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The B-chromosome polymorphism of the grasshopper *Eyprepocnemis plorans* in North Africa: II. Parasitic and neutralized B_1 chromosomes

M Bakkali, F Perfectti and JPM Camacho
Departamento de Genética, Facultad de Ciencias, Universidad de Granada, 18071, Granada, Spain

The transmission of the B_1 chromosome through females has been analysed in three Moroccan populations (Smir, SO.DE.A. and Mechra) of the grasshopper *Eyprepocnemis plorans*. We analysed transmission ratio (k_B) variation at two levels: intra-individual (to test female age effects) and inter-individual (to test for A chromosome effects). In 81.8% of females, k_B did not differ among successive egg-pods, suggesting no effect of female age. The remaining females (18.2%), showed significant differences in k_B values among egg-pods, but without clear temporal patterns. In Smir, k_B ranged between B elimination (0.244) and B accumulation (0.689) but there was no net accumulation (mean ± s.e. = 0.463 ± 0.045). In SO.DE.A., all females analysed transmitted B_1 at a Mendelian rate, with a mean k_B equal to 0.512 ± 0.020. In Mechra, k_B ranged from 0.341 to 0.972, with mean k_B (0.575 ± 0.029) showing a net B accumulation. All these observations suggest that the B_1 chromosome could be at a drive-suppression stage in Smir and Mechra, but that it has already been neutralised in SO.DE.A.
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Introduction

B chromosomes are large pieces of dispensable supernumerary DNA usually lacking functional genes, showing irregular modes of transmission and, in many cases, decreasing the fitness of individuals carrying them. In spite of being a frequent phenomenon (about 15% of described living species have been estimated to carry them; Beukeboom, 1994) discovered long ago (Wilson, 1907), their origin and evolution is beginning to be understood only recently (for review, see Camacho *et al*, 2000).

Two classical hypotheses have been proposed to explain the maintenance of B chromosomes in natural populations. The parasitic or selfish model (Östergren, 1945; Nur, 1966a, b; Nur, 1977; Jones, 1985; Shaw and Hewitt, 1990) proposes that B chromosomes maintain themselves by the action of accumulation mechanisms (eg, meiotic drive) that counterbalance their deleterious effects on host fitness. Most B chromosomes fit this model. The heterotic model (White, 1973), however, argues that B chromosomes lacking drive may be maintained because of their beneficial effects on the fitness of individuals carrying small number of Bs. Some B chromosomes are good candidates to be heterotic since they have an obvious beneficial effect on carrier fitness (eg, *Nectria haematococca* (Miao *et al*, 1991a, b) and *Avena sativa* (Dherawattana and Sadanaga, 1973)), but their population dynamics has been analysed only in *Allium schoenoprasum*, where Bs lack drive and are beneficial in increasing viability from seed to seedling (Holmes and Bougourd, 1989; Plowman and Bougourd, 1994).

Camacho *et al* (1997) have proposed a long-term model for the evolution of parasitic B chromosomes, in which the establishment of a B chromosome depends on the existence of an accumulation mechanism facilitating the initial increase in B frequency, surpassing possible deleterious effects on host fitness. The B frequency increase provides the conditions for the selective spread of host genes leading to the suppression of B drive and thus to the neutralisation of the B which, after a long stochastic decline, could be eliminated. The model finally suggests the possibility of regeneration of the polymorphism by the substitution of the neutralised B variant by a newly arisen B variant showing accumulation. Under this model, the same B could show drive or not, depending on its evolutionary status at the moment of analysis. In addition, differences in drive could depend, at least theoretically, on the existence of cryptic variation (ie, not recognisable by cytological techniques) in the B chromosomes.

The study of the B chromosome polymorphism of the grasshopper *Eyprepocnemis plorans* in the Iberian Peninsula has shown all the stages of this life cycle, ie driving and neutralised Bs (Camacho *et al*, 1997; Zurita *et al*, 1998) as well as polymorphism regeneration (Zurita *et al*, 1998).

