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From homothally to heterothally: Mating preferences and genetic variation within clones of the dinoflagellate *Gymnodinium catenatum*

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ABSTRACT

The chain-forming dinoflagellate *Gymnodinium catenatum* Graham is responsible for outbreaks of paralytic shellfish poisoning (PSP), a human health threat in coastal waters. Sexuality in this species is of great importance in its bloom dynamics, and has been shown to be very complex but lacks an explanation. For this reason, we tested if unreported homothallic behavior and rapid genetic changes may clarify the sexual system of this alga. To achieve this objective, 12 clonal strains collected from the Spanish coast were analyzed for the presence of sexual reproduction. Mating affinity results, self-compatibility studies, and genetic fingerprinting (amplified fragment length polymorphism, AFLP) analysis on clonal strains, showed three facts not previously described for this species: (i) That there is a continuous mating system within *G. catenatum*, with either self-compatible strains (homothallic), or strains that needed to be outcrossed (heterothallic), and with a range of differences in cyst production among the crosses. (ii) There was intraclonal genetic variation, i.e. genetic variation within an asexual lineage. Moreover, the variability among homothallic clones was smaller than among the heterothallic ones. (iii) Sibling strains (the two strains established by the germination of one cyst) increased their intra- and inter-sexual compatibility with time. To summarize, we have found that *G. catenatum*'s sexual system is much more complex than previously described, including complex homothallic/heterothallic behaviors. Additionally, high rates of genetic variability may arise in clonal strains, although explanations for the mechanisms responsible are still lacking.

1. Introduction

The ecology of bloom-forming alga, such as dinoflagellates, has been widely studied in order to understand, predict, and hopefully mitigate toxic blooms. A crucial phase in the development of blooms is the bloom termination phase. Dinoflagellate blooms typically end when dinoflagellates cells encyst to form resting stages and sediment out of the water column. Encystment, in turn, is tightly coupled to sexual reproduction (Taylor, 1980). Hence, the triggers, steps, and the control of sexual reproduction are of great interest for the understanding of harmful algal bloom ecology.

In dinoflagellates, sexual reproduction (in the laboratory) can occur either through self-fertilization (i.e. fusion of genetically identical gametes) or by outcrossing (fusing of gametes from genetically different strains). These processes are referred to as homothallism and heterothallism, respectively. In dinoflagellates (with the excepcion of Noctiluca spp.), the planktonic or "adult" life form is haploid.

In some dinoflagellate species, the diploid zygote develops into a resting cyst stage, which is resistant against extreme environmental changes, and undergoes a period of dormancy (Pfiester and Anderson, 1987). In the laboratory, cyst production occurs within a clone in homothallic reproduction, whereas two different clonal strains are required for cyst formation in heterothallic reproduction. Following dormancy, cysts are induced to germinate, a process which is often triggered by temperature (Anderson, 1980; Anderson and Rengefors, 2006). From one zygotic resting stage, typically two genetically different haploid cells with opposite mating types emerge (Walker, 1984). Because of these features, the two strains established by the products of single cyst germination will be able to form zygotes and cysts with each other. Such cultures are called sibling strains.

Homothally is a puzzling behavior, since the advantage of genetic recombination is supposedly lost, and thus, sexual reproduction would seem in no way different from asexual division. However, other advantages of sexuality, such as DNA repair or a

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potentially less harmful effect of mutations in the offspring may be kept in organisms with this type of sexuality (Bernstein et al., 1985; Burt, 2000). In addition, homothallic species have less problem of finding a partner than heterothallic species, which need to find the compatible mating type. Because of all these benefits, homothally is believed to have evolved from heterothally (Goodenough, 1985). Some dinoflagellate species might reflect this evolutionary development, given that both homothallic and heterothallic strains are found in the marine dinoflagellate species *Noctiluca scintillans* (Zingmark, 1970) and *Scrippsiella trochoidea* (Montresor et al., 2003), the freshwater species *Peridinium volzii* (Hayhome et al., 1987) and the chlorophyte *Gonium pectorale* (Fabry et al., 1999). Nevertheless, the extent of homothallic versus heterothallic reproduction in the field is not known.

Traditionally, it is agreed that sexual reproduction is especially beneficial because it allows for continuous adaptation in a changing environment due to genetic recombination (Williams, 1975; Maynard Smith, 1978). Moreover, it allows for DNA repair and avoidance of unfavorable mutations (Bell, 1982). Although sex is an obligatory part of the life cycle of many species, in prokaryotes and many eukaryotes (such as in dinoflagellates), sex is facultative, mainly occurring in response to stress, and often involving the formation of a stress-resistant dormant life stage (von Stosch, 1973; Pfiester, 1975; Dale, 1983). Because both sexual and asexual types of reproduction are possible, some compensatory advantage to sexual reproduction must exist (Williams, 1975).

In the first life-cycle description of the paralytic shellfish poisoning causative species *Gymnodinium catenatum*, a simple heterothallic behavior (+/-) seemed to be enough to explain resting cyst formation (Blackburn et al., 1989). Later, Blackburn et al. (2001) verified that more than two sexual types were possible (multiple heterothallic systems) and that clonal crosses differed in reproductive success (i.e. cyst formation ability, germination rate, and progeny viability). To explain these results, the same authors proposed that the mating type in *G. catenatum* might be regulated by a multiple loci system with several alleles. However, many facts remained unexplained; such as the complexity of the mating system, the high number of negative crosses for resting cyst presence, the observation of gamete mating and fusion in cultures where no cysts were formed, or the change in the sexual compatibility behavior of some strains with time. Although not previously considered, the variability recorded in these phenotypic traits might be explained by changes in the genotype of the strains. In fungi, organisms with a sexual behavior comparable to *G. catenatum* (Blackburn et al., 2001), a connection between intraclonal genetic variation and changes in the sexual behavior have been observed (Eden et al., 2001).

