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The application of species specific DNA-targeted probes and fluorescently tagged lectin to differentiate several species of *Pseudo-nitzschia* (Bacillariophyceae) in Chinhae Bay, Korea*

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SUMMARY: We applied molecular methods using a DNA probe and fluorescently tagged lectins to discriminate toxic *Pseudo-nitzschia multiseries* from the Chinhae Bay for a Korean harmfulalgae monitoring program. From the binding activity of lectins, *P. multistriata*, *P. subfraudulenta*, *P. pungens*, *P. multiseries* and *P. cuspidata* bound ConA, whereas *P. subpacifica* and *P. delicatissima* did not. Ribosomal RNA-targeted oligonucleotide probes (muD1, puD1 and deD1) specifically reacted to *P. multiseries*, *P. pungens* and *P. delicatissima*, respectively, whereas auD1, frD1 and amD1 probes did not bind to *P. multiseries*, *P. pungens*, *P. cuspidata*, *P. multistriata*, *P. subpacifica*, *P. subfraudulenta* and *P. delicatissima*. In particular, fluorescent FITC-conjugated WGA specifically bound to *P. multiseries* but not to *P. pungens*, indicating that this is a desirable method for their rapid and easy discrimination. In addition, we tested a species-specific oligonucleotide DNA probe (muD1) using the whole cell hybridization filter tube system, and the WGA lectin probe to discriminate *P. multiseries* in the field. The oligonucleotide probe and fluorescent WGA bound specifically to *P. multiseries* and these labelled cells were correlated to label cell abundance. These results imply that DNA and lectin probes are apporopriate tools for counting *P. multiseries* and distinguishing morphologically similar *Pseudo-nitzschia* species in natural samples, therefore, these methods are especially pertinent since rapid separation and quantitative estimation of cell abundance of *P. multiseries* are now important for a routine harmful algae monitoring program in Korean waters.

Key words: *Pseudo-nitzschia*, differentiation, DNA probes, FITC-conjugated lectins.

INTRODUCTION

The first Korean record of the blooms dominated by *Pseudo-nitzschia* spp. was from Masan Bay in 1975 (Yoo and Lee, 1980). Since then, *Pseudo-nitzschia* blooms have been annual features in Korean coastal waters (Lee, 1994; Kim *et al*., 1997; Lee and Baik, 1997). Recently, domoic acid (DA) causing amnesic shellfish poisoning (ASP) was detected in Korean shellfish after the implementation of a comprehensive biotoxin monitoring program following a suspected shellfish poisoning event. Recent serious instances of DA contamination in mussels have occurred in many parts of Chinhae Bay, a commercial mussel harvesting site. Both phytoplankton monitoring and shellfish flesh testing programs have led to a database which has helped to link *Pseudo-nitzschia* species to specific DA occurrences. It is known that DA-contaminated cultured blue mussels caused 107 cases of human poisoning

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and three deaths in Canada (Bates *et al*., 1989, 1991), but no fish kills or economic impacts were associated with blooms caused by *Pseudo-nitzschia* in Korean coastal waters (Kim *et al*., 1997).

Pseudo-nitzschia species that occur in Chinhae Bay include *P. pungens*, *P. subpacifica*, *P. multistriata* and *P. subfraudulenta* (Cho *et al*., 1999a). However, it is difficult to identify these species under the light microscope due to their morphological similarity (Hasle, 1994). This requires observations using an electron microscope, which are time-consuming and costly (Scholin *et al*., 1994). Attempting to solve these problems, some researchers have assessed FITC-conjugated lectins, which exhibit a special characteristic of binding to specific sugars on the cell surface, as a tool for differentiating toxic from non-toxic microalgae (Fritz, 1992; Rhodes *et al*., 1995, 1998a, b; Fraga *et al*., 1998). In 1999, we isolated toxic *P. multiseries* from Chinhae Bay, but when the light microscope was used, it was often misidentified in the field as non-toxic *P. pungens*. In a previous report, lectin probes enabled a risk assessment of biotoxin monitoring events in Korean coastal waters (Cho *et al*., 1998). Subsequently, we tested the application of fluorescently tagged lectins to discriminate four *Pseudo-nitzschia* species and then suggested that this method should be able to identify several species of *Pseudo-nitzschia* in the field (Cho *et al*., 1999a). We also showed that this was a promising tool for phytoplankton monitoring in Korean waters.

