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Chromosomal complements of two forms of *Neanthes japonica* (Polychaeta, Nereididae) with evidence of male-heterogametic sex chromosomes

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Abstract. Karyotypes of the brackish-water polychaete *Neanthes japonica* (Izuka) collected from five rivers in Japan from 1984 to 1988 were examined with air-drying and flame-drying methods using materials consisting of regenerating tails, clumps of spermatogonia and young *N. japonica* specimens (embryos, larvae or juveniles). A diploid number of 28 was determined in well-spread metaphase chromosomes of mitotic cells. The presence of an XX-XY (male heterogametic) sex chromosome system was established for the first time in polychaetes. The Y chromosome was larger than the X chromosome. Slight differences in karyotype were found between two forms (the small- and large-egg forms), which are very similar in adult morphology but can be distinguished by reproductive and developmental characteristics.

Introduction

Annelids are one of the animal phyla that are less well known from a cytological point of view (Christensen 1980). Previous karyological studies of nereidid polychaetes demonstrated that the diploid chromosome number is 28 in many species, with differing interspecific karyotypes (Christensen 1980). There has been no report of sex chromosomes in annelids.

In the present paper, we describe the chromosome complement of a brackish-water polychaete *Neanthes japonica* comprising two forms [1] (the small- and large-egg forms), which are very similar in adult morphology but different in egg size and reproductive and developmental characteristics (Sato and Tsuchiya 1987, 1991). Our recent studies have suggested that the two forms are in fact separate species with some morphological differences in mature adults (Sato unpublished data), although we use the terms small- and large-egg forms in this paper. In this study, we report on the presence of heteromorphic sex chromosomes in these forms and karyological differences between the two forms.

Materials and methods

Chromosome preparation using adult males and females

Adult worms (5 to 10 cm long) of *Neanthes japonica* were collected in 1984 from the sediment of the intertidal flats in the Kôtsuki River (1.5 km upstream from the mouth) and the Omoi River (1.3 km from the mouth) in Kagoshima Prefecture (Fig. 1, Table 1). In the laboratory, the posterior tip (ca. 5 mm long) of each worm was excised. Thereafter, each worm was kept at 17 to 20 °C in a Petri dish containing 50% seawater (salinity: 17‰) and sand. Worms were fed on commercially available fish food. Ca. 2 wk later, the regenerating tail developed to a length of 1 to 2 mm. To obtain well-spread chromosomes in metaphase from the regenerating tail, we modified the air-drying technique of Imai et al. (1977). Each worm with a regenerating tail was treated with 0.05% colchicine dissolved in 50% seawater for 15 to 24 h at 17 to 20 °C. The regenerating tail was then removed with a razor and placed in a hypotonic solution (1% sodium citrate) for 20 to 40 min. The tail was fixed in at least two changes of fixative (1:1 methanol:acetic acid) for a total time of 20 to 40 min, transferred onto a ice-cold precleaned slide using a Pasteur pipette, and teased apart using two dissecting needles and by adding several drops of the fixative to the material. Slides were air-dried and stained for 20 min in freshly-prepared 2% Giemsa solution (Merck solution diluted in 0.07 *M* Sorensen's pH 6.8 buffer). Slides were rinsed in distilled water for 1 s, air-dried, and observed under a microscope without a coverslip.

The sex of each individual was determined by examining coelomic content. Individuals having oocytes were judged to be females and those having clumps of spermatogonia or spermatocytes (Olive 1983, Ikeda and Sato 1991) were judged to be males. Oocytes and spermatogonial clumps have never occurred together in a single worm throughout our observations of more than a hundred worms.

Male worms were also collected from the Kôtsuki River, Kagoshima Prefecture, the Yahata River, Hiroshima Prefecture, and the Kominato River, Aomori Prefecture from 1984 to 1988 (Fig. 1, Table 1), and clumps of spermatogonia were used for chro-

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[1] The term "two forms" is used in this paper to be equivalent to "two types" used in our previous papers (Sato and Tsuchiya 1987, 1991)

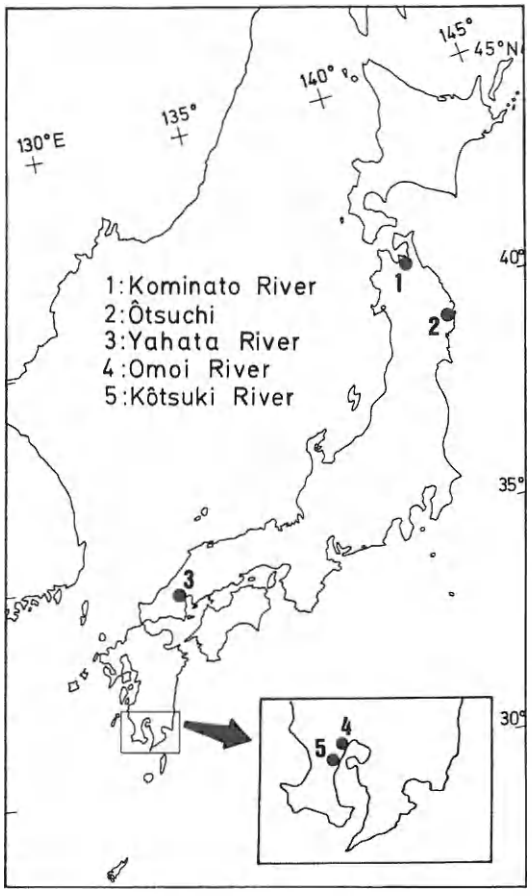

Fig. 1. *Neanthes japonica*. Localities of materials used for karyological studies

mosome preparation. Semitransparent clumps (ca. 10 µm in diameter) of spermatogonia were obtained by cutting the male body with forceps. Gonia were centrifuged (300 *g*, 2 to 3 min) and treated with 0.005% colchicine in 50% seawater for 30 min and then with 1% sodium citrate for 40 min. The materials were fixed in two changes of 3:1 methanol:acetic acid for a total time of 20 to 40 min. Slides with a few drops of cell suspension were flame-dried and stained with 2% Giemsa solution (Macgregor and Varley 1983).

