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Localization of *HSP* single-copy genes by inexpensive, permanent non-fluorescent *in situ* hybridization on meiotic chromosomes of the grasshopper *Schistocerca pallens* (Acrididae)

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ABSTRACT. There have been many studies on *Schistocerca gregaria* and *Locusta migratoria*, which are important grasshopper pests in many parts of the world. However, the main pest grasshopper species in Brazil, *S. pallens*, *Rhammatocerus schistocercoides* and *Stiphra robusta*, are very poorly characterized genetically. We adapted a permanent *in situ* hybridization method to extend the genetic characterization of *S. pallens* by mapping the single-copy genes *Hsp70*, *Hsp83*, *Hsp27*, and *Ubi* on meiotic chromosomes. *Hsp70* was mapped on the L_2 chromosome, in which 82% of the signals were observed. *Hsp83* was mapped on a medium-sized chromosome, on which 81% of the signals were observed, tentatively identified as M_7. The hybridization signals for the *Hsp27* gene were detected on the L_1 chromosome at a frequency of 58%. The main hybridization site of the *Ubi* probe was on the L_2 chromosome, with 73% of the signals. All

mapped genes also presented secondary hybridization signals, always at frequencies below 30%. These are the first single-copy genes mapped for *S. pallens* and also for the Acrididae family. Since the Acrididae generally present very similar karyotypes, these data are useful as new landmarks for chromosome identification and as a tool for phylogenetic studies on the genus *Schistocerca* and for comparison with other insects.

Key words: *Schistocerca pallens*, *In situ* hybridization, *Hsp*, Single-copy genes, Gene mapping, Acrididae

INTRODUCTION

The main species of pest grasshoppers found in swarms that damage crops, native vegetation and pastures around the world are *Locusta* or *Schistocerca* species. Swarm formation is a gradual process, along one or two decades, when environmental conditions favor population growth (Steedman, 1990). In polyphenic species, phase polymorphism is triggered by hormonal factors in the growing populations, promoting behavioral changes that cause solitary individuals to become gregarious, through mechanical, visual and chemical-contact stimuli (Hägele and Simpson, 2000; Tawfik and Sehnal, 2003). In Brazil, the main grasshopper species with potential for swarm formation and occasional attacks on cultivated areas are the Acrididae species, *Rhammatocerus schistocercoides* and *Schistocerca pallens*, and the Proscopiidae species *Stiphra robusta*. Although genetic approaches are important for knowledge of physiological and etiological features needed for efficient biological control planning, as has been demonstrated for *L. migratoria* and *S. gregaria* (Simonet et al., 2002a,b), there have been only a few genetic studies of Brazilian pest grasshopper species. Genetic knowledge on these species is limited to chromosome number, which in the Acrididae is typically 2n = 23 (XO, males) and 2n = 24 (XX, females). The karyotype is composed of chromosomes similar both in size and shape, which makes correct identification of each chromosome difficult. The use of banding patterns facilitates this task in *S. pallens* and *S. flavolineata* (Souza and Melo, 2007), although new markers are still needed. The best-characterized species in the genus *Schistocerca* is *S. gregaria*, from which several genes have been cloned, including *abd-A* (Tear et al., 1990) and *abd-B* (Kelsh et al., 1993), the *HOX* homeotic cluster (Ferrier and Akam, 1996), *FABP* (Wu et al., 2001), two neuroparsin genes (Janssen et al., 2001), five chemoreceptor genes (Angeli et al., 1999; Picone et al., 2001), and genes coding for two isoforms of the serine-protease pacifastins (Vanden Broeck et al., 1998; Simonet et al., 2002a). The genome size of grasshoppers, such as *S. gregaria*, is about 9.3 gigabases, about 52 times the genome of *Drosophila melanogaster*, which severely limits the use of a whole-genome-sequencing approach (Hoy, 1994), and even cloning of single genes has rarely been done. *In situ* gene mapping can generate markers to detect chromosomal rearrangements that occur during the evolutionary process, when compared to other species (Campos et al., 2007). We examined the localizations of the single-copy genes *Hsp70*, *Hsp83*, *Hsp27*, and *Ubi* by permanent *in situ* hybridization (PISH) on the meiotic chromosomes of the grasshopper *S. pallens*. This is an important first step towards genetic characterization, generating

physical markers that can individualize the chromosomes, which would also be useful as landmarks to guide a future genome-sequencing program for this species.

MATERIAL AND METHODS

Specimens and cytological preparations

Testes from eight adult males of *S. pallens* collected from the States of Pernambuco and Bahia in the northeast region of Brazil (Table 1) were dissected, fixed in ethanol and acetic acid (3:1) and stored in a freezer. Slides were prepared by the squash technique, with a testicular follicle in 45% acetic acid (Souza, 1991). After freezing in liquid nitrogen to remove the cover slip, the preparations were stored at 4°C until hybridization. The best preparations were used for PISH.

