Toxin profile of *Alexandrium tamarense* (Dinophyceae) from Hokkaido, northern Japan and southern Sakhalin, eastern Russia

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Abstract: To evaluate the variations in the toxin profiles of *Alexandrium tamarense* (Dinophyceae) from Hokkaido and southern Sakhalin, paralytic shellfish poison of 103 culture strains obtained from six areas (Funka Bay, Tomakomai, Urakawa, Akkeshi, Okhotsk Sea and Aniva Bay) was analyzed using high performance liquid chromatography-fluorescent detection. All of the strains, except for two culture strains, were classified by a hierarchical cluster analysis into clusters in which the strains had almost the same toxin composition as found in previous studies (major toxins: C2, gonyautoxin 4, neosaxitoxin and gonyautoxin 3). On the other hand, cellular toxin contents of the culture strains varied from 1 to 1,128 fmol cell⁻¹ and the cellular toxin content (y fmol cell⁻¹) was inversely proportional to the cell density (x cells mL⁻¹) ($y = 9942.2x^{-0.448}$, Spearman's rank correlation coefficient: $r_s = 0.539$, $p < 0.01$). It can be concluded that the toxin compositions of *A. tamarense* are almost the same in Hokkaido and southern Sakhalin, and high-toxicity blooms of *A. tamarense* as usually found possibly occur in the natural environment at low cell densities (10⁻²–10² cells mL⁻¹).

Key words: *Alexandrium tamarense*, Hokkaido, paralytic shellfish poison, Sakhalin, toxin profile

Introduction

The toxic dinoflagellate, *Alexandrium tamarense* (Lebour) Balech, has been recognized as a major causative organism of paralytic shellfish poisoning (PSP) throughout the world (Hallegraeff 1993). It is reported that *A. tamarense* occurs in coastal waters from the Pacific Ocean to the Okhotsk Sea in Hokkaido, and a large number of cysts of *A. tamarense* were found in the bottom sediments in the regions where PSP occurrences have been frequently recorded (Shimada & Miyazono 2005). It was found that C-toxin, gonyautoxin (GTX), neosaxitoxin (neoSTX) and saxitoxin (STX) were detected from culture strains of *A. tamarense* (e.g. Kaga et al. 2006), and the toxin compositions that varied among strains were inherited following the Mendelian rules (Sako et al. 1995). Recently, Nagai et al.

(2007) revealed that several diverse populations can be identified in *A. tamarense* collected along the coast of Japan using microsatellite markers, and inferred that gene flow has possibly occurred among the populations due to human-assisted dispersal (e.g. shipping of bivalve spat). Hallegraeff (1995) pointed out that dinoflagellate cells can be and have been dispersed by ballast water in ships. From these reports, it is supposed that diverse populations of *A. tamarense* have a complex distribution pattern in Hokkaido, so that various populations with different toxin profiles may occur because bivalve culture, especially scallops, is a major industry along the coast of Hokkaido. Furthermore, it was suggested that *A. tamarense* blooms originate by the germination of cysts on the continental shelf from Hokkaido to Sakhalin in the Okhotsk Sea (Shimada et al. 2010). However, there is a paucity of information about the variation in toxin profiles and cellular toxin content of *A. tamarense* and their relation to the geographical distribution. If *A. tamarense* occurs in the fishing grounds for bi-

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valves, this information is important to allow estimation of the potential toxin composition and content in bivalves. The aims of the present study are to clarify and compare the toxin composition and the cellular toxin content among culture strains of *A. tamarense* isolated from sediments or seawater from Hokkaido and southern Sakhalin.

Materials and Methods

Bottom sediment or seawater samples for establishing culture strains of *Alexandrium tamarense* were collected at six areas from Funka Bay, southwestern Hokkaido to Aniva Bay, southern Sakhalin in August 2000–May 2009 (Fig. 1, Table 1). Sediment samples collected using a Smith-McIntyre or Ekman-Birge grab sampler were preserved in airtight containers at 3°C in a refrigerator. To obtain vegetative cells of *A. tamarense* from cysts, sieved samples

Fig. 1. Map showing the six sampling areas in Hokkaido and southern Sakhalin.