In order to further discuss sexual behavior and intraclonal variation, the definition of the term clone must be scrutinized. A clone can be defined as a population obtained from the asexual division of a single organism. This is the terminology we are going to follow in this paper, although the term clone is often employed as synonymous of a strict genetic fidelity between the asexual offspring and the original founder. In fact, the veracity of this last definition is quite improbable, given that the existence of genetic variability within clones might be commonplace in some asexual systems (Lushai and Loxdale, 2002). Because clones are genetically unstable, they will adapt and evolve in accordance with environmental selection.

Since one aim of this study is to explain the phenotypic differences recorded between parallel clones of *G. catenatum* we needed to choose an appropriate molecular marker. Testing clonality based on genotypic identity depends on the genetic resolving power of the molecular marker used. The relatively rapid divergence rates of the internal transcriber spacer (ITS) region, makes this marker a useful tool for inferring genetic relationships at the intraspecific level of many microorganisms (e.g., Kooistra et al., 2001; LaJeunesse, 2001). However, *G. catenatum* strains have very similar, often identical, ITS sequences (Adachi et al., 1997; Bolch et al., 1999), as is also the case in the raphidophyte *Heterosigma akashiwo* (Connell, 2000), and several cold-water dinoflagellate species (Logares et al., 2007, 2008). Allozyme analysis of strains of *G. catenatum* from Japan, Spain, Portugal and Australia revealed very limited polymorphism (Bolch et al., 1999). Several studies have shown that where allozyme variation is small, random amplified polymorphic DNA (RAPD) analysis can provide fine-scale information (Fabry et al., 1999; Baillie et al., 2000), and thus, this analysis should provide higher resolution than other methods to examine the closest intraspecific relationships. In *G. catenatum*, all strains analyzed by RAPD had distinct genotypes (Bolch et al., 1999; Ordás et al., 2004). However, although RAPD analyses have been extensively used in the past, reproducibility has been claimed to be one of its major drawbacks. Amplified fragment length polymorphism (AFLP) technique, on the other hand, produces profiles that have shown extremely high reproducibility in plants (Jones et al., 1997), animals (Bensch and Åkesson, 2005), and microbes (Duim et al., 2000). John et al. (2004) first applied the AFLP technique for phylogenetic analysis of a marine dinoflagellate using the species *Alexandrium tamarense*, and showed that although it can provide useful information at the population level, the amount of variation was too high to compare strains from the same geographic clade. Later, it has been applied to studies of sexuality in the dinoflagellate *Gymnodinium nolleri* (Figueroa et al., 2006a), the raphidophyte *Gonyostomum semen* (Figueroa and Rengefors, 2006), and to distinguish two recently diverged dinoflagellate species (Logares et al., 2007).

The objective of this study was to clarify both the complex sexual behavior of *G. catenatum* and of the phenotypic variability in reproductive success observed among homothallic and heterothallic clonal strains. Thus, experiments on sexual reproduction were conducted, aimed at studying self- and intersexual compatibility among clonal and sibling strains. We also report on the application of AFLP analyses to identify intraclonal genetic variation, although how this variation arises and its possible implications need to be studied further.

2. Methods

2.1. Cultures

Clonal strains of *G. catenatum* isolated from single cells from the Spanish Atlantic coast were used in this study (Table 1). All isolates were grown at 20 °C, approx. 90 μmol photons m^{-2} s^{-1},

Table 1
Isolation and history of the *Gymnodinium catenatum* strains.

Strain name	Source	Year of isolation	Culture history	Clonal
VGO 22AM	Cádiz (Spain)	1999	Chain	Yes
VGO 27AM	Cádiz (Spain)	1999	Chain	Yes
VGO 29AM	Cádiz (Spain)	1999	Chain	Yes
VGO 38AM	Cádiz (Spain)	1999	Chain	Yes
VGO 42AM	Cádiz (Spain)	1999	Chain	Yes
VGO 49AM	Cádiz (Spain)	1999	Chain	Yes
VGO 51AM	Cádiz (Spain)	1999	Chain	Yes
VGO 53AM	Cádiz (Spain)	1999	Chain	Yes
VGO 55AM	Cádiz (Spain)	1999	Chain	Yes
VGO 56AM	Cádiz (Spain)	1999	Chain	Yes
VGO D7V	Ría de Vigo (Spain)	1993	Chain	Yes
VGO G9V	Ría de Vigo (Spain)	1986	Chain	Yes

using a 10:14 h light:dark (L:D) cycle. Culture stocks were grown in Erlenmeyer vessels filled with 50 ml of modified Atlantic seawater-based L1 medium (Guillard and Hardgraves, 1993). Modification included absence of additional silica, and reduction of salinity to 31 by the addition of sterile double-distilled water.

