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Toxic *Pseudo-nitzschia* spp. in the northwestern Adriatic Sea: characterization of species composition by genetic and molecular quantitative analyses

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Various genetic aspects of the toxic diatom *Pseudo-nitzschia* species in the northwestern Adriatic Sea were investigated. For this area, limited or no knowledge is available regarding the genetic diversity and geographical patterns of *Pseudo-nitzschia* spp., as well as the toxin content. Phylogenetic analyses identified strains belonging to *P. delicatissima*, *P. calliantha*, *P. pungens* and *P. mannii*. Networks of haplotypes inferred from Adriatic and worldwide strains revealed two main haplotypes in *P. delicatissima* from the Mediterranean and north Atlantic, with a single panmictic population in *P. calliantha*, and Adriatic *P. pungens* strains sharing the most frequent haplotype. The quantitative real-time PCR (qrt-PCR) assay was developed to estimate the number of rDNA copies and their variation among *Pseudo-nitzschia* species and strains. Qrt-PCR analysis showed that *P. delicatissima* and *P. calliantha* had different average rDNA copy numbers per cell ($P < 0.001$). It is suggested that different rDNA copy numbers among species might be used to discriminate

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between morphotypes identified using light microscopy. We also discuss how the rDNA copy number variability found among *P. pungens* strains from different months ($P < 0.001$) may relate to physiological activities and/or adaptative strategies. Northwestern Adriatic *P. delicatissima* strains produced domoic acid at low concentrations.

KEYWORDS: domoic acid; genetic identification; HAB; NW Adriatic Sea; *Pseudo-nitzschi*a spp.; qrt-PCR

INTRODUCTION

The diatom *Pseudo-nitzschia* is distributed worldwide as a common component of marine phytoplankton assemblages (Hasle, 2002). At least 12 *Pseudo-nitzschia* species are toxic, as they produce domoic acid (DA), the neurotoxin responsible for amnesic shellfish poisoning (ASP) in humans after the ingestion of DA-contaminated seafood, such as natural or cultivated bivalves (Bates and Trainer, 2006). DA can be transferred through marine food web, causing sickness, as well as mortality in different marine organisms (i.e. seabirds and mammals) that feed on contaminated prey (Scholin *et al*., 2000; Lelong *et al*., 2012). *Pseudo-nitzschia* spp. has been and is being extensively investigated with regard to ecological, morphological and genetic aspects, as well as the toxin content on a global scale (Trainer *et al*., 2012). Blooms of *Pseudo-nitzschia* spp. are defined as harmful algal blooms, and have often been related to nutrient regimes modified by anthropogenic pressure (Glibert *et al*., 2005).

The northwestern coastal Adriatic Sea (Mediterranean Sea) is a high-meso trophic area strongly influenced by inputs from the Po River, which initiate blooms dominated by diatom communities with high frequency and considerable biomass, including *Pseudo-nitzschia* spp. (DeGobbis and Gilmartin, 1990; Bernardi Aubry *et al*., 2004; Socal *et al*., 2008). In the northwestern Adriatic Sea, nothing is known regarding the genetic diversity and geographical pattern of *Pseudo-nitzschia* species, and relatively little is understood regarding the chemical toxin composition of *Pseudo-nitzschia* spp. populations (Ciminiello *et al*., 2005). Moreover, the frequency of toxic blooms characterized by different toxigenic species of *Pseudo-nitzschia* seems to be increasing in various coastal areas of the world (Trainer *et al*., 2012) and, therefore, adequate measures for the local monitoring of species-specific distribution, succession and occurrence are needed to contribute to mitigation strategies against the negative impacts caused by toxic *Pseudo-nitzschia* spp. blooms (Heisler *et al*., 2008).

The taxonomy of this genus is far from fully understood. The existence of cryptic and/or pseudocryptic species (i.e. *P. delicatissima* complex) makes species delineation difficult (Amato *et al*., 2007; Lundholm *et al*., 2012). Recently, along the northwestern Mediterranean Sea (Catalan Sea) coast, it was found that *P. delicatissima* complex also included the cryptic species *P. arenysensis.* Both species are morphologically indistinct, but differ with respect to physiological and genetic parameters (Quijano-Scheggia *et al*., 2009). In the Mediterranean Sea, *Pseudo-nitzschia* spp. have distinct distributions based on morphological and genetic characterization (Cerino *et al*., 2005; D'Alelio *et al*., 2009). Based on studies of seasonal succession (Zingone *et al*., 2006), *P. calliantha* is the most common species and is widely distributed in association with *P. delicatissima* and *P. pungens.* Concerning this latter species, very few genetic data are currently available in the Mediterranean Sea. However, *P. mannii* (ex *P. calliantha*2 genotype) has only been found in the western Mediterranean areas (Amato and Montresor, 2008; Quijano-Scheggia *et al*., 2010). In general, this accounts for differences in ecological niches in phytoplankton species, which tend to exhibit seasonal occurrence or persistent presence but with periodic bloom episodes (McDonald *et al*., 2007).

Genetic approaches can play a fundamental role in solving taxonomic issues, as well as in geographical distributions and the level of genetic differentiation of populations. These tools are especially valuable in the assessment of the occurrence, expansion and/or abundance of toxigenic specimens (Lundholm *et al*., 2006; Casteleyn *et al*., 2009; Penna *et al*., 2010). The choice of adequate molecular tools to target taxonomic resolution at the species, clade and population levels remains essential (Murray *et al*., 2009; Delaney *et al*., 2011). To date, the nuclear ribosomal gene sequences of phytoplankton species (i.e. *Pseudo-nitzschia* spp.) are one of the best candidates as molecular markers for phylogenetic and molecular quantitative characterization purposes (Hubbard *et al*., 2008).

Among the innovative molecular methodologies, quantitative real-time PCR (qrt-PCR) is able to provide an accurate and sensitive estimation of microalgal species in a marine coastal environment using standard

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curves of plasmids and genomic DNA extracted from cultured or environmental target cells in bloom conditions (Galluzzi *et al*., 2010; Perini *et al*., 2011). Although qrt-PCR studies have been widely used for the detection and quantification of target toxic microalgal species, the use of qrt-PCR in taxonomic and routine monitoring studies has been limited (Créach *et al*., 2006; Garneau *et al*., 2011; Zamor *et al*., 2012). However, qrt-PCR has the capacity to characterize the target rDNA copy number of a species or population in relation to physiological activities, such as life cycle or adaptation strategies to the environment (von Dassow *et al*., 2008). In fact, copy number variations in specific genes have been shown to be widespread in a variety of organisms, including plants (Ossowski *et al*., 2008; Maydan *et al*., 2010) and yeasts (Carreto *et al*., 2008). In some cases, copy number variation is highly frequent and great enough to encompass whole genes, and therefore, is likely to affect organism fitness (Zhu *et al*., 2005; Schrider and Hahn, 2010).

