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Chromosome Number of Sea Urchin Andromerogones during Early Development

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ABSTRACT—The chromosome number of the andromerogones obtained by fertilizing non-nucleate egg fragments of *Hemicentrotus pulcherrimus* was examined by an air-drying method during early development. The non-nucleate egg fragments were prepared by centrifuging unfertilized eggs in a stepwise saccharose density gradient with a purity of 99.9%, comprising from about one-tenth to one-fourth of the total egg volume. The andromerogones cleaved, hatched and then actively swam. Though their development tended to be slower compared with that of control whole embryos after hatching, most of them developed into larvae and the remainder developed as permanent blastulae. While the larvae had differentiated gut and pigment cells, they also had various skeleton types which varied from almost normal to irregular. The chromosome preparations were made from blastomeres dissociated from many andromerogones. The rate of cells having a haploid chromosome number of 21 was 73% at the two cell stage, 84% at the 8 cell stage, 75% at the morula stage, 76% at the hatching blastula stage and 87% at the swimming blastula stage, while that of cells having a diploid number of 42 was 2% if averaged out for total cells examined. A total of 73–87% of the andromerogones was found to develop with a haploid number of chromosomes.

INTRODUCTION

Sea urchin egg fragments with no female pronucleus can develop after fertilization with sperm (Morgan, 1896) and some are even known to develop into larvae (Boveri, 1889). Such egg fragments developing with only a male nucleus, “andromerogones”, have been actively studied since the late 19th century and early 20th century from the viewpoint of their developmental capacity or the role of the nucleus in heredity.

In those days, the egg fragments were prepared by shaking unfertilized eggs in a test tube containing a small amount of sea water to study merogones (Hertwig and Hertwig, 1887; Boveri, 1889; Morgan, 1896) and then by cutting them into two halves (Whitaker, 1929). The shaking, however, produced various sizes of non-nucleate fragments and the cutting could not supply enough non-nucleate fragments. On the other hand, the study of egg stratification by slightly centrifuging unfertilized eggs had already began (Lyon, 1907). When eggs were centrifuged furthermore, they separated into halves or quarters and non-nucleate fragments were located in the centrifugal area (Harvey, 1932, 1940, 1956). Many non-nucleate egg fragments could thus be constantly obtained and detailed research on them including their development became possible.

Andromerogones have been thought to develop with only a haploid number of chromosomes (Morgan, 1896), since a haploid chromosome set and a mitotic center are supplied to the non-nucleate fragments from sperm through fertilization. Morgan (1896), by counting the chromosome number at the two or four cell stage in the paraffin sections, actually reported that normal *Echinus* (*Psammechinus*) *miliaris* embryos had 22 chromosomes as a diploid number and that their andromerogones had from 8 to 12 chromosomes. According to Colombera (1974), however, the chromosome numbers of several species reported in the early 20th century are different from those obtained by using the squash method developed at mid-century. Since the chromosome number reported by Morgan is also too low, reexamination is necessary to clarify the chromosome number of andromerogones.

Harvey (1940) pointed out the existence of multinucleate forms in fragments of *Arbacia punctulata* as a great amount of heavier granular materials interfered with the cleavage planes. Von Ledebur-Villiger (1972) reported that parthenogenetic embryos of *Paracentrotus lividus* had diploid and tetraploid number of chromosomes by intranuclear division. Moreover, the irregular chromosome numbers caused by mitotic disturbance have also been found in the merogonic embryos of Triton (Fankhauzer, 1934). The possibility still remains from these reports that sea urchin andromerogones may have diploid and aneuploid number of chromosomes.

The aim of the present work is to ascertain whether andromerogones develop with a haploid number of chromosomes by accurately counting their chromosome numbers using the air-drying method (Saotome, 1982a, 1987). I used a modified centrifugation method to obtain a large number of andromerogones, from which a large number of metaphase

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plates could be analyzed.

MATERIALS AND METHODS

Hemicentrotus pulcherrimus was used as material. Adults *H. pulcherrimus* were collected from the Misaki Marine Biological Station, University of Tokyo, and Manazuru, Kanagawa Prefecture. Eggs and sperm were obtained by injecting 0.05 ml of 0.01 M acetylcholine chloride into the body cavity of mature adults. More than 20 pairs of females and males were used.

