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Toxin and molecular analysis of *Gymnodinium catenatum* (Dinophyceae) strains from Galicia (NW Spain) and Andalucía (S Spain)

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Nineteen strains of Gymnodinium catenatum were isolated from one bloom in Andalucía (S Spain) and from different blooms in Galicia (NW Spain). The PSP toxin profiles of 16 of the strains were analyzed, and although the saxitoxin was exclusive to the Galician strains, the corresponding dendogram showed no clustering of the isolates from this location. However, nine out of eleven Andalusian strains were included in the same cluster. In order to compare toxin with molecular analysis, a fragment of the large subunit ribosomal (LSU) RNA gene was partially sequenced for all of the strains and fully sequenced for the five strains that had shown most different growth curves. Since all the strains were identical in the LSU sequenced region, another fragment comprising the internal transcribed spacer 1 (ITS 1), 5.8S rRNA gene and ITS 2, was sequenced and compared among all the strains. Although this region has been used before for the detection of intraspecific variability, it was similar in all our strains. Finally, to detect molecular differences in the strains, a random amplified polymorphic DNA (RAPD) analysis was performed. The corresponding cluster analysis grouped the strains in three clusters: one of them comprised all the Galician strains plus three from Andalucía, another one included eight Andalusian strains, and the last one, more separated from the two previous, was constituted by two Andalucía isolates. Although the results of the toxin and RAPD analysis were different, seven Andalusian strains were clustered together in both dendograms. Since neither the toxin nor the RAPD analysis brought out a clear geographic signal, we can conclude that differences in toxin content and RAPD profile between the isolates of G. catenatum are probably not linked to the location in which the strains were collected.

INTRODUCTION

The identification and differentiation of marine dinoflagellates and, particularly, of toxin-producing species, has traditionally been carried out by morphological characterization using light and electron microscopy. Recently, molecular diagnostic tools have been developed that allow a more specific and sensitive identification of the organisms. For example, the variability of *Alexandrium* was first studied on the basis of morphology, bioluminescence capacity, mating compatibility and toxin composition (Anderson *et al.*, 1994). However, more recent work on the inter- and intraspecific variability of this organism was based on the sequencing and comparison of genomic fragments (Scholin *et al.*, 1994, 1995; Medlin *et al.*, 1998) and restriction fragment length polymorphism (RFLP) analysis (Adachi *et al.*, 1994; Scholin and Anderson, 1994). Another genus extensively studied using molecular techniques has been *Gymnodinium*. Species of the genus *Gymnodinium* can be discriminated by specific immunological assays (Vrieling *et al.*, 1994) or by molecular comparisons (Ellegaard and Oshima, 1998; Bolch *et al.*, 1999a; Kim *et al.*, 1999; Hansen *et al.*, 2000). In fact, the sequencing of partial large subunit ribosomal RNA (LSU rRNA) sequences have permitted, along with ultrastructural observations, the reconstruction of the phylogenetic relationships among major genera of dinoflagellates (Daugbjerg *et al.*, 2000).

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Gymnodinium catenatum, a species that produces Paralytic Shellfish Poisoning (PSP) toxins, has been described worldwide but with a localized distribution; there is no continuity in the dispersion of the dinoflagellate (Hallegraeff and Fraga, 1998). Variation in the genotype of strains from different countries has been studied by comparison of rRNA regions (Adachi *et al.*, 1997; Ellegaard and Oshima, 1998), random amplified polymorphic DNA (RAPD) analysis (Adachi *et al.*, 1997; Bolch *et al.*, 1999b) and allozymes (Ellegaard and Oshima, 1998; Bolch *et al.*, 1999b). The PSP toxin production of *G. catenatum* isolates from widely separated geographic regions has also been characterized and contrasted (Oshima *et al.*, 1993; Ellegaard and Oshima, 1998; Negri *et al.*, 2000).

The aim of this paper was the comparison of toxin and molecular methods for the analysis of strains of *G. catenatum*. For this purpose, we determined the toxin composition of isolates from different regions in Spain, as well as the sequence of several genomic regions with a potential taxonomic or differentiating value. In addition, a RAPD analysis was performed to detect genomic polymorphisms not present in the previously determined sequences. The strains of *G. catenatum* used in this study were isolated from the Atlantic coast of Galicia (NW Spain) and from the Gibraltar Strait in the Andalucía coast (SW Spain). The proximity of both locations has allowed the study of the local variability of this dinoflagellate species in terms of toxin content and DNA sequence.

METHOD

Cultures

The isolation date for six *G. catenatum* strains isolated from different blooms in the Ría de Vigo (Galicia, NW of Spain) and 13 strains from the same bloom in the Gibraltar Strait (Andalucía, S of Spain) (Figure 1), is shown in Table I. The two locations are separated by ~750 km. All the cultures were established from single cells or single chains, and were maintained in 125 mL Erlenmeyer flasks with L1 medium (Guillard and Hargraves, 1993) at 18°C (14:10 L:D, 90 μmol m^{-2} s^{-1}, 34 p.s.u.). The strains are deposited in the Culture Collection of Harmful Micro-algae of the Instituto Español de Oceanografía in Vigo (CCVIEO).