Chromosome preparation using embryos, larvae or juveniles

The small- and large-egg forms are distinguished according to differences in the gamete morphology and the developmental patterns (Sato and Tsuchiya 1991). Karyotypes of the two forms were compared by using young *Neanthes japonica* individuals (embryos, larvae or juveniles). Sexually-mature worms of the small-egg form were collected when they were swimming as reproductive swarming in the Omoi River (0.1 km upstream from the mouth) (Table 2). Sexually mature worms of the large-egg form were collected from the sediment of the intertidal flat in the Kôtsuki River (1.5 km from the mouth). Larvae or juveniles were obtained by artificial fertilization and rearing of the embryos (Sato and Tsuchiya 1987, 1991). An egg mass, with developing embryos (trochophore stage) of the large-egg form, was found in a burrow of *N. japonica* in the small creek (0.5 km upstream from the mouth) near the port of Ôtsuchi, Iwate Prefecture (Fig. 1). These embryos were also used for karyotype analysis. The embryos, larvae or juveniles were treated for 2 h at room temperature (17 to 20 °C) with 0.05% colchicine dissolved in 50% seawater (salinity: 17‰) for the large-egg form or 70% seawater (salinity: 24‰) for the small-egg form. Then, specimens were placed in 1% sodium citrate for 30 to 40 min and fixed in at least two changes of 3:1 methanol:acetic acid for a total time of more than 30 min.

Slides were prepared according to the modified air-drying technique of Pesch and Pesch (1980), which was adapted from Kliger-

Table 1. *Neanthes japonica*. Materials used for karyological comparison between males and females. More than five metaphase spreads were examined in each individual to determine the diploid chromosome number. Karyotypes of one to four spreads in the best condition were analysed in each individual to determine sex chromosomes

Locality	Sampling date	Tissue used	Sex of individuals	No. of individuals	No. of spreads for karyotype analysis
Kôtsuki River	8 Sep 1987	Regenerating tail	Female	1[a]	4
	30 Aug 1985	Spermatogonia	Male	3	3+3+2
Omoi River	23 Dec 1987	Regenerating tail	Female	3	1+1+1
			Male	3	1+1+1
Yahata River	27 Mar 1985	Spermatogonia	Male	1	4
	4 Jan 1988	Spermatogonia	Male	1	2
Kominato River	13 Dec 1984	Spermatogonia	Male	1	2

[a] The female was identified as the large-egg form by oocyte morphology

Table 2. *Neanthes japonica*. Materials used for karyological comparison between the small-egg and large-egg forms. More than ten metaphase spreads were examined in each brood to determine the diploid chromosome number. For karyotype analysis, chromosomes of one to six spreads in the best condition were measured in each brood. It is unknown how many individuals these spreads are derived from

Form	Locality	Sampling date of mature worms	No. of broods	Young *N. japonica* used	No. of spreads for karyotype analysis
Small-egg form	Omoi River	4 Mar, 18 Mar 1988	2	Trochophore larvae	2+2
Large-egg form	Kôtsuki River	24 Oct 1988	2	Embryos of trochophore stage or 3-setiger juveniles	6+1
	Ôtsuchi	15 June 1988	1	Embryos of trochophore stage	3

man and Bloom (1977). Using a Pasteur pipette, 5 to 50 of the embryos, larvae or juveniles with a small amount of fixative were placed on a clean slide heated to ca. 40 °C on a slide warmer. Immediately several drops of 50% acetic acid were added to cover the specimens. The specimens were smeared 2 to 4 min later by pressing a second slide on top of the first and then drawing the two slides apart horizontally with one smooth continuous movement. By use of this method, cells of the specimens were dissociated well and attached to each slide, which were air-dried and stained in 2% Giemsa solution. Arm length of well-spread metaphase chromosomes was measured on enlarged (×4000 to 5000) photographs with a digitizer. The chromosomal pairs were classified on the basis of the arm ratio (Levan et al. 1964): metacentric chromsomes with an arm ratio range of 1.0 to 1.7, submetacentric chromosomes with an arm ratio range of 1.7 to 3.0, and subtelocentric chromosomes with an arm ratio range of 3.0 to 7.0. The pairs were arranged in descending order of size except for the last pair of sex chromosomes.

Results

Karyotypes from adult males and females

Well-spread metaphase chromosomes were obtained from adults of four demes, using the regenerating tails of three males and four females and clumps of spermatogonia of six additional males (Table 1). A female from the Kôtsuki River could be identified as a large-egg form due to the morphology of its relatively large oocytes (maximum diameter: 170 µm) (see Sato and Tsuchiya 1991). For all other adults, discrimination between the small- and the large-egg forms was impossible.

A diploid ($2n$) chromosome number of 28 was observed in most spreads in all demes. In rare cases (less than 10%), we observed 27 or fewer chromosomes, probably due to artifacts during preparation. Chromsome sets of representative spreads are shown in Figs. 2 and 3. The length of chromosomes ranged from 1.5 to 4.8 µm. The morphology of one pair of chromosomes was different between males and females. The pair was small and monomorphic in females, but heteromorphic in males. These were considered to be heteromorphic sex chromosomes; females had two smaller X chromosomes, and males had a smaller X chromosome and a larger Y chromosome.

