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Studies on the Gametogenesis in Polyploid Ginbuna Carassius auratus langsdorfii

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Abstract

The structural changes of the chromosomes during meiosis of gynogenetic triploid female ginbuna *Carassius auratus langsdorfii,* and naturally produced or sex-reversed male ginbuna were studied cytologically. In triploid female ginbuna, at zygotene stage two homologue-like chromosomes out of three were sometimes adjacent each other in the neighbourhood. At pachytene stage almost all of the chromosomes were univalents, and at diplotene stage almost all of lampbrush chromosomes were also univalents. From these results it seems probable that the two homologue-like chromosomes undergo synapsis at zygotene stage, but they might be separated to univalents on and after pachytene stage. Thereafter, each univalent chromosome might be split to two chromatids and result in separation of one chromatids to one direction and the other to the opposite one. In this way, the egg may maintain the maternal chromosome number and karyotype throughout the oogenesis. On the other hand, in some specimens at diplotene stage at most two to three bivalent lampbrush chromosomes, connected with one chiasma, were observed. These facts seem to explain the hypervariabilities among the gynogenetic triploid ginbuna. On the other hand, male individuals were almost similar to the processes in meiosis, though in some chromosomes it observed a few bivalents and rod-shaped bi-, tri-, and multivalent chromosomes at metaphase.

Introduction

The ginbuna, the most popular teleost in Japan, shows unusual sex ratio which extremely declines to male in some districts. In 1969, NAKAMURA has reported that ginbuna is further divided into two types, the one is unisexual type (female only) and the other is ordinal bisexual type. KOBAYASHI *et al.* (1970) have reported from the cytological studies that the chromosome number of ginbuna collected from Miyazaki prefecture, belonging to bisexual type, is $2n=100$, on the other hand that of collected from Kanto district, belonging to unisexual type, is $3n=156$ or $4n=206$. Further,

KOBAYASHI (1971) and KOBAYASHI and OCHI (1972) have observed the facts that the egg of triploid ginbuna is only activated by the sperm of the other or subspecies of the fish, and the sperm nucleus is degenerated and disappeared from the blastomeres without offering any genetic functions on the offspring. Therefore, they have supposed that the triploid ginbuna must be reproduced gynogenetically. In 1976 KOBAYASHI has further studied cytologically the processes of meiosis of triploid ginbuna. According to the studies, it becomes apparent that the oocyte skips the first meiosis, undergoing a homotypic nuclear division as the somatic cells, and by these processes triploid ginbuna produces the eggs which have the same number of chromosomes and the same karyotype as the mother. OJIMA and ASANO (1977) have also observed the same processes in the triploid ginbuna of Lake Biwa.

In our laboratory, HIJIKATA *et al.* (1983) have examined the genetic constitution of triploid ginbuna of Lake Kasumigaura and Lake Suwa, both belonging to unsexual type, by the scale transplantation experiments and electrophoretic analyses of some enzymes and muscle protein. In these experiments they have observed the hypervariabilities in histocompatibility clones and electromorph clones. On the other hand, NAKAKUKI *et al.* (1983) have observed cytologically that in the eggs of triploid ginbuna inseminated artificially by the sperm of gold fish *Carassius auratus auratus,* the sperm nucleus is sometimes transformed into male pronucleus and conjugated with female pronucleus as in normal fertilization. Some of these eggs develop into tetraploid individuals. From these observations it becomes apparent that the male genome or genes may sometimes be brought into the genome of offspring even in the gynogenetic ginbuna.

In the present experiment, we tried to ascertain whether the homologous chromosomes undergo the synapsis in the meiosis and the chromosomes undergo the genetic recombination during the maturation division in triploid ginbuna. The cytological observations of spermatogenesis of triploid male ginbuna which bred by natural breeding and the sex-reversed individuals induced by the male gonadal hormone were also performed.

Materials and Methods

I. Materials

The experimental materials of ginbuna *Carassius auratus langsdorfii* were collected from Lake Kasumigaura and Lake Suwa. In Lake Suwa the other subspecies, nagabuna *C. a. buergeri* is distributed, and it is difficult to distinguish from ginbuna because they are very closely resemble each other. The chromosome number of nagabuna belongs only in diploid type ($2n=100$) (KOBAYASHI *et al.*, 1973 ; OJIMA and YAMANO, 1980). In the present experimental materials, some of the nagabuna may be contaminated in diploid ginbuna, then the fishes of diploid type were generally expressed as "diploid funa".

In addition to these wild-collected materials, two males obtained from the hybrids by triploid ginbuna with gold fish *C. a. auratus* (with heterozygous transparent scaled character, *Tt*), and the sex-reversed individuals induced by the male gonadal hormone (TASHITA *et al.*, 1983) were used as materials.

II. Estimation of Ploidy

The ploidy of experimental materials were determined by the microspectrophotometrical measurement of the DNA content of erythrocyte nucleus (MURAYAMA *et al.*, 1984) or by the counting of the chromosome number of cultured cells of the fin.

The chromosome preparations were prepared as follows. The fin was cultured according to the method of OJIMA (1978); after removal of posterior part of caudal fin, it was sterilized, chopped and cultured with 199 medium, supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin and 10 % fatal bovine gerum at 25°C. After proliferation, the cultured cells were treated with colchicine (0.025 μg/ml) for 4 h at 25° C. After 0.065 M KCl-hypotonization for 20 min, they were fixed by acetic alcohol, and the slideglasses were air-dried and Giemsa-stained as usual.

