Mol Genet Genomics (2014) 289:1209–1216
DOI 10.1007/s00438-014-0880-y

ORIGINAL PAPER

B chromosomes showing active ribosomal RNA genes contribute insignificant amounts of rRNA in the grasshopper *Eyprepocnemis plorans*

Mercedes Ruiz-Estévez · Liesbeth Badisco · Jozef Vanden Broeck · Francisco Perfectti · María Dolores López-León · Josefa Cabrero · Juan Pedro M. Camacho

Received: 20 January 2014 / Accepted: 14 June 2014 / Published online: 5 July 2014

Abstract The genetic inertness of supernumerary (B) chromosomes has recently been called into question after finding several cases of gene activity on them. The grasshopper *Eyprepocnemis plorans* harbors B chromosomes containing large amounts of ribosomal DNA (rDNA) units, some of which are eventually active, but the amount of rRNA transcripts contributed by B chromosomes, compared to those of the standard (A) chromosomes, is unknown. Here, we address this question by means of quantitative PCR (qPCR) for two different ITS2 amplicons, one coming from rDNA units located in both A and B chromosomes ($ITS2_{A+B}$) and the other being specific to B chromosomes ($ITS2_B$). We analyzed six body parts in nine males showing rDNA expression in their B chromosomes in the testis. Amplification of the $ITS2_B$ amplicon was successful in RNA extracted from all six body parts analyzed, but showed relative quantification (RQ) values four orders of magnitude lower than those obtained for the ITS_{A+B} amplicon. RQ values differed significantly between body parts for the two amplicons, with testis, accessory gland and wing muscle showing threefold higher values than head, gastric cecum and hind leg. We conclude that the level of B-specific rDNA expression is extremely low even in individuals where B chromosome rDNA is not completely silenced. Bearing in mind that B chromosomes carry the largest rDNA cluster in the *E. plorans* genome, we also infer that the relative contribution of B chromosome rRNA genes to ribosome biogenesis is insignificant, at least in the body parts analyzed.

Keywords B chromosome · Gene expression · qPCR · rDNA · rRNA genes

Communicated by S. Hohmann.

Electronic supplementary material The online version of this article (doi:10.1007/s00438-014-0880-y) contains supplementary material, which is available to authorized users.

M. Ruiz-Estévez · F. Perfectti · M. D. López-León · J. Cabrero · J. P. M. Camacho (✉)
Departamento de Genética, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain
e-mail: jpmcamac@ugr.es

L. Badisco · J. V. Broeck
Animal Physiology and Neurobiology, Zoological Institute, K.U. Leuven, Naamsestraat, 59, 3000 Louvain, Belgium

Introduction

Supernumerary (B) chromosomes are dispensable chromosomes which are mostly heterochromatic, for which reason they are usually considered to be genetically inert elements (for review, see Camacho et al. 2003; Camacho 2005). However, in the last decade, this view has been challenged by the finding of several cases of gene expression in B chromosomes. For instance, the existence of ribosomal RNA (rRNA) transcripts specifically coming from B chromosomes has been shown in the parasitic wasp *Trichogramma kaykai* (van Vugt et al. 2003), the plant *Crepis capillaris* (Leach et al. 2005) and the grasshopper *Eyprepocnemis plorans* (Ruiz-Estévez et al. 2012). In addition, Carchilan et al. (2009) showed, in rye, the presence of B-specific transcribed DNA sequences belonging to high-copy families with similarity to mobile elements, and Zhou et al. (2012) have characterized a B chromosome-linked scaffold that contains an actively transcribed unit. Recently, it has been shown the transcription of a protein-coding gene on B chromosomes of the Siberian roe deer (*Capreolus pygargus*) (Trifonov et al. 2013) and Banaei-Moghaddam

et al. (2013) have shown, by next generation sequencing, that about 15 % of the pseudogene-like fragments on B chromosomes are transcribed. All these results suggest that at least some of the DNA contained in B chromosomes are potentially functional. However, the biological meaning of the transcripts coming from B chromosomes remains unknown. Here, we attempt to shed some light on this question by quantifying rRNA transcripts specifically coming from B chromosomes in *E. plorans*, relative to all rRNA transcripts coming from either the standard (A) or B chromosomes.

