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Mendelian Inheritance Pattern and High Mutation Rates of Microsatellite Alleles in the Diatom *Pseudo-nitzschia multistriata*

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The diatom *Pseudo-nitzschia multistriata* exhibits a diplontic life cycle composed of an extensive phase of vegetative cell division and a brief phase of sexual reproduction. To explore genotypic stability, we genotyped seven polymorphic microsatellite loci in 26 monoclonal strains over 3–16 months in a culture maintenance regime. Moreover, to assess inheritance patterns of the microsatellite alleles, we genotyped 246 F1 strains resulting from four mating experiments between parental strains of know genotype. Results generally conformed expectations according to Mendelian inheritance patterns, but deviations were detected indicating mutations during sexual reproduction. A total of forty-two mutations were detected in the clonal cultures over time. Microsatellites with more core-repeats accumulated mutations faster. The mutation rate varied significantly across loci and strains. A binomial mass function and a computer simulation showed that the mutation rate was significantly higher during the first months of culture ($\mu \approx 3 \times 10^{-3}$ per locus per cell division) and decreased to $\mu \approx 1 \times 10^{-3}$ in the strains kept for 16 months. Our results suggest that genetic mutations acquired in both the vegetative phase and sexual reproduction add to the allelic diversity of microsatellites, and hence to the genotypic variation present in a natural population.

Key words: Asexual and sexual reproduction; diatom; Mendelian inheritance; microsatellites; mutation accumulation lines; mutation rate.

Introduction

Microsatellites are DNA sequences composed of a core of one to six nucleotides that are highly repeated in tandem (Li et al. 2002). Such sequences exhibit high mutation rates, which have been estimated to be as high as 10^{-3} and 10^{-4}/locus/generation in humans (e.g. Brinkmann et al. 1998; Hohoff et al. 2007), with changes following a nearly step-wise mutation model, i.e. addition/deletion of one or more core repeats (Hancock 1999; Kruglyak et al. 1998; Ouborg et al. 1999; Seyfert et al. 2008).

In recent years, microsatellite markers have been utilized to assess the genetic structure of populations of marine diatoms over different spatial (e.g. Casteleyn et al. 2010; Evans et al. 2005)

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and temporal (Härnström et al. 2011; Rynearson and Armbrust 2005; Rynearson et al. 2006) scales. High genetic and genotypic diversity was found in all species (e.g. Evans et al. 2005; Rynearson and Armbrust 2004). In natural populations of the centric diatom *Ditylum brightwellii*, up to 94% of genetically distinct strains were recorded in a sample size of hundreds of strains (Rynearson et al. 2006). Genetic diversity was high also during the peak phase of a bloom when rapid clonal propagation is supposed to occur (Rynearson and Armbrust 2005).

In unicellular organisms, such as diatoms, mitotic division prevails, but sexual reproduction occurs as well and has profound consequences for their population dynamics and genetic profile (Halkett et al. 2005). In such organisms, genetic changes can occur through the acquisition of somatic and meiotic mutations as well as due to genetic remixing during the sexual phase. During clonal propagation, the genetic variation in the gene pool may reach equilibrium between gains due to adaptive mutations, gene flow and selectively neutral mutations and losses due to random genetic drift (De Meester et al. 2006; Vanoverbeke and De Meester 2010). Genetic erosion and selection pressure play a role in the decrease of genotypic diversity. To restore high genotypic diversity, sexual reproduction is needed to induce genetic segregation and genetic remixing (Bengtsson 2003, Vanoverbeke and De Meester 2010). Hence, genotypic variation is enhanced principally during sexual reproduction.

Microsatellite alleles inherit following Mendelian rules, but departures from Mendelian segregation are known to exist (e.g. Dobrowolski and Tommerup 2002; Reece et al. 2004). Such deviations can be due to the presence of null alleles, or to non-disjunction at meiosis. In populations of diatoms with an extensive phase of vegetative cell division, deviations from Hardy-Weinberg equilibrium can also occur because the population is not panmictic (i.e. it is not randomly mating). Consequently, algorithms that estimate the null allele frequency cannot be employed, unless the rate of inbreeding (or selfing) is known (van Oosterhout et al. 2006), or when analysing a generation that arises from sexual reproduction.

Other sources of microsatellite genetic variation in natural populations are new alleles, which are generated through random mutations during both the vegetative phase and the sexual phase of the life cycle and can spread among populations through gene flow. It is unclear, however, which of these two phases is the principal source of novel microsatellite alleles. Even in higher plants, a group that has been well-studied using population genetic approaches, information on the frequency of microsatellite mutations during vegetative growth is rare (Cloutier et al. 2003; Douhovnikoff and Dodd 2003; O'Connell and Ritland 2004).

We developed seven microsatellite markers to study temporal changes in the population genetic structure of the planktonic diatom *Pseudo-nitzschia multistriata* (Takano) Takano (Tesson et al. 2011). The species can be enticed to reproduce sexually under controlled laboratory conditions, which means that the microsatellite mutation frequency over the sexual phase of the life cycle can be analysed by genotyping its F1-offspring. The species is heterothallic, i.e. only strains of opposite mating types can engage in sexual reproduction (D'Alelio et al. 2009a). In *P. multistriata*, sex can be induced when cells have become shorter than 55 μm. The initial F1 cells are distinguishable from their parents because they are markedly longer, namely between 82 and 72 μm (D'Alelio et al. 2009a). An additional way to distinguish cells of the parental strains from the F1 cells is by means of their internal transcribed spacer (ITS) sequence type. The population of *P. multistriata* in our study area, the Gulf of Naples, exhibits two markedly distinct types of ITS sequences and recombinants thereof (ITS rDNA, D'Alelio et al. 2009b). Individual cells and monoclonal laboratory strains generated from single cells possess either one sequence type, or the other, or a mix of both. Cells interbreed freely, irrespective of their ITS sequence types. This implies that the F1 cells resulting from a cross between parents with different ITS sequence types must exhibit both ITS sequence types.