Toxin analysis

Cultures were harvested in the stationary growth phase and filtered through a pre-combusted Gelman 13 mm type GA/E glass microfibre filter for the toxin extraction. Prior to filtration, a 1 mL aliquot of the culture was fixed with Lugol's solution in order to determine the cell concentration by direct counting on a Sedgewick–Rafter counting chamber under the light microscope. The filtered volume was also measured, so the number of cells extracted was quantified and the final toxin concentra-

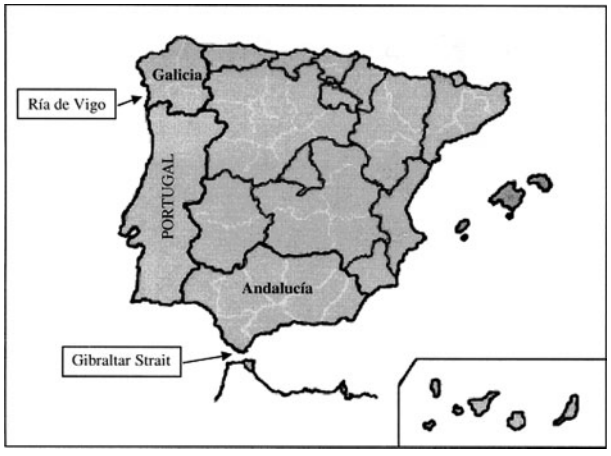

Fig. 1. Locations where the strains used in this study were isolated.

Table I: Strains of G. catenatum included in this study

Strain	Isolation date	GenBank accession no.	
		LSU D1–D3 domain	ITS 1- 5.8S- ITS 2
Galicia			
GC9V	Nov/85	AF375854	AY506574
GC11V	Sep/93		AY506575
GC12V	Sep/93	AF375855	AY506576
GC13V[a]	Sep/93		AY506577
GC19V	Nov/86	AF375856	AY506578
GC21V	Oct/86		AY506579
Andalucia			
GC22AM	Aug/99		AY506580
GC24AM	Aug/99		AY506581
GC26AM	Aug/99		AY506582
GC27AM	Aug/99		AY506583
GC31AM	Aug/99		AY506584
GC36AM	Aug/99	AF375857	AY506585
GC38AM[a]	Aug/99		AY506586
GC42AM	Aug/99		AY506587
GC43AM	Aug/99		AY506588
GC49AM[a]	Aug/99	AF375858	AY506589
GC51AM	Aug/99		AY506590
GC53AM	Aug/99		AY506591
GC56AM	Aug/99		AY506592

[a]Not included in the toxin analysis due to methodological problems.

tion for each strain could be expressed as molar percentage cell^{-1}:

molar percentage per cell of toxin X = (pmol cell^{-1} of toxin X/sum of pmol cell^{-1} of every toxin) × 100

The PSP toxins contained in the filter were analyzed by high performance liquid chromatography (HPLC) with a 5 μm LiChrospher 100 RP-18 (125 × 4 mm i.d.) column following the method of Oshima *et al.* (Oshima *et al.*, 1989) as modified by Franco and Fernández-Vila (Franco and Fernández-Vila, 1993). Most of the toxins were identified by retention times and absorption spectra identical to those of authentic standards, purchased from BCR (Brussels) [for saxitoxin (STX), decarboxysaxitoxin (dcSTX), and neosaxitoxin (neoSTX)] and NCR (Canada) [for gonyautoxins (GTX) 1, 2, 3 and 4]. Deoxysaxitoxin (doSTX) was determined based on the calibration line of STX. As no standards of C toxins, GTX5 and GTX6 were available, an aliquot of the sample was hydrolysed with the same volume of 0.4 N HCl at 100°C for 15 min, in order to convert the C toxins into the corresponding GTXs, and GTX5 and GTX6 into STX and neoSTX. The peak of the C isocratic corresponding to C5,6? [presumably C5 and C6 compounds recently described by (Negri *et al.*, 2000)] was impossible to quantify because of lack of the corresponding standard, so only absence or presence data could be reported.

DNA extraction

An aliquot of the same cultures processed for the toxin analyses were used for the DNA extraction. Cultures were filtered through a Whatman GF/C glass microfibre filter under low vacuum. Filters were transferred to Eppendorf vials, and incubated with 860 μL extraction buffer (SDS 1%, NaCl 150 mM, EDTA 2 mM, Tris–HCl 10 mM; pH 8), 100 μL of guanidine thiocyanate 5 M, and 80 μL of proteinase K (20 mg mL^{-1}), overnight at 56°C. The supernatant was then collected and DNA extracted with the Wizard DNA Clean-Up System Kit (Promega) following the manufacturer's instructions. DNA purity and concentration were determined spectrophotometrically, and DNA concentration was finally adjusted to 200 ng μL^{-1} in each sample.

