October 2006

Acrosome reaction is subfamily specific in sea star fertilization

Mia Nakachi
Keio University, Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan

Hideaki Moriyama
University of Nebraska - Lincoln, hmoriyama2@unl.edu

Motonori Hoshi
Keio University, Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan

Midori Matsumoto
Keio University, Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan

Published in *Developmental Biology* 298:2 (October 15, 2006), pp. 597–604. doi:10.1016/j.ydbio.2006.07.009

Submitted November 30, 2005; revised July 5, 2006; accepted July 11, 2006; published online July 14, 2006.

Acrosome reaction is subfamily specific in sea star fertilization

Mia Nakachi[a], Hideaki Moriyama[b,*], Motonori Hoshi[a], and Midori Matsumoto[a]

[a]*Department of Biosciences and Informatics, Keio University, Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan*

[b]*Department of Chemistry, e-Toxicology, and Biotechnology, University of Nebraska–Lincoln, 28 Hamilton Hall, Lincoln, NE 68588-0304, USA*

**Corresponding author. Email:* hmoriyama2@unl.edu

Abstract: In the fertilization process of sea stars, sperm is activated to go through the acrosome reaction before cell fusion. We focused on induction of the acrosome reaction as a key process in fertilization. Six species of sea stars were used in this study: *Asterias amurensis*, *Asterias rubens*, *Asterias forbesi*, *Aphelasterias japonica*, *Distolasterias nipon*, and *Asterina pectinifera*. Acrosome reaction assays indicate that the acrosome reaction can be induced across species within Asteriinae subfamily. However, cross-fertilization assays indicate that sea stars have species specificity in fertilization. Therefore, steps after the acrosome reaction are responsible for the species specificity. To explain acrosome reaction subfamily specificity at the molecular level, the sugar components of egg jelly were examined and analyzed by principal component analysis. *A. amurensis* and *A. forbesi* belong to the same induction group of the acrosome reaction. *D. nipon* and *An. pectinifera* are in a unique group. Enzyme-linked immunosorbent assays indicate that Asteriinae subfamily share a common glycan structure, the Fragment 1 of Acrosome Reaction-Inducing Substance from *A. amurensis*. Fragment 1 plays an important role in the subfamily specificity of acrosome reaction induction. In addition, *A. amurensis* sperm activating peptide was recognized by sperm from the same superorder. These results demonstrate that the specificity of acrosome reaction induction is present at the subfamily level in sea stars.

Keywords: Fertilization, Acrosome reaction, Species specificity, Egg jelly, *Asterias amurensis, Asterias rubens, Asterias forbesi, Aphelasterias japonica, Distolasterias nipon, Asterina pectinifera*

Sexual reproduction is an excellent survival strategy, in which genetic material from different individuals merge to provide genetic variety in descendants. In organisms that employ external fertilization, the process includes the risk of inter-species fertilization. To prevent this ill-fated outcome, successful fertilization requires many strictly defined steps (Vacquier, 1998) (also see Fig. 1). In one of the final steps in sea star fertilization, just prior to the penetration of a sperm into an egg, the sperm goes through an acrosome reaction. In the acrosome reaction, the plasma membrane of the front portion of the sperm head fuses with the acrosomal vesicle membrane. This exocytosis results in the exposure of devices essential for subsequent steps in fertilization (Hoshi et al., 2000).

Several key chemical components are determinants of species specificity in marine invertebrate fertilization, which occurs externally. In the sea urchin, the sulfated fucan in the egg's jelly coat, the extracellular matrix of egg (Biermann et al., 2004), the sperm protein bindin, and its egg receptor protein (Kamei and Glabe, 2003) are responsible for species-specific fertilization. Sulfated fucan induces the acrosome reaction, after which bindin on the newly exposed membrane of the sperm and bindin receptor in the vitelline coat of the egg dock with each other. In the abalone, the sperm protein lysin in the acrosomal vesicle and its receptor in the vitelline coat of the egg are responsible for species-specific fertilization. Lysin and its receptor bind each other and establish the physical path in the vitelline coat for sperm to penetrate (Galindo et al., 2003 and Kresge et al., 2001).

Sea star sperm undergo the acrosome reaction upon contacting the jelly coat of the egg (Ikadai and Hoshi, 1981a) (Fig. 1). In the Northern Pacific sea star, *Asterias amurensis*, the acrosome reaction is induced by the concerted action of three components of the jelly coat (Hoshi et al., 1994). These components include the sulfated proteoglycan-like molecule acrosome reaction-inducing substance (ARIS) (Ikadai and Hoshi, 1981a and Ikadai and Hoshi, 1981b), the sulfated steroid saponin Co-ARIS (Nishiyama et al., 1987a and Nishiyama et al., 1987b), and the asteroidal sperm activating peptide asterosap (Nishigaki et al., 1996). Of these, ARIS is the most important molecule for acro

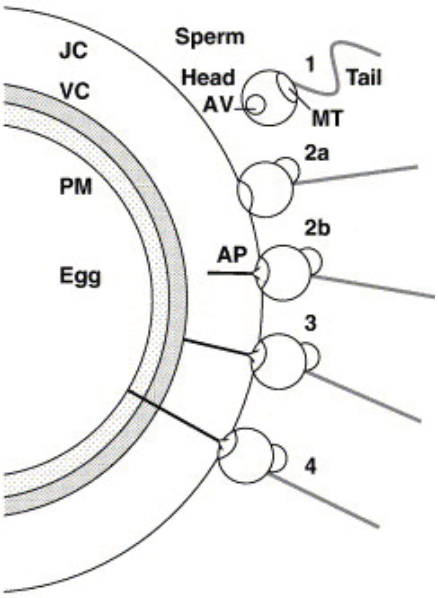

Fig. 1. The process of fertilization in the sea star. When sperm encounters a homogeneous egg, it is first attracted and activated by the asterosap (step 1). Then, the acrosome reaction is induced by ARIS, Co-ARIS, and asterosap. The acrosome reaction is characterized by the exocytosis of the acrosomal vesicle (step 2a), followed by the extension of a long acrosomal process (step 2b). The sperm then penetrates the jelly coat (step 3). Finally, sperm-egg membrane fusion takes place (step 4). Abbreviations: JC, jelly coat; VC, vitelline coat; PM, plasma membrane; AV, acrosomal vesicle; MT, mitochondria; AP, acrosomal process.

some reaction induction because ARIS alone can induce the acrosome reaction in alkaline or high Ca^{2+} seawater whereas neither Co-ARIS nor asterosap can. In normal seawater, however, ARIS requires either Co-ARIS or asterosap to induce the acrosome reaction (Hoshi et al., 1994 and Nishigaki et al., 2000).

