Pseudo-cryptic speciation in coccolithophores

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Coccolithophores are a group of calcifying unicellular algae that constitute a major fraction of oceanic primary productivity, play an important role in the global carbon cycle, and are key biostratigraphic marker fossils. Their taxonomy is primarily based on the morphology of the minute calcite plates, or coccoliths, covering the cell. These are diverse and include widespread fine scale variation, of which the biological/taxonomic significance is unknown. Do they represent phenotypic plasticity, genetic polymorphisms, or species-specific characters? Our research on five commonly occurring coccolithophores supports the hypothesis that such variation represents pseudocryptic speciation events, occurring between 0.3 and 12.9 million years ago from a molecular clock estimation. This finding suggests strong stabilizing selection acting on coccolithophorid phenotypes. Our results also provide strong support for the use of fine scale morphological characters of coccoliths in the fossil record to improve biostratigraphic resolution and paleoceanographic data retrieval.

Marine planktonic protists often present subtle morphological differences within species that are traditionally considered cosmopolitan, e.g., *Skeletonema costatum* (1). [By "species" we refer to a reproductively isolated group, i.e., the biological species concept (2).] This raises questions about the heritability of these differences, or if heritability is accepted, about their polymorphic or fixed nature. The answers to these questions may have important implications for various aspects of the study of marine plankton: in assessing biodiversity, in evaluating evolutionary and ecological diversification, and in interpreting the fossil record when the organisms involved have mineralized skeletons.

Coccolithophores are unicellular calcifying algae, members of division Haptophyta (3). In open oceanic environments they constitute a significant fraction of the phytoplankton and have an exceptionally rich fossil record spanning the last ≈200 million years (4). Coccolihophores impact greatly on marine ecosystems, and hence on the global carbon cycle (5). A comprehensive species-level taxonomy based primarily on coccolith morphology has been established (6–9) and widely applied over the past decade to studies of their ecology, biogeography, and sediment fluxes. When these morphological criteria are used, most species are well differentiated and have very broad, interoceanic distributions. However many exhibit fine-scale morphological variation: some are formally recognized as varieties, whereas others have been informally differentiated often after intensive morphometric study (e.g., ref. 10). Some authors, however, based on morphological grounds, have suggested that some of these taxa represent genuine species, or subspecies (e.g., refs. 11 and 12).

But overall, the biological significance of such fine-scale morphological variation has remained unclear, despite the considerable interest to palaeontologists seeking to maximize information recovery from their fossil record. Four scenarios can be envisaged to explain the morphological variation: (*i*) phenotypic variation as a response to environmental factors, (*ii*) variation associated with life cycle stages, (*iii*) genetic polymorphisms, or (*iv*) species-specific characters.

Although molecular techniques have rarely been used for unicellular planktonic organisms at the intraspecific level (13–17), they are essential to help us to distinguish the hypotheses. To date, single cell amplification of DNA has not proven possible on coccolithophores, primarily as a result of their minute size (5–20 μm in diameter). Consequently, clonal cultivated individual cells are a necessary precursor for molecular analysis. This time-consuming step has inevitably limited taxon sampling. Conversely, clonal cultures permit a parallel investigation of morphological variability within genetically homogeneous populations. In a collaborative effort, we and colleagues have produced the largest culture collection of coccolithophores to date, to address this and other issues (www.nhm.ac.uk/hosted_sites/ina/CODENET/caencultures.htm). Certain species in the genera *Calcidiscus*, *Coccolithus*, *Helicosphaera*, *Umbilicosphaera*, and *Pleurochrysis* were selected for study because they exhibited distinct variations in coccolith morphology recognized at the morphotype or varietal level. We attempt to provide a genetic interpretation for the fine-scale variation in coccolith morphology seen within these species, and if supporting of speciation events, to use our genetic data and the fossil record of the coccolithophores to date these divergences.

Materials and Methods

Coccolithophorid Strains and DNA Sequences. The list of clonal cultures used is given in Table 1, and were cultured as described in ref. 12. Morphotype identification of each culture was based on a qualitative scanning electrom microscope study and light microscopy-based morphometric study of the coccoliths applying the same criteria as used for field material. Cultures were harvested during logarithmic growth phase by centrifugation for 5′ at 2,500 × *g*. Pellets were resuspended in 1 ml of a lysis buffer (100 mM Tris·HCl, pH 8.0/100 mM NaCl/50 mM $Na_2EDTA{\cdot}2H_2O$) and immediately frozen in liquid nitrogen. Genomic DNA was purified with Dneasy Plant Minikit (Qiagen, Valencia, CA), following the manufacturer's instructions, but with a modification of the lysis step by adding a mixture (1:1) of acid-washed and sterilized 212- to 600-μm glass beads (Sigma) to the samples, followed by two grinding steps using a vortex for 20 s each at 2,500 rpm. DNA was eluted in 10 mM Tris·HCl (pH 8.0) from which 1–10 ng was used for PCRs, which were performed with an Amplitaq DNA Polymerase kit (Applied Biosystems) in a Master Cycler Gradient (Eppendorf), by using a 100-μl reaction volume.

