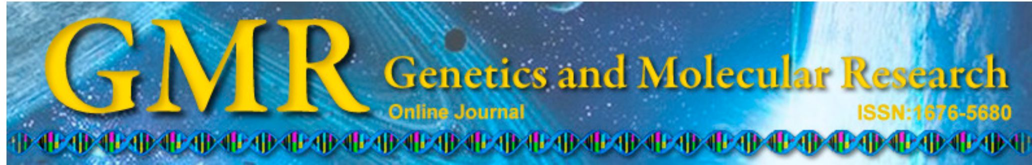

Physical mapping of the *Period* gene on meiotic chromosomes of South American grasshoppers (Acridomorpha, Orthoptera)

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ABSTRACT. The single-copy gene *Period* was located in five grasshopper species belonging to the Acridomorpha group through permanent *in situ* hybridization (PISH). The mapping revealed one copy of this gene in the L1 chromosome pair in *Ommexecha virens*, *Xyleus discoideus angulatus*, *Tropidacris collaris*, *Schistocerca pallens*, and *Stiphra robusta*. A possible second copy was mapped on the L2 chromosome pair in *S. robusta*, which should be confirmed by further studies. Except for the latter case, the chromosomal position of the *Period* gene was highly conserved among the four families studied. The *S. robusta* karyotype also differs from the others both in chromosome number and morphology. The position conservation of the single-copy gene *Period* contrasts with the location diversification

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of multigene families in these species. The localization of single-copy genes by PISH can provide new insights about the genomic content and chromosomal evolution of grasshoppers and others insects.

Key words: *Period* gene; Grasshoppers; Meiotic chromosomes; Permanent *in situ* hybridization (PISH); Evolution

INTRODUCTION

Sensu stricto grasshoppers are within the monophyletic superfamily grouping Acridomorpha of the Orthoptera order (Song, 2010). This group contains about 24 superfamilies, including the Acridoidea superfamily, which is the largest within Orthoptera and contains about 11 families and 7680 species defined by the phallic complex morphology, among other characteristics (Eades, 2000; Song, 2010). Among these families are Ommexechidae, Romaleidae, and Tristiridae, which are endemic to Neotropical regions and have a probable origin in South America. Although Acrididae has a worldwide distribution, 10 subfamilies are represented in the Neotropics, and five of them are exclusive to this geographical region (Carbonell, 1977). Recent molecular studies (Matt et al., 2008) support the removal of the Proscopiidae family from the Proscopioidea superfamily and its inclusion in the Eumastacoidea superfamily, to which it has a closer affinity, as proposed by Eades (2000).

The grasshopper species *Ommexecha virens* Thunberg, 1824 (Ommexechidae), *Xyleus discoideus angulatus* Stål, 1873 (Romaleidae), *Tropidacris collaris* Stoll, 1813 (Romaleidae), *Schistocerca pallens* Thunberg, 1815 (Acrididae), and *Stiphra robusta* Mello-Leitão, 1939 (Proscopiidae) are found in almost all of northeastern Brazil. The last three species are considered to be scattered pests, causing damage to various cultivars and associated native vegetation (Magalhães and Lecoq, 2007). They have symmetrical karyotypes showing slightly decreasing chromosome sizes with a predominant acrocentric morphology, conserved number (23 for males and 24 for females), and XO sex-chromosome system (Mesa et al., 1982). Exceptions are the submetacentric morphology of the largest chromosomal pair (L1) in *O. virens* (Mesa and Ferreira, 1977) and a lower diploid number (2n = 19 for males and 20 for females) in *S. robusta* (de Souza and Moura, 2000).

The physical positions of the clock gene *Period* were mapped in meiotic chromosomes of five South American grasshopper species using the permanent *in situ* hybridization technique (PISH) (Rieger et al., 2007). The data obtained for this single-copy gene were compared to those in the literature for other single-copy genes and repetitive-copy genes in order to obtain evolutionary insights.

MATERIAL AND METHODS

Specimens and cytological preparations

The species, number of specimens, and geographical coordinates of sampling points are presented in Table 1. All samples were registered and preserved as part of the insect collection of Departamento de Genética (Centro de Ciências Biológicas, UFPE, Recife, PE, Brazil), and all institutional and national guidelines for the care and use of laboratory animals were followed. After the capture, specimens were dissected, and the testes were fixed in Carnoy

solution (3:1 ethanol and acetic acid) and stored in a freezer. Slides were prepared by the classical technique of squashing a testicular follicle in 45% acetic acid. After freezing in liquid nitrogen to remove the coverslip, all preparations were stored at 4°C until hybridization. The best preparations were used for PISH according to Rieger et al. (2007). For the *in situ* hybridization procedures, five slides of representatives of each species were used.