Preparation of non-nucleate egg fragments

Non-nucleate egg fragments were obtained by centrifuging unfertilized eggs in a stepwise saccharose density gradient according to the method of Harvey (1932, 1956) with the following modifications: 1) four layers of stepwise density gradient were created by mixing 1 M saccharose and sea water in ratios of 1 to 2, 1 to 1, 2 to 1 and 1 to 0 from top to bottom in the 50ml centrifuge tube (Fig. 1a), 2) 0.5 ml of suspension of unfertilized eggs (4–10 $\times$ 10^4 eggs/ml) with jelly was layered over the gradient, and 3) eggs were centrifuged at 21,000 $\times$ *g* (in the middle of tube) for 20 min at 13°C (15,000 rpm, RPR 20–2 angle rotor, Hitachi CR20B2). The non-nucleate egg fragments located on the Band 2 in Fig.1b were removed with a fine pipette and transferred to 45 mm dish and then washed three times with sea water. The fragments were fertilized by adding a sperm solution (about 4 $\times$ 10^6 sperm/ml in final concentration) which is 10–20 times more concentrated than that used in normal fertilization. 3-Amino-1,2,4-triazole (Sigma Chemical Co. St Louis, Mo., USA) was added to the dishes at a final concentration of 0.1 mg/ml immediately after fertilization to prevent the hardening of the fertilization membrane (Showman and Foerder, 1979).

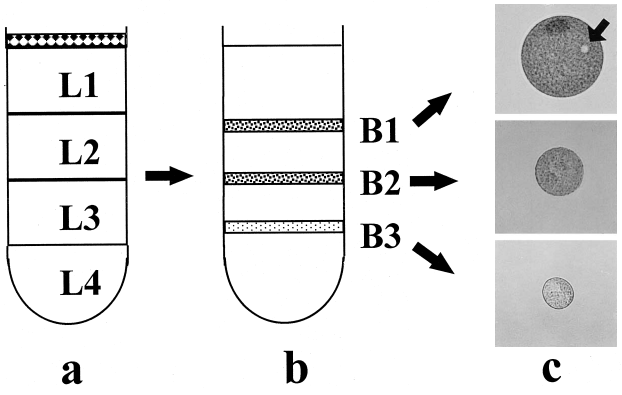

Fig. 1. Separation of eggs in a saccharose density gradient by centrifugal force. (a) Four layers (L1, L2, L3, and L4) were created by mixing 1 M saccharose and sea water. Details are described in Methods. (b) Separation pattern of egg fragments. The three major bands are indicated by B1, B2 and B3. (c) Phase contrast figure of the squashed fragments from each band. The small arrow indicates nucleus.

Chromosome preparation

Colchicine (Sigma Chemical Co. St Louis, Mo., USA) was added at a final concentration of 0.5 mg/ml to the dishes at the two cell, eight cell, morula, hatching blastula and swimming blastula stages to inhibit the next division. The embryos were collected by centrifugation and suspended with 1 M urea or Ca-Mg-free sea water (NaCl 31g, KCl 0.8g, $NaHCO_3$ 0.5g per liter of distilled water). The removal of the fertilization membrane and the dissociation of embryos into their component blastomeres were carried out at the same time by pipetting. The dissociated blastomeres were collected by centrifugation and then treated with 0.075 M KCl or 7% sodium citrate for 10 min. The blastomeres swollen were fixed with methanol-acetic acid (3:1) and washed three times with the fixative. Chromosome preparations were created by the air-drying method (Saotome, 1982, 1987). Chromosomes were stained for 10 min with 3% Giemsa (Merck, N.J., USA) solution diluted with 0.1 M phosphate buffer (pH 6.8).

RESULTS

Preparation of non-nucleate egg fragments

The stepwise saccharose density gradient in the present work is composed of four layers (L1, L2, L3, L4) as shown in Fig. 1a. When unfertilized eggs were centrifuged in the gradient, three bands (Fig. 1b) were obtained: the first band (B1) was located on the upper area of L2; the second band (B2) in the border area between L2 and L3; and the third band (B3) in the lower area of L3. While B1 and B2 were sharp bands, B3 was faint. The fragments from B1 were ascertained to be nucleates having nuclei (small arrow in Fig. 1c) and those from B2 and B3 were ascertained to be non-nucleates by observing the slightly squashed egg fragments with a phase contrast microscope (Fig. 1c).

