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## Experimental Hybridization among *Oryzias* Species. II. Karyogamy and Abnormality of Chromosome Separation in the Cleavage of Interspecific Hybrids between *Oryzias latipes* and *O. javanicus*

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**ABSTRACT**—In interspecific hybridization between *Oryzias latipes* and *O. javanicus*, all hybrid embryos failed to develop and died before hatching. Cytological examination of fertilization and early development was performed to discover the cause of lethal development. When *O. latipes* eggs were inseminated by sperm of *O. javanicus*, the cortical reaction was induced normally. Chromosomal material in the fertilized eggs was visualized using the DNA-specific fluorochrome Hoechst. The spermatozoon was capable of penetrating into the egg cytoplasm through the micropyle, and the sperm nucleus transformed to the male pronucleus. The female pronucleus that formed after extrusion of the second polar body migrated towards the male pronucleus. The female and the male pronuclei underwent DNA synthesis and encountered each other in the center of the blastodisc, fused with one another and formed a zygote nucleus before breakdown of the nuclear envelope. Metaphase chromosomes with electron dense chromatin regions were abnormally divided into each blastomere in cleavage. The abnormally separating chromatin masses were also labeled by BrdU. The abnormal separation resulting in partial loss of fragmented chromatin might be a cause of abortive development in the interspecific hybrids between *O. latipes* and *O. javanicus*.

**Key words:** *Oryzias*, interspecific hybridization, karyogamy, chromosome separation

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### INTRODUCTION

The genus *Oryzias* belonging to the family Oryziatidae (Rosen, 1964) inhabits in fresh water or brackish waters from India through Southeast Asia limited by Weber's line. At present, fourteen species of *Oryzias* have been described (Iwamatsu *et al.*, 1993).

Embryonic analysis of the differences between species and their interspecific hybrids may be valuable in clarifying the evolutionary and phylogenetic relationships within the genus *Oryzias*. Interspecific hybridization between *O. latipes*

and *O. celebensis* (Iwamatsu *et al.*, 1984), *O. melastigma* and *O. javanicus* (Iwamatsu *et al.*, 1986), or *O. latipes* and *O. curvinotus* (Hamaguchi and Sakaizumi, 1992; Sakaizumi *et al.*, 1993; Kurita *et al.*, 1995; Shimizu *et al.*, 1997, 2000) produces verifiable hybrid progeny. Females of interspecific hybrids between *O. latipes* and *O. curvinotus* were able to lay eggs, but males did not produce fertile spermatozoa (Hamaguchi and Sakaizumi, 1992). In contrast, the failure of interspecific hybridization between *O. latipes* and *O. javanicus* due to death of the embryos during early development has previously been reported (Hori and Iwamatsu, 1996; Iwamatsu *et al.*, 1994). However, the process of interspecific fertilization and development of hybrids between these species has not been documented cytologically. Although a number of early investigations employing interspecific

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hybrids in other fishes have so far been published, few have also cytologically analyzed the process of interspecific fertilization (Yamamoto, 1943). Nuclear behavior during fertilization and cleavage in lethal embryos resulting from interspecific hybridizations remains to be analyzed in detail. The aim of the present study was undertaken to clarify the mechanism of abortive development of interspecific zygotes.

We observed details of the nuclear behavior in eggs fertilized between *O. latipes* and *O. javanicus*, and found that the interspecific zygotes exhibited abnormal nuclear divisions during cleavage, although the fertilization process from sperm penetration to karyogamy was morphologically normal.

## MATERIALS AND METHODS

Sexually mature *Oryzias latipes* (orange-red type) were obtained from a local fish farmer (Amagun-Yatomi, Aichi), and wild *O. javanicus* were collected from Jakarta, Indonesia (Iwamatsu and Hirata, 1980). Unfertilized eggs were released from the ovarian cavity of the isolated ovary in medaka's saline (111.2 mM NaCl, 5.4 mM KCl, 1.1 mM CaCl<sub>2</sub>, 0.6 mM MgCl<sub>2</sub>, adjusted to pH 7.3 with NaHCO<sub>3</sub>; Iwamatsu *et al.*, 1976) by tearing the ovarian sac with fine forceps. Attaching filaments of each egg were cut off with the blunt tip of a glass rod. A sperm suspension ( $1\sim 2\times 10^6$  spermatozoa/ml) for insemination was prepared by incising the testes of five male *O. javanicus* or *O. latipes* in about 0.5 ml of medaka's saline within a depression glass slide.

