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Pseudo-nitzschia species, a possible causative organism of domoic acid in *Spondylus versicolor* collected from Nha Phu Bay, Khanh Hoa Province, Vietnam

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Abstract—Recently, it was reported that a significant level of domoic acid, a causative toxin of amnesic shellfish poisoning (ASP), was detected in bivalves belonging to the genus *Spondylus* collected from various tropical areas, including Vietnam, whereas no significant domoic acid was detected in other bivalve species. These facts suggest that the causative plankton species for domoic acid occur widely in tropical waters and that *Spondylus* spp. accumulate domoic acid more effectively than other bivalve species. In the monitoring on seasonal change of domoic acid levels in *S. vesicolor* and plankton net samples in Nha Phu Bay, Vietnam, domoic acid level of *S. versicolor* was found to increase when domoic acid was detected in the plankton net samples. Under light microscopic observation of the plankton samples, cells of *Pseudo-nitzschia* spp., the size of which was smaller than toxic *Pseudo-nitzschia* such as *P. multiseries*, were observed. When the plankton net samples were fractionated by successive filtration through the sieves and membrane filters with different pore sizes, most of the domoic acid was recovered in the particle fraction with smallest size (0.6–10 μm). Some strains of unicellular cultures established from the cells in this fraction showed the production of domoic acid, though the productivity was low. All the domoic acid-producing strains seemed to be the same species. These results indicate that *Pseudo-nitzschia* species is also causative for domoic acid in tropical bivalves.

Key words: domoic acid, amnesic shellfish poisoning, *Pseudo-nitzschia* sp., *Spondylus*, tropical bivalve

Introduction

Domoic acid is an excitatory amino acid responsible for amnesic shellfish poisoning (ASP) which was first found in Prince Edward Island, Canada in 1987 (Bates et al. 1989). Since this incident, accumulation of domoic acid in bivalve was reported from several areas in the world (Amzil et al. 2001, Horner and Postel 1993). However, these areas are limited in temperate waters. There is little knowledge on domoic acid accumulation in bivalve in tropical waters except those in *Spondylus versicolor* found widely in Southeast Asia (Takata et al. 2009).

Dao et al. (2009) found that during the period when domoic acid level of *S. versicolor* collected in Nha Phu Bay, Khanh Hoa Province, Vietnam was increasing, a significant level of domoic acid was detected in the plankton samples, showing the correlation between these two parameters. These findings show the occurrence of domoic acid-producing plankton in the bay, and that this plankton could be the source of domoic acid accumulated in *S. versicolor*. However, no significant number of *Pseudo-nitzschia* spp. was observed in the plankton samples containing domoic acid in the bay during 2004 and 2006. Generally, the width of *Pseudo-nitzschia* cells is narrow enough to pass through the 20 μm mesh size of the plankton net, suggesting that net hauling with 20 μm mesh is not suitable for the collection of *Pseudo-nitzschia* cells. It is suspected that small-sized plankton species, which are hardly trapped by plankton net with 20 mm mesh size are possibly involved in accumulation of domoic acid of *S. versicolor* in Vietnamese water. Thus, in this study, plankton cells collected by plankton net with 20 μm mesh were subdivided into fractions based on their sizes by successive filtration through sieves with different mesh sizes, and domoic acid in each fraction was analyzed. At the same time, unicellular cultures of several strains were prepared for toxin analysis.

Materials and Methods

Domoic acid in different—size plankton

Sample collection: From March to April, 2007, 5 specimens of *S. versicolor* (Fig. 1) were collected biweekly in Nha