(10–100 μm) of 2–3 g sediments were suspended with 100 mL of GeO_2 -added, filtered seawater (final conc. 1 mg L^{-1}), and 10 mL subsamples of the suspended samples were dispensed into culture plates (6 wells) and incubated for two weeks at 10°C under a 14 h : 10 h light : dark cycle with ca. $400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided as white fluorescent illumination (color temperature = 5,000 K).

For establishing clone cultures, vegetative cells of *A. tamarense* were washed with sterilized seawater using capillary pipettes and cultured for two weeks in 1 mL of SWIIIm medium (Sako et al. 1990) dispensed into culture plates (48 wells) under the same conditions as mentioned above. Clone cultures were obtained after the 1 mL cultures were transferred into the 10 mL of SWIIIm medium in test tubes and cultured for two weeks under the same conditions. Seawater samples were collected in Funka Bay using a Van-Dorn water sampler. Vegetative cells in the clone cultures obtained from the seawater were similarly washed and cultured as mentioned above. Axenic condition of the culture strains was not confirmed.

Identification of *A. tamarense* was performed on the basis of morphology after Balech (1995) under an epifluorescence microscope (XF-EFD2, Nikon, Tokyo, Japan) with UV excitation after calcofluor staining (Fritz & Triemer 1985).

Subsamples of the culture in log phase or stationary phase (approx. 10^2 – 10^4 cells mL^{-1}) were selected for toxin analysis. The 10 mL subsamples were centrifuged (450 G, for 10 min) and the supernatants were removed, and the cell pellets were fixed with acetic acid (conc. 0.5 M). Filtrate samples for high performance liquid chromatography-fluorescent detection (HPLC-FLD) were obtained after the fixed subsamples were sonicated using an ultrasonic homogenizer and then were centrifuged (25,000 G, for 10 min) and filtered through filter tubes (Ultrafree C3GC, Millipore, Massachusetts, USA) to remove high-molecular-weight substances ($\text{MW} > 10,000$). PSP were analyzed using HPLC-FLD (high pressure pump: L-6000, intelligent pump: L-6200, column oven: L-5030, fluorescent detector: F-1080, auto sampler: AS-2000, Hitachi, Tokyo, Japan) ac-

Table 1. List of sampling sites and culture strains.

Area	Location		Source of sample	Date of sampling (d/m/y)	Date of clone culture established (d/m/y)	Number of culture strains (abbreviated name in Fig. 3)	Research vessel*
	Latitude	Longitude					
Funka Bay	42°14.0'N	140°16.0'E	bottom sediment	24 Feb 2005	25 Jun 2006	16 (FKS)	Boat
Funka Bay	42°14.0'N	140°16.0'E	seawater	24 May 2006	24 May 2006	10 (FKW)	Boat
Tomakomai	42°29.9'N	141°33.9'E	bottom sediment	19 Apr 2005	23 Jun 2005	11 (TMS)	Kinsei Maru
Urakawa	42°07.8'N	142°44.8'E	bottom sediment	19 Apr 2005	23 Jun 2005	6 (URS)	Kinsei Maru
Akkeshi Bay	42°58.0'N	144°49.0'E	bottom sediment	8 Aug 2000	25 Jun 2007	30 (AKS)	Boat
Okhotsk Sea	45°30.1'N	142°49.8'E	bottom sediment	27 May 2009	30 Jun 2009	10 (OKS)	Hokuyo Maru
Aniva Bay	46°30.0'N	142°54.0'E	bottom sediment	28 Aug 2003	23 Jun 2005	20 (ANS)	Boat

* Boat: Light fishing boat <20 ton

Kinsei Maru: 151 ton, R/V of Hokkaido Hakodate Fisheries Research Institute

Hokuyo Maru: 237 ton, R/V of Hokkaido Wakkanai Fisheries Research Institute

cording to the method of Oshima (1995). Cell densities of *A. tamarens* in the subsamples were estimated by counting the number of cells of *A. tamarens* in 0.1 mL of the culture under a microscope. Cellular toxin contents of the strains were calculated from the toxin contents and the cell densities in the subsamples.

