Phycologia (2003) Volume 42 (3), 261–268 Published 19 June 2003

Identification of *Alexandrium affine* and *A. margalefii* (Dinophyceae) using DNA sequencing and LSU rDNA-based RFLP-PCR assays

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C.J. BAND-SCHMIDT, E.L. LILLY AND D.M. ANDERSON. 2003. Identification of *Alexandrium affine* and *A. margalefii* (Dinophyceae) using DNA sequencing and LSU rDNA-based RFLP-PCR assays. *Phycologia* 42: 261–268.

Restriction fragment length polymorphism (RFLP) patterns have been used in the past to distinguish *Alexandrium* species and strains. We report on the identification of *A. affine* and *A. margalefii* from Bahía Concepción, Mexico, by RFLP and DNA sequencing. Polymerase chain reaction amplifications of the D1–D2 fragment of the nuclear large-subunit (LSU) ribosomal RNA gene resulted in a single product of approximately 720 bp for both species. The restriction patterns for *A. affine* (strain AABCV-1) digested with *Nsp*I had fragments that were approximately 500, 125, and 75 bp long. With *Mse*I, fragments of approximately 350 and 300 bp were obtained; with *Apa*LI, two fragments, approximately 500 and 225 bp long, were observed. *Alexandrium margalefii* (strain AMBCQ-1) yielded fragments of 550 and 120 bp with *Nsp*I, and fragments of 300, 250, 95, and 45 bp with *Mse*I, but was not digested by *Apa*LI. Agreement between expected and observed RFLP patterns was found in *A. affine* and *A. margalefii* strains with *Nsp*I, *Mse*I, and *Apa*LI. Sequences of the D1–D2 domains of the LSU rDNA of *A. affine* and *A. margalefii* were compared with those of 18 other *Alexandrium* isolates, using maximum likelihood and maximum parsimony analyses. Strain AABCV-1 grouped within the *A. affine* clade, with 100% bootstrap support, and strain AMBCQ-1 was placed within the *A. margalefii* clade. The record of *A. margalefii* is the first for North America.

INTRODUCTION

Alexandrium Halim is the genus that includes the largest number of toxic species among the phytoplankton (Anderson 1998). Several species produce saxitoxin, the potent neurotoxin associated with paralytic shellfish poisoning (PSP). The geographical range of toxic dinoflagellates within the genus *Alexandrium* appears to be increasing on both regional and global scales (Scholin *et al.* 1995). Several hypotheses have been put forward to explain this trend: (1) an increased abundance of previously unnoticed endemic species; (2) natural dispersal through storms and currents; (3) human-assisted dispersal (e.g. via ballast water); (4) increased monitoring efforts, leading to the discovery of species that have always been present; or (5) a combination of the above (Anderson 1989; Smayda 1990; Hallegraeff & Bolch 1991, 1992).

Coincident with the global increase in harmful algal blooms (HAB), algal blooms have increased in Mexico during the last few decades. Of the blooms recorded, 78% have occurred on the coasts of the Gulf of California and most are innocuous (Morquecho *et al.* 2000). However, toxic species do occur, such as *A. catenella* (Whedon & Kofoid) Balech, *Cochlodinium polykrikoides* Margalef, *Pyrodinium bahamense* Plate var. *compressum* (Böhm) Steidinger, Tester & Taylor, *Gymnodinium catenatum* Graham, *Karenia brevis* (Davis) Gert Hansen & Moestrup, *Prorocentrum lima* (Ehrenberg) J.D. Dodge, and *Pseudo-nitzschia* H. Peragallo *in* H. & M. Peragallo spp., and these have been associated with health, economic, and environmental issues (Cortés-Altamirano *et al.* 1993; Cortés-Altamirano & Alonso-Rodríguez 1997; Sierra-Beltrán *et al.* 1998; Morquecho *et al.* 2000; Gárate-Lizárraga *et al.* 2001b, c; Heredia-Tapia *et al.* 2002).