2.2. Cyst formation

Intercrosses and intracrosses (self-crosses) among all strains were conducted in duplicate sterile polystyrene Petri dishes (Iwaki, Japan, 16 mm diam) filled with 3 ml of L1 medium with a 1:30 dilution of phosphates (P/30) and inoculated with exponentially growing cells (4000–6000 cells ml^{-1}) to a final concentration of 700 cells ml^{-1} (350 cells ml^{-1} from each strain). Light intensity, temperature, and L:D cycle was the same as for stock cultures. Cyst counts were performed at days 10, 15, 20 and 25 after crossing and the highest cyst production was recorded for each cross. All cysts in each well were counted. Negative results were verified in a duplicate cross. The average and standard deviation of cyst production between duplicates were calculated and the results scored according to the criteria shown in Table 2.

2.3. Cyst germination

Twenty cysts from each cross were individually isolated to sterile polystyrene Petri dishes (Iwaki, Japan, 6.4 mm diam) and checked for excystment every 2 days for a period of 30 days. Light intensity, temperature, and L:D cycle was the same as for stock cultures. Excystment was defined as the complete emergence of the protoplast from the cyst even if the germling remained non-motile (Anderson and Wall, 1978). The "minimum dormancy period" for each cross was defined as the minimum time from cyst formation to germination for any individual cyst. The number of germinated cysts divided by the number of germinated and non-germinated cysts was used to determine the germination percentage of cysts isolated on a given date. The viability of the germinated cells was tested every two days and progeny dividing beyond the eight-cell stage were considered viable, since this represents two successful rounds of mitotic division following meiosis (Blackburn et al., 1989).

2.4. Sexual behavior: sibling strains

A non-clonal strain obtained by the germination of a unique cyst was named as "sibling strain". Given that it is not well studied yet if genetic segregation in *G. catenatum* is at the division of the planomeiocyte (first meiotic division) or if there are two meiotic divisions, two or four different haplotypes could be present in each sibling. For the study of sexual behavior, the siblings were grown and maintained as described previously for culture maintenance. Intercrosses and backcrosses with the parental strains were performed as described above within 4 months after cyst germination, and the crosses were repeated one year and a half after cyst germination.

Table 2
Scoring criteria for cyst production in *Gymnodinium catenatum* and equivalent concentration (based on Blackburn et al., 2001).

Score	Cyst concentration (cysts L^{-1})
0	$0-3.0\times10^2$
1	$>3.0\times10^2-2.0\times10^3$
2	$>2.0\times10^3-1.0\times10^4$
3	$>1.0\times10^4-1.0\times10^5$
4	$>1.0\times10^5$

2.5. Homothallic strains

For the study of homothallism, five new single-cell isolations were made in 2002, from each of the clonal homothallic strains 38AM and 55AM, as well as from the clonal heterothallic strain 53AM (Table 1). The 15 new clonal strains were grown and maintained as described previously. Intracrosses were performed as described above within 4 months after cell isolation.

2.6. Reproductive compatibility

Three indices of reproductive success (Blackburn et al., 2001) were calculated from the data obtained in the studied crosses:

- *Compatibility index (CIs)*: The number of compatible pairings resulting in a score ≥ 1 divided by the total number of possible crosses other than self-crosses.
- *Average vigour (AVs)*: The average of the scores (0–5) (see Table 2 for ranges) for maximum cyst production for all successful crosses involving a particular strain.
- *Reproductive compatibility (RCs)*: This was calculated as the product of the CI and AV values.

2.7. Genetic analysis

Strains: DNA was extracted within 4 months after cyst germination from the clonal strains 53AM, 38AM and 55AM as well as from the 15 new clonal strains established from each of 38AM, 53AM and 55AM) (Table 1), within 4 months after the establishment of the clone.

DNA extraction: Cultures were harvested in exponential phase of growth by centrifugation (3000*g* for 10 min at 4 °C), and total DNA was extracted according to the method described previously by Bolch et al. (1999) and stored at −80°C.

AFLP analysis: For the AFLP analysis we followed standard protocols modified from Vos et al. (1995). Ten μL of the extracted DNA (10 ng/μL) from each sample was first digested with 2.5 units of EcoRI and TruI in a total volume of 20 μL for 1 h at 37 °C. Ligation to the ends of the DNA fragments was made by adding T-4 ligase (0.5 units per sample) and adaptors at a concentration of 0.01 μM for the E-adaptor and at 0.1 μM for the M-adaptor. Duplicate DNA templates were made for all cultures. The preamplification reaction was performed using a DNA Thermal Cycler (Perkin Elmer Applied Biosystems 9600), and carried out using 20 cycles (94 °C, 30 s; 56 °C, 30 s; 72 °C, 60 s). Following the preamplification step, the product was diluted (10 ×) with water and 2.5 μL were used for selective amplification. Six primer combinations were employed: 1: EcoRI-TCG and MseI-CGC; 2: EcoRI-TCT and MseI-CGA; 3: EcoRI-CGG and MseI-CGG; 4: EcoRI-TGA and MseI:CGG; 5: EcoRI-TCG and MseI-CGA; 6: EcoRI-TGA and MseI-CGA. The reaction mix contained 10 μL preamplified product, 1.8 μL of water, 0.4 units of Taq DNA pol., 4 μL 1 mM dNTPs, 0.06 μL E-primer (100μ), 0.06 μL M-primer (100μ), 2 μL $MgCl_2$ (25 mM) and 2 μCR buffer (10 ×). Amplification by touch-down PCR was performed with an initial denaturation at 94 °C for 2 min. and a first cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s. During the next 12 cycles the annealing temperature was reduced by 0.7 °C per cycle down to 56 °C, whereas the last 23 cycles were the same as described for preamplification.

