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B_1 Was the Ancestor B Chromosome Variant in the Western Mediterranean Area in the Grasshopper *Eyprepocnemis plorans*

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B chromosome · *Eyprepocnemis plorans* · FISH · Ribosomal DNA · Satellite DNA

Abstract
We analyzed the distribution of 2 repetitive DNAs, i.e. ribosomal DNA (rDNA) and a satellite DNA (satDNA), on the B chromosomes found in 17 natural populations of the grasshopper *Eyprepocnemis plorans plorans* sampled around the western Mediterranean region, including the Iberian Peninsula, Balearic Islands, Sicily, and Tunisia. Based on the amount of these repetitive DNAs, 4 types of B variants were found: B_1, showing an equal or higher amount of rDNA than satDNA, and 3 other variants, B_2, B_{24} and B_5, bearing a higher amount of satDNA than rDNA. The variants B_1 and B_2 varied in size among populations: B_1 was about half the size of the X chromosome in Balearic Islands, but two-thirds of the X in Iberian populations at Alicante, Murcia and Albacete provinces. Likewise, B_2 was about one-third the size of the X chromosome in populations from the Granada province but half the size of the X in the populations collected at Málaga province. The widespread geographical distribution of the B_1 variant makes it the best candidate for being the ancestor B chromosome in the whole western Mediterranean region.

B chromosomes are dispensable supernumerary elements frequently found in many eukaryote genomes in addition to the standard (A) chromosomes. They are mostly composed of repetitive DNAs such as ribosomal DNA (rDNA), satellite DNA (satDNA) and mobile elements [Camacho, 2005]. The grasshopper *Eyprepocnemis plorans* is an example that harbors all these components [López-León et al., 1994; Montiel et al., 2012]. In addition to the 22 + X0/XX standard chromosomes, more than 50 B chromosome variants have been described in the Iberian Peninsula on the basis of size and C-banding pattern [Henriques-Gil et al., 1984; Henriques-Gil and Arana, 1990; López-León et al., 1993; Bakkali et al., 1999], all of them being mostly made up of the same repetitive DNAs, i.e. rDNA and a 180-bp tandem repeat satDNA, thus suggesting their common descent [Cabrero et al., 1999].

B chromosomes are frequently polymorphic with many species showing 2 or more variants. For instance, Hewitt [1979] called attention on 10 grasshopper species showing more than one kind of B chromosomes, and Jones and Rees [1982], in their seminal B chromosome review, found more than 60 plant and animal species with 2 or more types of B chromosomes. Further cases have been found since then, the review of which is beyond the scope of the present study.

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On the basis of its widest distribution, Henriques-Gil et al. [1984] and Henriques-Gil and Arana [1990] suggested that B_1 was the ancestor variant for B chromosomes in the Iberian Peninsula, since it was found in populations from almost the whole Mediterranean coast, excepting the Granada and Western Málaga provinces, where it had been replaced by B_2, and Fuengirola, where it had been replaced by B_5. In addition, B_2 was replaced by B_{24} in the Torrox population [Zurita et al., 1998]. A comparison with B chromosomes found in eastern Mediterranean (Greece and Turkey) and Caucasian (Armenia and Dagestan) populations showed that B chromosomes from these latter populations were mostly composed of rDNA, with much smaller amounts of satDNA than western B chromosomes [López-León et al., 2008]. However, nothing was known about populations between these 2 extremes. Here, we analyze B chromosomes from Sicily and Tunisia in addition to 15 Spanish populations and conclude that the B_1 chromosome is the most widespread variant in the whole western Mediterranean region (including Sicily and Tunisia), which suggests that it was probably the ancestral B variant in this region.

Materials and Methods

Adult males of the grasshopper *E. plorans plorans* were collected at Palermo (Sicily, Italy) and Chott Mariem (Sousse, Tunisia) as well as in 15 Spanish locations: S'Albufereta and S'Esgleieta (Mallorca, Balearic Islands), San Juan (Alicante), Bullas and Cieza (Murcia), Mundo River (Albacete), Otivar and Salobreña (Granada), Maro, Nerja, Torrox, Algarrobo, Torre del Mar, Torremolinos, and Fuengirola (Málaga province).