The B chromosome polymorphism has recently been found in Moroccan populations of *E. plorans*, with the most widespread B variant being similar, in C-banding (Bakkali *et al*, 1999) and FISH (Cabrero *et al*, 1999) patterns, to the most widespread B variant in the Iberian Peninsula (B_1). Research in the Iberian Peninsula

Correspondence: Dr M Bakkali, Departamento de Genética, Facultad de Ciencias, Universidad de Granada, 18071, Granada, Spain. E-mail: mbakkali@ugr.es
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(Henriques-Gil and Arana, 1990; López-León *et al*, 1992; Camacho *et al*, 1997; Zurita *et al*, 1998) has shown that B_1 has been neutralised and replaced by another variant (B_2) in the Granada (where it has later been neutralised) and East-Málaga populations, and by B_5 in Fuengirola (Málaga) (also neutralised). In addition, B_2 has recently been replaced by B_{24} in Torrox (Málaga).

Since the status of the *E. plorans* B polymorphism in Morocco is unknown, the purpose of this paper is to analyse B_1 transmission through females (which is the driving sex in Iberian populations) from three Moroccan populations to ascertain its evolutionary status.

Materials and methods

We sampled three Moroccan populations of the grasshopper *Eyprepocnemis plorans*. In 1996, we collected 57 males and 34 females from Mechra (close to Mechra-bel-Ksiri) and 44 males and 30 females from SO.DE.A. (near to Ksar-el-Kebir). In 1997, we caught 34 males and 32 females from Smir (between Ceuta and Tetouan). The three populations were located on a north–south transect, with 129 Km from Smir to SO.DE.A. and 75 Km from SO.DE.A. to Mechra (see map in Bakkali *et al*, 1999).

To perform controlled crosses, the number of B chromosomes in males can be detected *in vivo* by means of the technique described in López-León *et al* (1993), one that is not possible in females. For this reason, many useless crosses are inevitably set up in all transmission analyses. To minimise these, and since B chromosome frequency was relatively low in these populations (Bakkali *et al*, 1999), we artificially increased B chromosome frequency in each population by collectively crossing two-thirds of females with all males carrying B chromosomes (high B frequency, or H crosses). The remaining females were crossed to males lacking B chromosomes (low B frequency, or L crosses). Grasshopper culture conditions were similar to those described in Herrera *et al* (1996).

Controlled crosses destined for the study of B chromosome transmission through females were performed by crossing, individually, F_1 0B males, from both L or H crosses, with virgin F_1 females from H crosses. Eighty such crosses were performed in each population. All crosses involved a male and a female from the same population.

Egg pods were incubated at 28°C for 11 days before egg dissection and embryo fixation, as described by López-León *et al* (1993). Females were dissected after being anaesthetised with ethyl acetate vapour, and the ovaries were fixed in 3:1 ethanol-acetic acid. The cytological analysis of all fixed materials was performed by the techniques described in Camacho *et al* (1991) and López-León *et al* (1992).

We calculated egg fertility as the proportion of eggs containing an embryo in an egg-pod. The B chromosome transmission ratio (k_B) was calculated as the mean number of B chromosomes in the progeny divided by the total number of Bs in the parents (in the present case, divided by the number of Bs in the female parent, since all fathers lacked Bs). To compare k_B with the expected Mendelian rate (0.5) we used the Z-test proposed by López-León *et al* (1992):

$$Z = \frac{(k_B - 0.5)}{\sqrt{\frac{0.25}{N}}}$$

where N is the total number of embryos. Z values higher than +1.96 indicate B accumulation, and Z values lower than −1.96 indicate B elimination.

Differences in k_B among pods laid by the same female were tested by a contingency χ^2 test performed with the R × C computer program, which employs the metropolis algorithm to obtain an unbiased estimate of the exact *P*-value (Raymond and Rousset, 1995). Twenty batches of 1000 simulated replicates were performed in all cases, and the sequential Bonferroni method was used to minimise type I errors.

The overall tendency for k_B variation in each population was analysed by means of a one-group *t*-test, with an expected mean equal to 0.5. Nested ANOVA was performed to partition the k_B variance into population and individual levels. One-way ANOVA was used to test for B effects on egg fertility, and correlation analysis was performed to investigate possible relationship between k_B and egg fertility.

Results

The transmission of the B_1 chromosome was analysed at two levels: among pods within females (intra-individual level) and among females within populations (inter-individual level). Thirty-eight out of 240 controlled crosses performed were informative for B transmission (ie, involving females harbouring the B_1 chromosome and yielding at least 25 analysable offspring): nine from Smir, six from SO.DE.A. and 23 from Mechra.