Table 1. Number of specimens of *Schistocerca pallens*, collection localities in Pernambuco (PE) and Bahia (BA) States (northeastern Brazil) and the geographical coordinates.

No. of specimens	Collection locations	Coordinates
3	Pesqueira (PE)	8 18' 45" S, 36 41' 15" W
2	Itamaracá (PE)	7 44' 52" S, 34 49' 32" W
1	Pombos (PE)	8 11' 15" S, 35 26' 15" W
1	Andaraí (BA)	12 48' 45" S, 41 18' 45" W
1	Sobradinho (BA)	9 26' 15" S, 40 48' 45" W

Plasmid amplification and probe preparations

The probes were prepared from conserved sequences of *D. melanogaster* genes *Hsp70* (Livak et al., 1978), *Hsp83* (Holmgren et al., 1981), *Hsp27* (Corces et al., 1980), and *Ubi* (Izquierdo et al., 1994), all cloned in pBR322-derived plasmids. Plasmids were transformed in the DH5α strain of *Escherichia coli* with the lithium acetate method and extracted by alkaline lysis (Sambrook et al., 1989). Whole plasmids were biotin labeled by nick translation using the BioNick DNA system, as indicated by the manufacturer (Gibco/BRL, Paisley, Scotland), to be used as probes.

In situ hybridization procedures

Hybridizations were carried out at 37°C in 30% formamide for 40 h, using 100 ng biotin-labeled probe for each slide. Stringent washes were made at room temperature with 2X SSC. The BluGene non-radioactive detection system (Gibco/BRL) was used for detection of the hybridization sites. After hybridization, the chromosome preparations were counterstained with lactic orcein (1% orcein in 20% lactic acid and 45% acetic acid) diluted 1:10 in 45% acetic acid, air dried and mounted in Entellan (Merck). The PISH signals in the chromosomes were analyzed and documented under phase-contrast microscopy. Since heterologous probes were used, we decided to quantify the signals, as described previously by Campos et al. (2007), and established a minimum of 30% signals at a site as a criterion of consistent marking.

RESULTS AND DISCUSSION

In the grasshopper, *Schistocerca pallens*, as in other representatives of the Cyrtacanthacridinae subfamily, the karyotype is composed of acro-telocentric chromosomes divided into three size groups: large (L_1, L_2 and L_3), medium (M_4 to M_8 and X chromosome), small pairs (S_9, S_{10} and S_{11}), and the sex determination system is XX, XO (Mesa et al., 1982). The medium and small chromosomes are especially difficult to distinguish. Although conventional cytogenetic markers, such as nucleolus organizer region and C-banding patterns, are very important for chromosomal individualization (Souza and Melo, 2007), they do not cover all chromosomes in the karyotype. Consequently, additional genetic markers can be very useful. The localization of single-copy genes can be of special interest to compare the karyotypes of close species or genera. While this can be accomplished by fluorescent *in situ* hybridization, the useful time for analysis is limited before signals disappear, and the cost of reagents and necessary equipment can be restrictive. To overcome these difficulties, we adapted a non-fluorescent and non-radioactive PISH procedure for meiotic chromosomes of the grasshopper *S. pallens*. The low cost and permanent marking makes it useful to generate signals on condensed meiotic and mitotic chromosomes.

Using biotinylated heterologous probes from *D. melanogaster*, the *Hsp70*, *Hsp83*, *Hsp27*, and *Ubi* loci were mapped by PISH on meiotic chromosomes of the grasshopper *S. pallens*. More than 750 nuclei after hybridization of probes of the four genes were analyzed, reaching a mean of marked nuclei of 60%, assuring that the technique was efficient. Since we used heterologous probes from a distant species, the hybridization signals were quantified and presented as frequencies. We established arbitrarily that 30% is the minimum frequency for a mark to be considered consistent, which makes the results still more reliable.

In each case, the probe hybridized mainly at a single site, with signal frequency always above the minimum of 30%, in different autosomal pairs (Table 2). The signal of the *Hsp70* gene probe hybridization was detected in 64% of the 313 nuclei analyzed, where 82% of the marks were on the L_2 chromosome (Figure 1). This is an indication that the *Hsp70* gene should be in single copy in *S. pallens*, as in *D. virilis* (Evgen'ev et al., 2004) and in the *willistoni* species group of *Drosophila* (Bonorino et al., 1993), although it is duplicated in several species of the *melanogaster* and *obscura* species groups (Segarra et al., 1996).

Table 2. Quantification of *in situ* hybridization signals of *Hsp70*, *Hsp83*, *Hsp27*, and *Ubi* gene probes on meiotic chromosomes of the grasshopper *Schistocerca pallens*.

Gene probe	Total nuclei analyzed	% Marked nuclei	% Marked chromosomes							
			L_1	L_2	L_3	M_4	M_5	M_7	S	X
Hsp70	313	64	-	82	-	-	17	-	-	1
Hsp83	211	77	-	18	-	-	-	81	1	-
Hsp27	78	60	58	-	-	34	-	-	-	8
Ubi	153	38	17	73	7	3	-	-	-	-

Percentage (%) of chromosomes presenting marks.