Fig. 2 shows the distribution of diameters of the egg fragments from B2 and B3, and that of whole eggs. The diameter of B2 ranged from 38 to 72 μm, that of B3 ranged from 23 to 42 μm and that of whole eggs ranged from 92 to 104 μm. The diameters of B2 fragments were heterogeneous and always larger than those of B3. The volume of fragments from B2 ranged from 1/10 to1/4 of that of whole egg and that of fragments from B3 was about 1/50. The egg fragments from B3 tended to cause cytolysis after being transferred to dishes and washed with sea water. The egg fragments from B2 were used as non-nucleates in this study with regard to volume size and the stability of fragments. The recovery of non-nucleate fragments was 10–30%, and 1,000–5,000 fragments were obtained from 2–5 $\times$ 10^4 eggs in a centrifugal tube. The con-

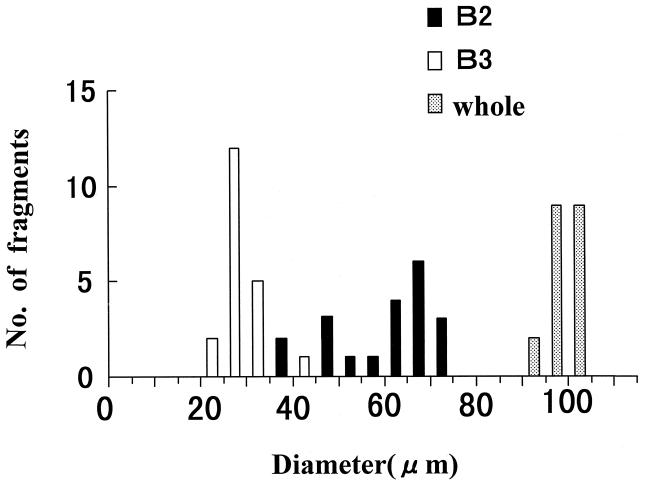

Fig. 2. Distribution of the diameter in non-nucleate egg fragments from B2 (–■–) and B3 (–□–), and whole eggs (–▨–).

tamination of nucleate egg fragments in the non-nucleates was less than 0.1%.

Development of andromerogones

When the non-nucleate egg fragments from B 2 were fertilized with a sperm solution similar in concentration to that used in normal fertilization, the cleavage rate of fragments ranged from 10 to 25%. The rate increased to 60–90% by fertilizing with a sperm solution 10–20 times higher than that used in normal fertilization. The difference (60–90%) in the percentage of cleavage rate depended on the batches of eggs. Moreover, the cleavage rate increased when using a more concentrated sperm solution, while polyspermy simultaneously took place at a higher frequency.

Whole embryos with normal male and female nuclei were used as “control” in this paper to compare with the development of andromerogones. Fig. 3 shows the development of control embryos (A-F) and andromerogones (a-f). Andromerogones cleaved successively into two (Fig. 3a), four and eight blastomers, developed to the morula stage (Fig. 3b), and then hatched and swam. Though the andromerogones had a similar developmental rate as the control embryos (Fig. 3A, B) in the early stages, the developmental delay in the andromerogones became large after hatching. Blastulae with small blastocoels and thick walls were formed after one day at 13°C (Fig. 3c). While the control embryos became gastrulae two days after fertilization (Fig. 3D), some andromerogones began to invaginate (Fig. 3d-1), though most were still blastulae with larger blastocoels and thin walls (Fig. 3d-2) and the remainder were blastulae containing many cells in the blastocoel (Fig. 3d-3). The andromerogones were still at the prism or blastula stage (Fig. 3e-1, 2, 3) even when the control embryos reached the early pluteus stage (Fig. 3E). When the control became pluteus after 5 days (Fig. 3F), the following two types were mainly observed in the morphology of andromerogones: 1) small plutei with differentiated gut and pigment cells, of which the skeleton formation is almost normal (Fig. 3f-1) or irregular (Fig. 3f-2,3), and 2) permanent blastulae having large blastocoels with or without many cells (Fig. 3f-4). The rate of the former was always higher than the latter.

Chromosome number of andromerogones during early development

A metaphase plate with a diploid chromosome number of 42 from the whole embryos at the early blastula stage is shown in Fig. 4a. Fig. 4b shows a typical metaphase plate from the andromerogones and the chromosome number of

