Identification of formalin-preserved eggs of red sea bream (*Pagrus major*) (Pisces: Sparidae) using monoclonal antibodies

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Catches of important commercial fish such as red sea bream, flat fish, and yellowtail are decreasing in Japan. In order to sustain these species it is especially important that their distribution and biomass at all life stages are known. However, information on the early life stages of these species is limited because identifying the eggs and larvae of such fish is sometimes extremely difficult.

Mito (1960, 1979) and Ikeda and Mito (1988) developed methods for identifying pelagic fish eggs based on morphological features. However, their methods have limitations because many unidentified eggs have similar features. In addition, eggs are usually fixed in formaldehyde solution just after collection in the field. This procedure may alter several egg characteristics and therefore prevent identification (Ikeda and Mito, 1988), or make identification difficult when the egg diameter measures 0.8–1.0 mm because so many kinds of eggs fall in that range. Thus, an alternative identification method would be useful.

Effective genetic analyses for identifying fish eggs or larvae (or both) have been developed by Graves et al. (1989), Daniel and Graves (1994), and Shao et al. (2002). However, their methods may have limitations if samples are preserved in formaldehyde for several years or if DNA must be extracted from numerous samples. In addition, we are lacking the DNA sequences for many species sequences that are necessary for identifying eggs in the field.

We have successfully produced monoclonal antibodies to differentiate harmful marine phytoplankton species from morphologically similar harmless species (Hiroishi et al., 1988; Nagasaki et al., 1991b; Sako et al., 1993; Vrieling et al., 1993; Hiroishi et al., 2002) as well as *Microcystis*, a toxic fresh water bloom-forming cyanobacteria (Kondo et al., 1998). These antibodies were obtained from a culture supernatant solution of hybridoma cells that was produced by a cell fusion procedure between myeloma cells and antibody-producing spleen cells. The specific antibodies described above could be used to detect and quantify harmful bloom-forming microorganisms that react with the monoclonal antibodies and that secondarily react with fluorescein isothiocyanate conjugated goat anti-mouse Ig(G+M) antibody. With fluorescence microscopy with B-exciting light, yellowish fluorescein coronas around the cells of the toxic species were observed, confirming a positive reaction. These antibodies can recognize different molecules distributed on the cell surface, even when the organisms have similar morphological features. One of the molecules distributed on *Chattonella* was determined to be glycoprotein (Nagasaki et al., 1991a). This method would help us to differentiate small marine organisms like fish eggs.

Red sea bream (*Pagrus major*) (Table 1) eggs can easily be distinguished from those of other sparids also found in Japan, such as *Acanthopagrus latus,* by differences in egg size and spawning seasons, and from those of *Evynnis japonica* by differences in spawning seasons (Ikeda and Mito, 1988; Kinoshita, 1988; Hayashi, 2000). However, eggs of some sparids, such as *Acanthopagrus schlegeli*, *Sparus sarba,* and *Dentex tumifrons* are extremely difficult to distinguish from eggs of *P. major*. Therefore, we developed monoclonal antibodies that allow *P. major* eggs to be clearly identified by immunostaining, thus differentiating them from other similar sparids.

This technique may be a useful new tool for identifying fish eggs. Here, we report a method for identifying *P. major* eggs using monoclonal antibodies developed to react specifically with the eggs.

Materials and methods

Eggs of *P. major* were obtained from adult female fish that had spawned in

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Table 1
Characteristics of Sparidae distributed in Japan.

Suborder	Species	Distribution	Spawning season	Egg size (mm)	Egg oil globule size (mm)
Pagrinae	*Pagrus major*	South of Hokkaido (Coastal)	Mar–May	0.90–1.03	0.19–0.25
	Evynnis japponica	South of Hokkaido (Coastal)	Oct–Dec	0.89–0.98	0.19–0.21
Sparinae	*Acanthopagrus schlegeli*	South of Hokkaido (Coastal)	Mar–Jun	0.83–0.91	0.20–0.22
	Acanthopagrus latus	South Japan (Coastal)	Oct–Nov	0.76–0.81	0.2
	Sparus sarba	South Japan (Coastal)	Apr–Jun	0.88–0.92	0.19–0.22
Denticinae	*Dentex tumifrons*	South Japan (Oceanic)	May–Jun	0.90–0.93	0.19

isolation tanks at several sea farming centers described in Table 2. Immediately after collection, fish eggs were fixed in a solution of 5% formaldehyde to sea water solution and stored. Before use, the eggs were thoroughly washed with distilled water and suspended in phosphate buffered saline (PBS) solution.

