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# Karyotyping of Female and Male *Hediste japonica* (Polychaeta, Annelida) in Comparison with Those of Two Closely Related Species, *H. diadroma* and *H. atoka*

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**ABSTRACT**—Karyotypes of females and males of the brackish-water polychaete *Hediste japonica sensu stricto*, collected from the Ariake Sea, Japan, were examined by using regenerating tails. We used the Giemsa staining method and a computer-assisted image-analyzing system for the identification of each chromosome pair. The somatic chromosome number was 2n=28. The presence of an XX–XY (male heterogametic) sex chromosome system was determined from well-spread metaphase plates of somatic cells. The type of sex chromosomes related with phenotype exactly. The metacentric Y chromosome was much larger than the submetacentric X chromosome. All autosomes were metacentric. The karyotype of this species was compared with those of the other two closely related species (*H. diadroma* and *H. atoka*). The karyotypes of all the three species were similar to one another.

**Key words**: karyotype, male heterogametic sex chromosome system, image-analyzing method, *Hediste*, polychaete

## INTRODUCTION

The genus *Hediste* Malmgren, 1867 (Polychaeta, Annelida) is one of the most dominant genera in shallow brackish waters in the North Temperate Zone and consists of five species: *H. diversicolor* distributed in both the European and the North American coasts of the Atlantic (Smith, 1977), *H. limnicola* in the North American Pacific coast (Smith, 1958), and three species in East Asia (Sato and Nakashima, 2003).

Previously, the Asian *Hediste* worms were regarded as belonging to a single species, *H. japonica*. However, the recent studies on the morphology, reproduction, early development and electrophoretic analysis of the allozymes revealed that "*H. japonica*" consists of three distinct species (Sato, 1999; Sato and Masuda, 1997; Sato and Nakashima, 2003; Sato and Tsuchiya, 1987, 1991): *H. diadroma* with a small egg size (130–170 μm in diameter) and a long pelagic larval life, *H. atoka* with a large egg size (200–250 μm) and no pelagic larval stage, and *H. japonica sensu stricto* with an intermediate egg size (180–210 μm) and a short pelagic larval life.

The same diploid chromosome number of 28 and similar karyotypes mostly composed of metacentric and submetacentric chromosomes are known to exist in three *Hediste* species, i.e., *H. diversicolor* (Christensen, 1980), *H. diadroma* and *H. atoka* (Sato and Ikeda, 1992). Sato and Ikeda (1992) showed that the latter two Asian species had a pair of heteromorphic sex chromosomes of male heterogamety (XX–XY system), where the Y chromosome was larger than the X chromosome. Once extreme sex chromosome differences evolve, the evolution of new sex-determining mechanisms may be prevented, and species descended from a common ancestor seem to share the same sex chromosome system (Bull, 1983). Therefore, the XX–XY system is likely to be shared also in the other congeneric gonochoristic species except for a hermaphrodite species, *H. limnicola* (Smith, 1950).

Previously, slight differences in karyotype were found between *H. atoka* and *H. diadroma*, but identification and numbering of homologous chromosome pairs were rather difficult because many chromosome pairs in a plate are similar to one another in size and shape (Sato and Ikeda, 1992). Identification and numbering of homologous pairs has become easier in some vascular plants by using an image-analyzing method that enhances the differences of Giemsa-staining intensity depending on the level of the chromatin condensation (Ito *et al*., 2000; Kato and Fukui,

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1998). In the present study, the karyotype of *H. japonica sensu stricto* was examined and compared with those of *H. diadroma* and *H. atoka* using this image-analyzing method for the first time in invertebrates.

## MATERIALS AND METHODS

### Materials

Adult worms of *Hediste japonica* were collected from sediment samples of muddy intertidal flats around the mouth of the Omuta-gawa River in Ariake Sea (Fukuoka Prefecture, Japan). Females and males of *H. diadroma* were collected from the Okinohata-gawa River in Ariake Sea (Fukuoka Prefecture, Japan) and the Omoi-gawa River in Kagoshima Bay (Kagoshima Prefecture, Japan), respectively. Females and males of *H. atoka* were collected from the Kotsuki-gawa River in Kagoshima Bay (Kagoshima Prefecture, Japan). The sex of each individual was determined by examining coelomic content. Individuals having oocytes and those having clumps of spermatogonia or spermatocytes were determined to be females and males, respectively. Oocytes and spermatogonial clumps never occurred together in a single worm.