The selective amplification was stopped by adding 10 μL of formamide dye (100% formamide, 10 mM EDTA, 0.1% xylene cyanol ff, 0.1% bromophenol blue) to the samples , which were stored at +4 °C over night before running on the gel. After three min denaturation at 95 °C, 3.5-μL samples were loaded onto a 6%

polyacrylamide gel. The fragments were separated at 30 W during 1–2 h, and detected by fluorescein labelled E-primers in a FlourImager (Vistra Fluorescens, molecular Dynamics Inc., Sunnyvale, California). Results were stored as TIFF files for further processing.

2.8. Data analysis

Each individual was amplified at least twice; reproducible, polymorphic bands (80 loci) were scored as 1 (band present) or 0 (band absent). The percentage of similarity was calculated with the Dice similarity coefficient (Dice, 1945). The relatedness coefficients between individuals (after Lynch and Milligan, 1994) were calculated based on 1000 random permutations of individuals using the software AFLP-SURV 1.0 (A program for genetic diversity analysis with AFLP population data, Vekemans (2002)). An UPGMA dendrogram representing the relatedness of the three groups and individuals was calculated using the software Treecon for windows (1.3b) (Van de Peer, 2001).

3. Results

3.1. Resting cyst formation

The results of the intercrossing experiments between the clonal strains of *G. catenatum* are shown in Table 3. Heterothallism (outbreeding) was most common, but 4 of the 12 strains were homothallic (self-cyst producers). However, the homothallic strains showed a considerable overall diversity in the degree of cyst formation, with the strains 55AM and G9 V producing higher cyst numbers than the strains 27AM and 38AM. The variation in compatibility among strains was studied using the reproductive compatibility indices shown in Table 4. High values of CIs in all strains except 22AM and 42AM were indicative of the large number of successful crosses that were possible among them. On the other hand, AV values, mainly below two, provided evidence of the typically low cyst concentrations produced in the compatible crosses.

3.2. Resting cyst germination

The period of minimum dormancy had a mean length of 8.8 ± 2.9 days and did not show significant (ANOVA, at $p < 0.05$) differences among the crosses studied. Cyst progeny was in general highly viable, even in the cases of homothallic origin (Table 5). However, the homothallic strain G9V was unusual, as only 10% of the cysts germinated and no single case of successful planomeiocyte division was recorded (0% of viability).

Table 3
Cyst production (Cysts ml^{-1}) for all possible crosses of *Gymnodinium catenatum*. (Strains names are abbreviated, see Table 1 for complete names).

	22	27	29	38	42	49	51	53	56	D7	G9	55
22	0	4	1	2	0	1	0	0	0	0	–	–
27	4	**0–1**	2	2	0	1	3	4	1	0	–	–
29	1	2	0	2	0	3	0	4	2	1	–	–
38	2	2	2	**1**	1	0	1	2	1	0	–	–
42	0	0	0	1	0	1	0	2	0	1	–	–
49	1	1	3	0	1	0	1	1	0	0	–	–
51	0	3	0	1	0	1	0	2	2	0	–	–
53	0	4	4	2	2	1	2	0	0	1	–	–
55	–	–	–	–	–	–	–	–	–	–	–	**3**
56	0	1	2	1	0	0	2	0	0	0	–	–
D7	0	2	1	2	1	0	0	1	0	0	–	–
G9	–	–	–	–	–	–	–	–	–		**3**	–

Values obtained in homothallic strains are indicated in "bold". Intercrosses were not performed in the highly homothallic strains 55AM and G9 V. See Table 2 for the scoring criteria for cyst production.

Table 4
Reproductive compatibility of each *Gymnodinium catenatum* strain measured by compatibility index (CIs), average vigour (AVs) and reproductive compatibility (RCs).

Strain	CIs	AVs	RCs
VGO 22AM	0.30	1.30	0.39
VGO 27AM	0.70	2.25	1.60
VGO 29AM	0.70	2.14	1.50
VGO 38AM	0.80	1.50	1.20
VGO 42AM	0.30	1.25	0.38
VGO 49AM	0.60	1.30	0.80
VGO 51AM	0.50	1.80	0.90
VGO 53AM	0.70	3.40	2.40
VGO 56AM	0.40	1.50	0.60
VGO D7V	0.50	1.40	0.70
Average	0.55	1.78	1.04

Homothallic strains were not analyzed.

Table 5
Viability of the F1 progeny in *Gymnodinium catenatum* (Strains names are abbreviated, see Table 1 for complete names).

	22	27	29	38*	42	49	51	53	56	D7	G9*	55*
22	–	4	–	–	–	4	–	–	–	–	–	–
27	4	**4**	4	–	–	2	4	4	4	–	–	–
29	4	4	–	–	4	4	–	3	3	4	–	–
38*	–	–	–	**4**	–	–	–	–	–	–	–	–
42	–	–	4	–	–	1	–	4	–	4	–	–
49	4	2	4	–	1	–	1	4	–	–	–	–
51	–	4	–	–	–	1	–	3	3	–	–	–
53	–	4	3	–	4	4	3	–	–	4	–	–
56	–	4	3	–	–	–	3	–	–	–	–	–
D7	–	4	4	–	4	–	–	4	–	–	–	–
G9*	–	–	–	–	–	–	–	–	–	–	**0**	–
55*	–	–	–	–	–	–	–	–	–	–	–	**4**

Scoring criteria: 1: 0–25%; 2: 25–50%; 3: 50–75%; 4: 75–100%.
In homothallic strains (*), only cysts from self-cyst production were studied.