Fig. 1. *Spondylus versicolor.*

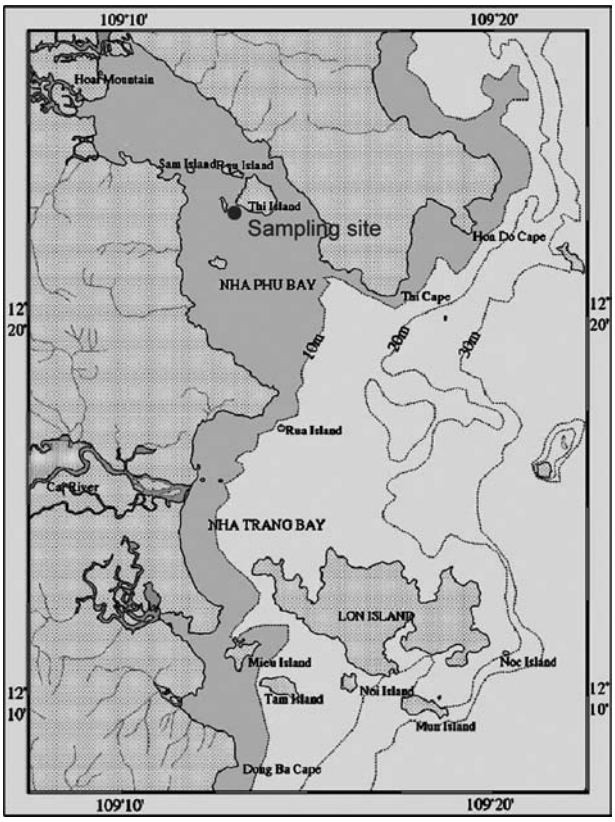

Fig. 2. Sampling site in Nha Phu Bay, Khanh Hoa Province, Vietnam.

Phu Bay (Fig. 2). When domoic acid level of *S. versicolor* started to increase, sampling frequency was increased to twice a week. At the same time of shellfish sampling, plankton samples were collected by vertical hauling from 2 m depth to the surface (20 times) using a plankton net (mesh size: 20 μm, diameter: 30 cm). On April 17, two sets of plankton samples were collected.

Extraction and analysis of domoic acid in plankton samples: The plankton cells of each net sample were collected by centrifugation (1,000 G, 15 min). Cell pellet samples thus obtained were extracted with an equal volume of water under boiling for 5 min. After centrifugation, domoic acid in the supernatant was analyzed by HPLC according to Kodama and Kotaki (2005). Domoic acid level of the plankton samples was expressed as ng l^{-1} of seawater. The minimum detectable concentration of domoic acid in the test solution required for S/N=3 at 20 μl injection was 9 ng ml^{-1} in HPLC applied in the present study. However, it was affected by the level of impurities which was different among the samples. In the present study, 90 ng ml^{-1} of the test solution was applied for quantification limit when 20 μl of samples was injected. Domoic acid in the plankton extracts was further confirmed by LC-MS/MS (API-2000, Applied Biosystems, CA, USA) with Wakosil Navi C18-5 column (2×150 mm, Wako) using a linear gradient elution from 0.1% trifluoroacetic acid to 100% acetonitrile within 15 min at a flow rate of 0.2 ml min^{-1}. The electrospray ionization (ESI) was operated in product ion scan mode in which N_2 was used as dissolvent, cone and collision gas (curtain gas: 50 psi, ion spray voltage: 5500 V, ion-source gas 1: 40 psi, ion-source gas 2: 60 psi, declustering potential: 20 V, focusing potential: 200 V, entrance potential: 10 V, collision energy: 30 eV, collision cell exit potential: 15 V). Presence of domoic acid in the extract was confirmed by comparison of the retention time of positive pseudo-molecular ion ($[M+H]^+=m/z$ 312) on LC and the fragment ions with those of domoic acid standard. The aqueous solution of domoic acid (Diagnostic Chemicals Limited, Charlottetown PEI, Canada), the concentration of which was calibrated using domoic acid standard (DACS-1C, National Research Council of Canada, Halifax, NS, Canada), was used for reference toxin for UV-HPLC and LC-MS/MS analysis.

