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Spatiotemporal changes in the genetic diversity of harmful algal blooms caused by the toxic dinoflagellate *Alexandrium minutum*

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Abstract

Organisms with sexual and asexual reproductive systems benefit from both types of reproduction. Sexual recombination generates new combinations of alleles, whereas clonality favours the spread of the fittest genotype through the entire population. Therefore, the rate of sexual vs. clonal reproduction has a major influence on the demography and genetic structure of natural populations. We addressed the effect of reproductive system on populations of the dinoflagellate *Alexandrium minutum*. More specifically, we monitored the spatiotemporal genetic diversity during and between bloom events in two estuaries separated by 150 km for two consecutive years. An analysis of population genetic patterns using microsatellite markers revealed surprisingly high genotypic and genetic diversity. Moreover, there was significant spatial and temporal genetic differentiation during and between bloom events. Our results demonstrate that (i) interannual genetic differentiation can be very high, (ii) estuaries are partially isolated during bloom events and (iii) genetic diversity can change rapidly during a bloom event. This rapid genetic change may reflect selective effects that are nevertheless not strong enough to reduce allelic diversity. Thus, sexual reproduction and/or migration may regularly erase any genetic structure produced within estuaries during a bloom event.

Keywords: bloom dynamics, clonality, linkage disequilibrium, population genetics, resting cyst, sexual reproduction

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Introduction

The demography and genetics of natural populations can vary according to the rate of sexual vs. clonal reproduction. These two modes of reproduction occur simultaneously in many eukaryotes. These species can benefit both from the advantage of sexual reproduction, which lies in the genetic recombination that it generates, and from the theoretical short-term demographic advantage of clonal reproduction (Maynard-Smith 1978). The relative contribution of sexual and asexual reproduction can vary within a species depending on environmental conditions (Eckert 2001; Silvertown 2008). For example in aphids, sexual reproduction is associated with the production of resting eggs that can survive harsh conditions, whereas populations living in milder environments may reproduce asexually throughout the whole year (Rispe *et al*. 1998). Sexual reproduction thus seems required to produce a resistant form, regardless of the genetic variability generated. In the freshwater microcrustacean *Daphnia pulicaria* that practices facultative sexual reproduction (alternation between parthenogenesis and sexual reproduction) during the growing season, selection on clonal lines results in an increase in

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genotypic adaptation and an erosion in clonal diversity (Deng & Lynch 1996). The overrepresentation of certain clonal genotypes at the end of the growing season leads to greater genetic structuring and linkage disequilibrium (LD). Similarly, most plankton species alternate between an active growing phase, during which the number of cells increases rapidly, and another phase, generally involving sexual reproduction, during which cells are in a dormant state. During the asexual reproduction phase, fitness differences among clonal lineages may cause rapid changes in genotype frequencies. In the cyclically parthenogenetic rotifer species *Brachionus plicatilis*, there is evidence that clonal selection occurs during the parthenogenetic phase (Gómez & Carvalho 2000). However, sexual reproduction as well as dormant stages may slow down the response to directional selection and may act to maintain genetic variation when selection fluctuates (Hairston *et al.* 1996). Thus, in the freshwater copepod *Diaptomus sanguineus*, the dormant cells may act as a reservoir of genotypes (similar to a seed bank) produced in past environments, but they also may interact as a part of the selection-response dynamics in the current environment (Hairston *et al.* 1996).

An extreme case of alternation of an asexual growing phase and a dormant sexual phase is exemplified by unicellular phytoplankton bloom species involved in harmful algal blooms (HABs). Compared to multicellular organisms, these unicellular organisms are characterized by a ubiquitous distribution, suggesting very large population sizes and broad dispersal abilities (Cloern 1991; Norris 2000; Finlay & Fenchel 2004). Nevertheless, dispersal of marine phytoplankton is a hotly debated issue, and published results are subject to controversies. Studies on marine phytoplankton dispersal differ vastly with respect to study scale, sampling strategy (e.g. sampling few individuals per global geographical region; population samples) and the resolution of the characters or markers used (e.g. morphological markers; highly variable, microsatellite-type molecular markers). For example, using morphological criteria, Cermeno & Falkowski (2009) argue that the biogeographical distribution of fossil diatom genera is ubiquitous and that these taxa thus have no geographical structure. However, more recent studies using microsatellite markers demonstrate that there is genetic differentiation within a single species, the toxic diatom *Pseudo-nitzschia pungens*, on a very large scale (i.e. samples from the Atlantic and Pacific Oceans; Casteleyn *et al.* 2010). The issue of connectivity between populations remains unresolved because there is little information on the distance that a single phytoplankton cell can cover and because there are only a few studies on how the marine physical environment influences dispersal. Exploring these factors is necessary for a better understanding of the ecology and evolution of plankton organisms (Palumbi 1994). Over the past several years, more and more studies have focused on spatiotemporal differentiation in dinoflagellate populations. For example, in the Gulf of Maine, genetic differentiation was found between two subpopulations investigated during offshore blooms, corresponding to the beginning and the end of a bloom event (which lasts about 2 months), but in different geographical locations (northern and southern, separated by hundreds of kilometre; Erdner *et al.* 2011). Other work (Richlen *et al.* 2012) shows spatiotemporal differentiation in the estuaries of Massachusetts (Nauset Marsh system and the Gulf of Maine) at two sites, but only one site was sampled throughout the whole bloom event (from beginning to end), precluding any comparison between dates of the same bloom at different sites. Whatever their scale, these previous studies demonstrate genetic differentiation between sites and high genetic diversity within a given bloom event. This differentiation of bloom populations and their high genetic diversity leads to questions on the role of the two life cycle phases on population dynamics and the importance of sexual reproduction relative to clonal reproduction.

