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FISH detection of ribosomal cistrons and assortment-distortion for X and B chromosomes in *Dichroplus pratensis* (Acrididae)

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Abstract. Assortment-distortion with respect to the X and NOR activity of a rare mitotically stable B chromosome (B_N), was examined in 16 males of *Dichroplus pratensis* (Acrididae: Melanoplinae) from Argentine populations. In 1B individuals, the X and B associate preferentially during prophase I reaching a maximum level of association at zygotene. Frequency of X/B association remains relatively high up to diplotene-diakinesis and decreases steeply towards metaphase I. The percent X/B association at each stage is positively influenced by association at the previous stage, and interindividual variability in X/B association decreases as the frequency of association increases. Both chromosomes tended to preferentially orientate toward the same pole at MI (mean ratio of 16 individuals, 1.50:1) which determined an excess of XB and 00 second spermatocytes over X0 and 0B ones (1.39:1). No significant differences occurred between the MI, AI and MII assortment ratios. Fluorescent in situ hybridisation (FISH) confirmed that the B chromosome carries ribosomal genes and helped to establish that, during spermiogenesis, both the B and the normal NOR-bearing chromosome (S_8) are clustered near the centriole adjunct region of spermatids. However, FISH failed to reveal the existence of inactive ribosomal cistrons in the X chromosome, as previously suggested, thus providing no support to a simple origin of the B from the X.

B chromosomes are an almost universal accessory component of eukaryotic genomes (Jones and Rees, 1982; Camacho et al., 2000). They have multiple dissimilar origins, DNA composition and genetic activity are diverse and their behaviour and effects are complex and sometimes, downright bizarre. It is difficult to generalise on B chromosomes but the consensus is that they are parasitic, selfish DNA entities that co-evolve with the A-genome to maximise their representation in the next generation despite their potential harmful effects (Bell and Burt, 1990; Camacho et al., 2000, 2003). Such effects may derive from their sole presence within the cell (through meiotic misbehaviour) or because of transcription of genes present in the Bs that could somehow interfere with normal cellular activity. Nevertheless, little is known about genetic activity of B chromosomes in most plants and animals, with few notable exceptions (Camacho et al., 2000). The fact that Bs are selfish genetic parasites generates genomic conflict between them and the A genome (Camacho et al., 2000, 2003) and thus, they are under natural selection against their spread throughout the population. Because of the former, B chromosomes have evolved a number of strategies to counteract the effects of negative selection and to persist within natural populations (Jones, 1991). As a result of these opposing forces, Bs may attain the status of near-neutral genomic entities (Camacho et al., 1997a, b, 2000).

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Table 1. Orientation of X and B chromosomes to the spindle poles at Metaphase I in 16 1B male carriers of *Dichroplus pratensis*

Individual	Locality	B freq.	X and B going to				
			Same pole	Opposite poles	Ratio	χ^2	*P*
1 (B_{N1})	Sierra de la Ventana 38º06'S 61º48'W	0.04	171	111	1.59	12.77	0.000352
2 (B_{N1})	Sierra de la Ventana 38º06'S 61º48'W	0.04	143	98	1.46	8.40	0.003752
3 (B_{N1})	Punta Indio 42º48'S 65º03W	0.03	423	311	1.36	17.09	0.000036
4 (B_{N1})	Villa Ventana 38º04'S 61º55'W	0.02	105	61	1.72	11.66	0.000639
5 (B_{N1})	Saldungaray 38º04'S 61º50'W	0.05	87	59	1.45	5.37	0.020486
6 (B_{N1})	El Atravesado 38º08'S 61º51'W	0.11	12	1	12.00	9.31	0.002279
7 (B_{N1})	El Atravesado 38º08'S 61º51'W	0.11	9	6	1.50	0.60	0.438578
8 (B_{N1})	El Atravesado 38º08'S 61º51'W	0.11	24	16	1.50	1.60	0.205903
9 (B_{N1})	El Atravesado 38º08'S 61º51'W	0.11	25	12	2.08	4.57	0.032537
10 (B_{N2})	Diadema Argentina 45º45'S 67º48'W	0.13	52	28	2.05	7.20	0.007290
11 (B_{N2})	Diadema Argentina 45º45'S 67º48'W	0.13	22	20	1.10	0.10	0.751830
12 (B_{N1})	Puerto Madryn 33º32'S 65º02'W	0.20	9	11	0.82	0.20	0.654721
13 (B_{N1})	Puerto Madryn 33º32'S 65º02'W	0.20	20	8	2.50	6.61	0.010141
14 (B_{N1})	Istmo Ameghino 42º27'S 64º28'W	0.20	29	23	1.26	0.69	0.406164
15 (B_{N1})	Istmo Ameghino 42º27'S 64º28'W	0.20	30	15	2.00	5.00	0.025347
	Manantiales 33º32'S 63º20'W	0.07	50	33	1.51	3.48	0.062115
Total			1211	810	1.50	79.56	0.000000