Monoclonal antibodies were developed according to the methods of Köhler and Milstein (1975), Garfré and Milstein (1981), and Hiroishi et al. (1984, 1988): 0.5 mL of egg suspension (200 eggs/PBS solution from Fukui Prefectural Sea Farming Center, Obama City, Fukui Prefecture) was mixed with 0.5 mL Freund's complete adjuvant (Nacalai Tesque, Inc., Kyoto, Japan). The mixture were then injected subcutaneously into BALB/c female mice (4 weeks of age). The female mice received second and third injections at 2-week intervals. For the final immunization, *P. major* eggs collected in the sea farming center of Kansai Environmental Engineering Center Co. (Miyazu City, Kyoto, Japan) were injected into the mouse after being emulsified with Freund's incomplete adjuvant (Nacalai Tesque, Inc.). Three days after the final immunization, the spleens of the mice were removed and passed through a mesh (mesh size: 100 μm). The spleen cells obtained by this procedure were fused with the myeloma cell line X63-AG8.653 at a ratio of 10:1 with 50% polyethylene glycol. After cell fusion, hybrid cells were incubated in a selective hypoxanthine-aminopterin-thymidine medium (Köhler, 1979; Garfré and Milstein, 1981).

The reactivity of the antibodies produced by the hybridomas was then determined. Eggs fixed with 5% formaldehyde in seawater were washed with PBS solution in a 96-well plate. Throughout the experiments, the principal eggs used were from the Fukui Prefectural Sea Farming Center. Normal horse serum solution (200 μL), diluted 100-fold with PBS, was added to the wells to prevent any nonspecific reactions. After incubation at room temperature for 20 minutes, the eggs were washed with 200 μL of PBS. After removing the PBS, 200 μL of the hybridoma culture supernatant solution was added to the wells and incubated at room temperature for 30 minutes. After washing with PBS (100 μL), biotinylated horse anti-mouse IgG (100 μL) was added to the wells and incubated at room temperature for 20 minutes. After the incubation, VECTASTAIN^R ABC reagent (avidin DH + biotinylated horseradish peroxidase/PBS, 100 μL) was added according to the direction of VECTASTAIN^R Elite ABC kit (ABC Mouse IgG Kit, Funakoshi Co., Tokyo, Japan). After immunostaining the eggs were observed by stereoscopic microscopy (SMZ-2T, Nikon Co., Tokyo, Japan). In a positive reaction, the surface of the fish egg was stained brown as a result of the oxidation of 3,3′-diaminobenzidine (substrate) by horseradish peroxidase bound to the egg surface by the antibody.

Unidentified pelagic fish eggs from open water were collected by using a plankton net (MTD net, NGG54 with mesh size of 0.344 mm, Rigo Co., Tokyo, Japan) from Wakasa Bay (Fukui Prefecture, Japan) in May 1997. They were fixed with 5% formaldehyde in sea water, either immediately or after incubation in seawater in finger bowls at 20°C for 24 hours, and identified by careful observation as described by Ikeda and Mito (1988) and Ikeda et al. (1991). The fixed eggs were transferred to net wells (mesh size 200 μm, diameter 24 mm, Corning Incorporated, Corning, NY) and washed with 10 mL of distilled water three times. Then the eggs in the netwells were immersed in 100 mL of PBS in a polystyrene tray (Corning Incorporated, Corning, NY) for 5 minutes. The egg suspension was placed into the wells of a six-well plate and incubated with 10 mL of normal horse serum solution for 20 minutes. After incubation, the eggs were incubated with 10 mL of MT-1 antibody solution (hybridoma culture supernatant) and then incubated with 10 mL of biotinylated horse anti-mouse IgG. The subsequent procedure was performed as described above.