Alexandrium catenella and *A. monilatum* (Howell) Balech have been reported in the Gulf of California and off the western coast of Baja California (Gárate-Lizárraga *et al.* 1990, 2001c; Licea *et al.* 1995; Sierra-Beltrán *et al.* 1996). In Bahía Concepción, several species of *Alexandrium* have been reported, including *A. minutum* Halim, *A. monilatum, A. pseudogonyaulax* (Biecheler) Horiguchi *ex* Yuki & Fukuyo, *A. catenella, A.* cf. *tamarense* (Lebour) Balech, *A. tamiyavanichi* Balech, and *A. affine* (Inoue & Fukuyo) Balech (Martínez-López & Gárate-Lizárraga 1994; Sierra-Beltrán *et al.* 1996, 1998; Verdugo-Díaz 1997; Lechuga-Devéze & Morquecho-Escamilla 1998; Lechuga-Devéze *et al.* 2000; Gárate-Lizárraga *et al.* 2001a, c; Góngora-González 2001). Paralytic shellfish poisoning toxins have been detected in excess of the maximum limit permitted for human consumption (80 μg saxitoxin equivalents per 100 g meat) in a mollusc fishery in Bahía Concepción (Sierra-Beltrán *et al.* 1996) and this has been attributed to the presence of dinoflagellates of the genera *Alexandrium* and *Gymnodinium* Stein (Gárate-Lizárraga 1995; Verdugo-Díaz 1997; Lechuga-Devéze *et al.* 2000; Gárate-Lizárraga *et al.* 2001c; Góngora-González 2001). The identification of *Alexandrium* species in this bay has hitherto relied on traditional microscopical methods, but this is not an easy task, because it is necessary to observe detailed cellular characteristics, such as the arrangement of plates and the presence or absence of a ventral pore. An alternative to these methods is to use molecular tools to differentiate strains or species.

Restriction fragment length polymorphism (RFLP) analysis of small-subunit ribosomal RNA (rRNA) genes has been used to discriminate species and strains (Scholin *et al.* 1994; Walsh

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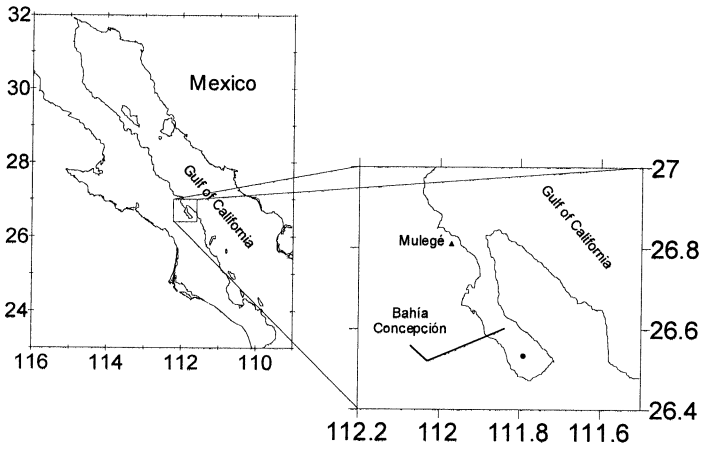

Fig. 1. Bahía Concepción, Baja California Sur, Mexico. (● = Collection site.)

et al. 1998), but the variation in large-subunit (LSU) rRNA gene sequences provides finer-scale species and clade resolution for *Alexandrium* species (Destombe *et al.* 1992; Scholin & Anderson 1994). Scholin & Anderson (1996) used sequencing and RFLP analysis to demonstrate that there are clear differences in LSU rDNA that can be used to distinguish closely related *Alexandrium* species or clades. Their results revealed a division of the *A. tamarense–catenella–fundyense* species complex into clusters that were defined by geographical origins rather than by morphological characters. LSU RFLP patterns have been reported for several *Alexandrium* species, including *A. affine* (Scholin & Anderson 1996) and *A. margalefii* Balech (LeinonenDuFresne *et al.* 2000).

Here, we provide RFLP and sequence characterization of LSU rDNA for two strains of *Alexandrium* from Bahía Concepción, identified morphologically as *A. affine* and *A. margalefii.* Because *A. margalefii* has not been identified previously in Mexico, and *A. affine* has only recently been reported, it was important to confirm the identification of these species and to establish linkages to other populations globally.