3.3. Sexual behavior of sibling strains with respect to age of cultures

Sexual reproduction of sibling strains from four parental crosses was studied at two different points in time, i.e. when strains were 4 and 18 months old (Table 6). Three main points summarize the results of this study: (i) With the exception of strain A2, newly-germinated sibling strains were able of self-cyst production. The cysts formed by strain A2 had a germination rate of 50% and a longer dormancy period than average (12.1 ± 3.7 days) (ANOVA, at $p < 0.05$). (ii) Sibling strains were typically able to produce cysts with only one of the parental strains (44% of the siblings). However, one strain was compatible with both parents (D2), while 4 sibling strains (B1, C1, C2 and D1) were not compatible with any of the parental strains. Important differences in the compatibility with the parents were recorded among sibling strains from the same parental cross (B1, B2 and D1, D2). For example, in the cross B:49 × 42, the sibling strain B2 was highly compatible with one of the parents, but the sibling B1 was not compatible with either. No significant differences (ANOVA, at $p < 0.05$) were found in the minimum dormancy period of cysts formed in the backcrosses (sibling strain × parental strains) (8.8 ± 3.5 days), except for those formed in the cross A1 × P2, which had a dormancy period double than that of the average

(16.5 ± 2.9 days). (iii) Sibling strains changed their self-compatibility with time. After 18 months in culture, all strains except one (C4) showed an increase in their self-cyst production capability, from absence of self-compatibility to values of self-cross cyst production between 1 and 3. The compatibility with both parental strains also increased. This feature was observed even in the case of C4, the only strain that remained non self-compatible.

3.4. Sexual behavior of homothallic strains

To study and confirm homothallism, we made 5 new clones from single-cell isolates from each of the homothallic clonal strains 55AM and 38AM (Table 3). All new clones (E1–E5) from strain 55AM were also homothallic (Table 7). In 38AM, two of the clones (F2 and F3) were apparently not homothallic. The number of cysts produced and the postmeiotic viability of germlings varied considerably among the new clonal isolates from strain 55AM. Cyst production varied between 2 (E2) and 4 (E4) (a 100-fold difference), and although the germination ability was high in all cases, viability ranged from less than 50% (E1) to more than 75% (E2, E3 and E5). On the contrary, similar results for germination rate and viability were recorded in the two homothallic clones (F1 and F5) from the strain 38AM.

Table 6
Viability of the F2 progeny in *Gymnodinium catenatum*. (see Table 1 for complete names).

Parental cross	Sibling strain	*T1 A.C.*	× *P1*	× *P2*	Viability	*T2 A.C.*	× *P1*	× *P2*
A: 49 × 29	A1	0	0	0–1	0	2	2	2
	A2	1	–	–	2	–	–	–
B: 49 × 42	B1	0	0	0	–	1	2	2
	B2	0	3	0	2	1	2	0
C: 49 × 51	C1	0	0	0	–	2	2	2
	C2	0	0	0	–	1	2	1
	C3	0	0–1	0	1	3	2	2
	C4	0	0–1	0	0	0	2	1
D: 49 × 53	D1	0	0	0	3	3	2	3
	D2	0	1	0–1	3/3	3	2	3

T1 A.C: Cyst production from auto-compatibility at time 1.
T2A.C: Cyst production from auto-compatibility at time 2.
× *P1*: Cyst production from the backcrossing of the sibling strain with the first parent.
× *P2*: Cyst production from the backcrossing of the sibling strain with the second parent.
See Table 2 for scoring criteria for cyst production.
Scoring criteria for viability: 1: 0–25%; 2: 25–50%; 3: 50–75%; 4: 75–100%.

3.5. Intraclonal variability

The AFLP analysis allowed the identification of several intraclonal differences. However, comparison between duplicates (originating from duplicate but separate DNA extractions) showed 100% similarity, supporting the fidelity of the technique. All clonal strains analyzed showed different AFLP fingerprints (13 examples are shown in Fig. 1) with all primer combinations tested. Furthermore, the genetic similarity between the clones was positively correlated with the homothallic behavior, since the similarity was greater among clonal strains 55AM than among the strains 38AM and 53AM (Fig. 2), which had more heterothallism. The percentages of similarity were 89.4 ± 10.8% for the three strains of 55AM, 68.4 ± 13.6% for the strains 38AM, and 63.5 ± 14.5% for the 53AM clones.

4. Discussion

This study has revealed hitherto unknown complexity of the sexual reproduction in the species *G. catenatum*, including homothally, intraclonal genetic variation, and changes in sexual compatibility with time. These observations suggest that the mating type system of this species is under a complex genetic regulation. Furthermore, this study adds new data to the scarcely studied sexual systems of dinoflagellates. Below we discuss the above summary in detail.