Slight differences of karyotypes were observed between demes. In karyotypes from the Omoi deme (Figs. 2a, b), Pairs 1 to 3 were distinctly larger than all other pairs. Pairs 5, 6, 7, 10, 13 and 14 (X, Y) were submetacentric, and all others metacentric. On the other hand, in karyotypes from the Kôtsuki deme including an individual identified as the large-egg form (Fig. 2c), Pairs 1 to 4 were distinctly larger. Pairs 6, 10, 13 and 14 (X, Y) were submetacentric and all others metacentric. A karyotype from the Yahata deme (Fig. 3a) was similar to that from the Kôtsuki deme except that Pair 5 was also submetacentric in the former.

Karyotypes from the young of the two forms

The embryos or larvae in the trochophore stage, or 3-setiger juveniles were used for comparison of karyotypes between the two forms in three demes (Table 2). A diploid chromosome number of 28 was observed in most spreads of both forms (Figs. 4, 5 and 6). In all demes, the sex of each individual was detemined by sex chromosomes. The X chromosome was a small (but not the smallest) submetacentric or metacentric chromosome. On the other hand, the Y chromosome was a relatively large submetacentric or subtelocentric chromosome.

Autosomes were classified into three categories: metacentric, submetacentric and subtelocentric (Tables 3, 4, 5). In the small-egg form collected from the Omoi River

Table 3. *Neanthes japonica*. Characteristics of chromosomes of the small-egg form collected from the Omoi River, Kagoshima. Four well-spread metaphase plates (one from a female and three from males, see Fig. 4) of trochophore larvae were used for measurement. Average length and range (in parentheses) are shown. Arm ratio equals length of long arm divided by length of short arm. m: metacentric; sm: submetacentric

Pair no.	Length of chromosomes (µm)	Arm ratio of chromosomes	Classification of chromosomes
1	5.8 (4.5–7.3)	1.2 (1.0–1.4)	m
2	5.2 (4.1–6.6)	1.3 (1.2–1.4)	m
3	4.8 (3.4–6.0)	1.3 (1.2–1.6)	m
4	4.0 (3.2–5.2)	1.1 (1.0–1.3)	m
5	4.0 (3.2–4.7)	2.1 (1.7–2.4)	sm
6	3.4 (2.6–4.1)	1.6 (1.3–1.8)	m
7	3.3 (2.4–3.9)	1.8 (1.6–1.9)	sm
8	3.2 (2.3–3.8)	1.3 (1.1–1.7)	m
9	3.0 (2.2–3.5)	1.5 (1.3–1.7)	m
10	2.9 (2.1–3.6)	1.9 (1.6–2.2)	sm
11	2.8 (2.1–3.3)	1.3 (1.0–1.5)	m
12	2.7 (2.1–3.2)	1.2 (1.0–1.6)	m
13	2.3 (1.7–2.8)	1.8 (1.5–2.1)	sm
14X	2.8 (2.2–3.4)	1.8 (1.4–2.1)	sm
14Y	4.3 (3.1–5.4)	2.5 (2.0–2.8)	sm

Table 4. *Neanthes japonica*. Characteristics of chromosomes of the large-egg form collected from the Kôtsuki River, Kagoshima. Seven well-spread metaphase plates (five from females and two from males, see Fig. 5) of embryos of trochophore stage or 3-setiger juveniles were used for measurement. Average length and range (in parentheses) are shown. Arm ratio equals length of long arm divided by length of short arm. m: metacentric; sm: submetacentric; st: subtelocentric

Pair no.	Length of chromosomes (µm)	Arm ratio of chromosomes	Classification of chromosomes
1	5.2 (4.5–6.7)	1.1 (1.0–1.1)	m
2	4.8 (4.0–5.8)	1.2 (1.0–1.3)	m
3	4.6 (3.9–5.7)	1.3 (1.1–1.4)	m
4	4.1 (3.5–4.5)	1.2 (1.0–1.4)	m
5	3.5 (2.9–4.0)	1.4 (1.1–1.7)	m
6	3.2 (2.9–3.5)	2.3 (1.7–3.1)	sm
7	3.0 (2.6–3.4)	1.6 (1.2–1.9)	m
8	2.9 (2.5–3.6)	1.2 (1.1–1.3)	m
9	2.8 (2.4–3.3)	1.5 (1.3–1.7)	m
10	2.7 (2.1–3.4)	2.2 (1.7–2.6)	sm
11	2.5 (2.1–2.9)	1.2 (1.0–1.5)	m
12	2.3 (2.0–2.8)	1.2 (1.1–1.3)	m
13	1.9 (1.5–2.5)	2.1 (1.6–2.6)	sm
14X	2.3 (2.1–2.7)	1.8 (1.5–1.9)	sm
14Y	3.6 (2.5–4.7)	3.2 (1.8–4.6)	st