To assess the microsatellite mutation frequency over the vegetative phase of the diatom's life cycle, we screened 26 monoclonal strains over various time intervals (3 – 16 months, ca. 45-240 vegetative divisions). We built a computer model that simulates mutation accumulation in our culturing protocol, with which we estimated the mutation rate (expressed as mutations per division) required to explain the observed number of mutations accumulated in our strains.

The results acquired from these various approaches provide insights in the inheritance mode of microsatellite markers in heterothallic diatoms. Furthermore, by tracking microsatellite patterns of clones over the vegetative part of their life cycle, and by assessing the patterns from one generation into the next, we are able to assess the relative importance of mutations acquired during the vegetative versus the sexual phase of the diatom life cycle. This study provides novel

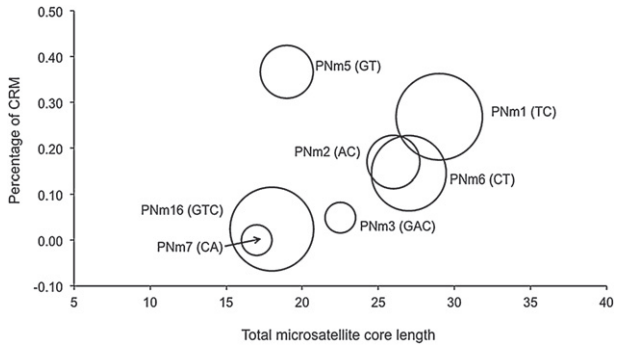

Figure 1. Percentage of core repeat mutations (CRMs) as a function of the microsatellite core length (in base pairs). Microsatellite core composition is indicated between brackets. The volume of the circles is proportional to the polymorphism of each locus.

insights into how genetic diversity is generated in microsatellite loci in diatoms, which is relevant for our understanding of microsatellite-based genetic diversity in natural populations as well as for the evaluation of genotypic stability of laboratory cultures.

Results

Genetic Changes in Mitotic Divisions

A total of 22 out of 26 *Pseudo-nitzschia multistriata* strains analysed exhibited differences in one or more of the seven microsatellite loci after different time intervals whereas none of these strains showed variations in their ITS rDNA types. The microsatellite variations, i.e. the core repeat mutations (CRMs), consisted of the addition/deletion of one to three core repeats (Supplementary Table S1). The number of CRMs recorded (i.e. fixed in the culture) for each strain varied between zero and three. In total, we recorded 42 CRMs in the 26 strains: 34 were single CRMs, i.e. only one allele mutated per locus, and eight were double CRMs, i.e. both alleles mutated in a given locus. CRMs were observed in all microsatellites besides *PNm7* (Fig. 1). In one strain three alleles were detected instead of two at *PNm16* (Supplementary Table S1). Loci differed significantly in the mean number of recorded mutations (bootstrap: $p<0.0001$). Significant variation in the number of CRMs was explained by microsatellite size expressed as the number of core repeats (Ordinal Logistic regression: $Z=2.10$, $p=0.035$) (Fig. 1). The analysis showed a mean odds ratio=5.23 (5-95% Confidence Interval (CI): 1.12 - 24.41), indicating that with the addition of each core repeat, the probability of adding a CRM increased by circa a factor 5. Remarkably, neither the repeat unit (di- or trinucleotide) of the locus ($Z=-1.83$, $p=0.068$), nor the time in culture ($Z=0.40$, $p=0.691$) explained significant variation in the number of recorded CRMs. The latter finding is particularly noteworthy, given that the time in culture varied considerably between strains (3 – 16 months), which should have resulted in a larger number of mutations in the oldest strains.

We analysed whether the rate with which mutations were fixed differed significantly between the three time intervals (Experiment A, B and C) in which we subdivided strains, which were kept in culture for an average of 13.7, 6.0 and 4.25 months, respectively. Table 1 shows the results of a binomial mass function. In this analysis, we used the average mutation rate in a particular experiment (expressed as mutations per strain per time in culture) to calculate the cumulative binomial probability of finding the number of mutations in another experiment. The results reject the null hypothesis of an equal mutation rate in 4 out of 6 pairwise comparisons (Experiment A vs. B, B vs. A, A vs. C and C vs. A). Only the mutation rates in Experiments B and C, i.e. strains that were kept in culture for a limited time period of 3 – 6 months, were not significantly different (Table 1). Apparently, the mutation rate during the vegetative phase was high at the start of a culture and then levelled off rapidly. Next, we designed a computer model to estimate the number of mutations accumulated in diatom strains, simulating the population demography (i.e. fluctuations in population size during the experiment and the time in culture) across a range of mutation rates ($10^{-4} \leq \mu \leq 10^{-2.5}$). Results of this analysis showed that a mutation rate of $\mu \approx 3\times10^{-3}$ is required to explain the large number of mutations accumulated in strains kept in culture for only 3 – 4 months (Fig. 2). A mutation rate of $\mu \approx 1\times10^{-3}$ is more representative of the number of mutations accumulated in the strains that have been cultured for the longest time (ca. 16 months).

