



Genetic load caused by variation in the amount of rDNA in a wasp

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Abstract

Extensive variation in the size of the short (heterochromatic) arm of chromosome 14 was found in the wasp *Trypoxylon (Trypargilum) albitarse*. Ten different variants were differentiated by size and C-banding pattern. Fluorescent *in-situ* hybridization (FISH) revealed that ribosomal DNA in this species is clustered in the darkly C-banded parts of the heterochromatic short arm of chromosome 14. On this basis, we got an indirect estimate of the amount of rDNA from the area of these dark C-bands. The significant absence in males of the three chromosome variants with lower amounts of rDNA indicates that these three variants are lethal in this sex, and suggests the existence of a threshold marking the minimum amount of rDNA which is tolerable in haploidy. This implies about 4% genetic load in the population caused by variation in rDNA amount.

Introduction

Intraspecific variation in the size and number of nucleolus organizer regions (NORs), and consequently in the number of rRNA genes, seems to be common in several animal groups, e.g. grasshoppers (White *et al.* 1982), fish (Moreira-Filho *et al.* 1984, Sánchez *et al.* 1990, Martínez *et al.* 1993, Castro *et al.* 1994, Viñas *et al.* 1996, Castro *et al.* 2001, Ferro *et al.* 2001), amphibians (Miller & Gurdon 1970, Macgregor *et al.* 1977, Schmid 1982, King *et al.* 1990), reptiles (Bickham & Rogers 1985) and mammals (Miller 1981). Given the important role these genes play in cell metabolism and their wide variation in natural populations, it is very

interesting to ascertain whether this variation is essentially neutral or, on the contrary, some variants are forbidden by natural selection. For instance, in the brown trout, the extensive size polymorphism of the principal NOR (on chromosome 11) seems to be inherited in a Mendelian fashion (Castro *et al.* 1998) and is essentially neutral (Martínez *et al.* 1993). In other cases, however, some kind of selection has been invoked to explain the absence of homozygous combinations (Schmid 1982).

The existence of a single rDNA cluster per haploid genome is usually considered plesiomorphic in most organisms. Variations on this starting point might take place in two directions: (1) the single locus may grow or decrease in gene

number, or (2) new loci may appear as a result of non-homologous recombination or transposition (Schubert & Wobus 1985, Phillips *et al.* 1988). In principle, the increase in rRNA gene number should be well tolerated, at least while most copies are usually active. High amounts of inactive rDNA seems, however, to be tolerated in some organisms, an extreme case being the grasshopper *Stauroderus scalaris* (López-León *et al.* 1999). But the reduction of rRNA gene number below a minimum might be deleterious whenever cell needs were not satisfied (Miller & Brown 1969, Miller & Gurdon 1970, Lyckegaard & Clarck 1991).

Trypoxylon (Trypargilum) albitarse, a solitary haplodiploid wasp ($n_{\text{males}} = 16$; $2n_{\text{females}} = 32$) inhabiting mud nests, is one of the few cytogenetically studied species in the Sphecidae family, with about 8000 species. Like the majority of the Sphecidae, *T. albitarse* is not an economically important species but recent studies have revealed the singular evolutionary value of this species by possessing the only known B chromosomes that have a chance of being integrated into the A genome (Araújo *et al.* 2001). The standard chromosome complement of *T. albitarse* (males $n = 16$ and females $2n = 32$) consists of fourteen pseudoacrocentric chromosomes, as Imai (1991) named hymenopteran chromosomes with a heterochromatic short arm, and two acrocentric chromosomes. In addition, most individuals carry one metacentric or acrocentric heterochromatic B chromosome per haploid genome (Araújo *et al.* 2001). In the standard complement, heterochromatic regions are restricted to the short arm of all chromosomes (Araújo *et al.* 2000).

In this report, we show that *T. albitarse* has a single rDNA cluster on chromosome 14, which is variable in size. This variation implies at least 4% genetic load due to the lethality of those chromosome variants with an amount of rDNA below a critical threshold in the haploid sex.