Amplification and DNA sequencing

The D1–D3 fragment of the LSU rRNA gene was amplified for all strains by polymerase chain reaction (PCR) using primers D1R and D3B (Table II). This fragment was partially sequenced for all of the strains with those two primers, and was fully sequenced for the strains GC9V, GC12V, GC19V, GC36AM and GC49AM with the additional primers D2Ra, D3A and D3B (Table II). For amplification of the internal transcribed spacer 1 (ITS 1), 5.8S rRNA gene and, partially, ITS 2, the forward primer Perk-ITS-S and the reverse primer Perk-ITS-AS, were used (Table II).

PCRs were performed in 25 μL volumes with 23 μL of PCR Supermix (Life Technologies), 0.5 μL of each primer (10 μM) and 1 μL of DNA. PCR conditions for the primer set D1R/D3B were one initial cycle of denaturation at 94°C for 3 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 3 min. The temperature profile was completed by a final extension cycle at 72°C for 6 min. For the other primer set, Perk-ITS-S/Perk-ITS-AS, the PCR temperature conditions were identical to those described by Kotob *et al.* (Kotob *et al.*, 1999).

Table II: List of primers used to amplify and sequence the D1–D3 region of the LSU rRNA gene and the genomic fragment ITS 1- 5.8 S rRNA- (partially) ITS 2 of several Spanish strains of G. catenatum

Primer (5′ to 3′)		Length (nucleotides)	Direction[a]	Reference
LSU				
D1R	ACCCGCTGAATTTAAGCATA	20	Forward	Scholin *et al.* (1994)
D3B	TCGGAGGGAACCAGCTACTA	20	Reverse	Nunn *et al.* (1996)
D2Ra	TGAAAAGGACTTTGAAAAGA	20	Forward	Scholin *et al.* (1994)
D3A	GACCCGTCTTGAAACACGGA	20	Forward	Nunn *et al.* (1996)
D2C	CCTTGGTCCGTGTTTCAAGA	20	Reverse	Scholin *et al.* (1994)
ITS 1- 5.8 S- ITS 2				
Perk-ITS-S	CTTAGAGGAAGGAGAAGTCGTAAC	24	Forward	Kotob *et al.* (1999)
Perk-ITS-AS	GCTTACTTATATGCTTAAATTCAG	24	Reverse	Kotob *et al.* (1999)

[a]Direction of DNA synthesis priming.

PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide in a UV transilluminator. A 100 bp ladder standard (Amersham Pharmacia Biotech) was also included on the gel.

PCR products were purified by digestion with the enzymes Exonuclease I, which removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR, and Shrimp Alkaline Phosphatase (SAP), to remove the remaining dNTPs from the PCR mixture which would interfere with the sequencing reaction. Both enzymes were purchased from Amersham Pharmacia Biotech. The digestion was carried on for 1 h at 37°C, and then the enzymes were denatured by incubation at 80°C for 15 min.

The sequencing reactions of purified PCR products were accomplished using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction DNA Sequencing Kit (Applied Biosystems) following the manufacturer's directions. The products resulting from the sequencing reactions were purified by ethanol precipitation, and the samples were resuspended in 3–5 μL of formamide:loading buffer 5:1 (vol/vol) and denatured at 94°C for 3 min. The sequencing was done using an ABI PRISM 377 DNA automatic sequencer (Perkin–Elmer). Nucleotide sequence data were analyzed with the software packages Gene Jockey and Clustal X.

RAPD analysis

All samples of total genomic DNA were subjected to RAPD analysis using the Ready to Go RAPD Analysis Kit (Amersham Pharmacia Biotech), which includes six arbitrary primers, following the manufacturer's instructions. For each primer combination, all the bands produced by all strains were recorded, and then the absence or presence of each band in each strain was scored as 0 or 1, respectively. The RAPD assay was repeated twice to ensure its reproducibility.

Statistical analysis

The toxin content of each strain, expressed as pmol cell^{-1}, was standardized by subtracting the mean and dividing by the standard deviation, and Euclidean distances among the strains were calculated. A cluster analysis was then performed by the UPGMA method (Sokal and Rohlf, 1995), and its reliability was assessed by the cophenetic correlation coefficient. For the RAPD analysis, a genetic distance matrix, based on the resulting binary matrix obtained by the combination of the results for each primer, was constructed following the method of Skroch *et al.* (Skroch *et al.*, 1992). Subsequently, a cluster analysis was performed and its reliability assessed as above. All the statistical analyses were carried out with the SAS program (SAS Institute, 1989).

RESULTS

Toxin profiles

The peaks in the three isocratics (STX, GTX and C) were well resolved for every toxin and strain, except for the last peak of the C isocratic, which was wide and not very high. It appeared at retention times of 30–38 min in the conditions of the analysis. Because this peak was present in 10 out of 16 strains analysed, and the same result was obtained when repeating the runs, it is unlikely that it was an artefact. The peak could correspond to the C5 and C6 compounds described by Negri *et al.* (Negri *et al.*, 2000), and therefore we applied the separation technique for the C toxins described in that study to some of our samples. No separation of the C5 from the C6 compound, or a better resolution of the peak, was obtained, and subsequently this peak will be referred to as C5,6?