The dinoflagellate *Alexandrium minutum* (Halim 1960) is one of the main species involved in paralytic shellfish poisoning (PSP) blooms off the coasts of France. This microalga produces saxitoxins that can accumulate along the trophic food web. They produce PSP events that cause cases of human poisoning, typically by ingestion of contaminated shellfish. This species shows an alternation of asexual and sexual phases. The vegetative cell is haploid, and sexual reproduction takes place when the environmental conditions become unfavourable (Figueroa *et al.* 2011). Sexual reproduction is the result of the fusion between two gametes, forming a planozygote (Probert *et al.* 2002). This stage changes into a resting cyst that sinks to the sediment (like seeds in a seed bank; Wyatt & Jenkinson 1997). After a dormancy period of several months (Garcés *et al.* 2004; Figueroa *et al.* 2007), cysts may germinate and give rise to vegetative haploid cells that reproduce asexually in the water column.

The objective of this study was to study the effect of asexual and sexual reproduction on the population genetic structure of this toxic dinoflagellate *A. minutum* on a small scale, both in space and in time. We used microsatellite markers to analyse monoclonal cultures of *A. minutum* isolated from samples taken at two sites located along the French coast of the English Channel, separated by approximately 150 km, for two consecutive annual bloom events and at three different periods during each bloom event. We specifically addressed the following questions: (i) To what degree are different

estuaries within the same geographical region connected? (ii) What role does sexual reproduction play in the dynamics of the bloom populations? (iii) Finally, is there any temporal genetic structuring during bloom events?

Materials and methods

Sampling sites and strain collection

More than 1240 strains of dinoflagellates (different species) were collected in the Penzé estuary (48°37′37.57″N, 3°57′13.17″W) and the Rance estuary (48°31′49.61″N, 1°58′21.81″W, Table 1). These two estuaries are separated by ~150 km (Fig. 1). *Alexandrium minutum* blooms occurred concomitantly in both ecosystems in early summer. In 2010, blooms extend from 17 May to 12 July in the Rance estuary (maximum density of 4.2×10^4 cells/L observed on June 22) and from 2 June to 7 July in the Penzé estuary (maximum density of 3.7×10^5 cells/L observed on 8 June). In 2011, blooms lasted from 11 May to 20 June in Rance (maximum density of 4.3×10^5 cells/L observed on 27 May) and from 1 June to 8 July in Penzé (maximum density of 2.6×10^5 cells/L observed on 23 June).

Plankton samples were collected from these two sites during the developmental phase of the bloom (beginning), during the maintenance phase (middle) and during the decline phase (end of the bloom event) whenever possible in each of two study years (2010 and 2011). All strains were obtained by micropipetting one single cell into fresh medium; 530 of these strains survived and 265 strains of *A. minutum* were genotyped. They were maintained at 19 °C with 70 μE/m^2/s of light in a 12:12 light:dark cycle. The medium F/2 (Marine Water Enrichment Solution; Sigma) was prepared using autoclaved natural seawater from the Penzé estuary. It was collected at least 3 months prior to use and stored in the dark. Strains used in this study (see Table S1, Supporting information) have been deposited at the Roscoff Culture Collection (www.sb-roscoff.fr/Phyto/RCC).

Flow cytometry

The DNA content of each monoclonal strain was checked by flow cytometry on whole *A. minutum* cells fixed in ethanol. A 250 μL aliquot of each culture was centrifuged at 1000 g for 10 min. The supernatant was removed, cells were then resuspended by vortexing in 250 μL of 70% ethanol and kept overnight at 4 °C. Cells were collected by centrifugation at 1000 *g* for 10 min to remove ethanol and then resuspended in 250 μL of PBS. Triton X-100 (0.1% final), RNase A (0.01%) and propidium iodide (PI, 30 μg/mL) were added. Samples were incubated at 37 °C for 30 min before flow cytometry analysis, performed using a FACS Canto II (Becton Dickinson, San Jose, CA, USA) flow cytometer equipped

Table 1 Characteristics of the spatiotemporal sampling. Sample sizes (*N*) are given for strains that were isolated for culturing and for the surviving strains that were genotyped. Codes for samples: PZ for Penzé, RC for Rance; 10 for 2010 and 11 for 2011; A, B, C and D indicate, respectively, the different periods of the blooms: the beginning, the middle 1, the middle 2 and the end of the bloom. Cells were counted in a counting chamber using an inverted microscope (Olympus CKX41)

