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GENETIC VARIABILITY AND MOLECULAR PHYLOGENY OF *DINOPHYSIS* SPECIES (DINOPHYCEAE) FROM NORWEGIAN WATERS INFERRED FROM SINGLE CELL ANALYSES OF rDNA[1]

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The objectives of this study were to determine rDNA sequences of the most common *Dinophysis* species in Scandinavian waters and to resolve their phylogenetic relationships within the genus and to other dinoflagellates. A third aim was to examine the intraspecific variation in *D. acuminata* and *D. norvegica*, because these two species are highly variable in both morphology and toxicity. We obtained nucleotide sequences of coding (small subunit [SSU], partial large subunit [LSU], 5.8S) and noncoding (internal transcribed spacer [ITS]1, ITS2) parts of the rRNA operon by PCR amplification of one or two *Dinophysis* cells isolated from natural water samples. The three photosynthetic species *D. acuminata*, *D. acuta*, and *D. norvegica* differed in only 5 to 8 of 1802 base pairs (bp) within the SSU rRNA gene. The nonphotosynthetic *D. rotundata* (synonym *Phalacroma rotundatum* [Claparède et Lachmann] Kofoid et Michener), however, differed in approximately 55 bp compared with the three photosynthetic species. In the D1 and D2 domains of LSU rDNA, the phototrophic species differed among themselves by 3 to 12 of 733 bp, whereas they differed from *D. rotundata* by more than 100 bp. This supports the distinction between *Dinophysis* and *Phalacroma*. In the phylogenetic analyses based on SSU rDNA, all *Dinophysis* species were grouped into a common clade in which *D. rotundata* diverged first. The results indicate an early divergence of *Dinophysis* within the Dinophyta. The LSU phylogenetic analyses, including 4 new and 11 *Dinophysis* sequences from EMBL, identified two major clades within the phototrophic species. Little or no intraspecific genetic variation was found in the ITS1–ITS2 region of single cells of *D. norvegica* and *D. acuminata* from Norway, but the delineation between these two species was not always clear.

Key index words: ***Dinophysis*; Dinophyta; genetic variability; phylogeny; ribosomal DNA; toxic algae**

Abbreviations: **bp, base pairs; G/C, guanine/cytosine; ITS, internal transcribed spacer; LSU, large subunit; ML, maximum likelihood; SSU, small subunit**

The marine dinoflagellate genus *Dinophysis* comprises nearly 200 species (Sournia 1986, Larsen and Moestrup 1992) and includes both phototrophic and heterotrophic species. Species with a protruded epitheca clearly visible in lateral view were separated previously, and still are by some authors (e.g. Steidinger and Tangen 1996), into the genus *Phalacroma* Stein. Because of overlapping morphology and identical plate tabulation, however, the two genera were united into a single genus, *Dinophysis* (Hallegraeff and Lucas 1988). Members of the genus *Dinophysis* belong to the order Dinophysiales, which is set apart from other major dinoflagellate groups by important features in thecal morphology (Taylor 1980). Members of Dinophysiales may possess a unique organelle called rhabdosome (Vesk and Lucas 1986) unknown in other dinoflagellates, a further indication of an early separation from the mainstream dinoflagellate evolutionary development (Lucas and Vesk 1990).

Dinophysis species occur in all seas, both in coastal and oceanic waters. Usually the cell densities are low (<100 cells$\cdot L^{-1}$), but seasonal blooms with a few 1000 cells$\cdot L^{-1}$ are recurrent in some areas such as in Europe and Japan (Hallegraeff and Lucas 1988, Dahl et al. 1996). *Dinophysis* species may produce toxins that cause diarrheic shellfish poisoning on consumption

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of mussels that have concentrated *Dinophysis* cells and become toxic. The most common *Dinophysis* species in Scandinavian waters are *D. acuta*, *D. acuminata*, *D. norvegica*, and *D. rotundata*, all of which can produce diarrheic shellfish toxins (Lee et al. 1989, Andersen et al. 1996). *Dinophysis* spp. occur in Norwegian waters throughout the whole year and cause problems for the shellfish production on the south and west coast of Norway, where they may prevent harvesting of mussels for several months per year (Aune et al. 1996, Dahl et al. 1996; also see www.algeinfo.imr.no).

Toxin content varies between *Dinophysis* species, but also within a species (Andersen et al. 1996, Dahl and Johannessen 2001). In southern Norway, *D. acuta* seems to be the main source of diarrheic shellfish toxin in blue mussels, whereas no clear correlation was found between diarrheic shellfish toxin content in blue mussels and the abundance of *D. acuminata* and *D. norvegica* in the water column (Dahl and Johannessen 2001). In Danish waters the toxin content in *D. acuminata* cells is highly variable, which may explain the discrepancy between the occurrence of *D. acuminata* and the toxicity of blue mussels (Andersen et al. 1996). The morphology is also highly variable within many species (Solum 1962, Larsen and Moestrup 1992, Zingone et al. 1998, Reguera and González-Gil 2001), and the delineation between species is at times unclear (Dodge 1982). Species identification is mainly based on cell form and cell size, form and length of sulcal lists, presence and absence of chloroplasts, plate pattern, and thecal ornamentation (Dodge 1982). Paulsen (1949) separated *Dinophysis acuminata* into several species (*D. baltica*, *D. borealis*, *D. lachmanni*, *D. granii*, *D. skagi*, *D. subcircularis*), but the morphologies of these were later found to be overlapping, and they are now all included in *D. acuminata* (Solum 1962, Balech 1976, Larsen and Moestrup 1992, Hasle and Heimdal 1998). However, because of the great morphological variation in *D. acuminata*, a species complex consisting of similar species or varieties has been suggested (Balech 1988). *Dinophysis norvegica* is also morphologically variable, and Paulsen (1949) distinguished between two forms (f. *crassior* and f. *debilior*) and even suggested separating them into *D. norvegica* and *D. debilior*. *Dinophysis acuminata* is best distinguished from *D. norvegica* and *D. acuta* by its small size and usually regular oval cell form (Larsen and Moestrup 1992). In *D. norvegica*, the hypocone is more pointed and has a straight to convex ventral side in the posterior part. *Dinophysis acuminata* and *D. norvegica* may, however, overlap in size, and both may have protrusions on the hypotheca. *Dinophysis acuta* may overlap in size with *D. norvegica* and are best identified by being widest below the middle of the cell, whereas *D. norvegica* is widest near the middle of the cell (Larsen and Moestrup 1992).

If these species embrace genetically different forms with different toxin contents, then this may explain the highly variable toxicity patterns seen in *D. acuminata* and *D. norvegica*, although other factors may also be involved. Despite several attempts to grow *Dinophysis* species in the laboratory (Maestrini et al. 1995) no one has up to now managed to keep growing cultures over time (>6 months). Thus, knowledge about, for example ecophysiology, life cycle, biochemistry, and genetics of *Dinophysis* species is very limited.

The nuclear rRNA operon, containing coding and noncoding regions with substantial differences in evolutionary rate, has been widely used to analyze higher level taxonomic relationships in algae as well as for intraspecific studies. The large subunit (LSU, 24S) and small subunit (SSU, 18S) rDNA have regions that are highly conserved and are therefore used extensively for phylogeny at higher taxonomic levels (Bhattacharya and Medlin 1995). The internal transcribed spacer (ITS)1 and ITS2, in contrast, are most useful at close phylogenetic (i.e. species and population) levels. The nucleotide sequence of an entire gene of a *Dinophysis* species has not previously been reported. However, partial sequences of LSU rDNA (Puel et al. 1998, Guillou et al. 2002b, Rehnstam-Holm et al. 2002) and SSU rDNA (Saunders et al. 1997, Janson et al. 2000, Ruíz Sebastián and O'Ryan 2001) are known.