ARIS is composed of polysaccharide and protein. One of the sugar fragments retains the biological activity of ARIS when liberated from the polysaccharide part. This fragment, Fragment 1 (Fr. 1), contains 10–11 repeats of the pentasaccharide unit, [(→4)-β-d-Xyl*p*-(1→3)-α-d-Gal*p*-(1→3)-α-l-Fuc*p*-4(SO_3^-)-(1→3)-α-l-Fuc*p*-4(SO_3^-)-(1→4)-α-l-Fucp-(1→)] (Koyota et al., 1997). Asterosap is composed of 34 amino acids; an intramolecular disulfide bond is essential for its biological activity (Nishigaki et al., 1996). The mRNA of the asterosap precursor encodes ten asterosaps tandemly interrupted by spacers of approximately 50 nucleotides (Matsumoto et al., 1999). The asterosap receptor is a membrane-bound guanylate cyclase with a single transmembrane domain (Matsumoto et al., 2003). Co-ARIS is thought to affect the sperm membrane directly, and its sulfate moiety and side chain of steroid are critical for its activity (Nishiyama et al., 1987a).

There has been little information with respect to the specificity of acrosome reaction and fertilization in sea stars. It was suggested that *A. amurensis* sperm does not recognize *Asterina pectinifera* ARIS (Matsui et al., 1986), whereas *A. amurensis* Co-ARIS retains its activity on *An. pectinifera* sperm (Amano et al., 1992). Further investigation was necessary to understand where and how speciation barriers occur in the sea star fertilization process. In this study, we investigated the specificity of fertilization of closely related sea star species (Table 1), focusing on the acrosome reaction. From our results we concluded that the acrosome reaction in sea stars is subfamily specific due to the polysaccharide structure of ARIS.

Materials and methods

Animals and gametes

Sea stars used in this study and their taxonomy are listed in Table 1. Sea stars were collected locally during their annual breeding seasons. *A. amurensis* were collected in Tokyo Bay and Otsuchi Bay, Japan from January to March, and in Tasmania, Australia from July to September. *Asterias rubens* were collected in the Millport area of Scotland from April to June. *Asterias forbesi* were collected in Woods Hole, Massachusetts, USA in June. *Aphelasterias japonica* and *Distolasterias nipon* were collected in Asamushi Bay, Japan from September to October and in April, respectively. *An. pectinifera* were collected in Japan: Tokyo Bay in May, Otsuchi Bay in September, and the Kagoshima area in December.

Mature eggs were collected by treating the ovaries with 1–10 μM 1-methyladenine, and then washing with artificial sea water (ASW) consisting of 423 mM NaCl, 9 mM KCl, 9 mM $CaCl_2$, 25 mM $MgSO_4$, 23 mM $MgCl_2$, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid (EPPS), pH 8.2, in deionized water (water purified with a Milli-Q system from Millipore Corp., MA, USA). The duration of 1-methyladenine treatment was approximately 2 h at room temperature, 20–25°C, for the fertilization assay and overnight at 4°C for the preparation of egg jelly. Sperm were collected immediately before use as 'dry' sperm by cutting the sperm ducts of the testes and collecting the contents of the testes. Sperm samples were stored on ice until use.

Preparation of egg jelly

Egg jelly was defined as the solution of the jelly coats of eggs in ASW and was prepared as described previously (Kawamura et al., 2002). Briefly, a suspension of mature eggs in ASW was gradually acidified, being mixed gently to pH 5.5 with 0.1 M HCl to dissolve the jelly coat, and returned to pH 8.2 with 0.1 M NaOH. Eggs without jelly coat were removed by centrifugation at 1000×*g* for 10 min. The supernatant was then centrifuged at 15,000×*g* for 30 min to remove cell debris. The resulting

Table 1
Taxonomy of sea stars

Superorder	Family	Subfamily	Genus	Species	Abbreviation
Forcipulatacea	Asteriidae	Asteriinae	*Asterias*	*amurensis*	*Aa*
			Asterias	*forbesi*	*Af*
			Asterias	*rubens*	*Ar*
			Aphelasterias	*japonica*	*Apj*
		Coscinasteriinae	*Distolasterias*	*nipon*	*Dn*
Valvatacea	Asterinidae	N.A.	*Asterina*	*pectinifera*	*Anp*

N.A., not applicable.

supernatant egg jelly was collected and stored at − 20°C until use. All above procedures were performed at 4°C. Sugar content of the egg jelly was determined by the resorcinol–sulfuric acid method (Monsigny et al., 1988) using l-fucose as the standard.

Acrosome reaction assay

The acrosome reaction was assayed as described previously (Kawamura et al., 2002) with some modifications. Briefly, 10 µl dry sperm were suspended in 1 ml ice-cold ASW and incubated on ice for five min. Twenty-microliter sperm suspension was added to 80 µl each of diluted series of egg jelly and incubated at room temperature for five min to induce the acrosome reaction. Then 20 µl 5% (v/v) glutaraldehyde in ASW was added to each sample to fix the sperm, and 2–3 µl 0.5% (w/v) erythrosine in 70% (v/v) ethanol were added to stain the sperm. The sperm were observed under a Nomarski differential interference microscope at 1000 times magnification. More than 100 sperm were scored in each sample.