We sequenced the plastid gene tufA, which encodes the elongation factor Tu, from 28 strains of pairs of morphovariants in selected species of *Calcidiscus*, *Helicosphaera*, *Umbilicosphaera*, *Pleurochrysis*, and *Coccolithus* (Table 1). For comparison, we also sequenced the slower-evolving gene 18S rDNA from one strain of each morphotype or variety of the first four mentioned

EVOLUTION

Abbreviations: Ma, million years (geologic age); S, small morphotype; I, intermediate morphotype; L*, large ambiguous morphotype; L, large morphotype; ITS, internal transcribed spacer.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ544115–AJ544134).

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Table 1. List of the strains sequenced

Species	Morphotype	Strain ID	Provenance	Isolation date	Isolated by	18S rDNA	tufA	ITS rDNA
Calcidiscus leptoporus ssp. *quadriperforatus*	L	ASM35	Alboran Sea (Western Mediterranean)	Oct-99	I.P.	AJ544115	AJ544124	
C. leptoporus ssp. *quadriperforatus*	L	ASM27	Alboran Sea (Western Mediterranean)	Oct-99	I.P.		=ASM35	
C. leptoporus ssp. *quadriperforatus*	L	N482-4	South Atlantic (Namibia)	Nov-00	I.P.		=ASM35	
C. leptoporus ssp. *quadriperforatus*	L	N482-3	South Atlantic (Namibia)	Nov-00	I.P.		=ASM35	
C. leptoporus ssp. *quadriperforatus*	L	NAP12	Mediterranean Sea (Italy)	Dec-00	I.P.		=ASM35	
*C. leptoporus**	L*	PC13*	Atlantic Shelf (Ireland)	July-98	I.P.	=ASM35	AJ544125	
*C. leptoporus**	L*	PC11M1	Atlantic Shelf (Portugal)	July-98	I.P.		=PC13*	
*C. leptoporus**	L*	PC11M3	Atlantic Shelf (Portugal)	July-98	I.P.		=PC13*	
C. leptoporus ssp. *leptoporus*	I	AS31	Alboran Sea (Western Mediterranean)	Oct-99	I.P.	AJ544116	AJ544126	
C. leptoporus ssp. *leptoporus*	I	N470-12	South Atlantic (Namibia)	Nov-00	I.P.		=AS31	
C. leptoporus ssp. *leptoporus*	I	N470-10	South Atlantic (Namibia)	Nov-00	I.P.		=AS31	
C. leptoporus ssp. *leptoporus*	I	N470-11	South Atlantic (Namibia)	Nov-00	I.P.		=AS31	
C. leptoporus ssp. *leptoporus*	I	NS10-2	South Atlantic (South Africa)	Sep-00	I.P.		=AS31	
Coccolithus pelagicus ssp. *pelagicus*	A	IBV 73	North Atlantic (Iceland)	July-99	I.P.	AJ544117	AJ544127	AJ544122
Coccolithus pelagicus ssp. *braarudii*	T	KL2	Atlantic (France)	Dec-99	I.P.	=IBV73	AJ544128	AJ544123
Coccolithus pelagicus ssp. *braarudii*	T	AS56T	Alboran Sea (Western Mediterranean)	Oct-99	I.P.		=KL2	=KL2
Coccolithus pelagicus ssp. *braarudii*	T	LK1c	East Atlantic (France)	Feb-99	I.P.		=KL2	=KL2
Coccolithus pelagicus ssp. *braarudii*	T	CC35	Atlantic (Portugal)	June-99	I.P.		=KL2	=KL2
Coccolithus pelagicus ssp. *braarudii*	T	PLY182G	English Channel	1958	M. Parke		=KL2	=KL2
Coccolithus pelagicus ssp. *braarudii*	T	NS31	South Atlantic (South Africa)	Sep-00	I.P.		=KL2	=KL2
Coccolithus pelagicus ssp. *braarudii*	T	N761	South Atlantic (Namibia)	Oct-00	I.P.		=KL2	=KL2
Coccolithus pelagicus ssp. *braarudii*	T	N476-2	South Atlantic (Namibia)	Oct-00	I.P.		=KL2	=KL2
Umbilicosphaera sibogae v. *sibogae*		ETH4728	Atlantic (USA)	1998	C. Klaas & L. Brand	AJ544118	AJ544129	
U. sibogae v. *foliosa*		ESP6M1	West Mediterranean (Spain)	Apr-99	I.P.	AJ544119	AJ544130	
Pleurochrysis carterae		HAP1	Atlantic (Morocco)	1983	J. Fresnel	AJ544120	AJ544131	
P. carterae v. *dentata*		HAP6	California (USA)	1989	R. Lewin	AJ544121	AJ544132	
Helicosphaera carteri v. *hyalina*		NAP11	Mediterranean Sea (Italy)	Dec-00	I.P.		AJ544133	
H. carteri v. *carteri*		NS10-10	South Atlantic (South Africa)	Sep-00	I.P.		AJ544134	

GenBank accession numbers are given only for differing DNA sequences. The sequences that were shown to be identical to any of those are indicated by ''=,'' followed by the strain to whose DNA sequence they are equivalent. However, five positions within the ITS rDNA of the sequenced strains of *Coccolithus pelagicus* ssp. *braarudii* presented two alternative nucleotides, but without fixed differences between those strains (see the text).

genera, as well as the fast-evolving spacer region, internal transcribed spacer (ITS) rDNA, from nine strains of *Coccolithus pelagicus* (Table 1). The ITS region from *Coccolithus pelagicus* was sequenced to compensate for the fact that only one culture of one of its two morphotypes was available for study.