The objectives of this study were to determine rDNA nucleotide sequences of the most common *Dinophysis* species in Scandinavian waters and to resolve their phylogenetic relationships within the genus and to other dinoflagellates. In particular, we examined the question of whether *Dinophysis* should once more be split into two genera, *Phalacroma* and *Dinophysis*. A third aim was to examine the intraspecific variation in *D. acuminata* and *D. norvegica* in different rDNA regions. If such variability could be proven, it would strengthen the possibility that the reported variation of morphology and of toxicity within either of these species has a genetic basis and that toxic strains can be distinguished from nontoxic strains by analysis of the rRNA operon. Finally, we wanted to identify regions within the rDNA operon with suitable variability for molecular probes. We addressed the following questions: Can we separate the *Dinophysis* species *D. acuminata*, *D. acuta*, *D. norvegica*, and *D. rotundata* by using parts of the rRNA operon as signature sequence? How much do they diverge in the different parts of rRNA operon? To achieve these objectives it was necessary to develop a method to obtain nucleotide sequences of coding and noncoding parts of the rDNA operon by amplification of *Dinophysis* cells, isolated from natural water samples.

MATERIALS AND METHODS

Isolation of algal cells. Water samples were taken by net haul at 0- to 2-m depths in the Oslofjord at different localities (approximately 59°50′N, 10°30′E) and off Fløde vigen, Arendal (58°25′N, 8°46′E) on the south coast of Norway (Tables 1 and 2). A water sample from the Baltic Sea, off Kalmar, Sweden, was collected from a subsurface maximum at approximately 15-m depths by Dr. Per Carlsson and was included in this study. Single live cells of *Dinophysis* were isolated under an inverted light microscope (Nikon TMS inverted microscope, Nikon Instech Co., Kanawaga, Japan) at 40 to 200× magnification by capillary isolation (Guillard 1973). Each cell was transferred to a small

TABLE 1. Origin of *Dinophysis* complete SSU and partial LSU (approximately 25–760 bp, including D1 and D2) rDNA sequences, sampling date and locality of the species, the number of cells used to obtain the sequence, and their EMBL accession numbers.

rDNA region	Species	Date	Locality	No. of cells	Accession no.
SSU	*D. acuminata*	29.03.99	Oslofjord	2	AJ506972
SSU	*D. acuta*[a]	20.08.98	Oslofjord	2	
SSU	*D. acuta*	17.11.99	Oslofjord	2	AJ506973
SSU	*D. norvegica*	26.06.98	Oslofjord	1	
		09.11.98	Baltic Sea	1	
SSU	*D. norvegica*	29.03.99	Oslofjord	2	AJ506974
SSU	*D. rotundata*	11.08.98	Oslofjord	1	AJ506975
		09.11.98	Baltic Sea	1	
LSU	*D. acuminata*	18.05.99	Fløde vigen	2	AJ506976
LSU	*D. acuminata*	17.11.99	Oslofjord	1	
LSU	*D. acuta*	24.08.99	Fløde vigen	1	AJ506977
LSU	*D. acuta*	17.11.99	Oslofjord	1	
LSU	*D. norvegica*	24.08.99	Fløde vigen	1	AJ506978
LSU	*D. norvegica*	24.08.99	Fløde vigen	1	
LSU	*D. rotundata*	17.11.99	Oslofjord	1	AJ506979

[a]Partial sequence, 1740 bp.

drop of autoclaved seawater of 30 psu and then washed in seawater twice. Each cell was examined under the microscope and identified to species. Ten microliters of Milli-Q water was added to each cleaned cell, and the cell and Milli-Q water were then transferred to a 0.5-mL Eppendorf tube and frozen to −20° C. The cells were stored up to several months before the PCR reaction. Before the final washing step, some of the cells used in the ITS sequence analyses were photographed in an inverted light microscope (Nikon Eclipse TE300, Nikon Instech Co., Kanawaga, Japan) at 200 to 400× magnification.

PCR amplification and sequencing. Double-stranded amplifications were performed in a thermocycler (Techne Genius Thermal Cycler, Techne, Cambridge, UK) in 0.5-mL Eppendorf tubes. As a rule, all PCR reaction mixtures (50 μL) contained one or two *Dinophysis* cells, 200 μM of dNTP, 200 nM of each primer, 5 μL of 10× PCR buffer (including 1.5 mM $MgCl_2$), and 1.0 units of DNA polymerase (DyNAzyme II, Finnzymes Inc., Espoo, Finland). The primers used in the PCR reactions and in subsequent sequencing reactions are listed in Table 3. The PCR program for each region is described separately below. Amplifications were examined for correct length, purity, and yield on 0.75% agarose gels, stained with ethidium bromide, visualized by UV-illumination (Sambrook et al. 1989), and photographed with Polaroid 667 film (Polaroid, St. Albans, UK).

SSU rDNA: Primers 1F+1528R were used to amplify the entire SSU rDNA region (Table 3). The amplifications were performed as described for ITS rDNA (see below) or, when this did not result in a useful PCR product, with an initial denaturation step of 95° C for 5 min, 35 cycles with the temperature profile as follows: 94° C for 2 min, 2 min ramp down to 37° C, 37° C for 2 min, 3 min ramp up to 72° C, 72° C for 6 min, followed by a 9-min extension step at 72° C. For *D. acuta* PCR products ready for sequencing were obtained by this first PCR reaction. For *D. norvegica*, *D. acuminata*, and *D. rotundata* a nested PCR procedure was performed. One microliter of the first PCR products (which were not visible on an agarose gel) was added as template to the PCR reaction mixtures as above, but with the primers 1F+690R for the first and 300F+1528R for the last part of the 18S rRNA gene. The nested amplification was performed with an initial denaturation step of 95° C for 5 min, 7 cycles with the temperature profile as above, followed by 20 cycles with the temperature profile as follows: 95° C for 1 min, 50° C for 1 min, 72° C for 2 min, and finally a 9-min extension step at 72° C.

LSU rDNA: The primer combination DIR+D2C was used to amplify the D1 and D2 domains of the LSU rRNA gene (Table 3). The initial denaturation step of 94° C for 5 min was followed by 35 cycles with the temperature profile as follows: 94° C for 1 min, 50 to 55° C for 1 min, 72° C for 2 min, followed by a 7-min extension step at 72° C.

ITS rDNA: The primer combinations 1400F+D1C or 1055F+D1C were used to amplify the ITS region (Table 3). The amplifications were performed with an initial denaturation step of 95° C for 5 min followed by 35 cycles with the temperature profile as follows: 95° C for 1 min, 50° C for 1 min, 72° C for 2.5 min, followed by a 7-min extension step at 72° C.

Sequencing. The PCR products were enzymatically purified (PCR product presequencing kit, Amersham Biosciences AB, Uppsala, Sweden), and both strands were sequenced directly with cycle sequencing (Thermo sequenase radiolabeled terminator cycle sequencing kit, U.S. Biochemical Corp., Cleveland, OH, USA) according to the manufacturer's recommendations. The primers used for sequencing are shown in Table 3. All se-

TABLE 2. Origin of *Dinophysis* ITS1, 5.8S, and ITS2 rDNA sequences and their EMBL accession numbers. A single cell per sequence was used.

Code	Species	Date	Locality	Region analyzed	Accession no.
DnorO3-24	*D. norvegica*	29.03.99	Oslofjord	ITS1, 5.8S, ITS2	AJ506980
DnorF5-6	*D. norvegica*	18.05.99	Fløde vigen	ITS1, 5.8S	AJ506981
DnorF6-3	*D. norvegica*	27.06.99	Fløde vigen	ITS1, 5.8S, ITS2	AJ506982
DnorF6-8	*D. norvegica*	27.06.99	Fløde vigen	ITS1, 5.8S, partial ITS2	AJ506983
DnorF6-2	*D. norvegica*	27.06.99	Fløde vigen	ITS1, 5.8S, partial ITS2	AJ506984
DacmO3-5	*D. acuminata*	29.03.99	Oslofjord	ITS1, 5.8S, ITS2	AJ506985
DacmF4-9	*D. acuminata*	20.04.99	Fløde vigen	ITS1, 5.8S, ITS2	AJ506986
DacmO8-24	*D. acuminata*	11.08.98	Oslofjord	ITS1, 5.8S	AJ506987
DacmO3-21	*D. acuminata*	29.03.99	Oslofjord	ITS1, 5.8S	AJ506988
DacmF6-14	*D. acuminata*	27.06.99	Fløde vigen	ITS1, 5.8S, partial ITS2	AJ506989
DrotF9-15	*D. rotundata*	20.09.99	Fløde vigen	ITS1, 5.8S, partial ITS2	AJ506990

TABLE 3. Oligonucleotide primers used for PCR and sequencing.