In the present study, we aimed to identify and characterize strains of *Pseudo-nitzschia* species sampled in a coastal northwestern Adriatic area not previously investigated with regard to phylogenetic relationships and geographical patterns. Furthermore, we characterize the *Pseudo-nitzschia* strains by comparing the amount of gene copies among and within species, as well as the toxin content of algal strains collected during 1 year of sampling. We then discuss how the variations in copy number could reflect the metabolic activities of strains throughout the year or could be used as taxonomic molecular markers to differentiate *Pseudo-nitzschia* species in marine coastal environments for species-specific identification.

METHOD

Sampling, isolation and culture conditions

The sampling site was located at Pesaro, 500 m off the coast, in the northwestern Adriatic Sea (43°55′50″N; 12°53′51″E) (Fig. 1). Net samples (10-μm mesh size) were collected throughout the water column and the surface seawater monthly from August 2009 to May 2010. Monoclonal strains of *Pseudo-nitzschia* species from net concentrated samples were isolated as described in Penna *et al.* (Penna *et al*., 2008) (Table I). A total of 108 strains of *Pseudo-nitzschia* spp. were established, and maintained in f/2 medium (Guillard, 1975) at 20°C on a 12:12 h light:dark cycle at an irradiance of 100 μmoL photons m^{-2} s^{-1}. All strains were processed

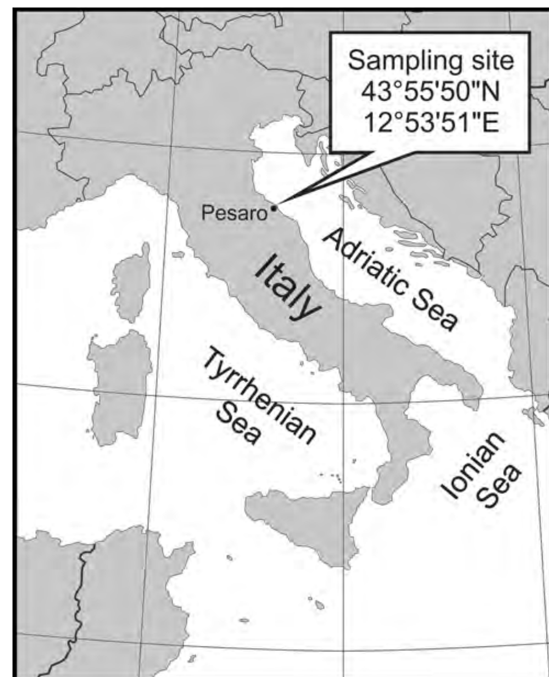

Fig. 1. Map of sampling site (Pesaro) of this study in the northwestern Adriatic Sea.

for genetic analyses, whereas some strains ($n = 21$) were used for toxin content analyses.

Microscopy and abundance of cultured cells and environmental samples

Subsamples (2 mL) of *Pseudo-nitzschia* spp. strains in the exponential growth phase were fixed with Lugol's solution and settled for 24 h in Utermöhl chambers. *Pseudo-nitzschia* spp. were counted in the entire sedimentation chamber under an inverted microscope (Axiovert 40 CFL, Zeiss) at ×200 or ×400 magnification. Phytoplankton counts were performed using an inverted microscope, equipped with phase contrast (model Zeiss Axiovert 35), at a final magnification of ×400. Environmental samples were fixed with formaldehyde to a final concentration of 2%. Subsamples from 5 to 50 mL were allowed to settle for 12–48 h and examined. A variable transect number was observed until at least 200 cells were counted for each sample. Species composition was defined according to Tomas (Tomas, 1997) and references therein. Undetermined organisms belonging to Cryptophyceans, Crysophyceans, Prymnesiophyceans (except Coccolithophores), Prasinophyceans and Chlorophyceans, with sizes varying between 3 and 4 μm, were all included in the flagellate group. Abundance in surface seawater samples was expressed as number of cells per liter (cells L^{-1}).

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Table I: List of Pseudo-nitzschia *spp. strains isolated from the nortwestern Adriatic Sea used in the phylogenetic and molecular analyses*

Sampling date	Species	Strain (MI ID code)
31 August 2009	*P. calliantha*	CBA38 (HE663413); CBA39 (HE663414); CBA40 (HE663415); CBA41(HE663416); CBA42 (HE663417); CBA44 (HE663418); CBA46 (HE663419); CBA52 (HE663420)
18 September 2009		CBA59 (HE663421); CBA61 (HE663422); CBA62 (HE663423); CBA63 (HE663424); CBA64 (HE663425); CBA65 (HE663426); CBA66 (HE663427); CBA67 (HE663428); CBA68 (HE663429)
24 October 2009		CBA69 (HE663430); CBA70 (HE663431); CBA71 (HE663432); CBA72 (HE663433); CBA73 (HE663434); CBA74 (HE663435); CBA75 (HE663436); CBA76 (HE663437); CBA77 (HE663438); CBA78 (HE663439)
19 November 2009		CBA79 (HE663440); CBA80 (HE663441); CBA81 (HE663442); CBA82 (HE663443); CBA83 (HE663444); CBA84 (HE663445); CBA85 (HE663446); CBA86 (HE663447); CBA87 (HE663448); CBA88 (HE663449)
30 December 2009		CBA93 (HE663450); CBA96 (HE663451)
20 January 2010		CBA113 (HE663452)
25 February 2010	*P. delicatissima*	CBA120 (HE650911); CBA122 (HE650912); CBA123 (HE650913); CBA124 (HE650914); CBA125 (HE650915); CBA126 (HE650916); CBA127 (HE650917); CBA128 (HE650918)
19 March 2010		CBA129 (HE650919); CBA130 (HE650920); CBA131 (HE650921); CBA132 (HE650922); CBA133 (HE650923); CBA134 (HE650924); CBA135 (HE650925); CBA136 (HE650926); CBA137 (HE650927); CBA138 (HE650928)
15 April 2010		CBA139 (HE650929); CBA140 (HE650930); CBA141 (HE650931); CBA142 (HE650932); CBA143 (HE650933); CBA144 (HE650934); CBA145 (HE650935); CBA146 (HE650936); CBA147 (HE650937); CBA148 (HE650938)
12 May 2010		CBA149 (HE650939); CBA150 (HE650940); CBA151 (HE650941); CBA152 (HE650942); CBA153 (HE650943); CBA154 (HE650944); CBA155 (HE650945); CBA156 (HE650946); CBA157 (HE650947); CBA158 (HE650948)
31 August 2009	*P. mannii*	CBA56 (HE650977)
18 September 2009		CBA60 (HE650978)
30 December 2009	*P. pungens*	CBA89 (HE650949); CBA90 (HE650950); CBA91 (HE650951); CBA92 (HE650952); CBA94 (HE650953); CBA95 (HE650954); CBA97 (HE650955); CBA98 (HE650956)
20 January 2010		CBA99 (HE650957); CBA100 (HE650958); CBA101 (HE650959); CBA102 (HE650960); CBA103 (HE650961); CBA104 (HE650962); CBA105 (HE650963); CBA106 (HE650964); CBA108 (HE650965); CBA109 (HE650966); CBA110 (HE650967); CBA111 (HE650968); CBA112 (HE650969); CBA114 (HE650970); CBA115 (HE650971); CBA116 (HE650972); CBA117 (HE650973); CBA118 (HE650974)
25 February 2010		CBA119 (HE650975); CBA121 (HE650976)

DNA extraction and sequencing

Total genomic DNA extraction and purification of *Pseudo-nitzschia* spp. strains and PCR amplification of the 5.8S gene and ITS regions were carried out according to Penna *et al.* (Penna *et al.*, 2008), with the following modifications: a preliminary denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension step of 10 min at 72°C. Sequencing of PCR fragments was carried out as described in Penna *et al.* (Penna *et al.*, 2008).

