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New insights into the six decades of Mesa's hypothesis of chromosomal evolution in Ommexechinae grasshoppers (Orthoptera: Acridoidea)

MYLENA D. SANTANDER[1,2], DIOGO C. CABRAL-DE-MELLO[3], ALBERTO TAFFAREL[1], EMILIANO MARTÍ[3], DARDO A. MARTÍ[1], OCTAVIO M. PALACIOS-GIMENEZ[4] and ELIO RODRIGO D. CASTILLO[1,*]

[1]*Laboratorio de Genética Evolutiva Dr. Claudio J. Bidau. Instituto de Biología Subtropical (IBS) CONICET-UNaM. FCEQyN. Posadas, Misiones, Argentina*
[2]*Departamento de Genética e Biologia Evolutiva. Instituto de Biociências, Universidade de São Paulo (USP). São Paulo, São Paulo, Brazil*
[3]*Departamento de Biologia Geral e Aplicada, Universidade Estadual Paulista (UNESP), Instituto de Biociências/IB. Rio Claro, São Paulo, Brazil*
[4]*Department of Organismal Biology – Systematic Biology Program, Evolutionary Biology Centre, Uppsala University.*

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In Acridoidea grasshoppers, chromosomal rearrangements are frequently found as deviations from the standard acrocentric karyotype (2n = 23♂/24♀, FN = 23♂/24♀) in either phylogenetically unrelated species or shared by closely related ones, i.e. genus. In the South American subfamily Ommexechinae, most of the species show a unique karyotype (2n = 23♂/24♀, FN = 25♂/26♀) owing to the occurrence of a large autosomal pair (L_1) with submetacentric morphology. In the early 1960s, Alejo Mesa proposed the hypothesis of an ancestral pericentric inversion to explain this karyotype variation. Furthermore, in Ommexechinae, extra chromosomal rearrangements (e.g. centric fusions) are recorded between the ancestral X chromosome and autosomes that originated the so-called neo-sex chromosomes. However, the evolutionary significance of the pericentric inversions and centric fusions in Ommexechinae remains poorly explored. Aiming for a better understanding of chromosomal evolution in Ommexechinae, we performed a detailed cytogenetic analysis in five species. Our findings support the hypothesis about the occurrence of an early pericentric inversion in the ancestor of Ommexechinae. Moreover, our results show a complex karyotype diversification pattern due to several chromosome rearrangements, variations in heterochromatin and repetitive DNA dynamics. Finally, the chromosomal mapping of U2 snDNA in L_1 provided new insights about the morphological evolution of this autosomal pair and revealed unnoticed chromosome reorganizations.

ADDITIONAL KEYWORDS: cytogenetic – differentiation – FISH – inversions – karyotype – multigene families – neo-XY – Ommexechidae – rDNAs – U2 snDNA.

INTRODUCTION

Among the modern grasshoppers of Acridoidea, i.e. the families Acrididae, Ommexechidae and Romaleidae (*sensu* Leavitt *et al.*, 2013), chromosomal stability is reported with high frequency of species harbouring a diploid number (2n) of 23♂/24♀ chromosomes (Hewitt, 1979; Mesa *et al.*, 1982). The presence of acrocentric chromosomes is also common in species of Acrididae and Romaleidae, with a fundamental number (FN; i.e. number of chromosome arms) of 23♂/24♀ (Mesa *et al.*, 1982). However, this presumptive karyotypic stability is contrasted with the observation of variability in specific groups, like variations in diploid numbers, sex chromosome systems and morphology of chromosomes, resulting from chromosomal rearrangements (CR) such as centric fusions and inversions (Hewitt, 1979; Mesa *et al.*, 1982). As in other eukaryotic groups, it may

*Corresponding author. E-mail: castillo.eliorodrigo@gmail.com

be presumed that these chromosome rearrangements have distinctive effects in the heterocygote, of which the suppression of recombination leading to reduced gene flow and accumulation of genetic differences in the affected genomic region has supported chromosomal speciation models (Kirkpatrick, 2010; Faria & Navarro, 2010).

In the Neotropical Ommexechidae, the available cytogenetic data support that 2n = 23♂/24♀ is the modal diploid number for the family (Mesa *et al.*, 1982). However, the presence of an autosome pair (the largest pair, L_1) with submetacentric morphology and a FN = 25♂/26♀ is described in high frequency (51% of karyotyped species) in the family Ommexechidae (Mesa *et al.*, 1982). According to Mesa, these submetacentric autosomes resulted from a pericentric inversion that putatively took place in the ancestor of Ommexechidae, which would represent the oldest shared CR in Acridoidea (Mesa, 1963; Mesa & Ferreira, 1977; Mesa *et al.*, 1982). The submetacentric L_1 autosome is even more frequent in Ommexechinae (the most species-rich subfamily of Ommexechidae), where most species share this trait. Besides the submetacentric L_1, other chromosomal variations are observed in Ommexechinae, such as reductions of 2n (Mesa *et al.*, 1982), neo-sex chromosomes (Mesa, 1961; Mesa & Ferreira, 1977; Mesa *et al.*, 1990), inversions involving sex chromosomes (Carvalho *et al.*, 2011) and B chromosomes (Souza *et al.*, 2015). These karyotypic features add complexity to an entangled scenario of chromosome evolution in the group that remain largely unexplored.