In this work, we apply a DNA fluorescent probe-based whole cell hybridization assay, as well as a fluorescently labelled lectin probe, for rapid detection and enumeration of several species of *Pseudo-nitzschia*. We also compared the ability of the DNA probe and the fluorescently labelled lectin probe to discriminate toxic *P. multiseries* from non-toxic *P. pungens* in the context of the Korean biotoxin monitoring program.

MATERIAL AND METHODS

Isolation

We isolated 7 species of *Pseudo-nitzschia* from Chinhae Bay (Table 1). They were collected by picking individual cells with a micropipette under the light microscope or by a serial dilution (Cho *et al*., 1998). After a clonal culture was established, the species were maintained and cultured in f/2+Si (Guillard and Ryther, 1962). These stains were grown in 100 ml polycarbonate flasks at 20°C, under an intensity of 100 μmol m^{-2} s^{-1} continuous light provided by cool-white fluorescent lamps.

TABLE 1. – List of *Pseudo-nitzschia* species used in this study and their toxicity.

Species	Toxicity[a]
Pseudo-nitzschia multistriata	-
P. subpacifica	-
P. subfraudulenta	-
P. pungens	-
P. multiseries	+
P. cuspidata	-
P. delicatissima	-

[a]Toxicity was analyzed by reversed-phase high-performance liquid chromatography with a fluorescence detector (Ex. 264 nm, Em. 313 nm) supported with UV detection. DA concentration in *P. multiseries* at day 20 was 221.8 ng/mL, 196.4 ng/mL and 25.4 ng/mL in the whole culture, cell-free medium and cell fraction, respectively. The cellular content of DA was estimated at 2.4 pg/cell.

Morphological analysis

For the scanning electron microscope (SEM) observations, 5 ml of *Pseudo-nitzschia* culture were fixed with Lugol's solution. They were acid-cleaned (1M HCL) and filtered through a 5μm pore-size polycarbonate membrane filter under no pressure (Lee and Baik, 1997). The washed samples on the filter were air dried, transferred to an aluminum stub, coated with deionized gold, and then examined with an Hitachi S-4200 SEM.

Application of fluorescent by labelled lectins probes

Fresh solutions of FITC-conjugated lectins (Table 2; Vector Lectin Kit, fluorescein FLK-2100, Vector Laboratories Inc., Burlingame, CA 94010) were prepared as described by Kim *et al*. (1995). Fluorescein isothiocyanate (FITC)-labelled lectins (Table 2) were added to 10 μl aliquots of 10^3 cells on glass slides for 40 min at room temperature. Slides were coated with a solution of 3-aminopropyltriethoxy-saline (3%). The treated cells were mounted on siliconised glass slides and examined for binding activity under an epifluorescence microscope: inverted Carl Zeiss MC-80 with an FITC filter set using blue light (excitation, 450-480 nm; emission, 515 nm). Binding activity was determined as described by Cho *et al*. (1998).

TABLE 2. – Lectin probes used in this study.

Lectin	Source	Specificity
ConA	*Canavalia ensiformis*	Methyl α-D-mannopyranoside; D-mannose; D-glucose
RCA	*Ricinus communis*	β-D-galactose
DBA	*Dolchis biflorus*	N-acetyl-D-galactosamine
PNA	*Arachis hypogaea*	α-lactose; D-galactose
SBA	*Glycine maxima*	N-acetyl-D-galactosamine; D-galactose; methyl α-D-galactopyranoside
UEA	*Ulex europaeus*	L-fucose
WGA	*Triticum vulgaris*	N-triacetylchitotriose; N-diacetylchitobiose; sialic acid