4.1. Intercrossing study

The *G. catenatum* strains used in this study originate from neighboring geographical areas off the Spanish coast and are expected to belong to the same species according to the biological species concept. Thus, the strains should be genetically similar enough in order to be able to reproduce, as some gene flow between the areas/populations is expected. This fact may explain both the generally high number of compatible crosses that are possible for each strain (CIs values, Table 4) and the high postmeiotic viability of the germlings (Table 5). The measures of reproductive success in our crosses (CIS, AVs and RCs in Table 4), were very similar to the values obtained by Blackburn et al. (2001) for inter-population crosses between Portuguese and Spanish strains (0.50, 2.00 and 1.00, respectively).

The average minimum dormancy period of the cysts for all crosses was of 8.8 ± 2.9 days, which implies 5–6 days of minimum dormancy, a figure significantly lower than the minimum 13 days estimated by Blackburn et al. (1989) for *G. catenatum*. However, median dormancies were considerably shorter in crosses involving Spanish strains in a comparative study among Japanese, Tasmanian, Portuguese and Spanish populations made by

Table 7
Cyst production and viability of clonal isolates from 2 homothallic strains.

Omothallic strain	Clonal isolate	Cyst production	Germination	Viability	Minimum dormancy (days) average ± SD
E: 55AM	E1	3	4	2	8.2 ± 1.4
	E2	4	4	4	7.2 ± 1.1
	E3	3	4	4	7.4 ± 1.1
	E4	2	4	3	7.2 ± 1.0
	E5	3	4	4	7.1 ± 1.2
F: 38AM	F1	1	3	3	6.5 ± 1.7
	F2	0	–	–	–
	F3	0	–	–	–
	F4	0–1	–	–	–
	F5	1	3	3	6.3 ± 1.0

Scoring criteria for viability and germinability: 1: 0–25%; 2: 25–50%; 3: 50–75%; 4: 75–100%.

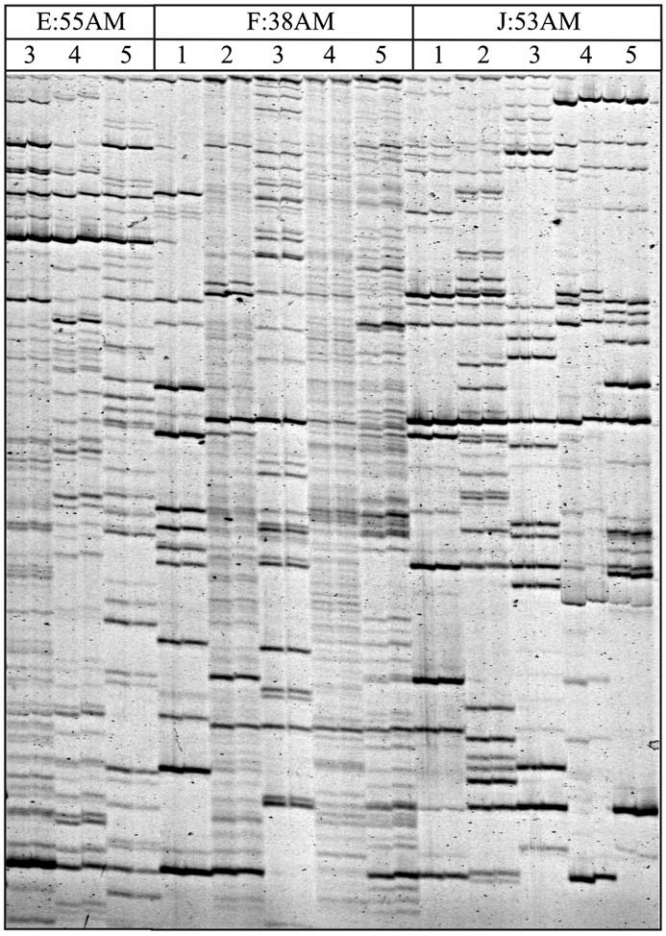

Fig. 1. Duplicate ALFP banding patters produced by the primer combination 1 in clonal isolates, E: 55AM strains, F: 38AM strains and J: 53AM strains.

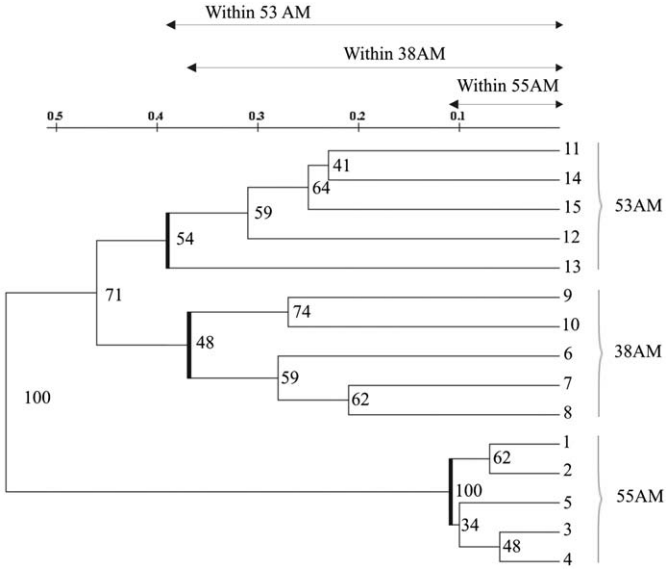

Fig. 2. Dendogram of UPGMA analysis of clonal isolates from strains 53AM, 38AM, and 55AM. Values on the scale bar are unbiased distance using (Nei and Li, 1979). Note that 55AM clones are genetically more similar to each other than the clonal isolates of strains 53AM and 38AM.

