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Chromosomes of the Lancelet *Branchiostoma belcheri* Gray

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ABSTRACT—We characterized the chromosomes of the lancelet *Branchiostoma belcheri* Gray. Testes were removed from adult males, cut up, treated with colchicine (25–50 μg/ml) for 3–6 hr, and dissociated into single cells by pippeting. The dissociated cells were swollen with 5.0–6.5% sodium citrate for 10 min and fixed with methanol-acetic acid (3:1). The fixed cells were dropped onto a glass slide and air-dried. The haploid chromosome number was 18 and diploid number was 36.

INTRODUCTION

The lancelets belong to the Cephalochordata, which occupy an important position in the evolution of invertebrates to vertebrates. A study of lancelet chromosomes should yield important information regarding chromosome evolution. About a century ago, Van der Stricht (1896), Sobotta (1897), and Cerfontaine (1906–1907) described lancelet chromosomes in the course of oogenesis or egg development. Later, Nogusa (1957) examined sections of *Brachiostoma belcheri* testes and reported a diploid chromosome number of 32. In the 1970s, Howell and Boschung Jr. (1971) reported that the diploid chromosome number of the western Atlantic lancelet *B. floridae* was 38, and Colombera (1974) showed the same number for *B. lanceolatum*. No clear karyotypes, however, had been shown as yet; an improved method for preparing lancelet chromosomes was necessary.

In Japan up to the 1950s, the major lancelet species, *B. belcheri*, was distributed widely from the middle of Honshu to the south of Kyushu (Nishikawa, 1981; Yamaguchi and Kikuchi, 1985; Nishikawa and Mizuoka, 1990). Since then, however, the numbers decreased owing to aquatic pollution and sludge in the sediment, and we had to search for populations for our chromosome research. We began our survey in 1994, after the lancelets were collected for the environmental assessment of the Ohkura coast in Akashi, and in 1996 and 1997 we obtained enough material for our research. In this paper, we describe an improved method for chromosome preparation and our findings on the chromosomes of *B. belcheri*.

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MATERIALS AND METHODS

Collection and maintenance

We collected *B. belcheri* specimens at Maruyama, on the west coast of Awaji Island, Seidan-cho, Mihara-gun, Hyogo Prefecture, Japan, on 15 June 1996 and 17 May 1997. We scooped the sand at a water depth of 7–8 m by hand into a 50×70 cm (about 10 liter) cotton bag and repeated this process 7–8 times. We obtained about 50 lancelets from about 70 liter of sand. We maintained them until use at 22°C in a beaker (200–300 ml) containing natural seawater.

Chromosome preparations

The testes from one or two adult males over 30 mm long were removed, cut into fine pieces with a knife, placed in a 35 mm dish containing 2 ml of seawater, and treated with colchicine (Sigma, St Louis, MO, USA) 25–50 μg/ml for 3–6 hr at room temperature. The suspension was transferred to microtubes, and the pieces were dissociated into single cells by pipetting. The dissociated cells were collected by centrifugation (300×*g* [1,500 rpm]×10 min), treated with 5.0–6.5% sodium citrate for 10 min, fixed with methanol: acetic acid (3:1), and washed twice with fresh fixative. The fixed cells were dropped onto a glass slide. The preparations were air-dried and stained with 3% Giemsa (Merck, Whitehouse Station, NJ, USA) diluted with a standard pH 6.9 solution (Horiba Ltd., Tokyo, Japan) for 10 min. We could make chromosome preparations from 6 out of 7 males in 1996 and from 8 out of 10 males in 1997. We examined the chromosomes with the aid of a microscope at a magnification of 1500 ×.

RESULTS

The air-drying method spread the chromosomes well. Fig. 1 shows the two types of metaphase plates we observed. Fig. 1a shows a meiotic metaphase from a spermatocyte with 18 chromosomes and Fig. 1b shows a mitotic metaphase from a spermatogonium with 36 chromosomes. The chromosomes ranged in length from 1 to 2 μm. The June 1996 specimens yielded mainly haploid cells. The August 1995 specimens had few metaphase plates but many figures of sperm heads, and

