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Karyotypic analysis of the hermaphroditic viviparous polychaete, *Hediste limnicola* (Polychaeta: Nereididae): possibility of sex chromosome degeneration

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Karyotypes of the hermaphroditic polychaete Hediste limnicola *were examined using an air-drying method and genetic material prepared from regenerating tail and newborn juveniles. Materials were obtained from a lineage of cultured worms originating from Watsonville Slough (California, USA) and maintained for six years in the laboratory. Giemsa-stained preparations were analysed by a computer-assisted image-analysing system for the identification of each chromosome pair. A diploid chromosome number of 26 was obtained from well-spread metaphase chromosomes of mitotic cells, consisting of metacentric (N = 11), submetacentric (N = 1) and telocentric (N = 1) chromosomes. It is possible that hermaphroditism in this species evolved through loss of a pair of sex chromosomes, which are present in closely related congeneric species.*

Keywords: Annelida, Nereididae, *Hediste*, image-analysing method, chromosome, karyotype, sex chromosomes, hermaphroditism

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INTRODUCTION

The genus *Hediste* (Nereididae: Polychaeta: Annelida), one of the most dominant genera in estuaries of the north temperate zone, contains five species: *H. diversicolor* distributed along the European and the North American coasts of the Atlantic (Smith, 1977), *H. japonica*, *H. diadroma* and *H. atoka* from East Asia (Sato & Nakashima, 2003), and *H. limnicola* from the North American Pacific coast (Smith, 1958). Although these five species are morphologically very similar, the reproductive characteristics of *H. limnicola*, which is hermaphroditic, self-fertilizing and viviparous, are markedly different from the other four gonochoristic, outcrossing species (Sato, 1999): the young of *H. limnicola*, which can inhabit fresh waters, develop into juveniles within the coelom of a parent and emerge by rupturing the body wall of the parent when they attain lengths of 4–8 mm with 20–30 segments (Smith, 1950). The viviparous hermaphroditism of this species appears to have evolved in response to the extreme environmental conditions associated with freshwater habitats (Bartels-Hardege *et al.*, 1996).

Christensen (1980) reviewed the cytogenetic characteristics of the Annelida and demonstrated that many members of the Nereididae have chromosome numbers of 28. Similarly, the four *Hediste* species, i.e. *H. diversicolor* (Christensen, 1980), *H. japonica* (Tosuji *et al.*, 2004), *H. diadroma* and *H. atoka*, have 28 chromosomes and karyotypes that consist mostly of metacentric and submetacentric chromosomes (Sato & Ikeda, 1992; Tosuji *et al.*, 2004). In addition, three Asian gonochoristic species (*H. japonica*, *H. diadroma* and *H. atoka*) had a pair of heteromorphic sex chromosomes and exhibited male heterogamety (XX–XY system), with the Y chromosome being larger than the X chromosome (Sato & Ikeda, 1992; Tosuji *et al.*, 2004).

Allozyme electrophoretic analyses revealed that *H. limnicola* is more closely related to *H. atoka* (=large-egg form of '*H. japonica*') than to *H. diversicolor* (Fong & Garthwaite, 1994). Hermaphroditism is a resource allocation strategy that is thought to have evolved to optimize gamete production and related fitness returns. When the allocation-return levels associated with being either male or female begin to diminish, individuals that shift additional reproductive resources to the other sex are selected and the simultaneous presence of both sexes in the same individual is considered favourable within an evolutionary context. As a reproductive strategy, self-fertilization is thus a potential means by which hermaphroditic taxa can ensure fertilization (Charnov, 1982). Moreover, in habitats such as estuaries, self-fertilization is commonly employed by sessile hermaphroditic organisms that are asynchronous broadcast spawners because sperm are not diluted or exposed to osmotic shock (Hsieh, 1997).