Fertilization assay

One microliter of dry sperm was suspended in 500–1000 µl ASW. Mature eggs were put onto a slide glass or a tissue culture plate with ASW and inseminated with the sperm suspension. After 3–20 min of insemination, the eggs were scored under an inverted microscope for the formation of the fertilization membrane around the cell as the indicator of fertilization. Between 100 and 118 eggs were scored from each sample.

Sugar composition analysis of egg jelly

The sugar composition of egg jelly was determined using the high-performance liquid chromatography (HPLC)-1-phenyl-3-methyl-5-pyrazolone (PMP) derivative method (Fu and O'Neill, 1995 and Honda et al., 1989). Egg jelly solution was concentrated by ultrafiltration with a cut-off molecular weight of 10,000, freeze-dried, and dissolved in deionized water by ultrasonication. Egg jelly was hydrolyzed with 3.75 M trifluoroacetic acid (TFA) at 120°C for 2 h. After cooling to room temperature, the solvent was evaporated and the remaining sample was washed with 2-propanol. Then, the sample was incubated with 0.25 M PMP and 0.15 M NaOH at 70°C for 2 h, followed by cooling to room temperature and neutralizing with HCl. PMP derivatives of sugar were separated as an aqueous layer and then applied to HPLC. Separation was carried out using the following conditions at room temperature: a C18 column (4.6 × 250 mm; Nacalai Tesque, Inc., Kyoto, Japan) was equilibrated with 10% acetonitrile in 100 mM ammonium acetate, followed by a linear gradient up to 25% acetonitrile in the same buffer over 55 min at a flow rate of 0.5 ml/min. The column eluate was monitored by absorbance at 245 nm. By comparing the peak patterns from equimolar mixtures of seven monosaccharides: fucose, xylose, galactose, glucose, mannose, *N*-acetylgalactosamine, and *N*-acetylglucosamine, each peak was identified as a monosaccharide and its proportion was calculated.

We then performed a principal component analysis (PCA) (Sokal and Rohlf, 1981) to identify the general pattern of association between sugar components of egg jelly from sea star species and acrosome reaction induction activity. The PCA was carried out using the statistical analysis package R by Robert Gentleman and Ross Ihaka of the Department of Statistics at the University of Auckland.

Enzyme-linked immunosorbent assay (ELISA) of egg jelly

Egg jelly was adsorbed on a polystyrene 96-well cell culture plate (Corning, NY, USA) by drying the diluted series of egg jelly in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4; pH 7.4). Sodium dextran sulfate 5000 (Wako, Osaka, Japan) was used as a negative control. After blocking with 300 µl 1% (v/v) human serum albumin (Baxter, IL, USA) in PBS for 30 min, the wells were incubated with 50 µl of five-fold diluted anti-Fragment 1 IgG (a mouse monoclonal IgG established previously in our laboratory (unpublished data)) in PBS for 1 h. The wells were washed with 300 µl of PBS three times and incubated with 50 µl of horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG (Chemicon, CA, USA) for 30 min. After washing with PBS, color was developed by incubating with 200 µl of *o*-phenylenediamine (PDA) solution (0.04% *o*-PDA, 0.012% hydrogen peroxide and 0.1 M sodium citrate buffer; pH 5.0) for 7.5 min. The reaction was quenched by the addition of 20 µl 2.5 M sulfuric acid. The absorbance at 492 nm was measured by Ultrospec Visible Plate Reader II 96 (GE, Uppsala, Sweden) 15 min after quenching.

Sperm activation assay

One microliter dry sperm was suspended in 100–1000 µl acidified ASW, at approximately pH 6, to repress their motility. Ten microliter of sperm suspension was put on a slide glass or a tissue culture plate and one µl of 10 µM P15, a synthesized isoform of asterosap from *A. amurensis* was added to the suspension. The change of sperm motility after the addition of P15 was observed under an inverted microscope.

Results and discussion

Specificity of the acrosome reaction

Species specificity is a key trait of the fertilization process. The goal of this study is to determine the role of the acrosome reaction in conferring species specificity to the fertilization process in the sea star. We investigated whether the acrosome reaction is responsible for the species specificity of fertilization. The acrosome reaction-inducing activity of egg jelly was measured among six closely related species. Sperm with an acrosomal process were defined as acrosome-reacted. We used concentrations of egg jelly up to 200 µg sugar/ml because previous studies have shown that the proportion of acrosome-reacted sperm plateaus before this concentration (Ikadai and Hoshi, 1981a).

In this experiment, induction of the acrosome reaction was less than 100% for many possible reasons. First, there was variation in sperm maturity levels. To obtain sufficient amounts of sperm, we used sperm from dissected testes rather than mature, naturally released sperm. In addition, isolated egg jelly lacks perfection in its components and its structure. The intact jelly coat may provide environmental support, in addition to acrosome reaction induction materials, which encourages the formation of the acrosomal process.