Blackburn et al. (2001). Both studies might indicate that genetic factors present in Spanish strains determine a shorter dormancy period than for other populations of this species. However, different experimental factors affecting cyst germination, as for example culture media (GPM versus L1 medium) or temperature (17 °C versus 20 °C) cannot be ruled out. Due to the short dormancy period, benthic cyst stages of *G. catenatum* appear not to play a role in seasonal bloom dynamics (Anderson and Rengefors, 2006), but their major function is to sustain this species through long periods when water column conditions are unfavorable for bloom formation (Hallegraeff, 1998).

4.2. Sexual behavior and genetic variability

The sexual behavior that we observed in *G. catenatum* can be summarized into three points. First, we report the existence of homothallic and heterothallic clonal isolates with different cyst production capabilities. Second, clonal and sibling strains of *G. catenatum* showed a high number of different phenotypes, whose expression, i.e. the production of cysts, changed with time in both self- and out-crosses. Third, the AFLP analyses detected the existence of intraclonal genetic variability, although it was smaller within homothallic isolates. Altogether, these results might be complementary to each other, as discussed below.

The species *G. catenatum* was placed within the complex heterothallic mating type behavior by Blackburn et al. (2001), who described a complex mating behavior. The same authors observed a very low level of homothallism, but did not consider it substantial enough to change the view of this species as being essentially heterothallic. However, we report the existence of a sexual compatibility gradient, which ranges from different degrees of homothallism to complex heterothallism, which in our opinion places *G. catenatum* in a complex homothallic/heterothallic behavior rather than simply heterothallic.

The existence of homothallic and heterothallic strains within a species has previously been recorded in the marine dinoflagellate species *N. scintillans* (Zingmark, 1970) and *S. trochoidea* (Montresor et al., 2003), although cyst production between strains was not compared. Some authors hypothesize that homothallism may have evolved from heterothallism (Goodenough, 1985), and that these species probably are a mid-step in the transition between the two sexual patterns. However, a combination of homo- and heterothallism is not necessarily a transition phase and could be an evolutionary end point.

A surprising result of this study is that our AFLP analyses showed intraclonal genetic variability in *G. catenatum* clonal strains, when we had expected them to be identical. Recent studies suggest that sequence fidelity between clones is improbable (Lushai and Loxdale, 2002), though the detection of genetic variability between organisms might depend either on the genetic resolving power of the molecular markers used (Lushai and Loxdale, 2002) or on the nature of the process that causes the variation measured (Powel et al., 1996). How has this observed variability then been generated? One explanation is extremely high mutation rates and the subsequent appearance of novel AFLP bands in the asexually produced offspring. It is well known that asexually reproducing viruses and prokaryotes mutate rapidly (for example Moran, 1996). In contrast, the eukaryotic clone is still often treated as if it is genetically homogeneous, although that fidelity has rarely been tested empirically (Loxdale and Lushai, 2003). With a generation time of 0.2–0.8 divisions per day, the clones of *G. catenatum* would have been separated for 360–1400 generations (given that the first isolation of the clones was done in 1999). It seems unlikely that so much variation should appear in such a short time. Nevertheless, intraclonal genetic differences were observed after only 5–14 generations in the grain aphid *Sitobion*

avenae F. (Lushai et al., 1997) and in the grape phylloxera *Daktulosphaira vitifoliae* Fitch (Forneck et al., 2001).

The fact that DNA sequence evolution appears to be constant on a scale of absolute time rather than generations has been considered as evidence that many substitutions are slightly deleterious rather than strictly neutral (Moran, 1996). However, the genome of dinoflagellates is peculiar in several aspects, such as enormous size (3000–215,000 Mb) (Spector, 1984), lack of nucleosomes, permanently condensed chromosomes, presence of 5-methylhydroxymuracil which sometimes replaces thymidine, and more (Hackett et al., 2004).

There is a need to investigate possible changes in chromosome number as cultures of dinoflagellates age. For example, Loper et al. (1980) reported polyploidy within cultured *Ptychodiscus brevis* (Davis) Steidinger populations. A polyploid or aneuploid (a chromosome complement that is not an exact multiple of the haploid number) progression in *Peridinium* species was also suggested by Shyam and Sarma (1978). Indeed, Dodge (1963) reported 44 chromosomes for *Peridinium trochoideum* (Stein) Lemm. (Plymouth 104) while 11 years later Fine and Loeblich (1974) reported 80–90 chromosomes for the same cultured strain. Also, Holt and Pfiester (1982) gave evidence for an aneuploid series in cultured *Peridinium* species. Although AFLP patterns do not change with an increment in the number of chromosomes, the mutation rate of both mitosis and meiotic processes does, which in turn may cause changes in the AFLP pattern. On the other hand, processes of chromosome deletion may change the AFLP pattern.