MATERIAL AND METHODS

Collection site

Bahía Concepción is a semienclosed bay on the east coast of Baja California Sur, Mexico, with an area of 275 km^2 (Fig. 1). The mean depth is 22 m, with a maximum of 34 m. The sediment at the bottom of the bay is mainly biogenic (Shumilin *et al.* 1996). The mean surface temperature is 24.5°C, with a minimum of 17.5°C in winter and a maximum of 34.8°C in summer (Mateo-Cid *et al.* 1993). The mean salinity is 35.3 psu. The region is hot and arid, with an annual rainfall of 112–155 mm (García 1988).

Cultures

Phytoplankton and sediment samples were collected twice a month during 2000 in Bahía Concepción (Fig. 1). Phytoplankton was collected by vertical tows using a 20 μm net. The cell concentrate was sieved through a 60 μm mesh and was enriched with modified f/2 medium (Anderson *et al.* 1984). Sediment cores (5–20 cm long, 1.3 cm diameter) were stored in darkness at 4°C. The upper first centimetre was separated and mixed with filtered seawater and processed using the sieving technique of Anderson *et al.* (1995). Vegetative cells and living cysts were isolated from the enrichments and sediment samples with micropipettes using a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Oberkochen, Germany). The cells were transferred to 96-well plates containing modified f/2 medium. Cultures from these wells were transferred into larger vessels for experimental purposes. Strain AABCV-1, morphologically identified as *A. affine,* was obtained from vegetative cells, isolated into clonal cultures by C. Band. Strain AMBCQ-1, morphologically identified as *A. margalefii,* was isolated following cyst germination by L. Morquecho. Strains were isolated during March 2000. The morphological identification of both cultures was based on previous descriptions (Fukuyo *et al.* 1985; Hallegraeff *et al.* 1991; Balech 1992). Both cultures were kept in modified f/2 medium (Anderson *et al.* 1984) at 20°C, with a 10 : 14 h light–dark cycle at 150 μmol m^{-2} s^{-1}; both are being maintained at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR).

DNA extraction

Cells in 50 ml of a culture in mid log growth phase were harvested by gentle centrifugation and the supernatant was removed by aspiration. The cell pellet was resuspended in 750 μl of filtered seawater, transferred to autoclaved disposable cryovials, and centrifuged at 3500 × *g* for 10 min at 4°C. The supernatant was removed and the sample was stored immediately in liquid nitrogen until further analysis.

The frozen cell pellet was thawed at room temperature. Cells were lysed in a nitrogen pump, using four cycles of 10 min at 2400 psi, followed by rapid release of pressure. The cells were then incubated with 500 μl of lysis buffer at 65°C for 30 min (Adachi *et al.* 1994). Nucleic acids were purified by double extraction with 100 μl of phenol–chloroform–isoamylic alcohol (25 : 24 : 1, v/v) with a centrifuge at 14,000 × *g* for 8 min at 4°C between extractions. This was followed by an extraction with a solution of chloroform–isoamylic alcohol

(24 : 1, v/v). The sample was then centrifuged at 14,000 × *g* for 8 min at 4°C. DNA was precipitated by adding 5 µl of mussel glycogen, 15 µl 3 M NaOAc and isopropanol, followed by incubation at −20°C for 30 min. The precipitate was collected by centrifugation at 16,000 × *g* for 60 min and rinsed with 1000 µl 70% ethanol, spun again, dried in a speed vacuum, and resuspended in 50 µl sterile deionized water.

PCR amplification of LSU rDNA

Amplifications were carried out in a Perkin–Elmer Gene Amp PCR system 2400 DNA thermal cycler. Reactions contained a total of 100 µl: 12 µl of total genome DNA (200–400 ng), 0.75 µl *Taq* DNA polymerase (Fisher Scientific, Pittsburg, PA, USA), 10 µl 10× reaction buffer, 4 µl of deoxynucleoside triphosphate (dNTP) (Takara Biomedicals, Otsu, Shiga, Japan), and primers D1R and D2C (Scholin *et al.* 1994). Thermocycling was as follows: denaturing at 94°C, 1 min; annealing at 50°C, 1.5 min; and extension at 72°C, 1 min (Scholin & Anderson 1994). This cycle was repeated 30 times, with an autoextension of the polymerization cycle of 5 s per cycle.