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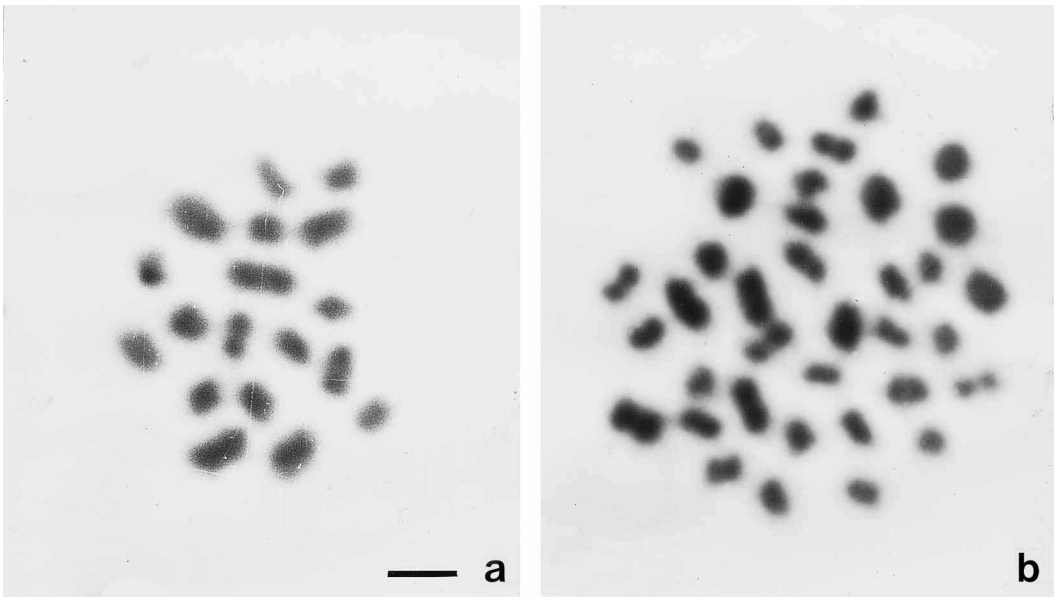

Fig. 1. Typical metaphase chromosomes from testes of *Branchiostoma belcheri* showing haploid (a) from a spermatocyte and diploid (b) from a spermatogonium. Bar, 2 μm.

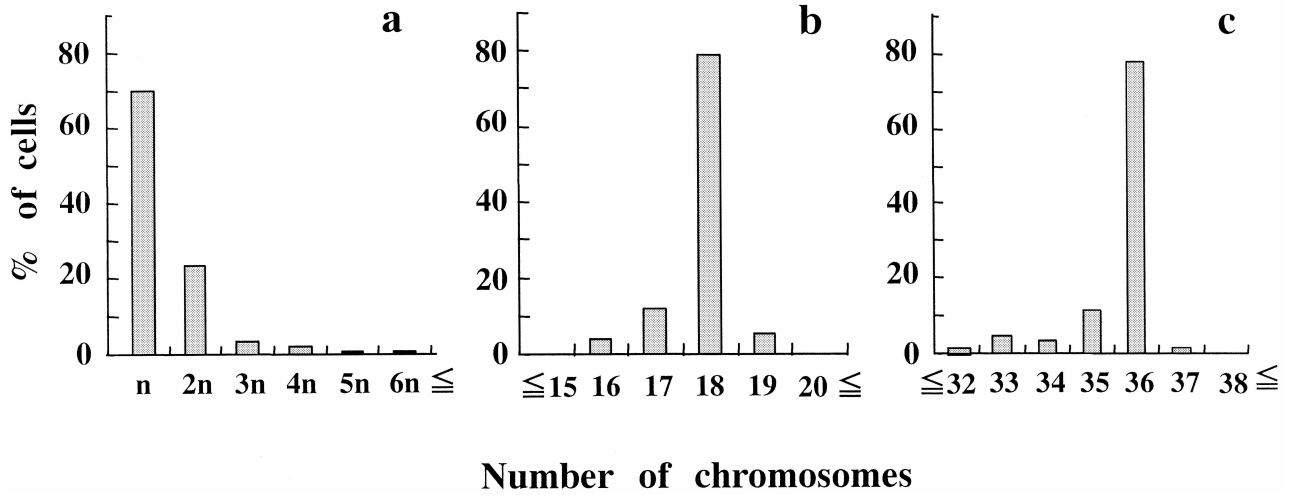

Fig. 2. Distribution of chromosome numbers in examined cells in May 1997(a, 468 cells), in haploid cells in June 1996 (b, 75 cells), and in diploid cells in May 1997 (c, 63 cells).

the July 1994 specimens had well-developed gonads. We obtained both haploid and diploid cells (ratio=3:1) from individuals collected in May 1997 (Fig. 2a), as well as polyploid (3n to 6n or more) cells. Fig. 2a shows the ploidy distribution in 468 cells taken from 8 males. Fig. 2b shows the distribution of chromosome number in haploid cells, and Fig. 2c shows it in diploid cells. The modal numbers were 18 and 36, respectively.