Intra-individual level

Several successive egg pods were analysed in 33 out of 38 controlled crosses. After applying the sequential Bonferroni method, four crosses from Smir (44.44% of females from this population) and two from Mechra (10.53%) showed significant differences in B_1 transmission among successive egg pods (Table 1). No cross in SO.DE.A. showed significant difference among successive egg pods. These temporal changes in B_1 transmission ratio showed no consistent temporal pattern (Table 1), which, in addition to the fact that they were absent in most crosses (81.8%), suggest that female age did not affect B_1 transmission ratio. The situation in Smir, however, needs further study since almost half of the crosses analysed showed temporal variation in B_1 transmission ratio.

Inter-individual level

One (11.1%) out of the nine females carrying one or more B_1 in Smir showed B_1 accumulation (k_B significantly higher than 0.5), three (33.3%) showed B_1 elimination (k_B significantly lower than 0.5), and the remaining five females (55.6%) showed a near-Mendelian transmission (k_B not significantly different from 0.5) (Table 2). In this population, k_B was between 0.244 and 0.689 (mean ± s.e. = 0.463 ± 0.045). In SO.DE.A., the six females analysed transmitted B_1 at a Mendelian ratio (Table 2), with a mean k_B equal to 0.512 ± 0.020. In Mechra, seven out of 23 females analysed (30.4%) showed B_1 accumulation, three

Table 1 B_1 transmission in six females where k_B (transmission ratio of B_1) varied between their pods. These females represented 18.2% of a total of 33 where more than one pod were analysed

Population	*Cross code*	*Bs in female*	*Pod*	*Eggs*	*Embryos*	*Fertility*	*Embryo offspring with*			k_B	Z	*Between-pod k_B comparison*	
							0B	*1B*	*2B*			χ^2	*P*
Smir	SM27	1	1	52	47	0.904	31	16	0	**0.340**	**–2.188**		
			2	48	48	1.000	31	17	0	**0.354**	**–2.021**		
			3	50	41	0.820	35	4	0	**0.103**	**–4.964**		
			4	47	46	0.979	39	7	0	**0.152**	**–4.718**	14.732	0.0021
	SM37	1	1	29	23	0.793	21	2	0	**0.087**	**–3.962**		
			2	28	27	0.964	12	10	0	0.455	–0.426		
			3	29	29	1.000	15	13	0	0.464	–0.378	9.439	0.0095
	SM38	1	1	42	40	0.952	16	23	0	0.590	1.121		
			2	48	48	1.000	2	45	0	**0.957**	**6.272**		
			3	20	20	1.000	15	5	0	**0.250**	**–2.236**	35.567	0.000
	SM75	2	1	30	28	0.933	18	10	0	**0.179**	**–3.402**		
			2	42	41	0.976	5	31	2	0.461	–0.487	17.777	0.0001
Mechra	M1	2	1	50	47	0.940	10	27	8	0.478	–0.298		
			2	39	39	1.000	1	19	18	**0.724**	**2.758**	12.097	0.002
	M51	1	1	42	38	0.905	28	10	0	**0.263**	**–2.920**		
			2	45	45	1.000	9	36	0	**0.800**	**4.025**	24.033	0.000

(13.0%) showed B_1 elimination and the remaining 13 (56.6%) showed a Mendelian ratio (Table 2). Mean k_B was 0.575 ± 0.029 (0.341 – 0.972).

As a whole, k_B did not differ significantly from the Mendelian ratio in Smir (one-group t-test, with expected mean equal to 0.5, $t_8 = –0.825$, $P = 0.433$) and SO.DE.A. ($t_5 = 0.629$, $P = 0.557$), but it indicated net accumulation in Mechra ($t_{22} = 2.600$, $P = 0.017$).

A nested ANOVA partitioned total k_B variance into population and individual levels (Table 3), showing significant differences among females within populations, but interpopulation differences did not reach significance.

There were no significant differences between the female k_{Bs} deduced from male and female embryo progeny in any of the populations analysed (Smir: $t_8 = 0.96$ and $P = 0.37$; SO.DE.A: $t_5 = 1.17$, $P = 0.30$; Mechra: $t_{22} = 0.31$, $P = 0.76$). This suggests that B_1 does not cause any sex-dependent mortality during early embryogenesis.

Table 2 shows that 1B females from the three populations yielded a total of 10 embryo offspring with 2B, among the 2425 embryos they produced. Since all male parents were 0B, these embryos should be derived from non-disjunction of the B univalent during female meiosis. This phenomenon thus occurred only in 0.41% of meioses.