For statistical evaluation of the variations in toxin composition in the culture strains, we performed hierarchical cluster analysis (unweighted pair group method with arithmetic mean) using “R” software (ver. 2.10) with “pvclust” package (Suzuki & Shimodaira 2006). Quotients of mean deviation (MD) and standard deviation (SD) of relative frequency (%) of PSP components in the culture strains were used in the dataset for the cluster analysis. Hierarchical clusterings were performed according to multi-scale bootstrap resampling (Shimodaira 2002) (number of resam-

plings=1,000). We recognized that the clusters with approximately unbiased p -values (AU) larger than 95% consist of culture strains with similar toxin profiles.

To analyze the relationship between the cell density and the cellular toxin content of the culture strains, power function curve fitting was performed using the least squares fitting method. Statistical significance of the regression equation was examined using Spearman’s rank correlation coefficient (r_s).

Results

The average composition of PSP in the culture strains of *Alexandrium tamarens* obtained from six areas are shown in Fig. 2. The culture strains of *A. tamarens* obtained from all areas contained C2, gonyautoxin 4 (GTX4), neoSTX

Fig. 2. Average composition of paralytic shellfish poison (mean ± standard deviation) in the culture strains of *Alexandrium tamarens* obtained from the six sampling areas. Error bars indicate standard deviation. “N” mean number of culture strains. Results of the culture strains obtained from bottom sediment and seawater in Funka Bay are described separately.

and gonyautoxin 3 (GTX3) as the major components and C1, gonyautoxin 1 (GTX1), gonyautoxin 2 (GTX2), decarbamoyl-gonyautoxin 3 (dcGTX3) and STX as the minor components. The results of the cluster analysis of the PSP compositions in the culture strains are shown in Fig. 3. The PSP compositions of the culture strains were respectively classified into a single cluster that shows the toxin composition as detailed above, while excluding only two culture strains (approximately unbiased p -value=99%). The toxin compositions of the two culture strains outside the cluster resembled the other cultures with respect to the major toxin components (C2, GTX4, neoSTX), though AKS-10 (from Akkeshi Bay) contained minimal GTX3 and STX, and TMS-09 (from Tomakomai) had a relatively high content of STX (Fig. 4). From these results, it is clear that the toxin compositions of the vegetative cells of *A. tamarens* obtained from the six areas in Hokkaido and southern Sakhalin can be classified into a single group with only a few exceptions.

The relationship between the cell density and the cellular toxin content of the culture strains of *A. tamarens* is shown in Fig. 5. Cellular toxin contents of the culture strains varied from 1 to 1128 fmol cell⁻¹ and the cellular toxin content (y fmol cell⁻¹) was inversely proportional to the cell density (x cells mL⁻¹) ($y=9942.2x^{-0.448}$, Spearman's rank correlation coefficient: $r_s=0.539$, $p<0.01$). A clear inverse relationship was found between cell density and cellular toxin contents, with extremely high cellular toxin levels at low cell densities.

