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Male chromosomes of sea urchin hybrid andromerogones created with cryopreserved sperm

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ABSTRACT—We developed a method for preparing male chromosomes from sea urchin hybrid andromerogones created with cryopreserved sperm. We obtained hybrid andromerogones by heterospermic insemination of *Hemicentrotus pulcherrimus* non-nucleate egg fragments produced by centrifuging unfertilized eggs in a stepwise saccharose density gradient. The hybrid andromerogones showed cleavage rates of 1%–93%, cleaved successively into two- and four- blastomeres and developed to early blastulae. The morulae or early blastulae were treated with colchicine (0.1–1.0 mg/ml), dissociated into single blastomeres by pippeting, swollen with 7%–10% sodium citrate for 10 min and fixed with methanol:acetic acid (3:1). The fixed cells were dropped on slides and air-dried. The andromerogones for 5 sperm species showed a half of their respective diploid chromosome numbers without chromosome elimination. This method is applicable for analysis of the haploid male chromosome complement in sea urchin species for which only sperm can be obtained.

Key words: chromosomes, sea urchins, hybrid andromerogones, cryopreserved sperm

INTRODUCTION

The echinoid fauna is especially abundant in Japan. Of about 260 genera (with subgenera) of the recent echinoid fauna over 110 (about 43%) are known to occur in Japan and the adjacent regions (Nisiyama, 1966, 1968). Though morphological (Durham and Melville, 1957; Philip, 1965; Shigei, 1974) and biochemical (Matsuoka, 1985, 1986) systematic studies have been carried out on the echinoids, cytogenetic one is not quite satisfactory. Sea urchin chromosomes prepared mainly from testes by sectioning or the squash method (Makino and Niiyama, 1947; Nishikawa, 1961; Colombera, 1974) were too small to be analyzed accurately. Clearer chromosome preparations can be obtained from embryos, by either the squash (Auclair, 1965) or air-drying method (German, 1966; Saotome, 1982), but we cannot always obtain mature eggs and sperm, fertilized eggs, or well-developed embryos for many species. Indeed we know the breeding seasons of only 11 of the known Japanese species, and one of the authors has already determined their chromosome numbers (Saotome, 1982, 1987, 1989, 1991).

Sea urchin egg fragments with no female pronucleus can develop after homospermic insemination, and some can develop to the larval stage (Boveri, 1889). Since these embryos, known as andromerogones, contain only a haploid male chromosome complement (Saotome, 1999), we can use them to analyze male chromosomes. Hybrids obtained by heterospermic insemination of the non-nucleate egg fragments are known as hybrid andromerogones, and some can cleave, hatch, and develop to the pluteus stage (Boveri, 1889; Hörstadius, 1936; von Ubisch, 1959; Chen and Baltzer, 1975). We can obtain sperm more easily than eggs in sea urchins because the male breeding season is longer than the female one. Moreover, sea urchin sperm can be cryopreserved (Dunn and McLachlan, 1973; Asahina and Takahashi, 1978; Kurokura *et al*., 1989), which facilitates the creation of hybrid andromerogones, and clear metaphase plates of male chromosomes may be obtained from hybrid andromerogones for many species.

In this paper, we describe a method for preparing male chromosomes from sea urchin hybrid andromerogones created with cryopreserved sperm.

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MATERIALS AND METHODS

Materials

We obtained *Strongylocentrotus intermedius,* and *Glyptocidaris crenularis* from the Asamushi Marine Biological Station, Tohoku University, Aomori Prefecture, and *Strongylocentrotus nudus, Pseudocentrotus depressus* from Kesengun, Iwate Prefecture. We obtained *Hemicentrotus pulcherrimus, Clypeaster japonicus,* and *Astriclypeus manni* from the Misaki Marine Biological Station, University of Tokyo, Kanagawa Prefecture.

Preparation of non-nucleate egg fragments

We obtained non-nucleate egg fragments by centrifuging *H. pulcherrimus* eggs in a stepwise saccharose density gradient of four layers (0.33 M, 0.50 M, 0.67 M, and 1 M saccharose) according to the method previously reported (Saotome, 1999). Unfertilized eggs ($2–5 \times 10^4$) with their jelly were layered over the gradient and centrifuged at 21,000 × g for 20 min at 13°C. The region containing the non-nucleate egg fragments was transferred to 35-mm dishes and then washed several times with seawater to remove saccharose.

