Furthermore, the genetic divergence of some of them (e.g., lineage D or E with the rest) exceeds that existing among traditional genera or even higher taxa. In fact, the high divergence time estimated for the first diversification of *M. pusilla* lineages, ~65 Myr, is even comparable to that of the divergence between the monocotyledonous and dicotyledonous plants (90–130 Myr as deduced from the fossil record). Therefore, although some (maybe even all) *Micromonas* lineages are ubiquitous in oceans supporting current assumptions about global microbial dispersal (Finlay 2002; Finlay and Fenchel 2004), their underlying genetic variance reveals that traditional eukaryotic morphospecies may hide a far larger (cryptic) species diversity, as it happens in prokaryotes (Rocap et al. 2002; Whitaker, Grogan, and Taylor 2003; Papke et al. 2004) and has been also reported for some eukaryotes (de Vargas et al. 1999; Saez et al. 2003). In consequence, traditional morphospecies must be viewed with extreme caution for protist species, especially those exhibiting limited number of morphological and structural features. Our results are in clear disagreement with the idea that "protists were never presented with the opportunity of evolutionary diversification, because they were never restricted by geographical barriers" (Finlay and Fenchel 2002). Moreover, because various *Micromonas* lineages have diversified much more recently, they may be still undergoing speciation. This may result from the adaptation to different ecological niches, such as light intensity or nutrient availability, as observed in marine cyanobacteria (Rocap et al. 2002, 2003) and the picoalga *O. tauri* (Rodriguez et al. 2005), or by adoption of different reproductive mechanisms or behavior, as it appears to occur in planktonic foraminifers (de Vargas et al. 1999). In accordance to molecular diversity studies that point to the existence of a large eukaryotic diversity (Moreira and López-García 2002), we anticipate that multilocus analysis of other small eukaryotes will also unveil a myriad of cryptic species indistinguishable by only superficial morphological traits.

Supplementary Material

Supplementary Tables S1 and S2 and Figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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