The molar percentage cell^{-1} of each toxin in the *G. catenatum* strain included in this study is shown in Table III. The strains GC13V, GC38AM and GC49AM were not analysed for the toxin production because of methodological problems (misuse of the filtering device). The most remarkable result was that, in contrast to the strains isolated in Andalucía, all strains from Galicia had STX. The toxins doSTX, C1 and C2 were present in all strains, whereas GTX1–4 were absent in almost all samples. On the other hand, the peak corresponding to C5,6? was most frequently observed in strains from Andalucía compared with those from Galicia.

The cluster analysis performed with the data of pmol of toxin per cell showed high reliability (cophenetic correlation coefficient of 0.87). In Figure 2, where the cluster analysis is represented, it can be observed that the Vigo isolates were not clustered together. In contrast, all of the isolates from Andalucía except GC31AM and GC22AM were grouped in the same cluster (in bold in Figure 2). In this 'Andalusian' cluster, one strain from Vigo, GC11V, was also included.

Amplification and DNA sequencing

The PCR products obtained using primer combinations D1R/D3B and Perk-ITS-S/Perk-ITS-AS resulted in bands of the expected length (data not shown).

The D1R/D3B PCR product from every strain was sequenced with primers D1R and D3B, and the approximately 420 bp obtained from the 5′-end and the 3′-end of the D1–D3 fragment of the LSU rRNA were identical in their sequence for all strains (data not shown). Those

Table III: Molar percentage cell^{-1} of each toxin in each G. catenatum strain included in this study

Strains	Toxins															
					Epimers		Epimers					Epimers		Epimers		
	STX	dcSTX	neoSTX	doSTX	GTX 1	GTX 4	GTX 2	GTX 3	GTX5	GTX6	dcGTX2,3	C1	C2	C3	C4	C5,6?
GC9V	10.40	11.78	13.88	20.02	–	–	–	–	21.37	14.68	–	1.92	5.96	–	–	–
GC11V	2.57	1.67	–	33.65	–	–	–	–	16.56	23.89	1.17	2.17	8.10	1.91	8.31	–
GC12V	1.68	0.87	–	16.37	–	0.91	–	–	16.13	40.49	0.95	4.15	9.99	1.99	6.48	–
GC19V	2.65	0.26	–	3.53	–	–	–	0.09	26.10	2.79	0.96	26.98	36.64	–	–	–
GC21V	4.29	0.61	–	32.69	1.23	–	–	–	12.42	23.08	1.22	2.02	8.17	0.80	13.48	+
GC22AM	–	–	50.92	11.89	–	–	4.35	–	15.33	–	1.08	1.93	14.50	–	–	–
GC24AM	–	0.97	–	12.74	–	–	–	–	7.75	23.13	1.25	11.81	26.69	5.10	10.54	+
GC26AM	–	1.26	1.85	11.15	–	–	–	–	12.34	25.39	1.29	6.97	25.57	2.84	11.34	+
GC27AM	–	0.47	3.98	18.45	–	–	–	–	22.39	18.21	0.74	6.71	15.98	3.94	9.12	+
GC31AM	–	0.42	2.34	15.14	–	–	–	–	18.30	31.89	0.92	4.21	12.40	2.60	11.78	+
GC36AM	–	0.27	6.50	29.35	–	–	–	–	22.97	16.46	2.56	5.49	10.58	1.65	4.18	+
GC42AM	–	0.51	6.79	24.85	–	–	–	0.03	19.85	27.19	0.73	4.30	5.07	2.55	8.13	+
GC43AM	–	1.86	–	50.23	–	–	–	–	–	–	3.84	5.63	21.23	3.58	13.64	+
GC51AM	–	–	–	30.25	–	–	–	–	–	–	1.80	15.28	52.67	–	–	–
GC53AM	–	0.11	–	38.76	–	–	0.38	–	1.60	10.24	1.49	10.33	24.17	3.31	9.61	+
GC56AM	–	0.60	1.63	45.69	–	–	–	–	2.44	10.48	2.21	4.84	20.41	1.66	10.04	+