Table 1. Number of grasshopper male specimens and geographical coordinates of collection places in the states of Pernambuco (PE), Piauí (PI), Paraíba (PB), and Bahia (BA) in northeastern Brazil.

Species	Number of specimens	Collection places	Coordinates
Ommexecha virens	2	Sobradinho, BA	09°26'15"S, 40°48'45"W
	3	Mucugê, BA	13°03'45"S, 41°18'45"W
Xyleus discoideus angulatus	2	Marcolândia, PI	07°26'15"S, 40°41'15"W
	1	Exu, PE	07°30'50"S, 39°42'33"W
Tropidacris collaris	3	Recife, PE	08°01'05"S, 34°56'48"W
	1	Andaraí, BA	12°48'45"S, 41°18'45"W
Schistocerca pallens	4	Itamaracá, PE	07°41'15"S, 34°48'45"W
Stiphra robusta	7	Fagundes, PB	07°21'18"S, 35°46'30"W

Plasmid amplification and probe preparations

The probes were prepared from the pTOP*Ziper*01 plasmid containing the flanking sequences of the threonine-glycine region of the *Period* gene of the drosophilid *Zaprionus indianus* cloned in the TOPO TA plasmid (Invitrogen, USA) (Müller et al., 2012). Plasmids were transformed into the DH5α strain of *Escherichia coli* with the calcium chloride method and extracted by alkaline lysis (Ausubel et al., 2002). 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.

PISH procedures

The *in situ* hybridization procedures were performed as described for the first time for grasshoppers by Rieger et al. (2007). Briefly, hybridizations were carried out at 37°C in 30% formamide for 36 h by using 200 ng biotin-labeled probe for each slide. Washes were performed at room temperature with 2X saline-sodium citrate buffer, adopting a stringency level of 65% (Schwarzacher and Heslop-Harrison, 2000). The streptavidin-alkaline phosphatase conjugate, nitro blue tetrazolium, and bromo-chloro-indolyl-phosphate were used to detect the hybridization sites as the visualization of dark blue spots over the chromosomes. 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. Because heterologous probes were used, the signals were quantified as described previously by Campos et al. (2007) and Rieger et al. (2007), establishing a minimum of 30% signals at a chromosome site as the criterion of consistent gene localization.

RESULTS

An arrangement of chromosomes in groups according to size is proposed here for the

S. robusta karyotype as shown in Figure 1. Their chromosomes are assigned to two groups as follows: four pairs of large chromosomes (L1-L4), five pairs of medium chromosomes (M5-M9), and a large X chromosome.

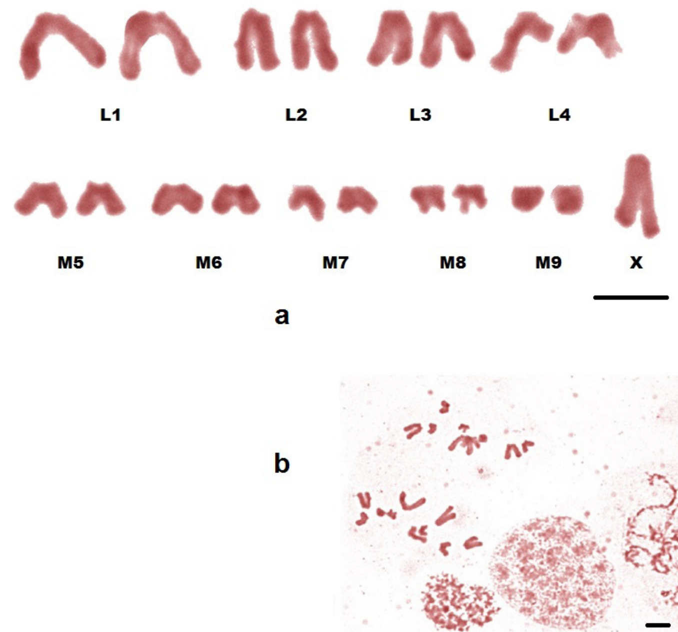

Figure 1. New arrangement of the *Stiphra robusta* karyotype according to chromosome sizes using the nomenclature used for other species of grasshoppers (**a**). Chromosomes were obtained from an anaphase I cell (**b**). Bar = 10 µm.

Using the PISH technique, the single-copy gene Period was located in meiotic chromosomes of *O. virens*, *X. discoideus angulatus*, *T. collaris*, *S. pallens*, and *S. robusta*. For all five species, at least 100 nuclei were analyzed (Table 2). The efficiency of the *in situ* hybridization ranged from 54 to 80%. Hybridization marks in these chromosomes are shown in Figure 2.

Table 2. Quantification of *in situ* hybridization signals for the *Period* gene on meiotic chromosomes of grasshopper species.