In eukaryotes, 45S ribosomal DNA (rDNA) is one of the most abundant tandem repetitive DNAs in the genome. Each cistron contains three ribosomal (rRNA) genes, 18S, 5.8S and 28S separated by the internal transcribed spacers 1 and 2 (ITS1 and ITS2) and flanked by the external transcribed spacers and the intergenic spacer (IGS) (Long and David 1980). After transcription, the rRNA gene products will form part of the two ribosome subunits and the ITSs are eliminated during transcript maturation (Sollner-Webb and Tower 1986). The rDNA array is placed at the secondary constrictions of the chromosomes, where the nucleolar organizer regions (NORs) are located (Heitz 1931; McClintock 1934). Activation of rDNA transcription is dependent on cell status, with high energetic requirements being accompanied by high transcription rates. However, only about 50 % of the rDNA repeats are usually transcribed (Reeder 1999) at a given moment. The phenotypic visualization of rRNA transcription is the nucleolus, which appears attached to the NORs. The size of the nucleolus is positively correlated with the rate of rRNA synthesis (Mosgoeller 2004). Since the nucleolus is constituted by acidic and highly argyrophilic proteins a simple silver impregnation technique is enough to visualize it (Rufas et al. 1982).

Eyprepocnemis plorans is a grasshopper species which possesses an extra amount of rDNA located in supernumerary (B) chromosomes. This species shows a standard (A) genome composed of 22+X0/XX chromosomes and a high variety of B chromosomes; B chromosomes are mainly composed of repetitive DNA sequences, such as rDNA, satellite DNA and transposable elements (López-León et al. 1994; Cabrero et al. 1999; Montiel et al. 2012). The activity of the rDNA contained in the B chromosome was first shown by Cabrero et al. (1987) in a male carrying the B chromosome fused to the longest autosome. Teruel et al. (2007) later showed that this was not a unique case, by finding recurrent B-NOR activity in the Torrox (Málaga, Spain) population, a fact that has been corroborated by detecting B-specific ITS2 rDNA transcripts (Ruiz-Estévez et al. 2012), based on a characteristic adenine insertion found only in ITS2 sequences obtained from microdissected B chromosomes (Teruel 2009; Teruel et al. 2014). The fact that 16 out of the 17 ITS2 sequences obtained from the B chromosome showed the adenine insertion, but none of the 35 sequences from microdissected A chromosomes (or from 0B genomic DNA) carried it, indicates that this adenine is exclusive of the B chromosome and is present in the immense majority of the rDNA units located in the B chromosome (Teruel et al. 2014). More recently, we have shown that B chromosomes from other Spanish populations also display B-NOR activity but at rates lower than that observed in Torrox (Ruiz-Estévez et al. 2013).

All previous studies of B-rDNA activity in *E. plorans* have been performed cytogenetically in testes, and molecularly in samples of the whole body without distinguishing among the body parts, so it was impossible to know whether B activity was a general characteristic or else there are differences among cell types. Here, we analyze rRNA gene expression in six body parts (head, gastric cecum, accessory gland, wing muscle, hind leg and testis) from nine males where we had cytologically detected B-rDNA activity in the testes. For this purpose, we designed two primer combinations allowing us to distinguish between the transcripts coming from the B chromosome and those coming from any (A or B) chromosome. This allowed quantifying the relative contribution of B chromosomes to total rRNA amount, since the adenine insertion by which one of these primers is based on is exclusive to the B chromosome and is absent from the A chromosomes (see above).

Materials and methods

Biological samples

A sample of 23 males of the grasshopper *E. plorans* was collected in the Torrox population (Málaga, Spain). We used males because, contrary to females, they have active meiosis in gonad cells where it is possible to visualize nucleoli attached to the chromosomes. At the laboratory, they were anesthetized and dissected to remove a portion of each testicle which was then fixed in freshly prepared 3:1 ethanol–acetic acid and stored at 4 °C for cytological analysis. At the same time, under a stereomicroscope, we dissected out six different body parts: head (H), gastric cecum (GC), accessory gland (AG), wing muscle (WM), hind leg (HL) and testes (T), which were frozen in liquid nitrogen and stored at −80 °C for molecular studies. Determination of B number in each male was performed by squashing two testis tubules in 2 % lacto-propionic orcein. rDNA expression of the B chromosome was first analyzed by submitting testis follicles to silver impregnation following the protocol described in Rufas et al. (1982), with an additional 1 % Giemsa stain step to differentiate the chromatin (blue–green) from the nucleoli (brown). In both techniques, we visualized primary spermatocytes at prophase or metaphase

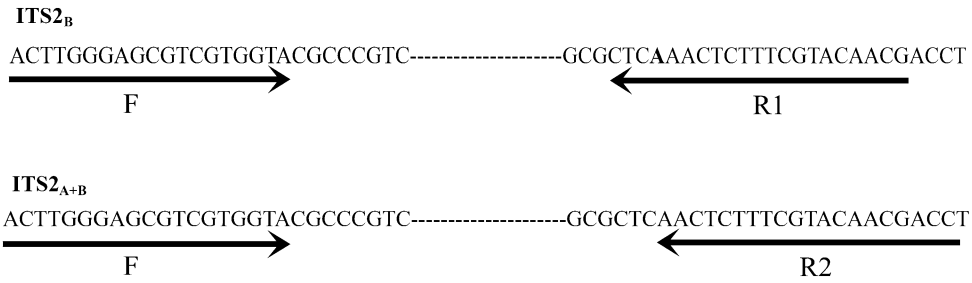

Fig. 1 Sequence of the primers employed to amplify the $ITS2_B$ and the $ITS2_{A+B}$ amplicons. The forward (*F*) primer was common but the two reverse (*R1* and *R2*) primers were anchored in the same region but whereas *R1* was anchored in the three adenine residues being exclusive of the B chromosome rDNA (Teruel 2009; Teruel et al. 2014), the *R2* one finished with two of these adenine residues, so that it could amplify the ITS2 region from both A and B chromosomes