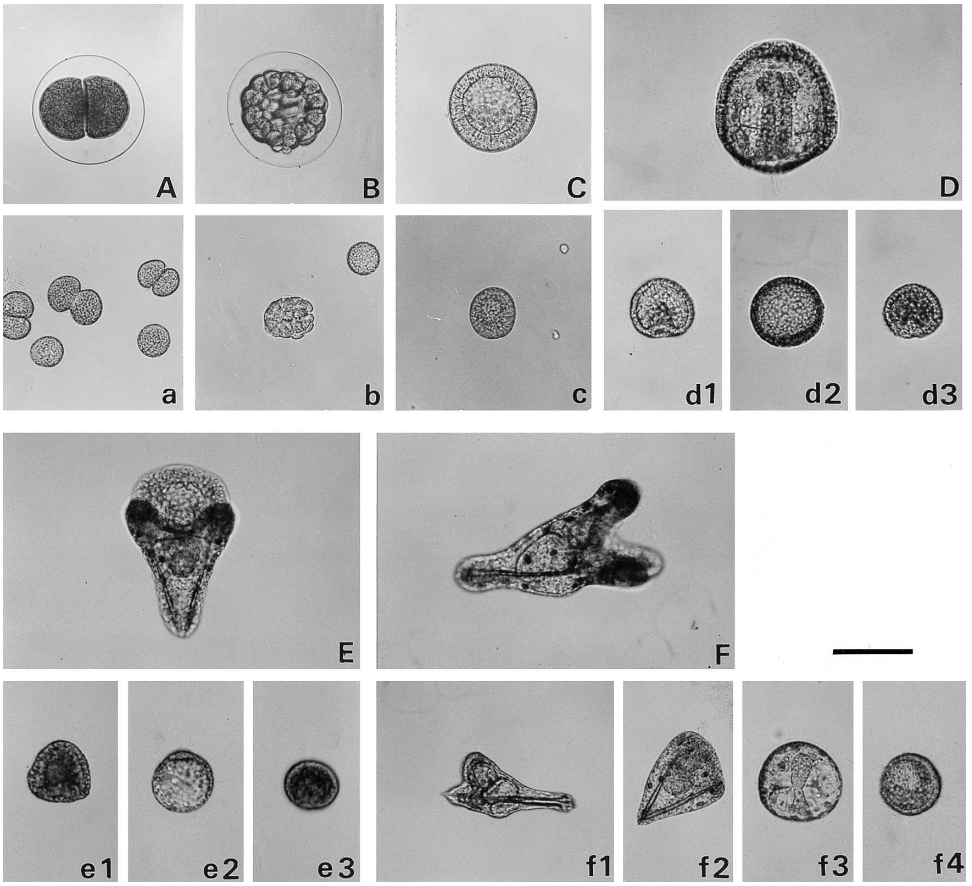

Fig. 3. Development of whole embryos (A–F) and andromerogones (a–f). (A, a), two cell stage; (B, b), morula stage; (C, c), swimming blastula stage; (D), gastrula stage; (E), early pluteus stage and (F), pluteus stage. Bar, 100 μm.

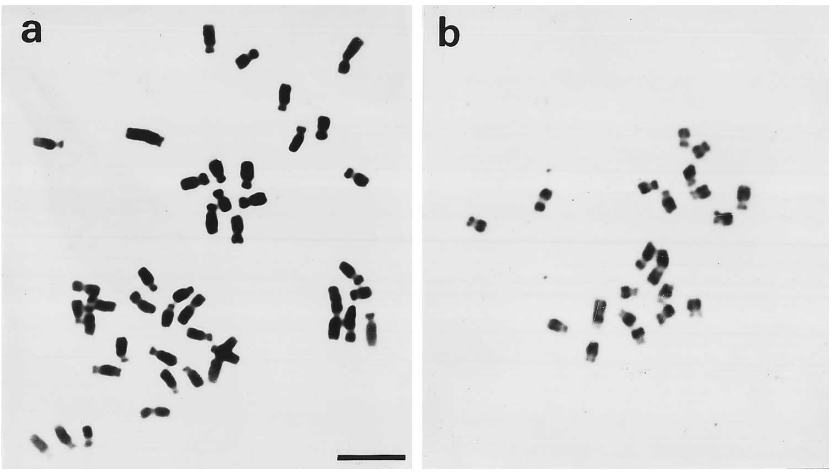

Fig. 4. Metaphase chromosomes from whole embryos (a) and andromerogones (b). Bar, 5 μm.

21 can be counted.

