SCI. MAR., 70 (1): 67-76 SCIENTIA MARINA 2006

Morphology and rDNA phylogeny of a Mediterranean *Coolia monotis* (Dinophyceae) strain from Greece*

NICOLAS P. DOLAPSAKIS [1], MICHAEL W. KILPATRICK [2], ATHENA ECONOMOU-AMILLI [1] and TRIANTAFYLLOS TAFAS [1,2]

[1] Department of Ecology and Systematics, Faculty of Biology, University of Athens, Panepistimiopolis 15784, Athens, Greece. E-mail: ndol@biol.uoa.gr
[2] Ikonisys Inc., 5 Science Park, New Haven 06511, Connecticut, USA

SUMMARY: Sequences of LSU and SSU ribosomal RNA genes and phylogeny have not been widely investigated for the dinoflagellate *Coolia monotis* Meunier, and no information is available on the small and large rDNA subunits of Mediterranean strains. A strain isolated from the Thermaikos Gulf in northern Greece was identified as *C. monotis*—a new record for the Greek algal flora—using thecal morphology by light, epifluorescence and scanning electron microscopy. The small subunit and partial (D1/D2) large subunit sequences were analyzed and compared to other strains of *C. monotis* and dinoflagellates from various regions. Thecal architecture showed that the Greek strain of *C. monotis* was phenotypically similar, but not identical, to other strains reported in literature. The partial LSU sequence (700 bp) was found to vary by 113 bp positions (16%) from the *C. monotis* strain from New Zealand, whereas the SSU (1757 bp) had 15 bp differences (0.85%) from the strain from Norway. Phylogenetic tree construction showed that the Greek strain fell within the *Coolia* clade and had a close relationship with the families Ostreopsidaceae and Goniodomaceae of the order Gonyaulacales. Preliminary findings suggest the existence of different genotype strains of *C. monotis* with large intraspecific genetic variability and minimal morphological differentiation (similar phenotypes). Certain ecological and evolutionary implications of these findings are discussed.

Keywords: *Coolia monotis*, Greece, microscopy, rDNA, taxonomy, phylogeny.

RESUMEN: MORFOLOGÍA Y FILOGENIA DEL RDNA DE UNA SUBESPECIE MEDITERRÁNEA DE *COOLIA MONOTIS* (DINOPHYCEAE) DE GRECIA. – Las secuencias de los genes del RNA de las subunidades ribosomales grandes y pequeñas (LSU y SSU, respectivamente) y la filogenia del dinoflagelado *Coolia monotis* Meunier han sido poco investigadas, y no hay información disponible sobre los genes LSU y SSU de subespecies mediterráneas. Una subespecie aislada del golfo de Thermaikos en el norte de Grecia fue identificada como *C. monotis* –una nueva aportación a la flora algal griega– por medio de la morfología de la teca observada a través de microscopía óptica, de epifluorescia y electrónica. Las secuencias correspondientes a la subunidad pequeña y a la parte (D1/D2) de la subunidad grande fueron analizadas y comparadas a las de otras subespecies de *C. monotis* y otras especies de dinoflagelados de diversas regiones. La arquitectura de la teca mostró que la subespecie griega de *C. monotis* era fenotípicamente similar, pero no idéntica, a otras subespecies registradas en la literatura. Se encontró que la secuencia parcial de la LSU (700 pares de bases o bp) difería de la de *C. monotis* de Nueva Zelanda en las posiciones de 113 bp (16%), mientras que la SSU (1757 bp) se diferenciaba en 15 bp (0.85%) de la subespecie de Noruega. La construcción del árbol filogenetico demostró que la subespecie griega se situaba dentro de la rama de *Coolia* y presentaba una relación cercana con las familias Ostreopsidaceae y Goniodomaceae del orden Gonyaulacales. Resultados preliminares sugieren la existencia de diversos genotipos de la subespecie de *C. monotis* con una importante variabilidad genética intraespecífica y una mínima diferenciación morfológica (fenotipos similares). Se comentan diversas implicaciones ecológicas y evolutivas de estos resultados.

Palabras clave: *Coolia monotis*, Grecia, microscopía, rDNA, taxonomía, filogenia.

*Received February 15, 2005. Accepted July 22, 2005.

INTRODUCTION

Coolia monotis Meunier is a cosmopolitan dinoflagellate species previously reported in the Mediterranean (e.g. Tolomio and Cavolo, 1985; Riobó *et al*., 2002). There have been no records to date of its presence in Greece. It is a benthic and hardy species observed under variable environmental conditions, mainly in sandy biotopes but also as epiphytic or planktonic (Taylor and Pollingher, 1987; Faust, 1992; Steidinger and Tangen 1997). It is a taxon that appears to consist of several genetically distinct geographical strain groups (Penna *et al*., 2002), thus it exhibits significant intraspecific genetic variation (IGV). These strains occur both allopatrically due to geographical isolation and in sympatry in the same geographic region (Schluter, 2001; Coyne and Orr, 2004). The allopatric Mediterranean strains appear to be non-toxic (Rhodes *et al*., 2000; Riobó *et al*., 2002).

There are currently a limited number of available sequences in GenBank concerning the large subunit (LSU) and small subunit (SSU) of *C. monotis*, which do not include any from the Mediterranean region and are not presented in any phylogenetic analyses. The phylogeny of *C. monotis* using these rRNA genes and the ones read for a Mediterranean strain allows a phylogenetic investigation to be done concerning *C. monotis* and the relation of the family Ostreopsidaceae (e.g. *Coolia*, *Ostreopsis*) to Goniodomaceae (e.g. *Alexandrium*, *Pyrodinium*) and other families of the order Gonyaulacales. The phylogeny and morphology of a Mediterranean strain also serves to present a preliminary study into its intraspecific diversity and to discuss certain ecological and evolutionary implications.

Widely varying intraspecific genetic variability among populations of potentially harmful benthic dinoflagellates has been observed for taxa such as *Ostreopsis* spp. (Pin *et al*., 2001; Penna *et al*., 2002) and *Gambierdiscus* spp. (Babinchak *et al*., 1996; Chinain *et al*., 1999). The intraspecific variability of potentially harmful benthic dinoflagellate taxa has raised interest in phenotypes as compared to genotypes from a taxonomic and biogeographic point of view. Benthic species typing is growing in importance with increasing interest in microalgal monitoring due to the problems encountered with potentially harmful benthic or bentho-planktonic species that impact seafood consumption (Faust *et al*., 1996; ICES/IOC, 1999, 2000; Lenoir *et al*., 2004).

In phylogeny, using the LSU is imperative for lower level taxonomic analysis in order to discriminate between closely related taxa at the genus, species and even strain level (Baroin-Tourancheau *et al*., 1992; Buchheim *et al*., 2001; Harper and Saunders, 2001; John *et al*., 2003). D1-D6 are phylogenetically useful variable domains ("D" regions; Hassouna *et al*., 1984; Michot *et al*., 1984) within the LSU sequence of dinoflagellates (Daugbjerg *et al*., 2000; Edvardsen *et al*., 2003; Murray *et al*., 2004). The SSU sequences evolve much slower and therefore provide information for comparing groups at a higher taxonomic level than the LSU permits; however, they are also used for distinguishing between species (Saunders *et al*., 1997; Grzebyk *et al*., 1998; Saldarriaga *et al*., 2001).