Site/Year	Sampling date	Sample code	Cells density × 10^3 cells/L	Temperature of water (air temperature min./max.) (°C)	Number of strains isolated	Number of surviving strains (%)	Number of *Alexandrium minutum* strains after screening	Number of strains genotyped
Penzé/2010	2 June	PZ10A	8	14.5 (8.6/22)	82	36 (43.9)	32	24
	30 June	PZ10D	102	19.0 (13.5/24.5)	29	16 (55.2)	09	09
	1 July	PZ10D	63	19.3 (12.2/25.9)	55	30 (54.5)	14	08
Penzé/2011	1 June	PZ11A	31	17.9 (2.7/18.6)	85	55 (64.7)	22	19
	15 June	PZ11B	88	18.0 (12.3/20.5)	93	46 (49.4)	24	14
	17 June	PZ11B	171	15.9 (8.4/16.3)	144	36 (25)	24	15
	22 June	PZ11C	402	16.0 (11.5/18)	119	49 (41.2)	32	24
	11 July	PZ11D	ND	17.5 (9/23.8)	195	30 (15.4)	26	18
Rance/2010	2 June	RC10A	36	16.3 (11.2/17.8)	65	43 (66.2)	43	40
Rance/2011	9 May	RC11A	13	15.4 (9.8/19.7)	45	26 (57.8)	19	15
	23 May	RC11B	168	16.8 (7.4/24.2)	96	55 (55.2)	40	25
	27 May	RC11C	288	17.0 (10.8/15.1)	96	57 (59.3)	38	25
	3 June	RC11D	66	18.5 (13/22.1)	83	22 (26.5)	17	10
	5 June	RC11D	106	18.8 (13.8/14.7)	53	29 (54.7)	24	19
				Total	1240	530 (42.7)	364	265

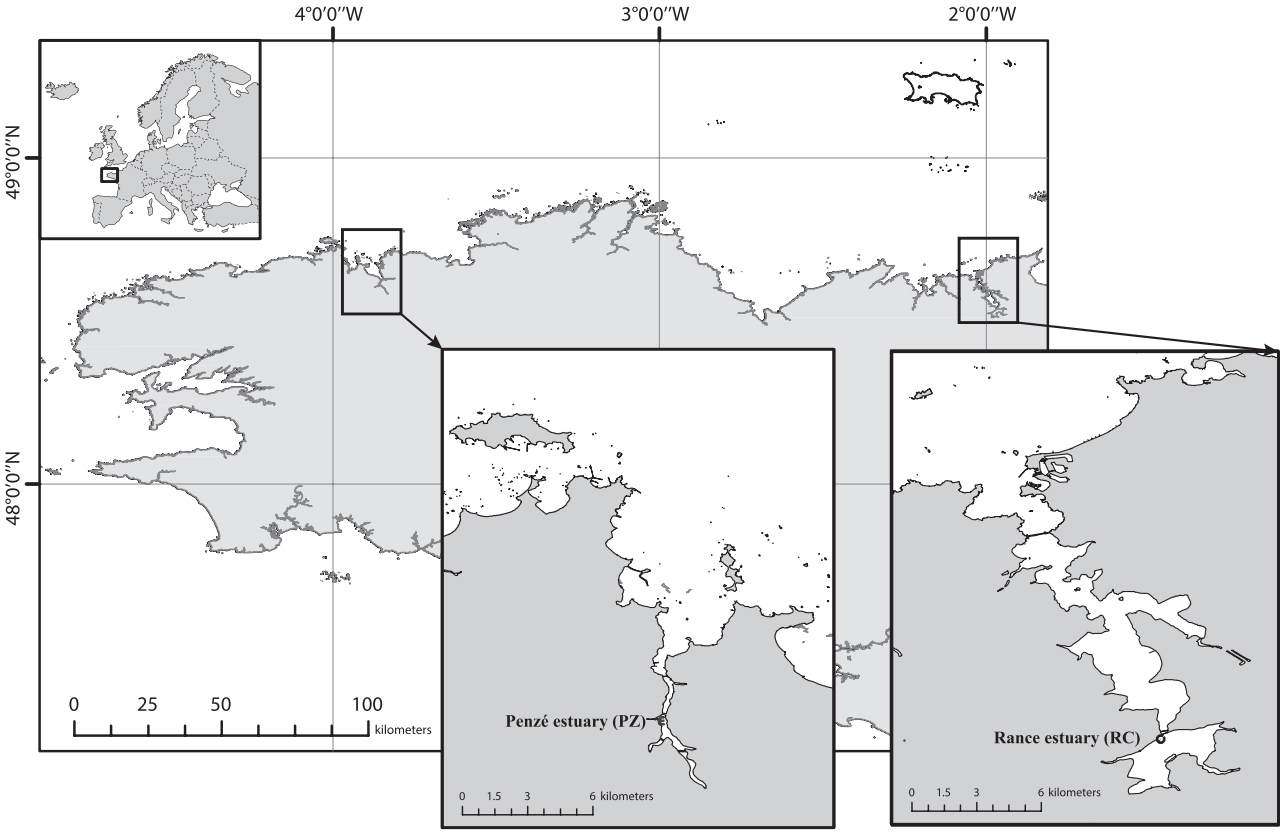

Fig. 1 Maps of the study area showing sampling stations in the two estuaries: Penzé (PZ) and Rance (RC).

with a 488-nm laser and the standard filter set-up. The DNA content of *A. minutum* strains was evaluated by comparison with human blood cells (HBCs, 2C = 6.4 Mbp). Isolated nuclei of HBCs were obtained by resuspending 10 μL of blood in 1 mL of NIB buffer (Marie *et al.* 2001). Then, 10 μL of this suspension was added to 250 μL of *A. minutum* culture resuspended in PBS buffer before staining. Then, all cultures were analysed by flow cytometry without HBC, and mean florescence of PI was compared to check for ploidy level differences.

DNA extraction, strain identification and genotyping

For DNA extraction, strains were collected during their exponential growth stage (strains listed Table 1). DNA was extracted using either the CTAB method (Lebret *et al.* 2012) or the Nucleospin 96 Plant kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions, using 5–10 mg of dried tissue resuspended in 100 μL elution buffer.