Amplification primers were as follows. (*i*) For tufA, tufAF: 5′-GCICATGTIGATTGTCCIGGICA(TC)G-3′; and tufAR: 5′-GTIGTIC(TG)IACGTAGAATTG(ACGT)GG-3′; (*ii*) for 18S rDNA as in (18); (*iii*) for ITS, 18S3F: 5′-GGGAAGCTGTCCGAACCTTATCATTTAGAG-3′; and PP874R: 5′-CCTCCGCTTAGTGATATGCTTAAGTTCAGC-3′. With the last primers the region amplified included ITS1, 5.8S rDNA and ITS2. The amplified DNA fragments were subsequently cleaned with a Qiaquick PCR Purification kit (Qiagen). Sequences from the PCR templates (both strands) were produced by Seqlab using an ABI 377 sequencer (Applied Biosystems) and dye terminator cycle sequencing kits (Perkin–Elmer). The quality of electrophoregrams was checked first as DNA sequences were edited,

with a subsequent revision for confirmation of newly observed substitutions. XESEE 3.2 software (Eric Cabot, personal communication) was used to manually align and edit the sequences. Electrophoregrams were viewed by using the CHROMAS 1.45 program (Conor McCarty, Griffith University, Queensland, Australia).

Phylogenetic and Molecular Clock Analyses. All age estimates based on a molecular clock were calculated by using the chloroplast tufA sequences. 18S rDNA was not used for this purpose because the low number of substitutions found in this gene between morphotypes or varieties made it less informative. For our molecular clock, we aligned the tufA sequences (Table 1) to 49 sequences from other Haptophyta (A.G.S. and L.K.M., unpublished data). The use of this extended data set allowed for the determination of a more robust nucleotide substitution model than would have been possible from the limited data set used here (19). The General Time Reversible model with gamma correction and a proportion of invariable sites (20) was determined to be the best fitting model of nucleotide evolution for our tufA data set as determined by Modeltest 3.06 (21), run with PAUP* 4.10b (22). However, because tufA codes for a protein, a better way to describe the heterogeneity in rates of evolution among sites is to assign separated substitution rates for the first, second, and third codon positions, which is not an option in MODELTEST, rather than by using the γ correction and an invariable proportion of sites. The software MRBAYES (23) was used for this purpose. When the General Time Reversible model is used with site specific rates of substitution, a Bayesian run of 10 million generations was performed by using our 15 different taxa of interest to build a consensus tree from trees of a higher posterior probability (23). The final tree was checked for molecular clock deviations (branches too fast or too slow in relation to the other branches in the tree) using the two-cluster test implemented in the LINTREE software (24). For this test, a neighbor-joining tree was constructed with the Tamura–Nei model of DNA substitution and γ correction (the most similar option to the General Time Reversible model with site-specific rates of nucleotide substitution, not found in LINTREE). The value of γ was calculated by PAUP* 4.10b for the Tamura–Nei plus γ model (22). The tree as a whole did not show a significant deviation from a clock-like evolution ($P = 0.5$), nor did any of its nodes ($P > 0.1$). A very similar result was obtained with the Branch Length test, also implemented in LINTREE. For dating the nodes of the tree the two-cluster test builds a "linearized tree," i.e., a tree with all tips equidistant from the root with the exception of the outgroup lineage (Fig. 2). In such trees, average lengths (i.e., distance to the tips) with their standard deviations are calculated for all nodes.

The linearized tree was then calibrated by using a fossil date. Both *Umbilicosphaera* and *Calcidiscus* have well documented continuous fossil records down to 23 million years (Ma) ago (Early Miocene), hence unambiguous minimum divergence time estimates could be made for these genera (25, 26).

Results

Calcidiscus leptoporus. Three morphotypes of *C. leptoporus* have been widely recognized: Small (S), Intermediate (I), and Large (L). They are usually distinguished by mean coccolith diameter (10, 27–29). However, this character has a quasi-continuous distribution (Fig. 1*A*); the assigned limits of coccolith diameter for each of the three morphotypes vary between authors (10, 27). Differences in qualitative morphological characters, such as the appearance of the coccolith central area and the shape of the suture lines on the distal shield of the coccolith (Fig. 1*B*), have also been used to distinguish the morphotypes (27, 28). The three *C. leptoporus* morphotypes overlap in their geographical range (10). Based on life cycle observations in natural samples and cultures, Geisen *et al.* (12) proposed that these minor morphological differences represent discrete taxa and emended the taxonomy, with the morphotype I becoming the subspecies *C. leptoporus* subspecies *leptoporus*; the morphotype L becoming *C. leptoporus* subspecies *quadriperforatus.* No life cycle observations were available for the S morphotype.

