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Morphology and Meiotic/Mitotic Behavior of B Cromosomes in a Japanese Harvestman, *Metagagrella tenuipes* (Arachnida: Opiliones): No Evidence for B Accumulation Mechanisms

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ABSTRACT—Earlier, it has been demonstrated that wild populations of a Japanese harvestman *Metagagrella tenuipes* (Arachnida: Opiliones) are polymorphic for B chromosomes. In this paper, we present results of a study of the morphology and mitotic and meiotic behavior of the Bs. The B chromosomes varied considerably in size and proportion of eu- and heterochromatin. The single nucleolus organizing region, found in males, was located on a chromosome of the A complement. Some intercell variation in number of Bs may be explained by accidental chromosome losses during chromosome preparation. We also found no intertissue variation in number of Bs. There were also no differences in mean number of B chromosomes per individual among males and females, adult and subadult harvestmen. Segregation of Bs in mitotic and meiotic divisions was nonrandom; B chromosomes tended to segregate equally between daughter cells. The results obtained provide no support for the hypothesis of existence of B accumulation mechanism in this species.

INTRODUCTION

Supernumerary B chromosomes are extra chromosomes which occur in many animal and plant species (Jones and Rees, 1982). Polymorphism for the B chromosomes usually remain stable over several generations (Nur, 1969; Hewitt, 1976; López-León *et al.*, 1992). Two principal hypotheses were suggested to explain retention of B chromosomes: "selfish" and "heterotic". The selfish hypothesis is based on two types of observations: i) Phenotypic effects of Bs are usually deleterious (for review, see Jones and Rees, 1982); ii) In many species there are B accumulation mechanisms leading to more-than-expected transmission of Bs (Nur, 1969; Romera *et al.*, 1991; Hewitt, 1976, Jones, 1991; Werren, 1991). According to the selfish hypothesis the frequency of Bs in the population depends on the equilibrium between the accumulation of Bs by more-than-random transmission and elimination of Bs by natural selection (Hewitt *et al.*, 1987; Jones, 1991). On the other hand, the heterotic hypothesis suggests that Bs confer some selective advantage to their carriers (White, 1973). It has been demonstrated that in some species selection in stressful environments may favor individuals with Bs (Hewitt and Rescoe, 1971; Holmes and Bougourd, 1989; Plowman and Bougourd, 1994). To accept one of the explanations it is crucial to know whether a B accumulation mechanism exists in the species.

Earlier, Tsurusaki (1993) showed that wild populations of a Japanese harvestman *Metagagrella tenuipes* (L. Koch) (Arachnida, Opiliones, Phalangiidae, Gagrellinae) are highly polymorphic for B chromosomes. To understand mechanisms which sustain the B chromosomes in the wild population of *M. tenuipes* it is very important to determine if this species has a B accumulation mechanism. The present paper describes the results of a cytological study of morphology and mitotic-meiotic behavior of B chromosomes in *M. tenuipes*.

MATERIALS AND METHODS

All specimens analyzed were collected in 1997 and 1998 from a site located on the coast of the Sea of Japan (35°31'N, 134°02'E), near Yatsukami Tunnel (Route 9), 30 km to the west of Tottori City (Tottori Prefecture, western Honshu, Japan).

In males, the chromosome complement was analyzed in cells of the following four types: spermatogonial mitosis, metaphase of first meiotic division (MI), metaphase of second meiotic division (MII) and mitotic metaphase cells of gut epithelium. In females, only cells of oogonial mitosis and cells of gut epithelium were analyzed. To estimate the number of B chromosomes we scored at least 15 cells from each individual.

For chromosome preparation of testes we used the air-drying technique with 30% acetic acid treatment for cell dissociation (Dietrich and Mulder, 1981) and general hypotonic treatment described earlier (Tsurusaki, 1985; Tsurusaki and Cokendolpher, 1990). For the preparation of chromosomes from the gut epithelium the following modifications were used: i) After dissection of a specimen under a stereomicroscope, the gut was removed from the body and rinsed three times in hypotonic solution (1% Na citrate); ii) 25% acetic acid was used to dissociate cells; iii) Cell suspension was rinsed three times with methanol/acetic acid (3:1 solution), by using re-suspen-

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sion and re-centrifugation. To stain nucleolus organizing regions (NOR) we used the method described by Howell and Black (1980). A total of 531 harvestmen were karyotyped.