RFLPs

All restriction digests followed manufacturer's recommendations and used the buffers and bovine serum albumin (BSA) extract supplied with restriction enzymes. The restriction enzymes used were: *Apa*LI, *Mse*I, and *Nsp*I (BioLabs New England, Beverly, MA, USA). Water, buffer, BSA, and the enzyme were prepared in a master mix; 4 µl of this were added to 10 µl aliquots of purified PCR product. Digests were prepared using 2–5 units of restriction enzyme and approximately 0.5–1 µg of PCR product per 12 µl reaction; digestion proceeded for 12 h at an incubation temperature of 37°C. Restriction products were stored at −20°C.

The restriction products were resolved on a 3.0% agarose gel of a 3 : 1 mixture of Nusieve and Low EEO agarose, 1× Tris-acetate-EDTA buffer, with ethidium bromide incorporated in the gel. Samples were mixed with 2 µl of loading dye. The entire sample was applied to the gel and then run at 75 V for 90 min. Gels were photographed using an MP-4 camera system and 667 print film, or the ChemiImager (Alpha Innotech, San Leandro, CA, USA) digital picture system. Sizes of PCR and digestion products were estimated by comparing their mobility with standards (BioMarker Low, BioVentures, Murfreesboro, TN, USA).

Expected RFLP fragment sizes were also calculated. The complete DNA sequence of each isolate (obtained as described below) was hand-checked for restriction sites for each enzyme. Fragment lengths were measured and used to generate a complete set of expected RFLP fragments, including those too small to be visualized on the agarose gels.

Sequence analysis

Twenty nanograms of the purified PCR products were added to 1.5 µM of either the D1R or the D2C primer (Scholin *et al.* 1994), depending on the desired direction of the reading, together with the manufacturer's recommended sequencing buffer and BigDye fluorescent sequencing mix (Applied Biosystems, Foster City, CA, USA). Volumes were brought to 10 µl with distilled deionized water. Reactions were topped with 15 µl mineral oil to prevent evaporation, and then run in a thermocycler (4800 series, Perkin–Elmer, Wellesley, MA, USA) for 30 cycles at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min, with a final hold at 4°C. Reactions were purified in Sephadex columns (Sigma Chemical Co., St Louis, MO, USA), and then vacuum dried and stored at −20°C. Reactions were later resuspended in 2 µl DNA-loading dye and run in acrylamide gels in an ABI automatic sequencer (Applied Biosystems) in 5% Long Ranger acrylamide gels (BioWhittaker, Walkersville, MD, USA).

Sequences were examined using ABI Sequencing Analysis 3.3 and ABI AutoAssembler 2.1 software (Applied Biosystems), and aligned with existing *Alexandrium* sequences obtained from GenBank using ClustalX 1.64b (Gibson *et al.* 2001). Strain designation, location of origin, and GenBank accession numbers are given in Table 1. The alignment was hand-checked, and ambiguously aligned regions were excluded, yielding a final data set with 598 included characters. Maximum likelihood (ML) and maximum parsimony (MP) analyses were carried out using PAUP 4.0b10 (Swofford 2002).

For ML analysis, the HKY85+G model was used, because our data adjusted better to its assumptions. Nucleotide frequencies and transition–transversion frequencies were estimated from the data; among-site rate variability was estimated with a gamma distribution with an alpha value of 0.5. For MP analysis, 189 of the 598 characters were parsimony informative. Multistate bases were treated as polymorphisms and gaps were treated as a fifth base. The addition sequence was random, with 1000 replicates. Branches were collapsed when the maximum branch length was zero. Bootstrap analysis included 1000 replicates under MP criteria as described above, with a resampling of the 598 character data set for each replicate.