I under an Olympus microscope (DP70). At least 20 diplotene cells per male were visualized to analyze B-rDNA expression, manifested by the presence of nucleoli attached to the B chromosomes.

Total RNA extraction and complementary DNA synthesis

Total RNA extractions from frozen body parts were performed using Lipid Tissue Mini Kit (Qiagen) following the manufacturer's recommendations. After extraction, we submitted the RNA to another 20U DNase post-treatment (RNase-Free DNase Set, Qiagen) to discard any genomic DNA contamination. Quantity and purity of the RNA were measured with a NanoDrop spectrophotometer (version 3.1.2., NanoDrop Technologies, Inc. Wilmington, DE, USA) and the quality was checked in a denaturing agarose gel to ensure the absence of nucleic acid degradation. We reverse transcribed 100 ng per sample of total RNA (PrimeScript™ RT reagent Kit, Perfect Real Time, Takara) using a combination of random and oligo dT primers. The resulting cDNA was diluted in RNase–DNase free water 1:10 (work solution).

B-rDNA expression in different body parts

Target and housekeeping gene primers

For rRNA gene expression analysis, we performed qPCR on the cDNA, obtained from total RNA extracted from each body part, using two primer pairs allowing us to distinguish whether transcription had taken place in an ITS2 unit specifically located in the B chromosome or in one located either in A or B chromosomes. This could be achieved by designing, with the Primer 3 v.0.4.0 software, a single forward (F: 5′ ACTTGGGAGCGTCGTGGTA 3′) and two reverse primers, one of them anchoring in the three adenine residues specific to B chromosomes (Teruel 2009; Ruiz-Estévez et al. 2012; Teruel et al. 2014) (R1: 5′ CGTTGTACGAAAGAGTTTGAG 3′) and the other (R2: 5′ AGGTCGTTGTACGAAAGAGTT 3′) finishing its anchoring in the second of these three adenines (Fig. 1). The F/R1 primer combination was assayed at different PCR annealing temperatures, in order that no amplification was observed in 0B individuals and thus assuring that the amplicon yielded by these primers is B-specific. The optimal temperature was 73.2 °C, and it was used in all qPCR experiments performed with the F/R1 and F/R2 primer combinations. This allowed performing quantitative analysis of those amplicons that were specifically transcribed from the B chromosomes ($ITS2_B$) and those potentially coming from any rDNA unit ($ITS2_{A+B}$), respectively.

To amplify the housekeeping genes (HKGs), we used the primer sequences provided by Van Hiel et al. (2009) and Chapuis et al. (2011) for Tubulin (TubA1), Armadillo (Arm), Actin (Act), GAPDH, Ubiquitin (Ubi), Elongation Factor 1α (EF1α), Ribosomal Protein 49 (RP49) and the homolog for CG13220. Primers for both target and HKGs were tested by qPCR at different conditions (see below). PCR products were visualized in a 1.5 % agarose gel, cleaned with Gen EluteTM PCR Clean-Up Kit (Sigma), sequenced by Macrogen Inc, and analyzed with BioEdit software (version 7.1.3.0.) (Hall 1999) before searching for sequence homologies at the NCBI site using BLAST (Basic Local Alignment Search tool).

Housekeeping reference genes' validations and relative quantification of B-rDNA expression

After ensuring the specificity of the HKG primers, we determined the most stable HKGs in our samples carrying out a geNorm analysis (Vandesompele et al. 2002). Standard curve analysis was used to determine the efficiency of the selected HKGs. Then we estimated the relative expression level of $ITS2_B$ and $ITS2_{A+B}$ in the body parts by means of qPCR. The reaction mixtures contained 5 µl 2× SensiMix™ SYBR Mastermix (SensiMix™ SYBR Kit,