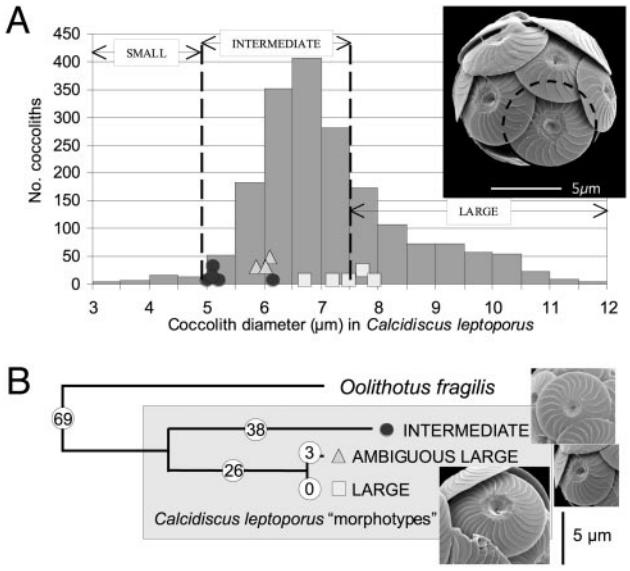

Fig. 1. (*A*) Mean coccolith diameter in *C. leptoporus* (from refs. 10 and 29, and our culture collection) with the strains that were genetically characterized [note that strains of Large (L) and Ambiguous Large (L*) fall into the Intermediate (I) morphotype size range]. The image is of *C. leptoporus* L* with a single coccolith indicated. (*B*) Most parsimonious tufA tree with branch lengths (circles) and coccolith morphologies. Notice the ring-like structure in the central area of morphotypes L and L*, which is absent from type I.

The gene tufA was sequenced from five strains of each *C. leptoporus* subspecies, *leptoporus* and *quadriperforatus*. Three additional *C. leptoporus* strains were selected because their identification was problematic: they show the qualitative morphological characters of *C. leptoporus* subspecies *quadriperforatus,* but their size range corresponds more to *C. leptoporus* subspecies *leptoporus*. This morphotype is referred to here as *C. leptoporus** (or L*, for ambiguous large). No cultures of *C. leptoporus* S were available for analysis. Both *C. leptoporus* subspecies *leptoporus* and *C. leptoporus* subspecies *quadriperforatus* revealed a specific tufA genotype, with strictly identical sequences within each morphotype. The additionally analyzed *C. leptoporus** shared a recent common ancestor with the *C. leptoporus* subspecies *quadriperforatus* (see Figs. 1*B* and 2, and Table 2). 18S rDNA sequences confirmed the genetic split between the subspecies *leptoporus* and *quadriperforatus*/*C. leptoporus**: four substitutions were found with the latter two having identical 18S sequences (Table 2). As a comparison, three substitutions only were found between *Calcidiscus* spp. and the closely related species *Oolithotus fragilis*. This lower genetic distance between genera is probably because of the low rate of nucleotide substitutions found at 18S, making this gene highly susceptible to the stochastic deviations of the molecular clock (30). By using the tufA sequences, our molecular clock estimations suggest that the *C. leptoporus* subspecies *leptoporus* and *C. leptoporus* subspecies *quadriperforatus*/*C. leptoporus** lineages diverged between 10 and 13 Ma (Table 2). This coincides well with the 10.8 Ma age for the split of these lineages based on analysis of coccolith morphology in the fossil record (31) and supports the accuracy of our molecular-clock calibration.

Table 2. Genetic distances between pseudo-cryptic coccolithophores

	No. of observed substitutions			Divergence, Ma (±SE)
	Morphotype	tufA	18S rDNA	
C. leptoporus ssp. *leptoporus*/ ssp. *quadriperforatus* + *C. leptoporus**	I/L+L*	64 + 67	4	11.57 (±1.61)
C. leptoporus ssp. *quadriperforatus*/*C. leptoporus**	L/L*	3	0	0.32 (±0.19)
Coccolithus pelagicus ssp. *pelagicus*/ssp. *braarudii*	A/T	18	0	2.15 (±0.57)
U. sibogae v. *sibogae*/v. *foliosa*		40	7 (2)†	5.59 (±1.15)
H. carteri v. *carteri*/v. *hyalina*		61	–	10.19 (±1.97)
P. carterae v. *carterae*/v. *dentata*		69	12 (1)†	12.89 (±2.63)

The divergence times and standard errors (24) are calibrated on the *Umbilicosphaera*/*Calcidiscus* node at 23 Ma (25, 26).
†Deletions or insertions are given in parentheses.

Coccolithus pelagicus. Two morphotypes for this species have been distinguished by using both cell and coccolith diameter (12, 32). Unlike the subspecies of *C. leptoporus*, the two morphotypes of *Coccolithus pelagicus* show discrete but partially overlapping geographical ranges: the smaller morphotype (6- to 11-μm coccolith length) occurs in the subarctic region, whereas the larger one (10–16 μm coccolith length) occurs in temperate latitudes. They are known as the subarctic (A) and temperate (T) morphotypes, respectively. Based on life cycle observations on cultures and wild samples, Geisen *et al.* (12) proposed that the two morphotypes are two distinct taxa. They have emended the taxonomy with the small, subarctic, becoming *Coccolithus pelagicus* subspecies *pelagicus* and the larger, temperate form, *Coccolithus pelagicus* subspecies *braarudii*.