Whole-cell probing of Pseudo-nitzschia species

At least 2,000 cells were captured on 25 mm, 0.8 μm pore-size Nuclepore Track-Etch membrane filters placed in custom made filter tubes. We filtered 10 ml of whole water per tube and used the least amount of vacuum possible (>100 mmHg). Once the cells were captured on the filter, the following was sequentially added to each tube: 5 ml of freshly prepared saline EtOH fixative, 3 ml 25 X SET (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris pH 7.8), 25 ml high-grade 95% ethanol, 2 ml dH_2O. The collected cells were allowed to fix at room temperature for 1 h. Afterward, samples were filtered and rinsed briefly with 1 ml hybridization buffer [(189.0 ml dH_2O, 48.0 ml 25 X SET, 2.4 ml 10% IGEPAL-CA630 (Sigma)], 0.6 ml Polyadenylic acid (Sigma). Cells were filtered and then 500 μl of hybridization buffer were added to each tube to resuspend cells before adding 12 μl of the appropriate probes, cap tubes then placed manifold into a waterbath or dry incubator at 45°C for 1 hr to hybridise. After hybridisation, filter and then risen briefly with 1 ml of 5 X SET (dilution of 25 x SET) to remove excess unbound probe for 10 minutes. After filtration, place membranes onto glass slides, add 20 μl Slow Fade Light (Molecular probes) to each membrane, mount cover slip, then viewed under a Nikon Optiphot microscope with a FITC optical filter set (excitation 450-490 nm; emission 529 nm). This study used 8 kinds of probes (Scholin *et al*., 1996); muD1 (specific probe for *P. multiseries*), puD1 (specific probe for *P. pungens*), auD1 (specific probe for *P. australis*), frD1 (specific probe for *P. fraudulenta*), amD1 (specific probe for *P. americana*), deD1 (specific probe for *P. delicatissima*), uniC (positive control for all eukaryotes), uniR (negative control to test for non-specific retention of probe) and no probe (negative control to compare uniR for autofluorescence versus non-specific retention of probe). To estimate cell density for discriminate *P. multiseries*, nine natural samples in this study were collected from different Chinhae Bay (Chinhae, Wuchong, Masan, Hapo, Nampo, Myongdong, Lidong, Dukdong and Jindong) and immediately returned to the laboratory and concentrated with a mesh size of 20 μm. To identify and enumerate toxic *P. multiseries*, we counted cells labelled with the fluorescent WGA lectin and the muD1 DNA probe using an epifluorescent microscope as above.

RESULTS

Morphological characteristics of *Pseudo-nitzschia* species by SEM

During this study, the genus of *Pseudo-nitzschia* occurred in Chinhae Bay was *P. pungens*, *P. multiseries*, *P. cuspidata*, *P. multistriata*, *P. subpacifica*, *P. subfraudulenta* and *P. delicatissima* (Table 3). *Pseudo-nitzschia pungens* showed 9-12 fibulae in 10 μm, two rows of poroids, and three to four poroids in 1 μm (Fig. 1a). The morphologically similar *P. multi-*

TABLE 3. – Morphometric data of *Pseudo-nitzschia* species occurred in Chinhae Bay.

Species	Apical axis (μm)	Central nodule	Fibulae in 10 μm	Row of poroids	Poroids in 1 μm
P. pungens	85-130	-	9-12	2	3-4
P. multiseries	70-100	-	13-14	3-4	4-6
P. cuspidata	60-65	+	15-16	1	5-6
P. multistriata	60-65	-	22-23	2	10-11
P. subpacifica	35-45	+	13-17	2	6-7
P. subfraudulenta	70-85	+	14-16	2	5-6
P. delicatissima	30-45	+	21-22	2	10-12