### Chromosome preparation

To obtain well-spread chromosome plates in metaphase from the regenerating tail, we modified the technique of Ikeda and Sato (1991) and Sato and Ikeda (1992). The posterior tip (ca. 5 mm long) of each worm was excised. Thereafter each worm was kept in 50% seawater (salinity: 16‰) at 17–20°C and fed on Tetramin Flakes fish food (Pfizer, NY, USA). After about a week, a regenerating tail developed to a length of 1–2 mm. The regenerating tail was cut with a razor and treated with 0.04–0.05% colchicine dissolved in 50% seawater for 15–24 hr at 17–20°C. Regenerated tails were placed in a hypotonic solution (1% sodium citrate) for 30–40 min at room temperature, and then fixed in at least three changes of fixations in methanol: acetic acid (3:1 vol/vol) for a total time of 40–60 min at room temperature. Fixed specimens were put onto ice-cold glass slides and then macerated by using dissecting needles. Slides were dried by use of an alcohol heater, then stained with freshly prepared 2% Giemsa solution (Merck, Germany) diluted with M/15 Sørensen's phosphate buffer (pH 6.8). The metaphase plates were examined under a light microscope, and taken with monochrome films (Minicopy HR II, Fuji Photo Film, Japan).

### Arrangement of each chromosome pair for karyotype analysis

Negative images of photographs were stored digitally in a personal computer (Power Macintosh G4; Apple Computer, USA), with a film scanner (Nikon Super Cool Scan 4000, Nikon, Japan). The differences of the Giemsa-staining intensity in the original monochrome image was enhanced by the image analysis software Mac SCOPE (Mitani Corp., Japan) as follows (see Fig. 2A): Firstly, the original image was inverted to negative image, then by application of a digital filter, the brightness of each pixel of the negative image was estimated. The brightest pixel on a image, which was a darkly stained part, was shown with the gray value of 255 (white), and the darkest pixel, which was the unstained background, was shown with the gray value of zero (black). Secondly, a three dimensional graph was drawn to take the Z-axis in the direction of the gray value. Thirdly, the Z axis was demonstrated by rainbow colors from red as the brightest pixel to purple as the darkest pixel. The original monochrome image of each chromosome was converted to the pseudo-colored image based on the rainbow colors of the Z axis. The length of arms of each chromosome was measured automatically by using a function of Mac SCOPE. Chromosome pairs were identified by comparison of their size, shape and pseudo-colored pattern, and arranged in descending order of size except for the last pair of sex chromosomes. Terminology of the karyotypic classification relating to centromeric position follows Levan *et al.* (1964).