Bioline), 0.7 µM of each forward and reverse primer and 5 ng cDNA, in a final volume of 15 µl. We performed two types of qPCR due to the need to use different PCR conditions for HKG and ITS2 amplification, since the latter required fine tuning of the reverse primer and high melting temperature to avoid unspecific amplification. We amplified the same calibrator sample (comprising cDNA synthesized from the RNA of different body parts) in each run as part of the quantification method and to ensure that data resulting from the experimental samples were comparable. qPCR assays were run in the Chromo4 Real-Time PCR thermocycler (BioRad) and PCR conditions were the following: an initial denaturation at 95 °C for 10 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s (HKGs) or 73.2 °C for 15 s (target sequences), 72 °C for 15 s and a melting curve step to check the specificity of the reaction. We included a negative control without cDNA to ensure that the reagents were free of contaminating DNA. Where possible, reagents were combined in mixed solutions to minimize the number of manipulations, and each sample was amplified in triplicate and the entire experiment was done in duplicate. Opticon Monitor v.3.1. software was used to export the qPCR raw data from the Chromo4 instrument. Relative Quantification (RQ) of the two transcripts was obtained following the "Efficiency calibrated mathematical method for the relative expression ratio in real-time PCR" (Roche Applied Science, Technical Note No. LC 13/2001), by referring the amplifications of both target genes to that of the $ITS2_{A+B}$ calibrator sample.

Statistical analysis

To compare RQ levels between the six body parts analyzed from nine males with two replicates, and the two different amplicons ($ITS2_B$ and $ITS2_{A+B}$), we used a generalized randomized block design, with individual as a random block and body part and amplicon as fixed factors. The statistical analysis was done in R (R Development Core Team 2008) by means of a linear mixed-effects model, with post hoc multiple comparisons by Tukey contrasts, using packages nlme (Pinheiro et al. 2013) and multicomp (Hothorn et al. 2008). We tested different models, including or excluding the number of B chromosomes as a covariate, using the AKAIKE information criterion.

Results

To analyze the relative B-rDNA expression between body parts by qPCR, we chose nine males with active B-NOR, i.e., showing a nucleolus attached to the B chromosome in diplotene cells (Fig. 2). Four of these males carried 1B, three 2B, one 3B and one 4B.

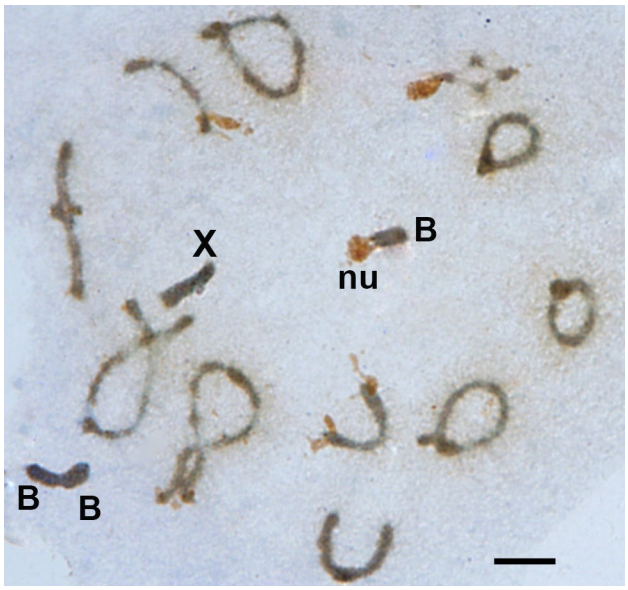

Fig. 2 Diplotene cell from a Torrox male carrying 3B chromosomes, one of them showing an attached nucleolus (*B* B chromosome, *X* X chromosome, *nu* nucleolus). *Bar* 5 µm

Total RNA extracted from the body parts of the nine males and subsequently treated with DNase showed high purity as was indicated by the absorbance ratio 260:280 nm (=1.9–2). Some of the HKGs showed two peaks (unspecific amplification) in the melting curve graph (results not shown), for which reason they were rejected as reference genes. After sequencing and confirming the three remaining HKGs (RP49, Tub1A and Armadillo), the geNorm analysis determined that Tub1A and Armadillo were the most stable HKGs in our samples. HKGs and target primers showed amplification efficiency values of 95–105 %. The target sequences ($ITS2_B$ and $ITS2_{A+B}$) showed homology with the ITS2 sequences reported by Teruel et al. (2014) for this species (Genbank accession numbers: JN811827–JN811902).

We obtained the $ITS2_B$ amplicon in the cDNA of each body part of the nine males, with the single exception of the gastric cecum of one of the 1B males. Sequencing of these amplicons showed that they corresponded to the expected ITS2 region. This suggests that the expression of the rDNA specific to B chromosomes takes place in all body parts analyzed, and is not restricted to the gonads only.

The statistical analysis of the RQ values observed for the six body parts and the two amplicons (see raw data in Table 1) was performed both including and excluding the number of B chromosomes as a covariate. In both cases, there were significant differences between amplicons and body parts, but the model excluding the number of Bs showed lower AIC values (379.6 vs 396.8) thus being the best model explaining the variation observed (Table 2).