Phylogenetic, molecular and statistical analyses

Alignments were made using ClustalX2 (Larkin *et al.*, 2007) using the default settings and then were edited manually; unalignable regions were excluded from the phylogenetic analyses. The sequences were deposited in the European Molecular Biology Laboratory (EMBL) and are listed in Table I. The ITS-5.8S rDNA sequences of northwestern Adriatic strains were aligned with other sequences of *Pseudo-nitzschia* spp. retrieved from GenBank (Supplementary data, Table SI). Supplementary data, Table SI shows the list of all *Pseudo-nitzschia* spp. ITS-5.8S rDNA sequences obtained from GenBank and used for different genetic analyses in this study.

Phylogenetic analyses were carried out with Modeltest ver. 3.7 (Posada and Crandall, 1998) using an evolutionary model that best fits the data according to Akaike's information criterion. The two-phase variable TrN+I+G substitution model was used for the 5.8S-ITS rDNA. The base frequencies were: $A = 0.1961$, $C = 0.2527$, $G = 0.2527$, $T = 0.2985$ with a gamma distribution among site rate ($Nst = 6$) and a gamma distribution shape parameter, $\gamma = 0.7616$. This model was used to calculate the distance matrix in neighbor-joining (NJ) analysis. Bootstrap values were

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calculated with 1000 pseudo-replicates. Maximum-parsimony (MP) analyses were performed using heuristic searches with tree-bisection-reconnection branch swapping. Branches were collapsed if their minimum length was 0; ambiguities were treated as polymorphisms, and gaps as missing data. The robustness of the MP tree was determined by means of bootstrapping with 1000 pseudo-replicates. These analyses were conducted with PAUP version 4.0b10 (Swofford, 2002). Bayesian analyses were performed using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001), with the following settings: four Markov chains were run for 2 000 000 generations with a sampling frequency of 100 generations. Log-likelihood values for sampled trees were stabilized after almost 200 000 generations. The last 18 000 trees were used to estimate Bayesian posterior probabilities, whereas the first 2001 were discarded as burn-in. Results from two-independent runs were used to construct a majority-rule consensus tree containing the posterior probabilities (Ronquist and Huelsenbeck, 2003). Maximum likelihood (ML) analyses were run with RaxML (randomized accelerated ML) software ver. 7.0.4 (Stamatakis *et al.*, 2005), which adopts a GTR substitution model and allows for the estimation of several parameters, such as the proportion of invariant sites and the alpha values of the gamma distribution for among-site rate variation. ML analyses were conducted on the 5.8S-ITS rDNA sequences. Bootstrap values were calculated with 1000 pseudo-replicates.

A statistical parsimony network (Templeton *et al.*, 1992) was obtained based on the ITS-5.8S rDNA sequences of the *Pseudo-nitzschia* strains with TCS ver. 1.18 software (Clement *et al.*, 2000). Standard and molecular diversity indices were estimated with Arlequin 3.1 (Excoffier *et al.*, 2005) and DnaSP ver. 5.10 (Rozas *et al.*, 2003). Standard deviations were obtained by 10 000 bootstrap pseudo-replicates.

Quantitative real-time PCR and statistical analyses of qrt-PCR data

The genus-specific primers used in this study were derived from Penna *et al.* (Penna *et al.*, 2007). The lysis procedure was carried out as described in Perini *et al.* (Perini *et al.*, 2011) without the sonication step. For the construction of the plasmid standard curve, the 5.8S-ITS rDNA region was cloned into the pCR 2.1 vector (Invitrogen, Carlsbard, CA, USA) and the pITSP plasmid obtained was purified using the Qiaprep Miniprep kit (Qiagen, Valencia, CA, USA). The plasmid DNA concentration was determined using a Qubit fluorometer (Invitrogen, Carlsbard, CA, USA) following the manufacturer's instructions. The plasmid standard curve for *Pseudo-nitzschia* was obtained by amplifying the genus-specific internal fragment from 10-fold scalar dilutions with copy number ranging from 10^5 to 10^1. Scalar cellular dilutions, generated from 2000 to 0.2 cells of *P. calliantha* CBA46, were used for the construction of a second curve only to compare the qrt-PCR efficiencies of the crude extract and the plasmid standard curve. In all experiments, negative controls containing Milli*Q* water were tested in triplicate.

Real-time PCR assays were performed in a final volume of 25 μL using the Hot-Rescue Real-time PCR KIT-SG (Diatheva, Fano, Italy) containing 1× Master Mix with SYBR Green dye, primers at a final concentration of 300 nM, 0.5 U of Hot-Rescue Plus DNA polymerase and 2 μL of crude extract diluted to 1:10 and 1:100. All reactions were carried out using a Step-One Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions consisted of 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. At the end of each run, a dissociation protocol was performed to ensure the absence of primer dimers or non-specific products. The qrt-PCR assays were performed in duplicate for each dilution factor of the lysate sample and in triplicate for each standard curve point. StepOne Software ver. 2.1 was used for data analysis of the qrt-PCR assays. The reaction percentage efficiency was calculated by the following formula: $(10^{(-1/\mathrm{slope})} - 1) \times 100$. *Pseudo-nitzschia* spp. rDNA copy number cell^{-1} was obtained by plotting sample threshold cycle (Ct) values versus the mean plasmid standard curve.

The normality of the rDNA copy number per cell data distribution was checked by means of Shapiro-Wilk tests. Differences between the median values of the above-mentioned variables were analyzed using the non-parametric Kruskal–Wallis test, with *a posteriori* Mann–Whitney pairwise comparisons. The Bonferroni correction was applied to all *P*-values in the *a posteriori* pairwise tests.

Toxin analysis

A total of 21 *Pseudo-nitzschia* cultured subsamples (100 mL) including strains of *P. calliantha* (CBA38, CBA62, CBA73, CBA81, CBA93 and CBA113), *P. delicatissima* (CBA134, CBA135, CBA137, CBA138, CBA143, CBA145, CBA152, CBA154, CBA157 and CBA158) and *P. pungens* (CBA94, CBA95, CBA99, CBA108 and CBA121) were harvested by filtration through a 3.0-μm membrane filter. Algal pellets were

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extracted with 90% MeOH in a final volume of 10 mL by sonication for 20 min. Algal suspensions were centrifuged and the supernatants were further purified using SPE SAX cartridges (Supelco, Inc., Bellefonte, USA) and a modified procedure previously described by Quilliam *et al*. (Quilliam *et al*., 1995). The elutes were concentrated under a nitrogen stream and analyzed by LC-MS/MS. The analytical instrument consisted of an Agilent 1290 LC coupled to a 6460 triple quad mass spectrometer equipped with a Jet Stream ESI (Agilent Technologies, USA). Two positive multiple reaction monitoring transitions were monitored: m/z 312→266 and m/z 312→161. Chromatographic conditions were similar to those previously described in Pigozzi *et al.* (Pigozzi *et al*., 2010). Quantification was achieved using an external standard method with DA calibration solutions in the concentration range of 1–20 ng mL^{-1}. A certified reference material of DA was obtained from the National Research Council of Canada (Halifax, Canada). In this study, all data represent the sum of DA and its isomer, epi-DA.