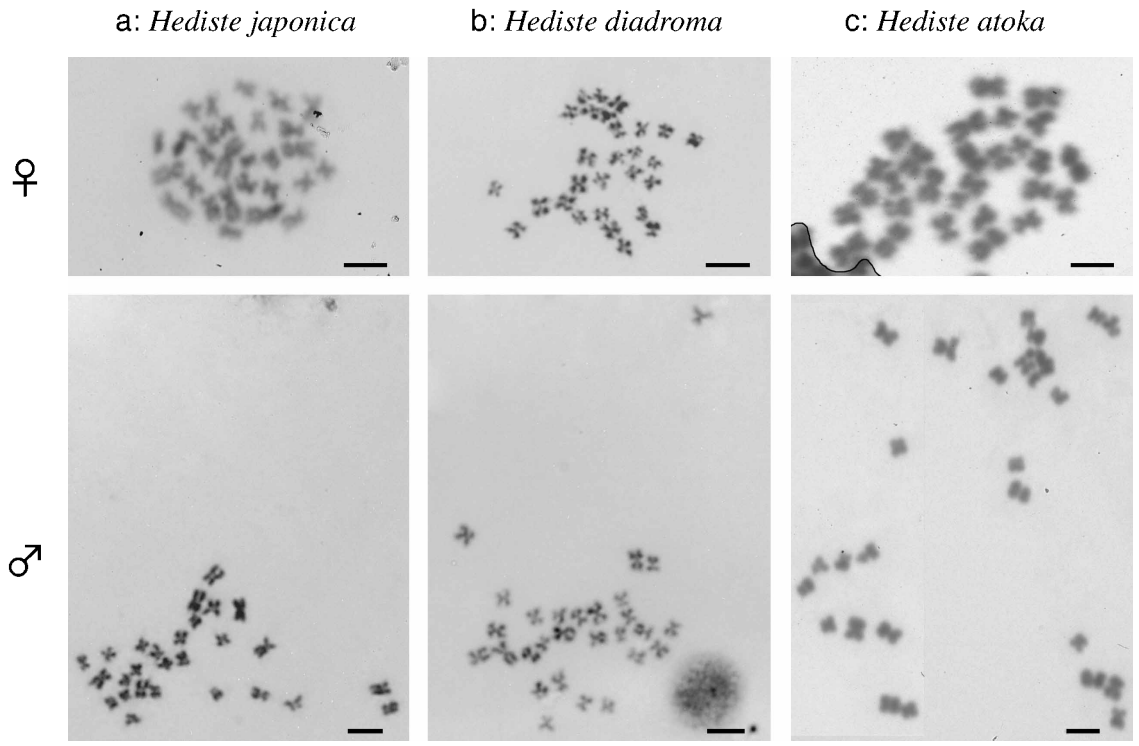


**Fig. 1.** Chromosome plates in mitotic metaphase of females (upper) and males (lower) in *Hediste japonica* (a), *H. diadroma* (b) and *H. atoka* (c). Each bar represents 5 μm.