Cryopreservation of sperm

We obtained dry sperm from 7 species by injecting 0.1–0.5 ml of 0.01M acetylcholine chloride (Wako Pure Chemical Industries, Osaka, Japan) or 1ml of 0.5 M KCl into the body cavity of mature adults and froze it as previously reported (Kurokura *et al*., 1989), with several modifications. About 1 ml of the dry sperm was suspended in 9 ml cryopreservation solution (seawater: distilled water: dimethyl sulfoxide = 2: 1: 0.4). We filtered the seawater with a Millipore filter or used artificial seawater instead (Jamarin U, Jamarin Laboratory, Osaka, Japan). The sperm suspension (0.5 ml) was sucked in plastic insemination straws (Fujihira Kogyo Co. Ltd., Tokyo, Japan). The straws were heat sealed, wrapped with several sheets of tissue paper, and inserted into test tubes with screw caps. The tubes were frozen with liquid nitrogen. The frozen straws were removed from the capped tubes and stored in liquid nitrogen. For use, we thawed the sperm rapidly by immersing the straw into a test tube containing room temperature seawater.

Insemination of non-nucleated egg fragments

Non-nucleate egg fragments ($2–7 \times 10^3$) were suspended in 5 ml seawater (35–mm dishes) and inseminated with thawed sperm

Table 1. Cleavage rates in the hybrid andromerogones obtaining by inseminating *H. pulcherrimus* non-nucleate egg fragments with cryopreserved sperm of 7 species

Sperm species	Cleavage rates* of *H. pulcherrimus* egg fragments (%)		
	Max.	Min.	Mean
Hemicentrotus pulcherrimus	70.0	20.0	46.3
Pseudocentrotus depressus	90.3	25.0	56.5
Strongylocentrotus nudus	92.9	13.3	52.6
Strongylocentrotus intermedius	23.1	5.0	12.6
Glyptocidaris crenularis	20.0	2.0	8.8
Astriclypeus manni	10.0	1.0	3.7
Clypeaster japonicus	0.0	0.0	–

* Cleavage rates were checked at the two cell stage.