Code	PCR	Sequencing	Sd	Nucleotide sequence 5′ to 3′	Position based on the *Saccharomyces cerevisiae* numbering system[a]
1F[b]	SSU	SSU	F	AACCTGGTTGATCCTGCCAGT	1–21 in SSU rDNA
300F[c]	SSU	SSU	F	AGGGTTCGATTCCGGAG	370–387 in SSU rDNA
528[c]		SSU	F	CGGTAATTCCAGCTCC	575–590 in SSU rDNA
690F[c]		SSU	F	(C/T)AGAGGTGAAATTCT	896–910 in SSU rDNA
1055F[c]	ITS	SSU	F	GGTGGTGCATGGCCG	1263–1277 in SSU rDNA
1400F[c]	ITS	SSU	F	TG(C/T)ACACACCGCCCGTC	1626–1642 in SSU rDNA
300R[c]		SSU	R	TCAGGCTCCCTCTCCGG	397–381 in SSU rDNA
690R[c]	SSU	SSU	R	AGAATTTCACCTCTG	910–896 in SSU rDNA
1055R[c]		SSU	R	CGGCCATGCACCACC	1277–1263 in SSU rDNA
1528R[b]	SSU	SSU	R	TGATCCTTCTGCAGGTTCACCTAC	1795–1772 in SSU rDNA
ITS 1[d]		ITS1	F	TCCGTAGGTGAACCTGCGG	1769–1787 in SSU rDNA
ITS 2[d]		ITS1	R	GCTGCGTTCTTCATCGATGC	49–31 in 5,8S/LSU rDNA
ITS 3[d]		ITS2	F	GCATCGATGAAGAACGCAGC	31–49 in 5,8S/LSU rDNA
ITS 4[d]		ITS2	R	TCCTCCGCTTATTGATATGC	218–199 in LSU rDNA
D1C[e]	ITS	LSU	R	ACTCTCTTTTCAAAGTCCTT	550–531 in LSU rDNA
DIR[e]	LSU	LSU	F	ACCCGCTGAATTTAAGCATA	184–203 in LSU rRNA
D2Ra[e]		LSU	F	TGAAAAGGACTTTGAAAAGA	527–546 in LSU rRNA
D2C[e]	LSU	LSU	R	CCTTGGTCCGTGTTTCAAGA	816–797 in LSU rRNA

[a] www-rrna.uia.ac.be/index.html.
[b] From Medlin et al. (1988).
[c] From Elwood et al. (1985).
[d] From White et al. (1990).
[e] From Scholin et al. (1994).
Sd, synthesis direction; F, forward; R, reverse.

quencing reactions were run manually on 6% polyacrylamide gels (glycerol tolerant) on sequencing gel electrophoresis apparatus (GIBCO BRL model S2, 40 cm). Sequences have been submitted to EMBL (Tables 1 and 2).

Sequence alignment and phylogenetic analyses. Sequences of coding and noncoding strands were aligned pair-wise, assembled, and edited using the software DNASIS (Hitachi, Pharmacia, Hitachi Software Engineering Company, Yokohama, Japan). Relative guanine/cytosine (G/C) content of the sequenced rDNA regions was determined using DNASIS.

SSU and LSU: Alignments of SSU and LSU *Dinophysis* sequences from this study were done manually in the software SeqApp (Gilbert 1996). Based on these alignments distance values (Kimura two-parameter analysis) and absolute number of nucleotide differences were calculated with PAUP* (Swofford 1999). A framework for our dinoflagellate SSU rDNA alignment was downloaded from rRNA WorldWide Web server (De Rijk et al. 1998), and additional sequences were added manually using SeqApp. Sequences of 56 dinoflagellate and 3 ciliate species (outgroup) were used in the SSU rDNA analyses (Table 4). The LSU rDNA alignment, with our 4 new and 11 dinoflagellate LSU sequences from EMBL, was done manually in SeqApp. The SSU and LSU alignments are available at EMBL under the accession numbers ALIGN_000507 and ALIGN_000506, respectively.

The existing dinoflagellate SSU data set has an uneven distribution of species within the taxonomic group. Further, the divergence (branch length) is not equal between the adjacent taxa in the phylogenetic tree, which increases the probability of divergent taxa grouping together because of homoplasious similarities rather than taxonomic affinity (Litaker et al. 1999). Maximum likelihood (ML) distances, calculated on the basis of gamma distribution of site rate variation, were therefore chosen over maximum parsimony analyses for the SSU and LSU sequences. LogDet analyses were implemented because it is considered to perform relatively well on data sets with A-T contents varying between the sequences and has been shown to perform well with dinoflagellate SSU rDNA data (Tengs et al. 2000). A large set of SSU sequences was included to resolve phylogenetic relationships of *Dinophysis* to other dinoflagellates. LogDet distances were determined by using proportion of invariable sites (pinvar) estimated from neighbor joining trees with Kimura two-parameter distances. In addition, ML distances were calculated using models selected by a likelihood ratio test implemented in the Modeltest program (Posada and Crandall 1998) on the alignments. Both the LogDet and the ML distance matrices were used to calculate minimum evolution trees with 10 heuristic searches with random additions of sequences and tree bisection-reconnection branch swapping. Tree topologies were tested with bootstrap analysis with 500 replicates, using one heuristic search with random addition of the sequences per replicate and the same model as the initial searches. All analyses were done using PAUP* (Swofford 1999).

ITS: The alignment of ITS sequences was made automatically using Clustal W followed by manual adjustments in SeqApp. Because of the very high similarity among all available *Dinophysis* ITS sequences, except for that from *D. rotundata*, no phylogenetic analysis was performed.

RESULTS

Single-cell PCR. Because of difficulties in keeping cultures of *Dinophysis*, we developed a method to obtain nucleotide sequences from one or two *Dinophysis* cells isolated from natural water samples. We used frozen cells and added the whole cell to the PCR tube without previous DNA extraction. For partial LSU and ITS sequences, useful PCR products were obtained with a single cell as template and with one PCR reaction only. For the longer SSU sequence, usually two cells as template and nested PCR were necessary to obtain a useful PCR product for sequencing. The PCR amplification of SSU rDNA could sometimes be improved by using a protocol with a slow ramp down from 94 to 37° C, followed by annealing at 37° C.

SSU rDNA sequences. The complete SSU rDNA sequence was determined for *D. acuminata*, *D. acuta*, *D. norvegica*, and *D. rotundata*. The nucleotide sequence of the SSU rDNA was determined twice for *D. norveg-*

TABLE 4. EMBL accession numbers for the SSU rDNA sequences used in the phylogenetic analyses.