Inheritance Pattern

In order to estimate the inheritance patterns and to evaluate the genetic variation arising during sexual reproduction, we performed crosses using four pairwise combinations of five parental strains. The parental strains possessed distinct microsatellite multi-locus genotypes (Table 2). The proportions of the various expected F1 genotypes were estimated per locus for each mating experiment, assuming Mendelian inheritance (Table 2). Expected

Table 1. Differences in mutation rate calculated using a binomial mass function.

Experiment	N	Time	Mutations	μ	Hypothesis and p-value	Hypothesis and p-value
A	7	13.71	15	0.1563	P(μA$\geq\mu$B) = 0.00016	P(μA$\geq\mu$C) = 0.01251
B	11	6	17	0.2576	P(μB$\geq\mu$A) = 0.00395	P(μB$\geq\mu$C) = 0.14168
C	8	4.25	11	0.3235	P(μC$\leq\mu$A) = 0.01092	P(μC$\leq\mu$B) = 0.15516

"N" denotes the number of strains in each experiment (A, B and C), "Time" indicates the time (in months) the strains were kept in culture, "Mutations" indicates the number of mutations summed across the strains in each experiment, "μ" stands for the mutation rate calculated by dividing the number of mutations by the total time the strains were kept in culture. The column "Hypothesis and p-value" gives the result of the binomial mass function which tests the hypothesis that the number of mutations observed in one line (A, B or C) is equal to, or less than, the number found in another line for each of the experiments.

proportions generally matched observed ones indicating that (+) and (-) parental strains contributed equally to the formation of the allelic combination of their progeny (Table 2). However, there was a single exception; one of the F1 strains in mating experiment #4 exhibited the same allelic pattern as its (-) parental strain SY379. Nevertheless, it was an F1 strain as shown by its mixed ITS-A/B type and its large cell size (74.6 $\pm$ 1.06 μm).

Several mutations and/or gene conversion events appear to have occurred also during the sexual cycle (indicated by a * in Table 2). In the F1 generation, 91.3% of the examined strains exhibited genotypes predicted by Mendelian inheritance rules (Table 2). The unexpected genotypes at a given locus (i.e. 148 loci out of 1705 tested) possessed new alleles (18 loci) or homozygote excess (3 loci) or deviated from the expected Mendelian patterns at one or more loci (96 loci, $p < 0.05$). The potential presence of a null allele was suggested by Micro-Checker at locus *PNm1*, although the null allele frequency was low (r=0.045, Brookfield1 estimator), (Supplementary Table S2). No evidence of large allele dropout or scoring errors was found in the seven microsatellite loci (Supplementary Table S2).

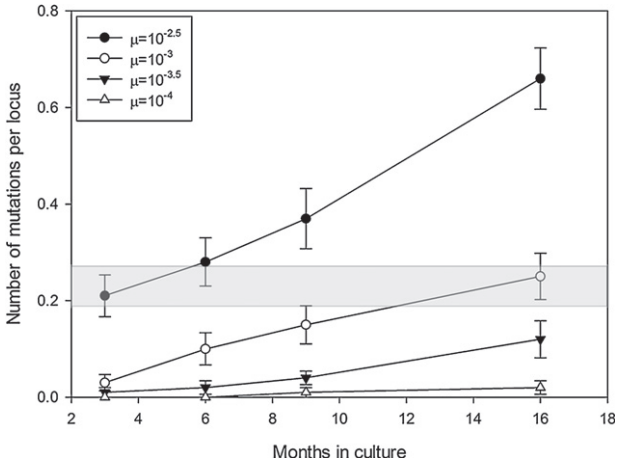

Figure 2. The mean ($\pm$SE) simulated number of mutations per microsatellite locus accumulated over time (in months) for different mutation rates. The mean ($\pm$SE) observed number of accumulated mutations (averaged across loci and strains) is shown in the grey envelope. Most consistent with the empirical data are a mutation rate of $\mu=10^{-2.5}$ ($\mu\approx3\times10^{-3}$) for the strains kept in culture for 3-6 months and a lower mutation rate of $\mu=1\times10^{-3}$ for the strains cultivated for 16 months.

The average genotypic diversity (G/N = number of distinct genotypes over the total number of individuals) in the F1 strains for the four mating experiments was 67.35% (G_o (168)/ N_{F1} (245); Table 3), indicating that identical genotypes were present in the F1 progeny. An unexpectedly low level of genotypic diversity (G/N=43.48%; Table 3) was observed in cross #4. In this cross we observed significantly fewer genotypes (G_o=26) than were expected based on the total number of unique genotypes possible (G_T=48) and the number of samples screened (N_{F1}=66; Table 3). Using a randomisation test with the sample size and the total possible number of genotypes, the expected mean ($\pm$99% CI) number of genotypes (G_E=38.9 ($\pm$31-49)) was considerably larger than the observed number G_o=26 (see Fig. 3). This suggests that the segregation of multi-locus genotypes, at least in this cross, was not strictly random. The genetic diversity generated by the four distinct crosses is visualized in Figure 4. The genotype of each individual has been mapped in a two-dimensional space resulting from a Factorial Correspondence Analysis (FCA).

A subset of F1 strains from the different crosses was characterized for ITS. All the progeny of the mating experiments #2 and #4, which involved parental strains of ITS-A and ITS-B types, exhibited an ITS-A/B type (Table 3), while in experiment #1, three out of 34 strains exhibited an ITS-A type. Mating experiment #3, which involved parental strains

Table 2. Estimation of the inheritance patterns of the seven microsatellite loci in the F1 strains obtained from the four mating experiments.