RESULTS

PCR amplifications for *A. margalefii* and *A. affine* resulted in a single product of approximately 720 bp (Fig. 2). The RFLP patterns for *A. margalefii* (strain AMBCQ-1) comprised fragments of 550 and 120 bp when digested with *Nsp*I, and fragments of 300, 250, 95, and 45 bp with *Mse*I, but the LSU rDNA was not digested by *Apa*LI (Fig. 2). *Alexandrium affine* (strain AABCV-1) digested with *Nsp*I yielded fragments of 500, 125, and 75 bp; fragments of 350 and 300 bp were obtained upon digestion with *Mse*I, and two fragments, approximately 500 and 225 bp long, were observed with *Apa*LI (Fig. 2). There was agreement between predicted and observed RFLP patterns (Table 2) in *A. affine* and *A. margalefii* strains. Sequences of the *A. affine* strain from Bahía Concepción revealed more cuts than other strains of *A. affine* digested with *Nsp*I and less cuts with *Apa*LI. The strains of *A. margalefii* from New Zealand and Bahía Concepción showed no differences in RFLP patterns digested with *Nsp*I, *Mse*I and *Apa*LI.

A phylogenetic tree was constructed from the LSU rDNA sequences of 20 isolates of *Alexandrium* species (Table 1). Of the 692 characters, 94 were excluded because of ambiguous alignment, 255 were constant, 154 were variable but parsimony uninformative, and 189 characters were parsimony informative. The phylogenetic placement of each of the two Mexican strains was identical under ML (Fig. 3) and MP cri-

Table 1. Strains, geographical origins and GenBank accession numbers of *Alexandrium* species used in phylogenetic analyses.

Species (strain)	Geographical origin	GenBank accession number
A. affine (X21)	Bay of Concarneau, France	AF318229
A. affine (Pa4V)	Galicia, Spain	L38630
A. affine (CU1)	Gulf of Thailand	U44935
A. affine (AABCV-1)	Bahía Concepción, Mexico	AY152706
A. andersoni (TC02)	Eastham, MA, USA	U44937
A. catenella (OF101)	Ofunato Bay, Japan	U44931
A. cohorticula (ACMS01)	Malaysia	AF174614
A. excavatum (Ge1V)	Spain	L38632
A. fundyense (AFNFA3.2)	Newfoundland, Canada	U44928
A. lusitanicum (AI18V)	Spain	L38623
A. margalefii (X12)	Bay of Concarneau, France	AF318230
A. margalefii (AGNZ01)	Bream Bay, New Zealand	AY152707
A. margalefii (Bream)	Bream Bay, New Zealand	AF033531
A. margalefii (AMBCQ-1)	Bahía Concepción, Mexico	AY152708
A. minutum (X20)	The Rance, France	AF318232
A. minutum (AI1V)	Spain	L38626
A. ostenfeldii[1]	New Zealand	AF033533
A. tamarense (ATBB01)	Bell Bay, Tasmania, Australia	U44933
A. tamarense (G. Hope 1)	ship ballast water, Samchonpo, South Korea	U44932
A. tamarense (OF041)	Ofunato Bay, Japan	U44929
Fragilidium subglobosum[1]	not provided	AF260387

[1] No strain designation provided.

teria. The identifications of strains AABCV-1 and AMBCQ-1 as *A. affine* and *A. margalefii,* based on morphological characters, were supported by the placement of these strains within the *A. affine* and *A. margalefii* clusters, respectively. Bootstrap analysis supported the placement of strain AABCV-1 within the *A. affine* cluster at 100%; there was no support for any subdivision of the species. The placement of strain AMBCQ-1 within the *A. margalefii* cluster also had 100% bootstrap support. Within the *A. margalefii* cluster, AMBCQ-1 grouped with a strain from France and one of two strains from New Zealand, with 92% bootstrap support. The second New Zealand strain branched off basal to this clade.