The immunoglobulin subclass of monoclonal antibodies was determined according to the directions of the mouse monoclonal antibody isotyping kit (Amersham Pharmacia Biotech Co., Uppsala, Sweden) as follows: 3 mL of monoclonal antibodies solution (hybridoma supernatant solution) obtained in this study was added to 0.3 mL of horseradish peroxidase-conjugated anti-mouse IgG in the kit. An isotyping stick in the kit was incubated with the above solution at room tem-

perature for 15 minutes. Then the stick was washed with 0.1% Tween 20/PBS, and incubated with 4-chloro-1-naphthol solution (substrate of horseradish peroxidase in the kit) containing 0.1% H_2O_2 at room temperature for 15 minutes. The immunoglobulin subclass of the monoclonal antibodies was determined by observing the positions of bands that appeared on the stick.

Results and discussion

After cell fusion, hybridomas were grown in 42 wells of 96-well plates. Supernatant solutions of the cultures were used for the immunostaining assay to select hybridomas producing antibodies reactive to *P. major* eggs. After the assay, positive reactions were observed in six wells. These hybridomas were cloned by the limiting dilution method, and finally three clones producing monoclonal antibodies reactive with *P. major* were obtained. Those antibodies were named MT-1, MT-2, and MT-3. The subclass of all antibodies was IgG_1. Specificity of the antibodies was examined by using the eggs shown in Table 2. As a result, the antibodies were reactive with all the *P. major* eggs in both the early and late stages (before or after tail-bud stage), but not with eggs of other species (Table 3, Fig. 1). Thus, it becomes possible to identify *P. major* eggs. The immunostaining assay took 2.5 hours.

The oldest eggs of *P. major* (20 April, 1995) could react with the antibodies obtained as clearly as the recently collected eggs of *P. major*, indicating that egg samples preserved for up to 7 years could be analyzed by this method.

The method was also successful with 102 eggs collected from Wakasa Bay (Table 4), which had been immediately fixed with 5% formaldehyde in seawater. Among them, only 11 eggs were identified as *Callionymoidei* spp.

Table 2

Fish eggs used in the experiment

Egg no.	Species	Sampling location	Stage of eggs	Sampling day
1		Kansai Environmental Engineering Center (Miyazu, Kyoto Pref)	Early	20 Apr '95
2		Kansai Environmental Engineering Center (Miyazu, Kyoto Pref.)	Late	21 Apr '95
3		Fukui Prefectural Sea Farming Center (Obama, Fukui Pref.)	Early	2 Jun '95
4	*Pagrus major*	Fukui Prefectural Sea Farming Center (Obama, Fukui Pref.)	Late	2 Jun '95
5		Kyoto Prefectural Sea Farming Center (Miyazu, Kyoto Pref.)	Early	31 May '96
6		Kyoto Prefectural Sea Farming Center (Miyazu, Kyoto Pref.)	Early	31 May '96
7		Faculty of Agriculture, Kyushu University (Fukuoka, Fukuoka Pref.)	Early	6 Jun '96
8		Fukui Prefectural Sea Farming Center (Obama, Fukui Pref.)	Early and late	21 May '97
9		Osaka Prefectural Fisheries Station and Sea Farming Center (Osaka Pref.)	Early	11 May '95
10		Osaka Prefectural Fisheries Station and Sea Farming Center (Osaka Pref.)	Late	19 May '95
11	*Acanthopagrus schlegeli*	Faculty of Agriculture, Kyushu University (Fukuoka, Fukuoka Pref.)	Early	6 Jun '96
12		Osaka Prefectural Fisheries Station and Sea Farming Center (Osaka Pref.)	Early	19 May '97
13		Osaka Prefectural Fisheries Station and Sea Farming Center (Osaka Pref.)	Late	19 May '97
14	*Acanthopagrus latus*	Himedo, Amakusa, Kumamoto (Kumamoto Pref.)	Unknown	21 Nov '98
15	*Sparus sarba*	Faculty of Agriculture, Kyushu University (Fukuoka, Fukuoka Pref.)	Late	3–5 Jun '96
16	*Dentex tumifrons*	Ohara, Chiba (Chiba Pref.)	Unknown	Oct '98
17		Japan Sea-Farming Association (Miyazu, Kyoto Pref.)	Early	20 Apr '95
18	*Paralichthys olivaceus*	Japan Sea-Farming Association (Miyazu, Kyoto Pref.)	Late	21 May '97
19		Fisheries Laboratory of Kinki University (Kushimoto, Wakayama Pref.)	Late	15 Jan '97
20		Japan Sea-Farming Association (Miyazu, Kyoto Pref.)	Early and late	23 Apr '97
21	*Engraulis japonica*	Wakasa Bay (Fukui Pref.)	Unknown	May '97