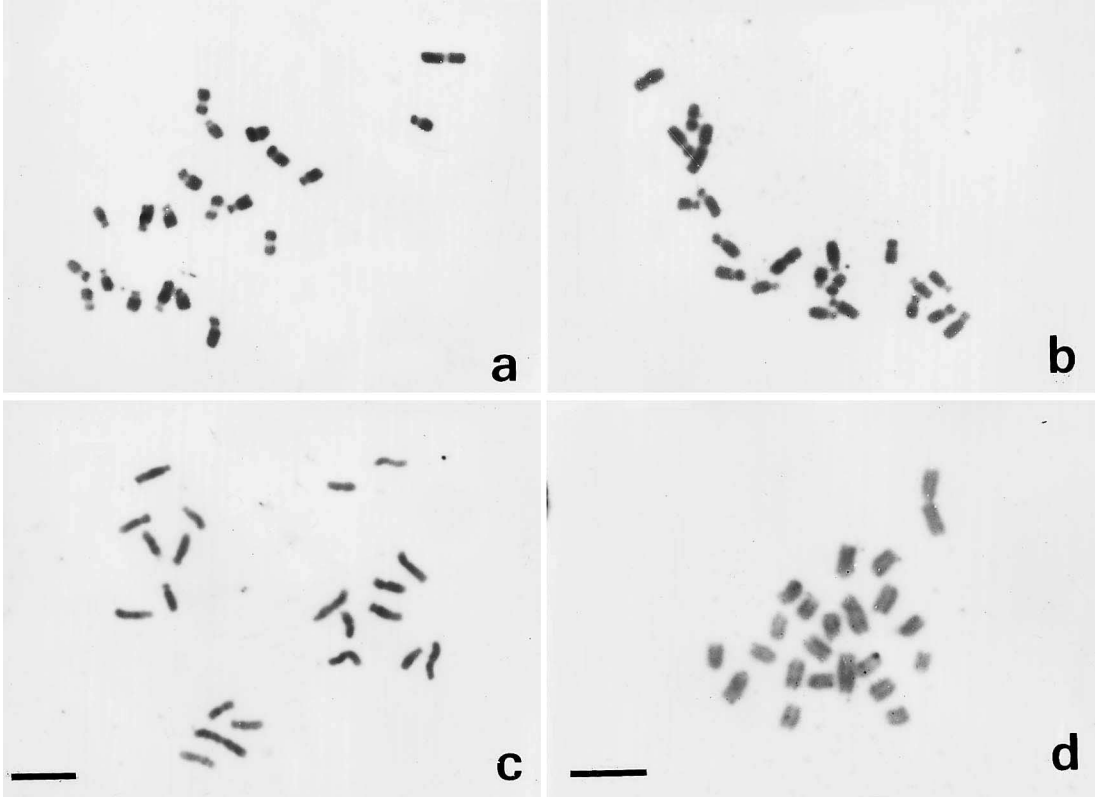

Fig. 1. Typical metaphase chromosomes from the hybrid andromerogones of *H. pulcherrimus* non-nucleate egg fragments and sperm of four species.
a) *Strongylocentrotus nudus,* b) *Pseudocentrotus depressus,* c) *Strongylocentrotus intermedius,* and d) *Glyptocidaris crenularis.* Bar, 5 μm.

suspension. We simultaneously added 3-amino-1, 2, 4-triazole (Sigma, St Louis, Mo., USA), 0.1–0.3 mg/ml, to inhibit hardening of the fertilization membrane (Showman and Foerder, 1979). The fragments were then washed several times with fresh seawater. When the fertilization rate was low, prior to insemination we suspended fresh non-nucleate egg fragments in 0.5 ml seawater, treated them with 0.002%–0.04% trypsin for 1–2 min, and then, added 10 ml seawater.

Chromosome preparation

Andromerogones at the morula or early blastula stage were treated with colchicine (Sigma, St Louis, Mo., USA) at a final concentration that stopped cell division (0.1–1.0 mg/ml), collected by centrifugation (300×g×5 min), suspended in 1M urea, and dissociated into their component blastomeres by pipetting. The dissociated blastomeres were collected by centrifugation, treated with 7%–10% sodium citrate for 10 min, fixed with methanol: acetic acid (3:1), and washed three times with the fixative. The fixed cells were dropped on slides and air-dried (Saotome, 1982, 1987). The preparations were stained for 10 min with 3% Giemsa (Merck, Whitehouse Station, NJ, USA) diluted with a standard pH6.9 solution (Horiba Ltd., Tokyo, Japan). The preparations were made twice-5 times in each species.

RESULTS

Formation of hybrid andromerogones

The sperm of all 7 species could be cryopreserved for about 1 year. When the sperm was thawed and diluted with seawater, the spermatozoa swam actively. Table 1 shows cleavage rates of the hybrid andromerogones obtained by inseminating *H. pulcherrimus* non-nucleate egg fragments with the cryopreserved sperm. The cleavage rate varied widely with the batch of egg fragments for heterospermic as well as homospermic (20%–70%) insemination, and also varied with sperm species: *S. nudus* showed highest cleavage rate (92.9%), while *C. japonicus* showed the lowest (0%). (When egg fragments were inseminated with *C. japonicus* sperm after trypsin treatment, the fertilization membranes were formed to some extent, but cleavage did not follow.)

The hybrid andromerogones cleaved successively into two-, four- and eight- blastomeres, and developed to the early blastulae.

Chromosomes of hybrid andromerogones

Figs 1a–d show typical metaphase chromosomes from the hybrid andromerogones obtained by inseminating *H. pulcherrimus* non-nucleate egg fragments with sperm of four species. The chromosomes spread over one layer, which made it possible to count the chromosome number precisely and analyze the karyotype.