RESULTS

Presence of *Pseudo-nitzschia* spp. in environmental samples

The phytoplankton assemblage was mainly composed of diatoms throughout the nearly year-long sampling period. This group represented > 60% of total abundance (mean 66 ± 12%) with dinoflagellates accounting for <10% (mean 6 ± 4%). Small flagellates and sporadic presence of Dictyochophyceae accounted for the remaining 28% of the total abundance. Among diatoms, *Skeletonema marinoi* was the dominant species (maximum abundance >10^{6} cells L^{-1}) and *Pseudo-nitzschia* spp. was always found in the seawater samples with the exception of June 2010; *Pseudo-nitzschia* spp. contributed up to 20% of the total diatom abundance with a maximum abundance >10^{6} cells L^{-1} in April 2010. The three species *P. calliantha*, *P. delicatissima* and *P. pungens* showed seasonality. *P. calliantha* was found in the spring–autumn period, *P. pungens* occurred exclusively in the winter and *P. delicatissima* appeared in the late winter and spring; these species occurred with a short period of overlap between *P. calliantha* and *P. pungens*, and between *P. pungens* and *P. delicatissima*. This evidence of species-specific *Pseudo-nitzschia* seasonal occurrence was confirmed by ITS-5.8S rDNA sequencing and phylogenetic analyses of the strains collected. Meanwhile, *P. mannii* was firstly identified on the northernwestern Adriatic coast based on phylogenetic relationships.

Molecular analyses

The final alignment was obtained from a total of 142 ITS-5.8S rDNA sequences of *Pseudo-nitzschia* spp. including 108 sequences derived from northwestern Adriatic *Pseudo-nitzschia* strains (40 sequences of *P. calliantha*, 38 sequences of *P. delicatissima,* 2 sequences of *P. mannii* and 28 sequences of *P. pungens*) and 34 sequences of various *Pseudo-nitzschia* species from GenBank. The final alignment with *P. turgiduloides* clone 3_19 as the outgroup was as follows: ITS-5.8S was 1013 bp in length (C 23.0%, T 31.17%, A 23.24%, G 22.60%) with 699 polymorphic sites and a transition/transversion ratio of 1.5. There were only minor differences between the NJ, MP, ML and Bayesian inference analyses; therefore, only the ML phylogenetic tree is presented (Fig. 2). Northwestern Adriatic Sea strains were included in the *P. calliantha, P. delicatissima, P. mannii* and *P. pungens* genetic lineages. In particular, northwestern Adriatic *P. pungens* strains were grouped within the clade I according to Casteleyn *et al.* (Casteleyn *et al*., 2008). All these phylogenetic groups were supported by high bootstrap and posterior probability values. *P. mannii* strains were not included in the following molecular statistical and qrt-PCR analyses due to their low number.

Parsimony networks were inferred within *Pseudo-nitzschia* species formed by strains from the northwestern Adriatic Sea, other Mediterranean and worldwide areas based on the ITS-5.8S rDNA sequences (Fig. 3). Within the Mediterranean, for *P. delicatissima* the different haplotypes were separated by a maximum of only four mutational steps from the most frequent Adriatic haplotype, CBA127, which was found in 43 strains. Furthermore, another distinct and separate frequent haplotype from cold seawaters was obtained sharing 11 strains separated by one distinct haplotype based on a single mutational step. The Mediterranean and North Atlantic haplotypes constituted two main independent outgroups that did not share any haplotype. The *P. calliantha* haplotypes were separated by a maximum of 12 mutational steps from the most frequent Adriatic haplotype, CBA65, which was found in 20 strains from both the northwestern Adriatic Sea and Virginia coast (eastern Atlantic, USA). Across this spectrum of variation there was no pattern of correlation with the geographical location of strains; the strains from the Mediterranean Sea and the Atlantic coast were widespread throughout the network. Some strains, such as ICMB 144, ICMB155 and ICMB118, were more divergent being separated by as many as 9–12 mutational steps from the most frequent haplotype, CBA65. Within *P. pungens* the most frequent haplotype was CBA121 of the northwestern Adriatic Sea, which shared the highest number

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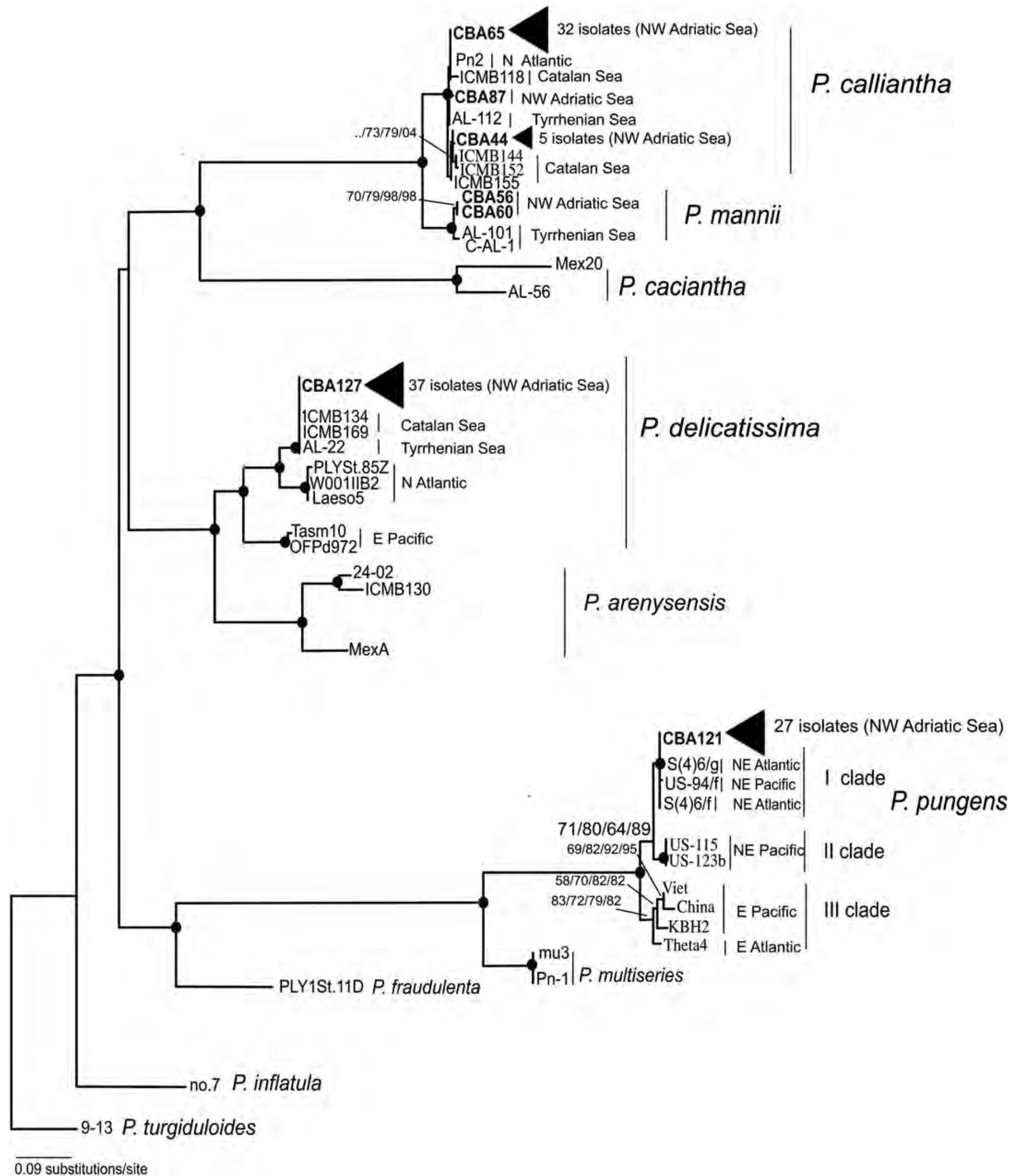