Here we report on the morphology (light, epifluorescence and scanning electron microscopy) and rDNA phylogeny (D1/D2 LSU, SSU) of a strain of *Coolia monotis* isolated from a mussel farming area in the north Aegean Sea, an area that has experienced frequent and diverse harmful dinoflagellate blooms within the last four years.

MATERIALS AND METHODS

Samples, isolation and cultivation

Sampling was carried out near the Loudias River delta at Malgara, in the Thermaikos Gulf, north Aegean Sea (40:32:08 N, 22:41:32 E). The sampling area was adjacent to a mussel farm's 'clean-dry' posts where mussels are hung up and naturally exteriorly cleaned by drying in the sunlight. Samples were obtained with a 1L Ruttner plankton sampler from various depths and up to 2.5 m near the sandy bottom at midday in June 2002. The water temperature was 22°C and salinity 33 ppt. The samples were concentrated with 8 μm-pore polycarbonate Nuclepore membrane filters and back-washed into sterile test tubes containing 8-10 ml of L-1 medium (Guillard, 1995). Samples were cultivated at 23°C, 14/10 hours light/dark, illumination at 60-70 μmoles m^{-2} sec^{-1} without aeration, and studied 7 days later to identify and isolate dinoflagellate species of interest. Secondary isolations produced four clones (C1, C2 - C3, C4) from two initial isolations of *C. monotis* cells made from cultured samples from 2.0-2.5 m depths; these were placed in individual tubes with 5 ml of L-1 medium for 1 month and subsequently grown to 100 ml cultures.

TABLE 1. – Primers used for PCR and sequencing.

Primer name	5'→3' sequence	Stage, position
LSU-D1R-For [1]	ACCCGCTGAATTTAAGCATA	PCR / sequencing, see text
LSU-D2C-Rev [1]	CCTTGGTCCGTGTTTCAAGA	PCR / sequencing, see text
LSU-SF2-For [3]	CATGAGGGAAATGTGAAAAGG	Sequencing, 329-349
LSU-SR2-Rev [3]	CTTTTCACATTTCCCTCATGG	Sequencing, 348-328
SSU-EU-For [2]	CAACCTGGTTGATCCTGCCAGT	PCR / sequencing, see text
SSU-EU-Rev [2]	CTGATCCTTCTGCAGGTTCACCTAC	PCR / sequencing, see text
SSU-SF2-For [3]	CAAGTCTGGTGCCAGCAGC	Sequencing, 515-533
SSU-SR2-Rev [3]	GCTGCTGTCACCAGACTTG	Sequencing, 515-533
SSU-SF5-Rev [3]	GCCCTTCCGTCAATTCCTTT	Sequencing, 1096-1115
SSU-SR3-For [3]	ATTCCGTTAACGAACGAGAC	Sequencing, 1272-1291
SSU-SF3-Rev [3]	GTCTCGTTCGTTAACGGAAT	Sequencing, 1272-1291
SSU-SR4-For [3]	CATCAGTTGTGTGATACGTCC	Sequencing, 1558-1581

[1], Scholin *et al*., 1994; [2], Medlin *et al*., 1988; Saldarriaga *et al*., 2001; [3], Specific for Greek C. monotis where the positions of the primers are numbered with respect to the sequences generated.

Microscopy

Clones were examined under light microscopy and digitally photographed using a Sony CCD Hyper HAD digital camera. For SEM, 10 ml culture (exponential phase) were initially filtered using a 60 μm Nitex net, then placed in glass centrifuge tubes with 4 ml of clean seawater and fixed in 2% OsO_4 buffered with PBS in the dark for 1h at 4°C. The cells were then pelleted at 400 g, and washed with 3 decreasing concentrations of seawater then with filtered tap water. Following centrifugation, the cell pellet was left to stand overnight in 1:1 [30% H_2O_2]:[glacial acetic acid], then rinsed 4 times with filtered tap water. An aliquot of the final pellet was mixed with acetone on a round cover slip and air-dried overnight at 30°C. The sample was gold-plated and observed in a Jeol JSM-35 SEM. For epifluorescence microscopy, 2 ml exponential culture were fixed with 2.5% glutaraldehyde in the dark for 1h at 4°C, buffered in PBS, washed twice for 5 minutes in 10% Triton-X, then stained. Cellulose thecal plates were stained with calcofluor white (Sigma-Aldrich), using the method of Fritz and Triemer (1985). Samples were viewed using an Olympus AX epifluorescence microscope, using a Chroma 730 nm emission filter s/n 11000 (Rockingham, VT). Images were acquired with an Optronics DEI 750 camera (Goleta, CA).

DNA extraction

Cultures of approximately $2x10^6$ cells in exponential phase of strains C1 and C3 (identical to C2 and C4 respectively since they were taken from the same primary clone) were filtered through 60 μm Nitex nets, then through 8 μm polycarbonate Nuclepore membrane filters, washed with 0.2 um filtered, sterilized seawater and transferred into 15 ml centrifuge tubes. The cells were pelleted at 400 g for 8 minutes and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA quality and quantity was assessed by spectrophotometry, and by agarose gel electrophoresis. Approximately 20 to 25 pg of DNA was extracted per cell.

Polymerase Chain Reaction (PCR) amplification

Primers used for PCR (Saiki *et al*., 1988) amplification of gene sequences were made by Invitrogen (Carlsbad, CA) and are listed in Table 1. LSU primer D1R-forward (Domain "D1") was targeted towards conserved core sequence positions 24 to 43, while primer D2C-reverse was for positions 733 to 714 relative to *Prorocentrum micans* LSU rRNA (Lenaers *et al*., 1989). The SSU eukaryotic universal forward primer EUF was targeted towards conserved core sequences 1 to 22 relative to the 18S sequence of dinoflagellates while the eukaryotic universal reverse primer EUR was for position 1795 to 1819. PCR was done using an Invitrogen AccuPrime kit following the manufacturer's instructions. An MJ Research (Waltham, MA) PTC-225 Peltier Thermal Cycler (DNA Engine Tetrad) was used for 50 μl PCR reactions with thin-walled 0.2 ml Eppendorf tubes. Approximately 40 ng of DNA template per 700 bp fragment was used with 100 pmol each of forward and reverse primer. Invitrogen AccuPrime (Carlsbad, California) and Roche (Basel, Switzerland) core kits were used. PCR cycles were: denaturation at 94°C for 4 min., 35 cycles of 94°C for 1 min., 41 to 45°C for 2.5 min., and 72°C

TABLE 2. – Dinoflagellates used for sequence comparisons and phylogenetic tree construction.