Testes were fixed in 3:1 ethanol:acetic acid and stored at 4°C until use. The number of B chromosomes in each male was determined by squashing 2 testis follicles in 2% lacto-propionic orcein and visualizing primary spermatocytes at prophase or metaphase under an optical microscope. This allowed selection of B-carrying males from each population, in which we then performed 2-color fluorescent in situ hybridization (FISH) on chromosome preparations obtained by squashing of 2 testis follicles in 50% acetic acid. We used 2 DNA probes, one for rDNA and the other for the 180-bp tandem repeat satDNA, which are the 2 major constituents of B chromosomes. The technique employed was essentially that described in Cabrero et al. [1999]. Chromosome preparations were analyzed under a BX41 Olympus epifluorescence microscope, and photographs were captured with a DP70 cooled camera. Images were composed and optimized for brightness and contrast with the GIMP freeware.

Results and Discussion

Dual-color FISH analysis with rDNA and satDNA probes showed that the B chromosomes found in all natural populations sampled were mostly made up of these 2 tandem repeat DNAs, with the exception of the small short arm (fig. 1), in consistency with previous observations [Cabrero et al., 1999]. Comparative analysis of the FISH pattern and relative size of the X and B chromosomes from the same cell allowed classifying B variants into 4 types (fig. 1; table 1). The first type, B_1, is characterized by the presence of similar amounts of both DNA types (see Bs from San Juan, Bullas, Mundo and Cieza in the Iberian Peninsula, and those from Sicily and Tunisia), or else a slightly higher amount of rDNA (see Bs from S'Albufereta and S'Esgleieta in the Balearic Islands). The relative size of the B compared to that of the X chromosome also differed between Balearic and Iberian Bs, the former being about half the size of the X chromosome (likewise those in Tunisia), whereas the Iberian Bs, and those from Sicily, were about two-thirds the X chromosome size. The differences between these 2 types of B_1 chromosomes could simply be explained by changes in the amount of satDNA.

The remaining B variants observed showed a higher amount of satDNA than rDNA. The second type, B_2, shows satDNA and rDNA in about a 2:1 ratio, and its size is about one-third that of the X chromosome in Salobreña and Otívar (Granada province), but about half the size of the X chromosome in Maro, Nerja, Algarrobo, Torre del Mar and Torremolinos (Málaga province). Again, size differences for B_2 chromosomes between populations could be explained by changes in the amount of the satDNA, although we cannot rule out the possibility of changes in the amount in rDNA, since the ratio between the 2 types of repetitive DNA remains roughly stable between the 2 types of B_2. The third type, B_{24}, is about half the size of the X chromosome and carries satDNA and rDNA in a 3:1 ratio. It was present in Torrox and Algarrobo (Málaga) populations, and it arose from the B_2 variant [Henriques-Gil and Arana, 1990; Zurita et al., 1998] after amplification of the satDNA region. The fourth type, B_5, is about two-thirds the size of the X chromosome and carries satDNA and rDNA in about a 2:1 ratio. It was only found in Fuengirola (Málaga). It could have arisen from B_1 (which is the prevalent B variant in populations surrounding Fuengirola) [Henriques-Gil and Arana, 1990] by amplification of the satDNA region. The former inferences about the size of the B chromosomes are based on the assumption that the size of the X does not vary among populations, since B chromosome size estimations were relative to X chromosome size.