Fig. 2. The ITS-5.8S rDNA phylogeny of *Pseudo-nitzschia* spp. The tree is obtained by Maximum Likelihood (ML) analysis. Numbers on the internal nodes represent, from left to right, Neighbor-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) bootstrap values and Bayesian posterior probability values. Only bootstrap values >50% are shown. Black circles indicate bootstrap values >80% with posterior probability values >0.9. Bold letters indicate NW Adriatic strains isolated and analyzed in this study.

of isolate sequences of clade I together with other 11 different haplotypes separated by one to three mutational steps from CBA121. Thus, clade II included fewer haplotypes that were segregated from the most frequent Adriatic CBA121 by a maximum of 11 mutational steps, whereas the clade III was represented by a few strains totally segregated from the most frequent Adriatic haplotype, CBA121 of clade I. Further,

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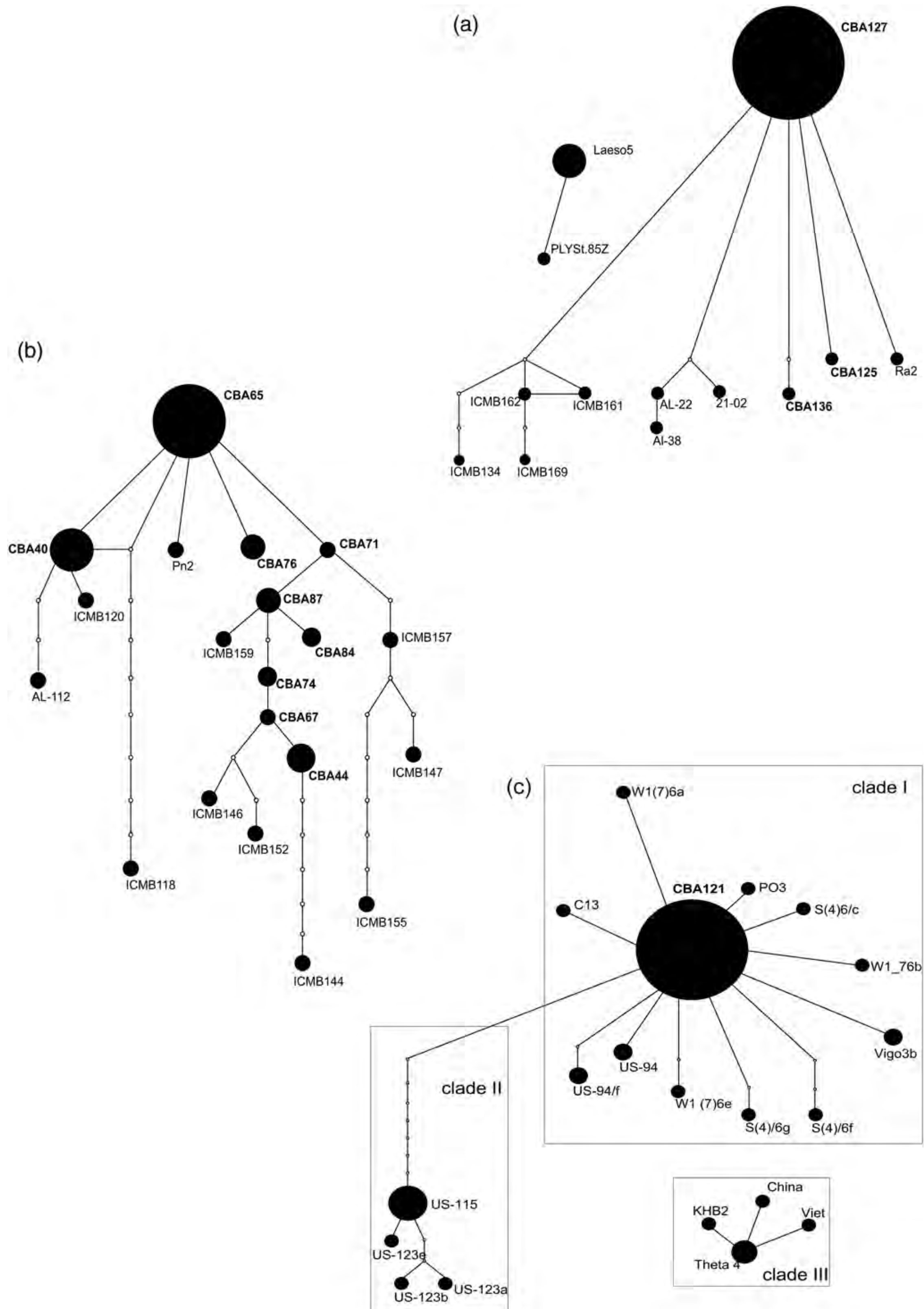

Fig. 3. Statistical parsimony network of haplotypes of *Pseudo-nitzschia delicatissima* (**a**), *P. calliantha* (**b**) and *P. pungens* (**c**) strains based on ITS and 5.8S ribosomal gene sequences. The sizes of the circles are proportional to the number of strains found to have that haplotype. Small white circles indicate missing haplotypes. Bold letters indicate NW Adriatic strains isolated in this study.