I. Species used for LSU sequence comparisons and accession number reference

Akashiwo sanguinea (Gymnodinium Sanguinea) [a]	AF042817
Alexandrium andersoni	U44937
Alexandrium margalefii	AY154958
Alexandrium minutum	AF318232
Alexandrium tamarense	AF200668
Ceratium fusus	AF260390
Ceratium lineatum	AF260391
Coolia sp. *(malayense?)*	AF244942
Coolia monotis	U92258
Fragilidium subglobosum	AF260387
Fragilidium subglobosum	AF033868
Gonyaulax baltica	AF260388
Heterocapsa triquetra	AF260401
Ostreopsis lenticularis	AF244941
Ostreopsis cf. ovata	AF244940
Peridinium bipes	AF260385
Prorocentrum micans	X16108
Protoceratium reticulatum	AF260386
Pyrodinium bahamense var. compressum	AY154959
Pyrodinium bahamense var. compressum	AY566194
Scrippsiella trochoidea var. aciculifera	AF260393

II. Species used for SSU sequence comparisons and accession number reference

Alexandrium margalefeii	U27498
Alexandrium minutum	U27499
Alexandrium ostenfeldii	U27500
Alexandrium tamarense	AF022191
Ceratium furca	AJ276699
Ceratium tenue	AF022192
Coolia monotis	AJ415509
Fragilidium subglobosum	AF033869
Heterocapsa triquetra	AF022198
Gonyaulax spinifera	AF022155
Karenia mikimotoi (Gymnodinium mikimotoi) [a]	AF009131
Ostreopsis ovata	AF244939
Pentapharsodinium tyrrhenicum	AF022201
Peridinium bipes	AF231805
Prorocentrum micans	M14649
Protoceratium reticulatum	AF274273
Pyrodinium bahamense	AF274275
Scrippsiella trochoidea	AF274277

[a] see Daugbjerg *et al*., 2000

for 4 min. Final extension was at 72°C for 10 min. Successful amplification was confirmed by agarose gel electrophoresis.

Sequencing

Amplified fragments were gel purified using a MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purified fragments were sequenced by dye terminator cycle sequencing using a DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. The sequencing primers used are listed in Table 1. Following ethanol precipitation, sequencing reactions were analyzed on a Beckman Coulter CEQ 2000.

Phylogenetic tree construction

Sequences were initially evaluated using the Basic Length Alignment Searching Tool (BLAST; Altschul *et al*., 1990) against deposited sequences (GenBank). Using *Prorocentrum micans* as an outgroup, sequences of 21 and 18 taxa of dinoflagellates (Table 2) were used to produce the LSU and SSU trees respectively. For the partial LSU, 626 characters were considered unambiguous and used for the alignment (see also John *et al*., 2003). The LSU genotype from Malaysia deposited as *C. malayense* (AF244942) was used as *Coolia* sp. (*C. monotis* ?) (see also Usup *et al*., 2002) in the absence of morphologic validation and as it exhibited 99 % similarity with *C. monotis* from New Zealand. Multiple sequence alignments were carried out using ClustalX (Thomson *et al*., 1997). Alignments were checked and improved using BioEdit manually (Hall, 1999). Using the PHYLIP 3.63 software package (Felsenstein, 2004), distance matrices were produced using the DNADIST module assuming Kimura's two-parameter model (Kimura, 1980) and then used to construct the phylogenetic tree topologies with the NEIGHBOR module (NJ, neighbor-joining algorithm of Saitou and Nei, 1987). In order to confirm robustness of the Ostreopsidaceae clades, the NJ topology was checked against the tree produced using the FITCH module (Fitch-Margoliash Least-Squares Distance method of Fitch and Margoliash, 1967) and the modules DNAPARS (Fitch, 1971) and DNAML (Felsenstein and Churchill, 1996) for Parsimony and Maximum Likelihood (ML) respectively. One thousand bootstrap resamplings (Felsenstein, 1985) were performed using the SEQBOOT module and the consensus tree was generated using CONSENSE. Trees were viewed with the Treeview software (Page, 1996).

RESULTS

Light Microscopy

In light microscopy (Fig. 1a, b) *Coolia monotis* cells from cultures were observed and the oblique axis seen when viewed from the side, and a compressed spherical shape when viewed ventrally with a strongly-lipped and defined cingulum and sulcus. Cells were variable in size, 30 to 40 μm in length. Smaller-sized cells were observed during reproduction. Cleaned cells under phase contrast microscopy (Fig. 2a, b)

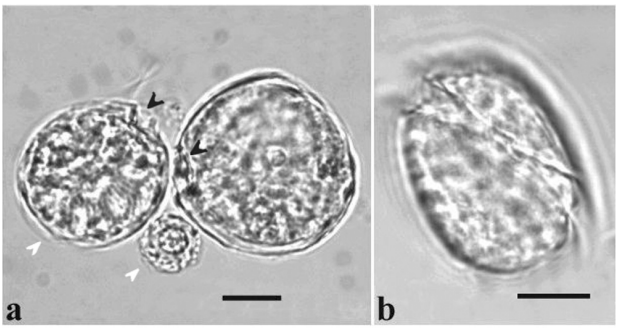

FIG. 1. – *Coolia monotis* cells (fixed) under light microscopy showing: a) Variability in cell size with smaller cells during reproduction (white arrowheads), and a strongly-lipped and defined sulcus (black arrowheads); b) The oblique axis when viewed from the side with strongly-lipped and defined cingulum. Scale = 10 μm.

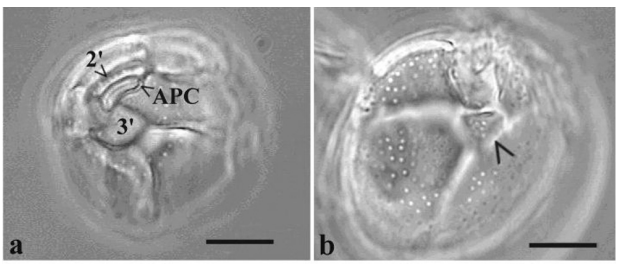

FIG. 2. – Cleaned cells of *Coolia monotis* in phase-contrast microscopy showing: a) plate 2' curving around the apical pore complex, along with the pentagonal and partly wedge-shaped 3' plate; b) the pentagonal 2'''' plate (arrowhead) at the end of the sulcus. Scale = 10 μm.

revealed a pentagonal and partly wedge-shaped 3' plate touching the APC. At the antapex, the pentagonal 2'''' plate was observed at the end of the sulcus.

Staining *Coolia monotis* with calcofluor revealed the general thecal architecture of 3 apical ('), 7 precingular (''), 5 postcingular (''') and 2 antapical (''''') plates (Fig. 3a-d). The existence of posterior intercalaries (p) could not be confirmed. Six cingulum (6c) plates were recorded. The APC was surrounded by plates 1', 2' and 3' (Fig. 3a, b). The lipped cingulum and sulcus were evident in the ventral view (Fig. 3c), while the antapical plates were seen dorsal-posteriorly (Fig. 3d).

