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ORIGINAL ARTICLE

Post-meiotic B chromosome expulsion, during spermiogenesis, in two grasshopper species

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Abstract Most supernumerary (B) chromosomes are parasitic elements carrying out an evolutionary arms race with the standard (A) chromosomes. A variety of weapons for attack and defense have evolved in both contending elements, the most conspicuous being B chromosome drive and A chromosome drive suppression. Here, we show for the first time that most microspermatids formed during spermiogenesis in two grasshopper species contain expulsed B chromosomes. By using DNA probes for B-specific satellite DNAs in *Eumigus monticola* and *Eyprepocnemis plorans*, and also 18S rDNA in the latter species, we were able to count the number of B chromosomes in standard spermatids submitted to fluorescence in situ hybridization, as well as visualizing B chromosomes inside most microspermatids. In *E. plorans*, the presence of B-carrying microspermatids in 1B males was associated with a significant decrease in the proportion of B-carrying standard spermatids. The fact that this decrease was apparent in elongating spermatids but not in round ones demonstrates that meiosis yields 1:1 proportions of 0B and 1B spermatids and hence that B elimination takes place post-meiotically, i.e., during spermiogenesis, implying a 5–25% decrease in B transmission rate. In *E. monticola*, the B chromosome is mitotically unstable and B number varies between cells within a same individual. A comparison of B frequency between round and elongating spermatids of a same individual revealed a significant 12.3% decrease. We conclude that B chromosome elimination during spermiogenesis is a defense weapon of the host genome to get rid of parasitic chromosomes.

Keywords FISH · Micronucleus · Microspermatids · Parasitic · Satellite DNA

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Introduction

Regular chromosome elimination from somatic cells has been reported in nematodes, insects, mites, finches, bandicoots, and hagfish, and has been interpreted as a mechanism for gene silencing, dosage compensation, sex determination, or germ line and soma differentiation (for review, see Wang and Davis 2014). Of course, this variety of adaptations is evolutionarily viable provided that the somatically eliminated chromosomes have granted their presence in the germ line. For the same reason, chromosome elimination from germ cells is most likely the result of a genetic conflict where the standard genome tries to get rid of a disturbing harmful element, e.g., a parasitic chromosome.

B chromosomes are considered genomic parasites which prosper in natural populations because they show an advantage in transmission (drive) counteracting their detrimental effects on host genome fitness (for review, see Camacho et al. 2000; Camacho 2005; Burt and Trivers 2006). The presence of B chromosomes evokes an evolutionary response in the host genome leading to suppress drive, and the two contending parts develop a true coevolutionary arms race (Camacho et al. 1997; Frank 2000) which may elicit the emergence of new adaptations in the host genome. A suggestive example of these adaptations are germ-line restricted B chromosomes, such as those in the marsupial *Echymipera kalabu* (Hayman et al. 1969) and the ant *Leptothorax spinosior* (Imai

1974), as this minimizes their harm to the somatic cells while still assuring their transmission to future generations.

The formation of aberrant meiotic products during spermatogenesis has been a recurrent subject in the literature on B chromosomes. The post-meiotic part of spermatogenesis is called spermiogenesis, during which the round-shaped spermatids resulting from meiosis generate a tail and undergo drastic nuclear changes to become spermatozoa. In grasshoppers, spermatozoa possess extremely elongated heads showing almost the same width as the tail. During spermatid nuclear elongation, DNA packaging changes to a highly condensed state facilitated by histone replacement with protamines. Electron microscopy studies have shown that grasshopper spermiogenesis can be divided into ten developmental stages (Szöllösi 1975). Under optical microscopy, however, it is only possible to differentiate between the immature round spermatids, the mature spermatozoa (with fibrillar heads), and the intermediate stages with elongating spermatids at several degrees of elongation.

In addition to the temporally differentiated round (immature) and elongating (maturing) spermatids, optical microscopy allows identifying other types of spermatids, on the basis of size. In addition to standard haploid spermatids, polyploid spermatids have frequently been reported in grasshoppers (named macrospermatids). For instance, Cabrero et al. (2013) showed the presence of 2C, 4C, 8C, and 16C macrospermatids in males that had been RNAi knocked-down for the *Ku70* gene. In addition, tiny microspermatids can sometimes appear within cysts of standard spermatids. However, the frequency of macro- and microspermatids has shown to be significantly higher in B-carrying males of several species (see below).

Nur (1969) was the first in claiming that the production of macro- and microspermatids could be related with the presence of B chromosomes in the grasshopper *Camnula pellucida*. This author suggested that lagging B chromosomes could block cytokinesis in both meiotic divisions leading to the formation of restitution nuclei and thus 2C or 4C macrospermatids. Alternatively, lagging B chromosomes could be excluded from the standard meiotic products giving rise to microspermatids. Other authors have later found aberrant spermatid formation in other species carrying B chromosomes (for review, see Teruel et al. 2009). Partial support to Nur's claiming and slightly different explanations were later given by other authors. For instance, Bidau (1986) reported that unequal cytokinesis in *Metalaptea brevicornis* gave rise to macrospermatids and a small nuclear bud which sometimes could include the B chromosome. Likewise, Suja et al. (1989) "observed the presence of condensed Bs outside the nuclei in both recently formed secondary spermatocytes and early spermatids" thus supporting the hypothesis that lagging Bs can be eliminated from "standard nuclei." However, based on the fact that spermatocytes within a same cyst are connected by cytoplasmic bridges as a result of incomplete cytokinesis (Phillips 1970), Suja et al. (1989) suggested that macrospermatids could also derive from B-provoked impairment of spermatid differentiation during early spermiogenesis, which would explain the lack of correspondence they observed between the number of centriolar adjuncts and ploidy level in spermatids of the grasshopper *Eyprepocnemis plorans*. In addition, Loray et al. (1991) found that the presence of B chromosomes in *Dichroplus elongatus* was associated with an increase in the frequency of macrospermatids even in testis tubules lacking this mitotically unstable B chromosome, and claimed for physiological effects of B's affecting meiosis even in cells lacking them. This kind of systemic response could also be explained through some kind of gene expression change due to B presence (included gene expression in the B itself) whose effects would be exported to B-lacking testis tubules. This kind of effect would be compatible with the spermiogenesis impairment suggested by Suja et al. (1989), but not with the odd-even effect frequently reported for the frequency of aberrant spermatids in the case of mitotically unstable B chromosomes, as they are most abundant in testis tubules carrying odd numbers of B chromosomes (see Camacho et al. 2004; Teruel et al. 2009) whereas the physiological effect should erase this difference.