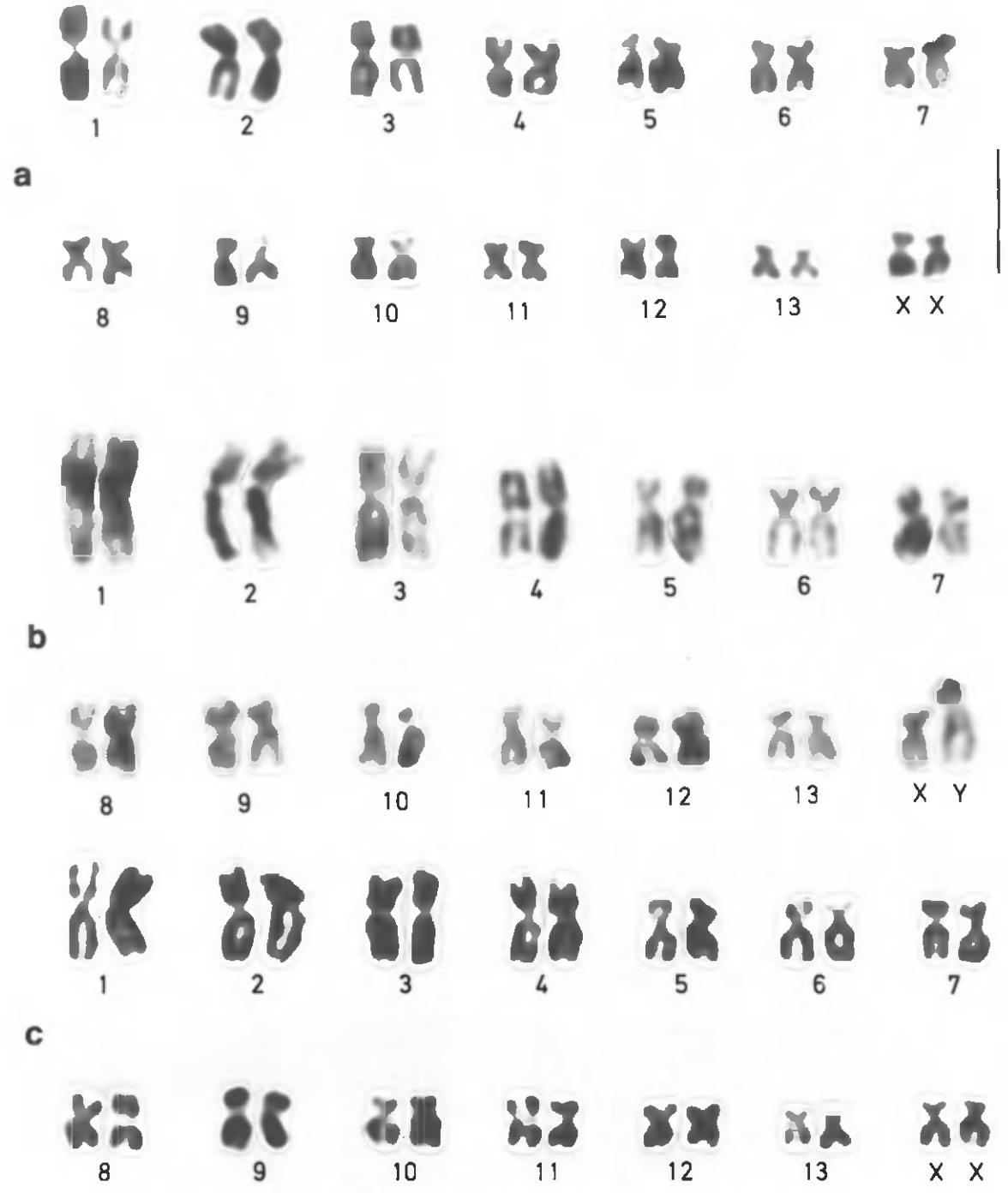

Fig. 2. *Neanthes japonica*. Karyotypes of females (a, c) and a male (b). Mitotic metaphase chromosomes were obtained from regenerating tails of adults collected from the Omoi River (a, b), and the Kôtsuki River (c), Kagoshima. The female from the Kôtsuki River (c) was identified as the large-egg form by oocyte morphology. Scale bar: 5 μm

(Figs. 4, 7a), the first three metacentric pairs (Pairs 1 to 3) were distinctly larger. All other pairs of autosomes descended in length little by little. Pair 5, which was as long as Pair 4, was consistently submetacentric, and Pairs 7, 10 and 13 were also submetacentric judging from their average arm ratios (Table 3).

In the large-egg form collected from the Kôtsuki River (Figs. 5,7b) and from Ôtsuchi (Figs. 6, 7c), the first four metacentric pairs (Pairs 1 to 4) were distinctly larger. Pairs 6 and 10 were consistently submetacentric or subtelocentric, and Pair 13 was also submetacentric judging from its average arm ratio (Tables 4, 5).

