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Diatom *Nitzschia navis-varingica* (Bacillariophyceae) and its domoic acid production from the mangrove environments of Malaysia

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ABSTRACT

The distribution of the toxic pennate diatom *Nitzschia* was investigated at four mangrove areas along the coastal brackish waters of Peninsular Malaysia. Eighty-two strains of *N. navis-varingica* were isolated and established, and their identity confirmed morphologically and molecularly. Frustule morphological characteristics of the strains examined are identical to previously identified *N. navis-varingica*, but with a sightly higher density of the number of areolae per 1 μm (4–7 areolae). Both LSU and ITS rDNAs phylogenetic trees clustered all strains in the *N. navis-varingica* clade, with high sequence homogeneity in the LSU rDNA (0–0.3%), while the intraspecific divergences in the ITS2 data set reached up to 7.4%. Domoic acid (DA) and its geometrical isomers, isodomoic A (IA) and isodomoic B (IB), were detected in cultures of *N. navis-varingica* by FMOC-LC-FLD, and subsequently confirmed by LC–MS/MS, with selected ion monitoring (SIM) and multiple reaction monitoring (MRM) runs. DA contents ranged between 0.37 and 11.06 pg cell^{-1}. This study demonstrated that the toxigenic euryhaline diatom *N. navis-varingica* is widely distributed in Malaysian mangrove swamps, suggesting the risk of amnesic shellfish poisoning and the possibility of DA contamination in the mangrove-related fisheries products.

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1. Introduction

Domoic acid (DA), a neurotoxin that binds irreversibly to the glutamate receptor in the vertebrate central nervous system (Pulido, 2008), was first discovered from the red alga *Chondria armata* (Kützing) Okamura (Takemoto and Daigo, 1958). The toxin was responsible for amnesic shellfish poisoning (ASP) (reviewed in Lelong et al., 2012; Fernandes et al., 2014) that can be fatal to humans after consuming DA-contaminated fishery products. The source of DA contamination was diatoms of the genus *Pseudo-nitzschia* (e.g., Bates and Bird, 1989; Lelong et al., 2012; Dao et al., 2014). The massive bloom of *Pseudo-nitzschia* along the west coast of North America in 2015 caused a closure of crab and molluscan shellfisheries for an unprecedented time. This affected not only the local economy but also the fishing communities (McCabe et al., 2016). DA has also disrupted the marine food web, resulting in mass mortalities of marine mammals (sea lions and whales) at higher trophic levels (Lefebvre et al., 2016).

Diatoms of the genus *Nitzschia*, on the other hand, have so far never been associated with any human poisonings, even though two species have been confirmed to produce DA: *N. navis-varingica* Lundholm and Moestrup (Lundholm and Moestrup, 2000) and *N. bizertensis* Bouchouicha Smida, Lundholm, Sakka and Hadj Mabrouk (Bouchouicha Smida et al., 2014). The toxigenic *N. navis-varingica* was discovered for the first time from shrimp-culture ponds in Vietnam (Kotaki et al., 2000), and subsequently was found in brackish waters and estuaries throughout the Southeast Asian region (Kotaki et al., 2004, 2005, 2006, 2008; Bajarias et al., 2006; Romero et al., 2008, 2011, 2012; Takata et al., 2009; Thoha et al., 2012; Suriyanti and Usup, 2015). Conversely, the distribution of *N. bizertensis* was limited to Bizerte Lagoon, Tunisia, the type locality (Bouchouicha Smida et al., 2014).

While most toxigenic *Pseudo-nitzschia* species are planktonic, *N. navis-varingica* was frequently found in the benthic brackish

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ecosystem (e.g., Kotaki et al., 2004, 2005; Romero et al., 2012). Mangroves are one of the tropical-substropical brackish ecosystems that play an important role as shelters for marine life. They often host many commercially valuable molluscan shellfish (e.g., benthic clams: *Polymesoda*; rock oysters: *Crassostrea*; razor clams: *Solen*) and crustacean species (mud crabs: *Scylla serrata*) (Rönnbäck, 1999; Honculada-Primavera, 2000), which may serve as vectors of DA (W.N. Noordin and P.T. Lim, per. comm.).

In the Western Pacific region, DA contamination in bivalves was limited to Vietnam (Dao et al., 2006, 2009; Huyen et al., 2006; Takata et al., 2009), Philippines and Japan (Takata et al., 2009). Only studies from Vietnam confirmed that DA contamination in bivalves was attributed to DA-producing *Pseudo-nitzschia* species (Dao et al., 2014, 2015). *Pseudo-nitzschia* species, including several novel and toxic ones, have been widely reported from coastal waters of the Straits of Malacca and South China Sea (e.g., Lim et al., 2013, 2014; Teng et al., 2014, 2015). Conversely, information on the occurrence and distribution of toxigenic *Nitzschia* species is very scattered. The aim of this study was to document the presence of toxigenic *N. navis-varingica* from the mangrove habitats of Peninsular Malaysia, and to further examine the morphology, genetics and toxin production of the Malaysian strains. Morphological characters of *Nitzschia* species were based on wild and cultured specimens, using light and electron microscopy. The phylogenetic relationship of *N. navis-varingica* was inferred based on the nuclear-encoded ribosomal DNA (rDNA) in the large subunit (LSU) and the internal transcript spacer (ITS) regions. Toxicity of some strains was analysed by liquid chromatography (FMOC-LC-FLD) and further confirmed by mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Sampling sites, sample collection and algal cultures

Sampling was undertaken at four mangrove areas (Fig. 1) where bivalve shellfish and crabs have been harvested by local populations for trading and personal consumption. Tok Bali (Kelantan; 5.8611° N, 102.5149° E), located at the north-eastern Peninsular Malaysia, is known for the production of benthic clams (*Polymesoda similis*) and rock oysters (*Crassostrea iredalei*); Linggi River (Negeri Sembilan; 2.3970° N, 101.9825° E and Malacca; 2.3883° N, 101.9713° E), on the west coast of the Peninsula, is an important site for the collection of mussels by artisanal fishermen; Muar (Johor; 2.0669° N, 102.5586° E), located at the south-western coast of the Peninsula, is well known for oyster harvesting; Pendas, situated at the Tebrau Strait (Johor; 1.3759° N, 103.6388° E), is a fishing village.