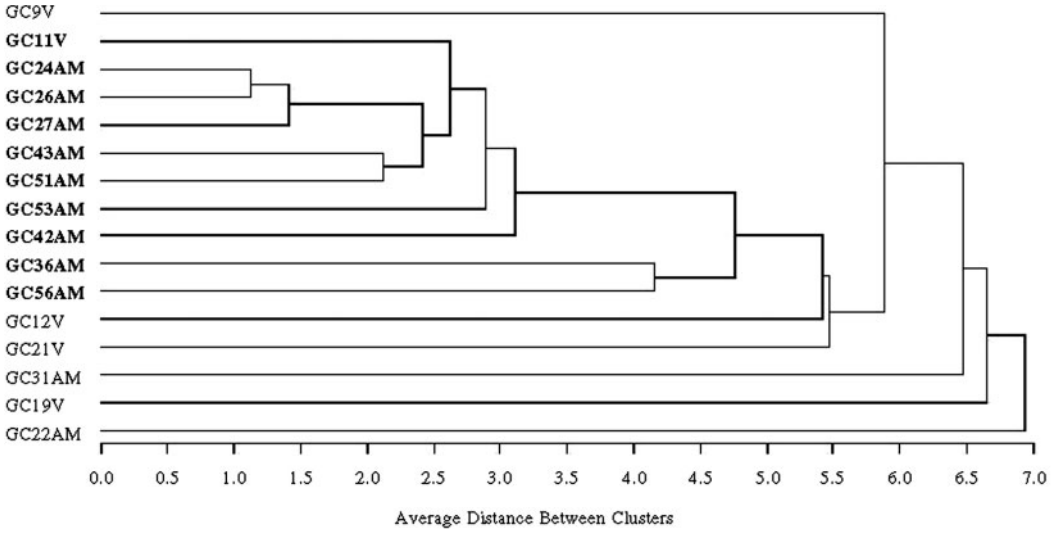

Fig. 2. Similarity dendogram of 16 strains of *G. catenatum* collected from Galicia (code ended in V) and Andalucía (code ended in AM), inferred from PSP toxin content. Strains in bold are grouped in the same cluster ('Andalusian' cluster).

strains that exhibited the most pronounced differences in growth curve (data not shown) were selected for the determination of the entire D1–D3 sequence, in order to check the presence of molecular differences in this region in physiologically distinct isolates. No differences were observed when comparing the resulting 907 bp from strains GC9V (AF375854), GC12V (AF375855), GC19V (AF375856), GC36AM (AF375857), and

GC49AM (AF375858) (GenBank accession numbers in brackets). Interestingly, these sequences had five base pairs different to another sequence of a *Gymnodinium catenatum* Spanish strain, also isolated from Vigo, deposited in the GenBank by Hansen *et al.* (Hansen *et al.*, 2000) with the accession number AF200672.

In order to compare another genomic fragment and to confirm the results obtained with the LSU gene, the fragment ITS 1-5.8S rRNA-(partial) ITS 2 was sequenced and compared in all of the strains. The sequences, 524 bp long (GenBank accession numbers AY506574–AY506592), were identical for all of our strains. This genomic region had already been sequenced by other authors for the strain GC19V (GenBank accession number AF208247). Similarly to what happened with the LSU fragment, this last sequence showed 3 bp different to the sequence we obtained for the same strain.

RAPD analysis

All of the primers produced different band profiles for each strain, and finally a total of 104 bands were checked for absence or presence in every strain. The band frequencies for each primer and strain are shown in Table IV. The RAPD reactions were highly reproducible since the profiles obtained in the two repeated amplifications with the same primer were similar. The value of the cophenetic correlation coefficient of the cluster analysis, although not very high (0.77), supported the reliability of the assay. The similarity dendogram (Figure 3) showed two large groups or clusters, one of them including all strains from Vigo plus GC42AM, GC49AM and GC53AM, and the other one (in bold in Figure 3) comprising exclusively strains from Andalucía. These two groups were equally separated from another one constituted by strains GC22AM and GC51AM.

Table IV: RAPD band frequencies of the strains included in this study

Primer[a]	Total bands	Band frequencies		
		$F = 1$	$1 < F > 0.50$	$F \leq 0.5$
1	18	2	6	10
2	23	0	8	15
3	15	0	5	10
4	19	0	4	15
5	14	0	3	11
6	15	0	8	7
Total	104	2	34	68

[a]Primers from the Ready To Go RAPD Analysis Kit (Amersham Pharmacia Biotech).

DISCUSSION

The strains of *G. catenatum* studied here were collected in relatively closely located areas, compared with other studies on the geographical variability of this dinoflagellate (Oshima *et al.*, 1993; Adachi *et al.*, 1997; Ellegaard and Oshima, 1998; Bolch *et al.*, 1999b; Negri *et al.*, 2000). However, we detected different PSP toxin profiles among the strains, except for STX, which was only present in the Galician ones. Despite the distinctive presence of STX in the isolates from Galicia, the dendogram performed with toxin data did not cluster these strains together. In contrast, all of the Andalusian strains were included in the same cluster except for the GC31AM and GC22AM isolates, whose high amounts of GTX6 and neoSTX, respectively, might have separated them from the resting AM strains.

The presence of doSTX in every Spanish isolate contrasts with previous studies in which no 13-deoxydecarbamoyl toxins were observed in this dinoflagellate species from Spain (Oshima *et al.*, 1993; Negri *et al.*, 2000). In our case, the doSTX was the only toxin present in every strain, and in some isolates it was the most abundant toxin. Some of the strains showed a final peak in the C isocratic, which could correspond to the recently identified C5 and C6 compounds (Negri *et al.*, 2000), described in *G. catenatum* from several countries including Spain and Portugal.

