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Physical mapping of 18S and 5S rDNA loci and histone H3 gene in grasshopper species of the subfamily Gomphocerinae (Acrididae)

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ABSTRACT. In this study, fluorescence *in situ* hybridization (FISH) analysis was used to determine and compare the numbers and chromosomal locations of two multigene families (rDNA and histone H3) in four Neotropical species of gomphocerine grasshoppers. FISH using the 18S rDNA probe identified a single site on the S9 chromosome of *Amblytropidia* sp and *Cauratettix borelli*, a single site on chromosome M6 of *Compsacris pulcher*, and two sites (chromosomes L1 and L2) in *Orphulella punctata.* By contrast, FISH with a 5S rDNA probe identified dispersion of this sequence in the genomes of the four species, with evidence of intraspecific variations. *Amblytropidia* sp had six to eight FISH signals on autosomal chromosomes, while *C. pulcher* exhibited a signal only on the M5 bivalent. The histone H3 gene was less variable and was restricted to a single pair in all species. The conservation of the numbers and locations of 18S rDNA and H3 genes in conjunction with data from the literature was useful

for evaluating karyotype evolution in this subfamily. The variation in the number and sizes of 5S rDNA sites indicates a process of recent dispersion that might have been mediated by transposition.

Key words: Chromosome; FISH; Multigene families

INTRODUCTION

Grasshoppers of the subfamily Gomphocerinae (Acrididae) correspond to one representative group of the orthopteran fauna of the Neotropical region (Otte, 1979; Otte and Jago, 1979). Molecular cytogenetic studies using fluorescence *in situ* hybridization (FISH) with repetitive DNA probes, such as rDNA and histone genes, have been carried out on many grasshopper species, particularly of the family Acrididae (Cabrero and Camacho, 2008; Loreto et al., 2008; Cabrero et al., 2009; Cabral-de-Mello et al., 2011a; Oliveira et al., 2011; Rocha et al., 2011; Jetybayev et al., 2012; Bueno et al., 2013; Palacios-Gimenez et al., 2013). The objective of physical mapping of these and other repetitive DNA sequences is to provide information on chromosome structure and gene distributions in a range of species that would serve as a basis for phylogenetic studies (Cabrero and Camacho, 2008; Cabrero et al., 2009; Cabral-de-Mello et al., 2011a,b; Jetybayev et al., 2012).

In the subfamily Gomphocerinae, previous studies using FISH analysis of 38 grasshopper species showed that the 45S rDNA sequence is present on more than one chromosome pair in 71% of cases (Cabrero and Camacho, 2008; Jetybayev et al., 2012). In contrast, studies of 5S rDNA sites in 14 species showed that they are present on more than one chromosome pair in 99.8% of cases; the only exceptions were *Chorthippus apicalis* and *Stauroderus scalaris* in which signals are limited to chromosome pairs 6 and 3, respectively (Loreto et al., 2008; Cabral-de-Mello et al., 2011a). These studies show the variation in dispersion of rDNA sequences, which in most species are present on two to five chromosome pairs. The distribution of histone sites is more conserved in the genomes of gomphocerine grasshoppers, with most species exhibiting a signal on only one autosomal pair (Cabrero et al., 2009).

In the present study, physical mapping of 18S and 5S rDNA and H3 histone sequences was performed to better understand the mechanisms of karyotype diversification in four Neotropical species of the subfamily Gomphocerinae, namely, *Amblytropidia* sp, *Cauratettix borelli*, *Compsacris pulcher*, and *Orphulella punctata*. Additionally, we examined the karyotypes of these species for possible intraspecific variations in the distribution of the genes analyzed. The mapping of these sequences will contribute to the understanding of the chromosome evolution in this less-studied Neotropical group of grasshoppers.

MATERIAL AND METHODS

Specimens of each of the four species of subfamily Gomphocerinae were collected from different localities in Brazil: three species, *Amblytropidia* sp, *C. borelli*, and *O. punctata* were collected in Pernambuco (PE) State, while *C. pulcher* was collected in Pará (PA) State (Table 1).

Cytological preparations were obtained from males of the different species by the classical technique of testicular follicle squashes. Slides containing well spread meiosis I preparations were selected for FISH.

Table 1. Localities and coordinates of the sampling sites for the gomphocerine species used in this study.

