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Androgenetic Reproduction in a Freshwater Diploid Clam *Corbicula fluminea* (Bivalvia: Corbiculidae)

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ABSTRACT—Two shell color types of the exotic bivalve *Corbicula fluminea* were collected in Kyoto city, Japan. DNA microfluorometry revealed that both types were diploids with non-reductional spermatozoa. Maternal chromosomes were found to be extruded as two polar bodies at the first meiosis, and the second meiosis could not be observed. Only the male pronucleus was present in the egg cytoplasm and became metaphase chromosomes at the first mitosis. The present study indicates that the diploid *C. fluminea* in Japan has the same mode of androgenetic reproduction as the triploid *C. leana*.

Key words: androgenesis, *Corbicula*, meiosis, clam, fertilization

INTRODUCTION

Corbicula fluminea (Müller 1774) is the most widely-distributed Asian clam species and is highly invasive and known also from North America, South America, Europe and Australia (Britton and Morton, 1986). *C. fluminea* reproduces by self-fertilization and broods its young in the inner demibranchs (Kraemer, 1978; Kraemer, 1983; Britton and Morton, 1986). Their spermatozoa have two flagella (Konishi *et al*., 1998).

Komaru and Konishi (1999) found three different shell color types of *C. fluminea* at the same location in Taiwan. They were composed of diploids and triploids with non-reductional spermatozoa (Komaru and Konishi, 1999).

In North America, it was voluntarily introduced from Asia and subsequently spread to many major river basins in North America. At present *C. fluminea* has become a major "pest" of industrial and domestic water supply systems (Britton and Morton, 1986; Balcom, 1994). *C. fluminea* was also introduced into Japan (Masuda and Habe, 1988). According to Nakai and Matsuda (2000), *Corbicula leana* (Prime, 1864) is an indigenous species to Japan, has been replaced by *C. fluminea* in some regions of Japan.

C. leana is a freshwater, hermaphrodite and triploid in Japan (Miyazaki, 1936; Habe, 1977; Ikematsu and Yamane, 1977; Okamoto and Arimoto 1986). Komaru *et al*. (1997) and Konishi *et al*. (1998) revealed that *C. leana* produces non-reductional spermatozoa with two flagella, which have the same DNA content as the somatic cells. *C. leana* reproduces by androgenesis (Komaru *et al*., 1997; Komaru *et al*., 2000) in which all the maternal chromosomes of eggs are extruded as two polar bodies at the first meiosis. After extrusion, only the male pronucleus forms the metaphase chromosomes at the first cleavage. It is indicated that *C. leana* is close relation to *C. fluminea* because they produces non-reductional spermatozoa with two flagella. However, little is known about egg developmental mode of *C. fluminea*.

In the present study, we examined the meiosis and fertilization of zygotes in *C. fluminea* by using DAPI staining for fluorescence microcopy.

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MATERIALS AND METHODS

Animals

Two color types of *Corbicula fluminea* were collected from the same point of Shishigatani creek, Kitashirakawa, Kyoto city, Kyoto Prefecture, Japan. 23 mature samples were collected and used in present study in June 1999. This creek is a part of the Lake Biwa-Yodo River system. We defined the two color types (n=23), as green (n=17) and pink (n=6). Triploid *Corbicula leana* samples were collected from the Amano River in Kitatawara, Ikoma city, Nara Prefecture, Japan, for determination of the ploidy of *C. fluminea*. Samples were collected in June 1999. The samples used in this study are deposited in the Lake Biwa Museum, Oroshimo, Kusatsu, Shiga Prefecture, Japan (green type: LBM-1300008873, pink type: LBM-1300008872).