It is, however, unknown whether microspermatids actually contain B chromosomes, as no direct evidence has hitherto been provided in animals. In contrast, using DNA probes specific to A or B chromosomes, Chiavarino et al. (2000) showed that "micronuclei formed during male meiosis in maize can include both A and B chromosomes." On this basis, and given that B-carrying males showed higher frequency of microspermatids than 0B ones in *E. plorans* (0.73 and 0.22%, respectively), Teruel et al. (2009) suggested that most microspermatids in this species presumably include B chromosomes, with a consequent decrease in B transmission rate.

The finding of repetitive DNAs which B chromosomes are very enriched for (e.g., ribosomal DNA) or else are specific to them (e.g., some satellite DNAs) allows getting an easy estimation of their transmission rate by simply visualizing them in the meiotic products by fluorescence in situ hybridization (FISH) for DNA probes being highly specific to B chromosomes. For instance, Milani et al. (2016) found U2 repeats in a B chromosome in the grasshopper *Abracris flavolineata* being useful for B-chromosome identification in interphase cells, and they can be also useful for B transmission studies.

Here, we analyze the presence of B chromosomes in standard and aberrant spermatids in two grasshopper species harboring B chromosome systems differing in mitotic stability. In *E. plorans*, B chromosomes are mitotically stable, meaning that they show the same number of Bs in all cells from a same individual. In *Eumigus monticola*, however, B chromosomes are mitotically unstable, so that the number of B chromosomes differs between the cells from different testis tubules but not

between cells within a same tubule (Ruiz-Ruano et al. 2016). In each species, we have used DNA probes for FISH analysis which allowed scoring the number of B chromosomes in standard spermatids and demonstrated the presence of B chromosomes in most microspermatids observed in both species. Remarkably, the standard spermatids showed a significant decrease in the frequency of B chromosomes between their round and elongating stages, suggesting that B chromosomes are eliminated during spermiogenesis.

Materials and methods

Adult males of the grasshoppers *E. plorans* and *E. monticola* were collected in natural populations from Spain, the former species in Alhama de Murcia (Murcia province), Salobreña (Granada), Otívar (Granada), and Torrox (Málaga), and the latter in Hoya de la Mora (Sierra Nevada, Granada). For the present analysis, we chose *E. plorans* males carrying a single B chromosome belonging to several different variants: B1 (four males from Alhama de Murcia and one from Torrox), B2 (four males from Salobreña and six from Otívar), and B24 (four males from Torrox). A description of these B chromosomes can be found in Cabrero et al. (2014). In the case of *E. monticola*, we used here one male carrying a mitotically unstable B chromosome, thus showing different B number in different cells.

Males were anesthetized with ethyl acetate vapors before dissection. Testes were fixed in 3:1 ethanol/acetic acid and stored at 4 °C. The number of B chromosomes was analyzed in squash preparations of testis tubules stained with acetic orcein. FISH, including DNA probe preparation and FISH reaction, was performed following the protocols described in Camacho et al. (2015a, b) and Ruiz-Ruano et al. (2016). The DNA probes employed in *E. plorans* were 18S ribosomal DNA (rDNA), which shows the largest cluster on B1 and B2 variants, and a B-specific satellite DNA recently found by us (Martín-Peciña et al., in preparation) which shows FISH signals only on B chromosomes. In *E. monticola*, we used a B-specific satellite DNA (EmoSat26-41) previously reported by Ruiz-Ruano et al. (2016). The electron microscope (Fig. 2f) was obtained by the methods reported in Teruel et al. (2009).

Statistical analysis of spermatid counts in *E. plorans* was performed by a goodness-of-fit χ^2 test with null hypothesis predicting that 1B males produce 0B and 1B standard spermatids at Mendelian 1:1 proportion. This test was separately applied to round and elongating spermatids and a heterogeneity χ^2 test was also employed to analyze within-population heterogeneity before testing the 1:1 proportion at the population level. In *E. monticola*, however, the mitotic instability of the B chromosome did not allow applying the same null hypothesis and we compared the number of spermatids with 0–3 B chromosomes, between round and elongating ones, by the RxC software (provided by G. Carmody, Ottawa, Ontario, Canada), which performs χ^2 tests in contingency tables, with permutation, and calculates *P* values by Monte Carlo methods. Twenty batches of 2500 replicates were performed.

Results

Mitotically stable B chromosomes in *E. plorans*

The B1 and B2 variants carry the largest block of rDNA in B-carrying genomes (Fig. 1a), so that FISH with an rDNA probe allows easy identification of B-carrying and B-lacking round and elongating spermatids (Fig. 1b–d). B24, however, carries a smaller rDNA block (see Cabrero et al. 2014), and this marker does not discriminate properly between B24+ and B24− meiotic products. However, the B-specific satellite DNA (EplSat115-11) shows conspicuous clusters on both ends of the B24 chromosome (Fig. 1e) and is clearly apparent in spermatids as one or two small dots (Fig. 1f–h).