Species	Nº of individuals analyzed	Localities	Coordinates
	2	Ipojuca - PE (Ipo)	08°24'00"S; 35°3'50"O
Amblytropidia sp	6	Lagoa do Carro - PE (Lca)	07°50'42"S; 35°19'12"O
	2	São Lourenço da Mata - PE (Slm)	08°00'07"S; 35°01'04" O
Cauratettix borelli	2	Moreno - PE (Mor)	08°07'07"S; 35°05'32"O
	3	Vitória de Santo Antão - PE (Vsa)	08°07'35"S; 35°18'27"O
	1	Acará - PA (Aca)	01°57'39"S; 48°11'49"O
Compsacris pulcher			
	4	Benevides - PA (Ben)	01°21'39"S; 48°14'42"O
	1	Santa Barbara - PA (Sba)	01°13'26"S; 48°17'38"O
	1	Bezerros - PE (Bez)	08°53'24"S; 36°29'34"O
Orphulella punctata	2	Gravatá - PE (Gra)	08°12'03"S; 35°33'54"O
	4	Moreno - PE (Mor)	08°07'07"S; 35°05'32"O

Probe synthesis

We synthesized the probes for 18S and 5S rDNAs and H3 histone genes by PCR amplification using genomic DNA of each species as the template and the following primers: 5S rDNA forward (5'-AAC GAC CAT ACC ACG CTG AA-3') and reverse (5'-AAG CGG TCC CCC ATC TAA GT-3'); 18S rDNA (Sca18S) forward (5'-CCC CGT AAT CGG AAT GAG TA-3') and reverse (5'-GAG GTT TCC CGT GTT GAG TC-3'); H3 histone (H3F-1) forward (5'-ATA TCC TTR GGC ATR ATR GTG AC-3' and reverse (5'-ATG GCT CGT ACC AAG CAG ACV GC-3').

The PCR products were visualized by agarose gel electrophoresis to verify amplification of the sequences and to determine the sizes of each fragment. The probes were labeled with digoxigenin (dig 11-dUTP) or biotin (bio 16-dUTP) according to Moscone et al. (1996).

FISH and photodocumentation

FISH was performed as described by Cabral-de-Mello et al. (2011c) with modifications. Hybridization was carried out at 37ºC in a moist chamber for renaturation and formation of the probe + target DNA hybrid. The slides were then submitted to post-hybridization washes in 2X SSC and 0.1X SSC, followed by immersion in blocking solution (1X PBS + 3% BSA + 0.5% Tween 20). The signal was detected by incubating the slides with avidin-FITC or anti-digoxigenin-rhodamine (or both in the case of double FISH) for 45 min, followed by washing in blocking solution. Next, the secondary antibody (biotinylated anti-avidin) was applied for 10 min, followed by avidin-FITC for 30 min to amplify the signal. After washing, the slides were counterstained with DAPI and mounted with Vectashield (Vector). Images were acquired with a Leica WILD MPS 52 microscope coupled to a video camera using the CW 4000 program. The images were assembled using Adobe Photoshop CS5 Extended.

RESULTS

Amblytropidia sp, *C. borelli*, *C. pulcher*, and *O. punctata* males have a karyotype of 2n = 23,X0. The chromosomes are acrocentric and divided into groups according to their size: large (L), medium (M) and small (S). The 18S rDNA site was located in the proximal region of chromosome S9 in *Amblytropidia* sp (Figure 1a), as previously reported by Loreto et al. (2008), and in *C. borelli* (Figure 1b). In *C. pulcher*, the 18S rDNA site was detected in the interstitial region of pair M6 (Figure

1c, g), but in *O. punctata* was located distally on chromosome pairs L1 and L2 (Figure 1d) (Table 2).