DNA Microfluorometry

Ploidy of the somatic cells was determined as follows: gill or mantle cells of *C. fluminea* (green: n=17, pink: n=6 and *C. leana*

n=5) were fixed with Carnoy's fixation and a cell suspension was prepared on a glass slide in 50% acetic acid with a scalpel and air-dried. Three or four samples of *C. fluminea* and triploid *C. leana* as a control were spread on the same slide. The slides were stained with the DNA-specific dye DAPI, and the relative DNA content per cell was measured by microfluorometry (Komaru *et al*., 1998). Ploidy of the spermatozoa was examined from the gonad and somatic cells from the gill or mantle of the same animal which were smeared and placed on the same slide. These slides were stained with DAPI using the above-mentioned method.

Spawning induction

Spawning was induced by rising the water temperature from 20°C to 27°C. Spawned eggs of the pink type were collected and mixed from three individuals, while those of green type were collected from one individual.

Observations of chromosome behavior in eggs with DAPI staining

Spawned eggs were incubated at 27°C. Both color types were kept in different aquarium. The eggs were released into the inner demibranch or into the water from the exhalant siphon. Spawning eggs were fixed with 99% cold ethanol at every 10 or 15 min after spawning.

Fixed eggs were rinsed with 0.2M phosphate buffer (pH 7.5) three times and stained with DAPI (0.25 μg/ml). The chromosome behavior was observed using fluorescence microscope (Nikon ECLIPSE E600-Y-FL).

RESULTS

Shell color

The samples consisted of two distinct shell color types. Green type: External surface was light or dark green, and inside was deep purple (Fig. 1A). Pink type: External surface was yellow or light brown, and inside was white, although the umbone areas are pink and was characterized by a purple flash along with the anterior and posterior lateral teeth (Fig. 1B).