Figs 2a–e show the distribution of chromosome num-

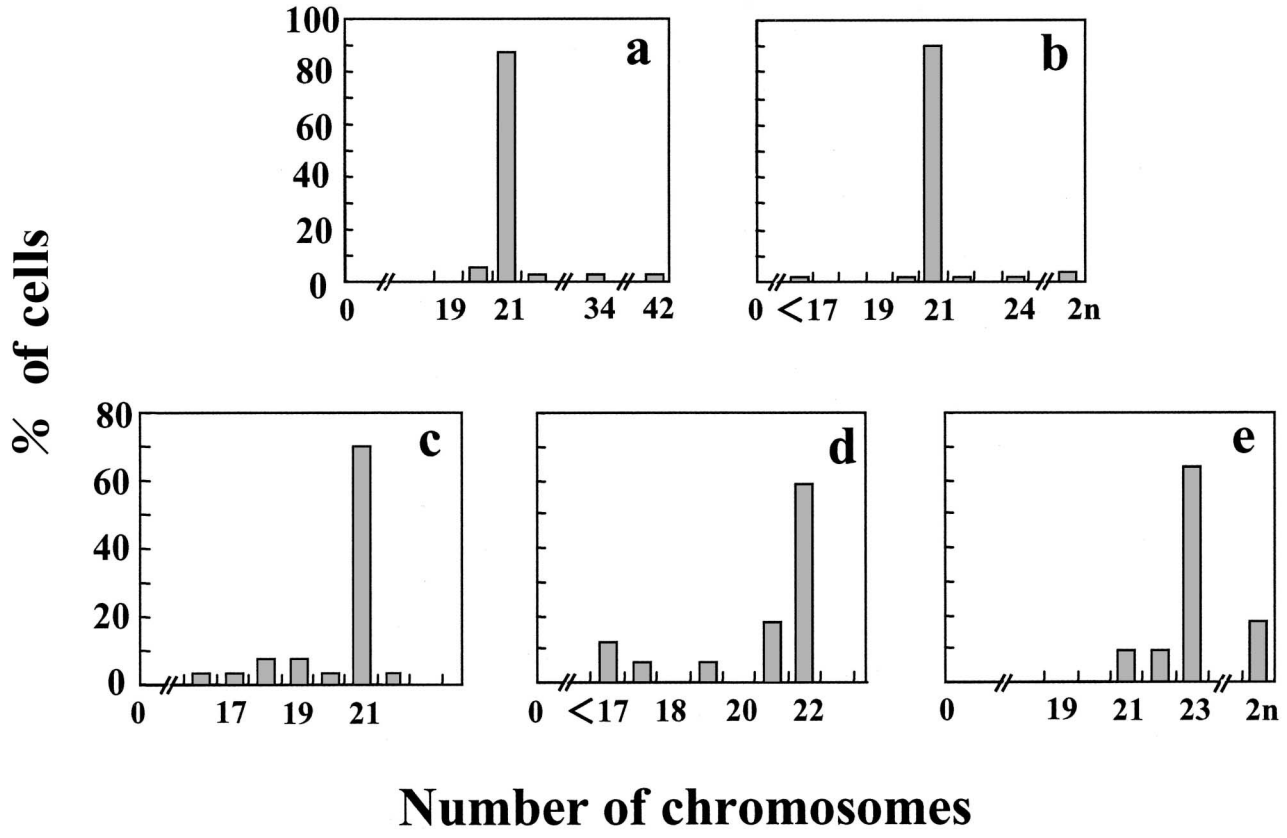

Fig. 2. Distribution of chromosome number in the hybrid andromerogones of *H. pulcherrimus* non-nucleate egg fragments and sperm of 5 species.
a) *Strongylocentrotus nudus* (analyzed cells, 39 cells), b) *Pseudocentrotus depressus* (64 cells), c) *Strongylocentrotus intermedius* (27 cells), d) *Glyptocidaris crenularis* (17 cells), and e) *Astriclypeus manni* (11 cells).

bers in the hybrid andromerogones of *H. pulcherrimus* non-nucleate egg fragments and the sperm of 5 species. The modal numbers were 21 in *S. nudus*, *P. depressus,* and *S. intermedius* (Fig. 2a–c), 22 in *G. crenularis* (Fig. 2d), and 23 in *A. manni* (Fig. 2e).

DISCUSSION

In this paper, we reported a method for preparing male chromosomes from hybrid andromerogones. We used *H. pulcherrimus* eggs for non-nucleate egg fragments. We were able to obtain viable eggs for 5 months (December to April) by growing adult females under appropriate laboratory conditions. We separated non-nucleate egg fragments by centrifugation, and found them easy to handle owing to their robustness. The sperm, on the other hand, were able to be cryopreserved until used in this study. Freezing was a simple procedure requiring only containers containing liquid nitrogen and could be applied anywhere fresh dry sperm can be obtained.