Table 1 Mean RQ values of the two replicates per individual for two amplicons: $ITS2_B$ being specific to the B chromosome and $ITS2_{A+B}$ anchoring in the ITS2 region of both A and B chromosomes, in 6 different body parts of the nine *E. plorans* males analyzed

Id	Bs	$ITS2_{A+B}$							
		H	GC	AG	WM	HL	T	Mean	SE
1	1	0.555651	0.038881	0.190512	1.093634	0.551344	0.722041	0.525344	0.110197
2	1	0.035403	0.060430	1.592289	1.253340	0.234632	1.456554	0.772108	0.214007
3	2	0.362195	1.165208	1.094372	0.518909	0.604521	1.147137	0.815390	0.102747
4	1	0.235731	0.007396	1.681369	1.304306	0.204594	1.079085	0.752080	0.203278
5	2		0.456946	1.663356	2.187745	0.220441	1.920493	1.289796	0.279800
6	4	0.688722	0.745317	2.294372	2.128115	0.584681	1.359290	1.300083	0.338754
7	3	0.306315	1.109483	2.711266	4.017098	0.653458	2.075294	1.812152	0.453731
8	2	0.199163	0.271810	2.110085	2.069849	0.530175	1.953074	1.189026	0.328143
9	1	0.657103	0.668395	1.168247	1.464664	0.908664	2.489012	1.226014	0.194838
Mean		0.380035	0.502652	1.611763	1.781962	0.499168	1.577998		
SE		0.060706	0.110952	0.280138	0.266972	0.065675	0.159362		
Id	Bs	$ITS2_B$							
		H	GC	AG	WM	HL	T	Mean	SE
1	1	0.000142	0.000018	0.000000	0.000121	0.000239	0.000015	0.000089	0.000028
2	1	0.000032	0.000003	0.000249	0.000089	0.000002	0.000256	0.000105	0.000035
3	2	0.000013	0.000180	0.000033	0.000041	0.000029	0.000094	0.000065	0.000019
4	1	0.000009	0.000000	0.000125	0.000036	0.000001	0.000045	0.000036	0.000014
5	2		0.000000	0.000155	0.000544	0.000001	0.000291	0.000198	0.000100
6	4	0.000072	0.000063	0.000161	0.000135	0.000048	0.000077	0.000093	0.000014
7	3	0.000012	0.000117	0.000170	0.000081	0.000087	0.000173	0.000107	0.000025
8	2	0.000004	0.000004	0.000256	0.000104	0.000047	0.000248	0.000111	0.000043
9	1	0.000018	0.000033	0.000032	0.000088	0.000016	0.000098	0.000048	0.000010
Mean		0.000038	0.000047	0.000131	0.000138	0.000052	0.000144		
SE		0.000014	0.000017	0.000028	0.000051	0.000019	0.000032		

These males carried active B_{24} chromosomes in the gonads

H head, *GC* gastric cecum, *AG* accessory gland, *WM* wing muscle, *HL* hind leg, *T* testis

Table 2 Linear mixed-effects ANOVA with RQ values as dependent variable and amplicon and body part as fixed factors

	df	*F*	*P*
Intercept	1, 192	68.40	<0.0001
Amplicon	1, 192	223.19	<0.0001
Body part	5, 192	13.95	<0.0001
Amplicon × body part	5, 192	14.05	<0.0001

The RQ values were extremely different between amplicons, the average RQ values for the six body parts being more than 11,000 times higher for $ITS2_{A+B}$ (mean = 1.07, SE = 0.0091, $N = 106$) than for $ITS2_B$ (mean = 0.000093, SE = 0.0000012, $N = 106$) ($t = 11.43$, $df = 210$, $P < 0.0001$) (Fig. 3, Fig. S1).

Separate comparison, for each amplicon, showed highly significant differences between body parts for the $ITS2_{A+B}$ amplicon ($F = 15.5$, $df = 5, 92$, $P < 0.0001$) and less significant differences for the $ITS2_B$ one ($F = 2.81$, $df = 5, 92$, $P = 0.0186$) (Fig. 3). In both cases, the model excluding the number of B chromosomes was also the best model (AIC values: 256.2 vs 263.8 for $ITS2_{A+B}$, and −1,479.4 vs −1,355.2 for $ITS2_B$).

Pairwise comparisons between the six body parts, by means of post hoc Tukey contrasts, revealed that, for the $ITS2_{A+B}$ amplicon, accessory gland, wing muscle and testis showed 3.6 fold higher RQ values than the three other body parts (head, gastric cecum and hind leg) ($P < 0.0001$ in all cases; Table S1) (Fig. 3). These same body parts showed threefold higher RQ for the $ITS2_B$ amplicon, thus reflecting the $ITS2_{A+B}$ pattern (Fig. 3), but all pairwise comparisons failed to reach significance for the $ITS2_B$ ($P > 0.05$ in all cases; Table S2).