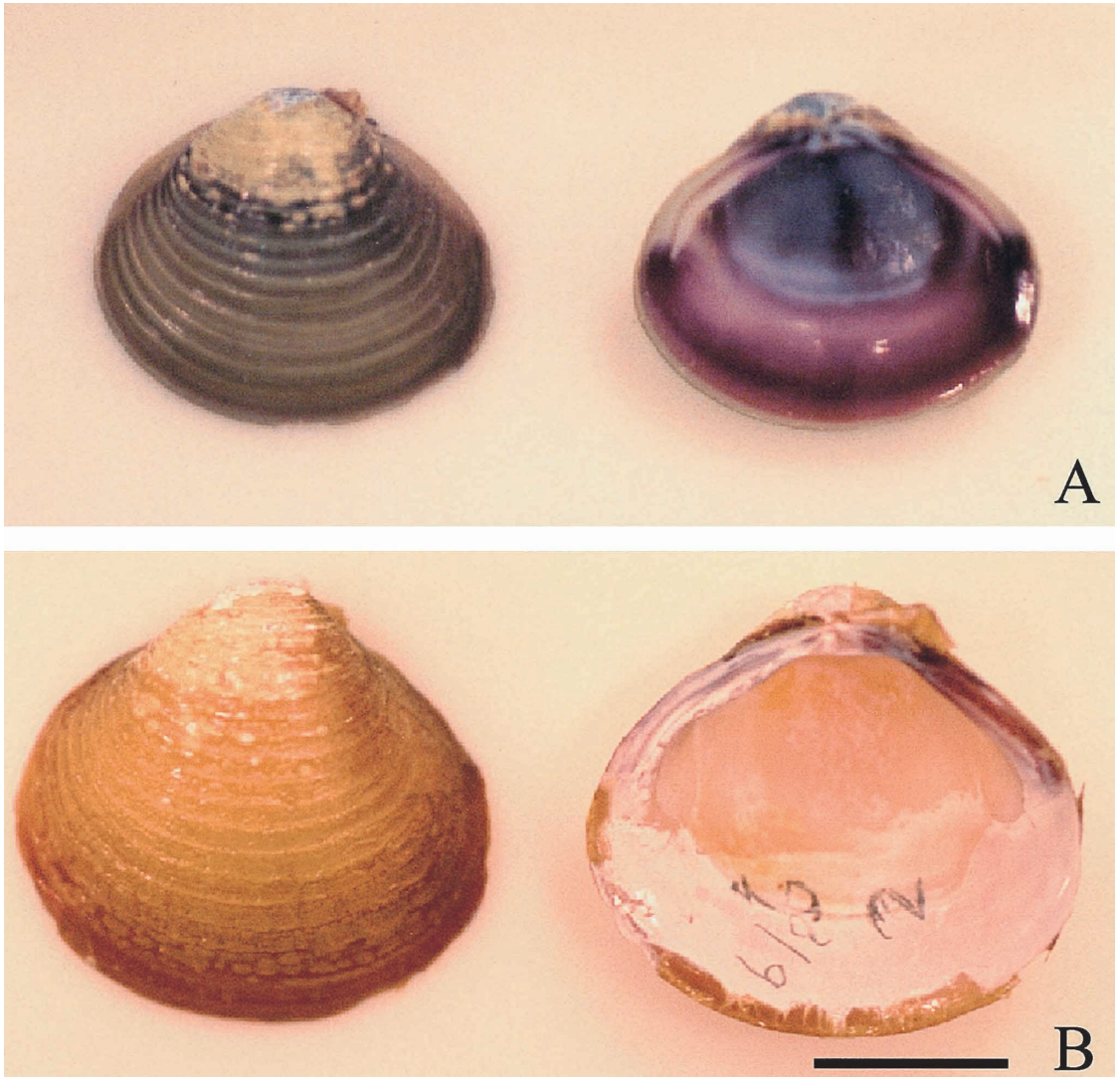

Fig. 1. External and internal view of the two types of *Corbicula fluminea* collected in Shishigatani creek, Kitashirakawa, Kyoto city. (A, green type:LBM-1300008873) (B, pink type:LBM-1300008872). Scale bar:10mm

Ploidy

The DNA content of the somatic cells is shown in Table 1. The mean relative fluorescence intensity of the green type of the *C. fluminea* compared to the triploid *C. leana* was 0.58 to 0.69. This showed that all the clams of the green type (sample Nos. 1–17) of *C. fluminea* were diploids. The mean relative fluorescence intensity of the pink type of *C. fluminea* compare to the triploid *C. leana* was 0.69–0.70, indicating those (sample Nos. 18–23) of pink type to be diploids.

Non-reductional spermatozoa

The mean DNA content of sperm and somatic cells of the two color types of *C. fluminea* is shown in Table 2. The relative DNA content of spermatozoa of both types was almost identical to those of the somatic cells. The relative fluorescence intensity from both types ranged from 0.98 to1.06. The ploidy of the spermatozoa of both the green and pink was the same as that of their somatic cells.

Egg size

The mean diameter of eggs from the green type just after spawning was 115.7±3.9 μm (n=105) and the pink type eggs was 127.9±4.9 μm (n=132). On the other hand, the egg diameter of triploid C. leana was 158.2±4.4 μm (n=98). Between the green and pink types, the egg diameter was not statistically different (*t*-test, $p>0.5$), however, the egg diameter of the two types of diploid *C. fluminea* was significantly smaller (*t*-test, $p<0.01$) than that of the triploid *C. leana*.

Process of androgenesis

Green type: Fig. 2 summarizes the process of androgenesis in the eggs. At 10 min after spawning, 20.7% of the eggs were unfertilized (Figs. 2A and 3) and 77.8% were fertilized and at the M-I stage (Figs. 2B and 3). At 20 min, most eggs were fertilized (87.9%). At 30 min, 81.5% of the eggs were at the anaphase of the first meiosis (A–I), and the maternal chromosomes were divided into two groups (Figs. 2C and 3). At 45 min, two polar bodies were formed in 75.3% of the eggs, but the female pronucleus was not formed (Figs. 2D and 3). All meiotic chromosomes were extruded as two polar bodies and only one male pronucleus was present in the egg cytoplasm. At 75 min, the male pronucleus became the metaphase chromosomes of the first cleavage (Figs. 2F and 3), and at 90 min, 66.3% of the eggs

Table 1. The relative DNA contents of somatic cells in *C. fluminea* compared to those of *C. leana* based on microfluorometry.