Strains were screened using the intergenic region of ribosomal DNA (ITS1, 5.8S and ITS2). PCR mixes (14 μL in total) included 20 ng of DNA, 1× GoTaq Flexi green buffer (Promega, Madison, WI, USA), 150 μM of each dNTP (Thermo Fisher Scientific Inc., Waltham, MA, USA), 20 pmol of the forward primer 329F (5′-GTG AAC CTG CRG AAG GAT CA-3′, complementary reverse of the eukaryote reverse primer 329-R (Moon-van der Staay *et al.* 2001), 20 pmol of the reverse primer DIR-R (5′- TAT GCT TAA AAT TCA GCA GGT-3′ (Scholin & Anderson 1994) and 1.25 U GoTaq® Polymerase (Promega). Amplification was carried out in a DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following cycling conditions: an initial denaturation at 95 °C for 5 min, followed by 35 PCR cycles (denaturation at 95 °C for 45 s, annealing at 53 °C for 45 s and extension at 72 °C for 45 s), followed by a final extension at 72 °C for 10 min. The PCR products were purified using the BigDye ® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) for subsequent bidirectional sequencing using a capillary sequencer type ABI 3130XL (Applied Biosystems).

The strains were genotyped using the 12 microsatellite markers developed by (Nagai *et al.* 2006). Amplification of microsatellite loci was carried out in a 10 μL final volume with the following components: 20 ng DNA, 1× GoTaq® Flexi buffer (Promega), 2 mM $MgCl_2$, 150 μM each dNTP (Thermo Fisher Scientific Inc.),

0.8 μg/μL bovine serum albumin, 30 pmol fluorescent-labelled forward primer (FAM, VIC, NED or PET), 30 pmol reverse primer and 0.35 U GoTaq® Polymerase (Promega). PCR amplifications were carried out in a Bio-Rad DNA Engine Peltier Thermal Cycler using the following cycling conditions: an initial denaturation at 95 °C for 5 min, followed by 10 PCR cycles (denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s) followed by 28 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s) and followed by a final extension at 72 °C for 10 min. Then, 2 μL of PCR products, diluted 1:10, was added to 10 μL of loading buffer containing 0.5 μL of SM594 size standard (Mauger *et al.* 2012) and 9.5 μL of Hi-Di formamide (Applied Biosystems). The loading mix was denatured at 95 °C for 3 min and run in an ABI 3130 XL capillary sequencer (Applied Biosystems) equipped with 50-cm capillaries. Genotypes were scored manually using GENEMAPPER version 4.0 (Applied Biosystems).

Genetic analyses

To increase sample size, close sampling dates were pooled (i.e. PZ10D, PZ11B and RC11D, Table 1) and ultimately only 11 samples with a minimum sample size of 15 strains were used in the genetic analysis (i.e. RC10D was excluded from the analyses; see Table 1).

Genetic diversity and multilocus genotype diversity

Because strains of *A. minutum* are haploid, the frequencies of null alleles were estimated by direct observation. After two reamplifications, strains for which more than half of the loci did not amplify correctly were removed from the data set. The absence of amplification at loci in other strains was considered as a null allele. Standard measures of genetic diversity [mean number of alleles per locus (N_a), Nei's unbiased estimator of genetic diversity (H_s; Nei 1987) and allelic richness (R_a)] for each sampling date and site were calculated using FSTAT (version 2.9.3.2; Goudet 2001). Allelic richness was estimated after randomly subsampling each sample (10 000 randomizations) to standardize the minimum sample size among populations. FSTAT (Goudet 2001) was used to test the difference between the H_s values obtained at the beginning phases (PZ11A, RC10A, RC11A) and at the end phases of bloom events (PZ10D, PZ11D, RC11D). Genclone (Arnaud-Haond & Belkhir 2007) was used to test the efficiency of the microsatellite marker set to discriminate the maximum clonal diversity available in the data set and to calculate the number of distinct multilocus genotypes observed (G).

Linkage disequilibrium

Linkage disequilibrium was assessed using a single multilocus measurement of LD that is provided by the association index $\bar{r}_d$ (Brown *et al.* 1980 modified by Agapow & Burt 2001) and was computed using MULTILOCUS ver 1.2. (Agapow & Burt 2001). Significance tests were based on the comparisons of the observed value to those of randomized data sets generated from 1000 permutations (Burt *et al.* 1996). The global *P*-value was calculated using a generalized binomial procedure to combine independent data (Teriokhin *et al.* 2007; De Meeûs *et al.* 2009).

Genetic differentiation

F_{ST} values were calculated between all pairs of populations using GENETIX 4.05 (Belkhir *et al.* 1996–2004), their significance was assessed using 1000 permutations and D (D_{est}; Jost 2008) was estimated with SMOGD 1.25 (Crawford 2010). Different levels of comparison were thus considered: among dates during a bloom event, among dates within a site and between sites. These data were summarized and statistically tested with a principal component analysis (PCA) performed using PCA-GEN software (version 1.2; Goudet 1999). The statistical significance associated with each axis was evaluated after 10 000 randomizations.