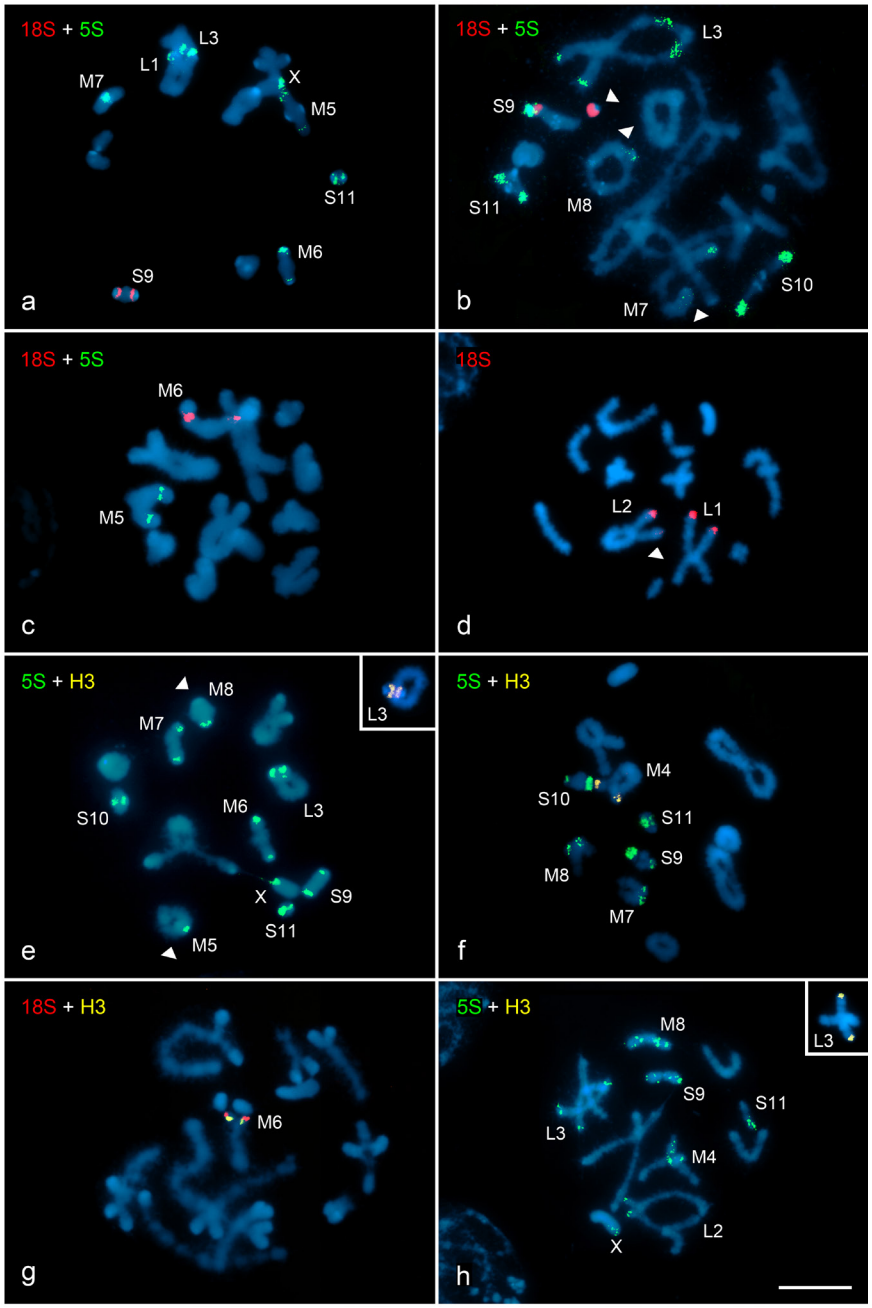

Figure 1. FISH analysis of 18S rDNA (red), 5S rDNA (green), and histone H3 (yellow) in representative species of the subfamily Gomphocerinae. **a. e.** *Amblytropidia* sp, **b. f.** *Cauratettix borelli*, **c. g.** *Compsacris pulcher*, and **d. h.** *Orphulella punctata*. Arrowhead indicates heteromorphism. Scale = 10 µm.

Table 2. FISH analysis of the 18S and 5S rDNAs and histone H3 gene in four Neotropical species of the subfamily Gomphocerinae.

Species	Site sequences	Locality codes	1	2	3	4	5	6	7	8	9	10	11	X	Nº of individuals analyzed
	18S	Ipo, Lca									pr				4
Amblytropidia sp	5S	see Table3			pc		pc*	pc	pc	pc*	pc	pc	pc	pc	5
	H3	Ipo, Lca, Slm			pr										6
	18S	Mor, Vsa									pr				3
C. borelli	5S	see Table3			pr,d				pc	pr*	pc	pc	pc		3
	H3	Mor, Vsa				pc									3
	18S	Aca, Ben, Sbp						i							5
C. pulcher	5S	Ben, Sbp					i								2
	H3	Ben						i							2
	18S	Mor	d	d*											3
O. punctata	5S	Gra		pc	pc,d	pc				pr,i	pc		pr	pc	1
	H3	Bez, Grav, Mor			d										3

pc = pericentromeric; pr = proximal; i = intersticial; d = distal. *Heteromorphism. In gray: chromosomes that carry the sites in all individuals. Aca (Acará - PA), Ben (Benevides - PA), Bez (Bezerros - PE), Gra (Gravatá - PE), Ipo (Ipojuca - PE), Lca (Lagoa do Carro - PE), Mor (Moreno - PE), Sbp (Santa Bárbara do Pará - PA), Slm (São Lourenço da Mata - PE), and Vsa (Vitória de Santo Antão - PE).