The hermaphroditic polychaete *H. limnicola* is an interesting subject for karyotype studies since, unlike closely related

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gonochoristic species with sex chromosomes, *H. limnicola* does not. In addition, to our knowledge, no karyotypic studies have been conducted on this species to date. In the present study, we describe the karyotypic characteristics of *H. limnicola* using a laboratory-cultured lineage.

MATERIALS AND METHODS

Materials

Adult worms of *Hediste limnicola* were originally collected from Watsonville Slough, a tidal creek flowing into Monterey Bay, California, USA in 1996, and subcultured in aquaria with 50% seawater (16 psu) at 18°C with a photoperiod under 12L/12D and fed on Tetramin Flakes fish food (Tetra Werke, Germany) by Dr Peter P. Fong at Gettysburg College in USA. Several worms of this laboratory-cultured lineage were then shipped alive to Kagoshima University in 1999 where they were maintained individually in Petri dishes containing 50% seawater until such time as chromosomes were prepared for the present study in 2001–2002.

Chromosome preparation and arrangement

To obtain well-spread chromosome plates in metaphase from the regenerating tail tissue, we used a modification of a previously described technique (Sato & Ikeda, 1992; Tosuji *et al.*, 2004). Terminal segments (~2–3 mm) of the tails of the small polychaete worms (body length approximately 20 mm) were excised and worms were maintained individually in 50% seawater at 18°C and fed on Tetramin Flakes. After approximately one week, the regenerating tails measuring 2 mm in length were removed using a razor blade and incubated overnight in 0.1% colchicine dissolved in 50% seawater at 18°C. The material was then immersed in a hypotonic solution (1% sodium citrate) for 1 hour at room temperature, and then fixed at least three times in a methanol–acetic acid mixture (3:1 vol/vol) for a total period of 40–60 minutes at room temperature. Newborn juveniles, with approximately 20 setigers at the time of parturition, were treated and fixed in the same manner as the regenerating tail. Fixed specimens (regenerated tail or juvenile) were placed onto glass slides and several drops of 50% acetic acid were added. After 5 minutes, specimens were transferred onto ice-cold glass slides and then macerated using dissecting needles. Slides were dried on a hot plate (~50°C), and then stained with a 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 (Nikon OPTIPHOT; Nikon, Japan), and photographed with Minicopy HR II film which was developed with Microfine developer (Fuji Photo Film, Japan).

The arrangement of chromosome pairs was performed using a computer-assisted image analysis system according to Tosuji *et al.* (2004) with slight modifications. Negative images of photographs were scanned using a film scanner (Nikon LS-4500AF, Nikon, Japan) and stored digitally. The original monochrome image was enhanced and converted to a pseudo-coloured image using the NIH imageJ 1.40 g public domain image processing and analysis program on a Macintosh computer (Power Macintosh G4; Apple Computer, USA). Chromosome pairs were identified by comparisons of their size, shape and pseudo-coloured patterns, and arranged in descending order of size. Terminology for karyotypic classification according to centromeric position follows Levan *et al.* (1964).

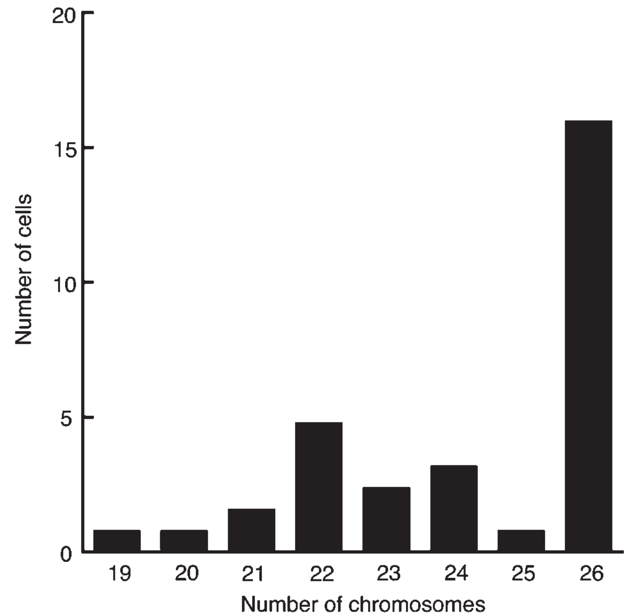

Fig. 1. Histogram of chromosome number distributions in *Hediste limnicola*. The abscissa shows the number of chromosomes and the ordinate shows the number of cells (N = 38 cells).