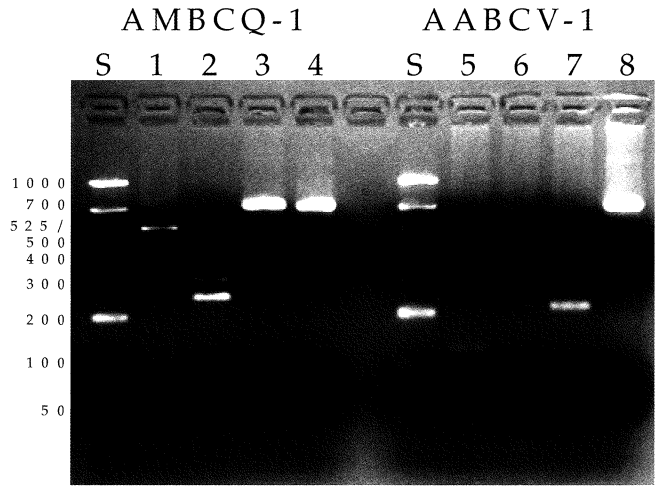

Fig. 2. Restriction patterns of *Alexandrium margalefii* and *A. affine* produced with three enzymes. S = size standard, *A. margalefii* (AMBCQ-1) digested with (1) *Nsp*I, (2) *Mse*I, (3) *Apa*LI, and (4) PCR amplification product. *Alexandrium affine* (AABCV-1) digested with (5) *Nsp*I, (6) *Mse*I, (7) *Apa*LI, and (8) PCR amplification product.

DISCUSSION

We have conclusively documented the presence of *A. margalefii* and *A. affine* in Mexican waters. *Alexandrium affine* has recently been reported from Bahía Concepción (Gárate-Lizárraga *et al.* 2001a; Band-Schmidt *et al.* 2003), but no previous record of *A. margalefii* exists for Mexico or the North American continent. It is possible that these species have been present in this bay for some time, but have gone unnoticed for lack of appropriate identification skills or technology. On the other hand, a recent introduction cannot be ruled out.

Identification of *Alexandrium* species has been largely based on visual observations by light microscopy and scanning electron microscopy. However, the size and shape of *Alexandrium* strains in cultures and wild populations vary considerably (Hallegraeff *et al.* 1991), making morphological identification difficult. Subcellular characteristics, including molecular data, have proved to be useful for reconstructing the evolution and geographical dispersal of these organisms (Scholin *et al.* 1995; Medlin *et al.* 1998). The D1 and D2 regions of LSU rDNA are among the most rapidly evolving regions of the eukaryotic rRNA genes (Michot *et al.* 1984; Michot & Bachellerie 1987; Lenaers *et al.* 1989, 1991) and accelerated rates of nucleotide substitutions within these domains have allowed identification of species-specific and, in some cases, strain-specific sequences for several *Alexandrium* species; they have also allowed the biogeographical dispersal of *Alexandrium* to be tracked (Scholin *et al.* 1994; Medlin *et al.* 1998).

The RFLP and DNA sequence data from our isolates correspond to the LSU rDNA ribotypes of *A. margalefii* and *A. affine* previously reported by other authors (Scholin *et al.* 1995, 1996; LeinonenDuFresne *et al.* 2000). The RFLP pattern places strain AABCV-1 in the *A. affine* cluster (Scholin

Table 2. Size of expected and observed restriction fragments of the D1–D2 LSU rDNA fragment of *Alexandrium affine* and *A. margalefii* strains. The *A. affine* strains are from the Gulf of Thailand (CU1) and Bahía Concepción (AABCV-1); those of *A. margalefii* from Bream Bay, New Zealand (AGNZ01), and Bahía Concepción (AMBCQ-1). bp = base pairs.

Species (strain)	Expected fragments (bp)[1]			Observed fragments (bp)[2]		
	*Nsp*I	*Mse*I	*Apa*LI	*Nsp*I	*Mse*I	*Apa*LI
A. affine (CU1)[3]	471	345	496	460	350	500
	159	309	491	160	300	225
	81	26	220	82		
		19				
		12				
A. affine (AABCV-1)	471	345	491	500	350	500
	129	309	220	125	300	225
	81	26		75		
	30	19				
		12				
A. margalefii (AGNZ01)	557	309	none	550	300	none
	119	252		120	250	
	37	94			95	
		45			45	
		12				
A. margalefii (AMBCQ-1)	557	309	none	550	300	none
	119	252		120	250	
	37	94			95	
		45			45	
		12				

[1] Hand computed from DNA sequence.
[2] Estimated from agarose gel photos comparing mobility of size standards with digestion products.
[3] Data from Scholin & Anderson (1996).