The genomic regions most frequently used for the comparison and establishment of phylogenetic relationships among *Gymnodinium* species and/or *G. catenatum* strains are the small subunit ribosomal RNA (SSU rRNA) gene (Takishita *et al.*, 1999), the internal transcribed spacers (ITS) 1 and 2, the 5.8S rRNA gene (Adachi *et al.*, 1997; Kim *et al.*, 1999), and different domains of the LSU rRNA gene (Ellegaard and Oshima, 1998; Bolch *et al.*, 1999a; Daugbjerg *et al.*, 2000). Although the SSU rRNA has been widely used for the search of variability because it has both conserved and not conserved regions (Medlin *et al.*, 1988), it is not suitable for species level distinctions (Rowan and Powers, 1991; McNally *et al.*, 1994). Therefore, we selected the D1–D3 domain of the LSU rRNA gene, as suggested by Michot *et al.* (Michot *et al.*, 1990), for the search of variability among our strains. Since all the sequences obtained corresponding to this domain were identical, we decided to use more variable gene regions, such as the ITS 1-5.8S-ITS 2, in order to look for more recent historical phylogenetic divisions (LaJeunesse, 2001). The primers used for amplifying and sequencing this genomic fragment had been described for the parasite *Perkinsus* (Goggin, 1994). The genus *Perkinsus*, although already included in

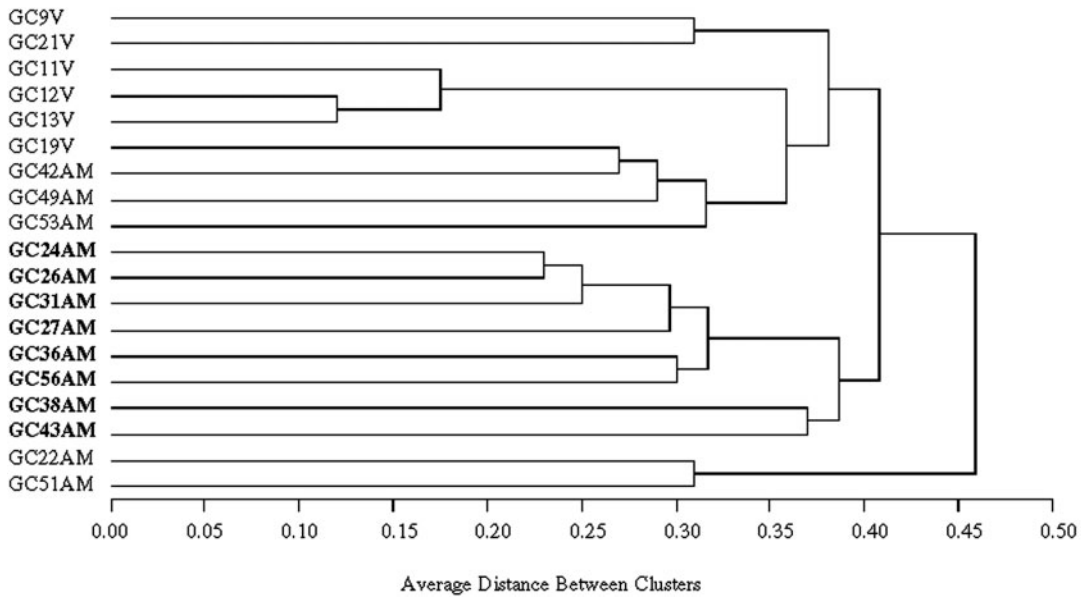

Fig. 3. Similarity dendogram of 19 strains of *G. catenatum* collected from Galicia (code ended in V) and Andalucía (code ended in AM), inferred from the presence/absence of bands in the RAPD analysis. The strains included in the second cluster (GC24AM–GC43AM) are in bold to simplify its visual separation from the first cluster (GC9V–GC53AM) and the third cluster (GC22AM and GC51AM).

the phylum Apicomplexa, is more closely related to the Dinoflagellata as demonstrated by phylogenetic analysis based on the sequence of several genomic regions (Saldarriaga *et al.*, 2003). Therefore, it is not surprising the correct amplification of the *G. catenatum* ITS 1-5.8S-ITS 2 region using such a primer pair. Again, no differences were detected in this genomic region among all of the strains used in the study, confirming that, as suggested by LaJeunesse (LaJeunesse, 2001), the level of genetic resolution offered by the ITS does not explain all morphological and physiological differences. Recently, a similar result was obtained by Tengs *et al.* (Tengs *et al.*, 2003) for several isolates of *Pfiesteria piscicida* with different toxin characteristics. In conclusion, the divergence in the toxin profiles among the *G. catenatum* strains was not associated with differences in the genomic regions compared.

In order to ascertain the molecular variability not detected by the DNA comparison, we decided to use the RAPD technique. This method rapidly detects genomic polymorphisms, and has been extensively used for gene mapping, population analysis, epidemiology, analysis of phylogenetic and taxonomic relationships and detection of strain diversity (Welsh *et al.*, 1995). In fact, the RAPD analysis has been applied before for the comparison of *G. catenatum* isolates from widely separated geographical regions (Adachi *et al.*, 1997; Bolch *et al.*, 1999b).