Species	Accession no.
Akashiwo sanguinea (Hirasaka) G. Hansen & Moestrup	AF276818
Alexandrium fundyense Balech	U09048
Alexandrium margalefii Balech	U27498
Alexandrium minutum Halim	U27499
Alexandrium ostenfeldii (Paulsen) Balech & Tangen	U27500
Alexandrium tamarense (Lebour) Balech	X54946
Amphidinium belauense Trench	L13719
Amphidinium carterae Hulburt	AF009217
Amyloodinium ocellatum Brown	AF080096
Cachonina hallii Freudenthal & Lee	AF033865
Ceratium fusus (Ehrenberg) Dujardin	AF022153
Ceratium tenue Ostenfeld & Schmidt	AF022192
Ceratocorys horrida Stein	AF022154
Colpoda inflata Stokes	M97908
Crypthecodinium cohnii Seligo	M64245
Cryptoperidiniopsis brodyi Marshall et al.	AF080097
Dinophysis acuminata Claparède & Lachmann	AJ506972
Dinophysis acuta Ehrenberg	AJ506973
Dinophysis norvegica Claparède & Lachmann	AJ506974
Dinophysis norvegica Claparède & Lachmann	AF239261
Dinophysis rotundata Claparède & Lachmann	AJ506975
Fragilidium subglobosum (von Stosch) Loeblich	AF033869
Gloeodinium viscum Banaszak, Iglesias-Prieto & Trench	L13716
Gonyaulax spinifera Diesing	AF022155
Gymnodinium béii Spero	U37365
Gymnodinium catenatum Graham	AF022193
Gymnodinium fuscum Ehrenberg	AF022194
Gymnodinium simplex Lohmann	U41086
Gyrodinium impudicum Fraga & Bravo	AF022197
Heterocapsa triquetra (Ehrenberg) Stein	AF022198
Karenia brevis (Davis) G. Hansen & Moestrup	AF72714
Karenia mikimotoi (Miyake & Kominami ex Oda) G. Hansen & Moestrup	AF022195
Karenia mikimotoi (Miyake & Kominami ex Oda) G. Hansen & Moestrup	AF172713
Karlodinium micrum (Leadbeater & Dodge) J. Larsen	AF172712
Lepidodinium viride Watanabe, Suda, Inouye, Sawaguchi & Chihara	AF022199
Lingulodinium polyedrum (Stein) Dodge	AF377944
Noctiluca scintillans (Macartier) Kofoid & Swezy	AF022200
Onychodromus quadricornutus Foissner, Schlegel & Prescott	X53485
Pentapharsodinium tyrrhenicum (Balech) Montresor, Zingone & Marino	AF022201
Pfiesteria shumwayae Glasgow & Burkholder	AF080098
Pfiesteria piscicida Steidinger & Burkholder	AF077055
Polarella glacialis Montresor, Procaccini & Stoecker	AF099183
Prorocentrum arenarium Faust	Y16234
Prorocentrum concavum Fukuyo	Y16237
Procentrum emarginatum Fukuyo	Y16239
Prorocentrum lima (Ehrenberg) Dodge	Y16235
Prorocentrum maculosum Faust	Y16236
Prorocentrum mexicanum Tafall	Y16232
Prorocentrum micans Ehrenberg	M14649
Prorocentrum minimum (Pavillard) Schiller	Y16238
Prorocentrum panamensis Grzebyk, Sako & Berland	Y16233
Pyrocystis noctiluca Murray & Schütt	AF022156
Scrippsiella nutricula Banaszak, Iglesias-Prieto & Trench	U52357
Scrippsiella trochoidea (Stein) Loeblich III	AF274277
Sterkiella nova Foissner & Berger	X03948
Symbiodinium corculorum Trench	L13717
Symbiodinium meandrinae Trench	L13718
Symbiodinium microadriaticum Freudenthal	M88521
Symbiodinium pilosum Trench & Blank	X62650

ica and *D. acuta* and was identical within a species. The SSU sequence of *D. norvegica* isolated from both the Oslofjord and the Baltic Sea in 1998 (one cell from the Oslofjord and one cell from the Baltic Sea pooled in one and the same PCR reaction) was identical to *D. norvegica* from the Oslofjord isolated in March 1999 (Table 1). Also, *D. acuta* isolated in August 1998 from the Oslofjord was identical to *D. acuta* isolated in November 1999 from the same region (Table 1). The absolute number of nucleotide differences and distance values (Kimura two-parameter) among *Dinophysis* SSU rDNA sequences in this study are shown in Table 5. The differences among the chloroplast-containing species *D. acuminata*, *D. acuta*, and *D. norvegica* were small, amounting to 5 to 8 of 1802 base pair (bp), which equals to approximately 0.3% distance. By contrast, the heterotrophic *D. rotundata* differed from the other three *Dinophysis* species by 53 to 56 bp (approximately

TABLE 5. Distance values (Kimura two-parameter, upper right) and absolute number of nucleotide differences (lower left), including adjustments for gaps and ambiguities, among SSU rDNA in *Dinophysis* spp.

	D. acuminata	*D. acuta*	*D. norvegica*	*D. rotundata*
D. acuminata	0	0.004	0.003	0.031
D. acuta	8	0	0.003	0.031
D. norvegica	6	5	0	0.029
D. rotundata	55	56	53	0

3% distance) (Table 5). The sequence of *D. acuminata* in this study was compared with a partial SSU rDNA sequence of *D. acuminata* (945 bp, AY027905) from a single cell isolated from South Africa (Ruíz Sebastián and O'Ryan 2001), and the two sequences were identical in all positions. Also, the sequences of *D. norvegica* from this study was compared with an almost complete SSU rDNA sequence of *D. norvegica* (1736 bp, AF239261) from material isolated from Sweden (Janson et al. 2000). The latter sequence differed from our *D. norvegica* sequences in 17 positions in addition to having 10 ambiguous positions. The large difference from all other available SSU *Dinophysis* sequences, the many ambiguities, and its origin from many cells suggest that the AF239261 sequence needs to be verified.

LSU rDNA sequences. The part of LSU rDNA including the domains D1 and D2, embracing 719 to 737 bp, was determined for *D. acuminata*, *D. acuta*, *D. norvegica*, and *D. rotundata* (Table 1). As a rule a single cell served as the template for each partial LSU sequence. The nucleotide sequence was determined twice for *D. acuminata*, *D. norvegica*, and *D. acuta* and was identical within each species. *Dinophysis acuminata* isolated in May 1999 from Fløddevigen, Skagerrak had exactly the same sequence as a *D. acuminata* isolated in November 1999 from the Oslofjord. Furthermore, a *D. acuta* cell isolated from Fløddevigen in August 1999 was identical in the partial LSU region to a cell of *D. acuta* isolated from the Oslofjord in November 1999. Also, the two *D. norvegica* cells were identical in the LSU region. As for SSU there was little variation in the partial LSU region between *D. acuminata*, *D. acuta*, and *D. norvegica*, with a difference of 3 to 12 bases of 733 bp (= 0.4%–1.6% distance). The difference between these species and *D. rotundata* amounted to 105 to 109 bp (approximately 14% distance, Table 6). The LSU sequences in this study were aligned and compared with more than 20 LSU *Dinophysis* sequences downloaded from EMBL and GenBank. *Dinophysis acuminata* LSU sequences from this study were identical to *D. acuminata* from France and South Africa (AF318243-6, Guillou et al. 2002b) and also to *D. sacculus* from the Mediterranean (AF318242, Guillou et al. 2002b). *Dinophysis rotundata* differed in three positions (0.4% distance) from *D. rotundata* from France (AF318235, Guillou et al. 2002b). *Dinophysis acuta* from Norway was most similar to *D. fortii* from France and South Africa (AF318236-7, Guillou et al. 2002b) and differed from those in only three bases. It differed from *D. acuta* from Portugal and the United Kingdom (AY040569-70, Guillou et al. 2002a) in 16 positions (2% distance). LSU sequences of gene clones from *D. acuminata*, *D. acuta*, and *D. norvegica* cells isolated from Sweden and the United States (AF414680-91, Rehnstam-Holm et al. 2002) showed intraspecific variability and differed from sequences in this study by 0.5% distance or more.

TABLE 6. Distance values (Kimura two-parameter, upper right) and absolute number of nucleotide differences (lower left), including adjustments for gaps and ambiguities, among partial LSU rDNA (25–758 bp from 5′ end) in *Dinophysis* spp.

	D. acuminata	*D. acuta*	*D. norvegica*	*D. rotundata*
D. acuminata	0	0.016	0.004	0.142
D. acuta	12	0	0.015	0.148
D. norvegica	3	11	0	0.144
D. rotundata	105	109	106	0

Phylogenetic relationships. The most optimal phylogenetic SSU rDNA tree that we generated with LogDet transformation is presented in Figure 1. The tree topology in the LogDet tree (pinvar = 0.58) showed a branching pattern similar to that of an ML distance tree (model selected by Modeltest: TrN+I+G, parameter values: base frequencies = A: 0.28, C: 0.18, G: 0.24, T: 0.30; substitution rate matrix [AC, AG, AT, CG, CT] = 1.00, 2.89, 1.00, 1.00, 5.17; proportion of invariable sites = 0.31; gamma shape parameter = 0.65). Thus, only the LogDet tree is shown but with additional ML distance bootstrap values for the *Dinophysis* branch. Maximum parsimony analysis was found not to be appropriate due to problems with long branch attraction in the SSU rDNA data set. The backbone of the tree in Figure 1 is highly similar to previously presented phylogenies (Saunders et al. 1997, Litaker et al. 1999, Saldarriaga et al. 2001, Jakobsen et al. 2002) in placing *Noctiluca* as the basal branch, as well as dividing the dinoflagellates into several well defined terminal clades, but with a poorly resolved or unresolved basal topology. The *Dinophysis* species formed a deeply divergent branch indicating an early separation from other dinoflagellates. All *Dinophysis* species were grouped into a common clade with 94/79% support (LogDet/ML-distance), in which *D. rotundata* diverged first (95/79%), whereas the three chloroplast containing species were more closely related. *Dinophysis norvegica* and *D. acuminata* grouped together and formed a sister clade to *D. acuta* with 63/80% support.