Species	Number of nuclei analyzed	Percent of nuclei marked	Percent of marked chromosomes														
			L1	L2	L3	L4	M3	M4	M5	M6	M7	M8	M9	S9	S10	S11	X
Ommexecha virens	169	62	48	12	13	*	*	28	-	-	-	-	*	5	-	-	5
Tropidacris collaris	164	63	51	13	*	*	14	9	11	-	-	-	*	9	-	-	9
Xyleus discoideus angulatus	250	54	45	19	13	*	*	9	10	-	-	-	*	5	5	-	10
Schistocerca pallens	100	56	39	12	12	*	*	3	-	-	-	23	*	5	-	-	5
Stiphra robusta	221	80	70	46	21	15	*	*	15	15	11	11	11	*	*	*	4

The asterisk indicates that the chromosome number does not apply to that species.

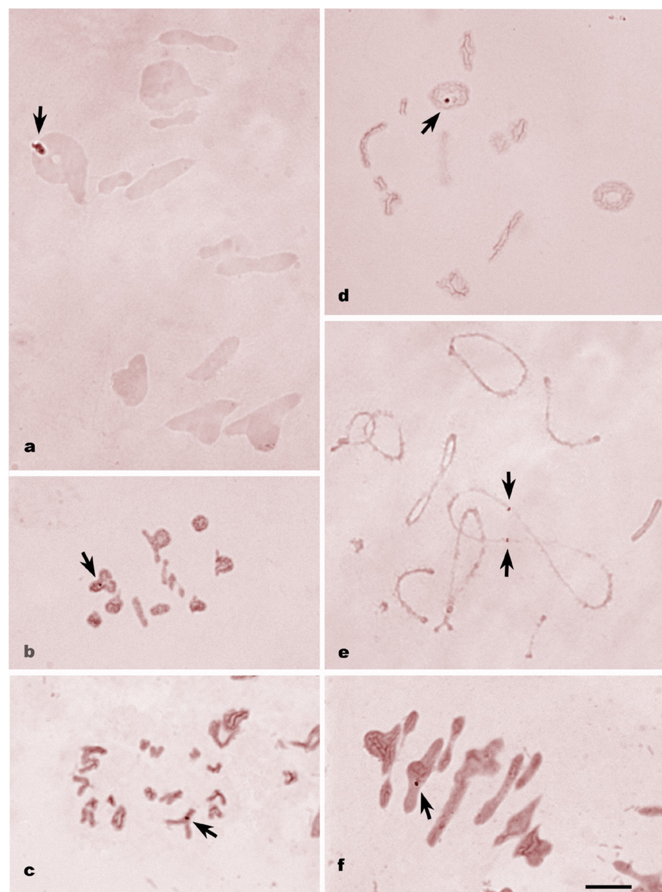

Figure 2. Chromosomal location of the *Period* gene on the L1 chromosome pair (arrows) by permanent *in situ* hybridization (PISH) in a *Tropidacris collaris* metaphase I cell (**a**), *Ommexecha virens* metaphase I cell (**b**), *Xyleus discoideus angulatus* metaphase I cell (**d**), *Schistocerca pallens* diplotene cell (**e**), *Stiphra robusta* anaphase I cell (**c**), and on the L2 chromosomal pair of a *Stiphra robusta* metaphase I cell (**f**). The photographs were taken at the same magnification. Bar = 10 µm.

DISCUSSION

The karyotypes of *O. virens* (Carvalho et al., 2011), *X. discoideus angulatus* (Souza et al., 1998), and *S. pallens* (Souza and Melo, 2007) were characterized by basic cytogenetic techniques, such as C banding and silver nitrate impregnation. In these species, 2n♂ = 23 chromosomes was found, and chromosomes were generally arranged as three pairs of large chromosomes (L1-L3), five pairs of medium chromosomes (M4-M8), three pairs of small chromosomes (S9-S11), and the X chromosome, which is considered to be a medium-sized chromosome. While *T. collaris* has the same chromosome number as most orthopterans, it differs in chromosome morphology, presenting two pairs of large chromosomes (L1-L2), six pairs of medium chromosomes (M3-M8), three pairs of small chromosomes (S9-S11), and a medium-sized X chromosome (Mesa et al., 1982). A similar chromosome grouping clas-

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sification was adopted in this study for *S. robusta* to facilitate chromosome and karyotype comparisons because there is not such a description in the literature. It is noteworthy that the *S. robusta* karyotype differs from other species in this study, both in chromosome number and in chromosome sizes, with four chromosome pairs in the large group, five pairs in the medium group, and a large X chromosome. This species did not have small chromosomes like those seen in other Proscopiidae members, such as *Scleratoscopia protopeirae* (Cabral-de-Mello et al., 2011a). The M3 chromosome pair of *T. collaris* corresponds to the L3 pair in the other species in this study, but it is placed in the medium chromosome group because of its smaller size. The homology of chromosomal content among species is yet to be examined. The same observations must be made regarding the L4 chromosome pair of *S. robusta*, which, although placed in the large chromosome group due to its bigger size, may correspond to the M4 pair in the other species.