The 5S rDNA sites were more diverse and were found dispersed throughout the genome of most species, and intraspecific variations were also observed. *Amblytropidia* sp showed the greatest dispersion of 5S rDNA sites (Figure 1a, e and Table 2); the five individuals analyzed had different staining patterns although the signals were only detected in pericentromeric regions. In all individuals, 5S rDNA signals were detected on the X chromosome and in autosomes. In the autosomes, we found that the number of signals varied from eight [with different chromosome distributions in three individuals from the Lagoa do Carro (Lga) population], to seven [one individual from the São Lourenço da Mata (Slm) population] and to six [one individual from Ipojuca (Ipo)] (Table 3).

Table 3. Chromosomal positions of 5S rDNA sites in different populations of *Amblytropidia* sp and *Cauratettix borelli*.

Species	1	2	3	4	5	6	7	8	9	10	11	X
Amblytropidia sp												
Slm 1	pc*	pc*	pc*			pc*	pc*			pc*	pc	pc
Ipo 1	pc		pc		pc	pc	pc				pc	pc
Lca1			pc		pc*	pc	pc	pc*	pc	pc	pc	pc
Lca 2	pc	pc	pc		pc	pc	pc			pc	pc	pc
Lca 3		pc*	pc			pc	pc	pc*	pc	pc	pc	pc
C. borelli												
Vsa 1			pr,d				pc	pr*	pc	pc	pc	
Vsa 2			pr,d				pc*	pr*	pc*	pc	pc	
Vsa 3							pc	pr*	pc	pc	pc	

pc = pericentromeric; pr = proximal; d = distal. *Heteromorphism. In gray: chromosomes that carry the sites in all individuals. Ipo (Ipojuca - PE), Lca (Lagoa do Carro - PE), Slm (São Lourenço da Mata - PE), and Vsa (Vitória de Santo Antão - PE).

In *C. borelli*, the 5S rDNA sequences were also found to be dispersed. Two of the three individuals from Vitória de Santo Antão (Vsa) population had signals in the pericentromeric regions of six autosomal pairs, except for the L3 and M8 bivalents in which the sites were located proximally (Figure 1b). In these individuals, in addition to the proximal signal, a FISH signal was also observed

in the distal region of the L3 pair. The third *C. borelli* individual analyzed showed a similar pattern of FISH signals except for pair L3, which lacked any signals (Figure 1f) (Table 2).

In *O. punctata*, 5S rDNA signals were detected on six autosome pairs and on the X chromosome (Figure 1h). The sites were located in the pericentromeric region of pairs L2, L3, M4, S9, and the X chromosome, and in the proximal region of the M8 and S11 chromosomes. An additional signal was exhibited in the distal and interstitial region of the L3 and M8 chromosomes, respectively, of *O. punctata*. *C. pulcher* was the only species studied in which the 5S rDNA site was restricted to a single autosome pair, with a signal in the interstitial region of the M5 chromosomes (Figure 1c and Table 2).

The histone H3 gene was located on a single chromosome pair in all species studied. FISH signals were detected in the proximal region of the L3 chromosomes in *Amblytropidia* sp (Figure 1e), in the pericentromeric region of the M4 chromosomes in *C. borelli* (Figure 1f), in the interstitial region of the M6 chromosomes in *C. pulcher*, juxtaposed with the18S rDNA (Figure 1g), and in the distal region of the L3 chromosomes in *O. punctata* (Figure 1h and Table 2).

Double FISH revealed partial co-localization of the 18S and 5S rDNA sites in *C. borelli* (Figure 1b) and of the 18S rDNA and H3 histone sites in *C. pulcher* (Figure 1g). Furthermore, heteromorphisms were observed in some autosomal pairs; these were characterized by the absence of a signal in one of chromosomes homologs, such as for the 5S rDNA sites of *Amblytropidia* sp (Figure 1e) and *C. borelli* (Figure 1b), and the 18S rDNA sites of *O. punctata* (Figure 1d).

DISCUSSION

The distribution of the 18S and 5S rDNA loci and the H3 histone genes in the species studied here indicated that these markers were located and distributed differently in the genomes of each species, although a preference for positioning near the centromere was observed. In general, the positions of the 18S rDNA and histone H3 sites were conserved compared to 5S rDNA sites, which exhibited different patterns of dispersion among species.