We sequenced tufA from eight *Coccolithus pelagicus* subspecies *braarudii* and one *Coccolithus pelagicus* subspecies *pelagicus*. No substitutions were found among the eight *braarudii* isolates, whereas 18 nucleotides differed between them and *Coccolithus pelagicus* subspecies *pelagicus*. Because only one *Coccolithus pelagicus* subspecies *pelagicus* culture was available, we sequenced a second genomic region, ITS rDNA, to confirm our results. There were 13 fixed substitutions plus one insertion/deletion between both subspecies. In addition, five ambiguous substitutions were found among the eight *Coccolithus pelagicus* subspecies *braarudii* isolates. These five polymorphic positions likely result from intraindividual variation, because most of them result from unequal double peaks in the sequencing electrophoregrams. We finally sequenced 18S rDNA from one *Coccolithus pelagicus* subspecies *braarudii* and one *Coccolithus pelagicus* subspecies *pelagicus* strain (Table 1), which showed to be identical (Table 2). The divergence time between the two lineages based on tufA was estimated between 1.6 and 2.7 Ma (Table 2). This is in agreement with the fossil record where the large form appears in the quaternary (1.8 Ma; our unpublished data).

Umbilicosphaera sibogae, Helicosphaera carteri,* and *Pleurochrysis carterae. The two varieties of *Umbilicosphaera sibogae* were originally described as separate species: *Umbilicosphaera sibogae* (Weber-van Bosse) Gaarder and *Umbilicosphaera foliosa* Kamptner. Although they show considerable differences in coccolith and cell morphology, they were recombined as variants by Okada and McIntyre (33) on the basis of rare observations of coccospheres bearing coccoliths of both types. In contrast, the two *Umbilicosphaera* varieties that we analyzed show all of the characteristics of their respective original species, which remained stable for >2 years.

Four extant species of *Helicosphaera* have been described: *Helicosphaera carteri*, *Helicosphaera hyalina*, *Helicosphaera pavimentum*, and *Helicosphaera wallichii* (33, 34). The status of *H. pavimentum* has not been disputed; the other three, however, are very similar, differing primarily in the presence and alignment of pores in the central area. Moreover, intermediate morphotypes occur, and coccospheres bearing coccoliths of more than one morphotype have been reported (e.g., refs. 33 and 35). So, in recent work on extant coccolithophores, they have been regarded as varieties; *H. carteri* v. *carteri*, *H. carteri* v. *hyalina*, and *H. carteri* v. *wallichii* (7, 8, 36). We isolated in culture 11 strains of *H. carteri* v. *carteri* and one strain of *H. carteri* v. *hyalina*. In each case the distinctive coccolith morphology remained entirely stable in culture over periods of 1–2 years. This supports the more traditional taxonomic approach of using relatively subtle morphological characters as species indicators.

Pleurochrysis carterae was described by Braarud and Fagerland in 1946 as *Syracosphaera carterae*, but was transferred to *Pleurochrysis* by Christensen (see ref. 37). *Pleurochrysis carterae* v. *dentata* was described by Johansen and Doucette (38).

For *Umbilicosphaera sibogae* (v. *sibogae* and *foliosa*), *P. carterae* (v. *carterae* and *dentata*), and *H. carteri* (v. *carteri* and *hyalina*), we sequenced tufA from one strain of each variety. Each variety showed a well-differentiated DNA sequence. For the *Umbilicosphaera* and *Pleurochrysis* varieties, high numbers of substitutions were found between the "variety" pairs in both the tufA and the 18S rDNA genes (Table 2). For *Helicosphaera,* high substitution numbers were found in tufA (the 18S rDNA could not be sequenced for *H. carteri* v. *hyalina*, despite numerous attempts). The large number of substitutions in the chloroplast gene tufA and the nuclear gene 18S rDNA, as well as the absence of heterozygous sites, strongly support that the three pairs of varieties within *U. sibogae*, *H. carteri* and *P. carterae* correspond to independent evolutionary lineages. Their divergence times, inferred from the tufA molecular clock go back to the Miocene (Table 2 and Fig. 2).

Discussion

The assessment of species boundaries using genetic markers is primarily based on the concept that coexisting sexual organisms

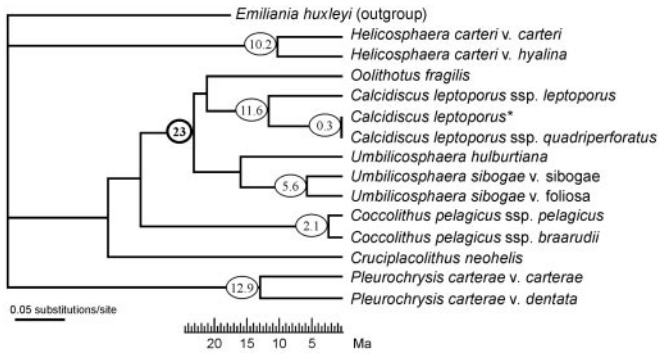

Fig. 2. Linearized tufA tree from which age estimations in Ma were obtained. The node used to calibrate this tree (i.e., relating genetic branch length to geological age) is 23 Ma old (25, 26). Nodes older than 25 Ma should not be dated from this tree, because from approximately that age unrecoverable multiple nucleotide substitutions were observed (data not shown).

will share a genetic pool, which generally is not available to organisms of other species. As a consequence, members of the same species are expected to differ genetically much less among themselves than with individuals from other species. Because evidence is increasing that coccolithophores undergo sexual reproduction as part of their heteromorphic life cycle (12, 39–41), we use here the biological species concept of reproductive isolation (2) to infer species limits in these organisms. The levels of DNA variation observed within and between the subspecies/morphotypes of *C. leptoporus* (tufA) and *Coccolithus pelagicus* (tufA and ITS) support their reproductive isolation: whereas there are no differences within subspecies, the genetic distances between them range between three and 67 DNA substitutions (Table 2).