Plankton samples were collected with a 20-μm mesh plankton net using the kick-net sampling method (Kotaki et al., 2000). Live samples were transferred into 500-ml bottles, and brought back to the laboratory to establish cultures. Single cells were isolated using a finely drawn Pasteur pipette under a Leica DM3000 microscope (Leica, Germany). Generally, *Nitzschia* appeared at low densities; thus, the field samples were first enriched (see below) and incubated in continuous light for 24–48 h prior to cell isolation.

Cultures were established and grown in f/2 medium (Guillard and Ryther, 1962), at 25 ± 0.5 °C, salinity of 27, and a light intensity of 100 μmol photons $m^{-2} s^{-1}$ under 12:12 h light:dark photoperiod. Clonal cultures of *N. navis-varingica* established and used in this study are shown in Supplementary Table S1.

2.2. Morphological observation

Live cells of *Nitzschia* (8–12 days after inoculation) were observed under a Leica DM3000 microscope (Leica, Germany), with a 400 × magnification. For TEM, samples were acid-cleaned as described in Teng et al. (2013). A drop of the acid-cleaned cells was then transferred onto a Formvar-coated copper grid. Samples were observed under a JEOL JEM-1230 TEM (JEOL, Tokyo, Japan). Morphometric measurements of valve length and width, density of interstriae, fibulae, and areolae of >30 cells were obtained.

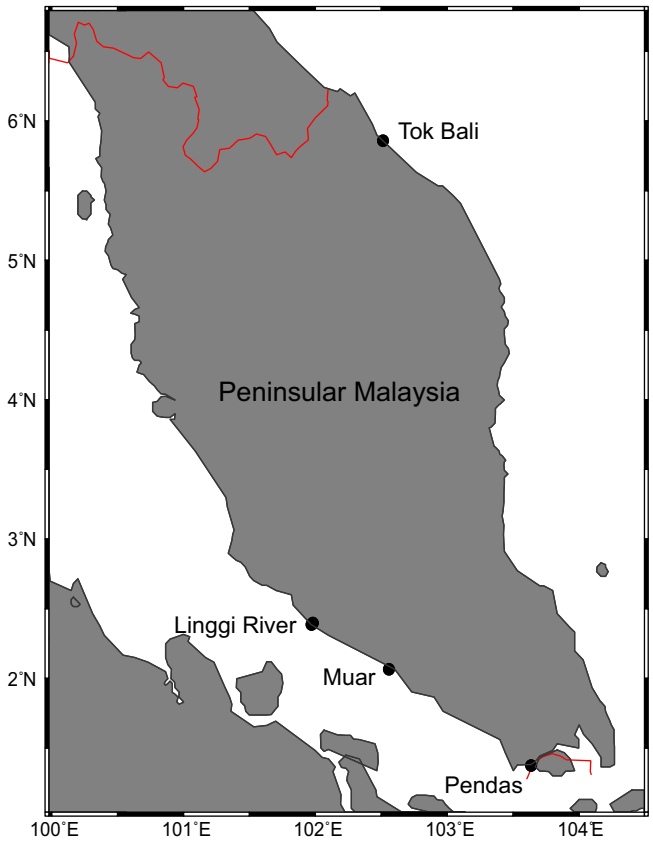

Fig. 1. Map of Peninsular Malaysia showing sampling sites of *Nitzschia* species.

2.3. DNA extraction, gene amplifications and sequencing

Cultures at mid-exponential phase were harvested by centrifugation (2600 × *g*, 10 min), lysed by adding 2 × Cetyl-trimetyl-ammonium-bromide (CTAB) lysis buffer, and the genomic DNA was extracted as described in Lim et al. (2012). Large subunit ribosomal DNA (LSU rDNA) in the domain D1–D3 was amplified using the primer pair DIR and D3Ca (Scholin et al., 1994), and the internal transcribed spacer (ITS) region was amplified using ITS1 and ITS4 (White et al., 1990). PCR was performed in a peqSTAR 96X universal gradient thermocycler (Erlangen, Germany) with the following PCR conditions: 94 °C for 5 min for the initial denaturation, followed by 35 cycles of denaturation (94 °C for 45 s), annealing (52 °C for 30 s) and elongation (72 °C for 90 s), and completed with a final extension at 72 °C 8 min. The amplicons were purified using the QIAquick DNA purification kit (Qiagen, Hilden, Germany). DNA sequencing was performed on an ABI 3770XL automated sequencer (PE Applied Biosystems, Foster City, CA) on both strands.

2.4. LSU rDNA analyses and phylogenetic reconstruction

For the LSU rDNA data set, 16 nucleotide sequences of *Nitzschia* were obtained in this study. These sequences, and 11 of *Nitzschia* retrieved from NCBI GenBank nucleotide database (Supplementary Table S2), were multiple-aligned using MUSCLE (Edgar, 2004).

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