The dendogram obtained with the RAPD data, contrary to that constructed with the toxin data, grouped all of the Galician strains in one cluster. However, and as we obtained before with the toxin data, this analysis did not reflect the collection sites, since three Andalusian strains were included in this cluster. In fact, the isolates from Andalucía, all coming from the same bloom, were distributed in three different clusters, as described in the Results section. This lack of homogeneity among the Andalusian strains agrees with previous studies on the genetic variation of *Alexandrium* and *Gymnodinium*, also performed with the RAPD technique (Adachi *et al.*, 1997; Bolch *et al.*, 1999b). These works concluded that the genetic variation was partitioned mainly within populations of the same species, compared with the variation between regions and between populations within regions. In spite of the apparent dissimilarity between the toxin and RAPD dendograms, we have to note that both analyses clustered together six Andalusian strains, GC24–26–27–43–36–56AM, probably due to a strong taxonomic proximity of these strains.

There was considerable distance in the RAPD cluster even among the most closely related strains (GC12V and GC13V). This points out the high local variability of *G. catenatum* and suggests that studies on the global distribution of this species should include a large number of strains from each location.

The fact that the dinoflagellate toxicity is mediated by external factors, such as the presence of bacteria in the cultures (Uribe and Espejo, 2003), suggests the weakness of this character for the establishment of taxonomic relationships. Since the RAPD analysis is based on genetic differences, it should be more reliable than the toxin analysis. In the present study, we have obtained different taxonomic

relationships among several strains of *G. catenatum* when using toxin or RAPD analysis. However, none of the methods applied brought out a clear geographic signal, suggesting the lack of connection between the strain characteristics (with respect to toxin content and RAPD profile) and the location in which they were isolated.

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REFERENCES

Adachi, M., Sako, Y. and Ishida, Y. (1994) Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). *J. Phycol.*, **30**, 857–863.

Adachi, M., Sako, Y. and Ishida, Y. (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.

Anderson, D. M., Kulis, D. M., Doucette, G. J., Gallagher, J. C. and Balech, E. (1994) Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. *Mar. Biol.*, **120**, 467–478.

Bolch, C. J. S., Negri, A. P. and Hallegraeff, G. M. (1999a) *Gymnodinium microreticulatum* sp. nov. (Dinophyceae): a naked, microreticulate cyst-producing dinoflagellate, distinct from *Gymnodinium catenatum* and *Gymnodinium nolleri*. *Phycologia*, **38**, 301–313.

Bolch, C. J. S., Blackburn, S. I., Hallegraeff, G. M. and Vaillancourt, R. E. (1999b) Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol.*, **35**, 356–367.

Daugbjerg, N., Hansen, G., Larsen, J. and Moestrup, Ø. (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 unarmoured dinoflagellates. *Phycologia*, **39**, 302–317.

Ellegaard, M. and Oshima, Y. (1998) *Gymnodinium nolleri* Ellegaard et Moestrup sp. ined. (Dinophyceae) from Danish waters, a new species producing *Gymnodinium catenatum*-like cysts: molecular and toxicological comparisons with Australian and Spanish strains of *Gymnodinium catenatum*. *Phycologia*, **37**, 369–378.

Franco, J. M. and Fernández-Vila, P. (1993) Separation of paralytic shellfish toxins by reversed phase high performance liquid chromatography, with postcolumn reaction and fluorimetric detection. *Chromatographia*, **35**, 613–620.

Goggin, C. L. (1994) Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Mol. Biochem. Parasitol.*, **65**, 179–182.

Guillard, R. R. L. and Hargraves, P. E. (1993) *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia*, **32**, 234–236.

Hallegraeff, G. M. and Fraga, S. (1998) Bloom dynamics of the toxic dinoflagellate *Gymnodinium catenatum*, with emphasis on Tasmanian and Spanish coastal waters. In Anderson, D. M., Cembella, A. D. and Hallegraeff, G. M. (eds), *Physiological Ecology of Harmful Algal Blooms*, NATO ASI Series, Vol G41. Springer-Verlag, Berlin, pp. 59–80.

Hansen, G., Daugbjerg, N. and Henriksen, P. (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.

Kim, G. Y., Ha, M. G., Cho, E. S., Lee, T.-H., Lee, S. J. and Lee, J.-D. (1999) Molecular identification of *Gyrodinium impudicum* and *Gymnodinium sanguineum* by comparing the sequences of the internal transcribed spacers 1, 2 and 5.8S ribosomal DNA. *J. Fish. Sci. Tech.*, **2**, 66–77.

Kotob, S. I., McLaughlin, S. M., Van Berkum, P. and Faisal, M. (1999) Discrimination between two *Perkinsus* spp. isolated from the softshell clam, *Mya arenaria*, by sequence analysis of two internal transcribed spacer regions and the 5.8S ribosomal RNA gene. *Parasitology*, **199**, 363–368.

LaJeunesse, T. C. (2001) Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region: in search of a 'species' level marker. *J. Phycol.*, **37**, 866–880.

McNally, K. L., Govind, N. S., Thome, P. E. and Trench, R. K. (1994) Small-subunit ribosomal DNA sequence analyses and a reconstruction of the inferred phylogeny among symbiotic dinoflagellates (Pyrrophyta). *J. Phycol.*, **30**, 316–329.

Medlin, L., Elwood, H. L., Stickel, S. and Sogin, M. L. (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, **71**, 491–499.