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Table II: Origin, number of the ITS-5.8S rDNA Pseudo-nitzschia *spp. sequences used from this study and GenBank, and haplotypes and nucleotide properties obtained from statistical parsimony analysis*

Species	Origin and sequence number (*n*)	Haplotype number generated	Fragment length (bp)	Polymorphic sites	Trans./transv. ratio
P. delicatissima	**NW Adriatic Sea (*n* = 38)**	13	615	38	1.3
	Tyrrhenian Sea (*n* = 4)				
	Catalan Sea (*n* = 10)				
	European Atlantic, North Sea				
	E Canada (*n* = 13)				
P. calliantha	**NW Adriatic Sea (*n* = 40)**	20	683	31	0.6
	Catalan Sea (*n* = 10)				
	Atlantic, Virginia (USA) (*n* = 10)				
P. pungens	**NW Adriatic Sea (*n* = 26)**	20	679	65	3.2
	Catalan Sea (*n* = 1)	Clade I (*n* = 12)[a]			
	European Atlantic (*n* = 11)	Clade II (*n* = 4)[a]			
	North Sea (*n* = 14)	Clade III (*n* = 4)[a]			
	W Canada (*n* = 10)				
	NE Pacific, USA (*n* = 6)				
	NE Pacific, USA (*n* = 10)				
	European Atlantic (*n* = 3)				
	E Asian (*n* = 3)				
	Mexico (*n* = 1)				

Bold letters indicate northwestern Adriatic strains isolated and analysed in this study.
[a]Number of generated haplotypes within clade.

Table III: Sequence divergence estimates in the ITS—5.8S rDNA among and within isolates of P. delicatissima *from the Mediterranean and north Atlantic areas*

Geographic region	NW Adriatic	Tyrrhenian	Catalan	N Atlantic
NW Adriatic (*n* = 38)	0.02 ± 0.01			
Tyrrhenian (*n* = 4)	n.r.	0.16 ± 0.10		
Catalan (*n* = 10)	0.03 ± 0.02	0.03 ± 0.02	0.40 ± 0.14	
N Atlantic (*n* = 13)	3.0 ± 0.66	3.0 ± 0.66	3.0 ± 0.67	0.02 ± 0.14

Table IV: Sequence divergence estimates in the ITS—5.8S rDNA among and within isolates of P. calliantha *from the Mediterranean and north Atlantic areas*

Geographic region	NW Adriatic	Catalan	N Atlantic
N Adriatic (*n* = 40)	0.39 ± 0.13		
Catalan (*n* = 11)	0.07 ± 0.04	1.15 ± 0.23	
N Atlantic (*n* = 10)	0.01 ± 0.01	0.14 ± 0.07	0.16 ± 0.08

Table V: Sequence divergence estimates in the ITS—5.8S rDNA among and within clades of P. pungens

	Clade I	Clade II	Clade III
Clade I (*n* = 68)[a]	0.08 ± 0.02		
Clade II (*n* = 10)	1.18 ± 0.4	0.19 ± 0.09	
Clade III (*n* = 7)	2.4 ± 0.56	2.7 ± 0.58	1.07 ± 0.25

Data are mean pairwise nucleotide differences (p-distance) within isolates (on the diagonal), and net pairwise nucleotide differences between isolates (below diagonal) expressed as percentage. SE calculated on 10 000 bootstrap replicates.
n.r., not reported as resulted not significant value.
[a]Included 28 northwestern Adriatic isolates.

haplotype and nucleotide properties of *Pseudo-nitzschia* species strains were shown in Table II.

Sequence divergence among strains of *P. delicatissima*, *P. calliantha* and *P. pungens* was assessed by means of the net number of nucleotide differences, based on the ITS-5.8S rDNA gene (Tables III–V). Within *P. delicatissima*, the ITS-5.8S rDNA sequences showed the greatest divergence between northwestern Adriatic, Tyrrhenian and Catalan strains with northern Atlantic strains (Fig. 3). The net pairwise nucleotide difference between Tyrrhenian and NW Adriatic strains was not significant, possibly because it was based on only four available Tyrrhenian sequences. Furthermore, the net pairwise nucleotide difference between Catalan and Tyrrhenian strains was lower than the mean pairwise difference within the four Tyrrhenian *P. delicatissima* strains, as well as within the Catalan strains. Among *P. calliantha* strains of the northwestern Adriatic Sea, Catalan Sea and northern Atlantic Ocean (USA), sequence divergence was lower than within the single groups, with the highest values for the Catalan strains and much lower values for northwestern Adriatic strains. Therefore, it seems that the highest genetic variability occurs in the

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Mediterranean groups. The molecular diversity of *P. pungens* showed clade I, II and III to be highly differentiated, with the greatest divergence between clade I and clade II with clade III.

Sensitivity, assay reproducibility of the qrt-PCR assay and rDNA copy number in northwestern Adriatic *Pseudo-nitzschia* spp. strains

The plasmid standard curve (pITSP) with a qrt-PCR mean efficiency of 92%, (mean equation $y = -3.52x + 34.71$, $n = 4$ experiments) and a sensitivity of 10 copies/reaction (Ct mean $= 31.14 \pm 0.59$) was used for the ITS-5.8S gene copy number quantification of northwestern Adriatic *Pseudo-nitzschia* spp. strains. The curve generated from cultured cells had a dynamic range of 5 $\log_{10}$ (average $r^2 = 0.99$) and an efficiency of 91%. As the equation of this latter curve was $y = -3.56x + 28.24$ ($n = 4$ experiments), the two curves showed the same efficiency ($\Delta s = 0.04$) and the quantification method using the plasmid standard curve was applied. To demonstrate the inter-assay reproducibility of both the plasmid and cellular curves, the coefficients of variations of cycle threshold and copy number were calculated (Supplementary data, Tables SII and SIII).

A total of 106 Adriatic strains of *Pseudo-nitzschia calliantha*, *P. delicatissima* and *P. pungens*, were processed for the quantification of rDNA copy number per cell. The qrt-PCR assay was performed on DNAs obtained from 20 and 2 cells of each *Pseudo-nitzschia* strain. The quantitative analysis of the ITS-5.8S gene of *Pseudo-nitzschia* strains showed that the three species of *P. delicatissima, P. calliantha* and *P. pungens* (clade I) had a different average rDNA copy number, 39 ± 22, 62 ± 30 and 83 ± 67, respectively. The results of the Shapiro–Wilk tests showed that the distribution of rDNA copy number per cell was significantly different from a normal distribution for two out of three *Pseudo-nitzschia* species, namely *P. pungens* ($n = 28$, $W = 0.9007$, $P = 0.0119$) and *P. delicatissima* ($n = 38$, $W = 0.8395$, $P < 0.0001$), while the hypothesis of normality could not be rejected for *P. calliantha* ($n = 40$, $W = 9593$, $P = 0.1589$). On the basis of these results, a non-parametric approach, i.e. the Kruskal–Wallis test, was selected for the statistical analysis of rDNA copy number per cell. Strains of *Pseudo-nitzschia* species were isolated monthly, and the rDNA copy number per cell did not show any differences among strains of either *P. calliantha* ($Hc = 8.027$, $P = 0.1554$) or *P. delicatissima* ($Hc = 5.477$, $P = 0.1405$), whereas a significant difference was found among *P. pungens* strains ($Hc = 10.22$, $P = 0.0061$) (Fig. 4). The pairwise *a posteriori* comparison of *P. pungens* rDNA copy number showed that the only significant difference was found between strains isolated in December and January (Mann–Whitney' $U = 21$, $P = 0.0150$ with the Bonferroni correction). As rDNA copy number per cell was not homogenous in only one single pairwise comparison between *P. pungens* strains, all the strains of each *Pseudo-nitzschia* species isolated in different months were cumulated before analyzing the differences among species. The Kruskal–Wallis test allowed rejection of the null hypothesis of equal medians of rDNA copy number per cell in the three *Pseudo-nitzschia* species ($Hc = 11.95$, $P = 0.0025$). However, *a posteriori* pairwise Mann–Whitney tests showed that the difference was significant ($U = 415$, $P = 0.0017$ with the Bonferroni correction) only in the case of the *P. calliantha*