Locus	MT +	MT -	F1 genotypes	Exp. (%)	Obs. (%)
Cross #1	SY278	SY017			
PNm1	115/127	127/127	115/127:127/127	50:50	0:100
PNm2	175/187	175/175	175/175:175/187	50:50	42:58
PNm3	205/208	205/208	205/205:205/208:208/208	25:50:25	23:54:23
PNm5	238/242	238/242	238/238:238/242:242/242	25:50:25	32:50:18
PNm6	267/271	261/267	261/267:261/271:267/267:267/271	25:25:25:25	25:19:31:25
PNm7	258/262	262/262	258/262:262/262	50:50	61:39
PNm16	325/337	325/337	325/325:325/337:337/337 (319/325/337)*	25:50:25	16:51:30 (3)
Cross #2	SY138	SY017			
PNm1	117/127	127/127	117/127:127/127 (119/127:117/117)*	50:50	45:49 (3:1)
PNm2	175/187	175/175	175/175:175/187	50:50	53:47
PNm3	208/208	205/208	205/208:208/208	50:50	47:53
PNm5	238/238	238/242	238/238:238/242 (238/240:238/248:240/240)*	50:50	55:42 (1:1:1)
PNm6	255/255	261/267	255/261:255/267	50:50	43:57
PNm7	258/258	262/262	258/262	100	100
PNm16	307/337	325/337	307/325:307/337:325/337:337/337	25:25:25:25	27:24:27:22
Cross #3	SY138	SY378			
PNm1	117/127	115/117	115/117:115/127:117/117:117/127 (117/119:119/119)	25:25:25:25	4:23:15:39 (4:15)
PNm2	175/187	175/187	175/175:175/187:187/187	25:50:25	19:69:12
PNm3	208/208	208/208	208/208	100	100
PNm5	238/238	238/238	238/238	100	100
PNm6	255/255	249/249	249/255	100	100
PNm7	258/258	262/262	258/262	100	100
PNm16	307/337	325/337	307/325:307/337:325/337:337/337	25:25:25:25	20:16:48:16
Cross #4	SY138	SY379			
PNm1	117/127	117/127	117/117:117/127:127/127	25:50:25	31:50:19
PNm2	175/187	175/175	175/175:175/187	50:50	37:63
PNm3	208/208	208/208	208/208	100	100
PNm5	238/238	230/238	230/238:238/238	50:50	55:45
PNm6	255/255	267/267	255/267 (267/267)*	100	98 (2)
PNm7	258/258	262/262	258/262 (262/262)*	100	98 (2)
PNm16	307/337	319/325	307/319:307/325:319/337:325/337 (319/325:307/319/337)*	25:25:25:25	24:15:19:39 (1.5:1.5)

For each locus and mating experiment (Cross #1, #2, #3, #4), the genotypes of the parental strains of opposite mating type (MT) are reported in the second and third column. In the subsequent columns, the recorded F1 genotypes, with their expected (Exp.) and observed (Obs.) percentage of occurrence are reported. Underlined numbers indicate a significant ($p < 0.05$) deviation from the Mendelian inheritance expectation. Unexpected allelic combinations and new alleles are indicated by (*) and their percentages are given between brackets.

Table 3. Microsatellite genotypic diversity and ITS-type diversity of the F1 progeny.

#	N_{F1}	G_o	G_T	G/N	P_G	MT −	MT +	$N_{F1\ ITS\text{-}typed}$	$N_{ITS\text{-}A}$	$N_{ITS\text{-}B}$	$N_{ITS\text{-}A/B}$
1	61	54	864	0.89	6.25	A	B	34	3	0	31
2	92	70	128	0.76	54.69	A	B	31	0	0	31
3	26	18	48	0.69	37.50	A/B	B	11	0	2	9
4	66	26	48	0.39	46.43	A	B	45	0	0	45
Tot	245	168						121	3	2	116

The mating experiment (#), the number of F1 initial cells isolated (N_{F1}), the number of observed genotypes (G_o), the theoretical number of possible genotype given the parental genotypes (G_T), the genotypic diversity (G/N), the percentage of recorded over possible number of genotypes (P_G), the ITS-type for each parental strain involved (MT − and MT +), the number of F1 strains for which the ITS-type is known ($N_{F1\ ITS\text{-}typed}$), and the number of strains recorded per ITS type ($N_{ITS\text{-}A\ /\ -B\ /\ -A/B}$), are shown.

of ITS-A/B and ITS-B types, gave rise to two F1 strains of ITS-B type out of the 11 strains analysed in total (Table 3).

The two 'sister cells', CRD1 and CRD2, generated by the same pair of parental cells exhibited the ITS-A/B type, which was as expected given that one parental strains had an ITS-A type and the other one an ITS-B type (Table 4). The microsatellite patterns of the sisters were identical and their allelic combination was in accordance with the hypothesis that they inherited one allele from each parental strain (Table 4).

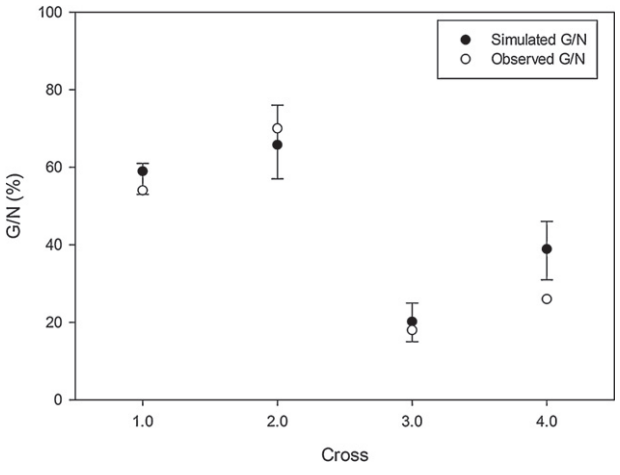

Figure 3. The observed and mean (±1-99%CI) simulated %G/N (number of distinct multi-locus genotypes (G) in a sample of N individuals screened). Note that Cross #4 has significantly fewer distinct genotypes than expected based on the number of screened F1 strains and the total possible number of multi-locus genotypes produced by this cross.