Two different types of B chromosomes have been described in the South American grasshopper *Dichroplus pratensis* (Melanoplinae, Acrididae) (Bidau, 1986, 1987). Nevertheless, and despite the enormous geographical distribution of the species and the large number of natural populations sampled to date (Bidau and Martí, 2002) these B chromosomes are exceedingly rare within the species. Their frequencies are very low in marginal populations hundreds of kilometers apart, while they are virtually absent in intermediate populations except in hybrid zones (Bidau and Martí, 2002). In a previous communication (Bidau, 1986) we described a mitotically stable B chromosome that exhibited NOR activity, as shown by silver impregnation, and also showed assortment-distortion with respect to the X chromosome in males. However, since the sample was small, the evaluation of assortment-distortion was provisional. NORs are relatively rare in B chromosomes (Green, 1990; Jones, 1995). Furthermore, silver impregnation does not necessarily reveal true NORs at least in mammals (Dobigny et al., 2002). Thus, after extensive sampling of almost 70 natural populations of *D. pratensis*, we found 16 males carrying the stable B chromosome, which were subjected to standard and FISH cytogenetic analyses to reanalyze the structure and behaviour of this rare supernumerary chromosome.

Material and methods

Sixteen males carrying one of two variants of a mitotically stable B chromosome were collected at the localities shown in Table 1. Non-B-carriers as well as carriers of a different B chromosome which is mitotically unstable (Bidau, 1987; Martí, 2002) from the same populations were used as controls for FISH analysis. One male carried both B chromosome types (individual 10; see Fig. 1c).

Standard testis preparations were performed by squash in propionic hematoxylin or lacto-propionic orcein. Female meiosis was studied as described in Bidau and Martí (2002).

Cytological preparations for FISH were performed without applying mechanical pressure in order to maintain tridimensional information according to a modification of the technique of Zhong et al. (1996). Briefly, methanol:acetic fixed testis follicles were disrupted onto glass slides containing a drop of 60% acetic acid. The material was then spread out on a thermal plate at 45°C, with circular movements and posterior addition of ice-cold fixative. Slides were left to air dry after several washes with ice-cold fixative, 10 min incubation in 60% acetic acid and a final wash with 100% ethanol.

The FISH protocol was adapted from Chiavarino et al. (2000) with modifications. Slides were pre-treated with RNAse (1 µg/ml, 37°C, 1 h), pepsin (0.1%, pH 2–2.5, 5 min, 37°C) and paraformaldehyde 4% (10 min, room temperature, RT) and ethanol dehydrated. Slides were denatured in 70% formamide/2× SSC for 1 min at 62°C and immediately dehydrated. The biotinylated probe pTa71, containing the 5.8S, 18S and 28S ribosomal DNA cistrons from *Triticum aestivum*, was used (Gerlach and Bedbrook, 1979). Probe denaturation (2 ng/µl) was performed for 10 min at 100°C in 50% formamide/10% dextran sulfate/2× SSC buffer. Hybridisation was allowed to proceed in a moist chamber at 37°C overnight. Slides were washed under agitation in 2× SSC (30 min at RT), twice in 1× SSC (5 min at RT and 30 min