Materials and methods

A total of 592 larvae of *Trypoxylon albitarse* were collected in the municipalities of Viçosa (20° 45' S, 42° 52' W), Porto Firme (20° 43' S, 43° 05' W) and Cajuri (20° 47' 17" S, 42° 47' 40" W) (Minas Gerais, Southeast Brazil) from January 1998 to December

2000. All cytogenetic studies were carried out on cells from the cerebral ganglia of post-defecating larvae, according to Imai *et al.* (1988). The C-banding procedure was that described by Pompolo & Takahashi (1990). For *in-situ* hybridization, the pDm 238 plasmid (Roiha *et al.* 1981), which contains the whole ribosomal unit (18, 28 and 5.8S) and the intervening sequences of *Drosophila melanogaster*, was biotin labelled by the nick translation reaction and used as a probe to analyse the chromosome location of ribosomal genes. Fluorescent *in-situ* hybridization (FISH) was performed according to the protocol of Viegas-Péquignot (1992).

C-banded preparations were analysed and photographed with an Olympus BX-60 microscope, with HQ, ISO 25 film, whereas the FISH preparations were analysed and photographed with an Olympus BX-60 epifluorescence microscope, equipped with a fluorescence filter (510–550 nm wavelength) for detection of propidium iodide and a fluorescence filter (450–480 nm wavelength) for detection of the fluorescein signal. Photographs were taken with Kodak Multispeed, ISO 400 film.

Since there exists a correlation between the number of rRNA genes and the size of the C-band harbouring them (White *et al.* 1982), we measured the C-band length in order to get an indirect estimate of the amount of rDNA for each chromosome 14 type. For this purpose, a total of 20 C-banded mitotic cells (two for each type of chromosome 14), at a similar level of chromosomal condensation, were observed with a 100× objective lens, photographed and digitalized. The length of the dark C-bands in the heterochromatic arm of chromosome 14 was measured in arbitrary units (a.u.) using the Image J program, version 1.20 s [<http://rsb.info.nih.gov/ij/>]. Hardy-Weinberg equilibrium for chromosome 14 variants was tested with the help of the Arlequin program (Schneider *et al.* 2000).

Results

Our sampling strategy was completely blind with respect to sex because sex was deduced from ploidy level after cytological analysis. Therefore, our cytological results provide a good estimate

of sex ratio in the analysed larval stage. As Table 1 shows, 54.6% out of the 592 individuals analysed cytologically were males, implying that the sex ratio is slightly male biased ($\chi^2_1 = 4.93$, $p = 0.026$).

Chromosome 14 showed extensive variation in the size of the heterochromatic short arm. The C-banding technique permitted us to differentiate ten variants of this chromosome, some of them including both dark and light C-bands in the short arm (Figure 1). All ten variants were observed in females. In males, however, only seven of them were present, since types #8, #9 and #10 (those with the smallest amount of darkly C-banded regions) were not detected. No homozygous females were found for these three variants. The frequencies of the ten variants (Table 1) differed between sexes ($\chi^2_9 = 31.36$, $p = 0.00026$) and among populations ($\chi^2_{126} = 207.07$, $p = 0.000007$). The most frequent variants were #2 (0.285), #4 (0.370) and #7 (0.185), the only variants that appeared in all populations analysed. Sex differences were mainly due to the absence of variants #8, #9 and #10 in males and to a difference in the frequency of variant #4 which was more frequent in males (see Table 1). Karyotypic frequencies in females fitted Hardy-Weinberg expectations in all populations

analysed (results not shown). In the total sample, however, there was a significant deficit of heterozygotes (0.700 observed versus 0.786 expected; exact test using a Markov chain: $p = 0.00039$), most likely reflecting population subdivision. The frequency of variants #8, #9 and #10 in females was, as a whole, 7.8% (see Table 1). Assuming that all chromosome 14 variants are equally viable in haploidy, about 25 males carrying any of these three variants would be expected among the 323 males analysed. The absence of males carrying these variants was thus significant ($\chi^2_1 = 27.35$, $p < 0.000001$).