We analyzed the presence of B chromosomes in round (strictly circular) spermatids (Fig. 1b, f) and also in elongating ones (i.e., showing elliptic to spearhead shape) (Fig. 1c–h). The four males from the Alhama de Murcia population, carrying one B1 chromosome, showed about similar proportions of B-carrying and B-lacking spermatids at both round and elongating stages (Table 1), thus showing a Mendelian rate of B chromosome transmission (k_B). In the Salobreña and Torrox populations, which harbor the B2 and B24 variants, respectively, no significant difference was observed between B-carrying and B-lacking round spermatids (Table 1). However, two males in each population showed a significant deficit of B-carrying elongating spermatids, and χ^2 tests applied to the totals in each population (supported by the heterogeneity χ^2 test) yielded significant decreases in the transmission rate of these 1B males (k_B being 0.455 for B2 in Salobreña and 0.463 for B24 in Torrox) (Table 1).

In the Otivar population, which also harbors the B2 variant, we scored only elongating spermatids in six males and all of them showed k_B lower than 0.5, but the difference with the Mendelian one was not significant. However, as a whole, they showed a significant tendency to B elimination (Table 1).

In the Torrox population, we found a male carrying one B1 chromosome, a very unusual event in this population where B24 is the most frequent variant. Remarkably, this male showed about similar proportions of B-carrying and B-lacking round spermatids, but a significantly lower proportion of B-carrying elongating spermatids rendering a low B transmission rate ($k_B = 0.375$) (Table 1).

The observed k_B in elongating spermatids implied only residual B loss in Alhama for the B1 variant (2.23%), but it

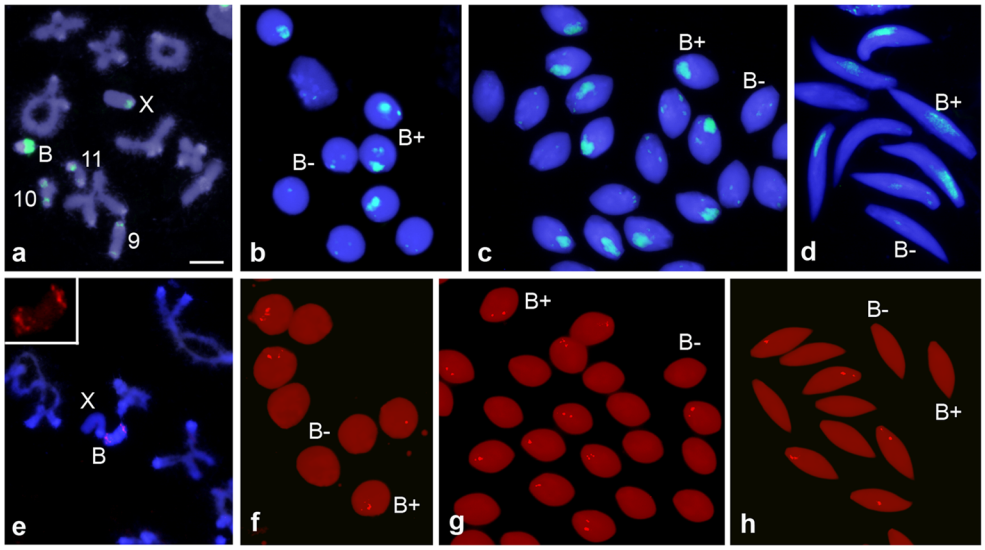

Fig. 1 Detection of B chromosomes in primary spermatocytes at diplotene (**a**, **e**), round spermatids (**b**, **f**), and elongating spermatids (**c**, **d**, **g**, **h**) of the grasshopper *Eyprepocnemis plorans* by means of FISH for 18S rDNA (**a**–**d**) and the B-specific EplSat115-11 satellite DNA (**e**–**h**) as DNA probes. Hybridization signals in (**a**)–(**e**) are merged with DAPI staining. *Inset* in (**e**) depicts the same B in the diplotene cell, at higher magnification, showing satellite location. In (**a**), note that the B1 chromosome in the Alhama de Murcia population carries the largest cluster for 18S rDNA and that this allows identifying B-carrying spermatids in (**b**)–(**d**). In (**f**)–(**h**), note that B24-carrying spermatids are identified by the presence of the B-specific satellite (*small dots*) in Torrox males. *Bar* in (**a**) indicates 5 μm for (**a**) and (**e**), and 10 μm for the remaining photographs

was higher in Otívar (4.9%) and Salobreña (9.04%) for B2, as well as in Torrox for B24 (7.41%) and B1 (25.1%).

FISH analysis showed that these decreases in B transmission rate (k_B) were paralleled by the presence of B-carrying macro- and microspermatids (Fig. 2a, b), and we scored them in cysts containing round spermatids in 13 males (excepting those from Otivar) and in cysts of elongating ones in all 19 males analyzed (Table 2). Multiple regression analysis, with k_B as dependent variable and the proportion of B-carrying macro- and microspermatids as independent variables, showed that k_B was independent of the frequency of these two types of aberrant gametes in the cysts containing round spermatids (round microspermatids—$r = 0.08$, $N = 13$, $t = 0.24$, $df = 10$, $P = 0.82$; round macrospermatids—$r = -0.12$, $N = 13$, $t = 0.35$, $df = 10$, $P = 0.73$). However, in the cysts of elongating spermatids, k_B was significantly negatively correlated with the frequency of B-carrying microspermatids ($r = -0.54$, $N = 19$, $t = 2.47$, $df = 16$, $P = 0.025$) but not with the frequency of B-carrying macrospermatids ($r = -0.25$, $N = 19$, $t = 1.12$, $df = 16$, $P = 0.28$). This suggests that microspermatid formation is related with a decrease in k_B whereas macrospermatids are not, confirming predictions by Teruel et al. (2009).