Fig. 5a-e are histograms of chromosome numbers in the andromerogones examined at 5 stages. The rate of cells having a haploid chromosome number of 21 was 73% in 86 cells at the two cell stage, 84% in 87 cells at the 8 cell stage, 75% in 115 cells at the morula stage, 76% in 201 cells at the hatching blastula stage and 87% in 46 cells at the swimming blastula stage. When the total of cells with chromosome number of 19, 20, 22 and 23 are defined as an aneuploid of a haploid chromosome number in this paper, the rate of an aneuploid was 16, 13, 13, 8 and 4% at the respective stages mentioned above. The rate of cells having a diploid number of 42 chromosomes was 5% at the two cell stage, 0% at the 8 cell stage, 2% at the morula stage, 2% at the hatching blastula stage and 4% at the swimming blastula stage. The rate was 2% if averaged out for total cells examined. The mode of chromosome number was found to be 21 at all stages.

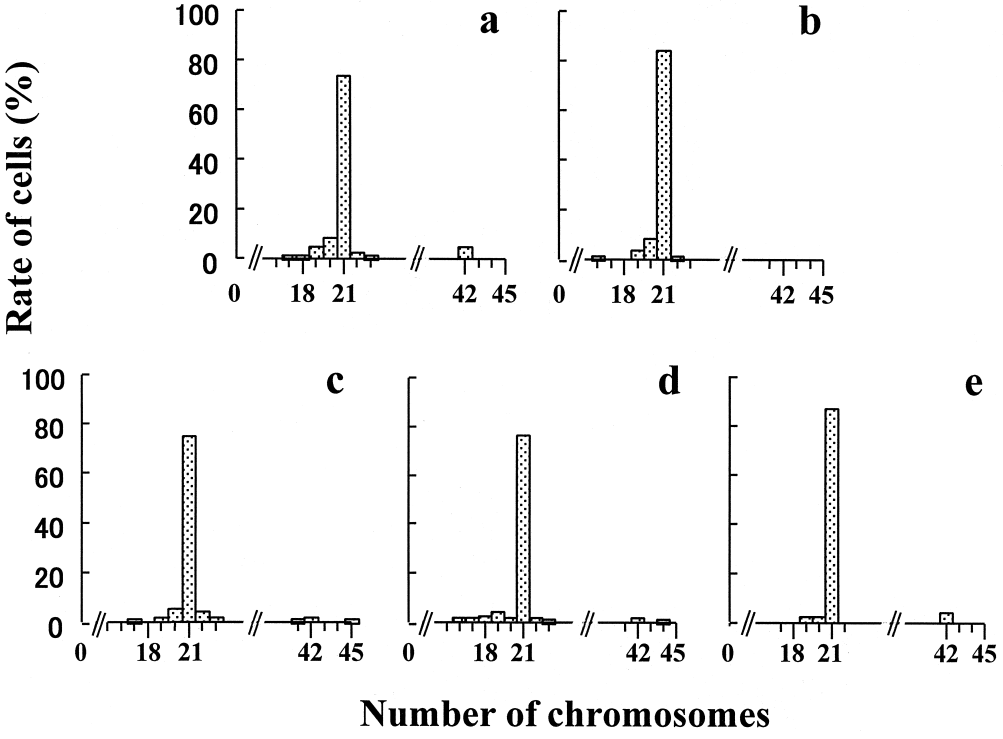

Fig. 5. Histogram of andromerogone chromosome numbers during early development. (a) two cell stage, (b) 8 cell stage, (c) morula stage, (d) hatching blastula stage and (e) swimming blastula stage.

DISCUSSION

H. pulcherrimus eggs were found to be separated into three bands by strong centrifugal force (21,000 × *g* for 20min) as shown in Fig. 1b and non-nucleate fragments could be obtained from Band2 (B2) with a purity of 99.9%. Harvey (1956) divided the egg halves after prolonged centrifugation (10,000 × *g* for 20-30 min) into quarters: clear, mitochondrial, yolk and pigment ones. Though these results can not be compared exactly with mine because of the difference in the centrifugation system and the species of sea urchins, examination of fragment size and their egg content suggests that the fragments from B2 in this paper correspond to the yolk quarter of Harvey (1956).

The diameter of egg fragments from B2 was heterogeneous compared to that of whole eggs or that of egg fragments from B3 (Fig. 2), which indicated that the points of constriction vary sensitively between B1 and B2 depending on individual eggs. B2 sometimes separated into two more bands. Since these bands did not appear by changes in egg number, but depending on individuals under the same centrifuge conditions (data are not shown), this pattern is thought to be related to the difference in the way of egg constriction depends on their batches. The fragments from both bands were non-nucleates and their development was similar to that of the fragments from B2 after fertilization.