FISH analysis showed that all rRNA genes are located in the heterochromatic arm of chromosome 14 (Figure 2). To ascertain whether there is a correspondence between the C-banding and FISH patterns, we performed FISH to the different chromosome 14 variants. The results showed that the rRNA genes are contained in the darkly C-banded regions of the heterochromatic arm of this chromosome. For example, compare the FISH of variant #6, in the inset of Figure 2a, with its C-banding pattern in Figure 1. This correspondence permitted an indirect quantification of the amount of rRNA genes contained in the different variants of chromosome 14, by measuring the length of dark

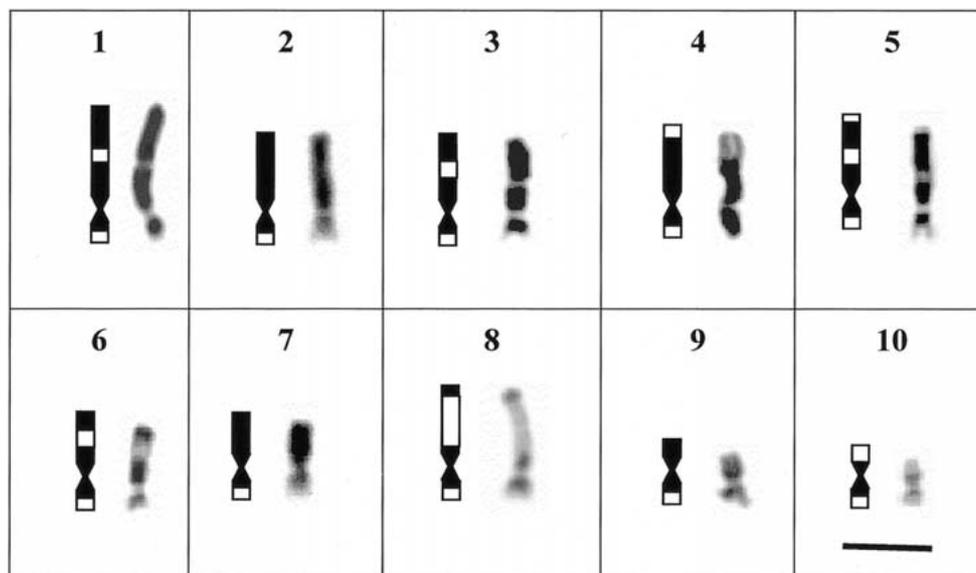


Figure 1. C-banding pattern and ideogram showing the ten distinct types of chromosome 14 of *T. albitalarse*. Bar = 5 μm .

Table 1. Frequency (%) of chromosome 14 variants in the sampled populations, with indication of number of specimens analysed

Population	Sex	n	Variant for chromosome 14									
			1	2	3	4	5	6	7	8	9	10
Amoras	Male	18	0	33	0	50	11	0	6	0	0	0
	Female	19	0	34	0	26	16	0	16	0	8	0
	Total	37	0	34	0	34	14	0	13	0	5	0
Campus	Male	28	4	32	0	32	7	4	21	0	0	0
	Female	22	5	41	7	30	0	2	2	2	11	0
	Total	50	4	38	4	31	3	3	10	1	7	0
V. Cristal	Male	27	4	37	0	30	11	0	19	0	0	0
	Female	28	4	39	2	20	5	0	16	4	11	0
	Total	55	4	39	1	23	7	0	17	2	7	0
Palmital	Male	35	3	31	6	34	9	3	14	0	0	0
	Female	33	9	27	2	35	2	0	21	0	3	2
	Total	68	7	29	3	35	4	1	19	0	2	1
Paraíso	Male	9	0	33	0	22	22	0	22	0	0	0
	Female	8	6	19	0	25	25	0	19	0	6	0
	Total	17	4	24	0	24	24	0	20	0	4	0
P. Firme	Male	30	0	23	7	50	0	0	20	0	0	0
	Female	32	0	30	6	33	2	0	25	2	3	0
	Total	62	0	28	6	38	1	0	23	1	2	0
Marrecos	Male	21	0	29	0	48	14	0	10	0	0	0
	Female	7	0	14	0	57	0	0	0	0	29	0
	Total	28	0	23	0	51	9	0	6	0	11	0
Silvestre	Male	8	0	50	0	50	0	0	0	0	0	0
	Female	10	0	15	0	35	5	10	25	0	10	0
	Total	18	0	25	0	39	4	7	18	0	7	0
V. Chaves	Male	20	0	45	0	30	5	0	20	0	0	0
	Female	12	0	33	4	29	8	0	17	0	4	4
	Total	32	0	39	2	30	7	0	18	0	2	2
Barrinha	Male	36	0	14	6	44	3	0	33	0	0	0
	Female	22	0	23	0	39	2	5	25	2	5	0
	Total	58	0	19	3	41	3	3	29	1	3	0
Fundao	Male	21	0	38	0	24	10	5	24	0	0	0
	Female	15	0	37	3	30	17	0	10	0	3	0
	Total	36	0	37	2	28	14	2	16	0	2	0
Cajuri	Male	30	0	20	0	67	0	0	13	0	0	0
	Female	19	0	21	3	29	8	0	26	0	8	5
	Total	49	0	21	2	46	4	0	21	0	4	3
A. Paragu	Male	16	0	38	0	31	6	0	25	0	0	0
	Female	11	0	36	0	46	5	0	14	0	0	0
	Total	27	0	37	0	40	5	0	18	0	0	0
M. Grande	Male	14	0	21	0	57	0	0	21	0	0	0
	Female	10	0	25	5	40	0	0	30	0	0	0
	Total	24	0	24	3	47	0	0	27	0	0	0
Piranga	Male	10	0	10	0	90	0	0	0	0	0	0
	Female	21	0	10	2	60	5	0	21	0	2	0
	Total	31	0	10	2	65	4	0	17	0	2	0
All populations	Male	323	1	29	2	43	6	1	18	0	0	0
	Female	269	2	28	3	34	6	1	19	1	6	1
	Total	592	2	29	2	37	6	1	19	1	4	1