Discussion

The gain of genetic diversity in the diatom *Pseudo-nitzschia multistriata* across different phases of its life cycle was demonstrated in our laboratory experiments. Sexual reproduction generated a plethora of new genotypes as expected based on the parental genotypes and Mendelian inheritance rules. Yet, our results reveal the fixation of several mutations in the clonal cultures, suggesting a high frequency of mutations and/or some form of positive selection. If mutations occur with such a high frequency in the field as well, then they will have a significant impact on the population genetic diversity. Novel mutations occur during cell division and during sexual reproduction induced in the laboratory and are fixed during the extensive phase of vegetative cell division. The computer model shows that the rate at which mutations get fixed in the

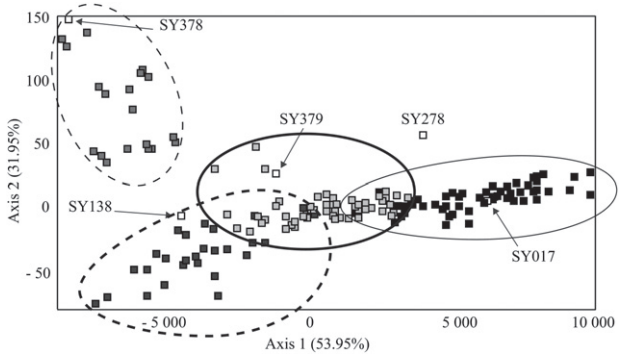

Figure 4. Results of Factorial Correspondence Analysis based on microsatellite allelic frequencies among the 246 F1-offspring obtained in the four crosses between the five parental strains. Clouds represent the F1 genotypes of different crosses (Cross 1 within thin circle, Cross 2 within bold circle, Cross 3 within dashed circle and Cross 4 within dashed bold circle). Parental cells are marked with arrows.

Table 4. Genotypes of the parental (SY017 and SY138) and F1 (CRD1 and CRD2) strains raised from two initial cells produced on the same gametangium ('sister-cell') of *Pseudo-nitzschia multistriata:* their ITS-types (ITS), and the microsatellite pattern over the seven loci.

Strain	ITS	*PNm1*	*PNm2*	*PNm3*	*PNm5*	*PNm6*	*PNm7*	*PNm16*
SY017	A	127 127	175 175	205 208	238 242	261 267	262 262	325 337
SY138	B	115 127	175 187	205 208	238 242	267 271	258 262	325 337
CRD1	A/B	127 127	175 175	205 208	238 242	267 271	258 262	325 337
CRD2	A/B	127 127	175 175	205 208	238 242	267 271	258 262	325 337

strains (and hence, the estimated mutation rate) differs between strains, and that the inferred mutation rate is highest in the newest strains.

Mutations during Mitotic Divisions

Our results reveal that microsatellite genotypes of *P. multistriata* strains change over periods of one to several months of vegetative maintenance in culture. The mutation rate during vegetative reproduction is high, particularly during the initial months of culturing. We estimated it to be about three times higher than that estimated in previous studies in human ($\mu = 10^{-3}$ to 10^{-4}/locus/generation, see e.g. Brinkmann et al. 1998; Ellegren 2000a; Hohoff et al. 2007), about one order of magnitude higher than reported in the lizard *Egernia sp.*, yet it is considerably lower than the rate of germline mutations in various organisms ($\mu = 1 \times 10^{-2}$ to 4×10^{-2} per meiosis, see Ellegren 2000a). Microsatellite DNA sequences show frequent additions and deletions of a core repeat by slippage of the DNA polymerase (Hancock 1999), although larger changes of multiple repeat units appear to be more common in some organisms (Seyfert et al. 2008). Changes can also arise from heteroduplex repair, which increases or decreases locus length in heterozygous conditions. The microsatellite mutation frequencies observed in *P. multistriata* corroborate observations by earlier workers that longer loci exhibit higher mutation rates (Ellegren 2000b, 2004). However, we did not detect a significant difference between di- and trinucleotides, possibly due to a lack of statistical power and the small number of loci analysed.

We do not believe that the high fixation rate of mutations in our clonal cultures is related to our culture transfer procedure. Cultures experience a severe bottleneck at each transfer into fresh medium, with only a few dozens of cells inoculated every 3–4 weeks. There is only a small probability that one or a few of those transferred cells exhibits a mutation. On the other hand, if one or more of them do, the likelihood that this novel mutation replaces the ancestral allele is increased relative to a culture that is refreshed by sampling many cells. Simulations indicated (data not shown) that the effects of a reduced probability of sampling a mutation versus an increased effect of drift cancel each other out, which indicates that this aspect of the culturing procedure does not affect the observed mutation rate. In rare cases, multiple alleles can be sampled when some cells contain the ancestral state allele and a few others the mutant allele. For example, strain SY411 possessed three alleles at locus *PNm16*. Reusch and Boström (2011) made a similar observation within individual clones of the seagrass *Zostera marina*, where somatic mutations accumulated over time in different parts of the clonal plant.