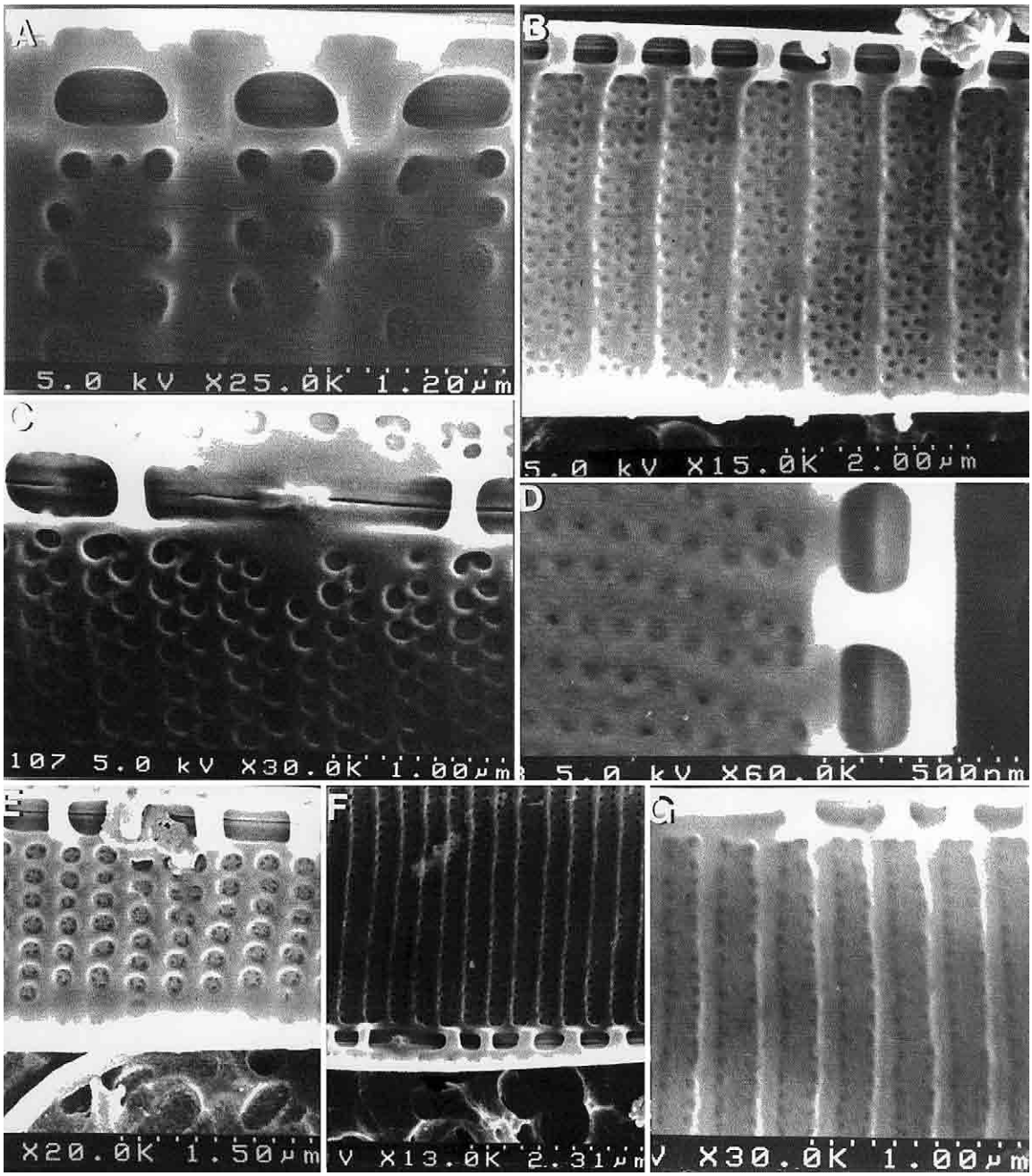

FIG. 1. The *Pseudo-nitzschia* species observed in Chinhae Bay: A: *P. pungens*, B: *P. multiseriese*, C: *P. cuspidata*, D: *P. multistriata*, E: *P. subpacifica*, F: *P. subfraudulenta*, G: *P. delicatissima*. The scale bar is represented on the bottom of each Figure.

series had 13-14 fibulae in 10 μm, three to four rows of poroids, and four to six poroids in 1 μm (Fig. 1b). However, both strains showed a similar characteristics of cell length (70-130 μm) and the absence of central nodule. *Pseudo-nitzschia cuspidata* and *P. multistriata* had a similar cell length (60-65 μm), but the number of fibulae in 10 μm, the number of rows of poroids, and poroids in 1 μm were different; 15-16, 1 and 5-6 in *P. cuspidata,* and 22-23, 2 and 10-11 in *P. multistriata*, respectively (Fig. 1c, d). Central

TABLE 4. – Binding of *Pseudo-nitzschia* species to FITC-labelled lectins.