C-bands in the heterochromatic arm. The results, expressed in arbitrary units provided by the measuring software (see Materials and methods),

showed a huge variation: 70, 55, 43, 41, 34, 31, 31, 26, 18 and 8 a.u. for variants #1 to #10, respectively.

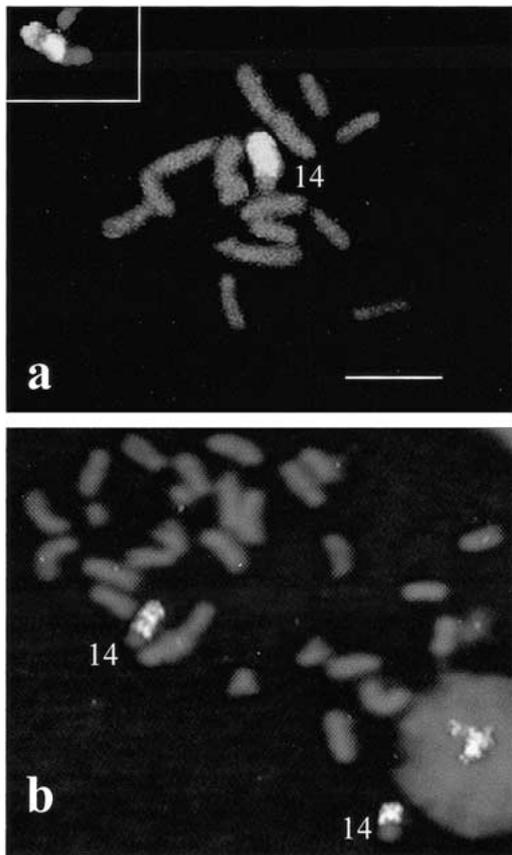


Figure 2. FISH on mitotic chromosomes of males and females of *T. albitarse*. (a) Male ($n=17$) with one rRNA gene-carrying chromosome. In detail, the chromosome 14 type #6. (b) Partial female metaphase ($2n=34$) showing two rRNA carrying chromosomes. Bar = $5\ \mu\text{m}$.

As expected, females (diploid) carried a mean amount of rDNA (84.19 a.u.) about double that of males (haploid) (43.78 a.u.) ($t=37.6$, $p < 0.000001$). No differences were observed among populations for the amount of rDNA in females ($F=1.55$; $df=14$, 254; $p=0.095$) or males ($F=0.77$; $df=14$, 308; $p=0.707$).

Discussion

FISH has shown the existence of a single chromosome, per haploid genome, carrying rRNA genes in *T. albitarse*. Since the occurrence of a single chromosome pair carrying the NOR appears to be a primitive character (Hsu *et al.* 1975), this situation is consistent with the taxonomic classification of the Sphecidae, which is considered one

of the most primitive groups in the order Hymenoptera (Bohart & Menke 1976).