Remarkably, the number of core-repeat mutations that are fixed in our strains did not increase linearly with time in culture, but instead, levelled off. Apparently, mutations become fixed especially during the initial months following culture establishment. We believe this trend is real, and not due to noisy data because the 26 strains analysed across a 3 – 16 month period guarantee sufficient statistical power.

A possible explanation for a decrease in the mutation fixation rate with time in culture is that back- or double-mutations erase previous mutations in the longer established strains. The initially high fixation rate could be a response to changes in the environment when strains are transferred from the field into culture condition. We do not believe that the elevated mutation rate found during asexual reproduction is due to a selective sweep or genetic hitchhiking per se. Each microsatellite variant, being a mutant or ancestral allele, will benefit equally from such a selective sweep. Rather, in the absence of sex and recombination, the adaptive evolution in an isolated culture can only occur through novel mutations. Given that the culture environment differs dramatically from the natural environment, it is likely that some mutations that would be neutral or even detrimental in the wild are beneficial under lab conditions (Lakeman et al. 2009). We propose that in our study, lineages

with a relatively elevated mutation rate proliferated because they accumulated novel beneficial mutations (some of which are adaptive in the novel laboratory environment) more rapidly than lineages with a lower mutation rate. This resulted in the relatively high microsatellite mutation rate recorded, which was particularly enhanced in the first few months after culture establishment.

Inheritance Patterns and Diversity Across Sexual Reproduction

Results of the observed microsatellite inheritance patterns in parents and their F1-offspring (Table 2) confirm the occurrence of heterothallic sexual reproduction in *Pseudo-nitzschia multistriata* (D'Alelio et al. 2009a) and reveal that, by and large, microsatellite inheritance patterns follow Mendelian rules. This result provides the 'genetic proof' for the occurrence of sexual recombination in diatoms (Schurko et al. 2009).

Significant deviations from Mendelian inheritance patterns were recorded in two microsatellites (*PNm1* #1 and *PNm16* #4). The recorded homozygote excess in *PNm1* may be explained by the presence of null alleles (Callen et al. 1993; Selkoe and Toonen 2006), or possibly gene conversion (Amos 2009; Duret and Galtier 2009). In a few cases, we also recorded the occurrence of new alleles, probably due to slippage of the DNA polymerase.

The term 'null allele' refers to an allele that cannot be visualized because of a mutation in one of the priming sites. In our dataset, the presence of a null allele may explain the excess of homozygotes at locus *PNm1*, cross #1 (Table 2). This null allele could be due to a mutation in the priming site of the 115 allele of *PNm1*. Consistent with this interpretation is the low frequency of a null allele (r=0.045) detected at this locus by Micro-Checker. Alternatively, the 115-allele may also have mutated to a 127 allele, although we think this is less likely because the addition of so many repeat units in a single event was not observed elsewhere. An alternative explanation for the observed homozygote excess is the occurrence of gene conversion, when one allele converts the alternative allele to its own state. In many loci, gene conversion appears to be more common than mutations, but it can only be rarely detected unambiguously (see e.g. Spurgin et al. 2011). We cannot exclude the possibility that gene conversion has converted the 115 to a 127 allele. In three other cases, an excess of homozygotes may be explained by gene conversion as well (e.g. Table 2, #2 *PNm1* and #4, *PNm6* and *PNm7*).

Mating experiments showed that the genetic diversity generated by sexual reproduction is vast, even with just four crosses. Given large enough numbers of offspring resulting from a crossing experiment between two parental strains, the F1 cells will exhibit all the genotype combinations possible given the parental genotypes and Mendelian inheritance of their alleles.

The situation in the field differs, of course, radically from that in a crossing experiment with only two parental genotypes. In the natural environment the microsatellite variation is much larger than in our experimental design, and sexual reproduction occurs randomly between any parental pair of the right mating type. Therefore, this system will maintain a huge genotypic diversity, given the biennial frequency of sexual reproduction events in *P. multistriata* inferred from cell size patterns recorded in the natural environment (D'Alelio et al. 2010). Thus, in case two years of on-going clonal reproduction combined with natural selection or random drift reduces the genotypic diversity of a population, then recombination through sexual reproduction boosts genotypic diversity again.

Interestingly, in one of the crosses (#4), the genotypic variation in the F1 was significantly lower than expected based on the total possible number of distinct multi-locus genotypes and the number of individuals screened. In that cross, the number of F1 genotypes recorded among these strains was about two times lower than the maximum number of expected genotypes (i.e. 26 observed versus 48). This suggests that the segregation of alleles was not strictly random, or that selection operated against (and/or favoured) certain genotypes. Selection could have taken place during the one week of growth in the culture plates, or during the 1-2 weeks that each of the F1 cultures were transferred to medium and incubated. Not all the picked cells survived into strains but the mortality rate did not differ from that in crossing experiments #1 and 2. Mortality was markedly higher in the F1 of cross #3 but here genotypic diversity was as expected.

At which Stage during Meiosis does Degeneration of Nuclei Occur?