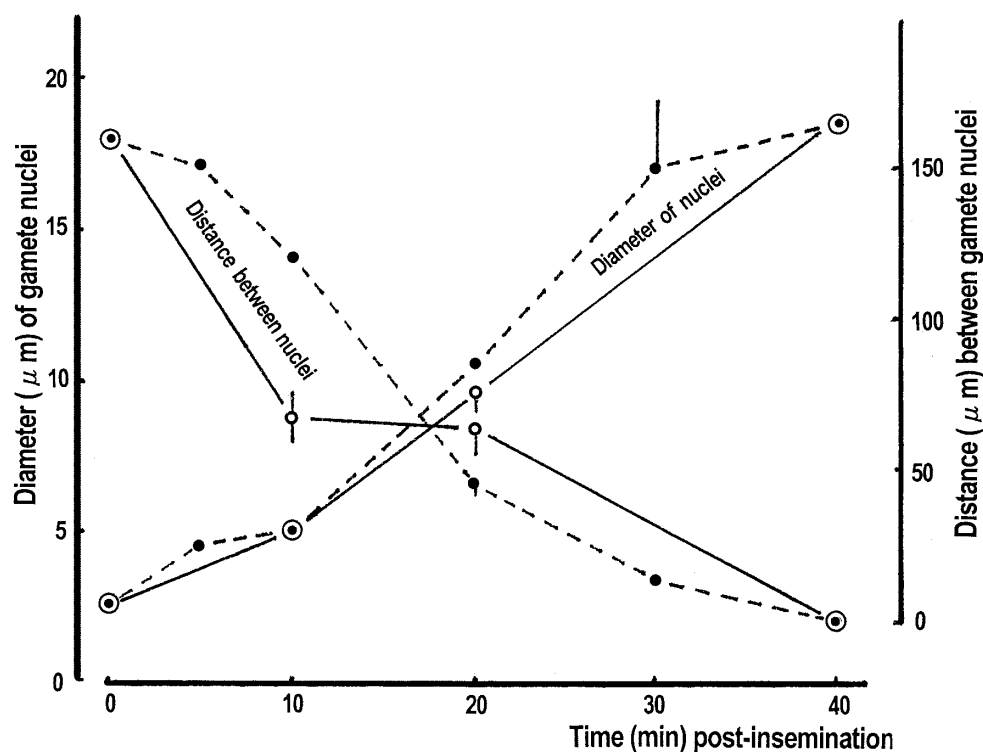
Unfertilized *O. javanicus* or *O. latipes* eggs were inseminated in sperm suspension of *O. javanicus* or *O. latipes* for about 60 sec

at room temperature and incubated at 27°C. Egg activation upon insemination was ascertained by cortical reaction (disappearance of cortical alveoli), followed by formation of the perivitelline space.

Fertilized eggs were fixed in 4% glutaraldehyde-0.1 M phosphate buffer (pH 7.0) before insemination and thereafter at 5–10 min intervals up to 140 min post-insemination (PI). For cytological observations, fixed samples were rinsed in distilled water and divided into the animal and vegetal hemispheres by cutting them with a pair of scalpels. The restricted cortical cytoplasm of the animal pole region including the gamete nuclei was dissected out of the spherical yolk mass with the scalpels and mounted on a clean glass slide with a small amount of water. A few drops of 5 µg/ml Hoechst 33582 (Wako Pure Chemical Industries, Ltd. Osaka) were dropped on it. After twenty minutes in a moist chamber, the Hoechst solution was withdrawn with a fine pipette, and one drop of 50% glycerin was placed on the pieces of cortical cytoplasm. A glass coverslip was laid over the mounting solution on the glass slide, and each sample was examined with an Olympus epifluorescence microscope (Olympus, Tokyo) using an Olympus UV-1 filter.