Fractionation of plankton sample: Plankton cells in one of the two plankton samples collected on April 17 were fractionated to four subsamples with different cell sizes. The sample was successively filtered through nylon gauzes each with the mesh size of 200, 100 or 10 μm, and a GF/F filter of 0.6-μm pore size. Cells on each sieve were rinsed with filtered seawater though GF/F filter (Whatman). A portion (1/10 to 1/20) of each fraction was fixed with formalin for quantitative observation of phytoplankton species under a light microscope. The rest of plankton cells in >200 μm, 100–200 μm and 10–100 μm fractions were centrifuged (1,000 G, 15 min) to obtain the cell pellets. Domoic acid in

these pellets was extracted with equal volume of water under heating for 5 min. After centrifugation (1,000 G, 15 min), the supernatant was analyzed for domoic acid by HPLC as described above. Cells in the 0.6–10 μm fraction were harvested by filtration through GF/F filter. The plankton cells in the 0.6–10 μm fraction retained on the filter were extracted together with the filter by 5 ml of water under heating for 5 min. After heating, the tube was centrifuged (1,000 G, 15 min) to obtain the extract. The supernatant was then ultrafiltered through a membrane (NMWL 5000, Millipore), and then analysed for domoic acid by HPLC as described above.

Domoic acid in Pseudo-nitzschia*-like strains*

Sample collection: Plankton net samples were collected by Van Dorn water sampler from 1 m layer of Nha Phu Bay on April 17, 2007 when domoic acid level of *S. versicolor* was increasing and a slight level of domoic acid was detected in the net sample collected on the same day.

Establishment of cultures of Pseudo-nitzschia *species:* A single cell or a chain of the *Pseudo-nitzschia*-like cell was isolated by capillary method under an inverted microscope, washed several times in filtered seawater through membrane with a pore size of 0.2 μm, and then inoculated to modified T1 medium (Ogata et al. 1987, Omura et al. 2003) with salinity 30 in a well of the 24 multi-well plated. The inoculated plates were incubated at 26 and 20°C under a light intensity of 150 μmol photons $m^{-2}s^{-1}$ with LD cycle of 12:12. When the cells grew, a part of culture was inoculated to larger tubes for culture to obtain a larger culture.

Analysis of domoic acid in the cultures: Ten mL of the culture was centrifuged after ultrasonication (10,000 *g*, 30 min). Domoic acid in the supernatant extract was analyzed by HPLC according to Kodama and Kotaki (2005). Briefly, the filtrate was acidified by addition of 1N HCl to pH 2–3. Domoic acid in the filtrate was trapped by Sep-Pak C18+ cartridge. After the cartridge was washed with water, the trapped domoic acid was eluted by 70% methanol. After dried up by evaporation, the residue was dissolved in one mL of water. Domoic acid in the extract was analysed by HPLC described above.

Results and Discussion

Domoic acid in small-sized plankton

Domoic acid level in *S. versicolor* increased from 8 ± 2 $\mu g\ g^{-1}$ (March 29) to 11 ± 3 $\mu g\ g^{-1}$ (April 13), and then to 17 ± 9 $\mu g\ g^{-1}$ (April 17), showing that domoic acid in *S. versicolor* was increasing during this period. The level of domoic acid in this bivalve in the present study was lower than that in previous study (Dao et al. 2009). Seasonal variation pattern of domoic acid in *S. versicolor* was repeated in the same manner as previous years, showing that the same phenomenon recurred in Nha Phu Bay. In other words, the domoic acid-producing plankton species bloom in Nha Phu Bay.

Table 1. Cell number of total plankton, *Pseudo-nitzschia* and *Nitzschia* species in the different-sized fractions of the plankton net sample.

Particle size fraction	Density of total plankton (cells l^{-1})*1	Density of *Pseudo-nitzschia* spp. (cells l^{-1})*1	Density of *Nitzschia* spp. (cells l^{-1})*1
More than 200 μm	1798	71	71
100–200 μm	1335	149	7
10–100 μm	1451	170	71
0.6–10 μm	354	71	131

*1: Cell number in the fraction is expressed as cells in 1 liter of seawater.

Table 2. Domoic acid level in the different-sized fractions of the plankton net sample.

Particle size fraction	Volume of extract (ml)	Level of domoic acid (ng l^{-1})*3
More than 200 μm	1.2*1	not detected
100–200 μm	0.5*1	not detected
10–100 μm	0.6*1	0.06
0.6–10 μm	5.0*2	0.6

*1: Plankton cells harvested by centrifugation were extracted with equal volume of water.
*2: Plankton cells harvested by filtration through GF/F filter were extracted with 5 ml of water.
*3: Domoic acid level is expressed as ng of domoic acid contained in 1 liter of seawater.