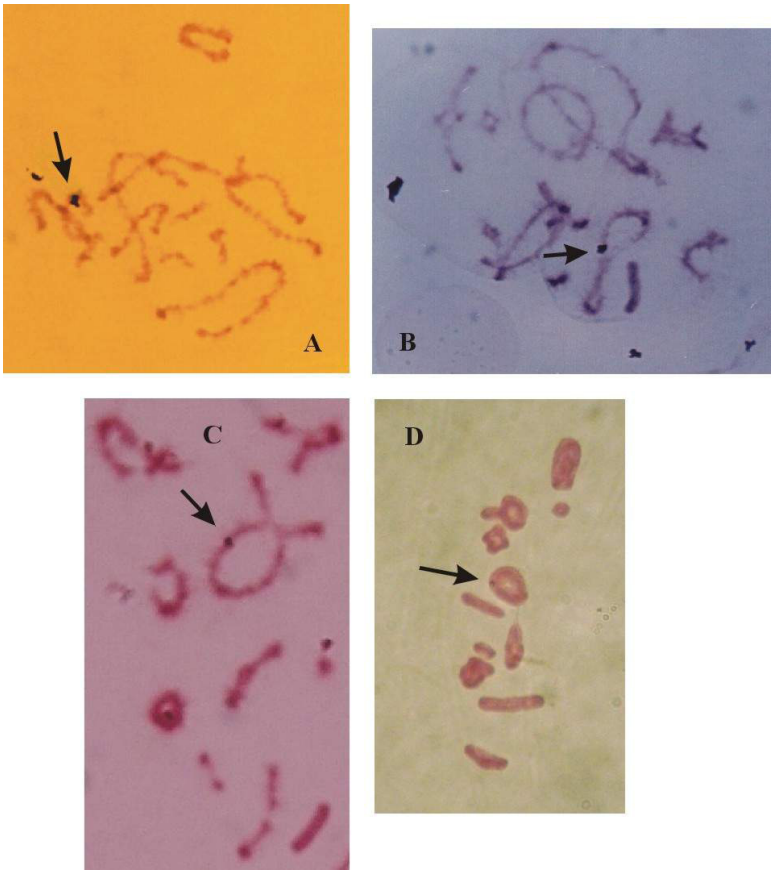

Figure 1. Localization of *Hsp* genes (arrows) on meiotic chromosomes of *Schistocerca pallens*. **A.** Pachytene with the *Hsp83* mark on the M_7 chromosome. **B.** Pachytene with *Hsp70* on the L_2 chromosome. **C.** Diplotene with the *Hsp27* mark on the L_1 chromosome. **D.** Metaphase with the *Ubi* gene mark on the L_2 chromosome. Bar = 10 µm.

The *Hsp83* gene probe hybridization signal was found in 77% of the 211 nuclei analyzed, with 81% of the marks on a medium-size chromosome, identified hereafter as the M_7 chromosome (Figure 1). The uniqueness of the *Hsp83* gene in *S. pallens* is consistent with the fact that in all insects investigated so far this gene is single-copy (Konstantopoulou and Scouras, 1998; Landais et al., 2001). For both *Hsp70* and *Hsp83*, other minor signals were also found that did not reach the minimal frequency of 30% to be considered as consistent marks.

The hybridization signals for the *Hsp27* gene probe were detected in 60% of the 78 nuclei analyzed, the main signal was observed on the L_1 chromosome (Figure 1) at a frequency of 58%, although another mark could be found on the M_4 chromosome at a frequency of 34%, which is just above the limit to be considered as a second consistent mark. It will be of interest to determine if this second mark represents a *bona fide* duplication of the *Hsp27* gene in *S. pallens*.

The probe for the *Ubi* gene hybridized mainly on the L_2 chromosome (Figure 1), at a frequency of 73%. This should be the polyubiquitin locus in *S. pallens*, and the minor signals putatively represent ubiquitin-fusion genes. Although marked at a frequency of 17% which is below the minimum of 30%, it is possible that a site on the L_1 chromosome represents another

polyubiquitin locus. Some strains of *D. melanogaster* present a polyubiquitin gene on the X chromosome, in addition to the main locus on chromosomal arm 3R (Izquierdo, 1994).

This PISH method can easily be applied to condensed meiotic and mitotic chromosomes of insects and other organisms, generating genetic markers additional to those of conventional cytogenetic techniques. The chromosomal landmarks established in this study will be useful as physical markers to guide an eventual genome-sequencing program for *S. pallens*. This can extend the possibilities of comparing the gene content of similar karyotypes of several species, probably expanding evolutionary knowledge concerning species for which whole-genome sequencing is still a prohibitive cost choice.

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