Scanning Electron Microscopy

The plates of *C. monotis* Meunier (Fig. 4a) had smooth surfaces perforated by large, round or oval pores. Plates were thick with clear overlapping (imbrication) and smooth intercalary bands of equal width. The oblong, six-sided apical 1' plate was situated on the left side of the cell, its right edge ran along the central cell axis. The large, deep and slightly curved APC was positioned dorsally on the left side of the cell, half-way between the apex and cingulum, with a single 10-12 μm pore plate (Po)

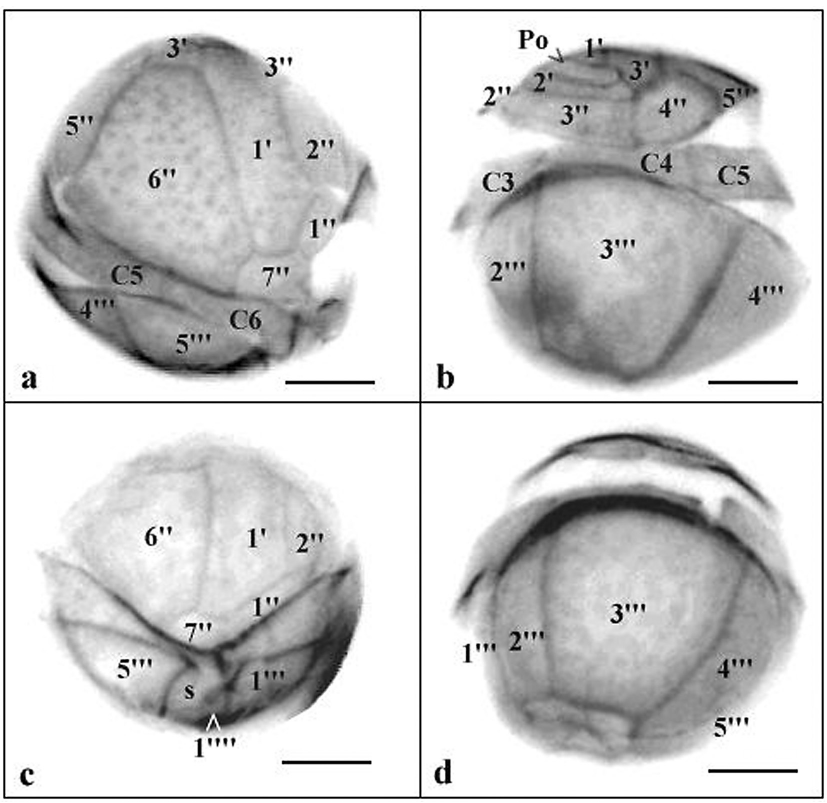

FIG. 3. – Calcofluor-stained cells of *Coolia monotis* in epifluorescence microscopy showing thecal plate architecture: a) anterior-ventral view of the right side; b) dorsal view showing the apical pore plate (Po); c) ventral view showing the sulcus (s) and the antapical 1''''plate (white arrow); d) dorsal-posterior view. Scale = 10 μm.

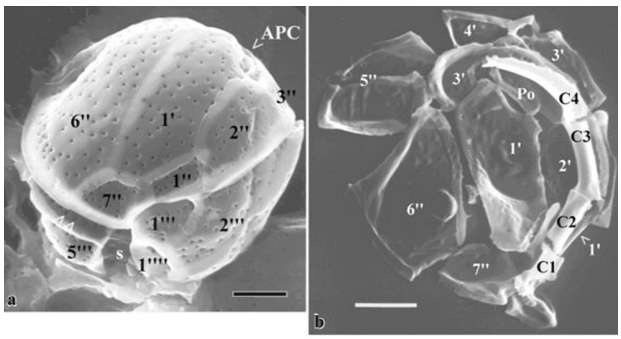

FIG. 4. – *Coolia monotis* cleaned specimen under SEM: a) left view of the anterior-ventral side showing thecal plate architecture, sulcus (s), cingulum pores (double arrowhead), the APC, the smooth plate surfaces and imbrication; b) interior view of collapsed epicone showing characteristic wedge-shaped 3' plate and part of the cingulum. Scale = 10 μm.

perforated by a clear row of small pores and containing the 7-9 μm, slit-like apical pore opening. Apical plate 2'' was wider than its neighboring precingular plates. The lists of the cingulum and sulcus were very prominent, with plate 1'''' extending into a sulcus list. The cingulum and sulcus were deep and wide in all specimens. The interior view of the collapsed epicone showed a characteristic wedge-shaped 3' plate (Fig. 4b).

DNA

Sequences of clones C1 and C3 were compared and found to be identical for both the partial LSU and SSU sequences.

For the LSU, a 700 bp fragment was generated and sequenced. This sequence aligned with the reported LSU sequences of *C. monotis* from New Zealand and *Coolia* sp. (*C. malayense*?) from Malaysia, both at 84% nucleotide similarity. The sequences of the Greek strain were highly similar in the D1 region (93% nucleotide similarity), but greater variation was observed after nucleotide 400, the D2 region (ca. 74% nucleotide similarity). Other LSU sequence similarity values ranged between 60% and 61% with *Alexandrium* spp., *Pyrodinium bahamense* var. *compressum*, and *Gonyaulax baltica*, 55 and 57% with *Fragilidium subglobosum*, and 54 and 55% with *Ostreopsis* spp..

A 1757 bp SSU sequence was generated and read. This sequence aligned with the reported SSU sequences of *C. monotis* from Norway at 99% nucleotide similarity. BLAST sequence alignment showed the closest related dinoflagellates to be taxa

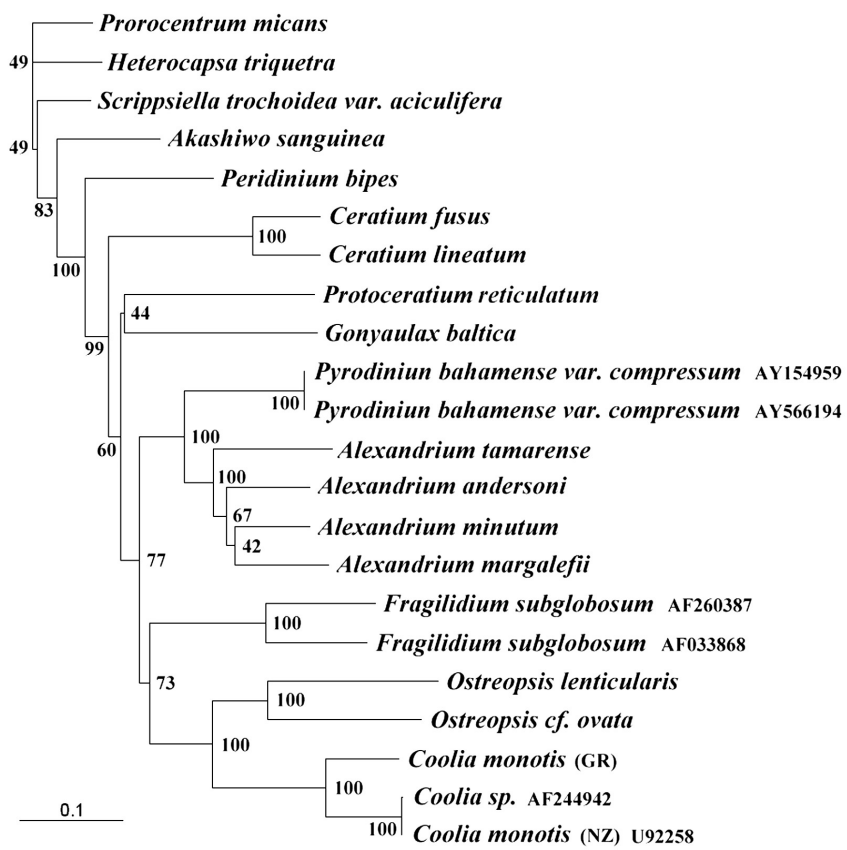

FIG. 5. – Partial LSU neighbor-joining distance phylogenetic tree comparing the Greek *C. monotis* with 21 dinoflagellates using *Prorocentrum micans* as an outgroup. Numbers at nodes are bootstrap consensus values (1000 replications).