(type II). The remaining 91 unidentified eggs were divided into three groups (types I, III, and IV) based on diameters. Of these 91, 51 type-II eggs reacted with MT-1. This finding is compatible with the possibility that the eggs were *P. major*, because the size was similar to that of *P. major* and each contained a single oil globule of a similar size (Tables 1 and 4). Another 43 eggs were collected from another area of Wakasa Bay (Table 5). None of the eggs fixed just after collection were morphologically identifiable. But, after incubation at 20°C for 24 hours until the late stage, all six eggs identified as *P. major* were reactive with the antibody MT-1, whereas the others were not. These findings strongly suggest that the method developed in this study is useful for identifying *P. major* eggs in seawater. Although only late stage eggs were used in this experiment, early stage eggs are also detectable because the antibody recognized both stages of *P. major* eggs from several sea farming centers (Table 2).

Table 3

Reactivity of monoclonal antibodies to fish eggs. + represents positive reaction; – represents negative reaction.

		Reactivity		
Egg no.	Species	MT-1	MT-2	MT-3
1	*Pagrus major*	+	+	+
2		+	+	+
3		+	+	+
4		+	+	+
5		+	+	+
6		+	+	+
7		+	+	+
8		+	+	+
9	*Acanthopagrus schlegeli*	–	–	–
10		–	–	–
11		–	–	–
12		–	–	–
13		–	–	–
14	*Acanthopagrus latus*	–	–	–
15	*Sparus sarba*	–	–	–
16	*Dentex tumifrons*	–	–	–
17	*Paralichthys olivaceus*	–	–	–
18		–	–	–
19		–	–	–
20		–	–	–
21	*Engraulis japonica*	–	–	–

Compared to genetic analysis of fish eggs, this method has the advantage of being able to assay many eggs simultaneously without the need to separate individual eggs in tubes and without extracting DNA from the individual egg in each tube. Further, this method works with formalin-fixed eggs, whereas extraction of DNA from formalin-fixed material is problematic. Plankton samples from field studies are typically fixed in formalin-seawater solution.

There was no problem obtaining a large amount of the monoclonal antibody required when identifying *P. major* eggs. The antibody can be easily obtained by large-scale cultures of hybridoma cells. About 50 mL of antibody solution was obtained after two weeks of cultivation. There was no technical problem assaying 43 or 102 eggs from natural waters. However, one assay of a field sample cost about 20 U.S. dollars. To keep costs down an assay kit cheaper than the VECTASTAIN[R] Elite ABC kit is needed when a large number of field samples are analyzed.

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Table 4

Reactivity of monoclonal antibody MT-1 to the pelagic eggs fixed with formaldehyde just after collection from Wakasa Bay. O.G. diameter = oil globule diameter

Fish egg type	Egg diameter (mm)	O.G. diameter (mm)	Reactivity (%) (positive egg no./ total egg no.)
I	0.72–0.79	0.16-0.19	0 (0/2)
II	0.75–0.82	no oil globule	0 (0/11)
III	0.81–1.02	0.19–0.28	58 (51/88)
IV	1.07	0.21	0 (0/1)