In each species, the acrosome reaction induced by conspecific egg jelly increased in a dose-dependent fashion, reaching up to 80% (Fig. 2). Among the species from Asteriinae subfamily, *Aa*, *Af*, and *Apj*, each egg jelly induced acrosome reaction in all species dose-dependently (Figs. 2A–C). Recently, gamete compatibility has been reported between the two species *Af* and *Ar* (Harper and Hart, 2005). This report is consistent with our results (Fig. 2B). On the other hand, *Dn* and *Anp* egg jelly did not induce the acrosome reaction in Asteriinae sperm (Figs. 2A–C). Interestingly, Asteriinae egg jelly induced the acrosome reaction in 20% of *Dn* sperm, which belongs to the same family but the different subfamily as Asteriinae (data not shown), although it completely failed to induce the acrosome reaction in *Anp* sperm, which belongs to the different superorder (Fig. 2D). These results indicate

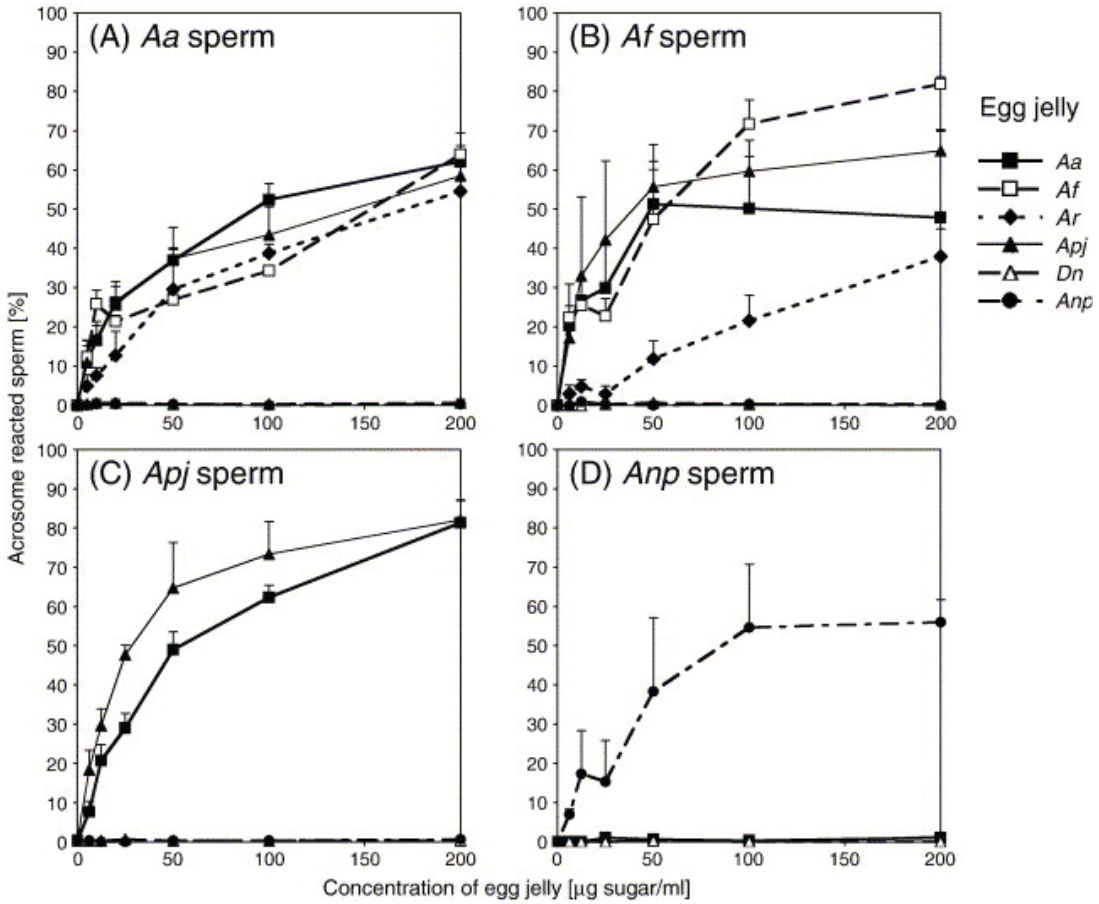

Fig. 2. Acrosome reaction among six sea star species. The acrosome reaction-inducing activity of egg jelly was scored as the proportion of acrosome-reacted sperm at each concentration of egg jelly. Each panel represents the acrosome reaction in *Aa* (A), *Af* (B), *Apj* (C), and *Anp* (D) sperm. In panels C and D, egg jelly of *Af* and *Ar* were not included due to the seasonal availability. The points represent means from three independent experiments. Error bars indicate standard deviation.

that the acrosome reaction in sea star is not species specific but rather subfamily specific.

However, it is noteworthy that in *Af* sperm, *Af* egg jelly induced the acrosome reaction at a higher rate than other Asteriinae egg jelly at the concentration of 200 µg/ml (Fig. 2B). This result suggests that the acrosome reaction exhibits species specificity in *Af* sperm at this egg jelly concentration. In cases of *Aa* and *Apj* sperm, the level of acrosome reaction induced by all Asteriinae egg jelly were equivalent. These observations are in contrast with the results obtained with *Af* sperm.

Because the induction of acrosome reaction can be interpreted as the recognition of egg jelly by sperm receptors, we interpret our data as follows: *Aa* and *Apj* sperm recognize egg jelly component(s) that commonly exist in all four species of Asteriinae. However, only the Asteriinae *Af* sperm recognize a unique component of *Af* egg jelly, giving *Af* some degree of species specificity. To our knowledge only ARIS is able to provide such specificity.

Species specificity of fertilization

We found that one of the processes of fertilization, the acrosome reaction, has subfamily specificity in sea stars. We extended our investigations to the specificity of the entire process of fertilization. Cross-fertilization tests were conducted using eggs and sperm from different species. A fertilized egg was defined by the presence of a fertilization membrane, which results from the exocytosis of cortical granules after sperm-egg membrane fusion. When *Aa* and *Ar* eggs and sperm were combined, 70% of eggs were fertilized by conspecific sperm whereas 40% of eggs were fertilized by heterospecific sperm (data not shown). In the combination of *Aa* and *Apj*, both from Asteriinae but from different genera, 80–90% of eggs were fertilized by conspecific sperm, whereas only 20% eggs were fertilized by heterospecific sperm. These results indicate that there is a significant degree of species specificity in the fertilization of sea stars (Fig. 3).