Discussion

The present study tried to clarify and compare the PSP profiles of vegetative cells of *Alexandrium tamarens* obtained from six areas around Hokkaido and Sakhalin. It has been reported that the culture duration for germination of the cysts of *A. tamarens* is ca. 10 days (Miyazono 2002a) and the maximum cell division rate is ca. 0.4 division day⁻¹ (Miyazono 2002b) under the same conditions as in the present study. It is therefore quite likely that the vegetative cells obtained in the culture for two weeks can be considered to be cells germinated from different cysts and are therefore a wide enough sample to be representative of sediment samples obtained from the six areas because the culture duration in the present study was probably not long enough for cell division in *A. tamarens*. On the other hand, considering the similar toxin compositions of the culture strains obtained from sediments and seawater in Funka Bay (Fig 2), the toxin compositions of the culture strains from sediments are a good representation of the regional characters in the six areas. Therefore, according to the results of the present study it is reasonable to conclude that the PSP profiles of culture strains of *A. tamarens* obtained from the six areas around Hokkaido and Sakhalin were almost the same.

The toxin compositions in the present study resembled

Fig. 3. Result of cluster analysis of the paralytic shellfish poison compositions of the 103 culture strains. Clusters with approximately unbiased p -values larger than 95% are highlighted by a broken-line rectangle, which are strongly supported by the data. “AU” and “BP” on the edges of the clustering mean approximately unbiased p -values and bootstrap probabilities (number of resamplings=1,000). “Height” on the scale on the top indicates branch length of the dendrogram.

Fig. 4. Paralytic shellfish poison compositions of the 101 culture strains in the clusters highlighted by the rectangle in Fig. 3 on average (mean ± standard deviation) and 2 culture strains (AKS-10 and TMS-09) outside the clusters.

those in the reports for southern Sakhalin (Orlova et al. 2007), Iwate Prefecture (Kaga et al. 2006), Sendai Bay (Ichimi et al. 2002) and Hiroshima Bay (Asakawa et al. 2005) with respect to the four major toxin contents (C2, GTX4, neoSTX and GTX3). These results suggest that the toxin compositions of *A. tamarens* are almost the same throughout Japan, though several different populations were found using microsatellite markers (Nagai et al. 2007) and the populations might be supposed to show different toxin compositions.

If *A. tamarens* have almost the same toxin compositions, it is reasonable to expect that the toxin composition accumulated in bivalves may reflect the typical toxin profile

Fig. 5. Relationship between the cell density (cells mL⁻¹) and the cellular toxin content (fmol cell⁻¹) of the culture strains of *Alexandrium tamarens*. Scatter diagram is plotted using different symbols to distinguish among the sources of the samples. Regression equation of curve fitting with Spearman's rank correlation coefficient (r_s) is described on the left side of the diagram.

of *A. tamarens*. It has been reported that the unstable β -epimer (C2, GTX4 and GTX3) in *A. tamarens* is gradually transformed into the α -epimer (C1, GTX1 and GTX2) in bivalves while the equilibrium point of the α/β ratio was 3 (Oshima et al. 1992). Takata et al. (2004) found the transformation from β -epimer into α -epimer in three bivalve species, oyster (*Crassostrea gigas* (Thunberg)), scallop (*Mizuhopecten yessoensis* (Jay)) and mussel (*Mytilus edulis* Linnaeus) in Kure Bay (Hiroshima Prefecture, western Japan), while the quantity of the toxin accumulated among bivalve species varied remarkably (scallop > mussel > oyster). Similarly, Murakami et al. (1998) reported that α -epimer was accumulated in surf clam (*Pseudocardium sachalinense* (Sehrenck)) in addition to the β -epimer in Kashima Nada, Ibaraki Prefecture, eastern Japan. In Funka Bay, it was reported that the major toxin contents of PSP-infested scallop were GTX1, GTX2, GTX3, GTX4, STX and neoSTX, while C-toxins were not analyzed (Asakawa & Takagi 1983). Considering the present results and those of previous studies, it can be assumed that bivalves generally accumulate β -epimer (C2, GTX4 and GTX3), α -epimer (C1, GTX1 and GTX2) and neoSTX as their major toxin contents when PSP infestation of bivalves by *A. tamarens* occurs in Hokkaido. On the other hand, PSP infestation of bivalves by *Alexandrium catenella* (Whedon et Kofoid) Balech has occurred in Funka Bay in autumn 1988 (Noguchi et al. 1990). It has been reported that the major toxin contents of *A. catenella* from northern Japan are C2 and neoSTX (Oshima et al. 1990, Noguchi et al. 1990, Kaga et al. 2006). Oshima et al. (1990) found that the major toxin contents of scallop infested with *A. catenella* were C1, neoSTX and C2 in Funka Bay in 1988. If PSP infestation of bivalves by *A. catenella* occurs, it can be supposed that the bivalves will accumulate C1 (α -epimer of C2), C2 and neoSTX as the major toxin contents, differing from