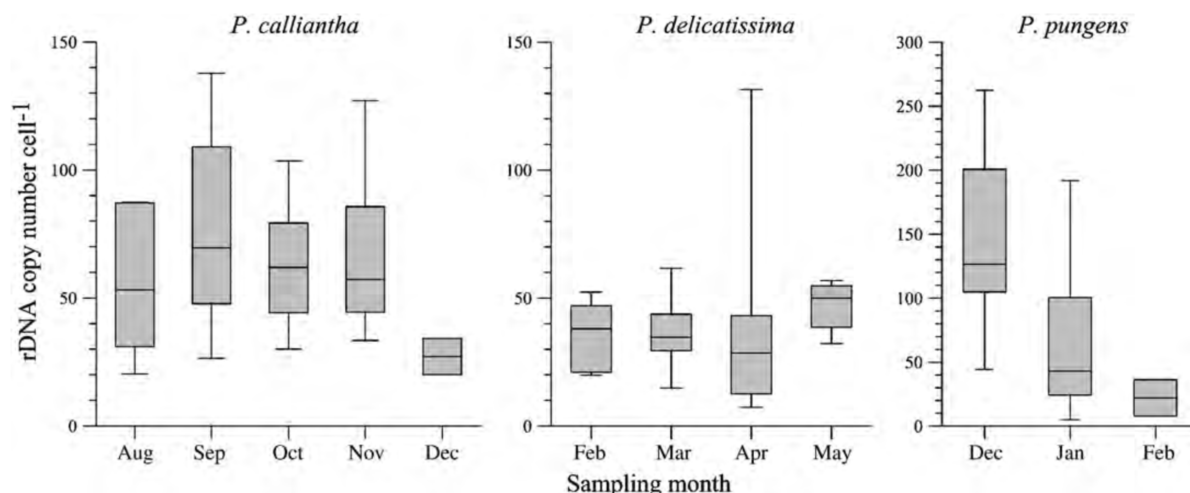

Fig. 4. The rDNA copy number per cell of northwestern *P. calliantha, P. delicatissima* and *P. pungens* strains determined by qrt-PCR and sampling period.

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versus *P. delicatissima* comparison. As for *P. pungens*, rDNA copy number per cell was not significantly different from other species, mainly because of its high variability in the ribosomal gene content.

Toxin content

In this study, the absolute limit of detection (LOD) estimated as signal-to-noise ratio 3:1 provided by the analytical method was 0.01 ng of DA injected per column. Taking into consideration sample preparation and differences in cell abundance among the 21 cultures analyzed ($1.2-8.9 \times 10^8$ cells L^{-1}), the LOD, converted into units of femtograms cell^{-1}, varied between 0.040 and 0.006 fg cell^{-1}. The presence of DA was detected in seven cultured strains of *P. delicatissima* and in one case DA reached the quantifiable amount of 0.063 fg cell^{-1}. None of the cultured strains of *P. calliantha* and *P. pungens* produced DA in detectable amounts.

DISCUSSION

The phylogenetic analyses based on ITS-5.8S rDNA showed that the northwestern Adriatic *Pseudo-nitzschia* spp. strains grouped within distinct genetic lineages corresponding to different species, such as *P. delicatissima*, *P. calliantha*, *P. mannii* and *P. pungens* clade I, previously described by Casteleyn *et al*. (Casteleyn *et al*., 2008); this held true even when the sample size was increased and included strains isolated during different periods of the year. All the phylogenetic groupings described within genus *Pseudo-nitzschia* were strongly supported by high bootstrap and posterior probability values. This allowed us to definitively assign northwestern Adriatic strains to different *Pseudo-nitzschia* species. Within *P. delicatissima* phylogenetic analyses placed the Mediterranean and Atlantic strains into two sister clades. The geographical pattern of Mediterranean *P. delicatissima* strains was represented by a principal haplotype that comprised northwestern Adriatic and some Catalan strains, but it was not related to northern Atlantic strains. Mediterranean and northern Atlantic strains were also differentiated based on mean pair sequence divergence that accounted for 3% of variability, representing a higher value than those between Mediterranean strains. Furthermore, the northwestern Adriatic strains showed a lower value of genetic variability in comparison with Catalan or Tyrrhenian strains, although a larger number of Adriatic strains were analyzed. In fact, the genetic differentiation value was similar to that within northern Atlantic strains, which included 13 strains from various geographical areas. The evident genetic differentiation within *P. delicatissima* can be approximately linked to the geographical origin of the strains. These data are preliminary and therefore additional investigations into the genetic variability of new strains from the Mediterranean and other areas should be carried out to investigate any potential geographical pattern of *P. delicatissima*.

Within *P. calliantha* the strains were widely dispersed from the western Atlantic to the Mediterranean Sea. Phylogenetic analyses placed these *P. calliantha* strains into a unique Atlantic/Mediterranean clade. In particular, the results of the statistical parsimony analysis of Atlantic/Mediterranean *P. calliantha* based on ITS-5.8S rDNA sequences showed at least four main haplotype groups. However, this grouping had no clear correspondence with the regional sea origin of the different strains. The haplotype CBA65 comprised strains from the northwestern Adriatic and Atlantic, whereas the others included strains from the northwestern Adriatic Sea and/or only the Catalan Sea. These four groupings of *P. calliantha* might be considered a single-large population with no clear geographical barriers inhibiting the gene flow. Moreover, the mean pairwise sequence divergence between various geographical *P. calliantha* strains was in the range of 0.01–0.14%, thus representing a value lower than those within strains.

Phylogenetic analyses of *P. pungens* ITS-5.8S rDNA sequences grouped the northwestern Adriatic strains in the clade I, which has a cosmopolitan distribution in the temperate waters of the Atlantic and Pacific Oceans, constituting a single globally homogeneous population based on rDNA genes (Casteleyn *et al*., 2008). In the parsimony network of *P. pungens*, three most frequent haplotypes were detected; this grouping corresponded to the distinct *P. pungens* clades. The northwestern Adriatic strains shared the most frequent haplotype (CBA121). This indicates that the *P. pungens* clade I is common in the Mediterranean Sea along with the Adriatic strains. Thus, the most frequent haplotype of clade II was still connected to the most frequent haplotype CBA121, whereas the four haplotypes representing clade III were segregated separately. The intraspecific variation of *P. pungens* strains was $\leq 1\%$ and clade I, which includes the largest number of strains from various geographical areas and now also the Adriatic strains, showed the lowest genetic variation, apparently making this cosmopolitan clade homogeneous based on ITS-5.8S rDNA. However, it is known that when more polymorphic markers, such as microsatellites, are used in the population analysis of clade I, significant geographical population differentiation is evident on a global scale suggesting the restricted gene flow among the different oceanic areas (Casteleyn *et al*., 2010).