The partitioning of genetic variation at different hierarchical levels was examined using molecular analyses of variance (AMOVA) in ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010). There was only one sampling date during the 2010 bloom in Rance (Table 1); it was therefore only possible to conduct the two following analyses. First, variation was assessed on the 2011 samples by determining the variance component attributable to between-sites differentiation and to among in-bloom phases within a site. Second, variation was examined in more detail only at Penzé by determining the variance component attributable to between-years differentiation and to among in-bloom phases within years. The significance ($P < 0.05$) of the fixation indices was evaluated based on 10 000 permutations.

Results

Isolation and identification of strains

Of the 1240 isolated cells, we obtained 530 usable strains for this study (i.e. 43% success rate). The survival rate by sampling date ranged from 27% to 66%. Of these 530 monoclonal strains, 364 (66%) assigned to *Alexandrium minutum* showing a unique haplotype corresponding to 100% similarity with a 550 bp of the

rDNA sequence (covering ITS1, the 5.8S rRNA gene and ITS2; McCauley *et al.* 2009; Casabianca *et al.* 2011) were selected (Table 1). Indeed, this unique sequence corresponds to an *A. minutum* sequence (AM1 strain) previously published in GenBank (EU707466). The comparison of *A. minutum* DNA content shows that whole cells of *A. minutum* analysed contain the same level of ploidy (32 Mbp, Fig. S1, Supporting information).

Genetic diversity

After reamplifying strains with dubious amplification, five of the 12 loci (Aminu39, Aminu44, Aminu10, Aminu15 and Aminu29) developed by Nagai *et al.* (2006) were removed from the data set. We also removed strains for which more than half of the loci did not amplify correctly. The final data set included seven loci amplified on 265 individual strains (Table S2, Supporting information). For these loci, the frequency of null alleles varied from 8% for locus Aminu11 to 22% for locus Aminu20. The efficiency of the microsatellite loci to discriminate the maximum clonal diversity available in the sample is given in Fig. S2 (Supporting information). Of the 265 strains analysed, 265 (100%) had unique seven-locus genotypes; there were therefore no repeated genotypes in any of the analysed strains.

The PCR products of these seven microsatellite markers showed one single band, that is, one allele. All loci were polymorphic. The genetic diversity indices are given in Table 2. The number of alleles varied among bloom populations, with some populations having twice as many alleles as other populations (cf. RC11A and RC10A, Table 2) and with sampling effort. Allele richness, corrected for sampling size, varied from 6.49 (RC11A) to 9.01 (RC11C). The per-locus and per-population numbers of alleles (N_a) ranged from 3 (locus A41) to 17 (locus A20) alleles. Whatever the sampling date during bloom events, Penzé and Rance showed high genetic diversities, with H_s values ranging from 0.79 to 0.88. These genetic diversity values did not differ between the beginning and the end phases of a bloom event within an estuary (permutation test of comparison among groups; *P*-value = 0.647).

Tests of LD revealed an overall *P*-value of 0.525. Of the 11 spatiotemporal samples, only one showed significant $\bar{r}_d$ (i.e. LD estimator) values. This sample from Penzé (PZ11D) had a *P*-value of 0.047, with $\bar{r}_d$ values of 0.068 (Table 3).

Genetic differentiation

The results of the PCA (Fig. 2) show a significant overall F_{ST} value of 0.073. The first two PCA axes were significant and explained 44% of the variance: axis 1 accounts for 26.39% of the variance (F_{ST} = 0.019, P = 0.001) and axis 2 accounts for 17.65% of the variance (F_{ST} = 0.013, P = 0.011). Axis 1 separates samples from the beginning of the 2010 bloom (PZ10A-RC10A) from the rest of the samples (Fig. 2). Axis 2 separates samples according to the sampling date during a bloom and their geographical origin. Samples from the beginning of the bloom were less genetically differentiated than samples from the end of the bloom events. These last samples formed distinct clusters according to their geographical origin (Fig. 2; cf. PZ11C, PZ11D compared to RC11D and RC11C).

Genetic differentiation between pairs of samples (F_{ST}) ranged from −0.0035 (RC11A vs. RC11B) to 0.0770 (RC10A vs. PZ11C), whereas D_{est} ranged from 0.0589 (RC11B vs. RC11C) to 0.4695 (RC10A vs. PZ11C; Table S3, Supporting information). The lowest F_{ST} values were observed for two samples from the same site in the same year at two consecutive in-bloom sampling dates, whereas the highest values were observed for samples between different sites and different years. The tests of differentiation at different spatial and temporal levels confirm the PCA results. They indicate that the early bloom samples in Penzé and Rance taken in 2010 (PZ10A and RC10A) differed significantly from all other samples (Table S3, Supporting information).

The first AMOVA helped gauge the importance of spatial variation with respect to phase variation within a bloom. This AMOVA could only be applied on the 2011 samples (Table 4a) and revealed that the genetic differentiation was significant between sites and among bloom phases within sites. More specifically, the per cent of variation explained by the genetic differentiation during the different phases of the bloom event was three times higher (3.87%) than the genetic differentiation between the Penzé and Rance sites (1.23%, Table 4a).

The second AMOVA was used to analyse the importance of temporal variation at two hierarchical levels: interannual and intrabloom (Table 4b). This temporal AMOVA could only be applied to the Penzé estuary. The results show again that the two levels of differentiation were significant. The per cent variation explained by genetic differentiation between the different in-bloom periods was weaker (4.27%, Table 4b) than that explained by genetic differentiation between 2010 and 2011 (10.01%, Table 4b).