The LSU phylogenetic analyses were performed on 11 *Dinophysis* and 1 *Karenia* (outgroup) sequences downloaded from EMBL (Hansen et al. 2000, Guillou et al. 2002a,b) and four sequences from this study to resolve the phylogeny within the genus *Dinophysis*. LogDet distances generated six equally optimal LogDet trees with essentially identical topologies (pinvar = 0.44, Fig. 2). The ML distance tree (model selected by Modeltest: TrN+G, parameter values: base frequencies = A: 0.25, C: 0.17, G: 0.29, T: 0.29; substitution rate matrix [AC, AG, AT, CG, CT] = 1.00, 1.80, 1.00,

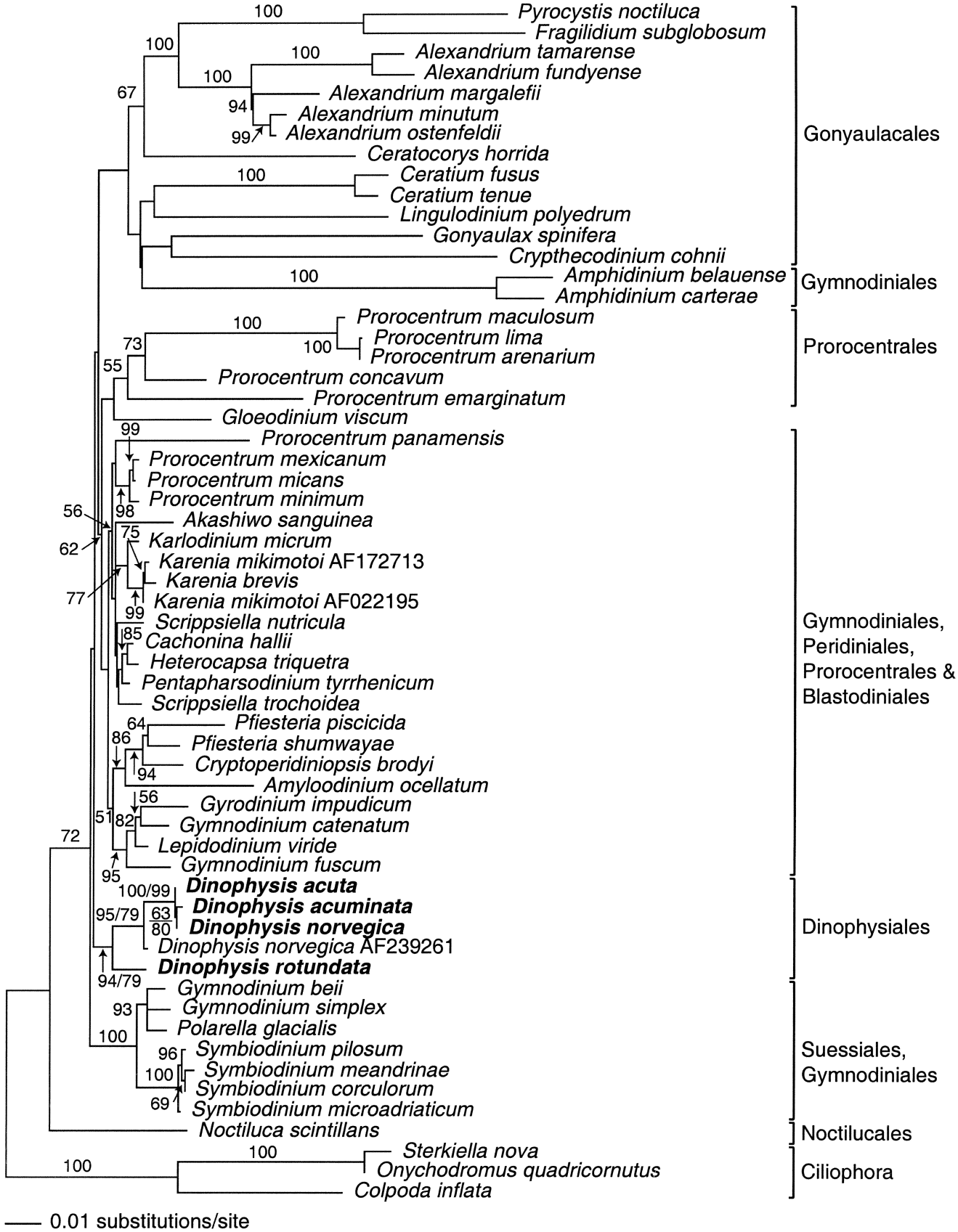

FIG. 1. Phylogenetic tree based on SSU rDNA sequences inferred from a LogDet distance matrix showing the relationships of *Dinophysis* within the genus and to other dinoflagellates. The tree is rooted on the branch leading to the ciliates (Ciliophora). The bootstrap values (500 replications) at the internal nodes are inferred from LogDet distance analysis (left) and for the *Dinophysis* clade also from ML distance analysis (right). Sequences from this study are in bold.

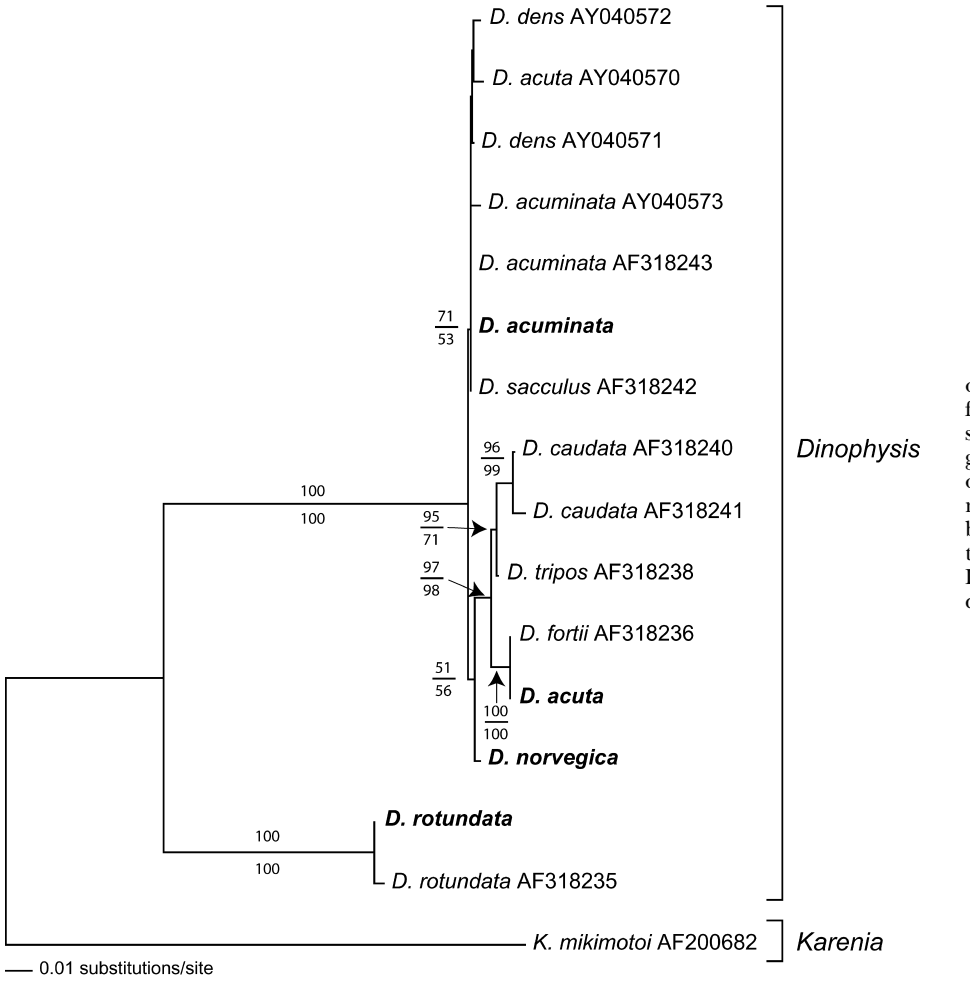

FIG. 2. Phylogenetic tree based on LSU rDNA sequences inferred from a LogDet distance matrix showing the relationship within the genus *Dinophysis*. The tree is rooted on the branch leading to the dinoflagellate *Karenia mikimotoi*. The bootstrap values (500 replications) at the internal nodes are inferred from LogDet/ML distance analyses. Sequences from this study are in bold.