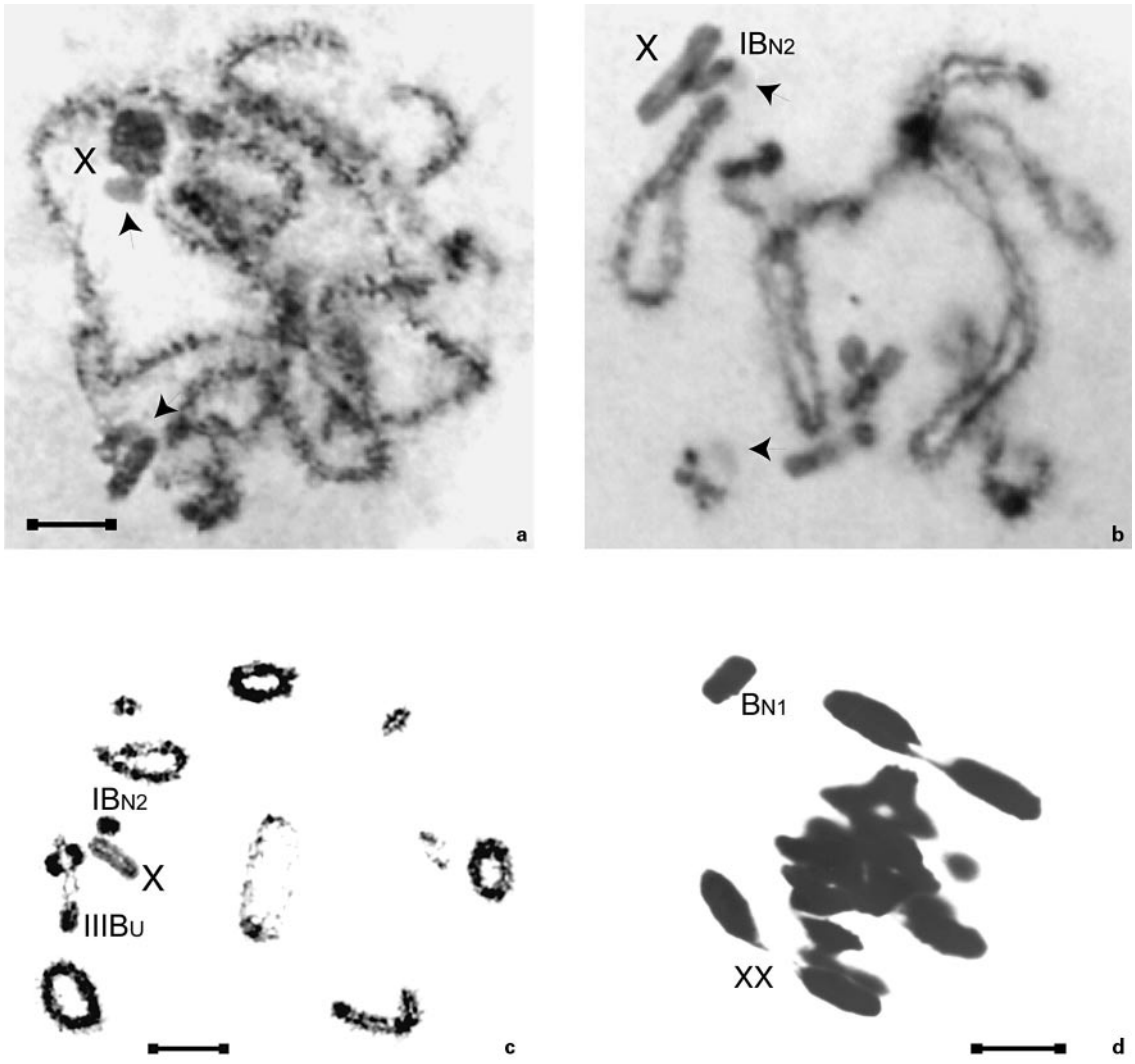

Fig. 1 . Meiosis in 1B carriers of *Dichroplus pratensis.* (**a–c**) Male meiotic stages. Propionic hematoxylin squashes. (**d**) Female metaphase I. Lacto-propionic orcein squash. (**a**) Mid-pachytene showing association of X and B_{N1} chromosomes. Arrows indicate nucleoli associated to the B_{N1} and the S_8 bivalent. (**b**) Early diplotene showing side-by-side alignment of the X and the B_{N2} chromosome. Arrows point to nucleoli. (**c**) Diakinesis of an individual carrying one B_{N2}. The cell also has a trivalent formed by an unstable B chromosome ($IIIB_u$). This individual was homozygous for a centric fusion. (**d**) Metaphase I from a standard (all-telocentric) female showing a B_{N1} chromosome at one of the spindle poles. Bars = 10 μm.

at 37 °C) and twice in 2× SSC (5 min at RT). Detection was performed by incubation with Avidin-Cy3 at 37 °C for 1 h, followed by two washes in 4× SSC, 0.2 % Tween 20 for 5 min at 37 °C under agitation. Preparations were counterstained and mounted with antifade-DAPI (DAKO), and analysed in an Olympus BX 50 microscope, with the appropriate filter set.

Results

Orientation and assortment of the B_N and X chromosomes during male meiosis

Two variants of the NOR-carrying B chromosome (B_N) occur in wild populations of *D. pratensis*. Both are telocentric, mitotically stable and X-like in their meiotic pycnotic behaviour and both show a paracentromeric secondary constriction which organises a nucleolus during premeiotic interphase and meiosis as suggested by silver impregnation and haematoxylin staining (Fig. 1a, b) (Bidau, 1986; Martí, 2002). However, one is about two-thirds of the X in length (B_{N1}) and the other, one-third (B_{N2}) (Fig. 1b–d; Fig. 2b). An unstable B chromosome (B_U) is also found in some populations of the species also carrying B_N (Fig. 1c).

The X and B_N chromosomes are expected to orientate and segregate independently of one another in meiosis of 1B individuals if during prophase I no association occurs between them, that is, if they behave as complete univalents. We analysed orientation of both univalents at metaphase I (MI) in 16 individuals carrying one or the other variant of the B_N chromosome and orientation of both chromosomes deviated signifi-