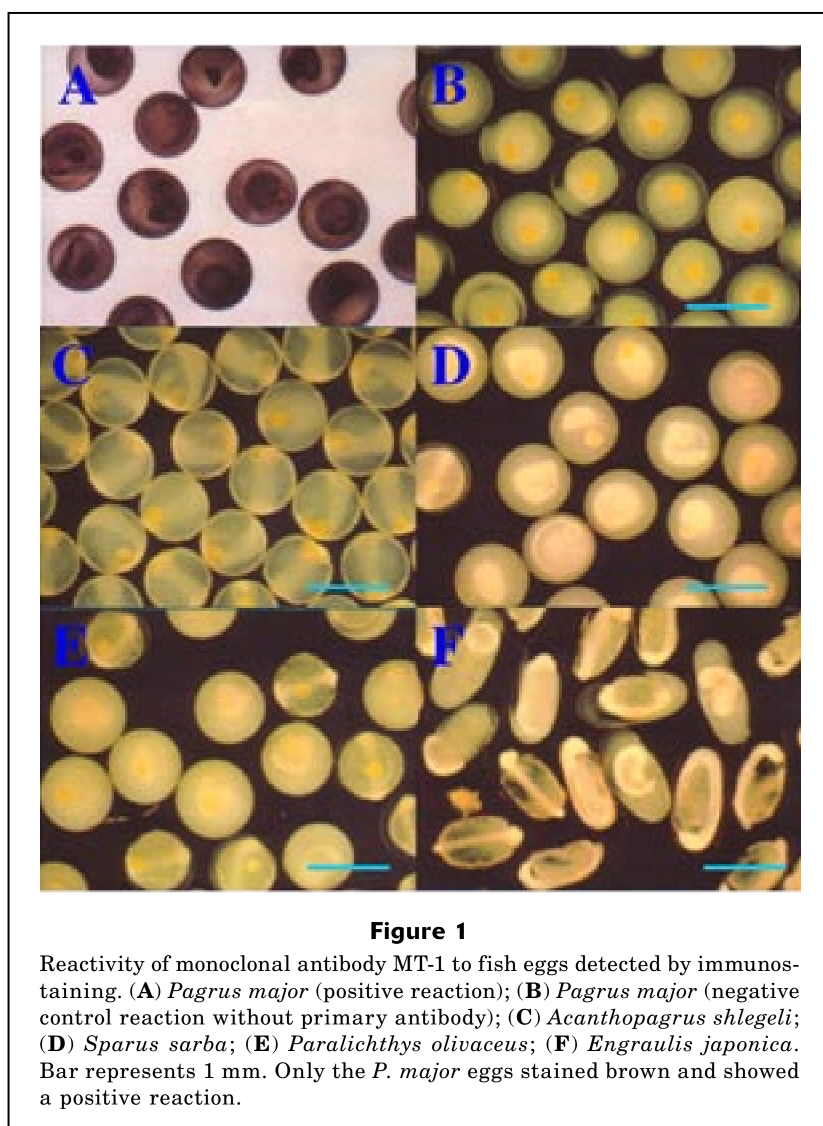

Figure 1

Reactivity of monoclonal antibody MT-1 to fish eggs detected by immunostaining. (**A**) *Pagrus major* (positive reaction); (**B**) *Pagrus major* (negative control reaction without primary antibody); (**C**) *Acanthopagrus shlegeli*; (**D**) *Sparus sarba*; (**E**) *Paralichthys olivaceus*; (**F**) *Engraulis japonica*. Bar represents 1 mm. Only the *P. major* eggs stained brown and showed a positive reaction.

Table 5

Reactivity of monoclonal antibody MT-1 to the pelagic eggs reared for 24 hours after collection from Wakasa Bay.

Egg	Species	Reactivity (%) (positive egg no./total egg no.)
Fish	*Pagrus major*	100 (6/6)
	Acanthopagrus shlegeli	0 (0/8)
	Paralichthys olivaceus	0 (0/1)
	Triglidae sp.	0 (0/1)
	Konosirus punctutus	0 (0/2)
	Soleoidei sp.	0 (0/7)
	Englauris japonicus	0 (0/13)
Decapod	*Enploteuthidae sp.*	0 (0/5)

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