III. Lampbrush Chromosome Preparations

The lampbrush chromosome preparations were prepared according to the method of GALL (1966). A small part of the ovary was removed, and oocytes with a diameter of 300 to 800 μm were obtained from the ovarian fragments. The oocyte was punctured with two forceps and squeezed gently in 5 : 1 solution, a mixture of 5 parts 0.1 M KCl and 1 part 0.1 M NaCl, following adjustment of pH to 6.8-7.2 by 0.01 M phosphate. After the nucleus was sucked in and out several times to remove the adherent yolk granules with a micropippete, the nuclear envelope was removed in a sitting drop of solution on the slideglass. The chromosomes were fixed in the vapor of buffered neutral formaldehyde for 20 to 70 min, and the slideglass was kept more than over night in a moisture-chamber at 25°C.

The observation of lampbrush chromosomes was carried out by an inverted microscope, NICON DIAPHOT-TMD.

IV. Chromosome Preparations of Germ Cells

The chromosome preparations were prepared according to the direct method of germ cells. The cell suspension obtained by the chopping of ovary was filtrated through 120 stainless mesh to gather oocytes during the pre-vitellogenesis. They were centrifuged at 1000 rpm for 10 min and the precipitate was fixed by acetic alcohol on the slideglass. They were airdried and Giemsa-, Feulgen-, and silver-stained. The silverstaining followed the ammonical silver (Ag-As) method of GOODPASTURE and BLOOM (1975), and KINGERMAN and BLOOM (1977). The Ag solution of 50 % $AgNO_3$ was pippeted on a slideglass, covered with a coverglass and incubated at 50°C for 20 min. The coverglass was rinsed off by deionized water and airdried. Next, the slideglass was

developed by adding As solution prepared by dissolving 4 g $AgNO_3$ in a solution of 5 cc deionized water and 5 cc ammonium hydroxide, followed by developed solution of acetate buffered 3 % formalin adjusted to pH 4.2, covered with coverglass. After proper development, the coverglass was rinsed off in deionized water and the slideglass was air-dried.

On the other hand, when the testis was used as materials, the supernatant of the chopped cell suspension was collected and centrifuged at 1000 rpm for 10 min. The pellet was treated with 0.065 M KCl-hypotonization for 70 to 80 min, and fixed on the slideglass by acetic alcohol. These preparations were airdried and stained with Giemsa.

For the study of earlier stages in meiosis of germ cells, colchicine treatment was avoided.

Results

1. *Early Meiotic Stage in Female Ginbuna*

The oocyte chromosomes were only faintly stained by Giemsa- or Feulgen-staining, then the silver-staining which reveals synaptonemal complex in mammalian spermatocytes (PATHAK and HSU, 1979 ; DRESSER and MOSES, 1979, 1980 ; FLETCHER, 1979) was utilized in the present experiment. The chromosomes from leptotene to early diplotene stage were observed in this method. Each stages in meiosis of diploid funa was judged from chromosome length and thickness, and from the appearance and number of amplificated nucleoli. The determination of each stages in meiosis of triploid ginbuna was followed by the characteristics of diploid funa. The number of nucleolus of oocytes from leptotene to pachytene stage of diploid funa was usually one, but in some cases two nucleoli were observed in a single cell. The number of nucleolus of oocytes from leptotene to pachytene stage of triploid ginbuna was also usually one, but in some cases two to four nucleoli were observed in a single cell.

A. *Diploid funa*

At zygotene stage, chromosomes and large nucleolus were darkly stained with silver (Plate 1-1). Two homologous chromosomes were sometimes adjoined each other only at certain points, forming Y-shaped structures. But further numerous homologous chromosomes were undergoing synapsis, forming bivalent chromosomes. However, in some of bivalent chromosomes, loop-shaped structures were observed. Other that the large nucleolus, some nucleoli which weakly stained with silver were observed (Plate 1-2).

At early pachytene stage, two homologous chromosomes formed bivalents in each other, but they were not still completely shortened and thickened. The chromosomes of oocyte were counted about 50. Many nucleoli were observed, and each of them showed heavy silver deposition (Plate 1-3).

B. *Triploid ginbuna*

At zygotene stage, chromosomes and large nucleolus were darkly stained with silver (Plate 1-4). Two homologue-like chromosomes out of three were sometimes adjacent each other in the neighbourhood. At pachytene stage, the shortened chromosomes, weakly stained with silver, were counted about 150. From these observations, it seemed probable that almost all of the chromosomes were univalents (Plate 1-5). Some small nucleoli were present, and almost all of them showed heavy silver deposition and adhered to chromosome.

2. *Lampbrush Chromosomes*

The lampbrush chromosomes can be observed during the period from October to May. As the chromosome of crucian carp is so numerous and small, it was difficult to count out the entire number of lampbrush chromosomes and to clarify their fine structures as in the amphibian oocytes (MÜLLER, 1974 ; OHTANI, 1975, 1978). So, in the present experiment, it was only tried to ascertain whether the homologous chromosomes were connected each other by chiasmata during the oogenesis or not.

A. *Diploid funa*

In almost all of the lampbrush chromosomes, the two homologous chromosomes of each pair formed bivalents, connested with one chiasma, but in some cases two or three chiasmata were observed (Plate 2-1). In a part of chromosomes univalentlike chromosomes were observed, but they may be fragments of bivalent chromosomes.