As Table 2 shows, not all microspermatids carried a B chromosome, the main exception being 29 round spermatids, all found in the m16 male from Salobreña, showing a nuclear bud containing a long chromosome carrying a small cluster of rDNA, which allowed identifying it as the X chromosome (Fig. 2c). All these 29 spermatids carried the B chromosome, and most of them showed the X chromosome still stuck to the nucleus, excepting one which was partially separated but still contacting by its end carrying rDNA, i.e., its centromeric region, and another nucleus showing the X chromosome completely separated from it (Fig. 2c). In elongating spermatids, we only observed four B-lacking microspermatids (Fig. 2c), one in m16 from Salobreña and three in m27 from Torrox, the 31 remaining microspermatids carrying the B chromosome.

It was highly remarkable that all B-carrying microspermatids observed by us were placed very close to a standard B-lacking spermatid (Fig. 2d, e), suggesting that the former derived from the same nucleus as the latter and that both share the same cytoplasm. This is also inferred from the fact that our preparations were made by squashing, so that the likelihood that the 47 B-carrying microspermatids were adjacent to a B-lacking standard spermatid would be negligible unless they share the same cytoplasm. In fact, some of the observed B-carrying microspermatids were physically in contact with an adjacent B-lacking nucleus whereas others did not contact with the nucleus and were found at different distance from the B-lacking nucleus (Fig. 2d, e), suggesting that microspermatids are finally expelled from the standard spermatids. Remarkably, a review of the photographs made by us in a previous analysis of spermatogenesis under electronic microscope (Teruel et al. 2009) revealed the presence of microspermatids sharing the same cytoplasm as standard spermatids and also the presence of very similar dense bodies outside spermatids which could correspond to remains of microspermatids extruded from the cytoplasm (Fig. 2f).

Table 1 Frequency of B-lacking (B−) and B-carrying (B+) spermatids in 19 males of the grasshopper *E. plorans* carrying 1B, collected at four Spanish populations

Population	Id	B type	FISH marker	ST	B−	B+	k_B	χ^2	*P* value
Alhama de Murcia	m1	B1	rDNA	R	127	131	0.508	0.06	0.80334
				E	168	180	0.517	0.41	0.52005
	m2	B1	rDNA	R	76	60	0.441	1.88	0.17007
				E	107	85	0.443	2.52	0.11235
	m4	B1	rDNA	R	48	46	0.489	0.04	0.83657
				E	156	140	0.473	0.86	0.35238
	m12	B1	rDNA	R	87	89	0.506	0.02	0.88017
Total				E	141	142	0.502	0.00	0.95260
				R	338	326	0.491	0.22	0.64144
Heterogeneity				E	572	547	0.489	0.56	0.45485
				R				1.79	0.6165
				E				3.24	0.3554
Salobreña	m8	B2	rDNA	R	144	138	0.489	0.13	0.72087
				E	186	156	0.456	2.63	0.10476
	m10	B2	rDNA	R	212	232	0.523	0.90	0.34254
				E	245	188	0.434	7.50	**0.00616**
	m16	B2	rDNA	R	46	49	0.516	0.09	0.75824
				E	63	67	0.515	0.12	0.72572
	m25	B2	rDNA	R	125	92	0.424	5.02	**0.02508**
Total				E	115	97	0.458	1.53	0.21637
				R	527	511	0.492	0.25	0.61946
Heterogeneity				E	609	508	0.455	9.13	**0.00251**
				R				5.90	0.11683
				E				2.65	0.44811
Otivar	m11	B2	rDNA	E	237	203	0.461	2.63	0.10504
	m12	B2	rDNA	E	212	188	0.470	1.44	0.23014
	m14	B2	rDNA	E	271	258	0.488	0.32	0.57193
	m17	B2	rDNA	E	109	94	0.463	1.11	0.29244
	m18	B2	rDNA	E	177	163	0.479	0.58	0.44770
	m21	B2	rDNA	E	194	182	0.484	0.38	0.53601
Total				E	1200	1088	0.476	5.48	**0.01921**
Heterogeneity				E				6.45	0.26446
Torrox	m02	B24	Sat115-11	R	125	122	0.494	0.04	0.84862
				E	121	110	0.476	0.52	0.46922
	m18	B24	Sat115-11	R	99	120	0.548	2.01	0.15588
				E	155	153	0.497	0.01	0.90927
	m21	B24	Sat115-11	R	193	206	0.516	0.42	0.51517
				E	207	166	0.445	4.51	**0.03376**
	m27	B24	Sat115-11	R	207	187	0.475	1.02	0.31365
Total				E	198	158	0.444	4.49	**0.03401**
				R	624	635	0.504	0.10	0.75655
Heterogeneity				E	681	587	0.463	6.97	**0.00830**
				R				3.39	0.33493
				E				2.57	0.46287
Torrox	m26	B1	rDNA	R	156	155	0.498	0.00	0.95478
				E	172	103	0.375	17.31	**0.00003**
Grand total				R	1645	1627	0.497	0.10	0.75301
				E	3234	2833	0.467	26.50	**<0.00001**

B chromosome presence was identified by FISH for 18S rDNA and a B-specific satellite (EplSat115-11). B transmission rate (k_B) was calculated as the proportion of B-carrying spermatids. *ST* spermatid type, i.e., round (R) or elongated (E). Goodness-of-fit χ^2 tested the null hypothesis that the B chromosome was transmitted at Mendelian rate ($k_B = 0.5$). Heterogeneity χ^2 is calculated as the sum of all individual χ^2 minus the χ^2 of total spermatid numbers. All χ^2 have one degree of freedom (*df*) except the heterogeneity one where they are calculated as the sum of individual *df* values minus 1 *df* of the χ^2 for the sum of all spermatids. *P* values <0.05 are noted in bold-type letter