& Anderson 1996). The *Apa*LI and *Mse*I patterns were identical to the published pattern, and the *Nsp*I pattern differed only slightly from the published pattern, with the second fragment closer to 125 than 160 bp. Sequence analysis revealed that the smaller size of the second band in strain AABCV-1 with the digestion with *Nsp*I was caused by a single base difference (A instead of T) at site 582. This caused the 160 bp fragment equivalent to that produced by strain CU-1 to be cut into two fragments, of approximately 125 and 30 bp. Strain AMBCQ-1 yielded fragments of 550 and 120 bp with *Nsp*I, and fragments of 300, 250, 95, and 45 bp with *Mse*I, but its LSU rDNA was not digested by *Apa*LI (Fig. 2). This placed strain AMBCQ-1 in the *A. margalefii* cluster. Both ribotypes are clearly distinct from the ribotypes of *A. andersonii, A. cohorticula, A. ostenfeldii,* and the *A. tamarensis–catenella–fundyense* and *A. lusitanicum–minutum* species complexes (Scholin *et al.* 1994; Medlin *et al.* 1998; Walsh *et al.* 1998; de Salas *et al.* 2001; Lilly *et al.* 2002). Expected fragments smaller than 37 bp for *A. affine* and *A. margalefii* strains cannot be seen in the RFLP image because of their small size.

The clade containing the *A. affine* isolates is related to *A. cohorticula* and the *A. tamarense–catenella–fundyense* group, whereas the *A. margalefii* clade clusters with *A. andersonii, A. lusitanicum–minutum,* and *A. ostenfeldii.* These major clusters are consistent with the results obtained for different *Alexandrium* strains from several geographical regions (Scholin *et al.* 1994; Medlin *et al.* 1998; de Salas *et al.* 2001). Previous work on *Alexandrium* strains from New Zealand (Spalter *et al.* 1997; Walsh *et al.* 1998), using LSU, SSU and intergenic rRNA sequences, found similar placements for *A. affine* and *A. margalefii.*

The available genetic data are not sufficient for us to be certain of the relationships between our strains and other strains of these two species collected from across their global distribution. The small number of rDNA sequences available makes it difficult to determine whether the Mexican strains represent distinct populations or recent introductions from another location. The rDNA sequences for the Mexican strains of *A. affine* and *A. margalefii* are unique, but there are only a few base-pair differences between them and sequences of strains from other areas. Genetic diversity is especially low in the *A. affine* clade, in which strains from Spain, France, Thailand and Mexico show little variation. It is thus possible that the global distribution of this species may be very recent. An alternative explanation is that the D1–D2 region of the LSU rDNA is not as variable for *A. affine* and *A. margalefii* as it is for members of the *A. tamarense–catenella–fundyense* clade. Additional analyses of strains from different geographical areas are needed to test this possibility. Unfortunately, the historical record of algal blooms in Bahía Concepción is limited: blooms have been documented only since 1992 (Sierra-Beltrán *et al.* 1996; Lechuga-Devéze & Morquecho-Escamilla 1998; Lechuga-Devéze *et al.* 2000). Moreover, the cysts of *Alexandrium* species are not preserved in the sediments and hence do not leave fossil records (Hallegraeff *et al.* 1997). Thus, at this time, there is no way to establish the historical presence of these species in Mexico.

A more detailed monitoring of *Alexandrium* species is needed along the entire Mexican coast, especially because several saxitoxin-producing species are found in this genus (Anderson 1998). Molecular biology, together with traditional taxonomic analyses, can provide accurate tools for their identification and can often reveal biogeographical patterns.