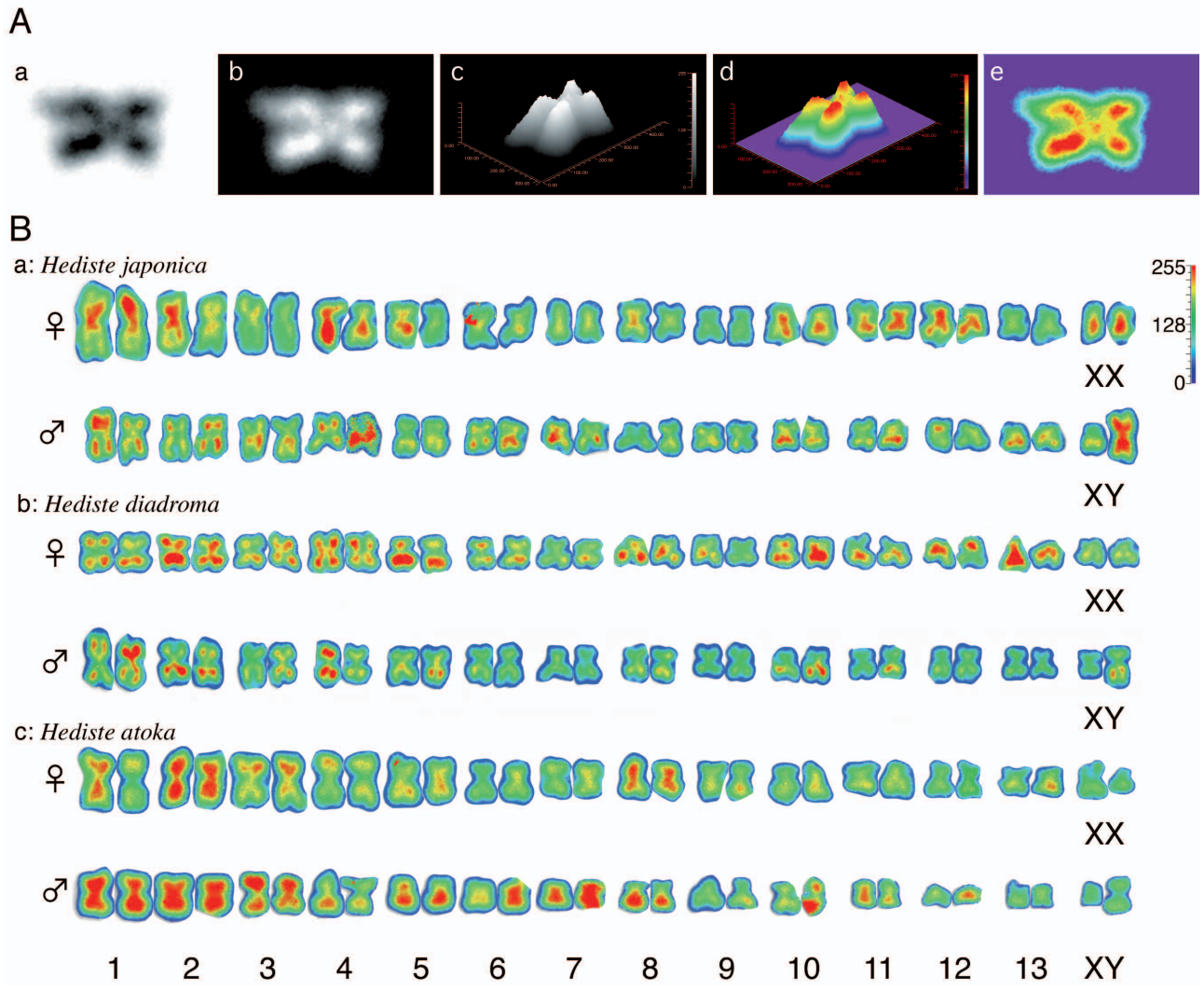


**Fig. 2.** A. Process of digital imaging. The chromosome image (a) was inverted to a negative image (b). A 3D graph was drawn to take the Z-axis in the direction of the gray value (c) and the surface was painted with rainbow colors (d). The chromosome image was pseudo-colored (e). B. The image-analyzed karyotyping using the pseudo-color method in three *Hediste* species. Karyotypes of females (♀) and males (♂) of *H. japonica* (a), *H. diadroma* (b), and *H. atoka* (c). The color bar at the top right corner indicates ranges of Giemsa-staining density.

## RESULTS

### The karyotype of *Hediste japonica sensu stricto*

Well-spread metaphase plates of 154 mitotic cells were obtained from eight worms (four females and four males). A diploid (2n) chromosome number of 28 was observed in most of the plates (27 or fewer chromosomes in 31% of the plates probably due to artifacts during preparation). Based on representative female (Fig. 1a upper) and male (Fig. 1a lower) plates, the pseudo-colored karyograms were produced (Fig. 2A). Using nine plates (five plates from two females and four plates from a male) containing relatively well-elongated chromosomes, the arm length of each chromosome was measured and the relative length of chromosome pairs (length of chromosome/average lengths of the longest pair of autosome), the arm ratio, and the centromeric indexes were calculated (Table 1).

The morphology of a pair of chromosomes was different between females and males (Figs. 2B and 3). The pair was small, and monomorphic in females, but heteromorphic in males. These were considered to be heteromorphic sex chromosomes; females had two smaller submetacentric X chromosomes, and males had a smaller X chromosome and a larger metacentric Y chromosome. The type of sex chromosomes exactly related with the phenotype decided by the existence of oocyte or spermatocyte. All of 13 pairs of autosomes were judged as metacentric.

### The karyotype of *Hediste diadroma* and *H. atoka*

A diploid chromosome number of 28 was observed in five well-spread plates obtained from a female (Fig. 1b upper) and three plates from two males (Fig. 1b lower) in *H. diadroma*, and also in five plates obtained from three females (Fig. 1c upper) and three plates from a male (Fig.

**Table 1.** Characteristics of chromosomes of *Hediste japonica* (a), *H. diadroma* (b) and *H. atoka* (c)

a: *H. japonica*

| Chromosome pair no. | Relateve length[1)] | Arm ratio[1)] | Chromosome type[2)] |
|---|---|---|---|
| 1 | 1.00 | 1.20 | m |
| 2 | 0.92 | 1.37 | m |
| 3 | 0.86 | 1.38 | m |
| 4 | 0.81 | 1.35 | m |
| 5 | 0.73 | 1.34 | m |
| 6 | 0.70 | 1.66 | m |
| 7 | 0.67 | 1.27 | m |
| 8 | 0.65 | 1.26 | m |
| 9 | 0.62 | 1.65 | m |
| 10 | 0.60 | 1.64 | m |
| 11 | 0.57 | 1.19 | m |
| 12 | 0.54 | 1.32 | m |
| 13 | 0.53 | 1.69 | m |
| X | 0.56 | 1.80 | sm |
| Y | 0.83 | 1.54 | m |