DNA synthesis was ascertained by detecting the fluorescent labeling of the nucleus of fertilized eggs from the time of sperm penetration. In this experiment, approximately 60 nl of saline containing 10 mM 5-bromo-2'-deoxyuridine (BrdU; Sigma) was microinjected into the cortical cytoplasm at the animal pole region, as soon as exocytosis of cortical alveoli started upon insemination. The specimens were then transferred into PMP fixative (4% paraformaldehyde, 10% methanol and 100 mM phosphate buffer, pH 7.0) at the stage of karyogamy or cleavage and fixed for 6 hr at room temperature. The BrdU-injected eggs were stained with an anti-BrdU monoclonal mouse antibody and the entire DNA counterstained with 0.1 µg/ml Hoechst 33582, according to a previous procedure (Iwamatsu *et al.*, 2002).

For fine structural observations, fertilized eggs 35, 40 and 43



**Fig. 1.** Nuclear behavior during cross-fertilization of *Oryzias latipes* and *O. javanicus*. The encounter of female and male pronuclei takes place within 40 min post-insemination (PI). Solid lines indicate the nuclear behavior of interspecific hybrids (○). Broken lines indicate the nuclear behavior of control *Oryzias latipes* eggs (●) fertilized by homologous sperm.

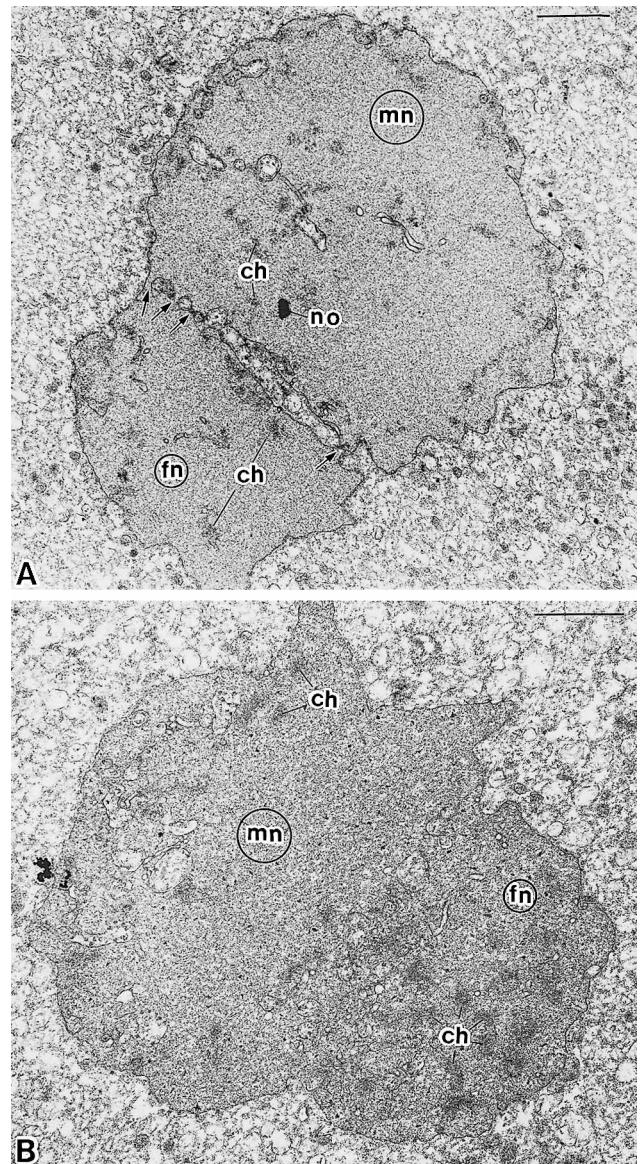
min PI were pre-fixed for 90 min at room temperature in a mixture of 4% paraformaldehyde, 0.5% glutaraldehyde and 1% picric acid (modified Karnovsky's fixative, pH 7.3; Ito and Karnovsky, 1968). After prefixation, the restricted cortical cytoplasm of the animal pole region of the egg was prepared by dissection with a pair of scalpels and post-fixed for 1 hr in aqueous 1% osmium tetroxide (0–4°C). Fixed samples were dehydrated in a graded series of ethanol solutions, and embedded in Spurr's low viscosity epoxy resin (Taab, Reading UK). Each sample was serially sectioned into 0.5 µm thick sections until a pronucleus was detected in the sections by staining with 0.5% toluidine blue in 1% sodium borate. The sections containing pronuclei were selected for examination by electron microscopy. Ultrathin sections were stained with uranyl acetate and lead acetate for observation with a transmission electron microscope (JEOL Ltd., Tokyo, Japan).