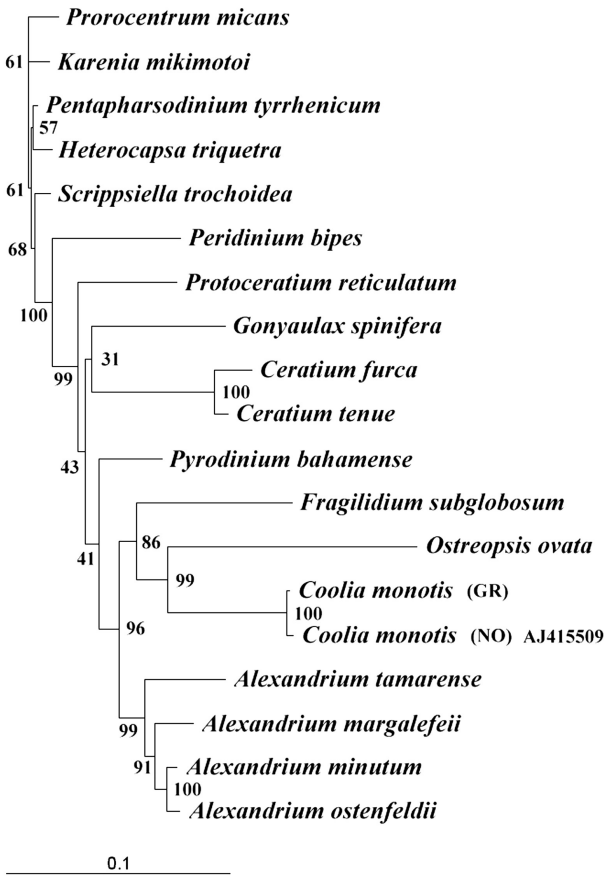

FIG. 6. – Neighbor-joining distance phylogenetic SSU tree comparing the Greek *C. monotis* with 18 dinoflagellates using *Prorocentrum micans* as an outgroup. Numbers at nodes are bootstrap consensus values (1000 replications).

of *Ostreopsis*, *Alexandrium* and *Pyrodinium*. Nucleotide similarity values were between 88% and 89% with *Alexandrium* spp., 87% with *Pyrodinium bahamense*, 84 % with *Gonyaulax spinifera*, 83 % with *Fragilidium subglobosum*, and 81% with *Ostreopsis ovata*.

Phylogeny

In both the LSU and SSU neighbor phylogenetic trees, *Coolia* strains clustered at 100% bootstrap values, while *Coolia* and *Ostreopsis* species (Ostreopsidaceae) formed a discrete cluster with decreasing affiliation with *Fragilidium* (Pyrophacaceae), and *Alexandrium* and *Pyrodinium* strains (Goniodomataceae). The tree determined from the LSU sequences showed that the *Coolia* cluster is related to that of *Ostreopsis* at a 100% bootstrap value (see Fig. 5), compared to a 99% bootstrap value for the SSU sequence tree (see Fig. 6). All other phylogenetic trees constructed (Fitch, Parsimony, ML; not shown here), for both the LSU and SSU sequences, gave very similar tree topology and bootstrap results. The differences were the positioning of *Fragilidium* with either the *Coolia*/*Ostreopsis* or the *Alexandrium* clade and the topology of *Ceratium* in relation to *Gonyaulax* and *Protoceratium*. The Greek *C. monotis,* however, was repeatedly confirmed in the *Coolia* clade at 100% bootstrap values, with a close relation to the *Ostreopsis* clade (99-100%).

DISCUSSION

Morphologically the Greek strain of *C. monotis* Meunier (Meunier, 1919; Balech, 1956; Fukuyo, 1981; Faust, 1992, 1995; Steidinger and Tangen, 1997; Ten-Hage *et al*. 2000), exhibits slight variations from other *C. monotis* strains of different geographical origins. The apical plate 1' is comparatively narrower with straight edges and the 2'' plate is much wider, similar only to Balech's (1956) findings. The APC is wider and the sulcus more curved than that of the Belizian *C. monotis* strain (Faust 1992). The characteristic partly wedge-shaped 3' plate is unlike any other reported in the literature, resembling more that observed for the species *C. tropicalis* (Faust, 1995). The cingulum and sulcus are wider in the Greek strain, resembling more those observed for the species *C. areolata* by Ten-Hage *et al*. (2000).

The partial LSU nucleotide sequence of the Greek *C. monotis* exhibited relatively low pairwise similarity (84%) to the LSU of *C. monotis* from New Zealand or *Coolia* sp. (*C. monotis*?) from Malaysia. Although it has nearly identical morphologically, the Greek strain's sequence varied by a very high number of 113 bp positions from the New Zealand one, mostly in the D2 region. This large number of character differences, however, is also observed when comparing the ITS rDNA sequences for various geographic-origin *C. monotis* strains deposited at GenBank (Penna *et al*., 2002). The SSU sequence of the Greek *C. monotis* had 15 base pair differences from the Norwegian *C. monotis*'s SSU sequence, which resulted in a very high sequence similarity between them (99%). The large similarity among the compared dinoflagellate SSU sequences contrasted with the lower similarity observed between the LSU sequences. However, using both sequences provided better phylogenetic analyses of *C. monotis* as has been done with *Dinophysis* (Edvardsen *et al*., 2003) and other algae (Huelsenbeck *et al*., 1996; Buchheim *et al*., 2001; Harper and Saunders, 2001).