Geographic isolation is unlikely to be responsible for the observed genetic divergence. When physical barriers separate populations, it is possible to observe high levels of molecular differentiation between them, even if they have not developed reproductive barriers. This is unlikely for the subspecies of *C. leptoporus*, because strains for both of them were actually collected in the same water-mass at the same time (Table 1). Moreover, the three morphotypes (subspecies) described in the literature show strongly overlapping geographic distribution (10). The subspecies of *Coccolithus pelagicus*, *pelagicus* and *braarudii*, differ in their biogeographical distribution; however, they coexist over a wide geographic area (32). Moreover, the geographic distances separating the North and South Atlantic sites from which the *Coccolithus pelagicus* subspecies *braarudii* strains were obtained are considerably larger than the distances between the temperate North Atlantic and Icelandic waters, from where four *Coccolithus pelagicus* subspecies *braarudii* and one *Coccolithus pelagicus* subspecies *pelagicus* were isolated, respectively (Table 1). The geographical distribution of the two subspecies of *C. pelagicus*, one subpolar and the other temperate, indicates another important aspect: that they exhibit different ecological preferences (32). Similarly, coccolith sizes of natural populations of the subspecies of *C. leptoporus* oscillate seasonally (29), which suggests that *C. leptoporus* subspecies *quadriperforatus* is more adapted to cooler mesotrophic conditions, whereas *C. leptoporus* subspecies *leptoporus*' abundance is more uniform throughout the year and possibly adapted to more oligotrophic conditions. Extensive biogeographical studies on foraminifera have shown that different genotypes, corresponding to different pseudocryptic species, exhibit different ecological preferences (14, 16, 17). A similar approach should be taken for the pseudocryptic species of coccolithophores, to confirm whether they also present different ecological adaptations. The geographical range of the identified pseudocryptic species of coccolithophores may also suggest that speciation could have occurred in sympatry (perhaps by ecological specialization), rather than in allopatry. Benton and Pearson (42) link sympatric speciation to gradualistic evolution, and suggest that planktonic protists, typically sustaining huge and widely disperse populations, are at the gradualistic extreme of an evolutionary gradient ranging to a purely punctuated equilibrium, of smaller and more structured freshwater populations, for example of fishes. This is a simple view of speciation, which is consistent with the subtle, and often continuous, morphological differences among the pseudocryptic species of coccolithophores, and with the extant distribution of the sympatric *C. leptoporus* spp. and, to a lesser extent, of the parapatric *Coccolithus pelagicus* spp.

Few molecular studies have addressed the species-limits problem in marine planktonic protists. Planktonic foraminifera are the most extensively studied group (14, 16, 17, 43). It had come as a surprise that foraminifera with highly similar morphologies could be subdivided into well defined genotypes. These molecular differences have been taken as evidence of reproductive isolation between morphologically indistinguishable (cryptic), or only *a posteriori* distinguished species (pseudocryptic) (44). Our data on coccolithophores parallel this observation and suggest that this may be a general phenomenon among marine protists. One obvious explanation is that some organisms, indeed foraminifera (14), have so few characters that phenotypes become almost "feature-free." This is, however, not the case in coccoliths, which, despite their very small size, show very complex structures. Among pelagic protists, diatoms also exhibit very elaborate morphologies, and there is emerging evidence of pseudocryptic speciation in this group as well. Mann (45) documented small-scale variation in the valve morphologies between demes of *Sellaphora pupula* that corresponded to reproductively isolated groups. He concluded that the diatoms were underclassified, and that small-scale variation in valve morphology, if consistent, likely represented pseudocryptic species.

Cryptic or pseudocryptic species may indicate optimal phenotypes subject to strong stabilizing selection (46). That is, the fact that they remain very similar, with just minute differences separating them morphologically, despite the lack of gene flow between these taxa during millions of years ($<$12.9 Ma; Fig. 2), is indicative of strong stabilizing selection acting on their overall phenotypes. This implies that the particular forms of their coccoliths are functionally relevant to their survival.

Although our sample size for each of the five recognized species of coccolithophores of this study is limited, we have confirmed, based on three different genes of two cellular organelles, that the morphological fine-scale variation observed within each of them correlates well with particular genotypes. This strongly supports previous views of reproductive isolation or genetic differentiation among recognized species of coccolithophores (e.g., refs. 11 and 47). Our results are also consistent with recent life cycle evidence (12) suggestive of the species limits of two of the taxa studied here, *C. leptoporus* (morphotypes L and I) and *Coccolithus pelagicus* (morphotypes A and T). For each of these "morphotypes," particular associations of hetero- and holococcoliths [two structurally different coccoliths present at different stages of the life cycle of coccolithophores (6)] have been found.

