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Pseudo-nitzschia simulans sp. nov. (Bacillariophyceae), the first domoic acid producer from Chinese waters

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ABSTRACT

The genus *Pseudo-nitzschia* has attracted attention because of production of the toxin, domoic acid (DA), causing Amnesic Shellfish Poisoning (ASP). *Pseudo-nitzschia* blooms occur frequently in Chinese coastal waters, and DA has been detected in several marine organisms, but so far no *Pseudo-nitzschia* strains from Chinese waters have been shown to produce DA. In this study, monoclonal *Pseudo-nitzschia* strains were established from Chinese coastal waters and examined using light microscopy, electron microscopy and molecular markers. Five strains, sharing distinct morphological and molecular features differentiating them from other *Pseudo-nitzschia* species, represent a new species, *Pseudo-nitzschia simulans* sp. nov. Morphologically, the taxon belongs to the *P. pseudodelicatissima* group, cells possessing a central nodule and each stria comprising one row of poroids. The new species is characterized by the poroid structure, which typically comprises two sectors, each sector located near opposite margins of the poroid. The production of DA was examined by liquid chromatography tandem mass spectrometry (LC–MS/MS) analyses of cells in stationary growth phase. Domoic acid was detected in one of the five strains, with concentrations around 1.05–1.54 fg cell^{-1}. This is the first toxigenic diatom species reported from Chinese waters.

1. Introduction

The genus *Pseudo-nitzschia* H. Peragallo is a group of pennate chain-forming diatom species globally distributed in marine waters, and known to produce the toxin domoic acid (DA) (Hasle, 2002). Following an intoxication incident in Canada in 1987, which was found to be caused by *P. multiseries* (Hasle) Hasle, species of *Pseudo-nitzschia* attracted attention and considerable progress has been made since then in our understanding of species diversity and toxicology. The number of described species in the genus has increased during the past two decades, and currently comprises 48, of which 24 are known to produce DA (Teng et al., 2016; Lundholm, 2017).

Identification of *Pseudo-nitzschia* often requires a combination of different methods, including colony traits and cell shape by light microscopy, frustule ultrastructure by electron microscopy, phylogenetic analyses based on molecular markers, ITS2 secondary structure comparison, sexual mating experiments and physiological traits (reviewed in Lelong et al., 2012; Trainer et al., 2012). Molecular tools have proved the presence of cryptic and pseudo-cryptic species in the genus (e.g. Amato et al., 2007; Lim et al., 2012; Lundholm et al., 2012; Orive et al., 2013; Percopo et al., 2016; Teng et al., 2016). Most recently described species of *Pseudo-*

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nitzschia are from the *P. pseudodelicatissima* complex, which is characterized by one row of poroids in each stria, and each poroid divided into a number of sectors (Lundholm et al., 2003, 2012; Amato et al., 2007; Lim et al., 2012, 2013; Teng et al., 2014, 2016).

In the past two decades, the toxicity of potentially toxic microalgae has been tested in several Chinese strains, especially focusing on dinoflagellates such as the genera *Alexandrium*, *Karenia* and *Dinophysis*, because most negative effects in Chinese coastal waters were related to blooms of these and related species (Lu et al., 2014). *Pseudo-nitzschia* blooms have also been found frequently in China (Qi et al., 1994; Lü et al., 2012), usually in early spring and summer. Some of the 24 potentially toxic *Pseudo-nitzschia* species have been recorded in China and appear to be relatively common and abundant (Qi et al., 1994; Lü et al., 2012). But no Chinese *Pseudo-nitzschia* strains have proved positive for DA production, in spite of, presently, no less than 186 strains have been tested (Li et al., 2005; Yang, 2007; Xu, 2015). Chinese *Pseudo-nitzschia* species were therefore considered not to produce DA. On the other hand, DA had been detected in a number of different marine organisms such as clams, shells and scallops (Chen et al., 2001; Li et al., 2002; Song et al., 2008; Ji et al., 2011; Wang, 2011), indicating the presence of DA producers in the water. It was therefore considered whether other diatom species could be responsible for DA production, as DA has been shown also in two additional pennate diatoms, *Nitzschia navis-varingica* and *N. bizertensis* (Lundholm and Moestrup, 2000; Romero et al., 2011; Smida et al., 2014), or whether unreported DA-producing *Pseudo-nitzschia* species could be present.

In this study, monoclonal *Pseudo-nitzschia* strains were established from Chinese coastal waters and examined using light microscopy, electron microscopy and molecular markers (both LSU and ITS of the rDNA), as well as information from comparison of the secondary structure of ITS2. Five strains were identified as belonging to the same species, but different from other species with regard to morphological and molecular data. The strains are described here and considered to represent a new species, *Pseudo-nitzschia simulans* sp. nov. The production of DA in these strains was examined by a liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis, and DA was detected in one of the five strains. This is the first known DA producer in Chinese coastal waters.