Discussion

Previous work has shown that, in *E. plorans* testis, total cell nucleolar area did not show significant intra-individual differences between cells where rRNA genes in the B chromosomes are active and those where they are inactive, suggesting that total cell demands for rRNA are tightly regulated, irrespectively of whether B chromosomes are

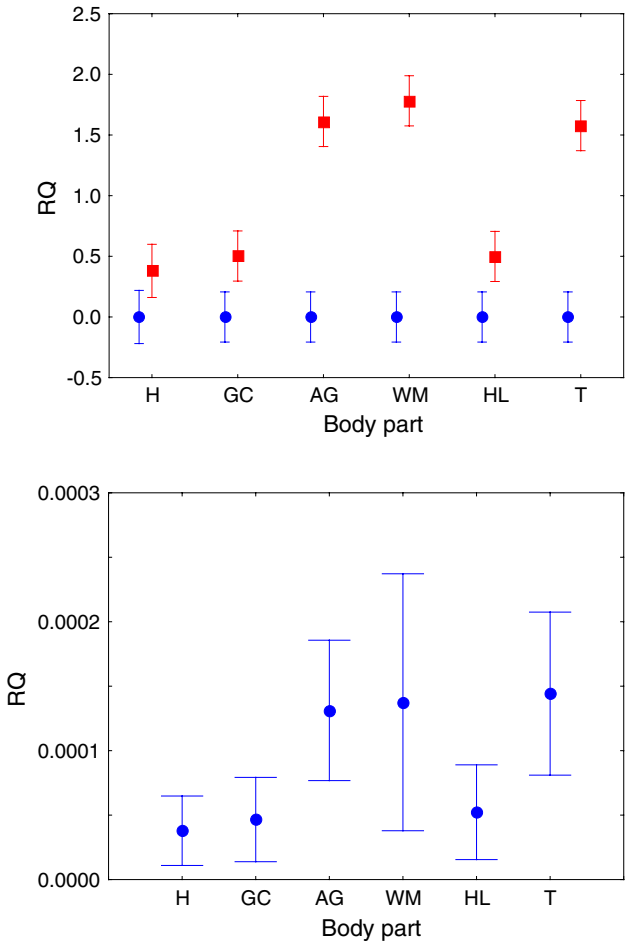

Fig. 3 Expression levels for the ITS2 region of rDNA in *E. plorans*. The *upper panel* shows the expression levels (RQ) of the $ITS2_B$ (*blue circles*) and the $ITS2_{A+B}$ (*red squares*) amplicons in six body parts of 9 males carrying active B_{24} chromosomes. The *lower panel* shows how the expression patterns for the $ITS2_B$ amplicon reflect those for the $ITS2_{A+B}$ one showed in the *upper panel*. (*H* head, *GC* gastric cecum, *AG* accessory gland, *WM* wing muscle, *HL* hind leg, *T* testis). *Bars* indicate 95 % confidence intervals

contributing rRNA or not (Teruel et al. 2007, 2009). It is thus conceivable that the observed differential expression of rRNA genes between body parts could have something to do with higher demands for rRNA in them, at least at the moment at which the individuals were frozen. Differential expression of rRNA genes was detected in *Plasmodium berghei*, where different rRNA "types" were transcribed depending on life cycle stage (Gunderson et al. 1987). Previous results have also shown that 18S rDNA transcription is regulated in different cell and tissue types, depending on the protein synthesis required by the individual (Hannan et al. 1998). Likewise, differential rDNA expression among tissues has been detected in abalone *Haliotis tuberculata* (Van Wormhoudt et al. 2011). In *E. plorans,* we found that three body parts (accessory gland, wing muscle and testis) showed about threefold higher RQ values than the three others. The finding of the same pattern for the $ITS2_{A+B}$ and $ITS2_B$ amplicons indicates that the observed upregulation in these three body parts is not a B chromosome-specific feature. No similar studies exist on animals, but differential expression of B chromosome sequences between plant tissues has recently been reported in rye in a study focused on pseudogene-like fragments residing in a B chromosome (Banaei-Moghaddam et al. 2013).

Our present results have revealed that the rDNA located in the B chromosomes of *E. plorans* males from the Torrox population is active not only in the gonad, but also in somatic body parts. The meaning of this B chromosome contribution to ribosome biogenesis will depend on the proportion of total rRNA transcripts coming from the B and whether they are functionally valuable. The fact that the same B-carrying individuals showing the $ITS2_B$ amplicon also showed nucleoli attached to the B chromosome in meiotic cells (Ruiz-Estévez et al. 2012, 2013; this paper) suggests that the rRNA molecules produced by the B chromosome are able to yield their phenotype, i.e., the nucleolus, thus being fully functional. This is also suggested by a recent analysis of the ITS2 region and part of the flanking 5.8S and 28S rRNA genes, by means of Roche 454 amplicon sequencing, which has shown that the 123 nt corresponding to these coding regions were fully conserved in the transcripts coming from the B chromosomes (Ruiz-Estévez et al. in preparation).