The recognition of extant pseudocryptic species with a fossil record may also have more practical consequences. Biostratigraphers have often used subtler morphological criteria than biologists in discrimination of species, and our results provide justification for this approach. Taxonomic subdivisions finer than previously assumed can thus provide useful biostratigraphic markers. To achieve this goal it is necessary to find morphological characters that can accurately discriminate "cryptic" species. Once these characters are found, then the cryptic species become "pseudocryptic" species, i.e., species that are morphologically recognized as such only after other methods have unveiled their existence (44). Our data suggest that the conventional morphological differences between the varieties of *U. sibogae* (38), *P. dentata* (38), and *H. carteri* (26) can now be used to separate them as species. As for the distinction between the subspecies of *C. leptoporus*, *C. leptoporus* subspecies *quadriperforatus* and *C. leptoporus** have a zone of obscured sutures around the central area, which is absent in *C. leptoporus* subpsecies *leptoporus* and *C. leptoporus* S (refs. 12 and 28; Fig. 1*B*). The relatively recent distinction between *C. leptoporus* subspecies *quadriperforatus* and *C. leptoporus** is only quantitative at present, and we have not been able to establish characters that permit a clear morphological separation. The subspecies of *Coccolithus pelagicus* are distinguished by the coccolith size, though some overlap exists (32).

Another practical consequence that may result from our study is the use of coccolithophorid pseudocryptic species as proxies to study past environmental conditions. This is practicable only if accurate morphological and ecological characters clearly de-

fine the pseudocryptic species. This approach, currently under investigation in pseudocryptic species of foraminifera (16, 48), should be further investigated in coccolithophores.

Taxonomical Considerations. Given the genetic divergences shown here coupled with life cycle studies (12), we conclude that the recognition of morphotypes as subspecies by Geisen *et al.* (12) was too conservative. Here we raise them to the species level and also list the recommended names for the varieties of *Helicosphaera*, *Umbilicosphaera*, and *Pleurochrysis*.

Calcidiscus leptoporus (Murray and Blackman) Loeblich and Tappan.

Calcidiscus quadriperforatus (Kamptner) Quinn and Geisen.

Basionym: *Syracosphaera quadriperforata*. (ref. 49, pp. 302, pl. 15, figures 15 and 16).

Coccolithus pelagicus (Wallich) Schiller.

Coccolithus braarudii (Gaarder) Baumann, Cachao, Young, and Geisen.

Basionym: *Crystallolithus braarudii* Gaarder (ref. 34, pp. 43, pl. 7).

Umbilicosphaera sibogae (Weber-van Bosse) Gaarder.

Umbilicosphaera foliosa (Kamptner) Geisen.

Basionym: *Cyclococcolithus foliosus* (ref. 50, pp. 167 and 168, Figure 38).

Pleurochrysis carterae (Braarud and Fagerland) Christensen.

Pleurochrysis dentata (Johansen and Doucette) Probert.

Basionym: *Pleurochrysis carterae* v. *dentata* Johansen and Doucette (ref. 38, pp. 81, figure 4).

Helicosphaera carteri (Wallich) Kamptner.

Helicosphaera hyalina Gaarder.