Sugar composition of egg jelly

Next, we investigated the composition of sugars in the egg jelly of the various sea stars to determine if sugar composition could be responsible for the specificity of acrosome reaction induction. The global sugar composition of egg jelly was determined by the HPLC-PMP derivative method (Table 2). *Aa* and *Af*, which share acrosome reaction inducing ability, had very similar sugar composition. Fucose was identified as the major sugar in these two species; this result is

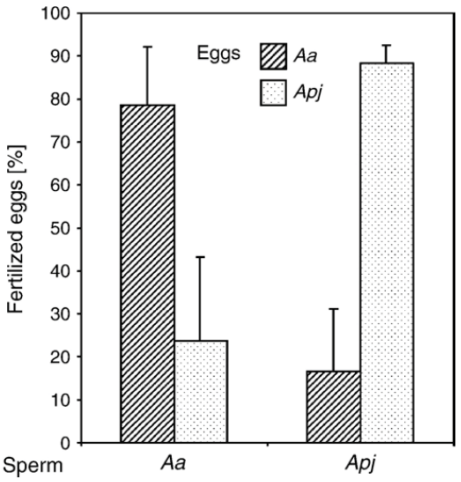

Fig. 3. Cross-fertilization between *Aa* and *Apj*. Fertilization rate was scored as the proportion of fertilized eggs in each combination of gametes. For *Aa* eggs, bars represent means from three experiments where total egg numbers were between 300 and 302. For *Apj* eggs, bars represent means from four experiments where total egg numbers were between 413 and 424. Error bars indicate standard deviation.

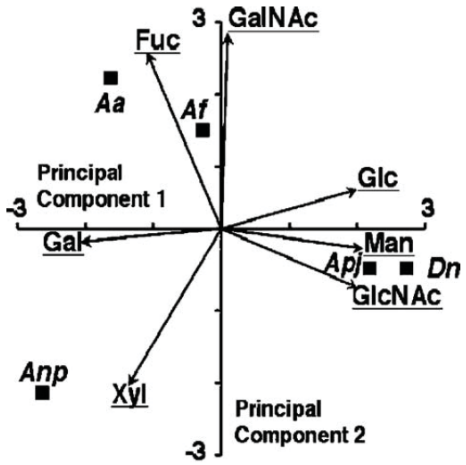

Fig. 4. Principle component analysis biplot on egg jelly components. Autovectors of five species of sea stars in the first two axis of a PCA are presented in a plane. Vectors indicate known chemical components. Solid boxes denote locations of each sea star. Geometrical locations were used to clarify the total chemical nature of the egg jelly.

consistent with the fact that in Fr. 1 of *Aa*, 3 residues in a pentasaccharide repeating unit are fucose (Koyota et al., 1997). *Anp* had a distinct sugar composition compared with the other species, and was also unique in its acrosome reaction-inducing ability. *Apj,* despite its acrosome reaction-inducing activity on *Aa* and *Af* sperm, had a distinct sugar composition from *Aa* and *Af*. However, the proportion of xylose:galactose: fucose, which is 1:1:3 in ARIS Fr. 1, was similar among *Aa, Af* and *Apj* compared to the other two.

These global differences did not specify the exact chemical nature of the egg jelly component. We therefore carried out an exploratory PCA, which is frequently used to distinguish groups of spectra. In the PCA biplot (Fig. 4), the first axis at 72.2% of the variance contrasts *Aa*, *Af*, and *Anp* against *Apj* and *Dn*. The second axis at 27.0% variance shows *Aa* associated with *Af* and exclusive of *Apj* with *Dn*. *Anp* is isolated in the plot. The difference contrasting *Aa* and *Af* against *Apj* with *Dn* is mostly due to the exclusive vectors, fucose, xylose, and galactose, on PCA biplot. These results indicate that the sugar composition of egg jelly is consistent with the phylogenic relationship of sea star species (Matsubara et al., 2004). However, components of the egg jelly did not necessarily correlate with acrosome reaction-inducing activity.

From these results we conclude that the global sugar composition of egg jelly does not necessarily reflect its acrosome reaction-inducing activity. We thus focused on a more specific part of egg jelly.

Specificity of ARIS structure

To investigate the factor responsible for the subfamily specificity of the acrosome reaction induction, we focused on the ARIS Fr. 1 structure in egg jelly. In *Aa*, ARIS is critical for the acrosome reaction induction, and its glycan part, Fr. 1, is essential for its activi-ty (Ikadai and Hoshi, 1981a and Ikadai and Hoshi, 1981b). We have previously established an anti-Fr. 1 monoclonal IgG that recognizes the intact sulfated form of Fr. 1 and inhibits egg jelly-induced acrosome reaction (unpublished data). We used this antibody to determine if the Fr. 1-like structure was present in the egg jelly of the six sea star species by ELISA. Anti-Fr. 1 bound dose-dependently and equally to all Asteriinae egg jelly. However, no specific binding was observed for *Dn* or *Anp* egg jelly (Fig. 5). This result demonstrates that Asteriinae subfamily share both the Fr. 1-like glycan structure and the acrosome reaction-inducing activity of egg jelly. This result thus indicates that the Fr. 1-like structure is likely responsible for the subfamily specificity of the acrosome reaction induction.