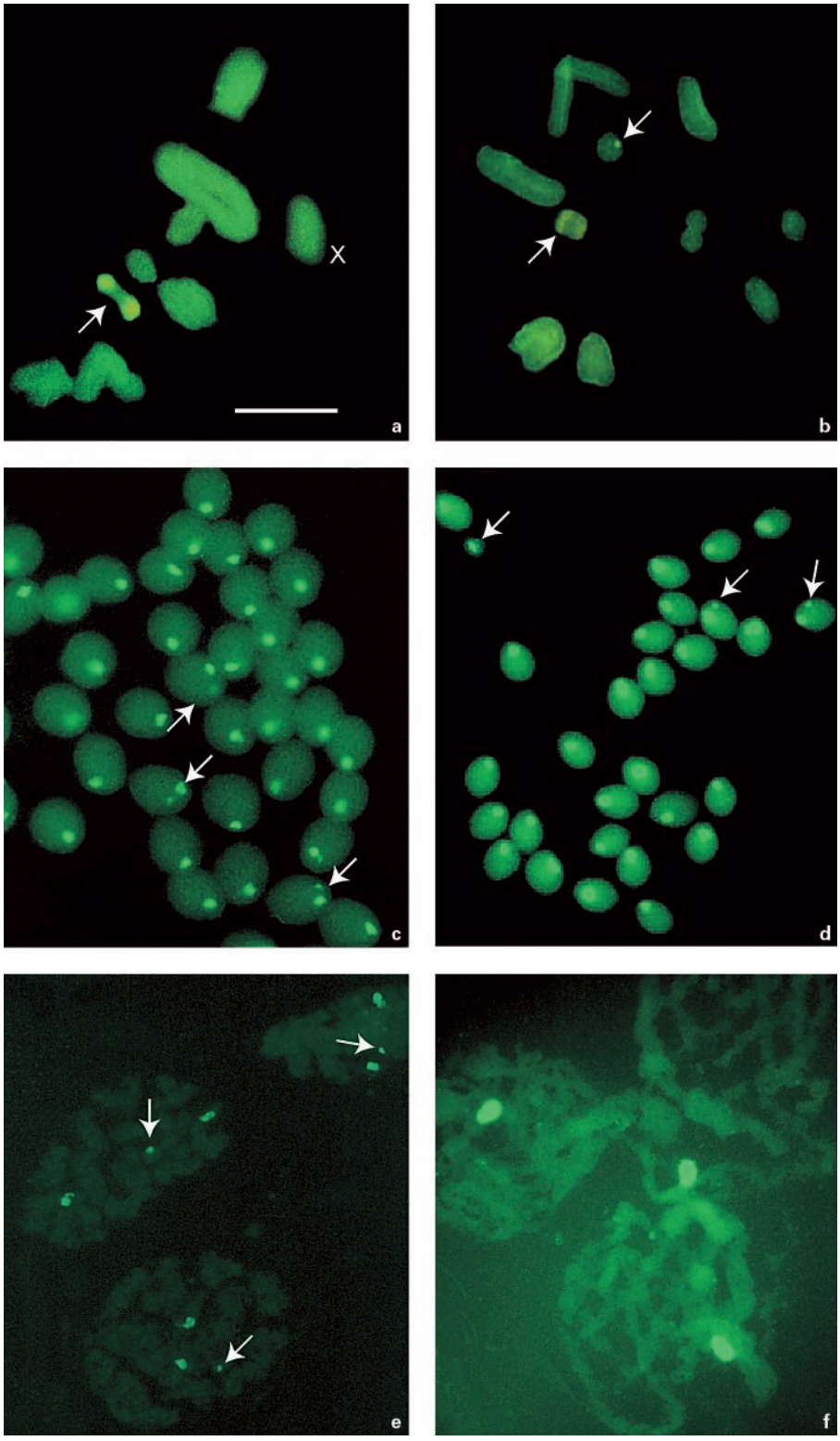

Fig. 2. FISH with the rDNA pTa71 probe. Meiosis and spermiogenesis of 0B and $1B_N$ males of *Dichroplus pratensis*. (**a**) Metaphase I of a 0B centric fusion homozygote. The arrow indicates the S_8 bivalent showing two strong hybridisation signals for the pTa71 probe in both homologues. (**b**) Metaphase I of $1B_{N2}$ carrier. Arrows point to the S_8 bivalent with two strong hybridisation signals, and to the B_{N2} chromosome showing a weaker paracentromeric signal. (**c**) Spermatids at the rounded stage from a $1B_N$ carrier. Arrows indicate two hybridisation signals of the pTa71 probe, clustered at the centriole adjunct region. (**d**) Spermatids from a $1B_N$ carrier at the beginning of the elongation stage. At right, arrows point to spermatids with both the S_8 and B_N signals. At left, the arrow indicates a microspermatid with a weak hybridisation signal. (**e**) Preleptotene nuclei of a $1B_{N1}$ carrier. Three hybridisation signals are visible in each nucleus. The arrow points to the signal from the B_N chromosome. (**f**) Zygotene nuclei of a 0B individual showing one strong signal on the S_8, resulting from pairing of both homologues. Bar = 10 μm.

cantly from the 1:1 expected ratio. That is, X and B tended to orientate preferentially toward the same spindle pole (Table 1). Variation occurred between individuals but it was not statistically significant (contingency $\chi^2 = 21.72$; df = 15; $P = 0.11536$).