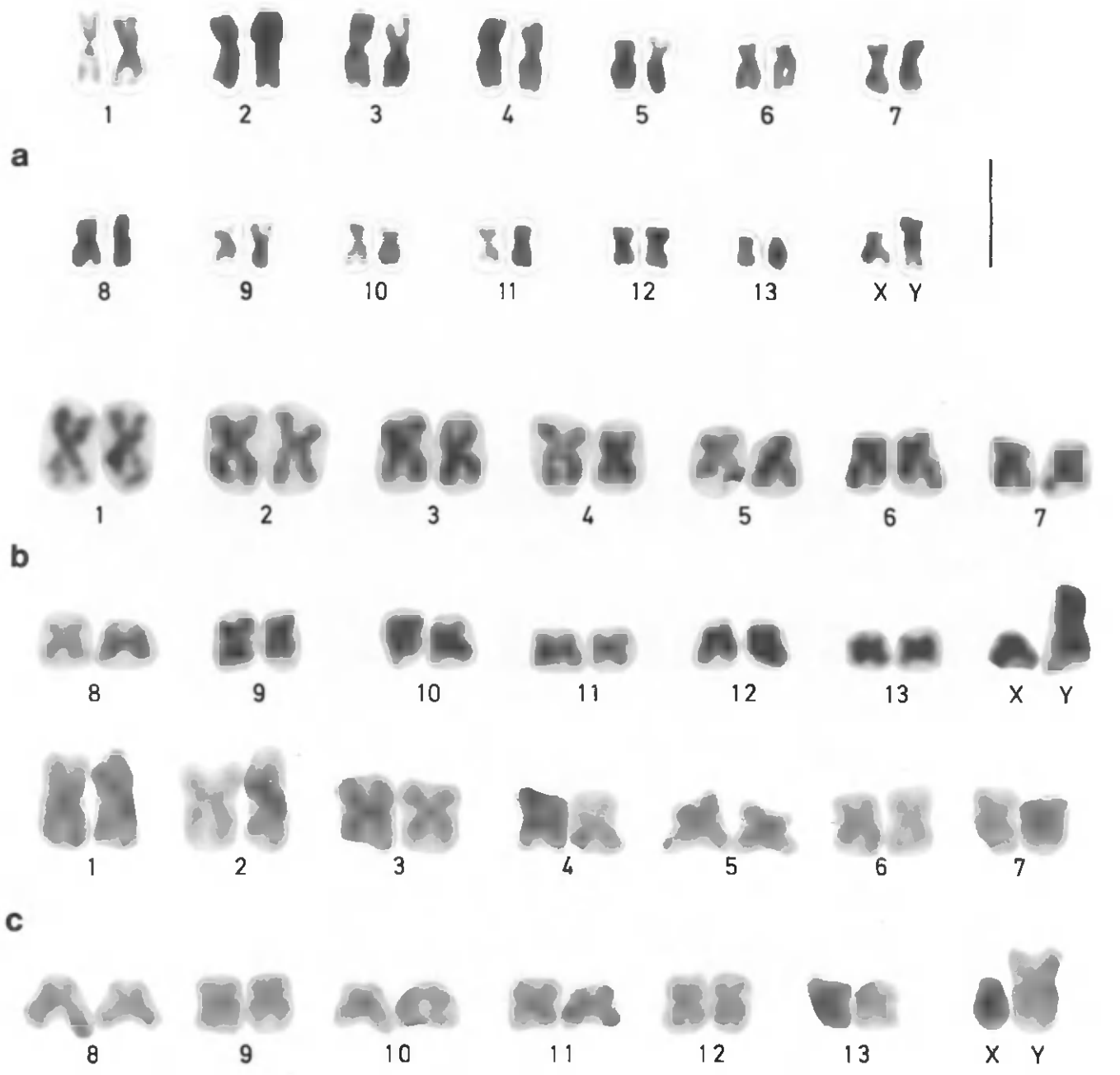

Fig. 3. *Neanthes japonica*. Karyotypes of males collected from the Yahata River, Hiroshima (a), the Kôtsuki River, Kagoshima (b), and the Kominato River, Aomori (c). Mitotic metaphase chromosomes were obtained from spermatogonia. Scale bar: 5 μm

Discussion

Chromosomal sex determination

This study is the first to document heteromorphic sex chromosomes in polychaetes. Sex in *Neanthes japonica* seems to be determined by a simple system of male heterogamety, i.e., male genotype is XY, while female genotype is XX. According to this system, simple outcrossing should produce a 1:1 sex ratio. We observed sex ratios of 20 females:24 males on 19 February 1988, and of 41 females:43 males on 1 March 1991, by random sampling of the small-egg form at the peak of reproductive swarming after evening high tide in the Omoi River.

Male heterogamety is prevalent in some groups of animals, such as nematodes, arachnids, most insects and mammals, where the X chromosome is usually longer than the Y (Bull 1983). In *Neanthes japonica*, however, the Y is longer than the X. Similar cases are known in angiosperms (flowering plants) (Westergaard 1958).

Annelids seem to have various mechanisms for sex determination. Aside from our finding for *Neanthes japonica*, *Dinophilus gyrocilatus* (Archiannelida) is the only case where the existence of sex chromosomes has