In the sea urchin, it has been reported that the differences in sulfation patterns and the positions of glycosidic linkages in the sulfated fucan of egg jelly ensure species specificity of

Table 2
Sugar compositions of egg jelly (percentage)

Species/sugar	Fucose	Xylose	GlcNAc	GalNAc	Mannose	Glucose	Galactose	Total
A. amurensis	29.0	11.5	10.5	11.0	14.0	4.0	20.0	100.0
A. forbesi	23.5	10.0	11.5	9.0	21.5	7.0	17.5	100.0
Ap. japonica	12.5	9.5	15.0	4.0	42.5	8.5	8.0	100.0
D. nipon	7.0	12.0	17.0	7.5	44.0	8.5	4.0	100.0
An. pectinifera	11.0	37.5	11.0	2.0	10.0	2.0	26.5	100.0

GlcNAc: *N*-acetylglucosamine; GalNAc: *N*-acetylgalactosamine.

acrosome reaction induction (Biermann et al., 2004 and Vilela-Silva et al., 2002). We therefore speculate that in sea stars, sulfation patterns and/or glycosidic linkage in ARIS Fr. 1 may determine acrosome reaction specificity. The sequence of sugar residues in ARIS Fr. 1 is also a possible determinant of acrosome reaction specificity.

Specificity of sperm activation

Asterosap activity is important for inducing the acrosome reaction in sea stars. Furthermore, it is known that synthetic P15, one of the asterosap isoforms from *Aa*, restores *Aa* sperm motility suppressed in acidic ASW. Thus, we performed sperm activation assays to examine the specificity of asterosap recognition by sperm between the species of sea stars. First, we suppressed sperm motility by exposing them to acidic conditions. Then we added *Aa* P15 and scored the restoration of motility. P15 activated all sperm except *Anp*, indicating that asterosap activity is shared within the superorder level (Table 3) and that sperm activation does not confer species-specificity in fertilization.

Fertilization in sea urchins is characterized by a discrete series of steps (Vacquier, 1998). First the attraction and activation of sperm by egg is induced via peptides (Kaupp et al., 2003, Neill and Vacquier, 2004 and Nishigaki et al., 2004). The sperm acrosome reaction is then induced by sulfated fucan (Hirohashi and Vacquier, 2002). Next, sperm-egg adhesion is mediated by bindin and its receptor (Kamei and Glabe, 2003). After fusion, a series of reactions including activation of the cytoplasm and pronuclear fusion take place.

The species specificity of bindin recognition has been studied in several sea urchins: *Echinometra lucunter* (Rock-boring sea urchin), *Arbacia punctulata* (punctuate urchin) (McCartney and Lessios, 2004), and *Heliocidaris erythrogramma* (Brown sea urchin) (Zigler et al., 2003). In the sea urchins *Strongylocentrotus franciscanus* (Red urchin) and *S. purpuratus* (Purple urchin), a specific domain of the bindin receptor governs species-specific sperm-egg adhesion (Kamei and Glabe, 2003). Whereas the binding mechanisms

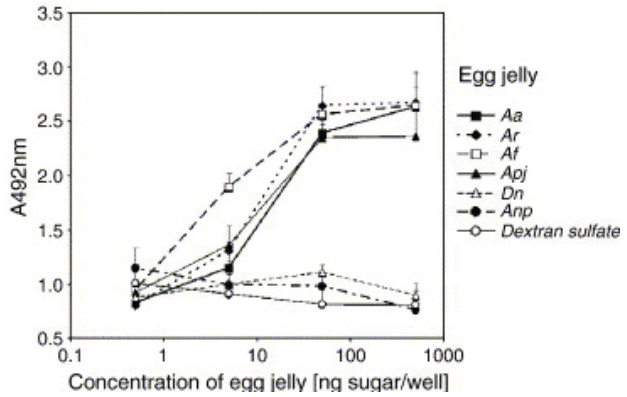

Fig. 5. ELISA of egg jelly. The affinity of anti-Fr.1 IgG for the egg jelly of each sea star species was detected as the absorbance at 492 nm at each egg jelly concentration. The points represent means from three measurements. Error bars indicate standard deviation.

Table 3
Sperm activation by *A. amurensis* asterosap P15 under acidic conditions

Sperm	Sperm activation
A. amurensis	+
A. rubens	+
A. forbesi	+
Ap. japonica	+
D. nipon	+
An. pectinifera	−

Experimental results: +, Sperm activation occurs; −, Sperm activation does not occur.

of bindin to its receptor(s) are not known, species specificity has been attributed to an alternation of primary structures. Many polymorphisms in bindin have been observed and are believed to result from positive selection and sequence rearrangements in genera (Metz and Palumbi, 1996). Bindin and its corresponding receptor(s) have not yet been identified in the sea star. Because the phylogenic locations of sea stars and sea urchins are far apart, it is not necessary that they share a common mechanism for species specificity of fertilization.

Recently, positive selection in the carbohydrate recognition domains of sea urchin sperm receptor for egg jelly (suREJ) proteins has been reported (Mah et al., 2005). suREJ1 binds sulfated fucan, which is responsible for the induction of the acrosome reaction. In this paper, we implicate ARIS Fr. 1 as a determinant of the subfamily-specific acrosome reaction induction in sea stars. Identification of the ARIS receptor is necessary for further studies. Subfamily-specificity of the acrosome reaction indicates that species specificity of fertilization is regulated in a later step(s).

In addition to the biochemical and genetic properties of eggs and sperm, environmental factors may also prevent cross-species fertilization. For co-existing species, different optimal temperature for reproduction is a possible barrier to cross-fertilization. For example, on the East coast of North America, American population of *A. rubens* co-exists with *A. forbesi* over a broad range (Franz et al., 1981 and Wares, 2001). However, it was reported that *A. rubens* begins spawning at 8°C, whereas *A. forbesi* reproduces at 15–16°C (Franz et al., 1981). Thus, differences in favorable temperatures for reproduction may results in different reproductive timing and reproductive isolation.

Separations in time and space also serve as a barrier to cross-fertilization. In Asamushi Bay, Japan, *A. amurensis* and *D. nipon* co-exist but do not share either the reproductive season (May and June for *A. amurensis*, April for *D. nipon*) or the acrosome reaction-inducing activity of egg jelly. In contrast, *A. amurensis* and *Ap. japonica* share acrosome reaction-inducing activity but do not co-exist in the same bay and have different reproductive season (September and October for *Ap. japonica* and from January to March for *A. amurensis*). These facts suggest that there are multiple barriers to cross-fertilization in sea stars.