When B variants are placed in a map of the western Mediterranean region (fig. 2), it is evident that B_1 is present in all geographical zones analyzed (Sicily, Tunisia,

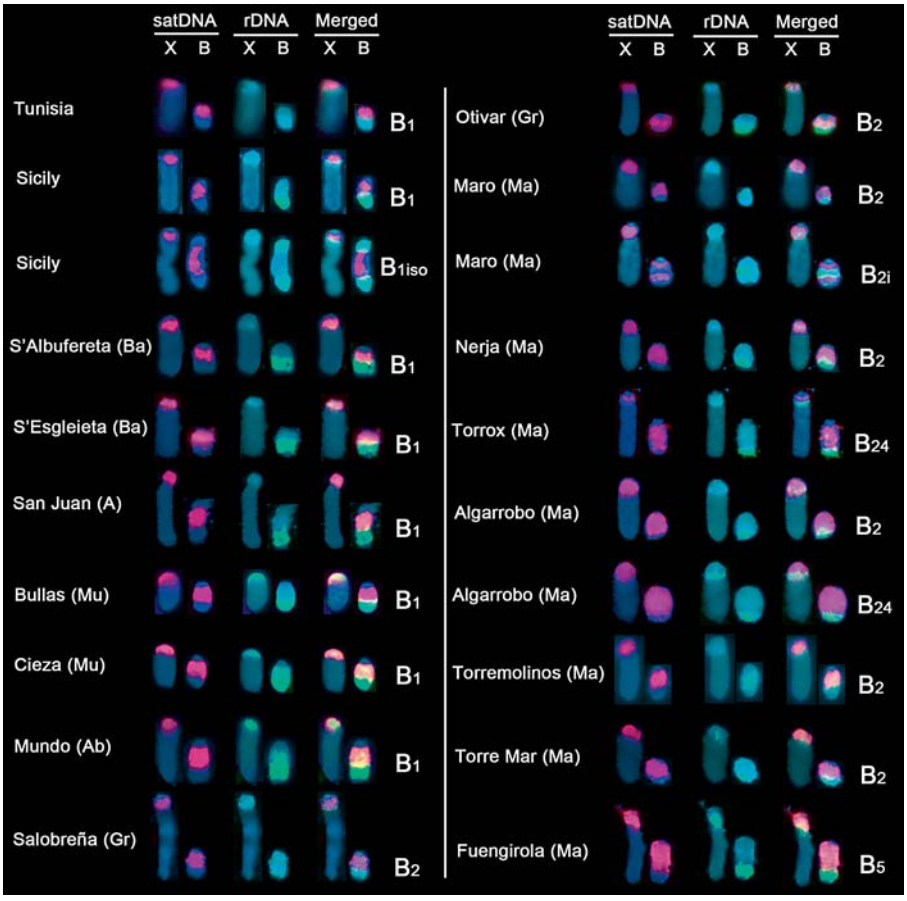

Fig. 1. Dual-color FISH patterns for the rDNA and satDNA probes found in X and B chromosomes from 17 natural *E. plorans* populations. X and B chromosomes depicted for each population are from the same diplotene cell. Given that X and B chromosomes show positive heteropycnosis during this meiotic stage, their relative sizes can be compared. Note that B size in respect to X size varies among populations, the B being about half the size of the X in S'Albufereta, S'Esgleieta, Nerja, and Algarrobo (B_2), about one-third in Salobreña and Otívar, and about two-thirds in San Juan, Mundo, Cieza, Algarrobo (B_{24}), Torrox, and Fuengirola populations. Note also that the relative amount of rDNA and satDNA varies among B types, being almost equal for the B_1 types, except for a slightly larger amount of rDNA in S'Albufereta and S'Esgleieta. The other variants show relatively smaller amounts of rDNA: one-third in B_2 and B_5 and one-fourth in B_{24}. A = Alicante; Ab = Albacete; Ba = Balearic Islands; Gr = Granada; Ma = Málaga; Mu = Murcia. Scale is variable for the different cells used, but the X chromosome in *E. plorans* is about 5 μm long.

Balearic Islands, and Iberian Peninsula). Bearing in mind that it is also present in Moroccan populations [Cabrero et al., 1999], we can conclude that B_1 is the most widely distributed B chromosome variant in the western Mediterranean region. In natural populations to the east of Sicily and Tunisia (e.g. Greece and Turkey), B chromosomes are mostly composed of rDNA, with considerably smaller amounts of satDNA than Western B chromosomes [Abdelaziz et al., 2007; López-León et al., 2008], so that, under the criteria employed here, they are rather different B types.