Statistical analyses were carried out with JMP (Ver. 3.15, SAS Institute, 1995). Nonparametric Kruskal-Wallis test was performed to compare the number of B chromosomes in different sex-age groups of harvestmen. Comparisons between observed and expected distributions were made using chi-square test.

RESULTS

Morphology of B chromosomes

B chromosomes in *M. tenuipes* vary considerably in size and amount of heterochromatin. Figure 1 represents the morphology of B chromosomes in some representative karyotypes. At prometaphase Bs were usually shorter, and more condensed as compared to chromosomes of the A complement. At the late metaphase, however, when both A and B chromosomes undergo strong condensation, the differences between A and B chromosomes decrease, so it is sometimes difficult to distinguish them by conventional staining.

Bs were almost completely heterochromatic, although some of them carried euchromatic segments located on terminal regions or (sometimes) in the middle of a chromosome (Fig. 1a). In contrast to Bs, most A chromosomes did not carry heterochromatin except minute blocks located near centromeres (Fig. 1b).

It has been shown that in some species B chromosomes carry nucleolus organizing regions (NOR) (Powell and Burton, 1966; Kirk and Jones, 1970; Mabuchi, 1991; Beukeboom, 1994). Taking into account that up to date there is no information on number and localization of nucleolus organizing regions in harvestmen, we analyzed NORs in *M. tenuipes*. Males exhibiting high mitotic index were used for the analysis. In the males studied a single NOR-carrying A chromosome was revealed. We never observed NORs located on B chromosomes (Fig. 2). There is a possibility that the NOR may be on a sex chromosome. To test this suggestion we tried to stain NORs in females; but, unfortunately, we failed to obtain slides with good staining of nucleolus organizing regions in females.