Strains	ConA	PNA	SBA	UEA	WGA	DBA	RCA
Pseudo-nitzschia multistriata	+	–	–	–	–	–	–
P. subpacifica	–	–	–	–	–	–	–
P. subfraudulenta	+	–	–	–	–	–	–
P. pungens	+	–	–	–	–	–	+
P. multiseries	+	–	–	–	+	–	+
P. delicatissima	–	–	–	–	–	–	–
P. cuspidata	+	–	–	–	–	–	–

TABLE 5. – Response of different *Pseudo-nitzschia* spp. to DNA probes. The symbols '+' and '-' represent binding and non-binding to DNA probes tested, respectively.

Species	muD1[1]	puD1[2]	auD1[3]	frD1[4]	amD1[5]	deD1[6]	uniC[7]	uniR[8]	no probe[9]
P. pungens	-	+	-	-	-	-	+	-	-
P. multiseries	+	-	-	-	-	-	+	-	-
P. cuspidata	-	-	-	-	-	-	+	-	-
P. multistriata	-	-	-	-	-	-	+	-	-
P. subpacifica	-	-	-	-	-	-	+	-	-
P. subfraudulenta	-	-	-	-	-	-	+	-	-
P. delicatissima	-	-	-	-	-	+	+	-	-

[1]specific probe for *P. multiseries*, [2]specific probe for *P. pungens*, [3]specific probe for *P. australis*, [4]specific probe for *P. fraudulenta*, [5]specific probe for *P. americana*, [6]specific probe for *P. delicatissima*, [7]positive control for all eukaryotes, [8]negative control to test for non-specific retention of probe, [9]negative control to compare against uniR for autofluorescence versus non-specific retention of probe

nodule was shown in *P. cuspidata*, whereas was not in *P. multistriata*. In *P. subpacifica* (cell length, 35-45 μm) there were 13-17 fibulae in 10 μm, two rows of poroids, and six to seven poroids in 1 μm (Fig. 1e). *Pseudo-nitzschia subfraudulenta* (cell length, 70-85 μm) had 14-16 fibulae in 10 μm, two rows of poroids, and 5-6 poroids in 1 μm (Fig. 1f). A smaller species, *P. delicatissima* was easily differentiated from the others by its smaller cell size (30-45 μm); 21-22 fibulae in 10 μm, two rows of poroids and 10-12 poroids in 1μm (Fig. 1g). Three species of *P. subpacifica*, *P. subfraudulenta* and *P. delicatissima* had central nodule.

Lectin binding to *Pseudo-nitzschia* species

The lectin response of the *Pseudo-nitzschia* species is given in Table 4. *Pseudo-nitzschia multistriata*, *P. subfraudulenta*, *P. pungens*, *P. multiseries* and *P. cuspidata* all bound with ConA, whereas *P. subpacifica* and *P. delicatissima* showed no binding with any of the different lectins in this study. The RCA probe bound with *P. pungens* and *P. multiseries*, which could also not be differentiated from each other under the light micscope because of their morphological similarity. However, WGA specifically conjugated to *P. multiseries*, which showed a fine fluorescent outline of the cell, whilst none bound to *P. pungens*.

DNA probe in the cultured *Pseudo-nitzschia* species

The *P. multiseries* probe (muD1), *P. pungens* probe (puD1) and *P. delicatissima* probe (deD1) were positive, whereas *P. cuspidata*, *P. multistriata*, *P. subpacifica* and *P. subfraudulenta* were not shown in 3 kinds of probes (Table 5). However, the auD1, frD1 and amD1 DNA probes did not bind to *P. multiseries*, *P. pungens*, *P. cuspidata*, *P. multistriata*, *P. subpacifica*, *P. subfraudulenta* and *P. delicatissima*.

muD1 probe and WGA lectin assay in field samples

Treatment of *P. multiseries* in nature with lectin WGA showed a green fluorescence signal on the cell surface against a background of other plankton and organic matter (Fig. 2a). In addition, the whole