The non-random orientation of X and B_N at MI distorts anaphase I (AI) assortment of both univalents. In a pooled sample of 406 first anaphases in which X and B assortment was recorded, both chromosomes had migrated to the same pole in 237 cells, and to opposite poles in 169 cells, in a 1.40:1 ratio

Table 2. Variation in the frequencies of association between the X and B chromosomes during early first meiotic prophase

	% X-B Association[a]				% X-B Association[a]			
Individual	L	Z	χ^2	*P*	Z	EP	χ^2	*P*
1	41.5	73.0	18.5	0.000017	73.0	52.0	11.1	0.000863
2	38.0	81.0	18.4	0.000017	82.0	72.0	1.2	0.273322
3	84.0	100.0	21.0	0.000005	100.0	91.0	9.5	0.002055
4	77.0	75.9	0.001	0.974773	75.9	66.1	1.6	0.205903
5	64.5	100.0	4.7	0.030162	100.0	78.0	1.9	0.168078
6	38.6	78.7	66.6	0.000000	78.7	87.4	2.0	0.157299
7	57.5	77.7	15.6	0.000078	77.7	81.1	0.5	0.479500
8	60.0	85.0	1.6	0.205903	85.0	63.1	1.5	0.220671
9	53.9	95.8	7.1	0.007708	95.8	96.2	0.4	0.527089
Total	60.9	80.9	82.7	0.000000	80.9	76.7	5.61	0.017858

[a] L, leptotene; Z, zygotene; EP, early pachytene.

Table 3. Correlation analysis between X-B association at different meiotic stages and the assortment ratio shown by both univalents

Comparison[a]	r	t	df	*P*[b]
a. L	0.69037	2.3375	6	0.02902*
b. Z	0.49332	1.3892	6	0.10707
c. EP	0.44668	1.2229	6	0.13309
d. LP	0.69192	2.3475	6	0.02863*
e. DD	0.50710	1.4412	6	0.09981
f. MI	0.79358	2.9163	5	0.01658*

[a] L, leptotene; Z, zygotene; EP, early pachytene; LP, late pachytene; DD, diplotene-diakinesis; MI, metaphase I.
[b] *Significant at the 5 % level.

significantly differing from the 1:1 ratio ($\chi^2 = 11.39$; df = 1; $P = 0.00074$). The result is an excess of secondary spermatocytes with either B and X, or none of them. This result is applicable to both B_{N1} and B_{N2}. Consistently, in 692 metaphase II (MII) cells, 198 were XB, 205 00, 138 X0 and 151 0B producing a 1.39 ratio ($\chi^2 = 19.41$; df = 3; $P = 0.00023$). No significant differences occurred between MI, AI and MII (contingency $\chi^2 = 0.78$; df = 2; $P = 0.67706$).

Behaviour of the B_N and X chromosomes during male prophase I

To determine the causes of B_N (both variants) and X assortment-distortion, their behaviour during prophase I was analysed in 9 males (Table 2; Fig. 3). Both the B and the X appeared as dense-staining bodies at the periphery of the nucleus during preleptotene and first prophase. Both elements could be associated (Fig. 1a–c) or free within the nucleus, but association was not random in all studied males that showed a parallel behaviour indicating a recurrent pattern: the frequencies of X/B association increased significantly from preleptotene to leptotene, and from this latter stage to zygotene where the maximum mean frequency was attained, reaching 100% in two individuals. From zygotene onwards, the X/B association frequency decreased slowly although maintaining high mean levels during early, middle and late pachytene. This pattern is repeated in all analysed males although slight differences occur (contingency $\chi^2 = 30.85$; df = 15; $P = 0.0092$). At diplotene/diakinesis X/B association fell to a level comparable to preleptotene, while a very steep decline occurred between the latter stage and M I. As shown in Table 2, in 7 individuals the difference in X/B association between leptotene and zygotene was highly significant. An opposite result was obtained when zygotene and early pachytene stages were compared (Table 2).