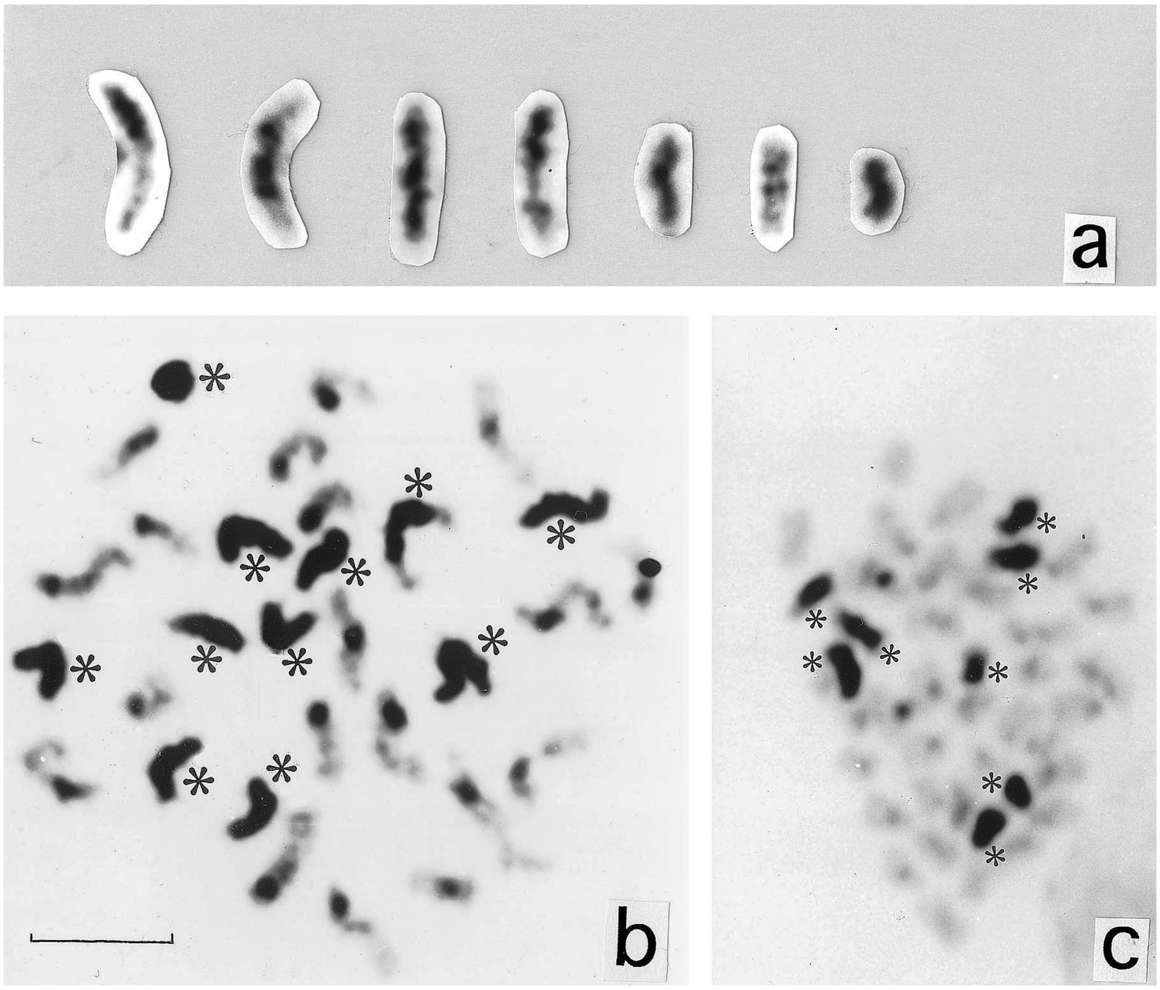

Fig. 1. Chromosomes of *Metagagrella tenuipes* from the Yatsukami population. a) Some variants of B chromosomes. Note differences in size and proportion of euchromatin. b) A spermatogonial metaphase in a male with 2n=29=18A+11Bs. c) A tetraploid cell from the gut epithelium of a male with 4 Bs in diploid cells. B chromosomes are asterisked in 1b–c. Scale = 5 μm.