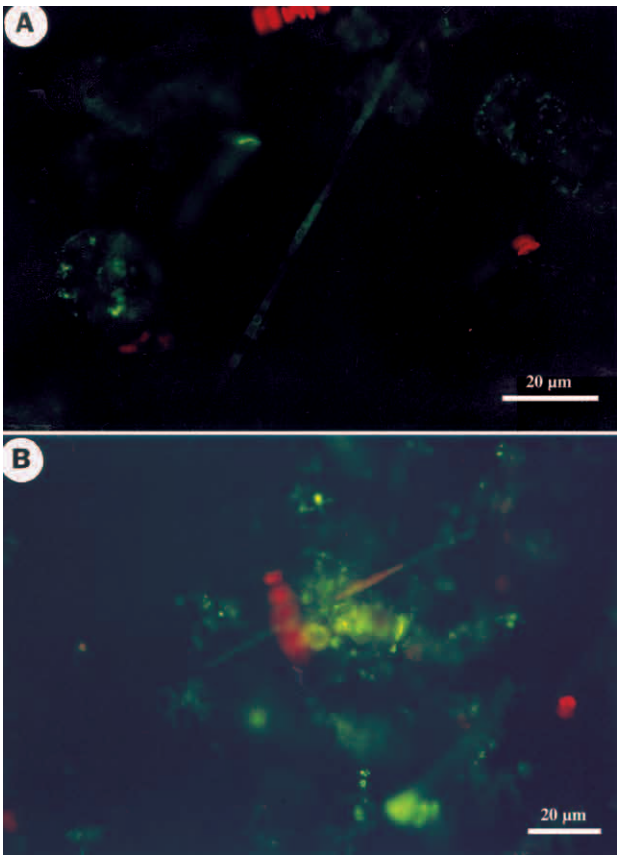

FIG. 2. Epifluorescence micrographs of toxic *P. multiseries* in the field. A: FITC-conjugated WGA lectin. B: Cells hybridized with species-specific probe muD1.

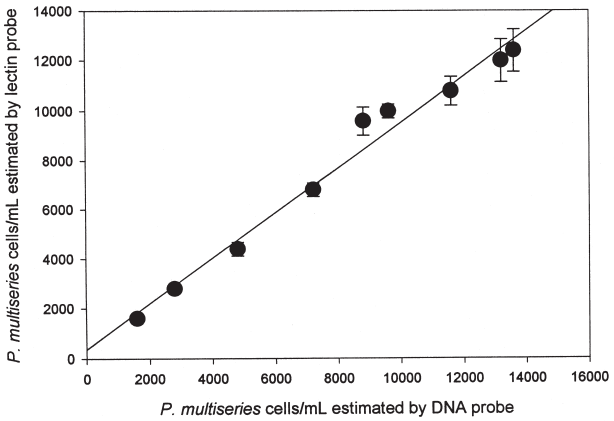

FIG. 3. Comparison of cell densities estimated by DNA probe and WGA lectin collected from the field.

cell muD1 probe successfully labelled only *P. multiseries* (Fig. 2b), and WGA lectin assay results were well correlated ($r^2 = 0.97$, $n = 9$, Fig. 3).

DISCUSSION

Five species of *Pseudo-nitzschia* (*P. cuspidata*, *P. multistriata*, *P. subpacifica*, *P. subfraudulenta* and *P. delicatissima*) are for the first time recorded in Korea. Although *P. cuspidata* had similarities to *P. multistriata* based on morphological features, the number of fibulae in 10 μm and poroids in 1 μm played on important role in differentiating *P. cuspidata* from *P. multistriata* (Table 3). However, discriminating *P. cuspidata* from *P. multistriata* depends on whether or not a central nodule is present. *Pseudo-nitzschia subpacifica* was morphologically similar to *P. heimii* as described by Hasle (1996), but *P. subpacifica* based on morphological characteristics of cell length, cell width, cell formation, *etc*, was shown in this study. However, the number of poroids in 1 μm are similar to *P. heimii*.