		DNA content (mean±S.D.)			
Sample No.	color type	*C. fluminea* (Cf)	*C.leana* (Cl)	(Cf)/(Cl)	ploidy
1	Green	22.17±1.40	32.39±1.41	0.68	2n
2	〃	22.22±1.34	32.39±1.41	0.69	2n
3	〃	20.42±0.97	31.54±1.30	0.65	2n
4	〃	20.92±1.24	31.54±1.30	0.66	2n
5	〃	20.82±0.84	31.54±1.30	0.66	2n
6	〃	21.38±1.42	31.54±1.30	0.68	2n
7	〃	20.34±0.99	30.24±1.20	0.67	2n
8	〃	19.90±0.91	30.24±1.20	0.66	2n
9	〃	19.00±1.17	30.24±1.20	0.63	2n
10	〃	18.46±1.01	31.51±1.19	0.59	2n
11	〃	18.25±0.91	31.51±1.19	0.58	2n
12	〃	20.12±0.91	29.58±1.28	0.68	2n
13	〃	19.45±1.07	29.58±1.28	0.66	2n
14	〃	20.41±1.26	29.58±1.28	0.69	2n
15	〃	20.78±1.10	31.56±1.50	0.66	2n
16	〃	20.81±1.32	31.56±1.50	0.66	2n
17	〃	18.57±1.18	31.35±1.12	0.59	2n
18	Pink	22.39±1.21	31.85±1.33	0.70	2n
19	〃	22.40±1.16	31.85±1.33	0.70	2n
20	〃	22.34±1.41	31.85±1.33	0.70	2n
21	〃	21.76±1.21	30.92±1.15	0.70	2n
22	〃	20.88±0.96	30.92±1.15	0.70	2n
23	〃	22.30±1.41	30.92±1.15	0.69	2n

Table 2. The relative DNA contents of spermatozoa and somatic cells (gill or mantle) in diploid *C. fluminea*.

Sample No.	color type	DNA content (mean±S.D.) Sperm (Sp)	Somatic cell (So)	(Sp)/(So)
1	Green	31.02±0.87	30.98±1.89	0.98
2	〃	32.63±1.64	30.67±1.54	1.06
3	〃	30.52±0.79	30.00±1.19	1.02
4	〃	30.54±0.62	30.13±0.71	1.00
5	〃	30.76±0.95	30.79±1.00	1.00
6	〃	30.71±0.92	30.72±1.10	1.01
7	〃	30.35±1.13	30.10±1.34	1.00
8	〃	30.35±1.27	30.26±1.23	1.01
9	〃	30.75±0.79	30.41±1.23	0.98
10	〃	30.49±1.08	31.32±0.92	1.00
11	〃	30.44±1.21	31.03±0.92	0.99
12	〃	30.38±1.05	30.33±1.00	0.99
13	〃	30.78±0.90	31.03±0.82	1.00
14	〃	30.94±1.11	30.13±0.66	1.00
15	〃	30.68±1.18	30.64±1.05	1.00
16	〃	30.66±1.33	30.50±1.22	1.01
17	〃	30.65±1.08	30.75±1.11	1.00
18	Pink	31.00±1.35	30.50±1.44	1.02
19	〃	30.62±1.79	30.53±1.40	1.00
20	〃	30.75±0.83	30.51±0.74	1.01
21	〃	30.74±0.79	30.79±0.76	1.00
22	〃	30.94±0.83	30.80±0.99	1.00
23	〃	30.63±1.02	31.02±0.87	0.99

were at the anaphase of the first cleavage.

Pink type: The eggs of the pink type also showed androgenetic development as shown in Fig. 4. All the maternal chromosomes were also extruded as two polar bodies. Thus, no female pronucleus was formed in the egg cytoplasm.