The cleavage rates varied widely among the 7 species. The sources of the variation were 1) non-nucleate egg fragment fertility, 2) sperm viability 3) hybrid formation ability, and 4) trypsin effect. 1) Non-nucleate egg fragment fertility varied with the batch of fragments, ranging from 60% to 90%, even with homospermic insemination, as was described previously (Saotome, 1999). Since fertilization between the different species are not apt to occur than that between the same one, there may have been wider variation in the hybrid andromerogones with heterospermic insemination. 2) Sperm viability after freezing and thawing should strongly affect cleavage rate. When whole eggs were inseminated with cryopreserved same-species sperm, fertilization rates varied from 5% to 95% (Dunn and McLachlan, 1973), 6% to 78% (Asahina and Takahashi, 1978), and 0% to 74.3% (Kurokura *et al.,* 1989). In the present study, cleavage rates varied widely in the case of *H. pulcherrimus* non-nucleate egg fragments inseminated with cryopreserved same-species sperm (Table 1), which may indicate the possibility of wider variation in the case of the non-nucleate fragments with heterospermic insemination. Even when there was good sperm motility, the cleavage rates were sometimes low. In such cases, the possibility may be thought that the sperm acrosome structure is destroyed after the cryopreserved sperm is thawed (Kurokura *et al.,* 1989). 3) Hybrid formation ability in whole eggs varies greatly with parental species. When whole *Paracentrotus lividus eggs* are fertilized with *Psammechinus microtuberculatus* sperm, numerous pluteus are formed (Hörstadius, 1936; Chen and Baltzer, 1975), but when they are fertilized with *Sphaerechinus granularis* sperm, very few hybrids become plutei owing to chromosome elimination (Chen and Baltzer, 1975). Also, hybrid formation ability may be lower in non-nucleate egg fragments than in whole eggs. Fertilization of *Genocidaris maculata* whole eggs with *Ps. microtuberculatus* sperm yields healthy hybrid offspring, while fertilization of the egg fragments with *Ps. microtuberculatus* sperm yields only embryos that do not survive past gastrulation (von Ubisch, 1959). 4) Trypsin facilitates hybrid formation (Chen and Baltzer, 1975). Trypsin treatment of *H. pulcherrimus* non-nucleate egg fragments prior to insemination with *A. manni* sperm increased the cleavage rate (from 1% to 7.3%). Facilitation of hybrid formation by trypsin, however, did not always take place in every hybrid combination. With *S. intermedius*, cleavage rates with trypsin treatment were lower (0.3%–5%) than without it (about 20%). Cleavage rates for *S. nudus* and *P. depressus* were high (about 50%, mean) even if non-nucleate egg fragments were not treated with trypsin. Variations in cleavage rates may be caused by a complex interaction of the above four factors.

The hybrid andromerogones from 5 species we examined could cleave normally, and all except *C. japonicus* could develop to early blastulae. Since *H. pulcherrimus* are located taxonomically far from *C. japonicus*, hybrid andromerogone formation ability may be low between them. We, however, do not understand clearly why the egg fragments fertilized with *C. japonicus* sperm did not cleave in spite of forming fertilization membranes. Though sperm diluted with seawater swam actively, they led to a low cleavage rate. A more concentrated sperm solution increased the cleavage rate with no apparent polyspermy. Cleavage was not synchronous; fertilization membranes were formed at once in some fragments and after 1 or 2 hr in others. Regrettably, development after the blastula stage was not researched because chromosomes were prepared from the blastulae.

The hybrid andromerogone modal chromosome numbers in this study were 21 (*S. nudus, S. intermedius*, and *P. depressus*), 22 (*G. crenularis*), and 23 (*A. manni*) (Fig. 2), corresponding to the haploid number of their respective species (Saotome, 1987, 1989). We observed a 3%–20% rate of aneuploidy. The possibility of chromosome elimination during cleavage may be low, because this scattering in number has been also observed in the preparations obtained from normal embryos (Saotome, 1987, 1989) and *H. pulcherrimus* andromerogones (Saotome, 1999).

This method is applicable to species in which dry sperm can be obtained and cryopreserved and whose andromerogone cleavage rates are greater than 10%. Alternative methods, such as the preparation from only cleaving hybrid andromerogones, are needed for andromerogones with lower cleavage rates. Moreover, if we are able to prepare chromosomes from hybrid andromerogone obtained by inserting directly a sperm into a non-nucleate egg fragment by microinsemination, chromosome analysis of rare species may be possible by obtaining only a small amount of sperm.

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