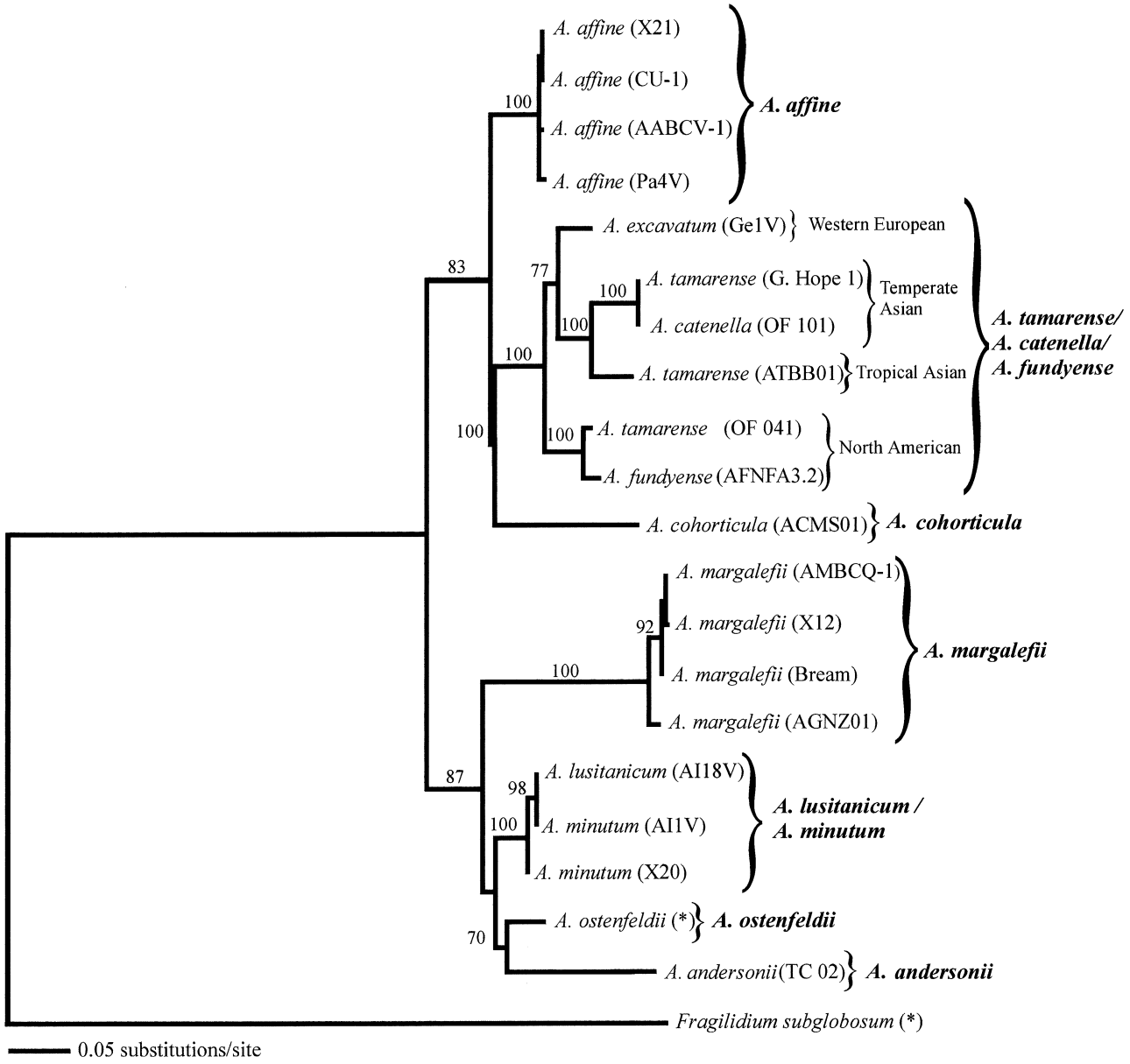

Fig. 3. Phylogenetic tree derived from *Alexandrium* LSU rDNA sequences generated by PAUP 4.0b10 under ML criteria, with bootstrap values generated from the MP analysis with 1000 replicates.

ACKNOWLEDGEMENTS

We thank the CIBNOR Graduate Program and the Mexican National Council of Science and Technology (CONACyT) for financial and logistical support, L. Morquecho for providing the strain of *A. margalefii,* C. Luxoro for technical advice, and Ira Fogel for editing the English text. This research was supported in part by CIBNOR Institutional Research Projects AYCG-8 (1999–2000), CONACyT grant R33598-B, National Oceanic and Atmospheric Administration (NOAA) grant NA96OP0099 (to D.M.A.), and National Science Foundation (NSF) grant OCE-9808173 (to D.M.A.). This is contribution number 52 of the US ECOHAB Program sponsored by NOAA, Environmental Protection Agency, NSF, National Aeronautics and Space Administration (NASA), and Office of Naval Research (ONR) and grant contribution 10743 from the Woods Hole Oceanographic Institution.

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Accepted 6 January 2003