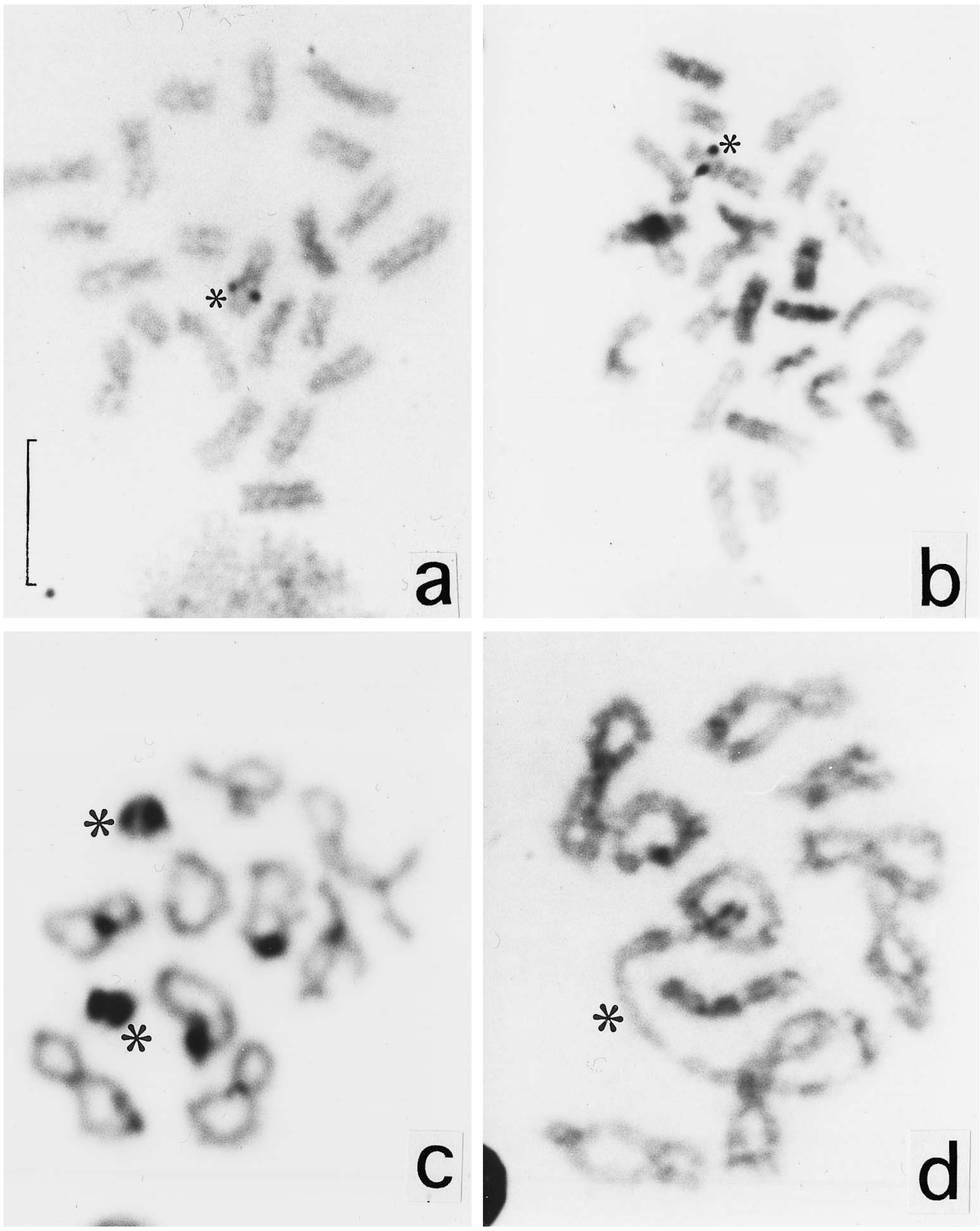

Fig. 2. Chromosomes of male *M. tenuipes* in mitotis (a-b) and meiosis (c-d). a-b) Mitotic metaphases (2n=22 and 25, respectively) after staining for nucleolus organizing regions (NORs). The single NOR carrying chromosome is marked by an asterisk. Note that all the Bs lack NORs. c) Meiotic chromosomes at MI in a male with 2n=20=18A+2Bs. Bs are revealed as univalents (asterisked). d) B chromosomes form a chain (asterisked) with end-to-end association between Bs in a male with 2n=21. Scale = 5 µm.

Inter-group and inter-tissue variation in numbers of B chromosomes

Data on the numbers of B chromosomes in harvestmen studied are shown in Table 1. We grouped all harvestmen studied according to the date of collection (from June to November) and number of B chromosomes from zero to twelve. An inspection of the data presented in Table 1 shows that individuals carrying 5–7 B chromosomes are most frequent and the frequencies of harvestmen with extremely high (11–12), or extremely low (0–2) number of Bs are very low. For example, the frequency of harvestmen without Bs was less than 0.004%, the same frequency has been found for harvesmen with 12 Bs in karyotype. Table 2 summarizes data on mean numbers of B chromosomes in four age-sex groups: immature males and females and adult males and females. Nonparametric Kruskal-Wallis test revealed no differences among the groups in number of Bs per individual (h=3.45, df=3, p=0.33).

It has been shown in some species that directed nondisjunction of B chromosomes at early stages of ontogenesis may result in accumulation of Bs in some tissues and exclusion from others (Hayman *et al.*, 1969; Hewitt, 1976; Mendelson and Zohary, 1972; Romera *et al.* 1989). To test if such an inter-tissue variation in number of B chromosomes exists in *M. tenuipes*, we scored Bs in cells of gut epithelium. We found that cells of gut epithelium were mostly tetraploid

Table 1. Number of *Metagagrella tenuipes* from the Yatsukami population with different number of B chromosomes in 5 (July to November) and 6 (June to November) succesive months in 1997 and 1998, respectively.