Assuming that every B-carrying microspermatid implied the conversion of a B-carrying standard spermatid into a B-lacking one due to B chromosome loss, we can calculate the expected frequency of B+ and B− standard spermatids and test whether this would explain the observed k_B in round and elongating ones. In the case of round ones, the analysis of 3272 standard spermatids indicated $k_B = 0.497$, and we found 16 B-carrying microspermatids (see Table 2). The expected frequencies of B+ and B− round spermatids is thus $3272 \times 0.5 - 16 = 1620$ B+ and $3272 \times 0.5 + 16 = 1652$ B− ($k_B = 0.495$), and a goodness-of-fit χ^2 test comparing these expected frequencies with the observed ones (1627 and 1645,

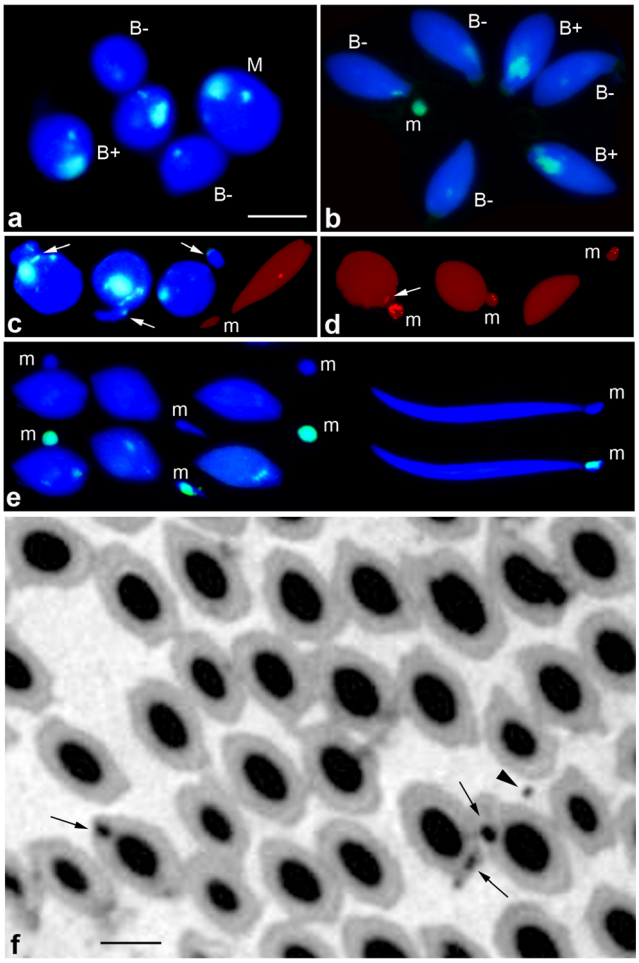

Fig. 2 Presence of macro- and microspermatids in the grasshopper *E. plorans*. **a** Two B-carrying (*B*+) and two B-lacking (*B*−) standard spermatids, and one macrospermatid (*M*). **b** Six standard elongating spermatids, four of which lack B chromosomes (*B*−) and two carry the B chromosome (*B*+). Note the presence of a B-carrying microspermatid (*m*). **c** Three round spermatids (on the *left*) showing a large chromosome being apparently extruded from the nucleus. Note that this chromosome carries a small rDNA cluster, which allows identifying it as the X chromosome (*arrow*). Note in the nucleus at the center that the centromere region, indicated by rDNA location, is still contacting the nucleus, whereas in the nucleus on the right, the whole X chromosome has lost contact with the main nucleus. The elongating B-carrying standard spermatid, on the *right*, was exceptional by lying beside a B-lacking microspermatid (*m*). **d** Examples of microspermatids (*m*) lying at different distances from a B-lacking standard spermatid. Note the presence of a small FISH signal in the main nucleus on the left (*arrow*). **e** Additional examples of microspermatids (*m*) laying by a B-lacking standard spermatid, showing DAPI staining (*upper row*) and FISH + DAPI (*lower row*). **f** Electron microscope photograph of cross-sectioned standard spermatid nuclei (dense bodies), showing the presence of small dense bodies appearing to be microspermatids, some of which share the cytoplasm with the main nucleus (*arrows*) and one is outside (*arrowhead*). *Bar* in (**a**) indicates 10 μm for (**a**)–(**e**), and that in (**f**) equals 1 μm

respectively) indicated the absence of significant difference ($\chi^2 = 0.06$, $df = 1$, $P = 0.8066$). On the contrary, we observed 2833 B+ and 3234 B− elongating spermatids ($k_B = 0.467$) plus 31 B-carrying microspermatids, and the expected frequencies, namely $6067 \times 0.5 - 31 = 3002.5$ B+ and $6067 \times 0.5 + 31 = 3064.5$ B− ($k_B = 0.495$), differed significantly from the observed ones ($\chi^2 = 18.94$, $df = 1$, $P = 0.00001$). This indicates that the observed amount of microspermatids does not explain the decrease in k_B observed in elongating spermatids. A possible explanation is that a fraction of the microspermatids produced are finally degraded and lost, so that we are able to visualize only part of those actually formed. We calculated that the loss of B chromosomes in 200 microspermatids (instead of the 31 B-carrying ones observed) would have yielded the observed $k_B = 0.467$, implying that we detected only 16% of the B chromosomes lost as microspermatids.