RESULTS

Well-spread metaphase plates of 38 mitotic cells were obtained from five regenerating tails and six juveniles (derived from six parents). A diploid (2n) chromosome number of 26 was observed in most of the plates (Figure 1). Instances where fewer chromosomes were observed (47%) are considered to have arisen due to losses encountered during preparation. Based on a representative plate (Figure 2), the chromosomes were analysed using the pseudo-colour method to produce ordinary karyograms (Figure 3); all of the chromosomes could be matched up in homologous chromosome pairs and no mismatching of chromosome pairs was observed. Using six plates containing relatively elongated chromosomes, the arm length of each chromosome was measured and the relative length of chromosome pairs (the average lengths of the longest pair was taken as 1), arm ratio, and centromeric indices were calculated (Table 1). The chromosomes were comparatively small, ranging from 1.04–3.87 μm in length. The diploid sets of this species consisted of 11 pairs of metacentric chromosomes, one pair of submetacentric chromosomes and one pair of telocentric chromosomes. The fundamental number (FN) was 50.

DISCUSSION

This is the first study to elucidate the karyotype of the hermaphroditic *Hediste limnicola*. The diploid chromosome number of 26 in this species is unique for the genus *Hediste*, the four other gonochoristic representatives of which have a

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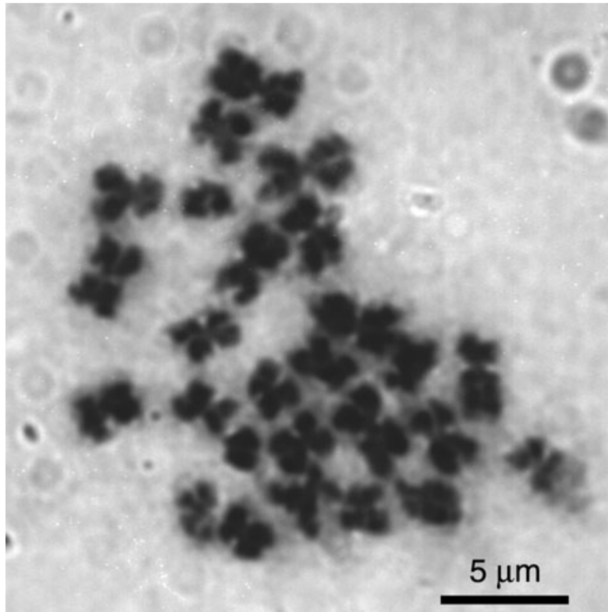

Fig. 2. Representative *Hediste limnicola* chromosomes in mitotic metaphase. The metaphase plates were stained with Giemsa solution. Bar represents 5 μm.

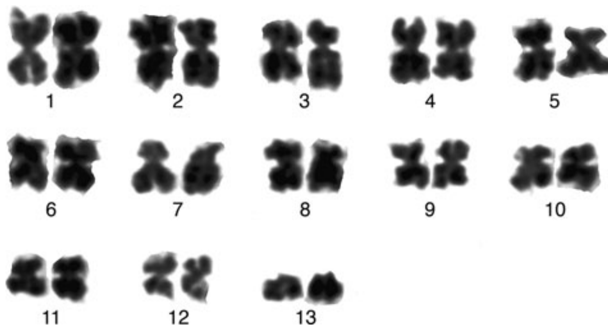

Fig. 3. Representative karyotyping of *Hediste limnicola*. The metaphase plate was stained with Giemsa solution and analysed using the pseudo-colour method and chromosomal arrangement.