Phylogenies inferred from both LSU and SSU neighbor-joining trees were similar, agreeing with the Dinophyceae phylogeny produced by others (e.g. Daugbjerg *et al*., 2000; Ellegaard *et al*., 2003; Saldarriaga *et al*., 2004). In overall, topologies in both of the phylogenetic trees support the morphology-based taxonomic system (Taylor, 1980; Taylor, 1987; Fensome *et al*., 1993; Steidinger and Tangen, 1997). The phylogenetic clade containing *Coolia* and *Ostreopsis* (see Figure 5 and Figure 6) was found to be very robust and in agreement with the identical plate formulae of these two genera (Balech, 1956; Besada *et al*., 1982; Faust *et al*., 1996; Steidinger and Tangen, 1997). Although these two Ostreopsidaceae taxa fall within the order Peridiniales in the NCBI taxonomic database (http://www.ncbi.nih.gov/Taxonomy/), Taylor (1987), Fensome *et al*., (1993) and Steidinger and Tangen (1997) place these genera, classically, in the order Gonyaulacales (Taylor, 1980). Our findings support the latter taxonomic structure in which a close relationship appears between the investigated Gonyaulacales families (Lindemann, 1928) such as Ostreopsidaceae (*Coolia*, *Ostreopsis*), Goniodomataceae (*Alexandrium*, *Pyrodinium*), Pyrophacaceae (Fragilidium), Ceratiaceae (*Ceratium*) and Gonyaulacaceae (*Gonyaulax*). This also agrees with the distant relationship observed in our phylogenetic analyses of the Peridiniales (e.g. *Heterocapsa*, *Pentapharsodinium*, *Scrippsiella*) to the Gonyaulacales, the latter also consistently forming a very robust phylogenetic group in the majority of extensive phylogenies carried out by other researchers. Genetic information concerning *Gambierdiscus* and other *Coolia* species could further elaborate Gonyaulacales phylogeny and structures of Ostreopsidaceae and Goniodomataceae families.

Our initial observations indicate the probable existence of intraspecific genetic variability (IGV, expressed as nucleotide differences) with significantly different LSU and SSU genotypes for *C. monotis*. High genetic variability with only slight morphological variations do not warrant new species designations, whereas the possible existence of cryptic species within populations cannot be ruled out (Montresor *et al*., 2003). Likewise, the possible existence of dominant strain types of *C. monotis* in various geographic populations (as with *Prorocentrum micans*; see Shankle, 2001; Shankle *et al*., 2004) needs to be assessed. Species genotypes from different geographical origins (populations) that have a high degree of conserved morphological features are also expected (Taylor, 1993; Medlin *et al*, 2000; Coyne and Orr, 2004). As the criteria used for species differentiation based on genetic information are continuously debated (Manhart and McCourt, 1992; Taylor, 1993; Coyne and Orr, 2004), it will continue to be necessary to use both morphotypes and genotypes when defining strains, especially when ascertaining the extent of speciation and strain development of an organism. Some taxonomical methods will probably need to be upgraded by using new genetic regions for strain identification, as proof exists that morphologically or toxically variable dinoflagellate strains such as *Karenia brevis* and *Pfiesteria piscida* (Loret *et al*., 2002; Tengs *et al*., 2003) have identical rDNA sequences. Open questions also remain as to how sexual reproduction and polyploidy of dinoflagellates (Pfiester and Anderson, 1987; Faust, 1992) compound genetic variability and speciation.

Intraspecific genetic variability observed in distantly-related Dinophyceae such as *Karenia*, *Gymnodinium*, *Amphidinium* and *Dinophysis* spp. appears to be minimal compared to that observed for Gonyaulacalean dinoflagellates (~10 to 50% IGV depending on the rDNA gene) such as *Alexandrium* (Scholin *et al*., 1994; Adachi *et al*., 1996; Guillou *et al*., 2002; John *et al*., 2003), *Coolia* (Penna *et al*., 2002), *Ostreopsis* (Pin *et al*., 2001; Penna *et al*., 2002), and *Gambierdiscus* (Babinchak *et al*., 1996; Chinain *et al*., 1999). This fact seems true even for the highly variable and quick evolving ITS region, but less evident for the slow evolving SSU. For example, compared to the 16% IGV found in this study for the D1/D2 LSU (700 bp) and 0.85 % for the SSU of *Coolia monotis*, intraspecific genetic variability for *Amphidinium* spp. was only 0 to 4.4% for the larger D1-D6 LSU fractions (ca. 1400 bp) (Murray *et al*., 2004), for *Karenia* and *Gymnodinium* spp. it was 0 to 0.3% for the D1/D2/D3 LSU fraction (ca. 700-1000 bp) (Adachi *et al*., 1997; Sako *et al*., 1998; Hansen *et al*. 2000; Guillou *et al*., 2002), and for *Dinophysis* spp. it was 0 to 2.0% for the D1/D2 LSU fraction, the SSU and ITS1-ITS2 regions(Guillou *et al*., 2002; Edvardsen *et al*., 2003). As numerous biological and ecological parameters affect intraspecific diversity (Medlin *et al*., 2000; Schluter, 2001; Montresor, 2003; Coyne and Orr, 2004), the above-observed large variations in species' IGV still remain to be confirmed and explained. Moreover, the evolution and the biogeography of strains need to be assessed in order to arrive at reasonable conclusions concerning dinoflagellate IGV. For example, the closely-related *Alexandrium* genus, like *Coolia*, apart from demon-

strating high IGV also seems to have evolved late in dinoflagellate history (see John *et al*., 2003; Saldarriaga *et al*., 2004), in comparison to the distantly-related and "older" dinoflagellates such as those of the order Gymnodiniales which coincidentally seem to have low IGV. Such work could also shed light on any relation of IGV with other dinoflagellate classification systems such as plate evolution models (see Saunders *et al*., 1997; Saldarriaga *et al*., 2004).

ACKNOWLEDGEMENTS

We greatly appreciate the help given to us by Nancy Skoura and Zoi Lada of the University of Connecticut for DNA sequencing and by Barry Elkind for PCR. Don Anderson and Dave Kulis of Woods Hole Oceanographic Institution, Mass. USA, provided valuable comments for dinoflagellate identification and taxonomy. This work represents part of a doctoral thesis funded by the IRAKLEITOS Fellowships for Research of NKUA-ENVIRONMENT and co-financed within Op. Education by the ESF (European Social Fund) and National Resources.

REFERENCES

Adachi, M., Y. Sako and Y. Ishida. – 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol*., 32: 424-432.

Adachi, M., Y. Sako and Y. Ishida. – 1997. Analysis of *Gymnodinium catenatum* Dinophyceae using sequences of the 5.8S rDNA-ITS regions and random amplified polymorphic DNA. *Fish. Sci*., 63: 701-707.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. – 1990. Basic local alignment search tool. *J. Mol. Biol*., 215: 403-410.

Babinchak, J.A., G.J. Doucette and R.M. Ball. – 1996. Partial characterization of the LSU rRNA gene from the ciguatoxic dinoflagellate, *Gambierdiscus toxicus*. In: T. Yasumoto, Y. Oshima and Y. Fukuyo (eds.), *Harmful and toxic algal blooms*, pp. 459-462. Intergovernmental Oceanographic Commission of UNESCO, Japan.

Balech, E. – 1956. Étude des dinoflagellés du sable de Roscoff. *Rev. Algol. (N. Ser.)*, 2: 29-52.

Baroin-Tourancheau A., P. Delgado, R. Perasso and A. Adoutte. – 1992. A broad molecular phylogeny of ciliates: identification of major evolutionary trends and radiations within the phylum. *Proc. Natl. Acad. Sci. USA*, 89: 9764-9768.