Mitotically unstable B chromosomes in *E. monticola*

The exclusive presence of the EmoSat26-41 satellite DNA in the B chromosome of the grasshopper *E. monticola* (Fig. 3a, b) (see also Ruiz-Ruano et al. 2016) allows scoring the number of B chromosomes in spermatids submitted to FISH (Fig. 3c–h). B chromosomes in this species are mitotically unstable, implying that B number varies among cells within a same individual, but not within a same testis tubule. For this reason, we analyzed round and elongating spermatids in the same six testis tubules and compared B frequency between these two kinds of standard spermatids. In total, we analyzed 911 round spermatids (355 with 0B, 465 with 1B, 89 with 2B, and 2 with 3B) and 442 elongating spermatids (210 with 0B, 193 with 1B, 34 with 2B, and 5 with 3B) and found a significant decrease in the mean number of B chromosomes between round (0.71) and elongating (0.62) spermatids (RxC contingency test—$P = 0.0004$, SE = 0.0002). This suggests that B chromosomes in *E. monticola* undergo about 12.3% elimination during spermiogenesis [$(0.71 - 0.62)/0.71 = 0.123$], as was also evidenced by the presence of 3% of B-carrying round microspermatids (Fig. 3c, d) and 5% of B-carrying elongating microspermatids (Fig. 3e, f). Likewise in *E. plorans*, the observed frequency of microspermatids was lower than the 12.3% decrease in B frequency, implying that we observed only about 42% (0.05/0.123) of B losses in the form of microspermatids, presumably because many of them are finally degraded.

Discussion

Population invasion by a parasitic B chromosome needs some kind of drive (Camacho et al. 1997). In *E. plorans*, we observed that B chromosomes show drive in some populations (Zurita et al. 1998) but not in others (López-León et al. 1992), as a consequence of drive suppression (Herrera et al. 1996; Camacho et al. 1997). In *E. monticola*, however, nothing is

known at this respect. However, our present results suggest that the loss during spermiogenesis would have impeded its birth as a B chromosome. It is thus likely that this B chromosome show drive at other stages of the reproductive cycle. Its mitotic instability suggests possible B accumulation based on mitotic non-disjunction during early cleavage divisions, with preferential destiny of mitotic products carrying more B chromosomes toward the germ line. This kind of accumulation has been reported for mitotically unstable B chromosomes of grasshopper species such as *Calliptamus palaestinensis* (Nur 1963), *Camnula pellucida* (Nur 1969), and *Locusta migratoria* (Nur 1969; Kayano 1971; Viseras et al. 1990). In the latter species, pre-meiotic accumulation of B chromosomes represents about a 30% increase in male B transmission, but it is counteracted by a 20% decrease during subsequent stages of the reproductive cycle, including the formation of microspermatids, the net B transmission thus implying about 10% accumulation in males (Pardo et al. 1994). In addition, this B chromosome shows 62% accumulation during female transmission (Pardo et al. 1994), which explains the worldwide distribution of B chromosomes in *L. migratoria*.

Our results have shown a significant decrease in B transmission rate (k_B) during spermiogenesis in two species of grasshopper carrying B chromosomes. In the case of *E. plorans*, males carrying one mitotically stable B chromosome yielded meiotic products at the Mendelian rate, given that about half of round spermatids carried the B chromosome. Therefore, spermiogenesis in these males begins with 1:1 proportion of B-carrying and B-lacking round spermatids. In contrast, most males showed a tendency to a decreased proportion of B-carrying elongating spermatids, which was significant in five males and, as a whole, in Salobreña, Otivar, and Torrox populations (see Table 1). Therefore, the k_B decrease takes place necessarily during spermiogenesis. We also demonstrate here that k_B was negatively correlated with the frequency of B-carrying microspermatids, suggesting that B chromosomes are lost during spermiogenesis in the form of microspermatids. Our FISH visualization of B chromosomes within microspermatids constitutes the first direct demonstration of Nur's claiming that microspermatids are a way of B chromosome loss (Nur 1969). Remarkably, we only observed B-carrying macrospermatids even though some B-lacking ones might be expected if cytokinesis failures would take place in B-lacking secondary spermatocytes. This suggests a direct role of lagging B chromosomes in the formation of macrospermatids, as was also suggested by Nur (1969).

In addition, the fact that B-carrying microspermatids were always found beside a B-lacking standard spermatid, even in squash preparations, along with the presence of microspermatids sharing a common cytoplasma with standard spermatids at electronic microscope images, suggests a causal relationship between microspermatid formation, B chromosome loss, and the decrease in k_B in standard spermatids. The conventional explanation for microspermatids is that they contain B chromosomes lagged during the precedent meiotic divisions, as was first suggested by Nur (1969) and later supported by other authors (Pearse and Ehrlich 1979; Viseras and Camacho 1985; Bidau 1987; Teruel et al. 2009; Abdel-Haleem et al. 2009). Our present results, however, challenge this hypothesis. Of course, we cannot rule out that some of the 16 B-carrying microspermatids found within cysts containing round standard spermatids could have derived from B chromosomes lagged during previous meiosis which failed to properly integrate into the main nucleus, and even some of the 31 B-carrying microspermatids observed in the cysts of elongating spermatids could actually have derived from them. However, if meiosis were the only source of microspermatids, we should observe similar values of k_B in round and elongating spermatids, and this was not the case in the two species analyzed here, thus clearly implying microspermatid formation during spermiogenesis.

The finding that micronuclei can be formed by nuclear budding in interphase cells could provide a mechanistic support to B chromosome elimination during spermiogenesis. Nuclear budding and micronucleus formation are common characteristics to many cell cultures frequently leading to chromosome elimination (Elston 1963; Longwell and Yerganian 1965). The classical mechanism of micronucleus formation claims that they incorporate lagging chromosomes during mitosis (Heddle and Carrano 1977; Schubert and Oud 1997; Fenech et al. 2011). However, recent findings have shown that nuclear budding and micronucleus formation can also occur in interphase cells (Gernand et al. 2005, 2006; Utani et al. 2011; Ishii et al. 2016). Similarly, round and elongating spermatids can form micronuclei (called here microspermatids) during spermiogenesis without the involvement of any additional cell division. In fact, our results show remarkable similarities with some characteristics of interphase micronucleus formation described by the former authors.