Medlin, L. K., Lange, M., Wellbrock, U., Donner, G., Elbrächter, M., Hummert, C. and Luckas, B. (1998) Sequence comparisons link toxic European isolates of *Alexandrium tamarense* from the Orkney Islands to toxin North American stocks. *Eur. J. Protistol.*, **34**, 329–335.

Michot, B., Qu, L.-H. and Bachellerie, J.-P. (1990) Evolution of large-subunit rRNA structure. The diversification of divergent D3 domain among major phylogenetic groups. *Eur. J. Biochem.*, **188**, 219–229.

Negri, A. P., Bolch, C. J. S., Blackburn, S. I., Dickman, M., Llewellyn, L. E. and Méndez, S. (2000) Paralytic shellfish toxins in *Gymnodinium catenatum* strains from six countries. Proceedings of the Ninth International Conference on Harmful Algal Blooms (Tasmania).

Nunn, G. B., Theisen, B. F., Christensen, B. and Arctander, P. (1996) Simplicity-correlated size growth of the nuclear 28S ribosomal RNA D3 expansion segment in the crustacean order Isopoda. *J. Mol. Evol.*, **42**, 211–223.

Oshima, Y., Sugino, K. and Yasumoto, T. (1989) Latest advances in HPCL analysis of paralytic shellfish toxins. In Natori, S., Hashimoto, K. and Ueno, Y. (eds), *Mycotoxins and Phycotoxins '88*. Elsevier, Amsterdam, pp. 319–326.

Oshima, Y., Itakura, H., Lee, K.-C., Yasumoto, T., Blackburn, S. and Hallegraeff, G. (1993) Toxin production by the dinoflagellate *Gymnodinium catenatum*. In Smayda, T. J. and Shimizu, Y. (eds), *Toxic Phytoplankton Blooms in the Sea*. Elsevier Science Publishers BV, pp. 907–912.

Rowan, R. and Powers, D. A. (1991) A molecular genetic classification of zooxanthellae and the evolution of animal–algal symbiosis. *Science*, **251**, 1348–1351.

Saldarriaga, J. F., McEwan, M. L., Fast, N. M., Taylor, F. J. and Keeling, P. J. (2003) Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. *Int. J. Syst. Evol. Microbiol.*, **53**, 355–365.

SAS, Institute (1989) *SAS STAT User's Guide*, Version 6, 4th edn, Vols 1 and 2. SAS Institute, Cary, NC.

Scholin, C. A. and Anderson, D. M. (1994) Identification of group- and strain-specific genetic markers from globally distributed *Alexandrium*

(Dinophyceae). I. RFLP analysis of SSU rRNA genes. *J. Phycol.*, **30**, 744–754.

Scholin, C. A., Herzog, M., Sogin, M. and Anderson, D. M. (1994) Identification of group- and strain-specific genetic markers from globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *J. Phycol.*, **30**, 999–1011.

Scholin, C. A., Hallegraeff, G. M. and Anderson, D. M. (1995) Molecular evolution of the *Alexandrium tamarense* 'species complex' (Dinophyceae): dispersal in the North American and West Pacific regions. *Phycologia*, **34**, 472–485.

Skroch, P., Tivang, J. and Nienhuis, J. (1992) Analysis of genetic relationships using RAPD marker data. *Applications of RAPD Technology to Plant Breeding*. Joint Plant Breeding Symposia Series (1 November 1992, Minneapolis, Minnesota), Crop Science Society of America/American Society for Horticultural Science/American Genetic Association, pp. 26–30.

Sokal, R. R. and Rohlf, F. J. (1995) *Biometry*, 3rd edn. W. H. Freeman and Co., New York, pp. 415 ff.

Takishita, K., Nakano, K. and Uchida, A. (1999) Preliminary phylogenetic analysis of plastid-encoded genes from an anomalously pigmented dinoflagellate *Gymnodinium mikimotoi* (Gymnodiniales, Dinophyta). *Phycol. Res.*, **47**, 257–262.

Tengs, T., Bowers, H. A., Glasgow, H. B., Jr, Burkholder, J. M. and Oldach, D. W. (2003) Identical ribosomal DNA sequence data from *Pfiesteria piscicida* (Dinophyceae) isolates with different toxicity phenotypes. *Environ. Res.*, **93**, 88–91.

Uribe, P. and Espejo, R. T. (2003) Effect of associated bacteria on the growth and toxicity of *Alexandriumn catenella*. *Appl. Environ. Microbiol.*, **69**, 659–662.

Vrieling, E. G., Peperzak, L., Gieskes, W. W. C. and Veenhuis, M. (1994) Detection of the ichthyotoxic dinoflagellate *Gyrodinium* (cf.) *aureolum* and morphologically related *Gymnodinium* species using monoclonal antibodies: a specific immunological tool. *Mar. Ecol. Prog. Ser.*, **103**, 165–174.

Welsh, J., Perucho, M., Peinado, M., Ralph, D. and McClelland, M. (1995) Fingerprinting of DNA and RNA using arbitrarily primed PCR. In McPherson, M. J., Hames, B. D. and Taylor, G. (eds), *PCR 2: A Practical Approach*. IRL Press, Oxford, pp. 197–218.

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