Boveri (1889) reported that perfect dwarf-larvae from non-nucleate fragments could be obtained by shaking. Harvey (1932, 1940, 1956) stated that several red half-eggs without nuclei obtained by centrifugation developed into normal larvae with lattice-like skeletons after fertilization, and that the larvae were very heavily pigmented and much smaller than those from whole eggs. While mitochondrial and pigment quarters prepared by Harvey (1956) developed into the swimming blastula without further development, yolk quarters formed pluteus with pigment and irregular skeletons. Most andromerogones in the present study developed into the larvae with differentiated guts, pigment cells and various types of skeleton, and the remainder developed into permanent blastula. The morphology of larvae depends on their skeleton formation: sufficient skeleton formation gives rise to almost normal larvae (Fig. 3f-1), and irregular skeleton formation causes abnormal ones (Fig. 3f-2, 3). The development of fragments from B 2 in this paper was similar to that of the yolk quarters of Harvey (1956).

The diploid number of *H. pulcherrimus* is 42 as shown in Fig. 4a (Saotome 1987). Since the chromosome numbers of sea urchins reported are mainly 42, 44, 46 (Colombera, 1974; Saotome, 1982a, 1987, 1989, 1991), the chromosome number 22 of *Echinus* (*Psammechinus*) *milialis* reported by Morgan (1896) is too low. This low value is thought to be caused by the sectioning method used at that time, because sea urchins have many small chromosomes. Chromosomes expanding into the monolayer could be obtained with accurate counts from andromerogones by using the air-drying method (Fig. 4b). Though the chromosome number could be examined until the blastula stage, that in the gastrula or pluteus stage could not because of the low frequency of mitosis. The distribution mode of chromosome numbers was found to be 21 at all stages examined in andromerogones indicating that most andromerogones develop with a haploid number of chromosome until the swimming blastula stage.

The fragments from B1 had nuclei as shown in Fig.1 and were about 60-70% of the total egg volume. Though the fertilized nucleate fragments developed into the blastulae, their further development was almost normal in certain cases and abnormal in other cases. They showed diploid number of chromosome in 70% of 195 cells (the data are not shown).

The cells having a diploid number of chromosomes in andromerogones were observed at the rate of 0–5% in the stages examined. Since the contamination of the nucleate fragments in the non-nucleates is less than 0.1%, the value can not be explained by only the existence of nucleate fragments. The following two possibilities, therefore, are considered: 1) only a few well developed embryos gain diploid numbers by duplicating chromosomes during early development and 2) multinucleate cells occur in a few andromerogones in which granular material interferes with cleavage. The analysis of chromosomes prepared from one almost normal andromerogone during development is necessary to clarify the possibility that duplication may occur. The chromosome preparations in this study are made from dissociated blastomeres of many embryos to obtain many and good metaphase plates in which chromosomes expand into monolayer. Though the preparation techniqe from one whole embryo by air-drying method is important to clarify the difference among embryos or that among areas in one embryo, the preparation trial from one whole embryo has not yet been successful because of inadequate expanding of chromosome. The second possibility (Harvey, 1940) is not considered to be possible because complete cleavage furrows were observed from the 2 to the 16 cell stages in the early stages of *H. pulcherrimus*. It may take place in a later stage if there is cleavage interference.

Though the rate of the cells showing an aneuploid chromosome number is 4–16% (Fig. 5), the following three reasons are thought to explain its existence:1) irregularity in the distribution of chromosomes, 2) polyspermy and 3) technical errors. Since abnormal chromosome numbers are known in merogones developed into abnormal embryos in Triton (Fankhauzer, 1934), the permanent blastulae in this paper may have aneuploid chromosomes. This possibility must be solved by analyzing chromosomes from abnormally developed permanent blastulae. Morgan (1896) pointed out that polyspermy is apt to take place in merogones. Abnormal cleavage derived from polyspermy was sometimes observed in this work because of use of a concentrated sperm solution to raise fertilization rate. This aneuploid number can be explained by polyspermy, since chromosomes are known to be abnormally divided by polypolar division (Saotome, 1982b). Technical error, however, is also possible, because the rate of cells having an aneuploid of a diploid chromosome number ranged

from 6% for the lowest in *Scaphechinus mirabilis* to 21% for the highest in *Temnopleurus hardwickii* even in normal development (Saotome, 1987). Though the rate of cells showing hypoploid numbers tends to be larger than that having hyperploid ones because of outward flow of chromosomes during air-drying in the technical error, a tendency was similar to that observed in this study. These results show that the existence of aneuploidy can be explained by polyspermy and technical error.

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