In spite of being functional, the fact that the frequency of the $ITS2_B$ amplicon was four orders of magnitude lower than that of the $ITS2_{A+B}$, indicates that the rRNA transcripts contributed by the B chromosome actually represent a very small proportion of all rRNA transcripts present in the body parts and cell types analyzed. This is consistent with the fact that, in the population analyzed, the frequency of B-carrying males showing expression of rRNA genes in the B chromosomes is usually lower than 50 % and, within these males, the proportion of cells showing nucleoli produced by the B chromosome is not higher than 29 % (Teruel et al. 2007, 2009; Ruiz-Estévez et al. 2012, 2013).

Taken together, these results suggest that the rRNA molecules produced by the B chromosome are fully functional, but that they represent only a minor fraction (0.01 %) of total rRNA molecules. Bearing also in mind that B chromosomes in this species can carry as many rDNA units as all A chromosomes together (Montiel et al. 2014), we can conclude that the effect of rRNA contribution by the B chromosome is only residual and of minor significance. This conclusion is valid for males, which were the subject of the current experiments, and it is consistent with the fact that B chromosomes in *E. plorans* are near-neutral with no significant effects on male fitness (Camacho et al. 1997).

Future studies should analyze the situation in females since a significant decrease in female fertility was reported in the population analyzed here (Zurita et al. 1998) and thus the meaning of the rRNA contributed by the B chromosomes could be different.

Acknowledgments We thank an anonymous reviewer for helpful suggestions that substantially improved the manuscript, and Karl Meunier for language revision. This study was supported by a grant from the Spanish Ministerio de Ciencia e Innovación (CGL2009-11917) and Plan Andaluz de Investigación (CVI-6649), and was partially performed by FEDER funds. M Ruíz-Estévez was supported by a FPU fellowship from the Spanish Ministerio de Ciencia e Innovación. The authors also gratefully acknowledge the KU Leuven Research Foundation (GOA/11/02) and the Research Foundation of Flanders (Belgium) for financial support.

Integrity of research All experiments comply with the current Spanish and Belgian laws. All institutional and national guidelines for the care and use of laboratory animals were followed. The authors declare that they have no conflict of interest.

References

Banaei-Moghaddam AM, Meier K, Karimi-Ashtiyani R, Houben A (2013) Formation and expression of pseudogenes on the B chromosome of rye. Plant Cell 25:2536–2544

Cabrero J, Alché JD, Camacho JPM (1987) Effects of B chromosomes of the grasshopper *Eyprepocnemis plorans* on nucleolar organizer regions activity. Activation of a latent NOR on a B chromosome fused to an autosome. Genome 29:116–121

Cabrero J, López-León MD, Bakkali M, Camacho JPM (1999) Common origin of B chromosomes variants in the grasshopper *Eyprepocnemis plorans*. Heredity 83:435–439

Camacho JPM (2005) B chromosomes. In: Gregory TR (ed) The evolution of the genome. Elsevier, San Diego, pp 223–286

Camacho JPM, Shaw MW, López-León MD, Pardo MC, Cabrero J (1997) Population dynamics of a selfish B chromosome neutralized by the standard genome in the grasshopper *Eyprepocnemis plorans*. Am Nat 149:1030–1050

Camacho JPM, Cabrero J, López-León MD, Bakkali M, Perfectti F (2003) The B chromosomes of the grasshopper *Eyprepocnemis plorans* and the intragenomic conflict. Genetica 117:77–84

Carchilan M, Kumke K, Mikolajewski S, Houben A (2009) Rye B chromosomes are weakly transcribed and might alter the transcriptional activity of A chromosome sequences. Chromosoma 118:607–616

Chapuis MP, Tohidi-Esfahani D, Dodgson T, Blondin L, Ponton F, Cullen D, Simpson SJ, Sword GA (2011) Assessment and validation of a suite of reverse transcription-quantitative PCR reference genes for analyses of density-dependent behavioural plasticity in the Australian plague locust. BMC Mol Biol 12:7

Gunderson JH, Sogin ML, Wollett G, Hollingdale M, de la Cruz VF, Waters AP, McCutchan TF (1987) Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. Science 238:933–937

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hannan KM, Hannan RD, Rothblum LI (1998) Transcription by RNA polymerase I. Front Biosci 3:376–398

Heitz E (1931) Die Ursache der gesetzmässigen Zahl, Lage, From und Grösse pflanzlicher Nukleolen. Planta 12:775–844

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biom J 50:346–363

Leach CR, Houben A, Bruce F, Pistrick K, Demidov D, Timmis JN (2005) Molecular evidence for transcription of genes on a B chromosome in *Crepis capillaris*. Genetics 171:269–278

Long EO, David IB (1980) Repeated genes in eukaryotes. Annu Rev Biochem 49:727–764