B. *Triploid ginbuna*

Almost all of lampbrush chromosomes were univalents (Plate 2-2), and the number was more than one hundred. Sometimes in these univalent chromosomes, two or three homologue-like chromosomes were adjacent each other in the neighbourhood. In some specimens, at most two to three bivalent chromosomes, connected with one chiasma, were observed (Plate 2-3).

C. *Tetraploid ginbuna*

The oocytes of two individuals of tetraploid ginbuna were used as materials. All of the lampbrush chromosomes of both individuals were univalents. In a part of univalent chromosomes, two homologue-like chromosomes were adjacent each other in the neighbourhood (Plate 2-4), but none of the bivalent was observed.

3. *Spermatogenesis in Males Ginbuna*

The structural changes of the chromosome during meiosis were observed by seven specimens. Each stages in meiosis of diploid funa was judged from chromosome length and thickness as in female, and the time of bivalent formation and chiasma formation were also refered. The determination of each stages in triploid male ginbuna and the sex-reversed individuals were followed by the characteristics of diploid funa. Sometimes in the spermatocytes of diploid funa, it observed a few aneuploid cells or some polyploid cells having a greater number of chromosomes than tetraploid. The number of nucleoli of spermatocytes from leptotene to pachytene stage was usually

one, but in some cases two nucleoli were observed in a single cell. As in diploid funa, in some of the spermatocytes of triploid or the sex-reversed male ginbuna, a few aneuploid cells or some polyploid cells having more than the hexaploid chromosomes were observed. The number of nucleoli of spermatocytes from leptotene to pachytene stage was usually one, but in some cases two to five nucleoli were observed in a single cell.

A. *Diploid funa*

At zygotene stage, elongated paired homologous chromosomes were observed. They were adjacent each other at certain points, forming bivalents (Plate 3-1). At pachytene stage, bivalent chromosomes began to contract and became thickness. Large nucleolus was observed (Plate 3-2). Thereafter, chromosomes became more short and thick (Plate 3-3). In metaphase of the first meiosis (metaphase I), 50 bivalent chromosomes were discerned. They were more contracted and classified into three from their forms; the one is a cross-shaped bivalent in which two homologous chromosomes are united at their two centers or other points, the one is a ring-shaped one in which two homologous chromosomes are united at their two ends, and the rest is a rod-shaped one in which two homologous chromosomes are united at one end (Plate 3-4). In metaphase of the second meiosis (metaphase II), 50 chromosomes were observed (Plate 3-5).

B. *Triploid ginbuna*

The spermatocytes of two triploid male ginbuna were used as materials. In early zygotene stage, two homologue-like chromosomes out of three underwent synapsis (Plate 4-1), and later, some of them were united at their each ends to form a ring-shaped bivalent chromosomes and the others were united at one end to form a rod-shaped bivalent chromosomes, while a part of chromosomes remained univalents (Plate 4-2), and later, chromosomes were more contracted (Plate 5-3). Almost all of the chromosomes were univalents, but some of them formed rod-shaped bivalents (Plate 4-4). At diplotene stage, each of univalent chromosomes became decondenced, and some rod-shaped bivalents were observed (Plate 4-5). In metaphase chromosomes, following two types were observed. The one is consisted of only univalent chromosomes (Plate 4-6), and the other is consisted of a few bivalent and univalent chromosomes, and many rod-shaped bi-, tri-, multivalent chromosomes, which two, three and multitude univalent chromosomes were united at one or two ends respectively (Plate 4-7). The average chromosome number in metaphase was 122 to 156, showing triploid range. In metaphase chromosome, shown in (Plate 5-6), the number of univalent chromosomes was 156,and three of them were longer than the others and one of the three was especially long.

C. *The Sex-reversed Individuals*

The spermatocytes of two individuals were used as materials. At zygotene stage, two homologue-like chromosomes out of three were adjacent each other at certain

points and formed Y-shaped structures (Plate 5-1). Later, some of the chromosomes underwent synapsis, and the others remained in univalents (Plate 5-2). At late pachytene stage chromosomes were further contracted. Almost all of the chromosomes were univalents, but a part of them formed ring-shaped bivalents (Plate 5-3). In metaphase chromosomes they were consisted only of univalent chromosomes. The average chromosome number was 143 to 153, showing triploid range. In the metaphase chromosomes, shown in (Plate 5-4), the number of univalent chromosomes was 153, and three of them were longer than the others and one of the three was especially long. Is some of metaphase chromosomes, they were consisted of many rod-shaped bi-, tri-, and multivalent chromosomes, as in those of spermatocytes of triploid male ginbuna (Plate 5-5).

Discussion

In the teleost fish, the gynogenesis has been reported in the following three genera, *Carassius* (*C. auratus gibelio, C. a. langsdorfii*), *Poecilia* and *Poeciliopsis* belonging to family Poeciliidae. CHERFAS (1966, 1972) has analyzed cytologically the process in meiosis of *C. auratus gibelio* and ascertained that the chromosomes are all univalents in later prophase of the first meiosis, and the chromosome is disposed among three poles of the tripolar spindle at metaphase I. But the first meiosis is abortive and the egg contains a nonreduced triploid number of chromosomes. On the other hand, in *Poeciliolsis* CIMINO (1972) have observed the formation of hexaploid primary oocytes through premeiotic doubling of the chromosome by endomitosis. Thereafter, the primary oocyte undergoes a conventional meiosis, and the triploid chromosomes are restored throughout meiosis.

In *Poecilia,* the meiotic mechanisms are not analyzed.