Discussion

Clonal reproduction vs. sexual reproduction

The reproductive system can play an important role in the functioning and evolution of populations by modifying their dynamics, their diversity and their

Table 2 Genetic diversity estimates in *Alexandrium minutum* at each of the 11 sampling sites and dates and for each of the seven microsatellite loci

Sample (*N*)	PZ10A (24)	PZ10D (17)	PZ11A (19)	PZ11B (29)	PZ11C (24)	PZ11D (18)	RC10A (40)	RC11A (15)	RC11B (25)	RC11C (25)	RC11D (29)
Aminu22											
N	19	15	14	26	23	16	34	12	23	21	27
N_a	4	7	4	9	6	5	9	5	7	8	8
R_a	3.74	6.58	3.85	6.62	5.05	4.60	6.36	4.96	5.63	6.33	5.97
H_s	0.57	0.84	0.57	0.73	0.75	0.68	0.76	0.74	0.63	0.62	0.68
Aminu41											
N	22	16	17	27	24	17	36	12	23	19	24
N_a	10	3	4	7	3	4	10	3	6	10	5
R_a	7.93	3.00	3.68	5.29	2.67	3.82	6.63	2.98	4.74	8.26	4.00
H_s	0.84	0.69	0.64	0.71	0.56	0.68	0.77	0.59	0.63	0.85	0.62
Aminu11											
N	22	16	17	28	22	17	33	14	23	25	28
N_a	12	9	9	12	12	11	14	10	11	14	13
R_a	10.02	8.20	8.54	9.04	9.55	9.82	9.85	9.48	9.34	10.67	10.38
H_s	0.94	0.90	0.93	0.89	0.91	0.94	0.91	0.95	0.93	0.93	0.94
Aminu20											
N	19	14	10	28	19	17	24	11	21	18	23
N_a	10	9	7	17	13	10	9	5	13	13	12
R_a	8.22	8.55	7.00	12.28	11.23	8.68	6.88	4.99	10.68	11.03	9.30
H_s	0.78	0.91	0.91	0.96	0.97	0.84	0.79	0.84	0.95	0.95	0.89
Aminu43											
N	19	16	18	24	23	14	28	14	22	19	20
N_a	13	10	13	12	13	8	15	8	14	9	10
R_a	10.92	9.17	10.90	9.84	10.05	7.63	10.90	7.70	10.96	8.00	8.99
H_s	0.95	0.93	0.94	0.93	0.92	0.89	0.93	0.91	0.94	0.89	0.93
Aminu48											
N	17	15	19	25	23	18	37	15	20	25	27
N_a	11	12	11	10	8	7	14	5	8	10	9
R_a	9.82	10.96	9.18	7.71	6.69	6.36	8.74	4.78	6.73	7.74	6.54
H_s	0.94	0.96	0.87	0.83	0.83	0.78	0.85	0.64	0.80	0.85	0.68
Aminu08											
N	22	15	17	27	23	14	28	13	23	24	28
N_a	9	10	11	13	10	11	13	11	13	15	11
R_a	7.38	9.36	9.82	9.18	8.22	10.33	9.67	10.58	9.90	11.07	7.91
H_e	0.83	0.94	0.94	0.87	0.88	0.96	0.91	0.97	0.88	0.93	0.83
Mean overs loci											
N_a (SE)	**9.86**	**8.57**	**8.43**	**11.43**	**9.29**	**8.00**	**12.00**	**6.71**	**10.29**	**11.29**	**9.71**
	0.30	0.34	0.42	0.28	0.41	0.35	0.22	0.44	0.32	0.24	0.28
R_a (SE)	**8.29**	**7.97**	**7.57**	**8.56**	**7.64**	**7.32**	**8.43**	**6.49**	**8.28**	**9.01**	**7.58**
	0.29	0.32	0.38	0.27	0.40	0.34	0.21	0.43	0.31	0.21	0.29
H_s (SE)	**0.84**	**0.88**	**0.83**	**0.85**	**0.83**	**0.82**	**0.85**	**0.81**	**0.82**	**0.86**	**0.79**
	0.16	0.11	0.19	0.11	0.17	0.14	0.09	0.19	0.17	0.13	0.17

N, number of individuals genotyped in each sample; N_a, number of alleles per locus; R_a, expected allelic richness based on the smallest sample size (10); H_s, Nei's gene diversity. Mean values are in bold.

Table 3 Multilocus measurements of linkage disequilibrium using $\bar{r}_d$ (Agapow & Burt 2001)

Sample (*N*)	PZ10A (24)	PZ10D (17)	PZ11A (19)	PZ11B (29)	PZ11C (24)	PZ11D (18)	RC10A (40)	RC11A (15)	RC11B (25)	RC11C (25)	RC11D (29)
$\bar{r}_d$	−0.008	0.068	−0.034	−0.017	0.037	0.005	0.003	0.027	−0.037	−0.049	0.009
P-value	0.566	0.047	0.870	0.730	0.063	0.416	0.423	0.240	0.902	0.971	0.348

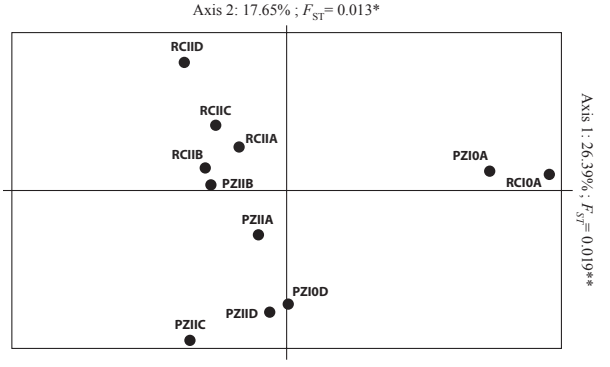

Fig. 2 PCA of genetic differentiation between samples taken from the Penzé and Rance estuaries in 2010 and 2011.