As in other pennate diatoms (Chepurnov et al. 2004), each sexualized parental cell (called gametangium) of *P. multistriata* generates only two haploid gametes, which conjugate with their counterparts generated by the gametangium of the opposite mating type. The resulting pair of 'true sister cells' can be identified as such because the auxospores from which they emerge have

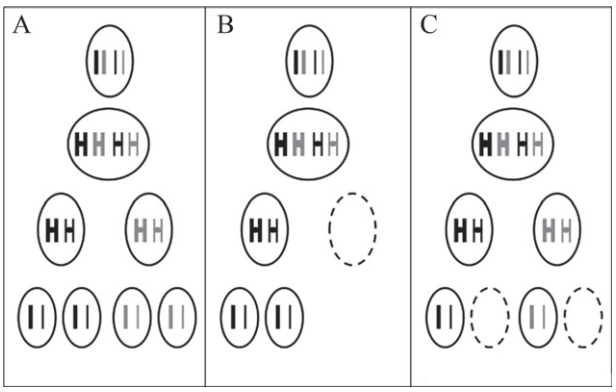

Figure 5. Schematic drawings illustrating gamete formation. Two chromosomes (one bold and the other thin) are represented, with one chromatid in black and its homologue in grey. Duplication of the genetic content (2nd row), first meiotic division (3rd row) and second meiotic division (4th row). Formation of four gametes, the rule in most Eukaryotes (**A**); one nucleus degenerates during the first meiotic division and two identical gametes are produced (**B**); two nuclei degenerate during the second meiotic division and two different gametes are produced (**C**).

developed side by side on the same gametangium (D'Alelio et al. 2009a).

The genotypic makeup of the resulting "true sister cells" may reveal the relative importance of crossing-over events in reshuffling the genetic information on the homologous chromosomes before the first meiotic division because the effect of crossing-over can be spotted in the genotypes of sister cells from the same gametangium. In addition, the genotypic makeup of these sisters may uncover how meiotic divisions proceed and at which meiotic division nuclei degenerate. The fact that only two gametes are produced per gametangium can be achieved in one of two ways (Round et al. 1990): i) one of the two nuclei degenerates following the first meiotic division (Fig. 5B; Hypothesis 1). In this case, the expectation is that, at least in the absence of crossing-over, the two gametes share the same alleles and that the two sister cells resulting from conjugation of these two gametes with the gametes of the partner gametangium are genotypically identical; ii) any combination of two of the four haploid nuclei degenerates after the second meiotic division (Fig. 5C; Hypothesis 2). In this case the expectation is that, again, in the absence of crossing-over, the pairs of gametes are genotypically identical in 50% of cases, and hence, the two sister cells resulting from conjugation of these two gametes with the gametes of the partner gametangium are genotypically identical in 25% of cases. Chepurnov et al. (2005) observed in *Pseudo-nitzschia pungens* that two nuclei are formed after meiosis I and that two of the four nuclei, formed by karyokinesis after meiosis II, are aborted before gamete formation (sensu Fig. 5C Hypothesis 2).

The fact that the two 'sister strains' in *P. multistriata* share an identical microsatellite fingerprint suggests that crossing over does not occur frequently or that the microsatellite loci are situated close enough to the centromeres of their chromosomes to have a low probability of being exchanged between sister chromosomes by means of crossing over. However, our results do not favour Hypothesis 1 above Hypothesis 2 because our result - genotypic identity of the daughter cells - is based on a single observation, meaning that a posteriori, the genotypic identity could be the one out of four cases predicted by Hypothesis 2.

Conclusions and Perspectives

We demonstrate that microsatellite markers within *P. multistriata* are inherited generally according to Mendelian rules, and that rare deviations are due likely to gene conversion and step-wise mutations. The occurrence of 'somatic' mutations in microsatellite regions in monoclonal cultures suggests that the microsatellite genotypic diversity observed in natural populations may result, at least in part, from mutations during the vegetative part of the life cycle. This rate at which these mutations are fixed is particularly high in the initial months of culturing when the diatoms cope with a dramatic change in their environment.

Methods

Culture isolation and maintenance: Twenty-seven strains of *Pseudo-nitzschia multistriata* were isolated from net samples collected at the Long Term Ecological Research station MareChiara (40°48.5′N, 14°15′E) in the Gulf of Naples (Supplementary Table S1). Individual cells or chains were isolated under an inverted light microscope using a micro-glass pipette and cleaned by sequential washing through several droplets of f/2 medium (Guillard 1975). Each cell or chain was placed in a separate well of a 12-well culture plate (Costar® CLS3513, Corning Incorporated, NY, USA) containing 2 ml f/2 sterile-filtered medium and grown under optimal conditions until enough biomass was obtained for DNA extraction. Backup cultures were grown at a low division rate (ca 0.5 division.day^{-1}) at 15 °C, under a sinusoidal light regime comprised between 40 to 80 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes, a 12 h:12 h light:dark photocycle and under constant orbital agitation (70 rpm; SSL1, International PBI S.p.A., Milan, Italy). Every month, culture plates were examined, cell density

was estimated semi-quantitatively and a volume of a few μL, to include ca. 80 cells, was transferred into new medium.

Accumulation of mutations in monoclonal cultures: Twenty-six strains were used to assess the genotype changes and the mutation rate in culture (see Supplementary Table S1). To obtain material for DNA extraction, a small volume of culture was inoculated in a culture flask (Corning® 430639, Corning Incorporated, NY, USA) containing 25 mL of f/2 medium and grown until the required biomass was obtained. DNA extractions were performed as described in Tesson et al. (2011) and were carried out at different time intervals, spanning from 3 to 16 months (Supplementary Table S1).