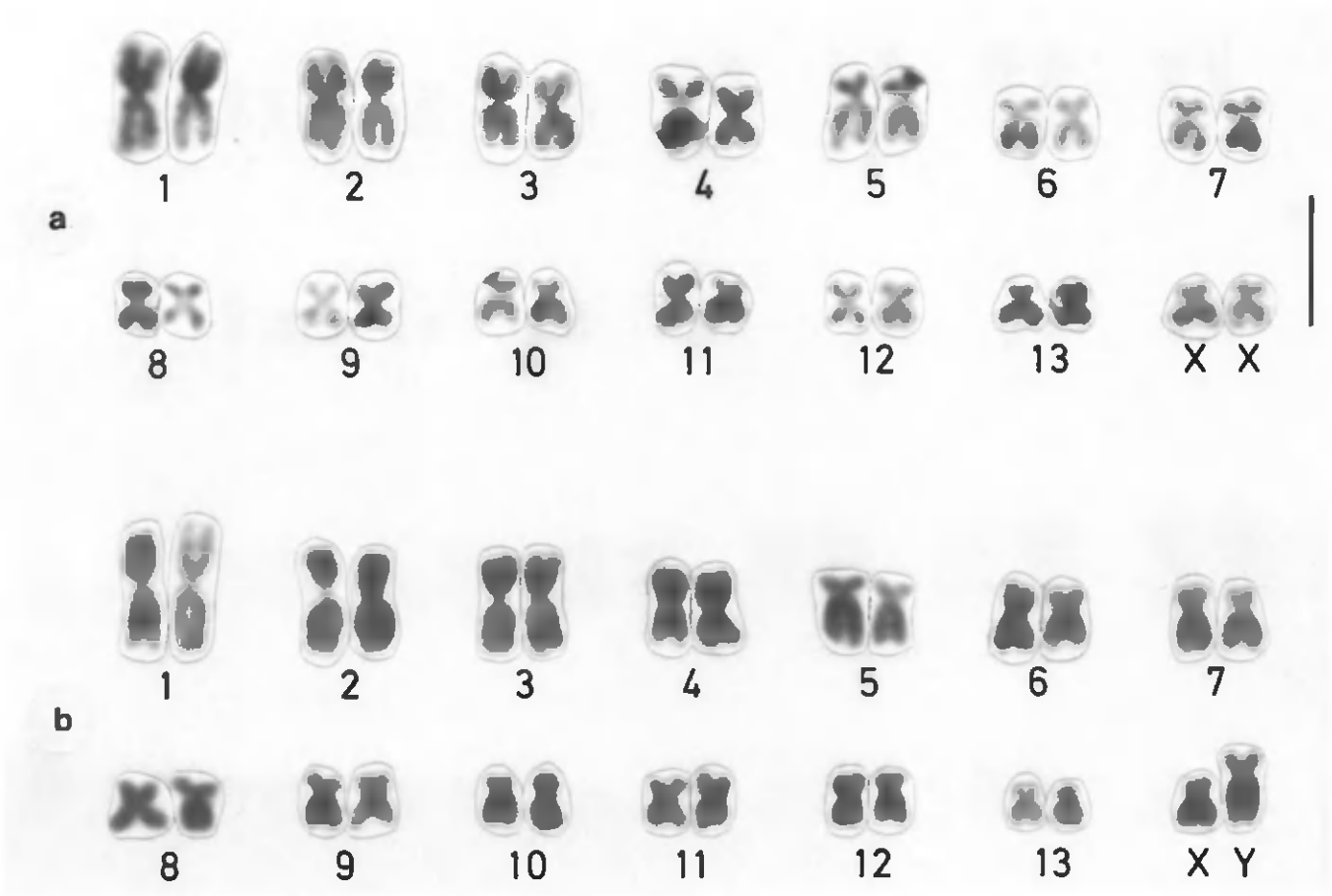

Fig. 4. *Neanthes japonica*. Karyotypes of the small-egg form collected from the Omoi River, Kagoshima. Mitotic metaphase chromosomes of a female (a) and a male (b) were obtained from trochophore larvae. Scale bar: 5 μm

Table 5. *Neanthes japonica*. Characteristics of chromosomes of the large-egg form collected from a small creek in Ôtsuchi, Iwate. Three well-spread metaphase plates (two from females and one from a male, see Fig. 6) of embryos in the trochophore stage were used for measurement. Average length and range (in parentheses) are shown except for 14Y, for which a single data is shown. Arm ratio equals length of long arm divided by length of short arm. m: metacentric; sm: submetacentric

Pair no.	Length of chromosomes (μm)	Arm ratio of chromosomes	Classification of chromosomes
1	8.0 (6.5–9.3)	1.1 (1.0–1.2)	m
2	7.0 (6.0–8.6)	1.2 (1.2–1.3)	m
3	6.7 (5.7–8.0)	1.2 (1.2–1.2)	m
4	6.1 (5.0–7.0)	1.2 (1.1–1.2)	m
5	5.0 (4.2–6.0)	1.6 (1.5–1.8)	m
6	4.7 (4.1–5.6)	2.0 (1.9–2.0)	sm
7	4.4 (4.0–5.1)	1.5 (1.5–1.5)	m
8	4.3 (3.9–5.0)	1.2 (1.1–1.3)	m
9	4.1 (3.4–4.7)	1.3 (1.1–1.6)	m
10	4.1 (3.5–4.7)	2.3 (2.0–2.9)	sm
11	3.7 (3.3–4.3)	1.1 (1.1–1.2)	m
12	3.6 (3.1–4.3)	1.2 (1.0–1.4)	m
13	3.0 (2.7–3.3)	2.0 (1.5–2.3)	sm
14X	3.8 (3.4–4.4)	1.5 (1.5–1.6)	m
14Y	4.4	1.9	sm

been considered. In this case, it was suggested that male heterogamety of the XX-XO type was included in a complex system for sex determination, though karyotypes of both sexes were not shown (Martin and Tràut 1987). Polyfactorial sex determination has been indicated in the dorvilleid *Ophryotrocha* spp., the syllid *Brania clavata* and *Bonellia viridis* (Echiuroidea) (reviewed by Schroeder and Hermans 1975, Pfannenstiel 1984). In the syllid *Typosyllis prolifera*, which is a partially protogynous hermaphrodite, sex at the first sexual phase was determined by homo-heterogamety in a local population, while determined by polyfactorial mechanisms in two other populations (Franke 1986). Petraitis (1985) experimentally showed sex determination by female heterogamety (i.e., males and hermaphrodites have a genotype of ZZ, while females have ZW) in crosses of the capitellid *Capitella capitata* (species type I). However, karyological analysis of this species (Grassle et al. 1987) did not demonstrate heteromorphic sex chromosomes. No heteromorphic sex chromosome was found in karyotype analyses of spermatocytes, oocytes or cleaving eggs in the serpulid *Hydroides elegans* (Vitturi et al. 1984). On the other hand, Pesch and Pesch (1980) found two types of a certain pair of chromosomes (i.e., a monomorphic pair of the two smallest telocentric chromosomes and a