diploid chromosome number of 28, e.g. *H. japonica* (Tosuji *et al.*, 2004), *H. diadroma*, *H. atoka* (Sato & Ikeda, 1992; Tosuji *et al.*, 2004) and *H. diversicolor* (Christensen, 1980). Karyotypes of the three Asian *Hediste* species, *H. japonica, H. diadroma* and *H. atoka*, were remarkably similar to one another, with all of these species having 13 pairs of metacentric or submetacentric autosomes and a pair of heteromorphic sex chromosomes. Specifically, these congeneric species exhibit male heterogamety (XX–XY system), in which the Y chromosome is larger than the X chromosome (Sato & Ikeda, 1992; Tosuji *et al.*, 2004) and in which sex determination appears to be determined by the chromosomal system simply. Once marked sex chromosome differences evolve, the evolution of alternate sex-determining mechanisms may be prevented, resulting in species that are descended from a common ancestor often sharing the same sex chromosome system (Bull, 1983). Consequently, the uniqueness of hermaphroditism in *H. limnicola* may be caused by the loss of sex associated with loss of a pair of sex chromosomes from the 28 chromosome set in the ancestral form.

Theoretically, the disappearance of heterozygosity in self-fertilizing hermaphrodites should be marked because self-fertilization is characterized by the absence or low levels of heterozygosity. Indeed, this disappearance of heterozygosity has been confirmed in numerous self-fertilizing terrestrial slugs and land snails (Selander & Kaufman, 1973; McCracken & Selander, 1980). However, an allozyme electrophoretic study in *H. limnicola* revealed that levels of heterozygosity remain relatively high, indicating that the strategy of self-fertilizing may be supplemented by frequent outcrossing (Fong & Pearse, 1992; Fong & Garthwaite, 1994).

It is possible to speculate that various karyotypic polymorphisms and reproduction modes may be maintained in wild populations of *H. limnicola*, i.e. some hermaphrodites have a chromosome set of 2n = 26 without a pair of sex chromosomes, while other members of the population are gonochoristic with a chromosome set of 2n = 28, including a pair of sex chromosomes. To determine whether this is likely, future karyotypic studies of wild populations are necessary. In addition, future investigations of chromosome number differentiation combined with analyses of

Table 1. Characteristics of *Hediste limnicola* chromosomes.

Chromosome pair No.	Length of chromosomes (average) (μm)	Relative length*		Arm ratio		Chromosome type
		Average	SD	Average	SD	
1	3.1–5.4 (3.87)	1.000	0.060	1.143	0.090	m
2	2.8–4.1 (3.30)	0.854	0.065	1.153	0.096	m
3	2.5–3.6 (2.98)	0.771	0.074	1.342	0.095	m
4	2.2–3.7 (2.86)	0.741	0.041	1.254	0.102	m
5	2.2–3.5 (2.68)	0.693	0.047	1.283	0.174	m
6	2.0–3.2 (2.50)	0.648	0.048	1.288	0.184	m
7	1.9–3.0 (2.38)	0.616	0.021	1.772	0.192	sm
8	1.7–3.1 (2.29)	0.592	0.037	1.390	0.207	m
9	1.7–2.8 (2.17)	0.562	0.041	1.367	0.138	m
10	1.8–2.6 (2.11)	0.546	0.043	1.368	0.188	m
11	1.5–2.5 (1.95)	0.504	0.031	1.295	0.107	m
12	1.4–1.9 (1.75)	0.452	0.055	1.581	0.471	m
13	0.7–1.6 (1.04)	0.270	0.040	–	–	T

*Value of 1 was assigned for the average length of chromosome Number 1; m, metacentric; sm, submetacentric; T, telocentric.

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chromosome structure among *Hediste* species may provide new clues to the phylogenetic and evolutionary relationships among the members of this genus.

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