In the ginbuna, *C. auratus langsdorfii,* KOBAYASHI (1976) has reported that he could not observed the prophase of the first meiosis in the triploid fish. On the other hand, OJIMA and ASANO (1977) have reported that the chromosome number of triploid ginbuna is approximately 150 at metaphase, and 300 at anaphase. In these observations they have confirmed that the polar body is extruded only once in triploid ginbuna, while twice in diploid ginbuna. From these studies, it has concluded finally that the oocyte in the triploid ginbuna skips the first meiosis, undergoing a homotypic nuclear division as somatic cells. Then the triploid ginbuna produces the same chromosome number and karyotype as the mother.

CIMINO (1972) has considered that the production of triploid offspring by gynogenesis requires a triploid egg and this might be produced by the one of following three processes; the one is direct transformation from a triploid oogonium, by vitellogenesis without synapsis and reductional division, the one is suppresion after synapsis of meiosis I, with concomitant suppresion of pairing, and the rest is an

endomitosis which increases the chromosome number to hexaploid and by a complete meiosis the triploid egg is produced. According to this hypothesis, *C. a. langsdorfii* belongs to the first type, and *C. a. gibelio* seems to belong to the first type or in some cases to the second type.

In the present experiment, it became apparent that two homologue-like chromosomes underwent synapsis at zygotene stage, but thereafter chromosomes became about 150 univalents throughout the pachytene to diplotene stage. These results seem to suggest the following hypothesis; the two homologue-like chromosomes undergo synapsis at zygotene stage, but after pachytene stage they might be separated to univalents, and each of them splits to two chromatids resulting in separation of one chromatid to one direction and the other to the opposite one. In this way, the egg might maintain the maternal chromosome number and karyotype throughout the maturation division. If it is true, the triploid ginbuna become to belong the second type of CIMINO's classification.

In the lampbrush chromosomes of triploid ginbuna, bivalent chromosomes were sometimes connected with chiasmata, though they were not always observed in all specimens, and their number were at most two to three pairs in one oocyte. These facts seem to suggest the possibility that the chiasmata might be formed after genetic recombination of two homologuelike chromosomes undergoing synapsis. Though we could not observed the separation processes of bivalent chromosomes, according to the hypothesis of IMAI and MORIWAKI (1980), it can not be denied the possibility that the bivalent chromosome splits into four chromatids, and the two of each pair separate out into both poles. On this way, the same chromosome number and karyotype might be maintained in the egg. But when the bivalent chromosomes are consisted with heterozygous-allele, the recombinant may be produced in these processes. HIJIKATA *et al.* (1983) have observed the hypervariabilities among the gynogenetic triploid ginbuna. These facts seem to be explained by the present assumption.

CHERFAS (1966, 1972) has observed the chromosome number of *C. a. gibelio,* and ascertained that the univalent gynogenetic triploid type is 141 while that of bisexual diploid type is 94. From these observations he has supposed that the chromosomes of triploid type are constituted by the three homologous chromosomes of each triplet. As to the karyotype analyses of triploid ginbuna, MURAMOTO (1975) has reported that the 157 chromosomes in nuclear plate reveal a partial trisomic constitution because there are one triplet of the largest submetacentrics and another acrocentric triplet with satellites. In the present experiment, at zygotene stage some of the chromosomes formed bivalents, while the other remained in univalents. At diplotene stage some of the bivalent chromosomes connected with chiasmata, though they were not always observed in all specimens, and the number was only two to three pairs in one oocyte. From these results, it was supposed that the chromosomes of triploid ginbuna are

constituted by the three homologous, allotriploid chromosomes.

On the other hand, KOBAYASHI *et al.* (1970, 1973) have reported that the 156 chromosomes of triploid ginbuna are consisted from 17 pairs of metacentric, 31 pairs of submetacentric and 30 pairs of acrocentric chromosomes. UEDA and OJIMA (1978) have investigated the C-banding patterns of chromosomes in triploid ginbuna and observed a pair of chracteristic C-band-positive on the short arms of the secondary large submetacentric chromosomes. From these results, they have considered that the chromosomes of triploid ginbuna might be constituted by the two homologous chromosomes of each pairs.

In the present experiment, the spermatogenesis of naturally produced triploid male and artificially sex-reversed male ginbuna were observed. With regard to the processes in meiosis, male individuals were almost similar to that of female ginbuna. But sometimes chromosomes were consisted of a few bivalents and rod-shaped bi-, tri- and multivalents at metaphase. BECAK *et al.* (1966, 1967, 1970) have considered that the synapsis and segregation of the multivalent chromosomes are regular and result in normal euploid gamete in autooctoploid *Ceratophrys dorsata* (8n=104). On the contrary, in the meiosis of artifical interspecific triploid hybrids (♂=33), the chromosome number is variable in metaphase II from 11 to 22. In this way, synapsis and segregation of chromosomes are not always regulated in triploid individuals. As mentioned above the chromosomes of triploid ginbuna seems to be constituted by the three homologous chromosomes of each triplet. If it is true, the uni-, bi- and trivalent chromosomes should be formed at metaphase. But actually many rod-shaped multivalent chromosomes were observed. Though the processes of the subsequent division is not still ascertained, if the chromosomes separate unequally, the aneuploid spermatids might be produced.