Effects on egg fertility

One-way ANOVA performed on all types of female (including 0B ones) to compare egg fertility between 0B, 1B and 2B females, showed the absence of B effects in Smir (F = 0.63, *df* = 2, 32, $P = 0.54$), SO.DE.A. (F = 0.01, *df* = 1, 30, $P = 0.92$) and Mechra (F = 0.19, *df* = 2, 52, $P = 0.83$). A correlation analysis between k_B and egg fertility showed a significant negative correlation in Mechra ($r = –0.445$, $P = 0.033$) but not in Smir ($r = –0.126$, $P = 0.746$) or SO.DE.A. ($r = –0.122$, $P = 0.817$). The significance in Mechra, however, was removed by the application of the sequential Bonferroni test. In fact, it depended crucially on a single outlier cross whose elimination would give a non-significant correlation in the remaining crosses ($r = –0.078$, $P = 0.729$). Therefore, there was no obvious relationship between B_1 transmission and egg fertility.

Discussion

B chromosome transmission ratio has been shown to decrease with female age in the grasshopper *Myrmeleotettix maculatus* (Shaw and Hewitt, 1984). In Moroccan *E. plorans*, most crosses where several successive egg pods were analysed showed no variation in k_B. A minority of these crosses (most of them from the Smir population; see Table 1) showed significant differences in k_B between pods, although with no obvious trend to increase or decrease with female age. This variation in k_B could be due to factors related to the reproductive biology of *E. plorans*, which were not controlled in these experimental crosses. For instance, it has been shown that females get proteinaceous nutrients from male ejaculate, which they incorporate into the eggs they lay, thus increasing their rate of egg production (Pardo *et al*, 1995). In addition, Herrera *et al* (1996) reported male effects on female k_B. These observations suggest that factors such as the number of matings performed by a female before laying each egg pod or the number of days between mating and laying, could modify female reproductive conditions and change k_B from pod to pod.

The variable transmission ratios observed between different females in the same population (significant female effect, see Table 3) could be explained by: (a) an effect of the genetic background in which Bs are transmitted and, presumably, a control of A chromosomes over B transmission; (b) cryptic B-chromosome variation responsible for k_B variation; or (c) an unidentified environmental effect. Since the experimental crosses were performed in the laboratory under a controlled environment, explanation (c) is the least feasible. Since the presence of some type of A chromosome genetic control over B accumulation has been shown in several plant and animal

Table 2 Transmission analysis of B_1 in each controlled cross performed with specimens from Smir (SM), SO.DE.A (SO) and Mechra (M). $k_B = B_1$ transmission ratio

Cross	*Bs in Female*	*Pods*	*Eggs*	*Embryos*	*Fertility*	*Embryo offspring with*			k_B	*Z*
						0B	*1B*	*2B*		
SM_7	1	4	142	90	0.634	47	40	0	0.460	–0.750
SM_{27}	1	4	197	182	0.924	136	44	0	**0.244**	**–6.857**
SM_{29}	1	6	194	145	0.747	63	72	0	0.533	0.775
SM_{37}	1	3	86	79	0.919	48	25	0	**0.342**	**–2.692**
SM_{38}	1	3	110	108	0.982	33	73	0	**0.689**	**3.885**
SM_{43}	1	3	98	77	0.786	41	34	2*	0.494	–0.114
SM_{72}	1	2	61	48	0.787	24	24	0	0.500	0
SM_{39}	2	5	92	81	0.88	5	59	15	0.563	1.125
SM_{75}	2	2	72	69	0.958	23	41	2	**0.341**	**–2.585**
SO_{13}	1	1	36	32	0.889	14	14	0	0.500	0
SO_{15}	1	2	62	51	0.823	20	22	1*	0.558	0.762
SO_{38}	1	3	95	82	0.863	42	34	0	0.447	–0.918
SO_{47}	1	5	223	211	0.946	88	115	0	0.567	1.895
SO_{57}	1	3	90	78	0.867	39	31	1*	0.465	–0.593
SO_{61}	1	2	78	55	0.705	23	27	0	0.540	0.566
M_9	1	1	39	37	0.949	17	18	2*	0.595	1.151
M_{11}	1	2	59	59	1	35	23	0	0.397	–1.576
M_{13}	1	2	47	47	1	17	25	0	0.595	1.234
M_{19}	1	2	58	57	0.983	26	31	0	0.544	0.662
M_{31}	1	1	55	55	1	18	36	1*	**0.691**	**2.832**
M_{40}	1	2	51	40	0.784	12	24	0	**0.667**	**2.000**
M41	1	3	84	76	0.905	27	49	0	**0.645**	**2.524**
M_{43}	1	2	50	36	0.72	1	35	0	**0.972**	**5.667**
M_{48}	1	5	153	135	0.882	87	45	0	**0.341**	**–3.656**
M_{51}	1	2	87	83	0.954	37	46	0	0.554	0.988
M_{55}	1	3	48	46	0.958	27	19	0	0.413	–1.180
M_{57}	1	2	68	67	0.985	28	38	0	0.576	1.231
M_{61}	1	2	88	88	1	29	58	1*	**0.682**	**3.411**
M_{64}	1	3	95	92	0.968	58	34	0	**0.370**	**–2.502**
M_{68}	1	3	124	122	0.984	73	49	0	**0.402**	**–2.173**
M_{71}	1	3	93	85	0.914	44	38	1*	0.482	–0.329
M_{74}	1	2	74	70	0.946	35	34	1*	0.514	0.239
M_{78}	1	2	73	70	0.959	22	47	0	**0.681**	**3.010**
M_1	2	2	89	86	0.966	11	46	26	0.590	1.646
M_7	2	1	58	50	0.862	10	19	18	0.585	1.167
M_{46}	2	2	97	92	0.948	0	58	32	**0.678**	**3.373**
M_{70}	2	2	32	32	1	0	23	9	0.641	1.591
M_{79}	2	1	32	27	0.844	2	16	8	0.615	1.177