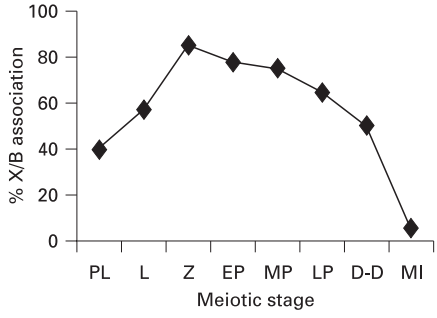

Fig. 3. Frequency of X-B_N association (%) from preleptotene to metaphase I. PL, preleptotene; L, leptotene; Z, zygotene; EP, early pachytene; MP, middle pachytene; LP, late pachytene; D-D, diplotene-diakinesis; MI, metaphase I.

A further suggestion of the non-randomness of the X/B_N association came from the data on correlation analysis shown in Table 3. Individual ratios of X/B_N assortment were positively correlated to prophase I association at all stages, correlations being statistically significant in three cases (Table 3a, d, f). Therefore, prophase I X-B_N association might condition their preferential assortment to the same pole in the first meiotic division.

Presence of a NOR in the B chromosome of D. pratensis revealed by FISH

In situ hybridisation with the rDNA pTa71 probe revealed the existence of two kinds of signal. A strong signal was present at the paracentromeric region of both homologues of the S_8 bivalent which is known, by silver impregnation, to be usually associated to a pair of nucleoli at mitosis and meiosis (Bidau, 1986; Martí, 2002) (Fig. 2a). A second, weaker, signal was always observed at the paracentric region of both B_N chromosomes (Fig. 2b). No other autosome nor the X chromosome or the unstable B chromosomes assayed showed a hybridisation signal at any meiotic stage.

The difference in intensity between the S_8 and B_N signals allowed their localisation and identification in all premeiotic, meiotic and spermiogenesis stages (Fig. 2). For example, spermatids of 1B individuals at different stages of maturation clearly showed one or two signals (of different size) at a 1:1 ratio, as expected from a Mendelian segregation rate for the B chromosome (Fig. 2c, d). It is interesting that when both signals were present, they were always observed near the centriole adjunct region of the spermatid (Fig. 2c, d) while no clustering of the Bs and S_8 signals was apparent at any previous stage (i.e. preleptotene nuclei of Fig. 2e). In 0B individuals, the S8 signal was also observed always at the same position in spermatids (Martí, 2002). Also, microspermatids with a weak signal were observed (Fig. 2d) indicating that they originated through anaphase lagging and micronucleus formation by the B chromosome. We cannot completely discard the possibility that some microspermatids carrying the fluorescent signals contained lagging S_8 chromosomes. However, this is doubtful due to the very regular meiotic behavior of S bivalents in this species. Anyway, the proportion of such microspermatids would be negligible.

Discussion

B chromosomes usually show non-Mendelian inheritance, so that their rate of meiotic transmission as univalents is higher than 0.5, which constitutes the basis for their parasitic nature (Camacho et al., 2000). A number of different B chromosome drive mechanisms have been described and these are the favoured explanation for B chromosome polymorphisms (Jones and Rees, 1982; Jones 1991; Camacho et al., 2000). Furthermore, some Bs may show meiotic distorted assortment with respect to other chromosomes of the A complement, especially sex chromosomes. Although few cases have been described (Fontana and Vickery, 1973; López-León et al., 1996; Nokkala et al., 2000), they involved achiasmate association and non-independent assortment of the X and B chromosomes to opposite poles. In *D. pratensis*, however, the association of both elements leads to their preferential migration to the same spindle pole (Bidau, 1986). Furthermore, the behaviour of the B_N chromosome is completely different from that of the mitotically unstable B chromosome also found in this species, since the unstable B shows no deviation from independent transmission with respect to the X (Bidau, 1987; Martí, 2002).