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In this study, we report the development of a qrt-PCR assay for the quantification of the ribosomal gene content of the genus *Pseudo-nitzschia*. The genus-specific primers were designed on the 5.8S rDNA sequence. The qrt-PCR assay was based on the SYBR Green dye that represents a widely used method for PCR product detection. The plasmid and cellular curves showed a similar mean efficiency ($\Delta s = 0.04$). Thus, it was appropriate to use the plasmid standard curve for the quantification of the rDNA copy number for each *Pseudo-nitzschia* isolate. The evidence that no significant differences in the 5.8S rDNA copy number per cell within *P. delicatissima* and *P. calliantha* strains can be found during the growth phase was in contrast to the trend in some dinoflagellate species where both ITS-5.8S and LSU rDNA content per cell is highly variable (Galluzzi *et al.*, 2010; Perini *et al.*, 2011); and contrasts with previous studies of *Pseudo-nitzschia* species abundance (Andrée *et al.*, 2011). However, in this study variations in the rDNA content indicated by the qrt-PCR assays of diatom field populations were not found, but the correct abundance estimation of species-specific diatoms or genus-specific *Pseudo-nitzschia* has been reported previously (Andrée *et al.*, 2011; Godhe *et al.*, 2008; Fitzpatrick *et al.*, 2010). In this study, strains were derived from young cultures in the early exponential growth phase to avoid potential variation in gene copy number in relation to the diatom growth stage (Godhe *et al.*, 2008). Significant differences in 5.8S rDNA copy number per cell were only found between *P. calliantha* and *P. delicatissima*, whereas *P. pungens*, which had the greatest ribosomal gene content variability, partly overlapped the other two *Pseudo-nitzschia* species. However, ribosomal gene content might be considered as a molecular taxonomic character for the qualitative discrimination of cultured species that contain cryptic or semi-cryptic taxa or are difficult to diagnose using light microscopy, such as *P. delicatissima* and *P. calliantha* morphotypes.

The *Pseudo-nitzschia* spp. represented a constant constituent of the central Adriatic diatom community occurring during the entire investigation period. In the western Adriatic Sea, the presence of potentially toxic *P. calliantha* and *P. delicatissima* (and *P. delicatissima* complex) has been documented, although both species are difficult to identify by optical microscopy (Socal *et al.*, 1999; Totti *et al.*, 2000; Penna *et al.*, 2006). In the Adriatic, *P. calliantha* and *P. delicatissima* can co-occur with potential seasonality in the winter and spring periods, respectively (Bernardi Aubry *et al.*, 2004; Caroppo *et al.*, 2005), and species-specific identification has been recently carried out using scanning electron microscopy (Maric *et al.*, 2011). Therefore, a fast and specific molecular qrt-PCR assay, exclusively distinguishing *P. calliantha* and *P. delicatissima* strains based on the significantly diverse ribosomal copy number, seems not only useful, but also potentially feasible with appropriate algorithms thanks to the significant differences shown in our results. Whereas, *P. pungens* was found in the winter period, as already observed (Bastianini unpublished data; Ljubesic *et al.*, 2011), and species-specific identification is easier using light microscopy based on clear morphology of this species.

Given the significant difference in rDNA copy number observed between *P. calliantha* and *P. delicatissima*, difference in rDNA copy number can be considered as a useful component of a multivariate approach. In fact, a model classification tree based on *P. calliantha* and *P. delicatissima* biovolume and the rDNA copy number was able to correctly classify 96.7% (29 out of 30) strains (data not reported).

The qrt-PCR assay was carried out on *Pseudo-nitzschia* strains isolated monthly. The rDNA gene content of *P. calliantha* and *P. delicatissima* strains did not show any significant variation within time, whereas significant variability in rDNA gene copy number was found among *P. pungens* strains. These strains were collected during the winter period and ribosomal gene variability was found between strains isolated in December and January. To minimize any artifact due to culture age and nutrient depletion, all strains, after a brief acclimation period from the net seawater samples, were promptly analyzed during the exponential phase. The gene content variability recorded may be due to the different life-cycle stages (Sarno *et al.*, 2010) and/or the physiological status of the *P. pungens* strains (Thessen *et al.*, 2009) in relation to the trophic conditions of the winter of 2009–2010 (data not shown). Differences in rDNA copy number between species that occur in different seasonal phases can be certainly regarded as the outcome of a selective process reflecting the overall fitness of the winning species. Other potentially relevant features, such as cell shape or biovolume, did not seem to play a meaningful role. In fact, shape was quite similar, whereas biovolume was different, as *P. calliantha* is larger than *P. delicatissima* (data not shown). However, this difference may not directly reflect species fitness, as smaller cells (*P. delicatissima*) should be favored between summer and early fall, with less energy and fewer nutrients in the water column, whereas larger cells (*P. calliantha*) should be better suited to the nutrient-rich mixed water column that was found between winter and early spring. As the observed pattern was the exact opposite, biovolume differences were probably not large enough as to play an ecological role. Therefore, only rDNA copy number seemed to play a role, and its role was possibly related to photosynthetic efficiency, as this

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property was the most important among those that may favor one species over another.

A total of 21 cultured strains belonging to three *Pseudo-nitzschia* species were analyzed to investigate their potential to produce DA. None of the cultured strains of *P. pungens* produced DA in detectable amounts, in accordance with the fact that this species is generally described as non-toxic in the Mediterranean Sea (Trainer *et al*., 2012). The lack of ASP toxin production has also been observed in *P. calliantha*, although other strains collected in Tunisia (Sahraoui *et al*., 2009) and in the Black Sea (Besiktepe *et al*., 2008) have been reported to be toxic. A bloom of this species in the eastern Adriatic Sea has recently been linked to ASP toxin accumulation in shellfish (Maric *et al*., 2011), although the authors recognized the need to conduct further experiments on cultured strains of the microalga. Only *P. delicatissima* was found to produce DA at low levels (0.063 fg cell^{-1} in one cultured strain).

The same species, collected at various locations in the Mediterranean Sea, has never proved to be toxic (Orsini *et al*., 2002; Quijano-Scheggia *et al*., 2010). This discrepancy could be related to culture conditions influencing the toxin production rate, variable sensitivity of the methods used for analysis and variations in the total number of cells analyzed. Several hypotheses have attempted to explain the origin of ASP toxins accumulation in Adriatic shellfish (Amato *et al*., 2010; Pistocchi *et al*., 2012), although at amounts well below the regulatory limit of 20 mg kg^{-1} in edible tissue (Ciminiello *et al*., 2005), and none has been unambiguously confirmed.

Given our results for the northwestern Adriatic region, we can conclude that the genetic characterization of toxic *Pseudo-nitzschia* species may greatly improve our understanding of the composition and diversity of the *Pseudo-nitzschia* complex and offer more specific and sensitive tools to investigate the relationships between diversity and the ecological function of a species.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://*plankt.oxfordjournals.org*.

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