Appendix. Supplementary data

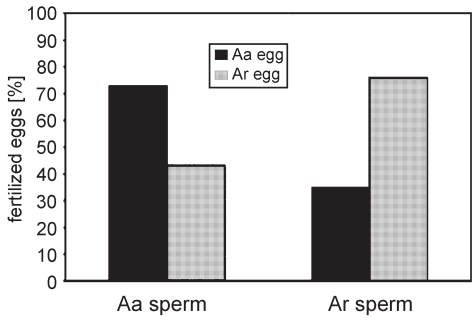

Fig. S1. Cross-fertilization tests between *A. amurensis* and *A. rubens* resulted in 70% fertilization within the same species and 40% fertilization in cross-combinations (data not shown).

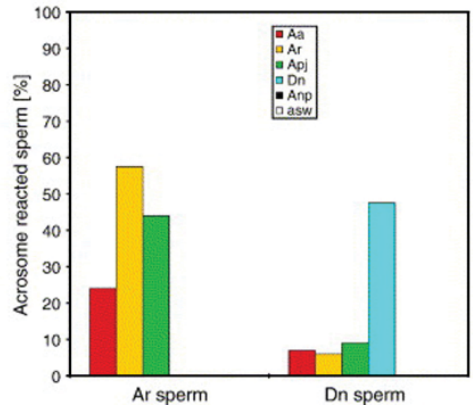

Fig. S2. While the sperm of *A. pectinifera* was not activated by the other egg jelly, the sperm of *D. nipon* was slightly activated (20%) by egg jelly of Asteriinae (data not shown).

Acknowledgments

We thank Dr. Hiroyuki Minakata at the Suntory Institute for Bioorganic Research for providing the synthetic peptide of asterosap. We also thank Dr. U. Benjamin Kaupp and members of his laboratory for their kind assistance in our experiment. We are grateful to the directors and staff of the following universities and centers for their assistance in collecting sea stars: the Research Center for Marine Biology (Tohoku University, Asamushi, Japan), the International Coastal Research Center (the University of Tokyo, Otsuchi, Japan), the Ushimado Marine Laboratory (Okayama University, Japan), the Marine Research Laboratories (University of Tasmania, Australia), the University Marine Biological Station (Millport, Scotland), and the Marine Biological Laboratory (Woods Hole, USA). We thank Mr. Todd Gardner at Atlantis Marine World for discussions on the geological distribution of sea stars in the Shinnecock Bay area. This research was supported by a grant from the Ministry of Education, Culture Sports, Science and Technology of Japan (M.M. and M.H.) and by an Interdisciplinary grant from the University of Nebraska-Lincoln (H.M). This research was partially supported by the Ministry of Education, Culture Sports, Science and Technology of Japan, Grant-in-Aid for the 21st Century Center of Excellence (COE) Program entitled 'Understanding and Control of Life's Function via Systems Biology (Keio University)' (M.N.).

References

Amano, T., Okita, Y., Matsui, T., and Hoshi, M. Pretreatment effects of jelly components on the sperm acrosome reaction and histone degradation in the starfish, *Asterina pectinifera*, *Biochem. Biophys. Res. Commun.* **187** (1992), pp. 268–273.

Biermann, C.H., Marks, J.A., Vilela-Silva, A.C., Castro, M.O., and Mourao, P.A. Carbohydrate-based species recognition in sea urchin fertilization: another avenue for speciation?, *Evol. Dev.* **6** (2004), pp. 353–361.

Franz, D., Worley, E., and Merrill, A. Distribution patterns of common sea stars of the Middle Atlantic continental shelf of the northwest Atlantic (Gulf of Maine to Cape Hatteras), *Biol. Bull.* **160** (1981), pp. 394–418.

Fu, D. and O'Neill, R.A. Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography, *Anal. Biochem.* **227** (1995), pp. 377–384.

Galindo, B.E., Vacquier, V.D., and Swanson, W.J. Positive selection in the egg receptor for abalone sperm lysin, *Proc. Natl. Acad. Sci. U. S. A.* **100** (2003), pp. 4639–4643.

Harper, F.M., and Hart, M.W. Gamete compatibility and sperm competition affect paternity and hybridization between sympatric Asterias sea stars, *Biol. Bull.* **209** (2005), pp. 113–126.

Hirohashi, N., and Vacquier, V.D. High molecular mass egg fucose sulfate polymer is required for opening both Ca2+ channels involved in triggering the sea urchin sperm acrosome reaction, *J. Biol. Chem.* **277** (2002), pp. 1182–1189.

Honda, S., Akao, E., Suzuki, S., Okuda, M., Kakehi, K., and Nakamura, J. High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives, *Anal. Biochem.* **180** (1989), pp. 351–357.

Hoshi, M., Nishigaki, T., Ushiyama, A., Okinaga, T., Chiba, K., and Matsumoto, M. Egg-jelly signal molecules for triggering the acrosome reaction in starfish spermatozoa, *Int. J. Dev. Biol.* **38** (1994), pp. 167–174.

Hoshi, M., Nishigaki, T., Kawamura, M., Ikeda, M., Gunaratne, J., Ueno, S., Ogiso, M., Moriyama, H., and Matsumoto, M. Acrosome reaction in starfish: signal molecules in the jelly coat and their receptors, *Zygote* **8** (2000) (Suppl. 1), pp. S26–S27.

Ikadai, H., and Hoshi, M. Biochemical studies on the acrosome reaction of the starfish, *Asterias amurensis*: I. Factors participating in the acrosome reaction, *Dev. Growth Differ.* **23** (1981), pp. 73–80.