## RESULTS

When 280 *O. latipes* eggs were inseminated with *O. javanicus* spermatozoa, 273 (ca. 98%) immediately began to undergo cortical reaction near the animal pole and it propagated towards the vegetal pole concomitant with formation of the perivitelline space. Each fertilized *O. latipes-javanicus* egg formed a morphologically normal blastodisc, and its female pronucleus migrated slowly to the male pronucleus (Fig. 1). Cleavages were delayed. Six (37.5%) of 16 *O. javanicus* eggs were also activated upon insemination with a suspension of *O. latipes* spermatozoa and began to develop, as observed in the previous studies (Iwamatsu *et al.*, 1994; Hori and Iwamatsu, 1996). All these fertilized eggs were ceased development before the stage of eye-vesicle formation, as found in the previous study (Iwamatsu *et al.*, 1994).

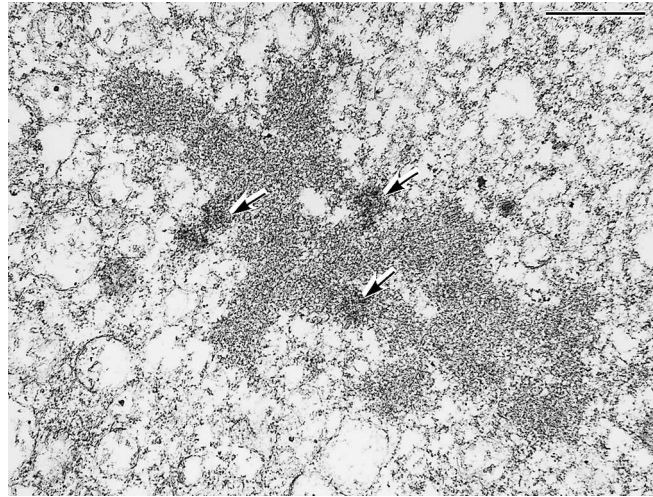
### Cytological observations:

Ten minutes PI, the enlarged *O. javanicus* sperm nucleus (4.9–5.8 µm in diameter) was observed about 438 µm from the egg nucleus, which was at anaphase of the second meiosis, in *O. latipes* eggs. The mean distance from the female pronucleus to the nucleus of the second polar body was about 25.3 µm. Twenty-five minutes PI, the female nucleus had migrated towards the position of the male pronucleus: the mean distances from the female pronucleus to the second polar body and to the male pronucleus were approximately 106.1 µm and 63.9 µm, respectively. Pronuclei were in contact with one another 35 min PI. The nuclear envelopes of large male and small female pronuclei began to fuse partially 38 min PI (Fig. 2A). Several nucleoli and condensing chromatin were present throughout the nucleoplasm. Forty minutes PI, the completely fused pronuclei had transformed to a single zygote nucleus. The zygote nucleus contained small vesicles, nucleoli and condensed chromatin throughout the nucleoplasm (Fig. 2B). Chromatin condensation took place later in the male pronuclear region than in the female pronuclear region, whereas both male and female pronuclei synchronously exhibited chromatin condensation in *O. latipes* eggs fertilized with *O. latipes* spermatozoa (Iwamatsu and Kobayashi, 2002). Forty-three minutes PI, an array of chromosomes was

