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ScienceDirect
Harmful Algae 6 (2007) 849–860
HARMFUL ALGAE
www.elsevier.com/locate/hal

Identifying *Pseudo-nitzschia* species in natural samples using genus-specific PCR primers and clone libraries

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Received 14 February 2007; received in revised form 13 March 2007; accepted 15 March 2007

Abstract

The diatom genus *Pseudo-nitzschia* contains a number of toxic and non-toxic species that are difficult to distinguish using light microscopy (LM) and at times even with electron microscopy (EM). In order to investigate the actual diversity and seasonal occurrence of *Pseudo-nitzschia* species, we developed genus-specific ribosomal DNA LSU primers to be used in PCR reactions with environmental DNA samples. Using this approach, we constructed clone libraries from samples collected in the Gulf of Naples (Mediterranean Sea) on six dates between April and October 2004 and compared molecular results with those obtained from counts using LM on the same dates. Thirteen distinct genotypes could be distinguished by their LSU sequence, against five species discriminated using the light microscope. Despite the limited number of samples, 10 out of 14 LSU genotypes known in the area were recovered. In addition, three new genotypes were retrieved, two of which were from within the *P. galaxiae* clade and one possibly corresponding to an undescribed *P. delicatissima*-like morph. Molecular results matched LM findings in the case of *P. multistriata*, whereas they provided a much higher resolution for morphs such as *P. delicatissima*- and *P. pseudodelicatissima*-like, which include several pseudo-cryptic species. Overall, the direct amplification with the primers developed proved to be an effective and useful tool to assess *Pseudo-nitzschia* diversity in the natural environment.
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Keywords: Clone libraries; Genus-specific primers; Molecular detection; LSU rDNA; *Pseudo-nitzschia*

1. Introduction

Pseudo-nitzschia is a diatom genus containing several potentially toxic species. Although the morphology of the frustule, as with all diatoms, yields a number of useful taxonomic characters, the identification of species within this genus is often problematic. In recent years, several new species have been circumscribed that are not easily distinguishable using light microscopy (LM) and often also using electron microscopy (EM) (Lundholm et al., 2002, 2003, 2006). These include *P. dolorosa* and *P. decipiens*, which were previously classified as *P. delicatissima*, and *P. calliantha* and *P. caciantha*, formerly included within *P. pseudodelicatissima*. *Pseudo-nitzschia cuspidata* and *P. inflatula* are also quite similar to *P. pseudodelicatissima*. All these species are referred to as pseudo-cryptic because of the subtle morphological dissimilarities between them, mainly consisting of slight differences in the ultrastructure of the poroids in the valve striae, a character that is only visible using TEM. Identification of pseudo-cryptic species is obviously critical when some of them are toxic, as in the case of *Pseudo-nitzschia*. In addition, lumping distinct taxa under a single name does not allow the

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doi:10.1016/j.hal.2007.03.003

recognition of species-specific patterns of occurrence, nor their relationship to environmental conditions, thus limiting ecological studies and bloom prediction capabilities.

Like other phytoplankton species, *Pseudo-nitzschia* species tend to exhibit a repeating seasonal occurrence (Fryxell et al., 1997; Fehling et al., 2006). In the Gulf of Naples (Mediterranean Sea), several *Pseudo-nitzschia* species regularly occur in restricted periods of the year (Zingone et al., 2003, 2006). However, species identified using LM as *P. delicatissima*, *P. pseudodelicatissima* and *P. galaxiae* seem to be present and bloom several times during the year. Prolonged periods of occurrence and multiple blooms could be due to single ecologically plastic species thriving in different seasons or, rather, to distinct pseudo-cryptic taxa hidden under these morphs.

A high genetic diversity in the Gulf of Naples was first demonstrated by Orsini et al. (2004), who found several distinct genotypes within *P. delicatissima*-like morphs during the late winter-spring bloom of the species, with a dominance of *P. delicatissima sensu stricto* (*ss*) at the peak of the bloom. In a more recent investigation on strains resembling *P. delicatissima* or *P. pseudodelicatissima*, eight taxa were identified and circumscribed based on morphological and molecular data and on mating incompatibility, including two new genotypes, *P. calliantha*2 and *P. delicatissima*2 (Amato et al., 2007). In this latter study, the presence of *P. cuspidata* was also revealed using ITS, while the LSU marker fails to separate this taxon from *P. pseudodelicatissima ss*. The two above-mentioned investigations required an intensive cultivation effort, with almost 200 strains isolated over several months. *Pseudo-nitzschia* species are frequent in the area in all seasons and often at high concentrations (million cells l^{-1}), which implies that the actual numbers of distinct taxa present in the area may have been so far under-sampled.

Problematic identification of microalgae in natural samples has in some cases been addressed using a molecular approach. PCR using specific primers has been performed on whole community DNA to recognise classes or other supra-generic taxa of marine picoeukaryotic assemblages (e.g. Díez et al., 2001a,b; Zeidner et al., 2003; Fuller et al., 2006), In a few cases, genera or species, mainly harmful, have been targeted with PCR, with the aim of detecting and quantifying known taxa (e.g. Gray et al., 2003; Galluzzi et al., 2004, 2005; Skovhus et al., 2004). Galluzzi et al. (2004, 2005) used genus-specific markers for 5.8S rDNA in real-time PCR to quantify the toxic dinoflagellate *Alexandrium minutum* in natural samples, whereas a species-specific *rbc*L primer was used in the case of *Karenia brevis* (Gray et al., 2003).

The aim of this study was to develop a molecular method to track different pseudo-cryptic or possibly cryptic species within *Pseudo-nitzschia*, thus overcoming problems posed by microscopy analyses and by the isolation/cultivation method. Genus-specific primers were developed to amplify a fragment from rDNA LSU from community DNA. LSU was chosen as the marker region because, with the exception of the case of *P. cuspidata*, it is a good marker to distinguish among species of the genus, and also because a high number of sequences were available for both *Pseudo-nitzschia* and other diatoms, making the design of primers more accurate. Clone libraries were constructed on selected dates in the Gulf of Naples, from samples collected at the long-term station MareChiara (MC, Ribera d'Alcalà et al., 2004) and compared to sequences from known strains and to the species abundance results from the phytoplankton counts. It was also investigated whether the *Pseudo-nitzschia* specific primers might be useful for Single Strand Conformation Polymorphism (SSCP) analyses, which would be a more effective method than clone libraries for routinely assessing diversity.

2. Materials and methods

2.1. Sampling

Samples of seawater were collected from 0 m at the MC sampling site (40°48.5′N, 14°15′E) between January and December 2004, approximately once a week, using a 12 l Niskin bottle mounted on an automatic Carousel sampler. Five litres were filtered onto a 3 μm filter at 200 mmHg using a vacuum pump. The filters were cut in sections, immediately frozen in liquid nitrogen and stored in Eppendorf tubes at −80 °C until DNA extraction. Phytoplankton samples, also collected from 0 m using a Niskin bottle, were fixed using 0.8% neutralised formaldehyde.

2.2. Species enumeration

Phytoplankton subsamples ranging from 1 to 50 ml were allowed to settle in combined chambers. Two transects of the chamber bottom were examined for cell counting using an inverted light microscope at 400×. Between 41 and 169 *Pseudo-nitzschia* specimens were counted in the six samples on dates when the libraries were obtained. Depending on the sample richness and the volume settled, it is estimated that species less abundant than 33,000 cells l^{-1} (for 1 ml settled) and

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