Single-copy gene mapping is routinely carried out in several dipteran species such as *Drosophila* (Ranz et al., 2001; Evgen'ev et al., 2004) and *Zaprionus* species (Campos et al., 2007) because of the facility of polytene chromosomes. Locating single-copy genes can be useful when comparing karyotypes of species, like the comparison of the *Drosophila* genus performed by Clark et al. (2007), or even to compare karyotypes in close genera or different species and families of dipterans. *In situ* hybridization of single-copy genes can be also applied to mitotic or meiotic chromosomes and used to compare the location of single-copy genes in orthopteran karyotypes (Rieger et al., 2007). There are not studies about the *Period* gene location in grasshoppers. Most of the research done with this gene was the molecular characterization of the threonine-glycine repeating regions in some groups, mainly Diptera and Lepidoptera, allowing the use of this gene as a marker in phylogeographic studies, molecular systematics, and evolution. These studies demonstrated the considerable level of evolutionary conservation and feasibility of using this gene, alone or combined with other markers, for applications in systematic, taxonomic, and population studies of insect groups (Rosato and Kyriacou, 2001; Miyatake et al., 2002; Barr et al., 2005; Mazzotta et al., 2005; Lankinen and Forsman, 2006; Sawyer et al., 2006; Matsumoto et al., 2008).

The most remarkable finding in this study was the conservation of the chromosomal location of the *Period* gene in the five species that were analyzed. This gene was located on the L1 chromosomal pair in all five analyzed species, displaying conservation in the interstitial chromosome position. The position of the *Period* gene was conserved in four families, which included three families (Romaleidae, Ommexechidae, and Acrididae) from the Acridoidea superfamily and the Proscopiidae family. This probably reflects the conservation of gene content in orthopterans. Only *S. robusta* seemed to carry a second copy of the *Period* gene near the centromere of the L2 chromosome, which, if confirmed in other Proscopiidae species, possibly represents a typical condition of this ancient grasshopper family (Flook et al., 2000). Thus, karyotype differentiation and gene content agree with the proposal of Eades (2000) to move the Proscopiidae family from the Proscopioidea superfamily to the Eumastacoidea superfamily.

A previous report by Rieger et al. (2007) described the mapping of single-copy genes in *S. pallens*. In this Acrididae representative, the *Hsp*70 gene was detected as a single locus on the L2 chromosome, although there are some species groups in *Drosophila* (Diptera) in which this locus is duplicated (Segarra et al., 1996). The *Hsp*83, *Hsp*27, and *Ubi* genes were also mapped to *S. pallens* chromosomes by Rieger et al. (2007). The *Hsp*83 gene was present as a single locus in all investigated insect species, and its position in *S. pallens* defined the first landmark that was available to identify the M7 chromosome pair. Duplication of the *Hsp*27

and *Ubi* loci was indicated in this species, and the main copy of *Hsp*27 and *Ubi* mapped to the L1 and L2 chromosomes, respectively.

The physical localization of repetitive-copy genes was determined for several grasshopper species. The 45S rDNA was observed in dispersed sites in the karyotypes of *O. virens* (Carvalho et al., 2011), *X. discoideus angulatus* (Souza et al., 1998), *S. pallens* (Souza and Melo, 2007), and others. The 5S and 18S families and the histone H3 gene presented several marks on medium chromosome pairs of *S. robusta* and other Proscopiidae species (Cabral-de-Mello et al., 2011a). The chromosomal location of 5S rDNA in a large number of Acrididae species revealed its position mainly on medium and small chromosomes. The 5S rDNA localization showed a variable number of signals, from a single chromosome pair in some species to all pairs in others (Cabral-de-Mello et al., 2011b). From the localization studies multigene families present dispersive location patterns, even in near species. This is in clear contrast to single-copy genes, such as *Period* (this study) and heat shock genes (Rieger et al., 2007), which maintain positions on the same chromosome in very distant grasshopper families.

An increased number of mapped genes in multiple grasshopper species will allow us to make important evolutionary inferences. Although it may seem premature, the positioning of the *Period* gene on the same chromosome pair in the five grasshopper species described in this study may indicate that, besides the similar karyotypes, grasshopper species may also share gene synteny, as demonstrated for drosophilids and other dipterans (Campos et al., 2007; Schaeffer et al., 2008).

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