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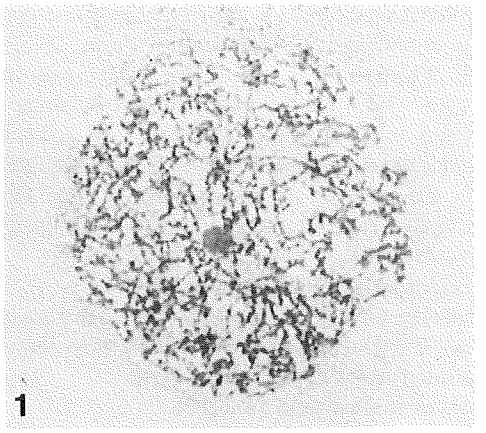
Plate 1 Photographs of oocyte in meiosis (× 1300).
1. At zygotene stage of diploid funa ; some of the homologous chromosomes begin to form Y-shaped structures, and large nucleolus is observed. 2. At late zygotene stage of diploid funa (the right side) ; numerous homologous chromosomes form bivalent chromosomes, but in some of bivalent chromosomes, loop-shaped structures are discerned. (the left side ; at early diplotene stage). 3. At early pachytene stage of diploid funa ; two homologous chromosomes form bivalents, and many nucleoli are observed. 4. At zygotene stage of triploid ginbuna ; some of the two homologue-like chromosomes out of three are adjacent each other in the neighbourhood, and large nucleolus is observed. 5. At pachytene stage of triploid ginbuna ; about 150 univalent chromosomes and some small nucleoli are observed.

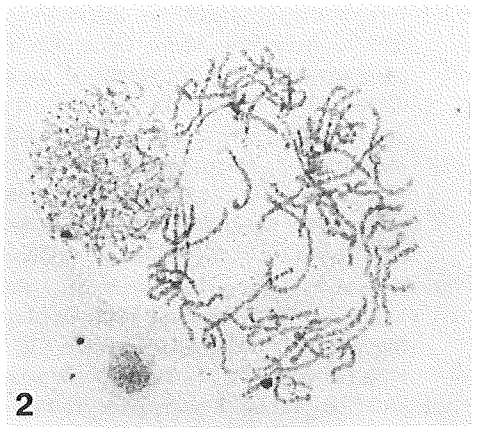
Plate 2 Photographs of lampbrush chromosomes of polyploid ginbuna (× 1300).
1. Diploid funa, forming bivalent chromosomes with chiasmato. 2. Triploid ginbuna, forming univalent chromosomes. 3. Triploid ginbuna, forming bivalent chromosome with chiasma. 4. Tetraploid ginbuna, forming univalent chromosomes.

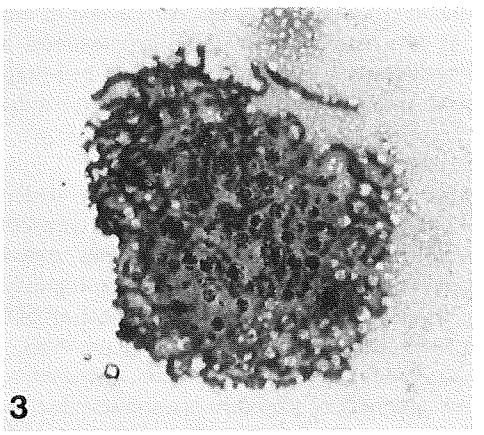
Plate 3 Photographs of spermatocyte in meiosis of diploid funa (× 5200).
1. At zygotene stage ; elongated paired homologous chromosomes, forming bivalents, and large nucleolus are observed. 2. At pachytene stage ; bivalent chromosomes begin to contract and become thickness. 3. At late pachytene stage ; chromosomes are further shortened and thicked. 4. At metaphase I ; cross-, ring-, and rod-shaped bivalent chromosomes are discerned, total chromosome number is 50. 5. At metaphase II ; 50 chromosomes are observed.

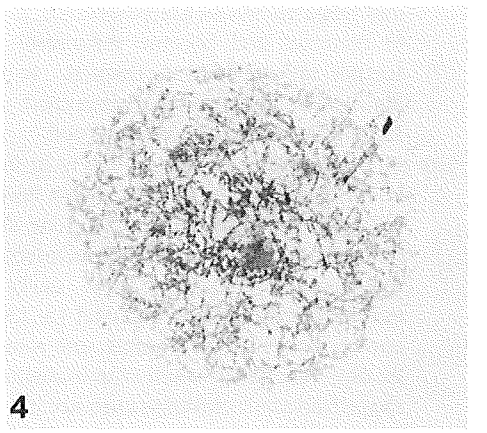
Plate 4 Photographs of spermatocyte in meiosis of triploid male ginbuna.
1. At early zygotene stage ; two homologue-like chromosomes out of three undergo synapsis (× 1300). 2. At zygotene stage ; some of two homologue-like chromosomes from ring- and rod-shaped bivalents, while a part of the chromosomes remains univalents (× 1300). 3. At early pachytene stage ; the two homologue-like chromosomes begin to contract (× 1300). 4. At late pachytene stage ; almost all of the chromosomes are univalents, and some of them form rod-shaped bivalents (× 2600). 5. At diplotene stage ; each of univalent chromosomes become decondenced, and some rod-shaped bivalent chromosomes are observed. A spherical structure is not nucleolus but a sperm head or spermatid (× 1300). 6. At metaphase ; the chromosomes are all univalents. Total chromosome number is 156 (× 2600). 7. At metaphase ; the chromosomes are consisted of a few bivalent and univalent chromosomes, and many rod-shaped bi-, tri-, and multivalent chromosomes are observed (× 5200).