1.00, 3.74; gamma shape parameter = 0. 61) showed a branch pattern similar to the LogDet tree. As was the case with SSU, the LSU analyses suggest that *D. rotundata* belongs to the basal part of the *Dinophysis* clade. The other *Dinophysis* species were separated in two main clades: one consisting of *D. caudata* Saville-Kent, *D. fortii* Pavillard, *D. tripos* Gourret, *D. acuta* (from Norway), and *D. norvegica*, and another consisting of *D. acuminata*, *D. dens* Pavillard, *D. sacculus* Stein, and *D. acuta* (from the United Kingdom). The placement of *D. norvegica* had low support (51/56% LogDet/ML distance) and is uncertain. *Dinophysis acuta* emerged in two different places in the tree.

Genetic variability within ITS1, 5.8S, and ITS2 rDNA. ITS1 and 5.8S rDNA were determined in five *D. norvegica* cells, five *D. acuminata* cells, and one *D. rotundata* cell isolated from Fløde vigen and the Oslofjord at different times during the year (Table 2). Entire ITS2 sequences were obtained for two *D. norvegica* cells and two *D. acuminata* cells (Table 2). Partial ITS2 sequences were obtained additionally for two *D. norvegica* cells and two *D. acuminata* cells. The alignment of the ITS1–ITS2 sequences is shown in Figure 3. All *D. norvegica* cells were identical in the ITS1–ITS2 region analyzed (except in three ambiguous positions), and no clear intraspecific variability was thus observed. In *D. acuminata*, three small rounded cells (DacmO8-24, DacmO3-21, and DacmF6-14) were mutually identical within the ITS1–ITS2 region, whereas two larger *D. acuminata* cells had an ITS1–ITS2 sequence almost identical to *D. norvegica* (DacmO3-5) or intermediate between *D. norvegica* and *D. acuminata* (DacmF4-9, Fig. 3).

Dinophysis acuminata (small cells) and *D. norvegica* differed in six positions within the ITS1 region. The 5.8S gene was identical in all cells analyzed, and in the ITS2 the two species differed in two positions. This shows that the two species were very similar even in a usually variable spacer region. *Dinophysis rotundata* was very different from the chloroplast-containing species in the ITS1 and ITS2 regions, to the degree that it was difficult to align reliably (Fig. 3).

ITS1–ITS2 sequences of *D. acuminata* and *D. norvegica* in this study were aligned and compared with available ITS sequences from EMBL and GenBank of chloroplast-containing *Dinophysis* species. Sequences of

```
          SSU ITS 1
DnorO3-24 CATTCGCACG CATCCAATAT CCATAACTTG AAATKTATTG TGTGAGCTTC TGGGTGAGGT
DnorF5-6  .......... .......... .......... .......... .......... ..........
DnorF6-3  .......... .......... .......... ....T..... .......... ..........
DnorF6-8  .......... .......... .......... ....T..... .......... ..........
DnorF6-2  .......... .......... .......... ....T..... .......... ..........
DacmO3-5  .......... .........K .......... .......... .......... ..........
DacmF4-9  .......... .........K .......... ....T.C... .......... ..........
DacmO8-24 .......... .......... .......... ....T.CC.. .......... ..........
DacmO3-21 .......... .......... .......... ....T.CC.. .......... ..........
DacmF6-14 .......... .......... .......... ....T.CC.. .......... ..........
DrotF9-15 .......... ......CATG AAT.G..C.. ...CT.GA.. GTGAG...-. ......G..A

DnorO3-24 TGAACAAAGT GTTGCCT--T CATGTGGAAG CTCGAGGGTA GATGAACTGA AGCAGTGTGG
DnorF5-6  .......... .......... .......... .......... .......... ..........
DnorF6-3  .......... .......... .......... .......... .......... ..........
DnorF6-8  .......... .......... .......... .......... .......... ..........
DnorF6-2  .......... .......... .......... .......... .......... ..........
DacmO3-5  .......... .......... .......... .......... .......... ..........
DacmF4-9  .......... .......... .......... .......... .......... ..........
DacmO8-24 .......... .......... .......... .......... .......... ..........
DacmO3-21 .......... .......... .......... .......... .......... ..........
DacmF6-14 .......... .......... .......... .......... .......... ..........
DrotF9-15 ..C.GTGCA. C.GCATCGC. .........T ....C...C. .CAA.TTG.G GTGGCGT..T

DnorO3-24 TCTTGCTGCT TCGTGGGCGC TACCGTCTGC TTGGCTCACA CTGCCTTGCG GTTGAACCTT
DnorF5-6  .......... .......... .......... .......... .......... ..........
DnorF6-3  .......... .......... .......... .......... .......... ..........
DnorF6-8  .......... .......... .......... .......... .......... ..........
DnorF6-2  .......... .......... .......... .......... .......... ..........
DacmO3-5  .......... .......... .......... .......... .......... ..........
DacmF4-9  ........T. .......... .......... .......... .......... ..........
DacmO8-24 ........T. .......... .......... .......... .......... ..........
DacmO3-21 ........T. .......... .......... .......... .......... ..........
DacmF6-14 ........T. .......... .......... .......... .......... ..........
DrotF9-15 G-.....TTG ATCCT.CT.. CGTT.CAGTT G..CAGTTGT G...A.GTTT ...TTG----

                                    ITS 1 5.8S
DnorO3-24 CATAAGTTTG TATGTGCATG CTGTATGTAT CACAATTTTC AGCGATGGAT GCCTCGGCTC
DnorF5-6  .......... .......... .......... .......... .......... ..........
DnorF6-3  .......... .......... .......... .......... .......... ..........
DnorF6-8  .......... .......... .......... .......... .......... ..........
DnorF6-2  .......... .......... .......... .......... .......... ..........
DacmO3-5  .......... .......... .......... .......... .......... ..........
DacmF4-9  T..TT..... .......... .......... .......... .......... ..........
DacmO8-24 T..TT..... .......... .......... .......... .......... ..........
DacmO3-21 T..TT..... .......... .......... .......... .......... ..........
DacmF6-14 T..TT..... .......... .......... .......... .......... ..........
DrotF9-15 T..T-..--. .T-....T.- ..C.------ T....C.... .......... .T........

DnorO3-24 GAACAACGAT GAAGGGCGCA GCGAAGTGTG ATAATCTTTG TGAATTGCAG AATTCCGTGA
DnorF5-6  .......... .......... .......... .......... .......... ..........
DnorF6-3  .......... .......... .......... .......... .......... ..........
DnorF6-8  .......... .......... .......... .......... .......... ..........
DnorF6-2  .......... .......... .......... .......... .......... ..........
DacmO3-5  .......... .......... .......... .......... .......... ..........
DacmF4-9  .......... .......... .......... .......... .......... ..........
DacmO8-24 .......... .......... .......... .......... .......... ..........
DacmO3-21 .......... .......... .......... .......... .......... ..........
DacmF6-14 .......... .......... .......... .......... .......... ..........
DrotF9-15 AG........ .......... .......... ....C..... .......... ..........
```

FIG. 3. Alignment of the ITS1–5.8S–ITS2 rDNA region flanked by SSU and LSU in cells of *Dinophysis acuminata*, *D. norvegica*, and *D. rotundata*. Coding regions are marked by a solid line. The origins of the analyzed cells are shown in Table 2.

the small *D. acuminata* cells in this study (DacmO8-24, DacmO3-21, and DacmF6-14; Fig. 3) were identical to most sequences of *D. sacculus* (AJ012007, AY040580-3, Giacobbe et al. 2000, Guillou et al. 2002a) and *D. pavillardii* (AJ404000, Penna et al. 2000) from France and Italy. *Dinophysis acuminata* from France and the United Kingdom (AY00574-8) differed from these in one position in this region. Also, sequences of *D. acuta* from France and the United Kingdom (AY040569-70, Guillou et al. 2002a) differed in only one position from sequences of small *D. acuminata* cells in this study.