The B_1 variant was considered the ancestral B chromosome type in the Iberian Peninsula because it shows the widest geographical distribution along the Mediterranean coast, from Tarragona to Huelva [Henriques-Gil et al., 1984; Henriques-Gil and Arana, 1990]. Our present results suggest that B_1 was the ancestral B variant for the whole western Mediterranean region. Recent molecular analysis of a 1,510-bp SCAR (sequence-characterized amplified region) marker specific to the B chromosomes has shown extremely scarce variation in its DNA sequence between Bs from both western (Spain and Morocco) and eastern (Greece, Turkey and Armenia) Mediterranean regions, which, for a dispensable chromosome, probably implies a very recent origin for these B chromosomes [Muñoz-Pajares et al., 2011]. Two additional facts point to the recent origin of B chromosomes in the Iberian Peninsula, both assuming that B chromosomes invaded the Iberian Peninsula through coastal populations in which B chromosomes are universally present. First, B chromosomes are absent around the headwaters of the Spanish Segura River basin, because abrupt geographical barriers have impeded the advance of B-carrying individuals [Cabrero et al., 1997; Manrique-Poyato et al., in preparation]. Second, the Otívar population, by the Verde River in the Spanish Granada province, is located 10 km from the coast and has been invaded by B chromosomes in the last 35 years [Camacho et al., submitted], whereas B invasion had been completed prior to 1977 in 4 other populations closer to the coast [Camacho et al., 1980]. The fact that B

Table 1. Geographical location of the 17 populations analyzed, relative size of their B chromosomes in respect to the X chromosome, relative proportions of rDNA and satDNA in the B chromosomes, and B chromosome type

Population	Province	Country	Latitude	Longitude	Altitude, m	B/X length	rDNA/satDNA[a]	Type
Chott Mariem	Sousse	Tunisia	35°52′44″N	10°35′55″E	6	1/2	rDNA = satDNA	B_1
Micciulla	Palerm	Italy	38°06′20″N	13°19′12″E	97	2/3 4/5	rDNA = satDNA rDNA = satDNA	B_1 B_{1iso}
S'Albufereta	Balearic Islands	Spain	39°51′41″N	3°05′43″E	1	1/2	**rDNA** > satDNA	B_1
S'Esgleieta	Balearic Islands	Spain	39°39′12″N	2°38′33″E	103	1/2	**rDNA** > satDNA	B_1
San Juan	Alicante	Spain	38°23′45″N	0°25′19″E	22	2/3	rDNA = satDNA	B_1
Bullas	Murcia	Spain	38°02′30″N	1°40′06″W	636	2/3	rDNA = satDNA	B_1
Cieza	Murcia	Spain	38°13′58″N	1°24′58″W	165	2/3	rDNA = satDNA	B_1
Mundo	Albacete	Spain	38°28′01″N	1°47′22″W	436	2/3	rDNA = satDNA	B_1
Salobreña	Granada	Spain	36°44′20″N	3°35′31″W	2	1/3	rDNA < **satDNA**	B_2
Otívar	Granada	Spain	36°48′57″N	3°40′59″W	234	1/3	rDNA < **satDNA**	B_2
Maro	Málaga	Spain	36°45′34″N	3°50′42″W	114	1/2 1/2	rDNA < **satDNA** rDNA < **satDNA**	B_2 B_{2i}
Nerja	Málaga	Spain	36°44′44″N	3°53′56″W	6	1/2	rDNA < **satDNA**	B_2
Torrox	Málaga	Spain	36°44′24″N	3°57′26″W	30	2/3	rDNA < **satDNA**	B_{24}
Algarrobo	Málaga	Spain	36°44′48″N	4°02′49″W	5	1/2 2/3	rDNA < **satDNA** rDNA < **satDNA**	B_2 B_{24}
Torre del Mar	Málaga	Spain	36°45′15″N	4°06′15″W	15	1/2	rDNA < **satDNA**	B_2
Torremolinos	Málaga	Spain	36°37′50″N	4°30′12″W	53	1/2	rDNA < **satDNA**	B_2
Fuengirola	Málaga	Spain	36°31′59″N	4°38′29″W	3	2/3	rDNA < **satDNA**	B_5

[a] The predominant repetitive DNA in each B chromosome is shown in bold.

invasion continues at current times and the recent origin of these B chromosomes suggest the possibility that B_1 spread across the western Mediterranean populations could occur in recent historical times, presumably aided by the increase of Mediterranean commerce, since *E. plorans* is very common in most Mediterranean cultivations and can be easily transported in plants [Cabrero and Camacho, pers. observation].