Date of collection	No. indiv*	Number of B chromosomes														
		0	1	2	3	4	5	6	7	8	9	10	11	12	mean	SD
July 97	85 (37m3f45j)			1	2	9	9	21	11	22	2	6	2		6.68	1.90
August 97	52 (m)			1	4	8	10	7	12	8		1	1		5.90	1.87
September 97	60 (m)		1	1	8	6	12	9	14	3	2	1	2	1	5.82	2.23
October 97	58 (m)			2	6	8	15	5	13	6	1	1		1	5.67	1.99
November 97	83 (m)	1		2	3	8	18	14	18	7	10		2		6.18	2.00
June 98	34 (j)	1		4	2	4	4	10	4	5					5.24	2.08
July 98	31 (10m21j)			1	3	8	3	3	6	4	2	1	1		5.91	2.26
August 98	10 (m)		1	1	1	2	3			1		1			4.70	2.67
September 98	54 (m)		1	3	1	6	7	9	18	6	2	1			6.02	1.89
October 98	25 (m)			2		4	3	7	6	2	1				5.76	1.74
November 98	63 (m)				1	6	7	8	7	4	3	1	1		6.29	1.86
Total	531	2	3	18	31	69	91	93	109	68	23	13	9	2	6.00	2.03

*m= males, f=females, j=juveniles.

Table 2. Mean number of B chromosomes in four sex-age groups of *M. tenuipes* from the Yatsukami population. Data for 1997 and 1998 were pooled as there was no difference in number of Bs per individuals between the years. Most of the juveniles were subadults. There was no difference in number of Bs between the four groups (Kruskal-Wallis test, P=0.33).

Sex-age group	N	Mean number of Bs	SE
female juveniles	42	5.71	0.35
female adults	3	6.67	1.33
male juveniles	59	6.20	0.30
male adults	427	6.00	0.09

(Fig. 1c); the number of Bs in cells of gut epithelium was two times as high as that in spermatogonial mitosis.

It has been shown in some species that Bs are accumulated in germ line and this leads to an increase in the number of B chromosomes in meiotic cells as compared to precedent mitotic ones (Rutishauser and Röthlisberger, 1966; Nur, 1969). To determine if such a process exists in *M. tenuipes* we assessed mean numbers of Bs at three successive stages of spermatogenesis: spermatogonial mitosis, MI, and MII. Numbers of Bs at these three stages in three representative karyotypes are shown in Table 3. Inspection of the data shows that the mean number of Bs per cell is quite stable trough all stages in all individuals analyzed. Some intercell variation in the mean number of B chromosomes may be attributed to accidental losses of chromosomes during slide preparation (see below).

We succeeded in obtaining chromosome spreads of good quality both from oogonial mitosis and from tetraploid cells of gut epithelium only for two females. In both females studied, the number of Bs in oogonial mitosis was half of that in cells of gut epithelium. Therefore, in females we also have found no differences in the number of B chromosomes between mitotic and meiotic cells.

Intercell variation in number of Bs

Intercell variation in the number of B chromosomes has been found in several species of grasshoppers (Hayman *et al.*, 1969; Mendelson and Zohary, 1972). This variation is believed to be a result of nondisjunction of Bs at early stages of ontogenesis (Nur, 1969; Hewitt, 1976, Romera *et al.*, 1991; Werren, 1991). In *M. tenuipes* we also found some intercell variation in number of Bs. As an example let's consider specimen No. 39 collected on 2 July 1997. We could unambiguously distinguish B chromosomes from A chromosomes in this individual because all the Bs carried large heterochromatic blocks, whereas A chromosomes were almost completely euchromatic. Among 50 spermatogonial mitoses analyzed we found: 39 cells containing 8 Bs, 9 cells containing 7 Bs and 2 cells containing 6 B chromosomes. Involvement of one of the following two factors is conceivable for this observed intercell variation: i) Specific nondisjunction of B chromosomes; ii) Nonspecific losses of B chromosomes during chromosome preparation. If first hypothesis is true, then the intercell variation in chromosome numbers should be found mainly among Bs but not among A chromosomes. If the variation in number

Table 3. Number of B chromosomes in three successive stages of spermatogenesis in three representative individuals collected in 1997.