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1. Hasle, G. R. (1973) *N. J. Bot.* **20,** 109–137.
2. Mayr, E. (1963) *Animal Species and Evolution* (Harvard Univ. Press, Cambridge, MA).
3. Edvardsen, B., Eikrem, W., Green, J. C., Andersen, R. A., Moon-Van-Der-Staay, S. Y. & Medlin, L. K. (2000) *Phycologia* **39,** 19–35.
4. Bown, P. R. (1998) *Calcareous Nannofossil Biostratigraphy* (Chapman & Hall, London).
5. Westbroek, P., Brown, C. W., Van Bleijswijk, J., Brownlee, C., Brummer, G. J., Conte, M., Egge, J., Fernandez, E., Jordan, R., Knappertsbusch, M., *et al.* (1993) *Global Planet. Change* **8,** 27–46.
6. Young, J. R. (1994) in *Coccolithophores*, eds. Winter, A. & Siesser, W. G. (Cambridge Univ. Press, Cambridge, U.K.), pp. 63–82.
7. Jordan, R. W. & Green, J. C. (1994) *J. Mar. Biol. Assoc. U.K.* **74,** 149–174.
8. Winter, A. & Siesser, W. G. (1994) *Coccolithophores* (Cambridge Univ. Press, Cambridge, U.K.).
9. Jordan, R. W., Kleijne, A., Heimdal, B. R. & Green, J. C. (1995) *J. Mar. Biol. Assoc. U.K.* **75,** 769–814.
10. Knappertsbusch, M., Cortes, M. Y. & Thierstein, H. R. (1997) *Mar. Micropaleontol.* **30,** 293–317.
11. Aubry, M.-P. (1988) *Paleobiology* **14,** 64–80.
12. Geisen, M., Billard, C., Broerse, A. T. C., Cros, L., Probert, I. & Young, J. R. (2002) *Eur. J. Phycol.* **37,** 531–550.
13. Lange, M., Chen, Y. Q. & Medlin, L. K. (2002) *Eur. J. Phycol.* **37,** 77–92.
14. de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W. & Pawlowski, J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2864–2868.
15. Medlin, L. K., Barker, G. L. A., Campbell, L., Green, J. C., Hayes, P. K., Marie, D., Wrieden, S. & Vaulot, D. (1996) *J. Mar. Syst.* **9,** 13–31.
16. de Vargas, C., Renaud, S., Hilbrecht, H. & Pawlowski, J. (2001) *Paleobiology* **27,** 104–125.
17. de Vargas, C., Bonzon, M., Rees, N. W., Pawlowski, J. & Zaninetti, L. (2002) *Mar. Micropaleontol.* **45,** 101–116.
18. Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. (1988) *Gene* **71,** 491–499.
19. Posada, D. & Crandall, K. A. (2001) *Syst. Biol.* **50,** 580–601.
20. Rodríguez, F., Oliver, J. L., Marín, A. & Medina, J. R. (1990) *J. Theor. Biol.* **142,** 485–501.
21. Posada, D. & Crandall, K. A. (1998) *Bioinformatics* **14,** 817–818.
22. Swofford, D. L. (2000) PAUP*: *Phylogenetic Analysis Using Parsimony (*and Other Methods)* (Sinauer Associates, Sunderland, MA), Version 4.
23. Huelsenbeck, J. P. & Ronquist, F. (2001) *Bioinformatics* **17,** 754–755.
24. Takezaki, N., Rzhetsky, A. & Nei, M. (1995) *Mol. Biol. Evol.* **12,** 823–833.
25. de Kaenel, E. & Villa, G. (1996) *Proc. Ocean Drill. Program Sci. Results* **149,** 79–145.
26. Young, J. R. (1998) in *Calcareous Nannofossil Biostratigraphy*, ed. Bown, P. R. (Chapman & Hall, London), pp. 225–265.
27. Kleijne, A. (1993) *Morphology, Taxonomy and Distribution of Extant Coccolithophorids (Calcareous Nannoplankton)* (Free University, Amsterdam).
28. Baumann, K.-H. & Sprengel, C. (2000) *J. Nannoplankton Res.* **22,** 185–193.
29. Renaud, S. & Klaas, C. (2001) *J. Plankton Res.* **23,** 779–795.
30. Hillis, D. M., Mable, B. K. & Moritz, C. (1996) in *Molecular Systematics*, eds. Hillis, D. M., Moritz, C. & Mable, B. K. (Sinauer Associates, Sunderland, MA).
31. Knappertsbusch, M. (2000) *J. Paleontol.* **74,** 712–730.
32. Baumann, K.-H., Young, J. R., Cachao, M. & Ziveri, P. (2000) *J. Nannoplankton Res.* **22,** 82.
33. Okada, H. & McIntyre, A. (1977) *Micropaleontology* **23,** 1–55.
34. Gaarder, K. (1970) *Nytt. Mag. Bot.* **17,** 113–126.
35. Nishida, S. (1979) in *Atlas of Pacific Nannoplanktons*, News of Osaka Micropaleontologists (Osaka City University, Osaka), Vol. 3, pp. 1–31.
36. Cros, L. & Fortuño, J.-M. (2002) *Scientia Marina* **66,** 1–186.
37. Tomas, C. R. (1993) *Marine Phytoplantkon: A Guide to Naked Flagellates and Coccolithophorids* (Academic, San Diego).
38. Johansen, J. R., Doucette, G. J., Barclay, W. R. & Bull, J. D. (1988) *Phycologia* **27,** 78–88.
39. Billard, C. (1994) in *The Haptophyta Algae*, eds. Green, J. C. & Leadbeater, B. S. C. (The Systematics Association, Clarendon Press, Oxford), Systematics Association Special Volume No. 51, pp. 167–186.
40. Green, J. C., Course, P. A. & Tarran, G. A. (1996) *J. Mar. Syst.* **9,** 33–44.
41. Cros, L., Kleijne, A., Zeltner, A., Billard, C. & Young, J. R. (2000) *Mar. Micropaleontol.* **39,** 1–34.
42. Benton, M. J. & Pearson, P. N. (2001) *Trends Ecol. Evol.* **16,** 405–411.
43. Darling, K. F., Wade, C. M., Stewart, I. A., Kroon, D., Dingle, R. & Brown, A. J. (2000) *Nature* **405,** 43–47.
44. Knowlton, N. (1993) *Annu. Rev. Ecol. Syst.* **24,** 189–216.
45. Mann, D. G. (1999) *Phycologia* **38,** 437–495.
46. Williamson, P. G. (1987) in *Rates of Evolution*, eds. Campbell, K. S. W. & Day, M. F. (Allen & Unwin, London), pp. 121–134.
47. Brand, L. E. (1982) *Limnol. Oceanogr.* **27,** 236–245.
48. Kucera, M. & Darling, K. F. (2002) *Philos. Trans. R. Soc. London A* **360,** 695–718.
49. Kamptner, E. (1937) *Arch. Protistenk.* **89,** 279–316.
50. Kamptner, E. (1963) *Ann. Nat. Mus. Wien* **66,** 139–204.