DISCUSSION

The present study showed that hermaphroditic freshwater diploid clam *C. fluminea* reproduces by androgenesis. The process was follows; androgenetic diploid *C. fluminea* were hermaprodite and produced non-reductional spermatozoa and its spermatozoa have two flagella. All the maternal chromosomes were extruded as two first polar bodies at the first meiosis. Only one male pronucleus was present in the egg cytoplasm and became the metaphase chromosomes at the first mitosis.

Komaru and Konishi (1999) and Qiu *et al*. (2001) reported that hermaprodite *C. fluminea* in Taiwan and China has various shell color types. Komaru and Konishi (1999) suggested that in *C. fluminea* from Taiwan, the shell color types might have different genetic background. *C. fluminea* consists of diploids, triploids and tetraploids and they produce non-reductional spermatozoa with two flagella in respective ploidy (Qiu *et al*., 2001). Diploid, triploid and tet-

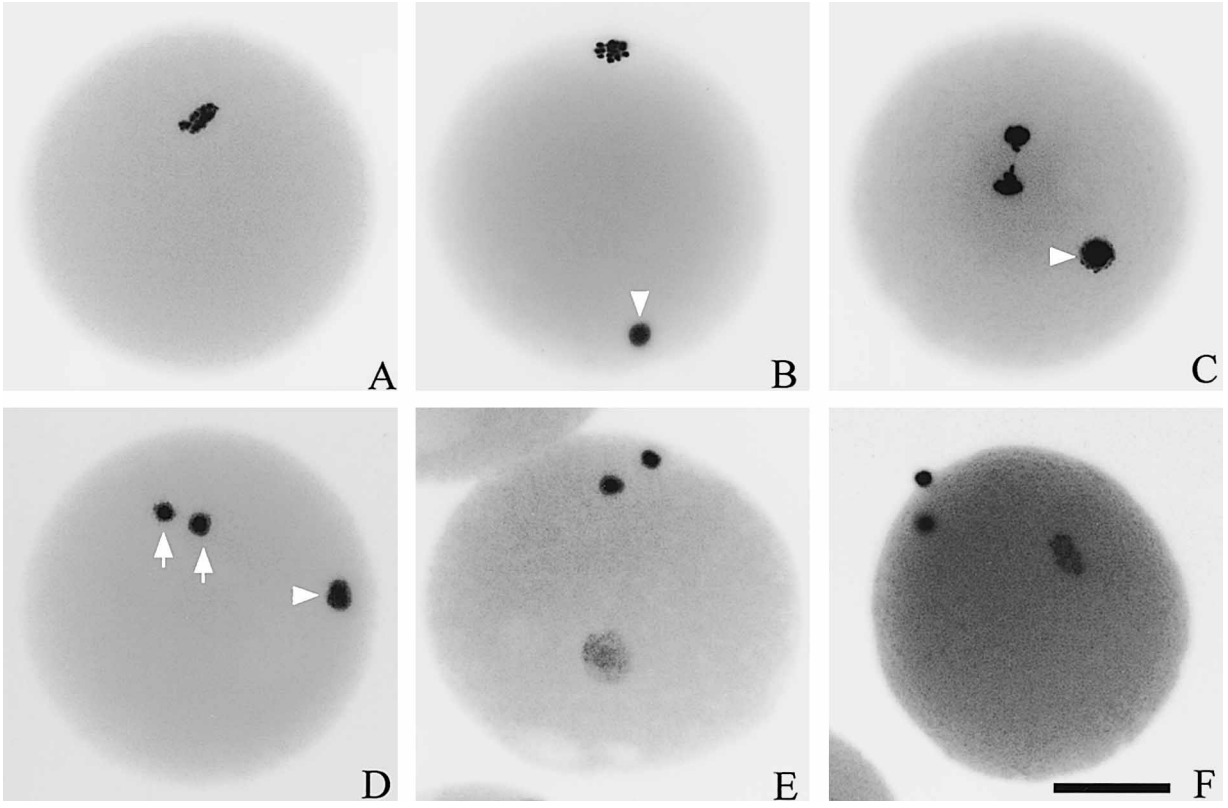

Fig. 2. DAPI images from the unfertilized egg stage to metaphase of the first cleavage in *C. fluminea* eggs. A: unfertilized at 10 min. B: fertilized at 20 min. C: anaphase of first meiosis at 30 min. D: polar body formation at 45 min. E: male pronucleus expanded at 60 min. F: metaphase of first cleavage at 75 min. Arrows indicate the polar body, arrow heads indicate the male pronucleus. Scale bar: 50 μm