Indiv. No.	Date collected	Spermatogonial metaphase		Meiotic metaphase I		Meiotic metaphase II	
		range	mean/mode	range	mean/mode	range	mean/mode
18	14 Jul.	3–5	4.8/5	1–5	4.4/5	1–4	2.3/2,3
7	25 Aug.	2–7	6.5/7	2–7	6.6/7	2–5	3.3/3,4
4	3 Nov.	1–7	5.6/6	2–6	5.4/6	1–5	2.8/3

of Bs is an artifact resulting from nonspecific losses of chromosomes during preparation, then both A and B chromosomes should have the same rate of chromosome losses and exhibit a similar level of intercell variation in the numbers of chromosomes.

Because the segregation of A chromosomes should be regular, intercell variation in the number of A chromosomes can be completely attributed to the accidental losses of chromosomes during preparation of chromosomes. This allows to assess the rate of accidental losses of chromosomes during preparation of chromosomes. Among 50 spermatogonial mitoses analyzed we found that 35 nuclei contained 18 A chromosomes, 11 with 17 A chromosomes, 3 nuclei with 16 and 1 with 15 chromosomes. The probability of a chromosome loss may be calculated as follows: fifty cells should contain 50×18=900 A chromosomes, however we found 880 chromosomes, so the probability of accidental chromosome loss is 20/900=0.022. Assuming the same probability of accidental losses for B chromosomes and using Poisson distribution, we can calculate the expected number of cells with 8 (no chromosome losses), 7 (one chromosome was lost), and 6 B chromosomes (two Bs were lost). We found that the observed distribution of cells doesn't differ from the distribution expected if the probability of loss of B chromosomes is the same as the probability of loss of A chromosomes (Fig. 3; chi square =1.62, df=2, p>0.05). Therefore, our results do not support the

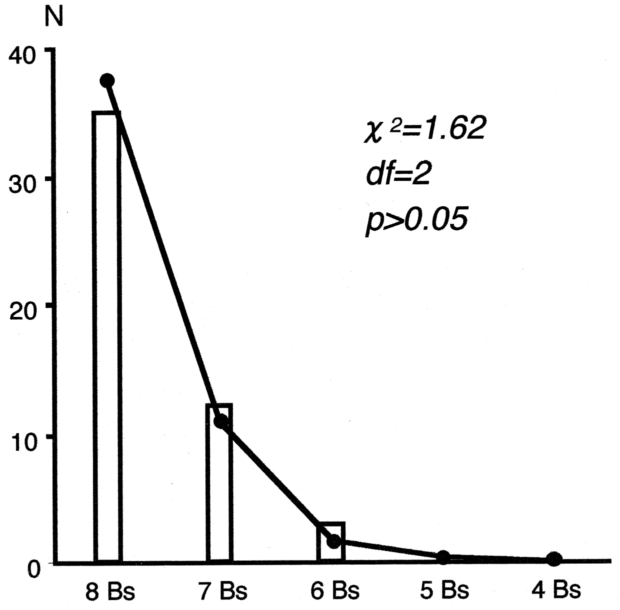

Fig. 3. The observed numbers of spermatogonial cells with different numbers of B chromosomes in the harvestmen with the mode of Bs equals to eight (bars). The number of cells expected if Bs are lost accidentally with the same frequency as A chromosomes (lines). The difference between the two distributions was insignificant (χ^2-test, p>0.05).