*Resulting from B-chromosome non-disjunction.

Table 3 Nested ANOVA partitioning total k_B variance into three levels: among populations, among females within populations and among egg-pods within females (error)

	df	*MS*	*F*	*P*
Among populations	2	0.121	3.17	0.054
Among females	34	0.039	1.71	0.038
Error	54	0.023		

species, including *E. plorans* (for review, see Camacho *et al*, 2000) we regard this possibility as the most plausible. Under this hypothesis, in Moroccan *E. plorans* populations, the A control over B transmission have been completed in SO.DE.A., where all females analysed showed k_B close to the Mendelian one. In Mechra, however, the A control seems to be in an earlier evolutionary stage since B_1 still accumulated in seven females (30.4%). In Smir, the A control was stronger since there was B_1 accumulation in a single female.

The observed differences in k_B among populations indicate that the B_1 polymorphism is at different evolutionary stages in these localities. The B seems to be near-neutral in SO.DE.A., it is almost neutralised in Smir, but is still selfish in Mechra. The demonstration that B_1 (the original B chromosome in *E. plorans* from Iberian and Moroccan populations) shows accumulation in Mechra, but not in the other two populations, confirms the existence of different evolutionary stages for a same B in different populations. Under the long-term evolution model (Camacho *et al*, 1997), the B_1 polymorphism in Morocco would be younger in the most southern population, Mechra, where B_1 still drives, and older in the two other populations where it is closer to neutralisation. In fact, in the three populations studied in this work, k_B values are roughly ordered in a north–south gradient, with the lowest value in the northern population (Smir, 0.463), an intermediate value at SO.DE.A. (0.512), and the highest k_B at the southern population (Mechra, 0.575). This gradient would coincide with an age gradient, with the B polymorphism being older in northern populations and younger in

southern populations. This would suggest a possible north–south invasion of the B in Morocco, and thus gives support to the hypothesis of the north Mediterranean origin of this B polymorphism (Bakkali *et al*, 1999).

One of the most widespread deleterious effects of parasitic B chromosomes is a decrease in host fertility (for review, see Jones and Rees, 1982). In *E. plorans*, the only significant effect of B chromosomes on host fitness was a decrease in egg fertility detected in Torrox for a selfish B (B_{24}) that had recently invaded the population to substitute a neutralised B (B_2) (Zurita *et al*, 1998). This effect, however, is not apparent in neutralised Bs as, for instance, B_2 (Camacho *et al*, 1997), but it can be forced to be manifest by limiting the number of female matings (Muñoz *et al*, 1998). This could suggest that deleterious effects of parasitic B chromosomes may disappear in parallel to drive neutralisation. The absence of obvious effects of the B_1 chromosome on egg fertility in all three Moroccan populations analysed in the present paper could indicate either a high tolerance to B chromosomes in these environments or an increase in tolerance that has evolved in parallel to the drive neutralisation. The introduction of B chromosomes and the analysis of egg fertility in Moroccan populations lacking B chromosomes, if they actually exist, could help to clear this point.

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