Besada, E.G., L.A. Loeblich and A.R. Loeblich III. – 1982. Observations on tropical, benthic dinoflagellates from ciguatera-endemic areas: *Coolia*, *Gambierdiscus*, and *Ostreopsis*. *Bull. Mar. Sci*., 32: 723-735.

Buchheim, M.A., E.A. Michalopulos and J.A. Buchheim. – 2001. Phylogeny of the Chlorophyceae with special reference to the Sphaeropleales: A study of 18S and 26S rDNA data. *J. Phycol*., 37: 819-835.

Chinain, M., M.A. Faust and S. Pauillac. – 1999. Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dynophyceae): *G. pacificus*, sp nov., *G. australes*, sp.

Coyne, J.A. and A.H. Orr. – 2004. *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Daugbjerg, N., G. Hansen, J. Larsen and Ø. Moestrup. – 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of naked dinoflagellates. *Phycologia*, 39: 302-317.

Edvardsen, B., K. Shalchian-Tabrizi, K.S. Jakobsen, L.K. Medlin, E. Dahl, S. Brubak and E. Paasche. – 2003. Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analyses of rDNA. *J. Phycol*., 39: 395-408.

Ellegaard, M., N. Daugbjerg, A. Rochon, J. Lewis, I. Harding. – 2003. Morphological and LSU rDNA sequence variation within the *Gonyaulax spinifera-Spiniferites* group (Dinophyceae) and proposal of *G. elongata* comb. nov. and *G. membranacea* comb. nov.. *Phycologia*, 42: 151-164.

Faust, M.A. – 1992. Observations on the morphology and sexual reproduction of *Coolia monotis* (Dinophyceae). *J. Phycol*., 28: 94-104.

Faust, M.A. – 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. *J. Phycol*., 31: 996-1003.

Faust, M.A., S.L. Morton and J.P. Quod. – 1996. Further SEM study of marine dinoflagellates: The genus *Ostreopsis* (Dinophyceae). *J. Phycol*., 32: 1053-1065.

Felsenstein, J. – 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution*, 39: 783-791.

Felsenstein, J. – 2004. PHYLIP (Phylogeny Inference Package) version 3.63. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Felsenstein, J. and G.A. Churchill. – 1996. A hidden Markov model approach to variation among sites in rate of evolution *Mol. Biol. Evol*., 13: 93-104.

Fensome, R.A., F.J.R. Taylor, G. Norris, W.A.S. Sarjeant, D.I. Wharton and G.L. Williams. – 1993. *A classification of living and fossil dinoflagellates. Micropaleontology special publication 7*. Sheridan Press, Hanover, PA.

Fitch, W.M. and E. Margoliash. – 1967. Construction of phylogenetic trees. *Science*, 155: 279-284.

Fitch, W.M. – 1971. Toward defining the course of evolution: minimum change for a specified tree topology. *Syst. Zool*., 20: 406-416.

Fritz, L. and R.E. Triemer. – 1985. A rapid simple technique utilizing Calcofluor White M2R for the visualization of dinoflagellate thecal plates. *J. Phycol*., 21: 662-664.

Fukuyo, Y. – 1981. Taxonomical study on benthic dinoflagellates collected in coral reefs. *Bull. Jpn. Soc. Sci. Fish*., 47: 967-978.

Grzebyk, D., Y. Sako and B. Berland. – 1998. Phylogenetic analysis of nine species of Prorocentrum (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological comparisons and description of *Prorocentrum panamensis* sp. nov.. *J. Phycol*., 34: 1055-1068.

Guillard, R.R.L. – 1995. Culture methods. In: G.M. Hallegraeff, D.M. Anderson and A.D. Cembella, (eds.), *Manual on harmful marine microalgae*. IOC Manuals and Guides No. 33, pp. 45-62. UNESCO, Paris.

Guillou, L., E. Nézan, V. Cueff, E. Erard-Le Denn, M.-A. Cambon-Bonavita, P. Gentien and G. Barbier. – 2002. Genetic diversity and molecular detection of three toxic dinoflagellate genera (*Alexandrium*, *Dinophysis*, and *Karenia*) from French coasts. *Protist*, 153: 223-238.

Hall, T.A. – 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser*., 41: 95-98.

Hansen, G., N. Daugbjerg and P. Henriksen. – 2000. Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, comb. nov. (= *Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. *J. Phycol*., 36: 394-410.

Harper, J.T. and G.W. Saunders. – 2001. Molecular systematics of the Florideophyceae (Rhodophyta) using nuclear large and small subunit rDNA sequence data. *J. Phycol*., 37: 1073-1082.

Hassouna, N., B. Michot and J.P. Bachellerie. – 1984. The complete nucleotide sequence of mouse 28S rRNA gene - implications for the process of size increase of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Res*., 12: 3563-3583.

Huelsenbeck, J.P., J.J. Bull and C.W. Cunningham. – 1996. Combining data in phylogenetic analysis. *Trends Ecol. Evol*., 4: 152-158.