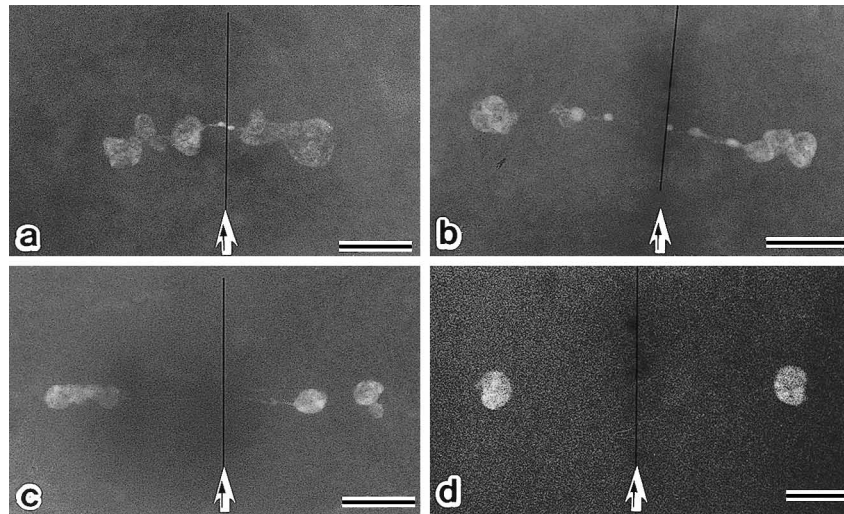


**Fig. 2.** Fine structure of the male (*O. javanicus*) and female (*O. latipes*) pronuclei at the time of karyogamy. A: Thirty-eight minutes PI, several regions (arrows) of the fusion between the nuclear envelopes of the female (fn) and male (mn) pronuclei with a tangentially sectioned nucleolus (no) are visible. Bar, 2 µm B: Forty minutes PI, a single zygote nucleus formed by complete fusion of the female (fn) and male (mn) pronuclei. In the small female pronucleus, many condensed chromatin bodies (ch) are seen, but the large male pronucleus has few. Bar, 2 µm

observed on the equatorial plate in the central region of blastodisc. The chromosomes had partially electron dense regions (Fig. 3) in all eggs examined. Fifty minutes PI, anaphase chromosomes were observed separating towards the centers of the two blastomeres. The clotted or irregular chromosomes looked like a loose ball of yarn in most cases. Fifty-five to sixty minutes PI, irregularly disentangled chromosomes were divided and scattered in two polar areas within the elongated blastodisc, but the furrow of the first cleavage was still not visible. Sixty-five to seventy-five min-



**Fig. 3.** Metaphase chromosomes in the first cleavage of a hybrid zygote. The chromosomes have electron dense regions (arrows). Bar, 1  $\mu\text{m}$



**Fig. 4.** Abnormalities of nuclear division in the first cleavage of hybrid zygotes. Sixty-five minutes PI, the different zygote nuclei (a-c) were abnormally divided into a few fragmented interphase nuclei with thread-like chromatin across the cleavage furrow (solid lines with arrows). (d) Normal interphase nuclei were observed in each blastomere of control *Oryzias latipes* eggs fertilized by homologous sperm. Bar, 25  $\mu\text{m}$

utes PI, small chromatin clots had a stretched, thread-like structure, and were divided by a cleavage furrow (Fig. 4).

Ninety minutes PI, the chromosomes began to separate abnormally into four poles of blastomeres for the second cleavage. One hundred minutes PI, the interspecific *O. latipes-javanicus* zygotes were at the 4 cell stage (Fig. 5), while the control *O. latipes* eggs fertilized with *O. latipes* spermatozoa were at the 8 cell stage (data not shown). The time sequences of development in *O. latipes* and *O. javanicus* eggs coincided approximately with those seen in previous studies (Iwamatsu *et al.*, 1994). Ten minutes later, each blastomere in most eggs had an interphase nucleus varying in size. Anaphase nuclei were observed in 4-cell stage eggs 120 min PI (90 min PI in control eggs). In most eggs at the 8 cell stage 140 minutes PI (110 min PI in control eggs), the sizes of the nuclei in the blastomeres varied. Development of all these cross-fertilized embryos ceased before formation

of eye-vesicles.