For instance, Gernand et al. (2005, 2006) reported that the chromosomes destined to elimination occupied a peripheral location in interphase cells of interspecific hybrids. This appears to be a general tendency since other chromosomes being regularly eliminated also occupy peripheral locations, such as E chromosomes in Cecidomyiidae (Kloc and Zagrodzinska 2001), the germ-line restricted chromosomes (GRC) in the zebra and the Bengalese finches (Schoenmakers et al. 2010; Del Priore and Pigozzi 2014), and even acentric, autonomously replicating extrachromosomal structures called double-minute chromosomes (Shimizu et al. 1998).

Interestingly, a tendency of B chromosomes to occupy peripheral locations in the nucleus during cell division was early noted by Avdulow (1933) in maize (see also Randolph 1941; Darlington and Upcott 1941; Carlton and Cande 2002). Subsequent research has reached the same conclusion for B chromosomes in *Poa alpina* (Hakansson 1948), *Dactylis*

Table 2 Frequency of B-carrying (B+) and B-lacking (B−) micro- and macrospermatids (M) in 19 males of the grasshopper *E. plorans* carrying 1B, collected at four populations

Population	Id	B	ST	k_B	Microspermatids					Macrospermatids		
					B+	B−	Total	frB+	frB−	B+	B−	frM
Alhama de Murcia	m1	B1	R	0.51	0	0	0	0	0	0	0	0
			E	0.52	0	0	0	0	0	3	0	0.9%
	m2	B1	R	0.44	0	0	0	0	0	2	0	1.5%
			E	0.44	0	0	0	0	0	5	0	2.6%
	m4	B1	R	0.49	0	0	0	0	0	0	0	0
			E	0.47	1	0	1	0.3%	0	2	0	0.7%
	m12	B1	R	0.51	0	0	0	0	0	2	0	1.1%
Total			E	0.50	1	0	1	0.4%	0	8	0	2.8%
			R	0.49	0	0	0	0	0	4	0	0.6%
			E	0.49	2	0	2	0.2%	0	18	0	1.6%
Salobreña	m8	B2	R	0.49	3	0	3	1.1%	0	14	0	5.0%
			E	0.46	0	0	0	0	0	3	0	0.9%
	m10	B2	R	0.52	0	0	0	0	0	5	0	1.1%
			E	0.43	4	0	4	0.9%	0	1	0	0.2%
	m16	B2	R	0.52	0	29	29	0	30.5%	4	0	4.2%
			E	0.52	0	1	1	0	0.8%	0	0	0
	m25	B2	R	0.42	0	0	0	0	0	3	0	1.4%
Total			E	0.46	0	0	0	0	0	5	0	2.4%
			R	0.49	3	29	32	0.3%	2.8%	26	0	2.5%
			E	0.45	4	1	5	0.4%	0.1%	9	0	0.8%
Otívar	m11	B2	E	0.46	0	0	0	0	0	0	0	0
	m12		E	0.47	0	0	0	0	0	0	0	0
	m14		E	0.49	1	0	1	0.2%	0	0	0	0
	m17		E	0.46	1	0	1	0.5%	0	0	0	0
	m18		E	0.48	1	0	1	0.3%	0	0	0	0
	m21		E	0.48	2	0	2	0.5%	0	0	0	0
Total			E	0.48	5	0	5	0.2%	0	0	0	0
Torrox	m2	B24	R	0.49	0	0	0	0	0	0	0	0
			E	0.48	0	0	0	0	0	0	0	0
	m18	B24	R	0.55	0	0	0	0	0	0	0	0
			E	0.50	0	0	0	0	0	0	0	0
	m21	B24	R	0.52	0	0	0	0	0	0	0	0
			E	0.45	0	0	0	0	0	0	0	0
	m27	B24	R	0.47	0	0	0	0	0	1	0	0.3%
Total			E	0.44	0	3	3	0	0.8%	13	0	3.7%
			R	0.50	0	0	0	0	0	1	0	0.1%
			E	0.46	0	3	3	0	0.2%	13	0	1.0%
Torrox	m26	B1	R	0.50	13	0	13	4.2%	0	10	0	3.2%
Grand total			E	0.37	20	0	20	7.3%	0	15	0	5.5%
			R		16	29	45	0.5%	1.0%	31	0	1.0%
			E		31	4	35	0.9%	0.1%	40	0	1.1%

ST spermatid type, i.e., round (R) or elongating (E); k_B B transmission rate estimated in normal spermatids (see Table 1); *frB+*, *frB−* frequency of B-carrying or B-lacking microspermatids calculated as the proportion between the observed number and the total number of normal spermatids (see values in Table 1); *frM* proportion of macrospermatids in respect to the total number of normal spermatids