Plate 5 Photographs of spermatocyte in meiosis of the sex-reversed ginbuna.
1. At early zygotene stage ; two homologue-like chromosomes out of three form Y-shaped structures. Two small spherical structures are not nucleoli but sperm heads or spermatids. A large one is nucleolus (× 1300). 2. At late zygotene stage ; some of the chromosomes undergo synapsis and the others remain in univalents (× 5200). 3. At late pachytene stage ; almost all of the chromosomes are univalents but a part of them formed ring-shaped bivalents (× 5200). 4. At metaphase ; the chromosomes are all univalents. Total chromosome number is 153 (× 520). 5. At metaphase ; the chromosomes are consisted of many rod-shaped bi-, tri-, and multivalent chromosomes (× 5200).









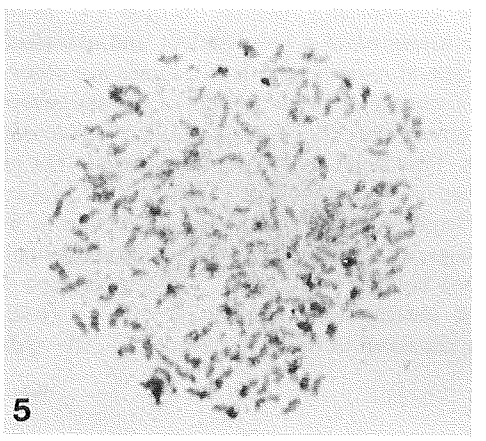


Plate 1

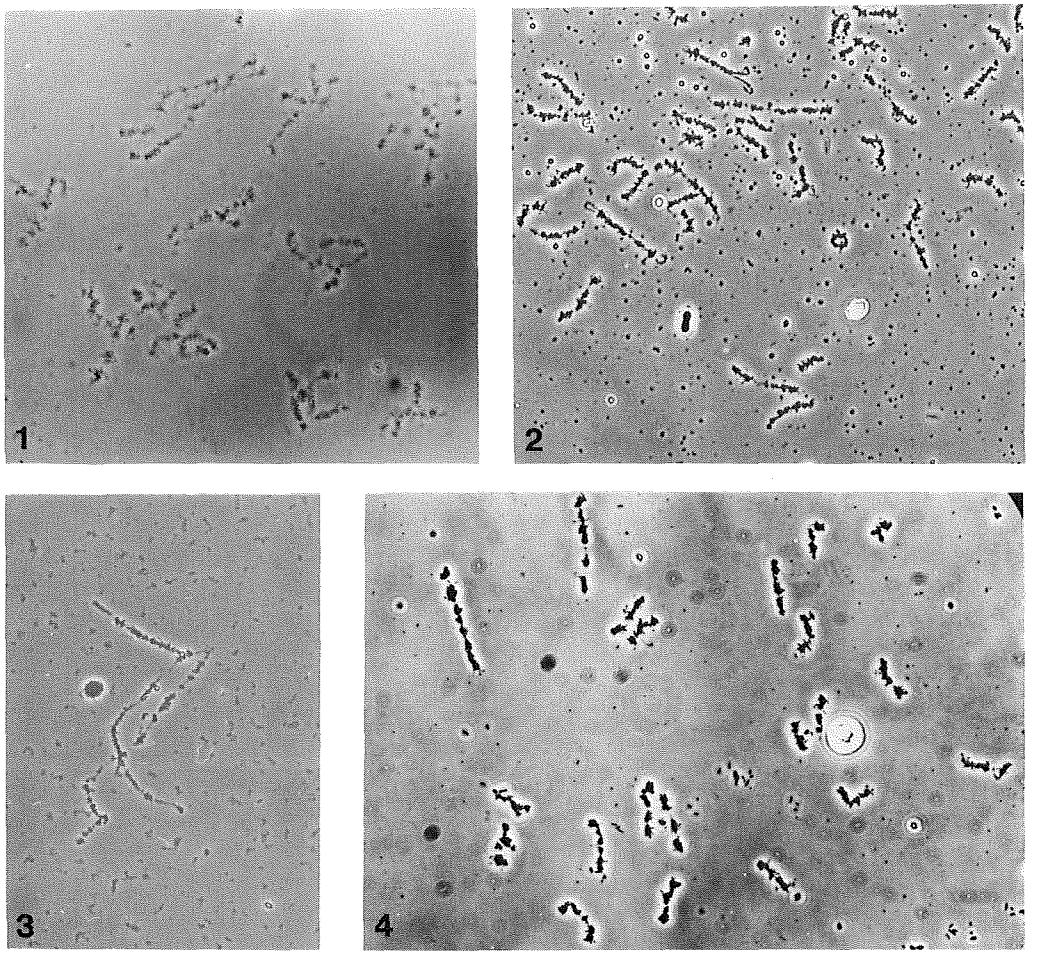


Plate 2

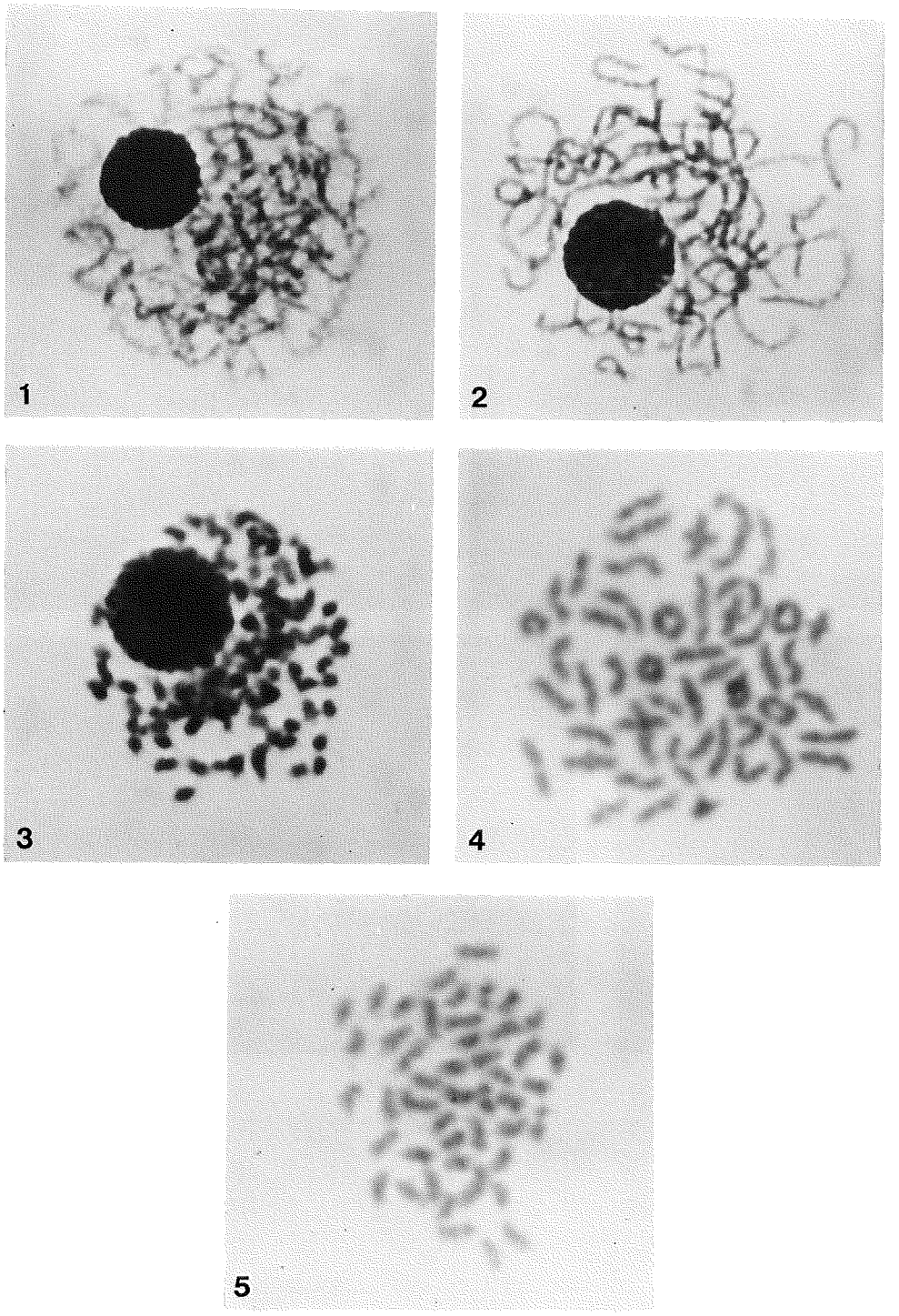
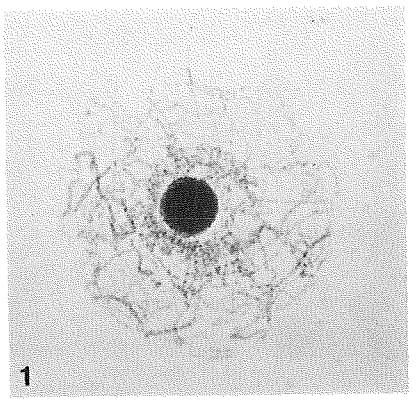
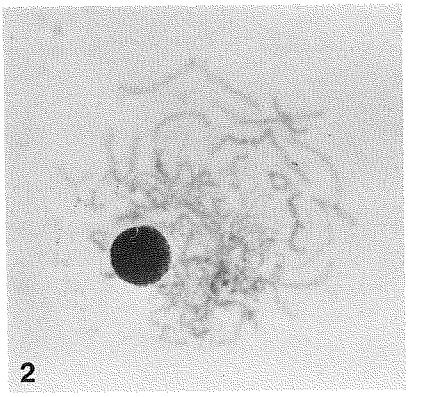
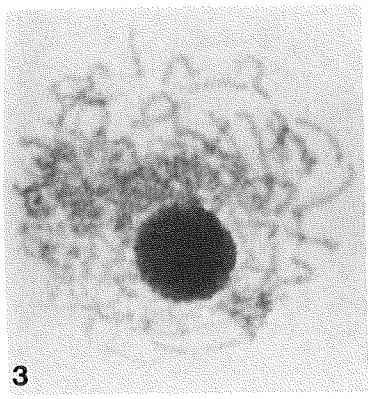
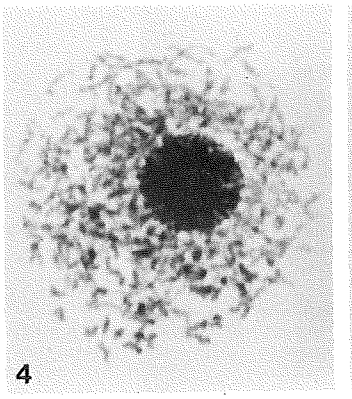