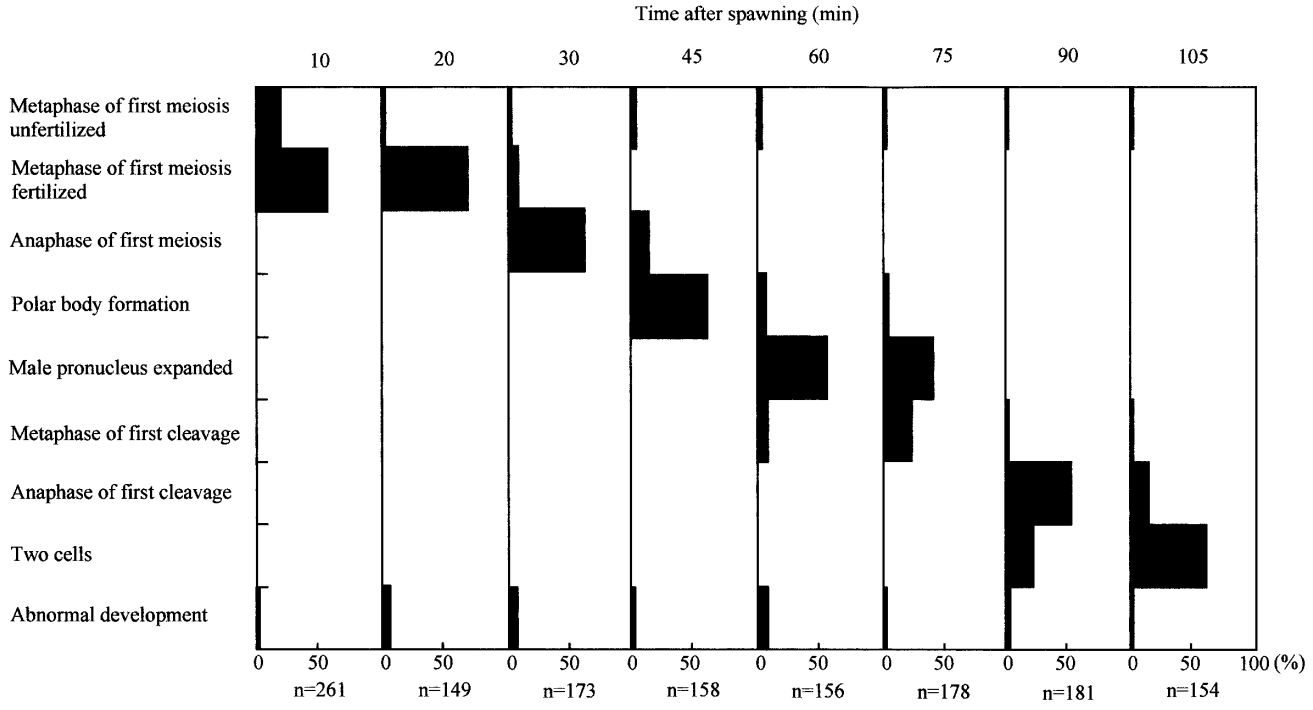

Fig. 3. Chromosome behavior from metaphase of first meiosis to two cell stages in the androgenetic *C. fluminea* eggs (green type).