It is remarkable that most of the changes that B chromosomes have experienced in the south of the Iberian Peninsula, giving birth to the B_{24} and B_5 variants, have mainly implied changes in the amount of satDNA. It has been shown that the replacement of B_2 by B_{24} in the Torrox population was based on significant drive for B_{24} but absence of it for B_2 [Zurita et al., 1998]. Cabrero et al. [1999] thus suggested that the replacement of ancestral

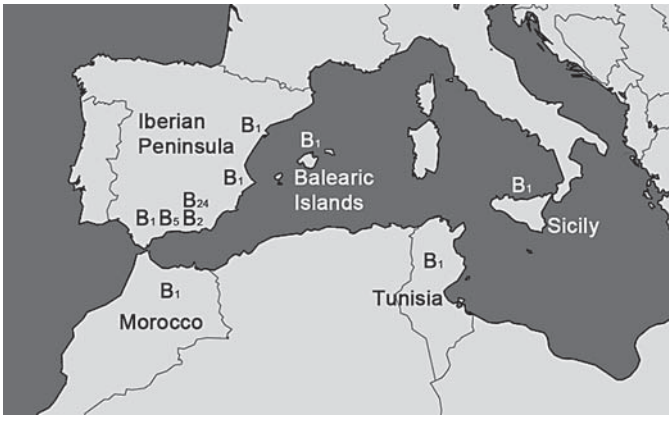

Fig. 2. Map of the western Mediterranean region showing the geographical distribution of the different B chromosome types found. Note the presence of the B_1 variant in all regions analyzed here and also in Morocco as previously shown by Cabrero et al. [1999].

variants (e.g. B_2 by B_{24} or B_1 by B_5) could be facilitated by a higher relative amount of satDNA in respect to rDNA. This could be valid for western Mediterranean Bs, where a relative increase in satDNA has been observed in the new variants in respect to the ancestral ones. In eastern Mediterranean Bs, however, this is less clear since Bs are mostly made up of rDNA, with very small amounts of satDNA, and this has not impeded their successful spreading to all regions analyzed [see López-León et al., 2008].

The appearance of new B chromosome variants in the progeny of controlled crosses, where none of the parents carried them, provides an estimate of the mutation rate of B chromosomes, since it can be safely assumed that this new variant arose by mutation of one of the Bs in the parents. Two different estimates point to the high mutability of B chromosomes in *E. plorans*, with rates ranging from 0.05 to 0.21% in Spanish populations [López-León et al., 1993] and from 0.21 to 9.6% in Moroccan populations [Bakkali and Camacho, 2004]. This explains how a young B chromosome system like this has given rise to so many different B types. Only in the Iberian Peninsula, Henriques-Gil et al. [1984] characterized 14 different B variants, and López-León et al. [1993] found additional variants. Including also the variants reported by Bakkali et al. [1999] in Morocco, Abdelaziz et al. [2007] in Greece, and López-León et al. [2008] in Turkey, Armenia, and Dagestan, more than 50 variants have hitherto been described in this species. Here we show a new B variant in the Maro population (B_{2i}) which presumably arose through a paracentric inversion changing the relative positions of the rDNA and part of the satDNA. Inversion is a frequent chromosome mutation affecting B chromosomes [López-León et al., 1993; Bakkali and Camacho, 2004]. The high incidence of mutations in the B chromosomes opens new evolutionary pathways to B chromosome polymorphism since some of the new variants can show higher transmission rates than their ancestor B and thus replace it, as documented for B_{24} in the Torrox population [Zurita et al., 1998].

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