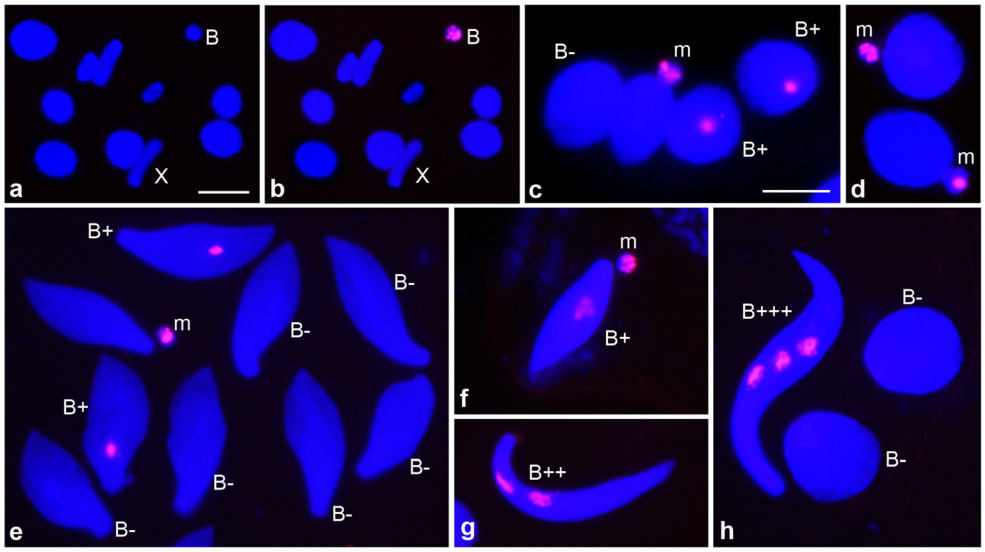

Fig. 3 Detection of B chromosomes in primary spermatocytes at metaphase I (**a**, **b**) and spermatids (**c**–**h**) of the grasshopper *Eumigus monticola*, submitted to FISH for the EmoSat26-41 B-specific satellite DNA. Examples of B-carrying (*B+*) and B-lacking (*B−*) standard round and elongating spermatids are shown in (**c**)–(**h**). In (**c**)–(**e**), note the presence of microspermatids (*m*) beside a B-lacking standard spermatid. Mitotic instability of B chromosomes in this species explains the presence of B-carrying standard spermatids beside a B-carrying microspermatid (**f**) since standard spermatids in this species can carry two (**g**) or three (**h**) B chromosomes. The number of B chromosomes within a sperm nucleus is indicated by the number of *plus signs*. *Bar* in (**a**) indicates 5 μm for (**a**) and (**b**), that in (**c**) indicates 10 μm for (**c**), (**f**), and (**g**), and that in (**e**) indicates 10 μm for (**d**), (**e**), and (**h**)

(Williams and Barclay 1972), and rye (Jones 1995; Morais-Cecílio et al. 1996; Langdon et al. 2000). In animals, the paternal sex ratio (PSR) is an extremely parasitic B chromosome which localizes to the outer periphery of the paternal nucleus and at the tip of the sperm nucleus, but in this case the B chromosome escapes from elimination which is focused on the paternal standard set (Swim et al. 2012). These authors visualized PSR by FISH in spermatids and mature sperm and about 98% of them, in both cases, carried the B chromosome, so that we can infer that PSR is not eliminated at all during spermiogenesis.

Sex chromosomes in animals also occupy peripheral locations (see Turner 2007; Finch et al. 2008; Calvente et al. 2013) and are inactivated during meiosis by means of epigenetic marks (Vaskova et al. 2010). Likewise, in *E. plorans*, X and B chromosomes are heterochromatic; they show frequent non-homologous association during the first meiotic prophase (Camacho et al. 1980) and are hypoacetylated for H3K9 during entire meiosis (Cabrero et al. 2007). They also tend to occupy peripheral location in meiotic nuclei, which probably facilitates their elimination in the form of microspermatids. It is tempting to speculate that the high similarity between X and B chromosomes during meiosis may lead to eventual X chromosome elimination, presumably because some of the epigenetic marks used for microspermatid formation are common to these two chromosomes.

Another resemblance of our present results with those in interspecific hybrids is that centromeric regions of pearl millet chromosomes are the last in being eliminated in wheat-pearl millet hybrids (Gernand et al. 2005). We observed this same fact in the case of the X chromosome elimination in m16 from Salobreña (see Fig. 2c). Interestingly, Gernand et al. (2005) suggested that micronucleus formation can eventually leave the centromeric region of the expulsed chromosome in the main nucleus, thus opening the possibility to de novo formation of B chromosomes in interspecific crosses. Our Fig. 2d shows a round microspermatid, beside a round standard spermatid, which harbors most of the B-specific satellite except a small FISH signal remaining in the main nucleus, indicating that B chromosome extrusion can be incomplete, thus giving indirect support to Gernand et al.'s claims.

In addition, Gernand et al. (2005) suggested that post-translational histone modification might play a role in chromosome elimination, as differential acetylation of histones H3 and H4 and methylation of histone H3 had been reported in chromosome elimination in sciarid flies (Goday and Ruiz 2002) and in programmed DNA elimination in *Tetrahymena* (Taverna et al. 2002). In addition, the GRC chromosome in the zebra finch is silenced from early leptotene onwards and is eliminated through micronucleus formation following metaphase I (Schoenmakers et al. 2010). It is thus presumable that the observed H3K9 hypoacetylation of X and B chromosomes in *E. plorans* (Cabrero et al. 2007) may serve as a signal for elimination through the evolutionary conserved mechanism suggested by Gernand et al. (2005, 2006).

Our present results suggest that even in organisms where chromosome elimination occurs only sporadically, e.g., B chromosome loss during spermiogenesis, interphase cells

appear to show the ability to eliminate chromosomes through micronucleus formation. The parasitic nature of B chromosomes makes them an elimination target with high fitness reward for the host genome. Ideally, the best situation for a B chromosome would be to remain limited to the germ line by being eliminated from somatic cells, thus minimizing harmful effects on the host. Examples of germ-line restricted B chromosomes have been found, for example, in the marsupial *E. kalabu* (Hayman et al. 1969) and the ant *L. spinosior* (Imai 1974). Even in this case, B chromosome presence in the germ line is still a load for the host genome, as it has to replicate extra DNA without a reward, except in the case that the B chromosome carries a gene whose activity result is profitable for the host (e.g., see Miao et al. 1991). In most cases, however, it is expected that the host genome continues trying to get rid of the parasitic element. The existence of post-meiotic elimination mechanisms like that shown here might help in this task, but it does not always work. Suggestive examples are germ-line restricted chromosomes like those reported in diptera (Bauer and Beermann 1952; Staiber 1988; Herrick and Seger 1999; Goday and Esteban 2001) or zebra finches (Pigozzi and Solari 2005; Schoenmakers et al. 2010; Del Priore and Pigozzi 2014), as they could actually be the last face of obstinately resistant parasitic B chromosomes.

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Compliance with ethical standards

Integrity of research All experiments comply with the current Spanish laws.

Conflict of interest The authors declare that they have no competing interests.

Human and animal rights and informed consent All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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