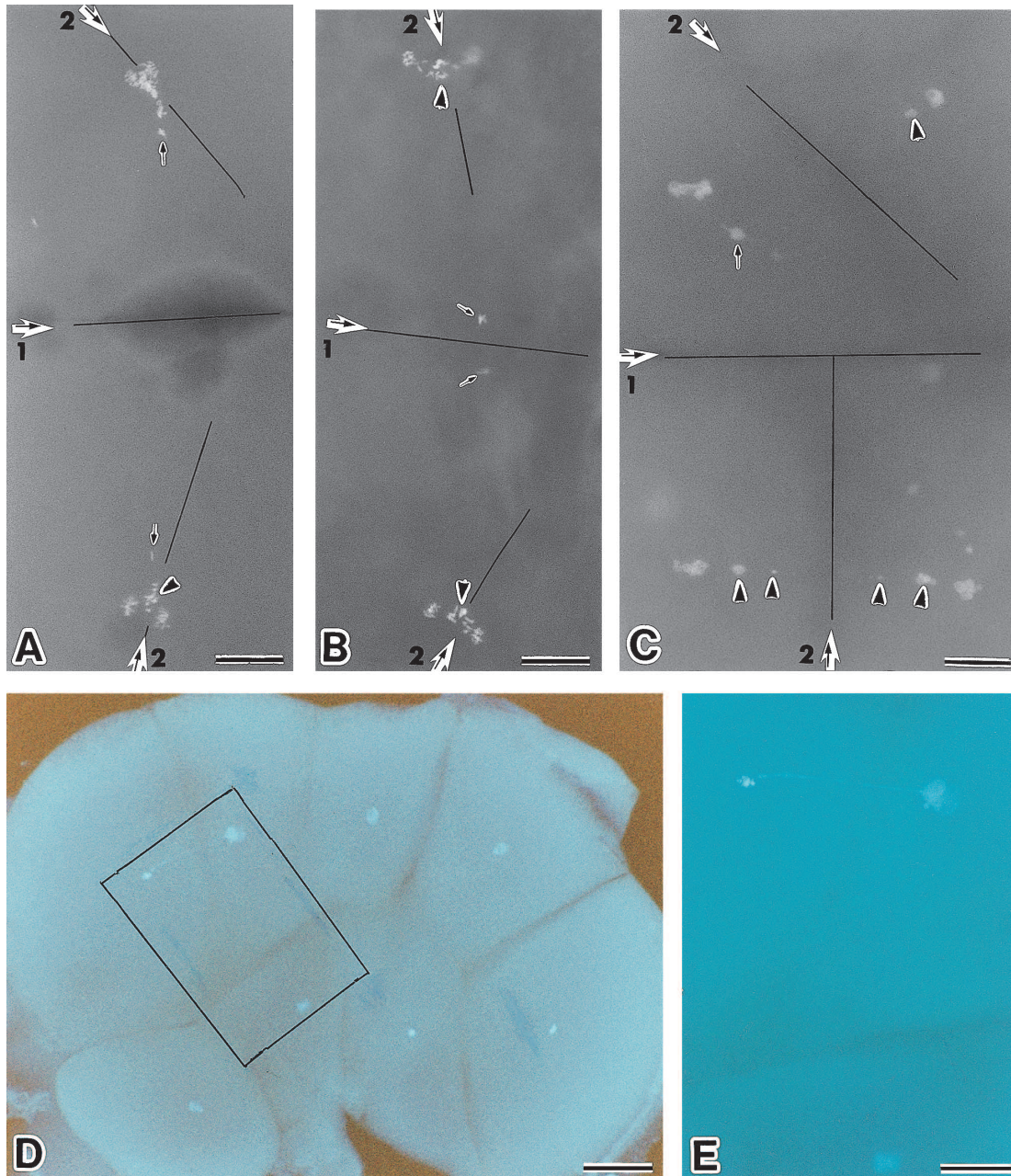
#### DNA synthesis:

To examine DNA synthesis in *O. latipes* eggs inseminated with an *O. javanicus* sperm suspension, BrdU was microinjected into the cortical cytoplasm near the animal pole at the beginning of exocytosis of cortical alveoli. Fifty minutes PI, the fluorescent label for BrdU was observed in abnormally separating chromatin undergoing the first cleavage. Undivided or stretched chromatin masses in dividing nuclei similar to those in the second cleavage (Fig. 5A, B) were also labeled by BrdU (Fig. 6).

## DISCUSSION

The type of karyogamy in which a single zygote nucleus is formed by fusion of pronuclear envelopes has been observed in normally fertilized eggs of *Arbacia* by Longo and



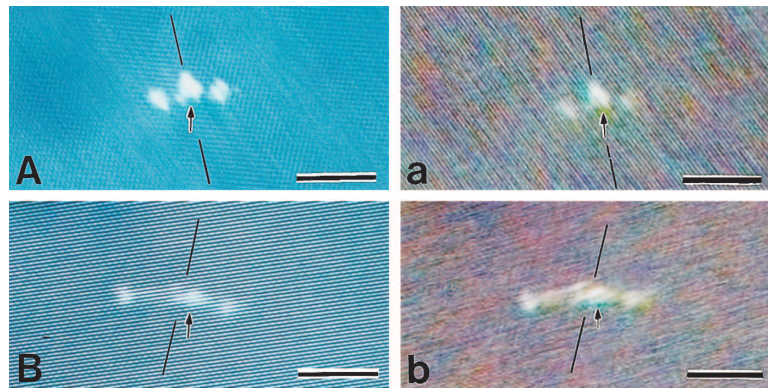


**Fig. 5.** Abnormal nuclear division in early cleavages of hybrid zygotes. A and B: Ninety minutes PI, small fragments (small arrows) of the chromatin frequently were abnormally separated near (B) or in the faraway distance (A) from cleavage furrows (solid lines) during the first cleavage (1 large arrows) and the second cleavage (2 large arrows). Bar, 37  $\mu\text{m}$ . C: One-hundred minutes PI, some fragments (arrowheads) of chromatin were unequally divided into each blastomere in the second cleavage. Bar, 37  $\mu\text{m}$ . D and E: One-hundred and forty minutes PI, a cross-fertilized egg at the 8 cell stage. Bar, 200  $\mu\text{m}$ . E: High magnification of a part (box) of figure D reveals unequal separation of the nucleus with a thread-like chromatin. Bar, 100  $\mu\text{m}$

Anderson (1968). Longo (1977) did not, however, report the direct evidence of pronuclear fusion in hybrids produced by cross-fertilization between *Arbacia* ♀ and *Mytilus* ♂. Therefore, the question as to whether or not pronuclear fusion in cross-fertilization occurs remained unanswered for a long time. In intraspecific fertilization of *O. latipes*, the pronuclei form a single zygote nucleus before breakdown of nuclear envelope by fusion of the nuclear envelopes (Iwamatsu and Kobayashi, 2002) in the same manner as in the sea urchin.

Thus, the present study confirms fusion of the nuclear envelope at karyogamy in this fish and is the first to demonstrate pronuclear fusion in interspecific hybrid zygotes produced by cross-fertilization between *O. latipes* and *O. javanicus*.