Ikadai, H., and Hoshi, M. Biochemical Studies on the Acrosome reaction of the Starfish, *Asterias amurensis*: II. Purification and characterization of acrosome reaction-inducing substance, *Dev. Growth Differ.* **23** (1981), pp. 81–88.

Kamei, N. and Glabe, C.G. The species-specific egg receptor for sea urchin sperm adhesion is EBR1, a novel ADAMTS protein, *Genes Dev.* **17** (2003), pp. 2502–2507.

Kaupp, U.B., Solzin, J., Hildebrand, E., Brown, J.E., Helbig, A., Hagen, V., Beyermann, M., Pampaloni, F., and Weyand, I. The signal flow and motor response controlling chemotaxis of sea urchin sperm, *Nat. Cell Biol.* **5** (2003), pp. 109–117.

Kawamura, M., Matsumoto, M., and Hoshi, M. Characterization of the sperm receptor for acrosome reaction-inducing substance of the starfish, *Asterias amurensis*, *Zool. Sci.* **19** (2002), pp. 435–442.

Koyota, S., Wimalasiri, K.M., and Hoshi, M. Structure of the main saccharide chain in the acrosome reaction-inducing substance of the starfish, *Asterias amurensis*, *J. Biol. Chem.* **272** (1997), pp. 10372–10376.

Kresge, N., Vacquier, V.D., and Stout, C.D. Abalone lysin: the dissolving and evolving sperm protein, *BioEssays* **23** (2001), pp. 95–103.

Mah, S.A., Swanson, W.J., and Vacquier, V.D., Positive selection in the carbohydrate recognition domains of sea urchin sperm receptor for egg jelly (suREJ) proteins, *Mol. Biol. Evol.* **22** (2005), pp. 533–541.

Matsubara, M., Komatsu, M., and Wada, H. Close relationship between Asterina and Solasteridae (Asteroidea) supported by both nuclear and mitochondrial gene molecular phylogenies, *Zool. Sci.* **21** (2004), pp. 785–793.

Matsui, T., Nishiyama, I., Hino, A., and Hoshi, M. Acrosome reaction-inducing substance purified from the egg jelly inhibits the jelly-induced acrosome reaction in starfish: an apparent contradiction, *Dev. Growth Differ.* **28** (1986), pp. 349–357.

Matsumoto, M., Briones, A.V., Nishigaki, T., and Hoshi, M. Sequence analysis of cDNAs encoding precursors of starfish asterosaps, *Dev. Genet.* **25** (1999), pp. 130–136.

Matsumoto, M., Solzin, J., Helbig, A., Hagen, V., Ueno, S., Kawase, O., Maruyama, Y., Ogiso, M., Godde, M., Minakata, H., Kaupp, U.B., Hoshi, M., and Weyand, I. A sperm-activating peptide controls a cGMP-signaling pathway in starfish sperm, *Dev. Biol.* **260** (2003), pp. 314–324.

McCartney, A., and Lessios, H.A. Adaptive evolution of sperm bindin tracks egg incompatibility in neotropical sea urchins of the genus Echinometra, *Mol. Biol. Evol.* **21** (2004), pp. 732–745.

Metz, E.C., and Palumbi, S.R. Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin, *Mol. Biol. Evol.* **13** (1996), pp. 397–406.

Monsigny, M., Petit, C., and Roche, A.C. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod, *Anal. Biochem.* **175** (1988), pp. 525–530.

Neill, A.T., and Vacquier, V.D. Ligands and receptors mediating signal transduction in sea urchin spermatozoa, *Reproduction* **127** (2004), pp. 141–149.

Nishigaki, T., Chiba, K., Miki, W., and Hoshi, M. Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs, *Zygote* **4** (1996), pp. 237–245.

Nishigaki, T., Chiba, K., and Hoshi, M. A 130-kDa membrane protein of sperm flagella is the receptor for asterosaps, sperm-activating peptides of starfish *Asterias amurensis*, *Dev. Biol.* **219** (2000), pp. 154–162.

Nishigaki, T., Wood, C.D., Tatsu, Y., Yumoto, N., Furuta, T., Elias, D., Shiba, K., Baba, S.A., and Darszon, A.. A sea urchin egg jelly peptide induces a cGMP-mediated decrease in sperm intracellular Ca(2+) before its increase, *Dev. Biol.* **272** (2004), pp. 376–388.

Nishiyama, I., Matsui, T., Fujimoto, Y., Ikekawa, N., and Hoshi, M. Correlation between the molecular structure and the biological activity of Co-

ARIS, a cofactor for acrosome reaction-inducing substance, *Dev. Growth Differ.* **29** (1987), pp. 171–176.

Nishiyama, I., Matsui, T., Y. Fujimoto, N. Ikekawa and M. Hoshi, Purification of Co-ARIS, a cofactor for acrosome reaction-inducing substance, from the egg jelly of starfish, *Dev. Growth Differ.* **29** (1987), pp. 161–169.

Sokal, R.R. and Rohlf, F.J., Biometry: The Principles and Practice of Statistics in Biological Research, W.H. Freeman, San Francisco (1981).

Vacquier, D. Evolution of gamete recognition proteins, *Science* **281** (1998), pp. 1995–1998.

Vilela-Silva, A.C., Castro, M.O., Valente, A.P., Biermann, C.H., and Mourao, P.A., Sulfated fucans from the egg jellies of the closely related sea urchins *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* ensure species-specific fertilization, *J. Biol. Chem.* **277** (2002), pp. 379–387.

Wares, J.P. Biogeography of Asterias: North Atlantic climate change and speciation, *Biol. Bull.* **201** (2001), pp. 95–103.

Zigler, K.S., Raff, E.C., Popodi, E., Raff, R.A., and Lessios, H.A., Adaptive evolution of bindin in the genus Heliocidaris is correlated with the shift to direct development, *Evol. Int. J. Org. Evol.* **57** (2003), pp. 2293–2302.