In the present study, a low level of domoic acid (0.2 ng l^{-1} of seawater) was detected only in the plankton sample collected on April 13. No domoic acid was found in the samples of March 29 and April 17, suggesting that the toxic species appeared in a low density and then soon disappeared.

In contrast, when analyzed the domoic acid in size-fractionated plankton, a certain amount of domoic acid was detected in the 0.6–10 μm fraction of the plankton net sample on April 17 (0.6 ng l^{-1}). Whereas a small amount of domoic acid was also detected in the 10–100 μm fraction (0.06 ng l^{-1}). In Table 1, the results of domoic acid analysis of all the particle fractions are summarized. These results indicate that most of the plankton species that contained domoic acid were trapped in the 0.6–10 μm fraction. In other words, the size of the toxic plankton species was small enough to pass through the sieve with 10 μm pore.

Table 2 shows the composition of *Pseudo-nitzschia* spp. and *Nitzschia* spp. in each size fraction. Cells belonging to both genera were observed in all the fractions. However, they are dominant in the 0.6–10 μm fraction in which most of do-

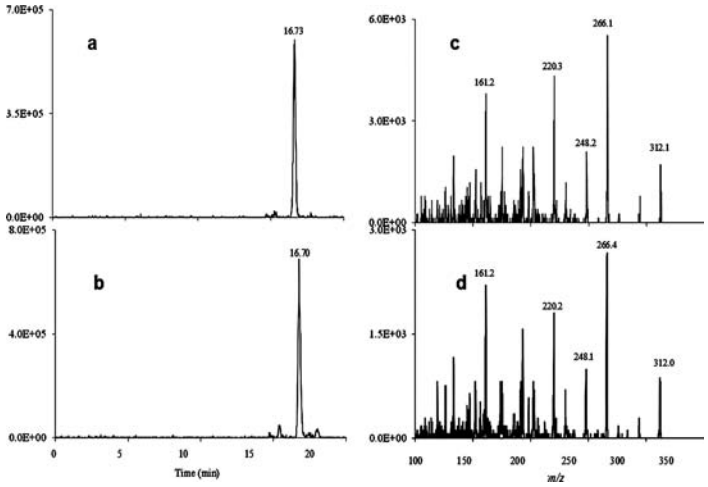

Fig. 3. Confirmation of domoic acid in *Pseudo-nitzschia* sp. by LC-MS/MS. a: Elution pattern of domoic acid standard in LC scanned by $(M+H)^+$ 312 *m/z*. b: Elution pattern of *Pseudo-nitzschia* sp. in LC scanned by $(M+H)^+$ 312 *m/z*. c: Fragmentation pattern of DA standard, d: Fragmentation pattern of *Pseudo-nitzschia* sp.

Table 3. List of the species and the number of strains in culture experiment.

	Species	Number of strains
1	*Amphiprora alata*	1
2	*Bacillaria* sp.	2
3	*Cylindrotheca closterium*	7
4	*Entomoneis* sp.	1
5	*Gyrosigma* sp.	1
6	*Navicula* sp.	5
7	*Nitzschia* sp.	5
8	*Pleurosigma* sp.	1
9	*Pseudo-nitzschia* spp.	11
10	*Rhizosolenia setigera*	3

moic acid was concentrated, though at least 15 species of phytoplankton mostly consisting of small-sized diatom species were observed in the fraction (data not shown). These strongly suggest that small sized species belonging to *Pseudo-nitzschia* and/or *Nitzschia* observed in the 0.6–10 μm fraction are causative for domoic acid production in the bay.

Pseudo-nitzschia *as domoic acid-producer*

In the analysis by HPLC, the culture strains of seemingly belonging to a genus *Pseudo-nitzschia* showed the presence of domoic acid. In the further analysis by LC-MS/MS, the occurrence of domoic acid was confirmed (Fig. 3). These results clearly show that the origin of domoic acid accumulated in tropical bivalves is also species belonging to *Pseudo-nitzschia*.

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