population genetic structure. As in many other phytoplankton species, the dinoflagellate species *A. minutum* is characterized by high rates of clonal reproduction during the development of bloom events, followed by a period of sexual reproduction that produces resting cysts, coinciding with the maximum and the decline of cell concentrations, respectively (Garcés *et al.* 2004; Bravo *et al.* 2010). During periods of asexual division, differences in selective values between clonal lines can rapidly lead to differences in genotype frequencies. Numerical simulations show that selective sweeps can occur when rapidly growing clones become more abundant within a population (Rynearson & Armbrust 2000). During a bloom event, advantageous genotypes may increase in frequency, leading to an overall decrease in genetic diversity via genetic hitchhiking over the whole genome (Maynard-Smith *et al.* 1993). Even in the absence of selection, low clonal diversity and linkage equilibrium provide evidence that sexual reproduction occurs in a population with clonal reproduction. At the end of a bloom, the most frequent clones would thus preferentially participate in the production of the following generation via sexual reproduction. However, in rapidly growing organisms with short generation times, the huge population sizes can be considered as virtually infinite and genotypic diversity can thus be maintained during the bloom events, even if organisms primarily reproduce clonally (Bengtsson 2003).

In this study, all the analysed clones were genetically distinct, suggesting that bloom events involve huge effective population sizes. The high genetic diversity observed here indicates that asexual reproduction does not predominate within *A. minutum* populations. It, however, does suggest that blooms were initiated by numerous different sexual cysts. Our results corroborate the recent observations reported for *Alexandrium fundyense* showing unique multilocus genotypic frequencies of 81–97% (Erdner *et al.* 2011; Richlen *et al.* 2012). Likewise, a study on *Alexandrium tamarense* has shown that the probability of sampling clones with the same genotype is practically nil due to the large population sizes of blooms (Nagai *et al.* 2007). The high genotypic diversity values as well as the high gene diversity values observed in our study ($0.78 < H_s < 0.87$) are comparable to those of Mediterranean populations of the same species

Table 4 Spatial (a) and temporal (b) analyses of bloom events

Source of variation	d.f.	Sum of squares	Variance component	Percentage variation (%)	*P*-value
(a) Spatial AMOVA: Variation among bloom phases between the Penzé and Rance sites sampled in 2011 (F_{ST} = 0.05. $P < 10^{-5}$)					
Between the Penzé and Rance sites sampled in 2011	1	13.91	0.04	1.23	0.008
Among bloom phases within site	6	44.51	0.12	3.87	0.002
Within bloom phase	318	896.08	2.82	94.88	
Total		954.49	2.97		
(b) Temporal AMOVA: Variation among bloom phases between years 2010 and 2011 at Penzé site (F_{ST} = 0.14. $P < 10^{-5}$)					
Between years 2010 and 2011 in Penzé	1	41.24	0.33	10.01	$<10^{-5}$
Among bloom phases within year	4	33.04	0.14	4.27	$<10^{-5}$
Within bloom phase	226	648.12	2.86	85. 72	
Total		722.41	3.34		

($0.62 < H_s < 0.89$; Casabianca *et al.* 2011). There are reports of similar high genotypic diversity values in other phytoplankton species, such as the haptophyte (*Prymnesium parvum*) and the Raphidophyceae species (*Gonyostomum semen*) using AFLP genetic markers (Barreto *et al.* 2011; Lebret *et al.* 2012).

The continuous germination of resting cysts during the entire bloom event (Lebret *et al.* 2012), as well as the specific cohort of these cysts produced during successive annual blooms and stored in the cyst bank, may enhance genetic diversity (in terms of genotypes and genes; Alpermann *et al.* 2009). Furthermore, we cannot exclude the possibility that sexual reproduction maintains genetic diversity, even during a bloom event. Observations in *in vitro* cultures suggest that the zygote (planozygote) may undergo meiosis without forming a resting cyst (Figueroa *et al.* 2007), thereby producing new vegetative cells. This type of reproduction may be promoted by the presence of the parasite *Parvilucifera sinerae* (Alveolata), and short-circuiting the resting cyst stage may possibly constitute a strategy to avoid the parasite (Figueroa *et al.* 2010). Two different species belonging to genus *Parvilucifera* were detected during the 2010 and 2011 sampling cruises (Lepelletier *et al.* 2013). However, unfortunately, there have been no investigations to identify this type of reproduction.

In our study, no global multilocus LD was detected, suggesting that recombination is sufficient to break down linkages between loci, confirming that *A. minutum* reproduces sexually. Only one sample taken during the late bloom period in Penzé in 2011 (PZ11D) showed significant multilocus LD, as estimated using $\bar{r}_d$).