Intraspecific variation in NOR size has been reported in diverse organisms, including hymenopterans, and it seems to be associated with alterations in the number of ribosomal genes (see Introduction). In the grasshopper *Warramaba virgo*, it has been shown that rRNA gene counts are proportional to the size of the corresponding C-bands (White *et al.* 1982). In *T. albitarse*, the correspondence of dark C-bands in the short arm of chromosome 14 with the heterochromatin containing the rRNA genes allows us to infer that the variation observed in this chromosome signifies variation in the amount of rDNA. A total of ten different chromosome variants were found in the 15 populations analysed, with three of them (#2, #4 and #7) being the most frequent and three others (#8–#10) being absent from males.

The absence of males carrying those variants with the smaller amounts of rDNA suggests the existence of a threshold below which the number of rRNA genes would not be enough to satisfy cells' needs for rRNA. This threshold could be about 30 a.u. since the minimum amounts found in both sexes were 31 a.u. in males and 39 a.u. in females. Therefore, the variants #8–#10, which contain rDNA below the threshold, seem to be lethal in males. Since these variants cannot be transmitted through males, no homozygous females can exist (and they were not observed). In consequence, the genetic load derived from the existence of chromosome variants bearing rDNA amounts below the threshold, is due to the appearance of males carrying these variants among the progeny of heterozygous females. The overall frequency of these males is the product of the frequency of these three variants in females (7.8%) and the male sex frequency in these populations (54.6%). Therefore, the overall genetic load for all populations analysed was 4.26%.

The existence of a minimum threshold for rDNA amount suggests the existence of a rigid control on the extensive variation observed for rDNA performed by natural selection acting against males carrying, in their single chromosome 14, amounts of rDNA below the threshold. In general, slight variations in the amount of rRNA genes, caused by rearrangements, do not seem to affect individual fitness (Miller & Brown 1969, Miller & Gurdon

1970, Zhang *et al.* 1990). Nevertheless, large deletions reducing the number of rRNA genes below a minimum could be harmful and even lethal (Lyckegaard & Clark 1991), resulting in small nucleolar organizers that form small nucleoli or do not form nucleoli at all (Miller & Brown 1969, Miller & Gurdon 1970). For instance, in *Drosophila melanogaster*, the gene *bobbed*, which contains the rDNA on sex chromosomes, does not require physical integrity but only a critical number of functional units (rDNA cistrons) (Karpen *et al.* 1988, Gatti & Pimpinelli 1992). The minimum number of rRNA genes necessary to satisfy cell needs is quite variable but it is generally assumed that reductions in the number of rRNA genes to less than the haploid number (i.e. in diploid organisms, 50% reductions) frequently affect the viability of individuals (Miller & Gurdon 1970, Long & Dawid 1980). In haplodiploid organisms, the haploid sex acts as a filter for the variation of rDNA amount, with all those variants below the minimum being eliminated by natural selection. *T. albitarse* is a good example, with the rDNA-richest variant of chromosome 14 (#1) bearing about double the estimated threshold of 30 a.u. Therefore, as a consequence of haplodiploidy, natural populations of *T. albitarse* suffer, on average, a minimum genetic load of about 4% due to the lethality of variants #8–#10 in males. In spite of this lethality, however, the sex ratio is slightly male biased, suggesting that the primary sex ratio (that in the zygotes) might be more male biased.

The main mechanism producing NOR size variation seems to be unequal crossing over (Macgregor *et al.* 1977, Butler & Metzenberg 1990, Chindamporn *et al.* 1993). In the brown trout, the formation of new NOR size variants has even been reported among the progeny of a controlled cross (Castro *et al.* 1998). The variants of chromosome 14 in *T. albitarse*, with different amounts of rDNA, have most likely arisen through recombination in females heterozygous for two different variants or through unequal crossing over in any kind of females. On the resulting variation, natural selection acts by eliminating those variants carrying amounts of rDNA below the minimum resulting viable in the haploid sex. We have no evidence of possible inviability of chromosome 14 variants with increased rDNA amounts (e.g. variant #1), which

are derived from the same crossover processes, but we cannot rule out the possibility that selection also marks an upper limit for the rDNA amount in chromosome 14. The frequent loss of NORs in plants after polyploidization (Vaughan *et al.* 1993) might be indicative of such control. The action of natural selection might thus modulate the amount of rDNA, helping to explain phenomena such as, for instance, clinal variation of the number of rRNA genes (Strauss & Tsai 1988) or the existence of an optimum rRNA gene copy number for growth in *Neurospora crassa* (Russell & Roland 1986).

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