Mating experiments: Five strains, three of the (-) mating type and two of the (+) mating type (Table 2), were used to carry out four mating experiments to analyse the allelic inheritance of microsatellite and ITS markers in the F1 generation. Mating types (− and +) were assigned based on the results of pilot experiments in which strains of different cell size were crossed in pair-wise combinations: the strain bearing the auxospores was defined as the (-) mating type. The average cell size of the parental strains ranged between 46.8 μm and 25 μm in length. Each cross was repeated at least sixty times, inoculating about 5,000 cells ml^{-1} of each exponentially growing parental strain in culture plate wells filled with 4 ml of filtered f/2 medium. Culture plates were incubated at 18 °C, 12 h:12 h light:dark photocycle and an irradiance of 80-90 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes. A few days after the start of the experiment, all the four crosses generated numerous auxospores and large-size F1-cells, spanning between 80 and 68 μm in length. Two to four F1 initial cells or post-initial cells (i.e. long cells that arose from vegetative division) were randomly isolated from each well, in order to avoid resampling bias. The isolation was performed using an inverted light microscope and drawn-out glass Pasteur pipettes. Each F1 cell isolated was transferred into a new well of a 12-well culture plate (Costar® CLS3513, Corning Incorporated, NY, USA) and incubated at the same conditions as indicated above. Among the 1247 F1-cells isolated, 298 strains survived among which 246 were genotyped for ITS rDNA and microsatellite sequences. Among them, two cultures were obtained by isolating a pair of 'true sister cells', that is, the two cells resulting from conjugation of each one of the two gametes generated by the two gametangia of opposite mating type (CRD1 and CRD2). After one week of growth in the culture plates, each F1 monoclonal culture was transferred in 30 ml f/2 medium and incubated for about 1-2 weeks before DNA extraction.

ITS (Internal Transcribed Spacer) - rDNA analysis: The ITS region of the nuclear rDNA (*ITS-1*, *5.8S* rDNA, and *ITS-2*) of cultured cells, F1 and parental strains was PCR-amplified. Amplifications were performed in 96-wells plates in a 25 μl reaction volume containing 25 pmol of each primer (ITS-1 as forward primer, 5′-TCCGTAGGTGAACCTGCGG-3′, (White et al. 1990) and a new reverse primer named ITS-4b, 5′-TCCTCCGCTTAATTATATGC-3′), 1 mM Tris-HCl, 0.15 mM $MgCl_2$, 5 mM KCl, pH 8.3 20 °C, 0.2 mM dNTP, 6 U Taq polymerase, 1.10 mg of DMSO, about 20 ng of DNA, and 14 μL H_2O. An initial denaturation step of 2 min at 96 °C, was followed by 40 cycles (1 min at 94 °C, 1 min at 46 °C and 2 min at 72 °C) and by a final extension step of 5 min at 72 °C. PCR products were purified and sequenced as in D'Alelio et al. (2009b). The software BioEdit (version 7.0.9.0) was used to infer ITS sequences from the electropherograms. The ITS A-type, B-type and A/B-type were attributed as described by (D'Alelio et al. 2009b).

Microsatellite analysis: Microsatellite loci were amplified and screened as described in Tesson et al. (2011). Allele designation was performed with the CEQ™2000XL (version 4.3.9, Beckman Coulter™) software. Homogeneity of the resulting data set and scoring errors were assessed with MicroChecker (version 2.2.3, van Oosterhout et al. 2004). Genotypic diversity was obtained with Gimlet (version 1.3.3, Valière 2002).

Statistical analyses: Z-tests and the Pearson Chi-square tests (Chi^2) were used to compare observed versus the predicted proportions of genotypes assuming Mendelian inheritance (Frontier et al. 2007; Zar 1999). In order to examine which factors explained significant variation in mutation accumulation, we used an Ordinal Logistic Regression with the number of mutations per microsatellite locus per strain being response variable, and the core repeat unit (di- or tri-nucleotide) as factor. In this model, time in culture (in months) and microsatellite core length (in bp) were used as covariates. In order to obtain maximum-likelihood estimates of all parameters, the model was fitted using an iterative re-weighted least squares algorithm. The log-likelihood was used to test whether the coefficients of the predictors were significantly different from zero. A logit link function was employed to calculate the odds ratio and its 95% confidence interval.

A Monte Carlo procedure was used to simulate the mutation accumulation of microsatellite loci in strains kept in culture for a period of 3 – 16 months. In the computer model, the cultures were started from a monoclonal strain and each month (15 divisions) the diatom population was bottlenecked to N=50 individuals, simulating the refreshing and inoculation of the cultures. Simulations with bottleneck sizes of N=20, 100 and 500 were also run to examine the effect of culture procedure on the observed number of mutations. Subsequently, the population grew exponentially through clonal division, with each individual contributing to the next generation according to a Poisson distribution (Mean = Var = 2). At the end of the experiment, the strain was genotyped by taking a small subsample of 100 diatoms. We assumed that a microsatellite peak needs to have a minimum height (number of allelic copies PCR amplified) to be observed, and hence, the model scored only alleles with relative frequency ($p>0.1$) in this subsample. Microsatellites mutated according to a stepwise mutation model, and the mean and the standard error of the mean (SE) were calculated over 1000 simulations. The code for this model was written for Minitab 12.1 and is available from the authors upon request.

Variation in the mutation rate between time periods (i.e. strains kept in culture for 9-16 months (*ca.* 189-336 vegetative divisions), 6 months (*ca.* 126 vegetative divisions) and 3-5 months (*ca.* 63-105 vegetative divisions), referred to as experiment A, B and C, respectively) was calculated using a binomial mass function. The mean genotypic diversity (G/N = number of distinct genotypes over the total number of individuals) in the F1 strains was calculated for the four mating experiments. The observed G/N value was compared to the distribution of the simulated values. The mean G/N (±1-99%CI) was calculated using a Monte Carlo procedure that simulated distribution of multi-locus genotypes that could be generated by the cross, and drawing from this (with replacement) a sample of N individuals screened.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2012.07.001.

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