ICES/IOC. – 1999. *Report of the Working Group on Harmful Algal*

Bloom Dynamics, Jena, Germany, 16–20 March 1999. Oceanography Committee, International Council for the Exploration of the Sea, CM 1999/C:4, ACME. Copenhagen, Denmark.
ICES/IOC. – 2000. *Report of the Working Group on Harmful Algal Bloom Dynamics, Barcelona, Spain 20-24 March 2000*. Oceanography Committee, International Council for the Exploration of the Sea, CM 2000/C:06, ACME, E. Copenhagen, Denmark.
John, U, R.A. Fensome and L.K. Medlin. – 2003. The application of a molecular clock based on molecular sequences and the fossil record to explain biogeographic distributions within the *Alexandrium tamarense* "species complex" (Dinophyceae). *Mol. Biol. Evol*., 20: 1015-1027.
Kimura, M. – 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*., 16: 111-120.
Lenaers, G., L. Maroteaux, B. Michot and M. Herzog. – 1989. Dinoflagellates in evolution. A molecular phylogenetic analysis of large subunit ribosomal RNA. *J. Mol. Evol*., 29: 29-40.
Lenaers, G., C. Scholin, Y. Bhaud and D. Saint-Hilaire. – 1991. A molecular phylogeny of dinoflagellate protists (Pyrrhophyta) inferred from the sequence of 24S rRNA divergent domains D1 and D8. *J. Mol. Evol*., 32: 53-63.
Lenoir, S., L. Ten-Hage, J. Turquet, J.P. Quod, C. Bernard and M.C. Hennion. – 2004. First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. *J. Phycol*., 40: 1042-1051.
Lindemann, E. – 1928. Neue Peridineen. *Hedwigia*, 68: 291-296.
Loret, P., T. Tengs, T.A. Villareal, H. Singler, B. Richardson, P. McGuire, S. Morton, M. Busman and L. Campbell. – 2002. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. *J. Plankton Res*., 24: 735-739.
Manhart, J.R. and R.M. McCourt. – 1992. Molecular data and species concepts in the algae. *J. Phycol*., 28: 730-737.
Medlin, L., H.J. Elwood, S. Stickel and M.L. Sogin. – 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene*, 71: 491-499.
Medlin, L.K., M. Lange and E.M. Nothig. – 2000. Genetic diversity in the marine phytoplankton: a review and a consideration of Antarctic phytoplankton. *Antarct. Sci*., 12: 325-333.
Meunier, A. – 1919. Microplancton de la mer Flamande. 3. Les Peridiniens. *Mem. Mus. R. Hist. Nat. Bruxelles*, 8: 1-116.
Michot, B., N. Hassouna and J.P. Bachellerie. – 1984. Secondary structure of mouse 28S rRNA and general model for the folding of the large RNA in eukaryotes. *Nucleic Acids Res*., 12: 4259-4279.
Montresor, M., S. Sgrosso, G. Procaccini and W.H.C.F. Kooistra. – 2003. Intraspecific diversity in *Scrippsiella trochoidea* (Dinophyceae): evidence for cryptic species. *Phycologia*, 42: 56-70.
Murray, S., M.F. Jørgensen, N. Daugbjerg, and L. Rhodes. – 2004. *Amphidinium* revisited II. Resolving species boundaries in the *Amphidinium operculatum* species complex (Dinophyceae) including the descriptions of *Amphidinium trulla* sp. nov. and *Amphidinium gibbosum* comb. nov. *J. Phycol*., 40:366-382.
Page, R.D.M. – 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comp. Appl. Biosc*., 12: 357-358.
Penna, A., M. Vila, S. Fraga, M.G. Giacobbe, E. Gangemi, F. Andreoni, E. Bertozzini, E. Garcés, M. Masò, P. Riobò and M. Magnani. – 2002. *Population diversity of Ostreopsidaceae: a preliminary study on the genetics, morphology and toxicity*. 10th International Conference on Harmful Algae, October 21-25, 2002, St. Pete Beach, Florida, USA.
Pfiester, L.A. and D.M. Anderson. – 1987. Dinoflagellate reproduction. In: F.J.R. Taylor, (ed.), *The biology of dinoflagellates. Botanical Monographs Vol. 21*, pp. 611-648. Blackwell Scientific, Oxford,
Pin, L.C., L.P. Teen, A. Ahmad and G. Usup. – 2001. Genetic diversity of *Ostreopsis ovata* (Dinophyceae) from Malaysia. *Mar. Biotechnol*., 3: 246-255.
Rhodes, L., J. Adamson, T. Suzuki, L. Briggs and I. Garthwaite. – 2000. Toxic marine epiphytic dinoflagellates, *Ostreopsis siamensis* and *Coolia monotis* (Dinophyceae), in New Zealand. *NZ J. Mar. Fresh. Res*., 34: 371-384.
Riobó, P., B. Paz, M. Fernández, S. Fraga and J.M. Franco. – 2002. *Lipophylic toxins of different strains of Ostreopsidaceae and Gonyaulacaceae*. 10th International Conference on Harmful Algae, October 21-25, 2002, St. Pete Beach, Florida, USA.
Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich. – 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239: 487–491.
Saitou, N. and M. Nei. – 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 4: 406-425.
Sako, Y., M. Rokushima, M. Yamaguchi, Y. Ishida and A. Uchida. – 1998. Phylogenetic analysis and molecular identification of red tide dinoflagellate *Gymnodinium mikimotoi* using ribosomal RNA genes. In: B. Reguera, J. Blanco, M.L. Fernández and T. Wyatt (eds.), *Harmful Algae*, pp. 295-298. Intergovernmental Oceanographic Commission of UNESCO, Vigo, Spain.
Saldarriaga, J.F., F.J.R. Taylor, P.J. Keeling and T. Cavalier-Smith. – 2001. Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple plastid losses and replacements. *J. Mol. Evol*., 53: 204-213.
Saldarriaga, J.F., F.J.R. Taylor, T. Cavalier-Smith, S. Menden-Deue and P.J. Keeling. – 2004. Molecular data and the evolutionary history of dinoflagellates. *Eur. J. Protistol*., 40: 85-111.
Saunders, G.W., D.R. Hill, J.P. Sexton and R.A. Andersen. – 1997. Small-subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. In: Bhattacharya, D. (ed.), *Origins of algae and their plastids*, pp. 237-259. Springer-Verlag, New York.
Schluter, D. – 2001. Ecology and the origin of species. *Trends Ecol. Evol*., 16: 372-380.
Scholin, C.A., M. Herzog, M. Sogin and D.M. Anderson. – 1994. Identification of group and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *J. Phycol*., 30: 999-1011.
Steidinger, K.A. and K. Tangen. – 1997. Dinoflagellates. In: C.R. Tomas (ed.), *Identifying marine phytoplankton*, pp. 387-589. Academic Press, San Diego.
Taylor, F.J.R. – 1980. On dinoflagellate evolution. *Biosystems*, 13: 65-108.
Taylor, F.J.R. – 1987. Taxonomy and classification. In: Taylor, F.J.R. (ed.), *The biology of dinoflagellates. Botanical Monographs Vol.21*, pp. 723-731 (Appendix). Blackwell Scientific, Oxford.
Taylor, F.J.R. – 1993. The species problem and its impact on harmful phytoplankton studies. In: T.J. Smayda and Y. Shimizu (eds.), *Toxic Phytoplankton Blooms in the Sea*, pp. 81-86. Elsevier, New York.
Taylor, F.J.R. – 1999. Charles Atwood Kofoid and his Dinoflagellate tabulation system: an appraisal and evaluation of the phylogenetic value of tabulation. *Protist*, 150: 213-220.
Taylor, F.J.R. and U. Pollingher. – 1987. Ecology of dinoflagellates, A. General and marine ecosystems. In: F.J.R. Taylor (ed.), *The biology of dinoflagellates. Botanical Monographs Vol.21*, pp. 388-502. Blackwell Scientific, Oxford.
Ten-Hage, L., J. Turquet, J.P. Quod and A. Couté. – 2000. *Coolia areolata* sp. nov. (Dinophyceae), a new sand-dwelling dinoflagellate from the southwestern Indian Ocean.. *Phycologia*, 39: 377-383.
Tengs, T., H.A. Bowers, H.B. Glasgow, J.M. Burkholder and D.W. Oldach. – 2003. Identical ribosomal DNA sequence data from *Pfiesteria piscicida* (Dinophyceae) isolates with different toxicity phenotypes. *Environ. Res*., 93: 88-91.
Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. – 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*., 25: 4876-4882.
Tolomio, C. and F. Cavolo. – 1985. Presenza di *Coolia monotis* Meunier (Dinophyceae, Perediniales) nelle acque della Laguna di Venezia. *Oebalia*, 11: 849-852.
Usup, G., L.C. Pin, A. Ahmad and L.P. Teen. – 2002. Phylogenetic relationship of *Alexandrium tamiyavanichii* (Dinophyceae) to other *Alexandrium* species based on ribosomal RNA gene sequences. *Harmful Algae*, 1: 59–68.

Scien. ed.: M. Estrada