López-León MD, Neves N, Schwarzacher T, Heslop-Harrison JS, Hewitt GM, Camacho JPM (1994) Possible origin of B chromosome deduced from its DNA composition using double FISH technique. Chromosom Res 2:87–92

McClintock DJL (1934) The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. Z Zellforsch Mikrosk Anat 21:294–328

Montiel EE, Cabrero J, Camacho JPM, López-León MD (2012) Gypsy, RTE and Mariner transposable elements populate *Eyprepocnemis plorans* genome. Genetica 140:365–374

Montiel EE, Cabrero J, Ruiz-Estévez M, Burke WD, Eickbush TH, Camacho JPM, López-León MD (2014) Preferential occupancy of R2 retroelements on the B chromosomes of the grasshopper *Eyprepocnemis plorans*. PLoS ONE 9(3):e91820

Mosgoeller W (2004) Nucleolar ultrastructure in vertebrates. In: Olson MOJ (ed) The Nucleolus. Kluwer, New York, pp 10–20

Pinheiro J, Bates D, DebRoy S, Sarkar D, The R Development Core Team (2013) nlme: linear and nonlinear mixed effects models. R package version 3.1-113

R Development Core Team (2008) R: a language and environment for statistical computing. In: R Foundation for Statistical Computing. Vienna, Austria. ISBN 3-900051-07-0. http://www.R-project.org

Reeder RH (1999) Regulation of RNA polymerase I transcription in yeast and vertebrates. Prog Nucl Acid Res Mol Biol 62:293–327

Rufas JS, Iturra P, de Souza W, Esponda P (1982) Simple silver staining procedure for the localization of nucleolus and nucleolar organizer under light and electron microscopy. Arch Biol 93:267–274

Ruiz-Estévez M, López-León MD, Cabrero J, Camacho JPM (2012) B-chromosome ribosomal DNA is functional in the grasshopper *Eyprepocnemis plorans*. PLoS One 7(5):e36600. doi:10.1371/journal.pone.0036600

Ruiz-Estévez M, López-León MD, Cabrero J, Camacho JPM (2013) Ribosomal DNA is active in different B chromosome variants of the grasshopper *Eyprepocnemis plorans*. Genetica 141:337–345

Sollner-Webb B, Tower J (1986) Transcription of cloned eukaryotic ribosomal RNA genes. Annu Rev Genet 55:801–830

Teruel M (2009) Origen, expresión y efectos fenotípicos de un parásito genómico. Universidad de Granada, PhD

Teruel M, Cabrero J, Perfectti F, Camacho JPM (2007) Nucleolus size variation during meiosis and NOR activity of a B chromosome in the grasshopper *Eyprepocnemis plorans*. Chromosom Res 15:755–765

Teruel M, Cabrero J, Perfectti F, Camacho JPM (2009) Quantitative analysis of NOR expression in a B chromosome of the grasshopper *Eyprepocnemis plorans*. Chromosoma 118:291–300

Teruel M, Ruíz-Ruano F, Marchal JA, Sánchez-Baca A, Cabrero J, Camacho JPM, Perfectti F (2014) Disparate molecular evolution of two types of repetitive DNA in the genome of the grasshopper *Eyprepocnemis plorans*. Heredity 112:531–542

Trifonov VA, Dementyeva PV, Larkin DM, O'Brien PCM, Perelman PL, Yang F, Ferguson-Smith MA, Graphodatsky AS (2013) Transcription of a protein-coding gene on B chromosomes of the Siberian roe deer (Capreolus pygargus). BMC Biol 11:90

Van Hiel MB, Van Wielendaele P, Temmerman L, Van Soest S, Vuerinckx K, Huybrechts R, Broeck JV, Simonet G (2009) Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different development conditions. BMC Mol Biol 10:56

van Vugt JFA, Salverda M, de Jong H, Stouthamer R (2003) The paternal sex ratio chromosome in the parasitic wasp

Trichogramma kaykai condenses the paternal chromosomes into a dense chromatin mass. Genome 46:580–587

Van Wormhoudt A, Gaume B, Le Bras Y, Roussel V, Huchette S (2011) Two different and functional nuclear rDNA genes in the abalone *Haliotis tuberculata*: tissue differential expression. Genetica 139:1217–1227

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3. RESEARCH0034.1-0034.11

Zhou Q, Zhu H, Huang Q, Zhao L, Zhang G, Roy SW, Vicoso B, Xuan Z, Ruan J, Zhang Y, Zhao R, Ye C, Zhang X, Wang J, Wang W, Bachtrog D (2012) Deciphering neo-sex and B chromosome evolution by the draft genome of *Drosophila albomicans*. BMC Genom 13:109

Zurita S, Cabrero J, López-León MD, Camacho JPM (1998) Polymorphism regeneration for a neutralized selfish B chromosome. Evolution 52:274–277