Nuclear behavior from sperm penetration to pronuclear assembly in cross-fertilized eggs was morphologically normal compared with control *O. latipes* eggs following homologous fertilization (Iwamatsu *et al.*, 1999). In *Oryzias* eggs, no species-specificity in sperm-egg binding and fusion has



**Fig. 6.** BrdU-labeled nuclei in the first cleavage of hybrid zygotes produced by cross-fertilization between *O. latipes* and *O. javanicus*. Forty-five minutes PI, fertilized eggs were fixed and stained with Hoechst (A and B) and FITC-conjugated fluorescent antibody to BrdU (a and b). In dividing nuclei of the first cleavage, a non-divided mass or stretched mass (arrows) of chromatin remaining in each cleavage furrow (solid lines) was also labeled by BrdU. Bar, 42  $\mu$ m

been recognized, as observed in previously published experiments on interspecific fertilization (Iwamatsu *et al.*, 1994). Factors that mediate the fusion of the sperm with the eggs may not be species-specific, and they induce normal cortical reaction in this fish. The inducer for cortical reaction seems to be shared, or absent between different species. While eggs of the sea urchin are penetrated by spermatozoa of the mussel (Longo, 1977) or the oyster (Osanai and Kyozuka, 1982), such sperm penetration fails to induce the cortical reaction. In sperm from completely different family, the inducer for cortical reaction may be functionally different.

The present investigation, revealed not only a delay of cleavage in interspecific hybrids between *O. latipes* and *O. javanicus*, but also cytological evidence of abnormality during nuclear division in each cleavage, although chromatin condensation was not retarded and occurred within 5 min concomitant with that of homologous fertilization (Iwamatsu and Kobayashi, 2002). The presence of *Rana pipiens* nuclei in the *R. palustris* egg cytoplasm leads to developmental arrest due to the nucleocytoplasmic incompatibility (Hennen, 1964). This is noteworthy to recall verifying the cause of the failure of the male pronucleus of *O. javanicus* to separate normally in the egg cytoplasm of *O. latipes* during the cleavage. In lethal hybrids between the frogs *Rana pipiens* and *R. sylvatica*, mitotic behavior of the chromosomes is also abnormal. It has been demonstrated that structural chromosome aberrations are frequently induced in embryos when nucleoplasmic incompatibility occurs between two species (Hennen, 1963).

As shown in the present data, DNA synthesis was also observed in nuclei that divided abnormally in each cleavage. In several cleavages, abnormal separation of very thin, thread-like or fragmented chromatin and incomplete formation of chromosomes were observed, possibly related to the electron dense regions (Fig. 3) of chromosomes. This is very similar to the behavior of chromosomes in *O. latipes* eggs fertilized in the presence of aphidicolin or topoisomerase I inhibitor (Iwamatsu *et al.*, 2002). In eggs treated

with these inhibitors, the formation and behavior of pronuclei were also morphologically normal, as seen in the present interspecific hybrids. Therefore, it is still unclear whether the abnormal nuclear division and cytokinetic delay are due to defects in incomplete DNA synthesis or chromosome formation following DNA synthesis. Further investigations from the molecular aspects of relationships between DNA synthesis and chromosome structures will necessarily elucidate the mechanism of chromosome separation.

According to Kurita *et al.* (1995), when interspecific hybrids between *O. latipes sinensis* and *O. curvinotus* were back crossed to *O. latipes sinensis*, several backcross offspring had a chromosome number different from the expected number. They thought the unexpected chromosome numbers might have occurred because the eggs laid by hybrid females were not always completely diploid and/or because of mosaicism resulting from non-disjunction to produce hypodiploidy during ontogenesis. Non-disjunction during early development in interspecific hybrids of the genus *Oryzias* may result in irregular chromosome numbers, although the number of chromosomes in lethal embryos was not examined in the present study. In another experimental observation (Yamashita, Unpublished data) on hybrids between *O. latipes* ♀ and *O. javanicus* ♂, only paternal chromosomes remain to be segregated at the equatorial plate region in cleavages, and consequently the embryos fail to reinstate the diploid chromosome number. In the early experiment on *Strongylocentrotus* ♀ × *Shaerechinus* ♂ hybrids (Baltzer, 1910), it has been also shown that at the time of the first cleavage, some of the paternal chromosomes failed to separate and had been eliminated. These facts imply that abnormality of chromosome separation in *O. latipes-javanicus* hybrids may be caused by a species-specific deficiency or enzymatic incompatibility in the process of chromosome formation (condensation) during the pronuclear stage. Further investigation of the possible causes of abortive development of interspecific hybrids is required.



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