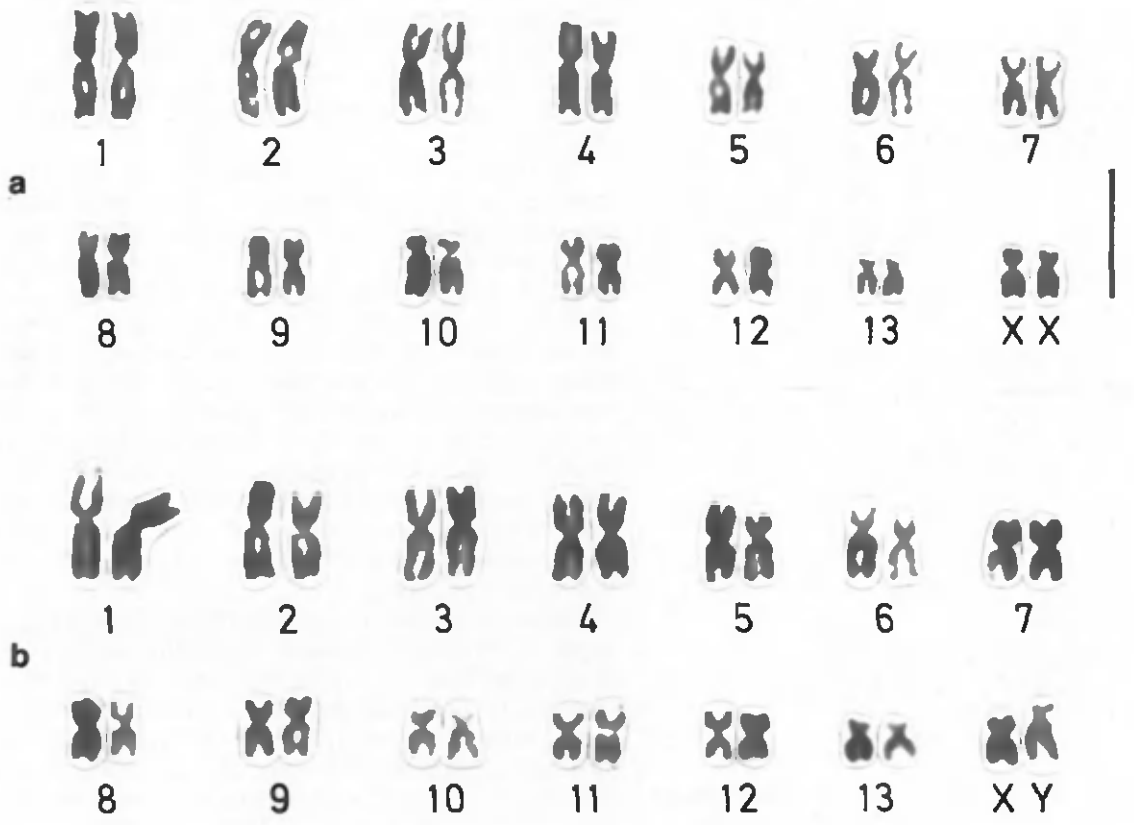

Fig. 5. *Neanthes japonica*. Karyotypes of the large-egg form collected from the Kôtsuki River, Kagoshima. Mitotic metaphase chromosomes of a female (a) and a male (b) were obtained from embryos of trochophore stage. Scale bar: 5 μm