The position of the 18S rDNA site in the proximal region of chromosome pair 9 in *C. borelli* and *Amblytropidia* sp corresponds to that observed in five other gomphocerine species and in 16 species of the family Acrididae (Cabrero and Camacho, 2008; Loreto et al., 2008; Rocha et al., 2011; Bueno et al., 2013), all with a karyotype of 2n = 23,X0. This finding indicates that the ancestral site was located on S9 bivalent in of Neotropical gomphocerine grasshoppers (Loreto et al., 2008). However, 18S rDNA sites have been identified on large autosomes and the X chromosome in species with a derived karyotype (2n = 17,X0) (Cabrero and Camacho, 2008). On the other hand, *C. pulcher* and *O. punctata* exhibited different patterns, with signals on medium and large chromosomes. This variation in the positioning of the 18S rDNA sites may be due to chromosome rearrangements or to transposition (Nei and Rooney, 2005; Eirín-López et al., 2012).

The cytogenetic marker showing the most dispersed FISH signals was the 5S rDNA gene, with a single site in *C. pulcher* and widely distributed signals in the genomes of other species. According to the literature (Cabral-de-Mello et al., 2011a; Oliveira et al., 2011), extensive variation in the number of 5S rDNA sites is common in grasshoppers of the families Acrididae and Romaleidae (Anjos et al., 2013; Neto et al., 2013), although they can be restricted to a single pair of chromosomes as in the Proscopiidae (Cabral-de-Mello et al., 2011b). Acrididae show a range of distribution patterns, from species with single sites to species with sites dispersed throughout all chromosomes, in addition to intermediate situations (Cabral-de-Mello et al., 2011a; Oliveira et al., 2011). Intraspecies variations in the distribution of 5S rDNA sites have so far been observed

in *Amblytropidia* sp and *C. borelli*, indicating the mobility of this marker in the genome of these species. The dynamics of repetitive sequence dispersion is often associated with the presence of active transposable elements in the genome. Bueno et al. (2013) suggested the presence of transposable elements in the H3 sequences of the grasshopper *Abracris flavolineata* to explain the dispersion of this repetitive family in the genome of the species.

The dispersion of sites may also be the result of ectopic recombination. The occurrence of this simple event is favored by the formation of chromocenters, which are observed in *Amblytropidia* sp, and involve chromosomes with and without 5S rDNA signals. In addition to 5S rDNA dispersion in most of the species studied, we also found evidence of heteromorphism, such as heterozygosity between homologous chromosomes for a marker, or differences in the sizes of the sites between homologs. These polymorphisms may be related to variations in copy number of these genes.

The restriction of histone H3 to only one chromosome pair in all species analyzed is in agreement with previous reports on the Acrididae (Cabrero et al., 2009; Palacios-Gimenez et al., 2013) and for other families such as Proscopiidae (Cabral-de-Mello et al., 2011b) and Romaleidae (Anjos et al., 2013; Neto et al., 2013). The dispersion of H3 histone sites in the genomes of members of the family Acrididae has only been reported in three species: *Rhammatocerus brasiliensis* (Oliveira et al., 2011), *A. flavolineata* (Bueno et al., 2013), and *Dichromatos lilloanus* (Palacios-Gimenez et al., 2013).

In the gomphocerine species studied here, the H3 histone locus was identified on the L3, M4 or the M6 chromosomes, in contrast to other acridid species. In studies of 24 acridid species with a karyotype of 2n = 23,X0 (including three gomphocerine species), 18 (75%) had a signal only on chromosome pair 8 (17 at interstitial positions and one at the pericentromeric position). Signals on pairs 2, 5, or 7 were detected in other species (Cabrero et al., 2009; Oliveira et al., 2011; Palacios-Gimenez et al., 2013). On the other hand, in acridid species with a karyotype of 2n = 17,X0 (14 species, all Gomphocerinae), the histone loci were located in the interstitial region of chromosome pair 3. Although the gomphocerine species studied here have a conserved karyotype (2n = 23,X0), it is possible that the ancestral site is present in chromosome 3, as observed for other representative species, and that simpler rearrangements such as translocation or inversion have modified the position in some species without causing the dispersion of the sequence in their genomes.

Regarding the localization of the18S rDNA and H3 histone sites in *O. punctata*, the pattern of distribution of these sequences should be highlighted, since it is similar to the distribution seen in Palearctic gomphocerine species with a derived karyotype (2n = 17, X0). This suggests that these are the preferential chromosomal locations of these sequences, irrespective of the occurrence of subsequent rearrangements. Another less likely possibility is that the Neotropical group originated from species with derived karyotypes. However, this would require the occurrence of new rearrangements to reverse the karyotype of 2n = 17,X0 to 2n = 23,X0, reestablishing the original pattern in these species. Broader cytogenomic and phylogenetic studies involving the subfamily Gomphocerinae should help to clarify this condition.

Conflicts of interest

The authors declare no conflict of interest.

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