Lectins are differentiated by their specific carbohydrate affinity for glucose/mannose, galactose/N-acetyl-D-galactosamine, N-acetyl-glucosamine, fucose or sialic acids (Slifkin and Doyle, 1990). Because of their ability to bind with different carbohydrates at the cell surface, fluorescently tagged lectins have been used as probes for species identification (Costas and Lopez-Rodas, 1994; Rhodes *et al*., 1995; Cho *et al*., 1998; Fraga *et al*., 1998). In particular, when it is difficult to distinguish species under the light microscope, each could be easily differentiated according to its individual lectin binding affinity (Rhodes *et al*., 1995). Following this, several researchers have suggested that fluorescently tagged lectin probes are a potential tool for species identification in harmful algae monitoring programs (Costas *et al*., 1993, 1994, 1995; Rhodes *et al*., 1995). For example, Cho *et al*. (1998) reported that ECA, HPA and WGA could differentiate the harmful algal *Cohlodinium polykrikoides* from the nontoxic *Gyrodinium impudicum*, whose morphologies are so similar that it was difficult to identify them under the light microscope.

From Table 3, FITC-conjugated lectin probes allowed some differentiation of the diatoms *P. multistriata*, *P. subpacifica*, *P. subfraudulenta, P. pungens*, *P. multiseries*, *P. delicatissima* and *P. cuspidata* in this study. *Pseudo-nitzschia multistriata*, *P. subfraudulenta*, *P. pungens*, *P. multiseries* and *P. cuspidata* bound ConA, with a fine fluorescent outline of the cell being observed, whereas *P. subpacifica* and *P. delicatissima* did not bind ConA. ConA is suitable for differentiating *P. subpacifica* and *P. delicatissima* from the other tested *Pseudo-nitzschia* spp., but *P. multistriata*, *P. subfraudulenta* and *P. cuspidata* were not differentiated by fluorescent lectins. Meanwhile, *P. pungens* and *P. multiseries* both showed binding activity with ConA and RCA, suggesting that RCA could be used to easily discriminate *P. pungens* and *P. multiseries* from the other tested *Pseudo-nitzschia* species.

Pseudo-nitzschia multistriata, *P. subfraudulenta*, *P. pungens*, *P. multiseries* and *P. cuspidata* therefore have glucose and mannose like sugar moieties at the cell surface (Table 4), but are lacking glucosamine and galactosamine, compared to some other Korean coastal red tide microalgae (Cho *et al*., 1998). *Pseudo-nitzschia multiseries* and *P. pungens* have similar morphological features, as is the case with *C. polykrikoides* and *G. impudicum*, but different nucleic acid sequences (Douglas *et al*., 1994; Scholin *et al*., 1994; Manhart *et al*., 1995; Cho *et al*., 2001). However, WGA might play an important role in differentiating Korean *P. multiseries* from *P. pungens* (Table 3). This result agrees with those of Fraga *et al*. (1998) and Rhodes *et al*. (1998b) .Meanwhile, Rhodes (1998a) has reported that SBA lectin also could play a taxonomic tool as species identification of *P. multiseries* and *P. pungense*. However, *P. multiseries* and *P. pungens* showed no binding affinity with SBA in this study, it is likely that the production of surface sugars by *Pseudo-nitzschia* varies depending on geographical separation and environmental conditions.

Miller and Scholin (1996) suggested that the species-specific DNA probe method is a rapid, simple and cost-effective technique for discriminating among cultured *Pseudo-nitzschia* species. Additionally, Scholin *et al*. (1996) have reported a sandwich hybridization method for detecting *Pseudo-nitzschia* species in plankton samples, which was simpler than whole cell hybridization and was able to be automated. Scholin *et al*. (1997) suggested that both whole cell and sandwich hybridization were useful methods for detecting targeted species of *Pseudo-nitzschia* in culture and natural samples. In this study, *P. multiseries* treated with the DNA probe was readily identified and enumerated in field samples, although it had a weaker fluorescence signal intensity than cells treated with WGA lectins (Fig. 2). Therefore, detection and enumeration of toxic *P. multiseries* using this DNA fluorescent probe-based whole cell hybridization assay and the WGA lectin binding assay will be carried out in the near future for the Korean harmful algae monitoring program.

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