Spatiotemporal genetic structure of bloom events

The results of the microsatellite analysis clearly indicate that *A. minutum* blooms are structured both in space and in time. Our study shows that there is a temporal genetic differentiation during and between *A. minutum* blooms as well as spatial differentiation between sites. Thus, the overall genetic differentiation observed between the Rance and Penzé estuaries separated by approximately 150 km was significant. This result suggests that gene flow via tidal currents is limited in *A. minutum*, as demonstrated in *A. tamarense* (Nagai *et al.* 2007). Given the short duration of *A. minutum* bloom events (30 days on average) and that they are geographically restricted to estuaries, the gene flow between populations is probably limited. Genetic differences observed between the two estuaries late bloom samples confirm the genetic isolation between estuaries during the bloom period. However, early bloom samples showed weaker spatial differentiation (Table 4). This observation suggests that the Penzé and Rance estuaries communicate during spring tides, potentially transporting vegetative cells or cysts via the residual west–east tidal currents. During the bloom season, which preferentially occurs during neap tides, the two estuaries are probably isolated and the accumulation of bloom biomass is mainly due to a response to water flow and to constant, suitable environmental conditions (i.e. salinity temperature, irradiance and nutrient supply; Anderson *et al.* 2012). In our study, interannual genetic differentiation was greater than intrabloom differentiation, suggesting that blooms are not only made up of newly formed cysts produced during the preceding year, but also of recruitment from migrant vegetative cells and/or from an older cyst bank (Estrada *et al.* 2010). Even if a multigenerational cyst bank gives rise to a bloom event, the germination of cysts may be influenced by environment conditions and, by chance or selection, samples become more differentiated than the more homogeneous cyst bank that produced them (McCue & Holtsford 1998). The constant germination of the cyst bank may thus lead to a decrease in the interannual F_{ST}. Nevertheless, the cyst bank does not appear to contribute regularly to bloom events because the 2010 Penzé sample PZ10D was not genetically different from the early 2011 Penzé bloom sample (PZ11A). Therefore, the 2011 early bloom sample may have been composed at least partly of cysts produced in the preceding year.

In isolated estuaries, there were significant genetic changes during a bloom event (see AMOVA, Table 4a). Although no additional signatures of selection, such as multilocus LD, could be detected with the neutral markers used in this study, we believe that, in these very large populations, genetic changes may nevertheless be driven by selection (see below). These genetic changes are concordant with those reported for *A. fundyense* blooms in Massachusetts where temporal intrabloom differentiation has also been observed (Richlen *et al.* 2012). Two factors can account for this temporal differentiation. First, changes may be due to delayed germination of different cohorts of cysts buried in the sediments and/or continuous encystment by a small percentage of motile cells (Pitcher *et al.* 2007). In *G. semen* (Raphidophyceae), Lebret *et al.* (2012) suggest that continuous germination of cysts during a bloom is a key element behind rapid genetic changes. Second, these changes may be the result of local abiotic or biotic selection as suggested for the diatom *Ditylum brightwellii* (Rynearson & Armbrust 2005; Rynearson *et al.* 2006). In these large populations, the effects of natural selection may predominate over those of genetic drift on genetic structure. Thus, in *D. brightwellii*, the succession of genetically distinct populations in different environmental conditions appears to reflect a tight coupling between environment and genetics (Rynearson *et al.* 2006). Of the various abiotic factors, seasonal temperature variation is one

parameter that can explain the maintenance of genetic diversity (Maynard-Smith & Hoekstra 1980; Bell 1982). Recent studies on the diatom *Asterionella formosa* have demonstrated that variation in temperature may contribute to maintaining genetic diversity in blooms (Gsell *et al.* 2012). In this species, reaction norms of clone growth studied as a function of environmental temperature reveal considerable genetic diversity within a bloom. In our study, fluctuating temperatures may explain not only the maintenance of genetic diversity, but also genetic changes during a bloom event. However, biotic factors, such as predation and/or parasitism, may also play a role in determining population structure and dynamics (Montagnes *et al.* 2008). In *A. minutum*, eukaryotic parasites such as *Parvilucifera* spp. and *Amoebophrya* spp. can regulate blooms (Chambouvet *et al.* 2008; Figueroa *et al.* 2010; Lepelletier *et al.* 2013). The impact of parasitism can be tested by analysing the fitness of different strains and their resistance to parasites according to date and sampling site. Whatever the factors involved, our study illustrates how sexual reproduction and/or migration are likely to erase any selective effects that arise each year during the vegetative phase of a bloom event.

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A.D. conceived the study, performed isolation and culture of strains, molecular data, analysis and drafted the manuscript, S.M. contributed to molecular analyses; E.B. performed isolation and culture of strains, D.M. performed cytometry analyses, M.V. participated in the design of the study, performed some statistical analysis and revised the manuscript, L.G. and C.D. coordinated the study, helped draft and revised the manuscript. All authors contributed to sample collection, read and approved the final manuscript.

Data accessibility

Data used in this study (Table S1, Supporting information) have been deposited at the Roscoff Culture Collection (www.sb-roscoff.fr/Phyto/RCC), and sequences data (Table S4, Supporting information) have been deposited in GenBank.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Roscoff Culture Collection (RCC) number, origin, population code and date of isolation of strains used in this study.

Table S2 Microsatellite data amplified on 265 individual strains at seven loci.

Table S3 Pairwise differentiation between the 11 populations of *Alexandrium minutum*.

Table S4 List of sequences (ITS1, 5.8S and ITS2) of *A. minutum* strains with their GenBank Accession nos.

Fig. S1 Quantification of DNA in *Alexandrium minutum* cultures in comparison with nuclei isolated from human blood cells (HBC, 6.4 Mbp), after staining with propidium iodide in PBS buffer.

Fig. S2 Resolving power of the microsatellite marker set to discriminate the maximum clonal diversity available in the data set.