b: *H. diadroma*

| Chromosome pair no. | Relateve length[1)] | Arm ratio[1)] | Chromosome type[2)] |
|---|---|---|---|
| 1 | 1.00 | 1.17 | m |
| 2 | 0.91 | 1.23 | m |
| 3 | 0.87 | 1.22 | m |
| 4 | 0.80 | 1.18 | m |
| 5 | 0.77 | 1.71 | sm |
| 6 | 0.74 | 1.24 | m |
| 7 | 0.73 | 1.61 | m |
| 8 | 0.68 | 1.50 | m |
| 9 | 0.68 | 1.30 | m |
| 10 | 0.64 | 1.62 | m |
| 11 | 0.63 | 1.37 | m |
| 12 | 0.60 | 1.30 | m |
| 13 | 0.54 | 1.60 | m |
| X | 0.54 | 1.61 | m |
| Y | 0.78 | 1.71 | sm |

c: *H. atoka*

| Chromosome pair no. | Relateve length[1)] | Arm ratio[1)] | Chromosome type[2)] |
|---|---|---|---|
| 1 | 1.00 | 1.22 | m |
| 2 | 0.96 | 1.14 | m |
| 3 | 0.89 | 1.25 | m |
| 4 | 0.87 | 1.23 | m |
| 5 | 0.75 | 1.42 | m |
| 6 | 0.69 | 1.68 | m |
| 7 | 0.68 | 1.40 | m |
| 8 | 0.64 | 1.37 | m |
| 9 | 0.63 | 1.59 | m |
| 10 | 0.63 | 1.69 | m |
| 11 | 0.57 | 1.35 | m |
| 12 | 0.52 | 1.39 | m |
| 13 | 0.49 | 1.76 | sm |
| X | 0.52 | 1.77 | sm |
| Y | 0.62 | 1.77 | sm |

[1)] Average.
[2)] m, metacentric; sm, submatacentric.

1c lower) in *H. atoka*. Each of the diploid sets of these species consisted of 13 pairs of metacentric and submetacentric autosomes and a pair of sex chromosomes; females had a pair of smaller metacentric (*H. diadroma*) or submetacentric (*H. atoka*) X chromosomes, while males had a heteromorphic pair of a smaller X chromosome and a larger submetacentric Y chromosome (Figs. 2B, 3 and Table 1).

## DISCUSSION

Our results show the karyotype of *Hediste japonica sensu stricto* for the first time. The chromosome number of 2n=28 is the same as that of the other *Hediste* species, i.e., *H. diversicolor* (Christensen, 1980), *H. diadroma* and *H. atoka* (Sato and Ikeda, 1992; present study), and many other nereidids (Christensen, 1980; Jah *et al.*, 1995).

Karyotypes of the three Asian *Hediste* species, *H. japonica, H. diadroma* and *H. atoka* were considerably similar to one another: all of these species have 13 pairs of metacentric or submetacentric autosomes and a pair of sex chromosomes of an XX–XY (male heterogametic) system, where the Y chromosome is larger than the X chromosome. Our results on karyotypes of *H. diadroma* and *H. atoka* were similar to those of the previous report for the same species (Sato and Ikeda, 1992) except for different classification of metacentric, submetacentric or subtelocentric chromosomes for some chromosomal pairs probably due to a slight variability of arm ratio in relation to different extents of chromosomal condensation during preparations.

*Hediste japonica* is morphologically distinguishable from the other two species even at the immature stage by three independent characteristics, while *H. diadroma* and *H. atoka* are indistinguishable, suggesting that the latter two species are most closely related (Sato and Nakashima, 2003). However, no marked karyological difference corresponding to the morphological differences was found among the three Asian *Hediste* species in the present study. This presents a striking contrast to the case of the "*Nereis acuminata*" species group where several morphologically similar species show marked differentiation in diploid chromosome number (2n=18, 22, 24, 28) and in karyotype (Pesch and Pesch, 1980; Pesch *et al.*, 1988; Weinberg *et al.*, 1990, 1992).