Characteristics of the rRNA operon in the four Dinophysis *species.* In Table 7 the lengths of the different rDNA regions and their G/C content are shown. The length

```
                                                                                                                    
DnorO3-24  ATCAATAGAG CTTTGAACGT GTCTTGCGCT TTCGGGCTAT ACCTGAAAGC ATGCCTGCGT
DnorF5-6   .......... .......... .......... .......... .......... ..........
DnorF6-3   .......... .......... .......... .......... .......... ..........
DnorF6-8   .......... .......... .......... .......... .......... ..........
DnorF6-2   .......... .......... .......... .......... .......... ..........
DacmO3-5   .......... .......... .......... .......... .......... ..........
DacmF4-9   .......... .......... .......... .......... .......... ..........
DacmO8-24  .......... .......... .......... .......... .......... .....?????
DacmO3-21  .......... .......... .......... .......... .......... .....?????
DacmF6-14  .......... .......... .......... .......... .......... ..........
DrotF9-15  .......... .........C ..G...T... G.....A... ......C... .......AT.

           5.8S ITS 2
DnorO3-24  TGGTGTCTGT ATGGCCTCAT TCAGTCAGCA ACACTGAACC TCATTGGTAG TGTGATGTCT
DnorF5-6   .......... ........?? ?????????? ?????????? ?????????? ??????????
DnorF6-3   .......... .......... .......... .......... .......... ..........
DnorF6-8   .......... .......... .......... .......... .......... ..........
DnorF6-2   .......... .......... .......... .......... .......... ..........
DacmO3-5   .......... .......... .......... .......... .......... ..........
DacmF4-9   .......... .......... .......... .....A.... .......... ..........
DacmO8-24  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmO3-21  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmF6-14  .......... .......... .......... .....A.... .......... ..........
DrotF9-15  .....C..TA T..TGTGAC. ..C.GTG.T. TA.GCAC... .TC.GT.CTT GACTGCAA.C

DnorO3-24  GTGTGTCGGT GTGTGCAAGG TTCACCCTTG TTACACAGAA TRCATGTTAA TTATCGTTGC
DnorF5-6   ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DnorF6-3   .......... .......... .......... .......... .A........ ..........
DnorF6-8   .......... .......... .......... .......... .......... ..........
DnorF6-2   .......... .......... .......... .......... .A......?? ??????????
DacmO3-5   .......... .......... .......... .......... .G........ ..........
DacmF4-9   .......... .......... .......... .......... .G......T. ..........
DacmO8-24  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmO3-21  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmF6-14  .......... .......... .......... .......... .G......T. ..........
DrotF9-15  TGTGTG.ATG TGCATGCCA. GGTGAA..GC A.G.C.TTGC .ATGCAAAGT GA..GCGAAG

DnorO3-24  CAACMGCTTG CTGTGCCTCT GTATAGTGGT TAACCTTCTG TTGGCACGAA ATCCAAGCAC
DnorF5-6   ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DnorF6-3   ....A..... .......... .......... .......... .......... ..........
DnorF6-8   ....A....? ?????????? ?????????? ?????????? ?????????? ??????????
DnorF6-2   ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmO3-5   .......... .......... .......... .......... .......... ..........
DacmF4-9   .......... .......... .......... .......... .......... ..........
DacmO8-24  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmO3-21  ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
DacmF6-14  ....A....? ?????????? ?????????? ?????????? ?????????? ??????????
DrotF9-15  .TTTGTG.GC ?????????? ?????????? ?????????? ?????????? ??????????

                      ITS 2 LSU
DnorO3-24  ATGTCCCAAT GTGTGATTAA TTAGACATGA CGTTAGGCTA
DnorF5-6   ?????????? ?????????? ?????????? ??????????
DnorF6-3   .......... .......... .......... ..........
DnorF6-8   ?????????? ?????????? ?????????? ??????????
DnorF6-2   ?????????? ?????????? ?????????? ??????????
DacmO3-5   .......... .......... .......... ..........
DacmF4-9   .......... .......... .......... ..........
DacmO8-24  ?????????? ?????????? ?????????? ??????????
DacmO3-21  ?????????? ?????????? ?????????? ??????????
DacmF6-14  ?????????? ?????????? ?????????? ??????????
DrotF9-15  ?????????? ?????????? ?????????? ??????????
```

FIG. 3. *(continued).*

of ITS1 was 204 bp in both *D. acuminata* and *D. norvegica* but was slightly less in *D. rotundata* (189 bp). The length of ITS2 was 197 bp in both *D. acuminata* and *D. norvegica*. There was nothing unusual about their G/C content, which ranged from 45% to 51% in all rDNA regions and in all species analyzed. The genetic variation among the *Dinophysis* species was larger in the LSU (0.4%–15% distance) than in the SSU (0.3%–3%) rDNA region (Tables 5 and 6). The D2 domain was more variable than the D1 domain within the LSU. The 5.8S gene was identical in *D. norvegica* and *D. acuminata*, and the ITS1 was more variable than ITS2

TABLE 7. Number of isolates sequenced and length and G/C content of different rDNA regions of *Dinophysis* species. LSU part starting at 25 bp from the 5′ end.

rDNA region	Species	No. of isolates	Base pairs	G/C content (%)
SSU	*D. acuminata*	1	1802	45.6
	D. acuta	2	1802	45.3
	D. norvegica	2	1802	45.5
	D. rotundata	1	1801	45.5
ITS 1	*D. acuminata*	5	204	47.0
	D. norvegica	5	204	47.5
	D. rotundata	1	189	51.3
5.8S	*D. acuminata*	5	154	48.0
	D. norvegica	5	154	48.0
	D. rotundata	1	154	47.4
ITS 2	*D. acuminata*	2	197	44.7
	D. norvegica	2	197	44.7
LSU	*D. acuminata*	2	736	46.7
	D. acuta	2	737	46.6
	D. norvegica	2	736	46.7
	D. rotundata	1	719	45.7

when comparing *D. acuminata* and *D. norvegica* (Fig. 3). Comparison between available *Dinophysis* rDNA sequences deposited in GenBank and EMBL indicates some intraspecific variability within the LSU and ITS regions.

One of the objectives of this study was to identify DNA regions with suitable variability to allow construction of molecular probes for detecting *Dinophysis* cells. The variability between the three chloroplast containing *Dinophysis* species that are common in Norwegian waters was low, both in coding and noncoding rDNA regions, but signature positions at the species level were found for all four species examined. No or low (ITS in *D. acuminata*) intraspecific variability was found in *Dinophysis* material from Norway.

DISCUSSION

Single-cell analysis. In this study we used one or two *Dinophysis* cells as templates for the PCR to obtain nucleotide sequences of *Dinophysis* species. It was possible to obtain PCR products of all regions by one single PCR reaction, but nested PCR often improved the result. This method can be used to obtain molecular data of other members in this genus, which has resisted culturing up to now. Single-cell PCR has previously been used to obtain SSU rDNA sequences from other dinoflagellates (Tengs et al. 2000) and from *D. acuminata* (Ruíz Sebastián and O'Ryan 2001) and of ITS rDNA sequences from *D. acuminata* (Marín et al. 2001). Other molecular studies of *Dinophysis* used multicell isolates from the field (Puel et al. 1998, Giacobbe et al. 2000, Rehnstam-Holm et al. 2002). It is also possible to use ethanol-preserved cells of *Dinophysis* as templates in single-cell PCR (Marín et al. 2001, Edvardsen unpublished observations) or Lugol-fixed cells (Guillou et al. 2002b). Using single-cell PCR of fixed *Dinophysis* cells (or other dinoflagellates) to obtain nucleotide sequences opens up the possibility of an investigation of the worldwide geographical distribution of *Dinophysis* species and genotypes as well as of their phylogenetic relationships.