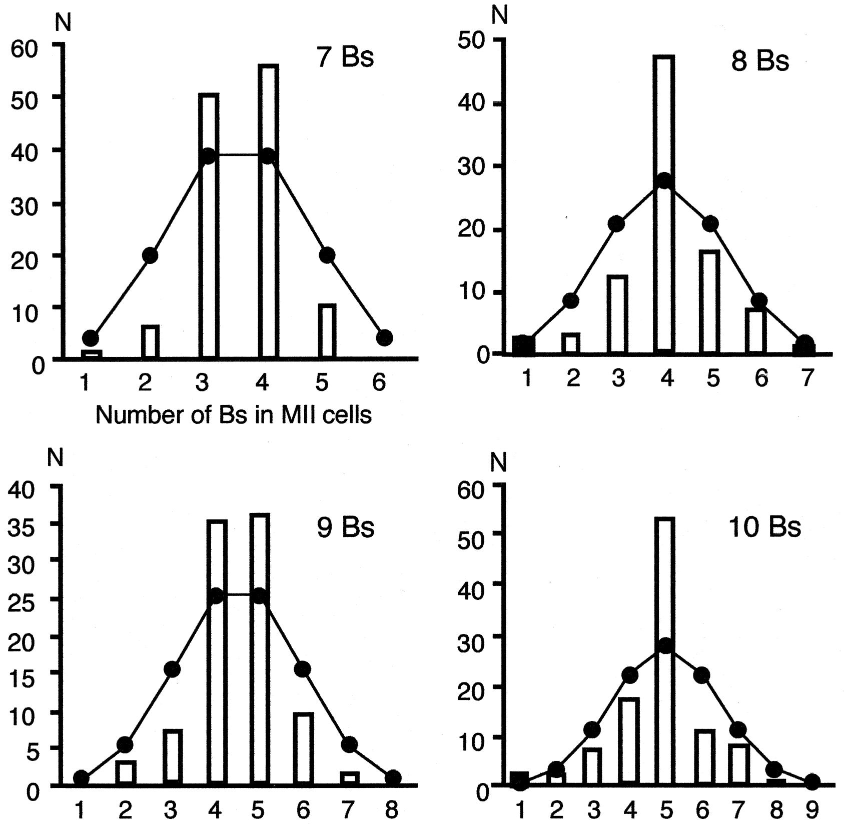

Fig. 4. The distribution of MII cells grouped by the number of B chromosomes in the first meiotic division. The differences between the expected under the condition of random segregation of Bs (lines) and observed (bars) numbers of Bs were significant in all cases.

hypothesis of nondisjunction of Bs in spermatogonial mitosis. It is most likely that the observed intercell variation in spermatogonial mitosis of *M. tenuipes* is an artifact of chromosome preparation.

Segregation of Bs in the first meiotic division

Comparison of the observed distribution of Bs among MII, with the distribution expected under hypothesis of random segregation of Bs, demonstrated that disjunction of B chromosomes in the first meiotic division is nonrandom (Fig. 4). If Bs segregate at random, it would result in binominal distribution of MII according to the number of Bs with the mean being equal to half of the number of Bs in the spermatogonial mitosis. Figure 4 represents expected and observed distributions of MII. For all the individuals observed the distribution of MII differed from the expected. In all the cases, the frequency of MII with the mean number of B chromosomes was higher than the expected frequencies of MII with low or high numbers of B. These differences were statistically significant (chi square test, $p<0.05$).

DISCUSSION

In *M. tenuipes*, behavior of Bs in mitosis and meiosis resembles that of A chromosomes in some respects. Number of Bs remains stable through the three successive stages of spermatogenesis. Some intercell variation in number of Bs is observed and may be explained rather by accidental chromosome losses during chromosome preparation than by abnormal segregation. Coincidence of the number of B chromosomes in different cell types (mitotic, meiotic cells and cells of gut epithelium) demonstrated that segregation of Bs in *M. tenuipes* is regular from the very beginning of development when spermatogonial and gut epithelium cell lines are separated.