DISCUSSION

In this paper, we report an air-drying method for preparing chromosomes from testicular cells of lancelets and the chromosome analysis. The collecting season (May), the concentration of colchicine (25–50 μg/ml) and duration of treatment (3–6 hr), and the concentration of hypotonic sodium citrate (5.0–6.5%) were important factors in obtaining good metaphase plates. The developmental state of the gonads and the appearance of the chromosomes for the 4 annual samples of lancelets from Awaji Island suggest that mitosis and meiosis take place in those organisms in May, meiosis in June, and sperm formation in July and August.

We are considering three possibilities for the origin of the polyploid cells (Fig. 2a): a technical artifact, chromosome polyploidization, or individual variation. Artifacts could arise when mitotic cells aggregate and pieces of testes with mitotic cells do not dissociate during pipetting; indeed we sometimes observed apparent polyploidy showing a combination of different mitotic stages such as prometaphase and metaphase. Polyploidy could also arise if colchicine inhibits cell division but not nuclear division. Odd ploidies, such as triploidy and

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pentaploidy, however, could not be explained by this mechanism. Concerning the second possibility, duplication and polyploidization of chromosomes are important mechanisms of chromosome evolution (Atkin and Ohno, 1967) and may have occurred during cell division in this study. The third possibility, individual variation, may not be low, because polyploidy was observed in every preparations from 1997, even in those obtained from one specimen.

The chromosomes of *B. belcheri* showed a gentle gradation in size (Fig. 1). Although Nogusa (1957) reported XY sex chromosomes in the male, heteromorphic sex chromosomes were not apparent in this study, nor were they found in *B. floridae* (Howell and Boschung Jr., 1971) or *B. lanceolatum* (Colombera, 1974). Though we provisionally consider that *B. belcheri* has several meta- or submetacentric chromosomes (Fig. 1b), we can not yet deny that morphology of those chromosomes is similar to that of meiotic bivalent chromosomes. On the other hand, there are also reports that 15 to 17 pairs of *B. floridae* chromosomes are subtelocentric or telocentric, and 2 to 4 pairs are meta- to submetacentric (Howell and Boschung Jr., 1971) while most chromosomes of *B. lanceolatum* are telocentric and 2 pairs are metacentric (Colombera, 1974). Karyotype analysis by FISH and banding methods may be necessary to clarify the position of the centromeres. Detailed analysis of *B. belcheri* chromosomes was limited in the preparations from testes owing to their smallness. One solution for this problem is to obtain preparations from somatic cells, embryos, or cultured cells because clear metaphase plates can be obtained from them (Ojima, 1983). Since collection of metaphase plates from lancelet gill or fin epithelium is difficult (Howell and Boschung Jr., 1971), preparations from embryos should be tried to confirm *B. belcheri* chromosome morphology. If we can obtain a supply of lancelet embryos, we will do that using sea urchin embryo methodology (Saotome, 1987).

Using an air-drying method we found the diploid chromosome number of *B. belcheri* to be 36, whereas Nogusa (1957), using a sectioning technique, found it to be 32. Studies in the 1970s showed the diploid number to be 38 in *B. floridae* (Howell and Boschung Jr., 1971) and in *B. lanceolatum* (Colombera, 1974). Cephalochordates are reported to be the subphylum closest to vertebrates on the basis of molecular (Wada and Satoh, 1994; Spruyt *et al*., 1998), anatomic and taxonomic studies. We found it interesting that lancelet chromosomes are close to the hagfish (jawless fishes) somatic chromosomes (mainly 2n=34) in number but not in size (Kitada and Tagawa, 1975; Kohno *et al*., 1998), and that they are similar to lamprey chromosomes in size (under 3 μm) but not in number (Sasaki and Hitotsumachi, 1967; Potter *et al*., 1968; Howell and Denton, 1969; Robinson and Potter, 1969, 1981; Potter and Rothwell, 1970; Howell and Duckett, 1971; Kitada and Tagawa, 1975). On the other hand, chromosome numbers of the lancelets are close to those of the family Perophoridae (2n=34), noted as primitive within ascidians (Colombera, 1982). The lancelets, the primitive jawless fishes, and the primitive ascidians may have been derived from an ancestor with a small amount of DNA (Atkin and Ohno, 1967) and haploid numbers around 20 (Colombera, 1982). From the ancestor, the lancelets may have evolved into several species without a major change in chromosome number, the tunicates may have diverged to many kinds of species by decreasing or increasing chromosome number, the hagfishes may have evolved by increasing DNA content without changing chromosome number, and the lampreys may have evolved by an extensive increase in chromosome number. Further chromosome investigation including karyotypes, however, is necessary to discuss lancelet phylogeny.

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