2. Material and methods

2.1. Sampling, isolation and culturing

Live samples were collected by a plankton net haul (10 μm mesh size) at four localities along the Chinese coast, representing a temperate, a subtropical and two tropical localities (Fig. 1). Using a glass micro-pipette under an inverted microscope (Mshot MI12, Guangzhou, China), single cells or chains of *Pseudo-nitzschia* were isolated into a 96-well cell culture plate (Greiner Bio-One GmbH, Frickenhausen, Germany), each well containing ca. 250 μL L1-medium with a salinity of 30 (Guillard and Hargraves, 1993). The plate was incubated at about 22 °C in a 12:12 light:dark (L:D) cycle with illumination provided by cool fluorescent lamps. When cell abundance reached >100 cells mL^{-1} (manually counted by inverted light microscopy), the culture was transferred into a triangular glass flask. The clonal cultures of *Pseudo-nitzschia* are listed in Table 1. For DNA sequencing, culture aliquots were concentrated by centrifugation and frozen at −20 °C.

2.2. Morphological observations

Cultures in mid-exponential phase were harvested for morphological studies. Light microscopical observations were performed using an Olympus BX-53 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP27 camera. The length of the valve and the ratio of overlapping valve part relative to total valve length were measured by LM on at least 20 randomly selected cells from each culture.

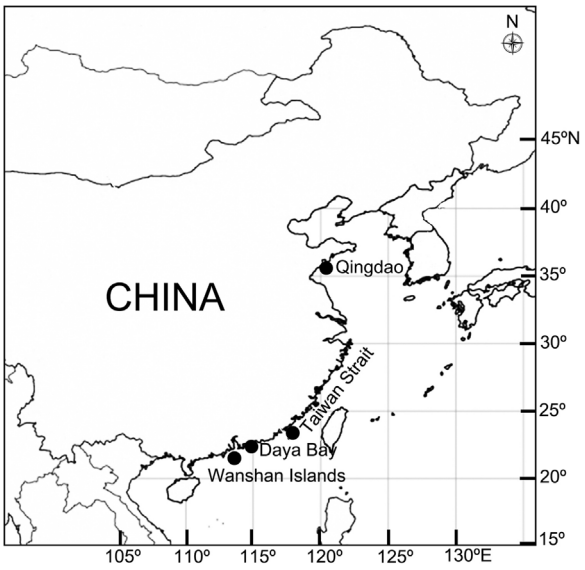

Fig. 1. Sampling sites along the Chinese coast.

Diatom frustules were cleaned by transferring about 10 mL culture aliquots into 75 mL Erlenmeyer flasks; an equivalent volume of concentrated H_2SO_4 was added and the material was boiled for 20–30 min in a water bath. Samples were subsequently rinsed several times with distilled water until a neutral pH was obtained. For transmission electron microscopy, the acid-cleaned material was dried onto Formvar-coated copper grids for examination in a JEOL-1010 transmission electron microscope (TEM) (Jeol, Tokyo, Japan). Morphometric characters, such as the width of valve, the density of striae, fibulae and poroids on the valve, the density of poroids on valvocopula, and the number of sectors within each poroid, were measured on the TEM micrographs.

For statistical analyses on the morphometrics, one-way ANOVA with Bonferroni-Holm post hoc tests were done using Daniel's XL Toolbox add-in for Excel, version 6.22 (Kraus, 2016).

2.3. Molecular and phylogenetic analyses

Genomic DNA extraction was performed as described in Lundholm et al. (2002), and the D1–D3 region of the large subunit ribosomal DNA (LSU rDNA) was amplified using the primers D1R-F (Scholin et al., 1994) and D3B-R (Nunn et al., 1996). The internal transcribed spacers ribosomal DNA (ITS rDNA), comprising ITS1, 5.8S and ITS2, was amplified using the primers ITS1 and ITS4 (White et al., 1990). Polymerase chain reaction (PCR) products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer, and sent to BGI Corporation (BGI Co. Ltd., Guangzhou, China) for sequencing, using the same primers as for PCR.

For phylogenetic analyses inferred from LSU rDNA and ITS rDNA, sequences of the five *Pseudo-nitzschia* strains obtained in this study, plus those selected by taxon sampling in NCBI GenBank, were included in the alignment. Sequences were aligned and edited manually in BioEdit (Hall, 1999), and for both alignments *Bacillaria paxillifer*, *Cylindrotheca closterium* and *Nitzschia navis-*