Phylogenetic relationships. In the phylogenetic analyses based on SSU rDNA, all *Dinophysis* species were grouped into a common clade in which *D. rotundata* diverged first. The results also indicate an early divergence of *Dinophysis* within the Dinophyta. However, due to an unresolved basal topology, it is uncertain whether the *Dinophysis* species are placed as a part of the so-called GPP complex composed of species in the orders Gymnodiniales, Peridiniales, Prorocentrales, and Blastodiniales or rather forming a sister group to it. Saunders et al. (1997) found *D. acuminata* to fall in a common branch with *Karenia mikimotoi* (*Gymnodinium mikimotoi*) within the GPP complex in a phylogenetic analysis based on partial SSU rDNA sequences and including only one *Dinophysis* species, *D. acuminata*. Daugbjerg et al. (2000) presented a phylogeny based on partial LSU rDNA sequences of 41 dinoflagellate species. Their analysis, based on 326 bp and including one *Dinophysis* species (*D. acuminata*), indicated an early divergence of *Dinophysis* in accordance with our SSU results. The heterotrophic *D. rotundata* differed from the chloroplast-containing species in 3% of the positions in SSU and 14% to 15% of the positions in the analyzed part of LSU. This supports the previous distinction between *Dinophysis* and *Phalacroma*, which placed *Dinophysis* species, such as *D. rotundata* with a clearly visible dome-shaped epitheca, most of them heterotrophic, in the genus *Phalacroma* (von Stein 1883, Taylor 1980). If these forms are removed from *Dinophysis*, some species still remain in the genus that appear to be heterotrophic, such as *D. hastata* (Larsen and Moestrup 1992). Conversely, a few "*Phalacroma*" species, for example, "*P.*" *rapa* Jorgensen, are photosynthetic, although the chloroplasts and pigmentation differ from those of photosynthetic *Dinophysis* species, which have cryptophyte-like chloroplasts with two thylakoid bands and orange fluorescence (Schnepf and Elbrächter 1999). The chloroplasts in "*P.*" *rapa* have three thylakoid bands and golden brown coloration (Hallegraeff and Lucas 1988). There are also ecological arguments for the generic distinction between *Dinophysis* and *Phalacroma*. The *Dinophysis* species are mostly neritic, whereas "*Phalacroma*" species are mostly oceanic (Hallegraeff and Lucas 1988). Reinstatement of the genus *Phalacroma* would depend on future phylogenetic studies of additional species within that group and might require some additional nomenclatural decisions. The LSU phylogenetic analyses, including 15 *Dinophysis* sequences, placed *D. rotundata* at the basal part of the *Dinophysis* clade, suggesting an ancestral position. The phototrophic *Dinophysis* species were separated in two major clades: one consisting of the larger species *D. caudata*, *D. fortii*, *D. tripos*, *D. acuta* (from Norway), and *D. norvegica*, and the other clade consisting of *D. acuminata*, *D. dens*, *D. sacculus*, and *D. acuta* (from the United Kingdom). This is essentially in accordance with the results recently presented by Guillou et al. (2002b). In our study, which includes additional

species, *D. acuta* emerged in two different places in the tree, suggesting that it is difficult to delineate and identify correctly or, alternatively, that this is a composite species. The placement of *D. norvegica* in the first clade had low support and is uncertain. In the SSU phylogenetic analyses *D. norvegica* was grouped together with *D. acuminata*. This intermediate position is reflected also by its intermediate morphology and cell size.

Intraspecific variability. The toxin content varies considerably within many *Dinophysis* species. The toxicity of *Dinophysis* cells was not analyzed in this study, but previous studies from Scandinavian waters indicate that it is highly variable within a species (Andersen et al. 1996). We found no or little intraspecific genetic variability within the rRNA operon of *Dinophysis* cells isolated from two localities off the Norwegian south coast at different times of the year. However, comparison with available *Dinophysis* sequences deposited in EMBL and GenBank indicated some intraspecific variability within the LSU and ITS regions. Further research may show whether it will be possible to distinguish toxic from nontoxic or less toxic strains within a species by using the part of rRNA operon analyzed in this study (SSU, ITS1, 5.8S, ITS2, D1 and D2 of LSU) as a marker.

The morphology of *D. acuminata* is variable, and some earlier authors considered it as representing several distinct species (Paulsen 1949, Balech 1988). Three small rounded cells of *D. acuminata* had identical ITS1 rDNA sequence, whereas the larger cells, which we also referred to this species based on morphological grounds, had ITS1–ITS2 sequences either identical to *D. norvegica* or intermediate between *D. norvegica* and *D. acuminata*. This suggests that the delineation between these two species is not always clear and that morphologically intermediate forms may also be genetically intermediate, perhaps even hybrids. Morphologically intermediate forms between *D. sacculus* and *D. acuminata* were reported by Zingone et al. (1998) and Truquet et al. (1996). Marín et al. (2003) analyzed ITS1–ITS2 rDNA sequences of *D. acuminata* and *D. sacculus* and found identical ITS1 sequences, suggesting that these are two morphotypes of the same species. On the other hand, Guillou et al. (2002a) found that *D. acuminata* from France and the United Kingdom differed in one position from *D. sacculus* from France within the ITS1–ITS2 region. Our small *D. acuminata* cells had an ITS1–ITS2 sequence identical to that of *D. sacculus* from France and Italy, as well as of *D. pavillardii* from Italy, analyzed by others (Giacobbe et al. 2000, Penna et al. 2000, Guillou et al. 2002a). The ITS1–ITS2 sequences of *D. sacculus* and *D. acuminata* from Galicia, Spain analyzed by Marín et al. (2003) differed slightly from the sequences in this study and those by Guillou et al. (2002a) and Giacobbe et al. (2000). It should be possible in future studies to complement the ITS sequence with an SSU sequence in the same PCR reaction and resolve these conflicting ITS sequences. Current information does not support the concept of *D. acuminata* as a composite species as suggested by, for instance, Balech (1988). Rather, it may possibly be conspecific with *D. sacculus* and *D. pavillardii* and possibly hybridize with closely related species, such as *D. norvegica*. No intraspecific variation was found in ITS1–ITS2, SSU, or partial LSU of *D. norvegica* isolated from southern Norway at different times of the year. Paulsen (1949) distinguished between *D. norvegica* f. *crassior* and *D. norvegica* f. *debilior*, based on overall cell morphology. However, Solum (1962) found *D. norvegica* from Norwegian coastal waters to be very variable in form and size even within one and the same water sample, with numerous transitional forms. Identical ITS1 rDNA sequences in the two forms of *D. norvegica* support Solum's conclusion that we have one form-variable species, *D. norvegica*.

Interspecific variability. Dinophysis acuminata, *D. acuta*, *D. norvegica*, and *D. rotundata* could be distinguished both in coding and noncoding regions of rDNA, although the difference between the phototrophic species was very small, with a distance of 0.3% to 0.4% in SSU and 0.4% to 1.6% in LSU. The coding region 5.8S was identical in *D. acuminata* and *D. norvegica*, and the two species differed in only 8 bp within the usually variable noncoding spacers ITS1 and ITS2. This suggests that the three chloroplast-containing species in our study have separated recently or that the rate of evolution is low within this group. When comparing *D. acuminata* and *D. norvegica* rDNA sequences, the variability (distance value) decreased in the order ITS1, ITS2, SSU, LSU, and 5.8S.

The toxin content per cell seems to vary considerably between and within *Dinophysis* species (Andersen et al. 1996, Dahl and Johannessen 2001). In the Norwegian algal monitoring program, the warning concentrations are presently 500 cells·L^{-1} for *D. acuta*, 900 cells·L^{-1} for *D. acuminata*, and 2000 cells·L^{-1} for *D. norvegica* (www.algeinfo.imr.no). It is therefore of great importance to be able to distinguish the different *Dinophysis* species from each other. We identified regions in both the LSU and SSU rDNA sequences as signature sequences for oligonucleotide probes at and above species level for identification and quantification of the four most common toxic *Dinophysis* species in Norway. The design of these probes and testing of their specificity represents the next step in our research on *Dinophysis*.

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