In our previous (Sato and Ikeda, 1992) and present study, the type of sex chromosomes (female=XX, male=XY) exactly related with the phenotype decided by the existence of oocyte or spermatocyte. Furthermore, Korablev *et al.* (1999) reported another example of the XX–XY male-heterogametic sex chromosome system in the spionid polychaete *Polydora curiosa*, in which the Y chromosome was also larger than the X. Jablonka and Lamb (1990) and Korablev *et al.* (1999) suggested that the larger Y chromosome may accumulate multiple transposable elements, which are less important for individual viability. On the other hand, the XX–XO male-heterogametic sex chromosome system has been demonstrated in the cosmopolitan interstitial polychaete

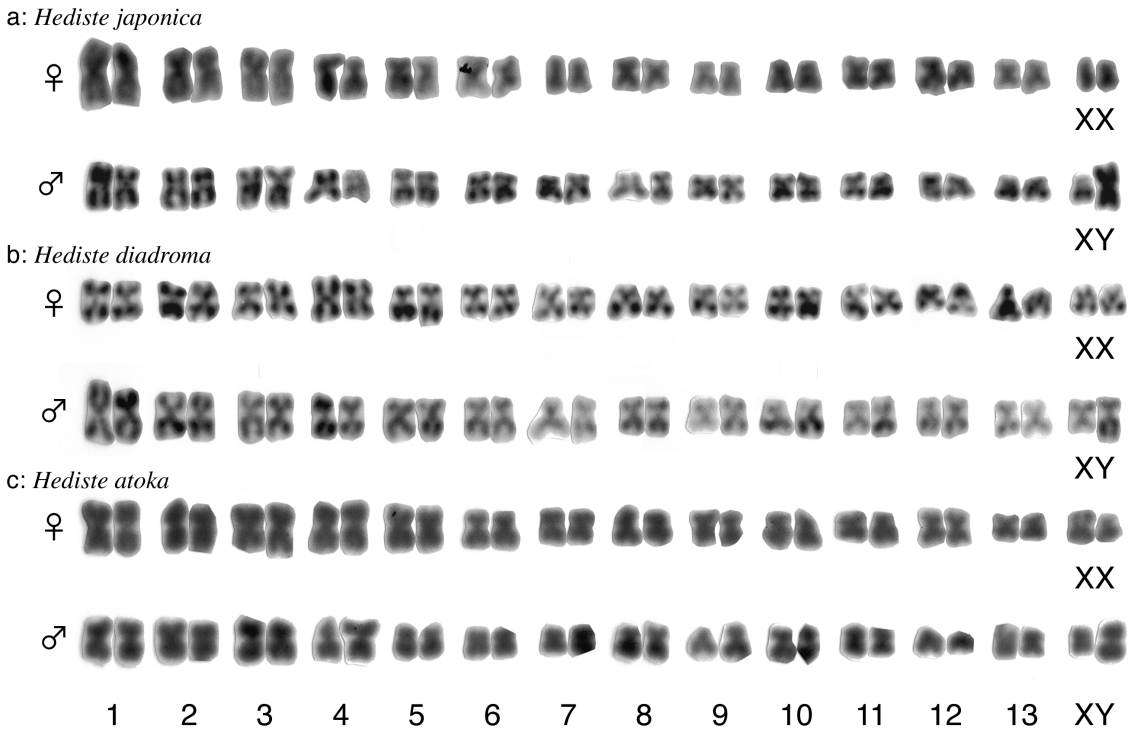


**Fig. 3.** The ordinary karyotyping in three *Hediste* species. Karyotypes of females (♀) and males (♂) of *H. japonica* (a), *H. diadroma* (b), and *H. atoka* (c).

*Dinophilus gyrociliatus* (Martin and Traut, 1987; Simonini *et al*., 2003). These results suggest that the sex determination system of polychaete depends on the combination of sex chromosomes.

In the present study, we used an computer-assisted image-analyzing system that enhanced the differences of Giemsa-staining intensity. The image-analyzing system has previously been applied for karyological studies of plants (Fukui, 1986; Miyamoto *et al*., 1991). This method is helpful for the identification of chromosome pairs, which are very similar in size and shape in a plate as shown in the present study, although it is not effective for the comparison of each chromosome pair between different plates.

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