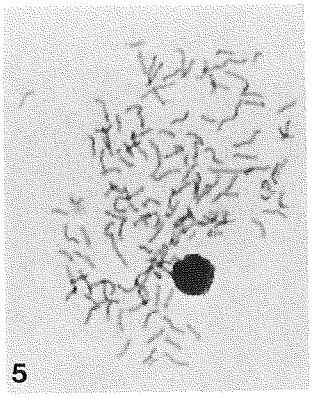
Plate 3

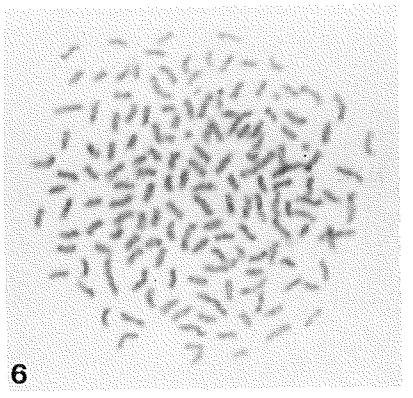












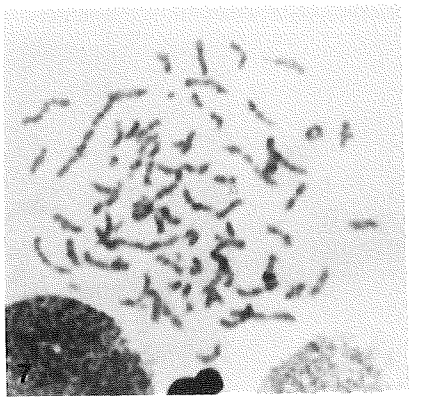


Plate 4

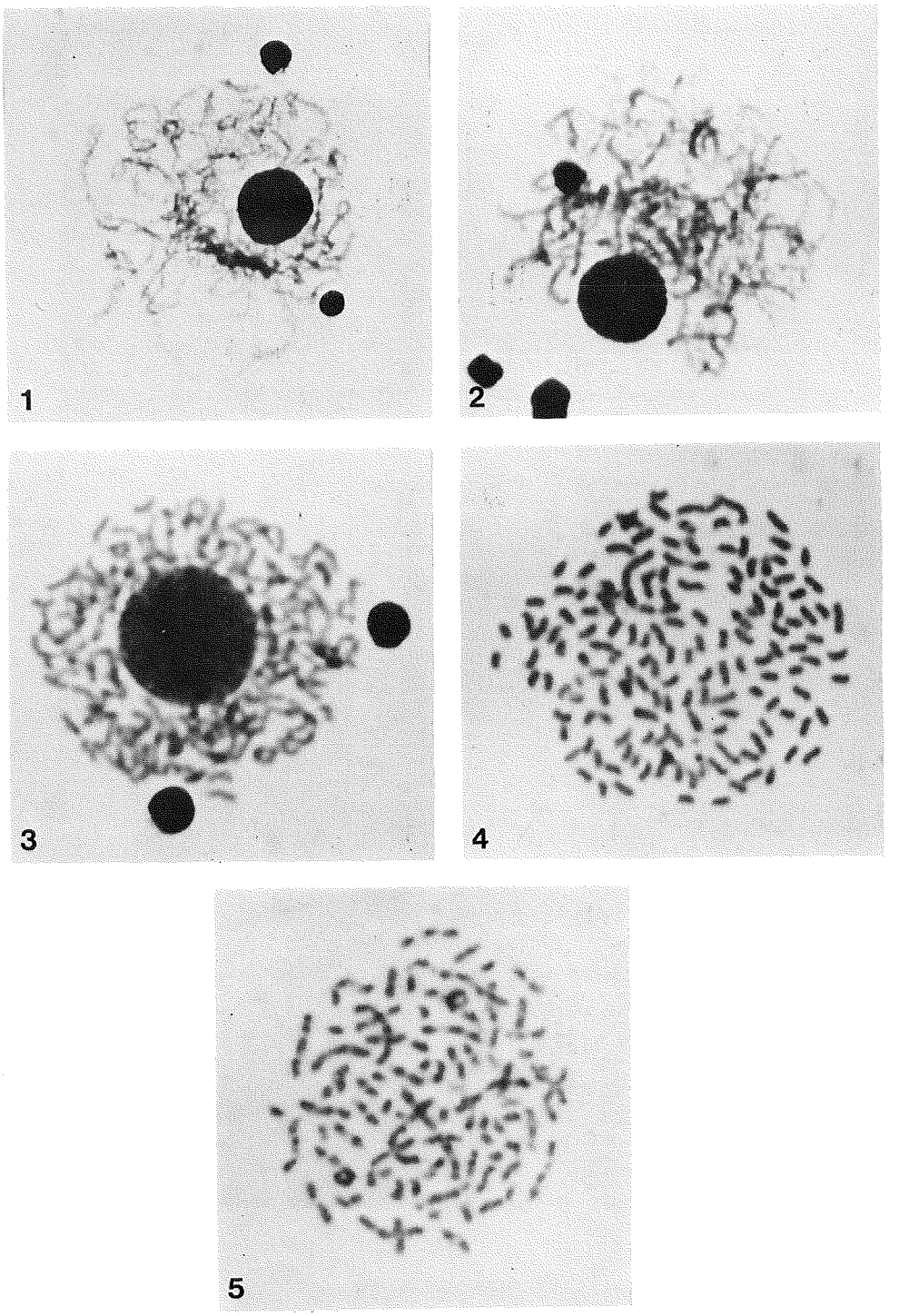


Plate 5