Some peculiarities of the species *G. catenatum* could also be very important in explaining our results. For instance, the chromosome number and DNA content is considered to be unusually high (Figueroa et al., 2006b), high interclonal genetic variation has been found in *G. catenatum* based on RAPD patterns (Ordás et al., 2004), and the life cycle processes are probably not fully described (Figueroa et al., 2008). The latter suggests that the complexity of the *G. catenatum* sexual cycle may include fusion of gametes and subsequent meiosis at a low percentage in some clonal cultures in which no resting cyst are produced (Figueroa et al., 2007, 2006b). Consequently these strains would be considered heterothallic although self-crossing is possible at a gamete or planozygote level. However, we have no data on whether meiosis without cyst formation occurs in the heterothallic strains we have studied. DNA repair during meiosis may explain why the variability detected between homothallic isolates was lower than between heterothallic ones (in which potential meiotic events are likely not happening at an equivalent percentage.

Blackburn et al. (2001) showed that self-mating of *G. catenatum* sibling clones is unusual, and that cultures established from a single resting cyst are seldom self-compatible, which could indicate the existence of partial barriers to this form of outbreeding. Although our results initially supported this conclusion, sibling strains increased their inter- and intra- sexual compatibility with time. Blackburn et al. (2001) further proposed that the *G. catenatum* mating type may be influenced by a multiple-locus system rather than by a single one. This hypothesis could explain the change in compatibility that we observed. Recombination events during meiosis may have generated new genotypes with better compatibility, which in turn have increased in number with each generation, and eventually lead to a change in self- and parental compatibilities.

In the present study, we do not know if two or four different haplotypes are present per sibling. However, if the mating type were controlled by only one locus, the compatibility preferences would unlikely increase with time regardless of the number of haplotypes. In other words, several loci are necessary to explain such a big increase in the self-compatibility of the strains or in their compatibility with the original parental strains. Similar behavior has been reported for the dinoflagellate *Alexandrium tamarense*, whose gametes tended to increase their frequency of self-recognition compared to when the clone was first isolated (Destombe and Cembella, 1990). Nevertheless, planomeiocytes produced as a result of self-fertilization died rapidly in that study. In contrast, in the present study *G. catenatum* planomeiocytes from self-fertilization generally had high levels of viability. The only exception to this pattern was the homothallic clone G9 V. In this strain, the germlings were 100% non-viable while cyst production was high, which might be analogous to syngens found in clorophytes. Coleman (2001) showed that both the genes controlling mating and those active postzygotically might be accumulating differences that ultimately result in elimination of any possible gene flow. However, the multiple-locus theory does not explain the different phenotypes of the parallel clonal isolates nor the enormous differences in cyst production recorded in the sexual crosses, and therefore, a different mechanism should be responsible for these results as well as for the homothallic condition.

The homothallic strains of *G. catenatum* showed a variable response in both cyst production and progeny viability, which was also observed in successive clonations of the same strain. In homothallic systems, every gamete must contain the genetic potential for becoming one mating type (+) as well as the other (−), and subsequently environmental and/or genetic factors determine the phenotypic sex. For example, in *Saccharomyces cervisiae* the homothallic condition is under an allelic control (Harashima et al., 1974; Hicks et al., 1984; Tanaka et al., 1984), and the homothallic cells switch from one mating type allele to the other by transposition of genetic information. This is possible when diploidization by zygote formation is complete, and pre-existing information is removed by the action of the gene products of four unlinked genes (Tanaka et al., 1984). However, this mechanism would not explain the different mating affinities found in the re-clonations of *G. catenatum* homothallic strains observed in the present work. If instead, sexual differentiation is under an epigenetic regulation, the sexual phenotype may result in a continuous distribution of sex phenotypes rather than into two discrete non overlapping classes (+/-).

Epigenetic control of gene expression can occur through several but interconnected mechanisms, such as DNA methylation, RNA-based processes, and interactions between redundant DNA sequences, which can trigger the formation and the transmission of inactive genetic states and DNA modifications (Wolffe and Matzke, 1999). For example, during the sexual cycle of fungi, there is an extensive mutagenesis that generates missense and nonsense codons, a process known as repeat-induced point of mutation (RIP) (Rossignol and Faugeron, 1995). Although these genes behave as epimutant alleles, RIP leads to genetic mutations (Selker, 1997), which can affect the sexual phenotype and genotype (and thus, the AFLP pattern) after many generations of inbreeding (Eden et al., 2001; Kakutani et al., 1996).

Nevertheless, the two possibilities (allelic versus epigenetic control) may be not mutually exclusive, which is corroborated by the fact that homothallism was a characteristic present in only some strains of *G. catenatum*, and that this capacity was maintained by all cells of the homothallic culture. The inheritance of this behavior might indicate that there is an allele responsible for homothallism, and thus, that this condition is probably not acquired in heterothallic clones without outcrossing. An explanation for the sexual behavior of this dinoflagellate might require a multiple loci system with polygenic modifiers permitting a progression of mating response, as suggested for *Chlamydomonas* (Gillham, 1969) and Desmids (Blackburn and Tyler, 1987). This regulation would allow the possible mating types to be

theoretically unlimited, as has also been suggested for protists (Bell, 1982). At this point, however, we don't know whether this is the mechanism behind the complex mating type system in *G. catenatum*.

5. Summary

Our findings can be summarized into two main conclusions:

(i) The mating type system of *G. catenatum* is more complex than previously known and a continuum between homothallism and heterothallism was found.
(ii) The genotype of *G. catenatum* may be prone to change, possibly through mutations in culture. Although both the relevance of this statement in nature and the mechanism responsible of these genetic changes must be further studied, this finding probably reflects some relevant aspect of the dinoflagellate genome.

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