Although our data on numbers and behavior of Bs in females of *M. tenuipes* are more limited than for males, the results obtained allowed us to conclude that behavior and transmission of Bs in cell divisions of females is also regular. We found no differences in mean number of Bs per individual between males and females. It was also found that the level of intercell variation in number of Bs in females is similar to that in males (data are not shown), besides, in females there are no differences in number of B chromosomes in oogonial mitosis and in cells of gut epithelium (in their tetraploid condition). In other words, we found no evidence for nondisjunction or B accumulation mechanism in either male or females *M. tenuipes*.

It is known that progressive accumulation of Bs during ontogenesis may lead to differences in the numbers of B chromosomes among different stages of ontogenesis (Rutishauser and Röthlisberger, 1966; Hayman *et al.*, 1969). In *M. tenuipes*, however, we found no differences between immature (mostly subadult) and adult harvestmen in the number of B chromosomes.

Imai (1974) found that in the ant *Leptothorax spinisior* the presence of B chromosomes is restricted to the germ cells in males and Bs are practically absent in females. Similar associations between Bs and a particular sex have also been found in other animals (Jackson and Cheung, 1967; Hayman *et al.*, 1969). In *M. tenuipes*, we found no differences between males and females in the number of B chromosomes. This observation also supports the conclusion that there is no nonsegregation of Bs in both males and females. In summary, our observations do not provide any support for the existence of Bs accumulation mechanism in *M. tenuipes*.

Note that at first meiotic division, B chromosomes segregate more regularly than expected if segregation is random. Bs tend to segregate equally in the first meiotic division. The search for the mechanism of such a nonrandom distribution of Bs was not the goal of this study and therefore was not studied further. Segregation of chromosomes is complex and multistep processes with many factors can influence it (Wells, 1996). It has been demonstrated that tension of microtubes attached to the kinetochore is essential for segregation (Buss and Henderson, 1971). In the absence of the tension, cell division is delayed (Li and Nicklas, 1995). In order to complete segregation of chromosomes, it is conceivable that B-carrying cells must provide some type of association between B chromosomes. Such a pairing would allow to complete cell division and promote equal distribution of B chromosomes between daughter cells. It is interesting to note that in some diakinetic cells in *M. tenuipes* we indeed found bivalent-like and multivalent-like associations between B chromosomes (see Fig. 2d).

In females of the grasshopper *Myrmeleotettix maculatus*, it has been demonstrated that B accumulation occurs in the first meiotic division through preferential transmission of B chromosomes to the daughter cells that are destined to become eggs (Hewitt, 1976). In maize preferential fertilization of eggs by B-carrying male gametes seems to be the main factor responsible for the accumulation (Roman, 1948; Carlson, 1978). Although we cannot completely exclude that such, or some other B accumulation mechanisms also exists in *M. tenuipes*, results of the analysis of the dynamics of B chromosome polymorphism in our study (Yatsukami population) seem to argue against the existence of a B accumulation mechanism. We found no differences between mean numbers of Bs per individual in two successive years. Mean number of Bs per individual also did not change during the six months from June to November, the period which completely covers the lifespan of adult harvestmen (unpublished data). If B accumulation exist, a cycle of B accumulation would be followed by a cycle of selection decreasing number of Bs per individual in order to keep the number of Bs per individual stable. Because we have found no traces of such a selection during the two years (1997–1998) in the Yatsukami population, we believe that either B accumulation mechanism doesn't exist, or it is too weak to be detected.

In short, in this study we failed to obtain any evidences for the "parasite" nature of B chromosomes in *M. tenuipes*. Therefore, one can suggest that B chromosomes in this species should confer some selective advantages to be boosted by natural selection. We have found an association between the number of Bs and susceptibility of the harvestmen to gregarine parasites and also between number of Bs and phenotype (Gorlov and Tsurusaki, 2000). The observation that some B chromosomes in *M. tenuipes* carry large segments of euchromatin which may be (at least potentially) genetically active, also provides some cytological basis for the hypothesis of selective advantages conferred by Bs in this species.

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