The most obvious explanation for X-B assortment-distortion in *D. pratensis*, is the non-random association of X and B during prophase I. Due to their usual heterochromatic nature, B and X chromosomes in the grasshopper *Metaleptea brevicornis* tend to associate achiasmatically during male first prophase, although random association and non significant differences between meiotic substages were observed (Grieco and Bidau, 1999). Moreover, in other grasshopper species, such as *Phaulacridium vittatum* and *Eyprepocnemis plorans*, where significant associations between B and X have been observed, both elements either segregated randomly (John and Freeman, 1975) or preferentially to opposite poles independently of previous degree of association (López-León et al., 1996). In *D. pratensis* however, the association between both univalents clearly followed a recurrent pattern by which, in all individuals, the maximum degree of association was attained during zygotene-early pachytene. Although the percent of X-B association decreased steadily toward MI, at which moment most associations had lapsed, a positive correlation seemed to occur between percent association at all stages and X-B assortment ratio. It is worth noting that, in the case of the only 2B individual found to date (Bidau, 1986), both B's associated non-chiasmatically with high frequency during prophase I and segregated regularly at anaphase I, while associations between both Bs and the X were random.

The frequent X-B meiotic association in *D. pratensis* might be interpreted as evidence that the B_N chromosome derived from the X chromosome. If residual homology between both elements existed, for example, if both shared repetitive DNA sequences as in the grasshopper *Eyprepocnemis plorans* (López-León et al., 1996), X-B associations could be partly homologous and thus, not random. A likely candidate for a repetitive sequence on the Bs is ribosomal DNA, which has been found in B chromosomes with certain frequency (Bidau, 1986; Green, 1990; Cabrero et al., 1999; Camacho et al., 2000). In a previous paper (Bidau, 1986) it was described that the B_N chromosome of *D. pratensis* was frequently associated to a nucleolus at its secondary constriction near the centromere in 1B and 2B individuals. The B thus provided an extra NOR in addition to the standard NOR of the species located in the S_8 chromosome. The technique used was silver impregnation which, in grasshoppers, reveals nucleoli but not NORs during meiosis. Thus, inactive NOR sequences, which could be widespread within the genome, would have gone completely undetected by this method, whereas inactive rDNA sequences are sometimes widespread within the genome (López-León et al., 1999; Dobigny et al., 2002). Our FISH analysis allowed us to determine that (1) both B_N variants carry rDNA sequences which, as shown by silver impregnation (Bidau 1986), represent active NORs, (2) since the strength of the hybridisation signal is much weaker in B_N than in S_8, B_N seems to carry less rDNA repeats than S_8, and (3) the X chromosome does not harbour rDNA sequences despite showing a secondary constriction in an equivalent paracentomeric position as B_N. The latter observation makes a direct derivation of B_N from the X unlikely, despite their overall similarity and behaviour, and points to either a B origin from the only A chromosome harbouring rDNA (S_8), by means of polysomy which is a widely accepted mechanism for B chromosome origin (Hewitt, 1979; Camacho et al., 2000), or else from a different A chromosome and later acquisition of the

rDNA through translocation. NOR regions are prone to continuous rearrangement and are likely places for breakage (Camacho et al., 2000). An interesting observation was that, in spermatids, the rDNA sequences of B_N and S_8 were localised very near one another in a specific nuclear region, although no evidence of co-localisation of both was obtained for meiotic or premeiotic stages. Such a close location might, in principle, facilitate chromosomal rearrangement between an ancestral B and S_8. Recently, Cabrero et al. (2003) produced evidence that in the grasshopper *Eyprepocnemis plorans* although B chromosomes from Spanish and Moroccan populations derive from the X, those from Caucasian populations may have derived from the small S_{11} chromosome. Thus, further meiotic and molecular analyses are needed to establish the origin of B chromosomes of *D. pratensis*.

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