those of *A. tamarens*e. Thus the results of the present study are useful to deduce the causative organism based on the toxin composition in bivalves.

The present study elucidated that the cellular toxin content (y fmol cell⁻¹) of *A. tamarens*e was inversely proportional to the cell density (x cells mL⁻¹) ($y=9942.2x^{-0.448}$). This result suggests that a vegetative cell in a low cell density environment (10⁻²–10 mL⁻¹) as usually found *in situ* has a very high toxicity (approx. $\geq 10^4$ fmol cell⁻¹). Oshima et al. (1990) found that the cellular toxin content of *A. tamarens*e collected from Funka Bay fluctuated from 35 to 280 fmol cell⁻¹ during the culture experiment. Sakamoto & Kotani (1998) pointed out that the cellular toxin content of *A. tamarens*e obtained from Kure Bay became higher at lower cell densities. In addition, Matsuda et al. (2006) elucidated that the cellular toxin content of *A. catenella* was much higher in the culture experiment under phosphate stress applied for maintaining a low growth rate. Our result supports these previous studies showing that the cellular toxin content of *A. tamarens*e fluctuates with the cell density in culture experiments. However, there have been no reports published to date about high toxin content ($>10^3$ fmol cell⁻¹) in clone cultures of *A. tamarens*e such as found in the present study. To explain the high toxin production of the several culture strains in our study, it seems necessary to examine the genetic character of the culture strains. Oh et al. (2010) reported that PSP contents of the culture strains of *Gymnodinium catenatum* Graham ranged between 56.6 and 740 fmol cell⁻¹ in Inokushi Bay (Ooita Prefecture, western Japan) and some strains with different toxin profiles probably originated from other areas. This report suggested that research initiatives are needed to investigate the population structure based on molecular biological methods. Genetic studies on high-toxicity strains of *A. tamarens*e should be done in the near future. Nishihama (1982) reported that a bloom (approx. 10³ cells L⁻¹) of *A. tamarens*e occurred and the PSP toxicity of cultured scallop drastically increased to 550 mouse unit g⁻¹ digestive diverticula in only two weeks in Funka Bay in 1979. The mechanism of PSP infestation in such a case can be partially explained by assuming that, as found in the present study, vegetative cells of *A. tamarens*e *in situ* may have extremely high toxicity (approx. $\geq 10^4$ fmol cell⁻¹). However, there are no previous reports about the occurrence of high-toxicity cells of *A. tamarens*e with a cellular toxin content of 10⁴ fmol cells⁻¹ along the coast of Japan. White (1986) estimated that the cellular toxin content of *Gonyaulax excavata* (Braarud) Balech (= *A. tamarens*e) collected *in situ* ranged from 2.7×10^{-6} to 1.1×10^{-3} μ g STX cells⁻¹, which can be converted to “from 9.0×10^{-6} to 3.7×10^{-3} nmol STX cells⁻¹” in the Bay of Fundy, southeastern Canada. This highest toxicity value is the only instance reported of the occurrence of *A. tamarens*e with such high toxicity, namely approximately 10 times larger than the highest cellular toxin content in our results. It is necessary to prove the occurrence of *A. tamarens*e with high toxicities

such as in our assumption and the report of White (1986), in the coastal waters in Japan through *in situ* sampling, and to analyze the cellular toxin content of *A. tamarens*e in the future.

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