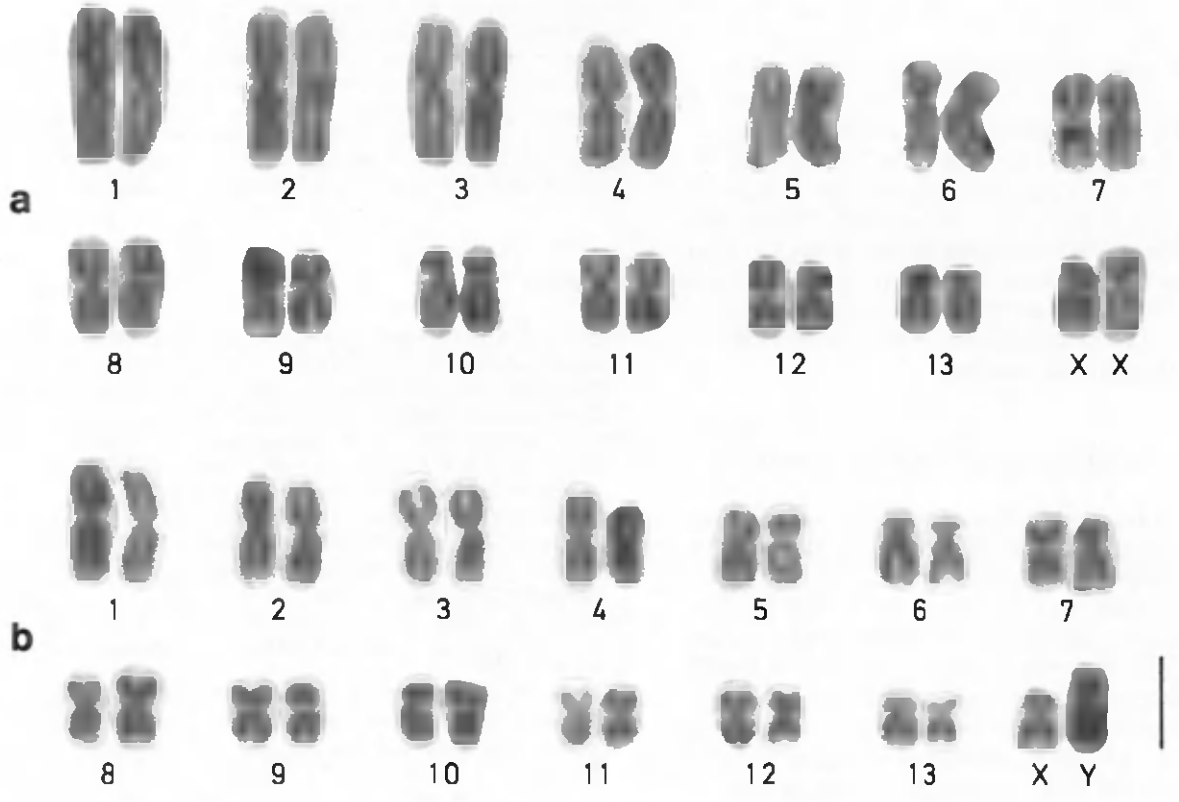

Fig. 6. *Neanthes japonica*. Karyotypes of the large-egg form collected from a small creek in Ôtsuchi, Iwate. Mitotic metaphase chromosomes of a female (a) and a male (b) were obtained from embryos of trochophore stage. Scale bar: 5 μm

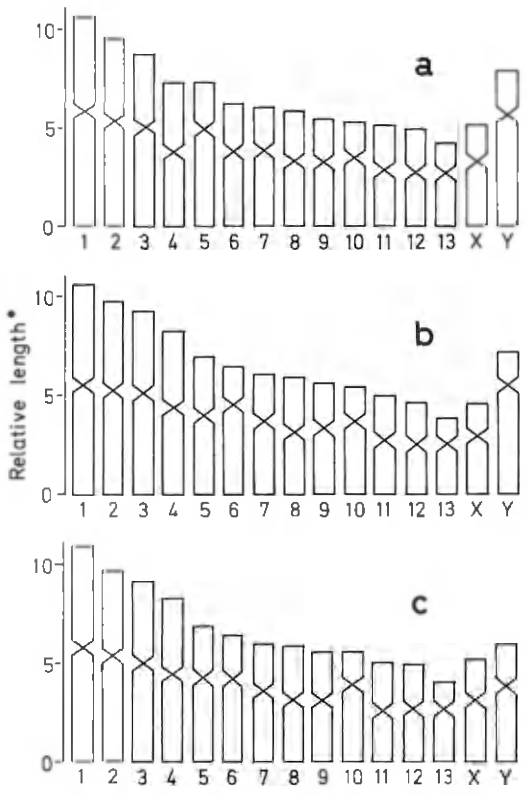

Fig. 7. *Neanthes japonica*. Idiograms drawn from the data in Tables 3, 4 and 5. (a) The small-egg form collected from the Omoi River, Kagoshima. (b) The large-egg form collected from the Kôtsuki River, Kagoshima. (c) The large-egg form collected from Ôtsuchi, Iwate. Relative length *: 100 × length of chromosome/[total length of the autosomes (haploid) + lengths of both X and Y chromosomes]

heteromorphic pair of the smallest telocentric chromosome and a larger subtelocentric one) in mitotic cells of larvae of the nereidid *Neanthes arenaceodentata*. Our results suggest that the chromosomes of this pair of *N. arenaceodentata* are sex chromosomes similar to those of *N. japonica*. Once extreme sex chromosome differences evolve, the evolution of new sex-determining mechanisms may be prevented, and species descended from a common ancestor will share the same sex chromosome system (Bull 1983). The heteromorphic sex chromosomes may be conserved in nereidid polychaetes.

Karyotypes of the two forms of "*Neanthes japonica*"

The small-egg form and the large-egg form are identified as *Neanthes japonica* according to the key of Imajima (1972), but differ in reproductive and developmental characteristics (Sato and Tsuchiya 1987, 1991). Both forms had the same diploid chromosome number of 28, which is common in nereidid species (Christensen 1980). Karyotypes of the two forms were considerably similar to each other. However, slight differences between them were demonstrated in the autosomes of young specimens. The small-egg form had autosomes characterized by three distinctly larger chromosome pairs and by four submetacentric pairs (Pairs 5, 7, 10 and 13), while the large-egg form had autosomes characterized by four distinctly larger pairs and by three submetacentric pairs (Pairs 6, 10 and 13). As for the large-egg form of the Kôtsuki deme, the karyotypic characteristics of embryos and juveniles (Fig. 5) were in accord with those of an adult worm (Fig. 2c).

Our results may add evidence to show that the two forms are in fact two reproductively isolated sibling species. Judging from the karyotypic differences between the two forms, a female and a male from the Omoi River (Figs. 2a, b) were presumed to be of the small-egg form, while a male from the Yahata River (Fig. 3a) was presumed to be of the large-egg form. Karyological differences involving different chromosome numbers have been demonstrated in such sibling species as the *Ophryotrocha* species group (Dorvilleidae) (Åkesson 1984, Robotti et al. 1991), *Capitella*, *Capitellides* and *Capitomastus* species groups (Capitellidae) (Grassle et al. 1987) and "*Neanthes arenaceodentata*" (= "*Nereis acuminata*") species group (Nereididae) (Pesch et al. 1988, Weinberg et al. 1990).

However, the karyotypic differences between the two forms of "*Neanthes japonica*" are slight, and not sufficiently elucidated by simple chromosomal morphology. Identification and numbering of chromosomal pairs are rather difficult because of the fact that many of the chromosomes are similar to one another in size and shape in both forms and that values of arm ratio are variable by several factors (Bentzer et al. 1971). The differences between the two forms should be proved more clearly in a further study, e.g. by means of banding techniques.

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