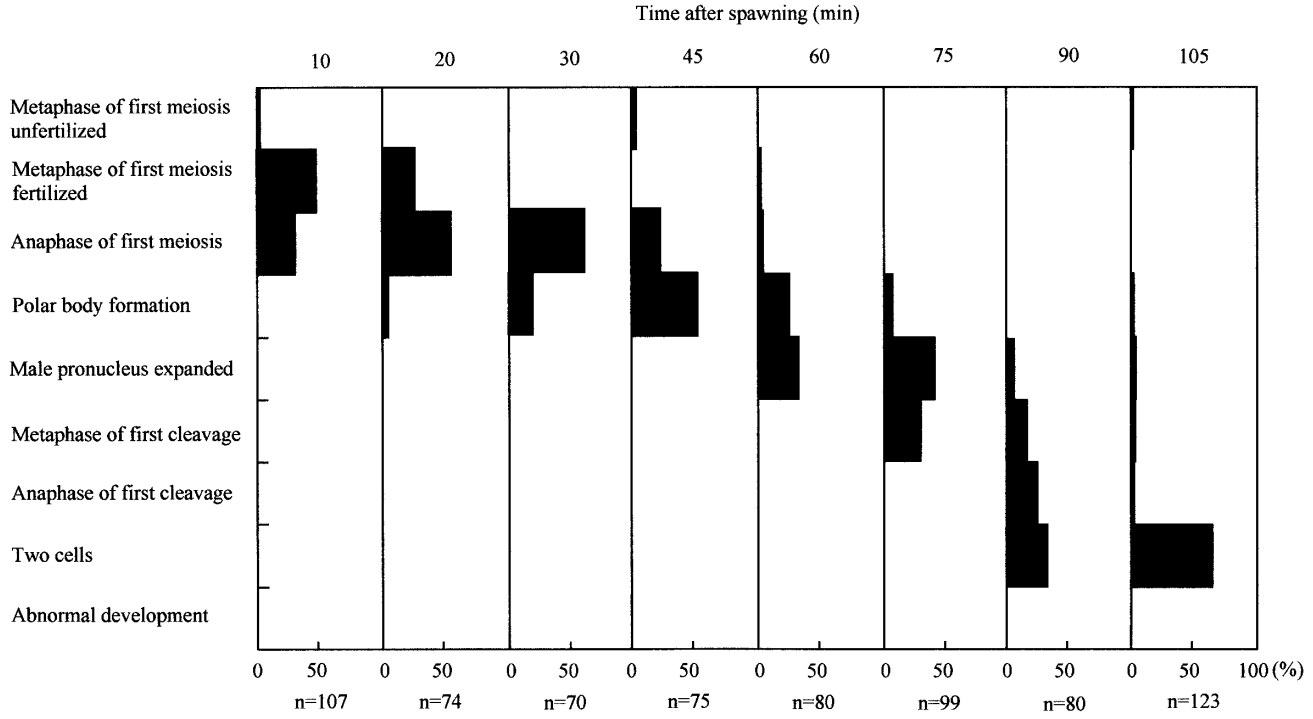

Fig. 4. Chromosome behavior from metaphase of first meiosis to two cell stages in the androgenetic *C. fluminea* eggs (pink type).

raploid *C. fluminea* in Taiwan and China may reproduce androgenesis because they were produced non-reductional spermatozoa such as *C. fluminea* in Japan.

Ishibashi *et al.* (2002) demonstrated that in androgenetic *C. leana* eggs treated with Cytocalasin D (CD) to inhibit polar body formation, the second meiosis occurred. Second meiosis proceeded normally in CD treated eggs when maternal chromosomes and centrosomes existed in egg cytoplasm, and typical meiosis system still proceeded. It is suggested that androgenetic form may have appeared from the meiotic form.

Ishibashi *et al.* (2002) showed the possibility that an

androgenetic triploid form may originate from the meiotic form in *Corbicula*. Triploid *C. fluminea* may elevate the ploidy level by accidental formation of the female haploid pronucleus (Komaru *et al*., 2001). So Diploid androgenetic form may arise from the meiotic form. Ploidy may have been elevated to the triploid levels. It is likely that the polyploid *C. fluminea* may have originated from the diploid androgenetic *C. fluminea*.

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