In the context of the occurrence of multiple chromosomal rearrangements, the study of Ommexechinae species could provide information about the karyotype evolution of modern grasshoppers. Until now, studies in the group were performed mostly based on conventional cytogenetic techniques with a reduced taxonomic sampling, giving only limited information about the karyotype variability (Mesa & Ferreira, 1977; Mesa *et al.*, 1990; Carvalho *et al.*, 2011; Souza *et al.*, 2015). The analysis of chromosomal markers based on repetitive DNA has been useful for understanding genome organization and chromosomal evolution in grasshoppers (Cabrero & Camacho, 2008; Cabrero *et al.*, 2009; Cabral-de-Mello *et al.,* 2011a,b; Anjos *et al.*, 2015). Likewise, the use of repetitive DNA as chromosomal markers in Ommexechinae could provide information to support chromosomal homologies and shed light on the CRs involved in karyotype evolution, particularly for the L_1 pair.

The main goal of this work is to test Mesa's hypothesis of chromosomal evolution in Ommexechinae grasshoppers (Mesa, 1963; Mesa *et al.*, 1982). To this goal, we combined classical and molecular cytogenetic techniques and chromosomal mapping of repetitive DNA in five species of Ommexechinae grasshoppers. Based on cytogenetic information, we proposed a series of chromosomal rearrangements that might be involved in the evolution of the L_1 chromosome, causing the observed variations in morphology compared to other modern grasshoppers. The data provide new information about chromosomal evolution in Ommexechinae and the reinterpretation of Mesa's hypothesis.

MATERIAL AND METHODS

Specimen collection, cytogenetic preparations and chromosomal staining

Male and female adults of five Ommexechinae species – *Clarazella bimaculata* Giglio-Tos, 1894, *Calcitrena maculosa* Eades, 1961, *Ommexecha macropterum* Blanchard, 1836, *O. virens* Serville, 1831 and *Pachyossa signata* Rehn, 1913 – were collected, based on their distribution range, from different localities in Argentina and Paraguay (Table 1). Specimens were etherized, dissected and deposited in the entomological collection of Laboratorio de Genética Evolutiva *Dr. Claudio J. Bidau*, Instituto de Biología Subtropical (IBS; CONICET-UNaM). Male testes and female gastric caeca were obtained and conserved in a mixture of ethanol:acetic acid (3:1). Muscular tissue was conserved in cold absolute ethanol at –20 °C.

Male meiotic preparations were performed by squashing testes' follicles in ferric haematoxylin, as described by Núñez (1968), or by crushing follicles with acetic acid (65%). Mitotic metaphase chromosomes were obtained from female gastric caeca, following the procedure described by Castillo *et al.* (2011). Some slides were stained with phosphate-buffered Giemsa solution (5%, pH 6.8) and others were used for C-banding, according to Sumner's protocol (1972). In addition, the base-pair richness was studied by staining with base-specific fluorochromes, chromomycin A_3 (CMA_3, Sigma-Aldrich, USA) for C+G-rich regions and 4', 6-diamino-2-phenylindole (DAPI, Sigma-Aldrich, USA) for A+T-rich regions, according to Schweizer's protocol (1980).

DNA extraction, probes obtaining and fluorescence *in situ* hybridization

Abracris flavolineata De Geer, 1773 genomic DNA was extracted by the phenol-chloroform protocol (Sambrook & Russel, 2001). The extracted DNA was used as template for the isolation of repetitive DNAs, which was carried out by polymerase chain reaction (PCR) with specific primers previously described for

Table 1. Locality of collection, coordinates, number of specimens, banding and FISH results for the analysed species

Species	Locality	Lat (S) / Lon (W)	N.º (♀/♂)	ID number (LGE-O)	C-banding	CMA_3	DAPI	18S	5S	U2	*H3*	TEL
Ca. maculosa	Caucete, San Juan, ARG.	31.614058/ 68.235714	1/3	1066 1075 1076 1071	C	C	-	M_{8p} X_p	L_{2p} M_{3d} M_4	L_{1d}	L_{2p}	T
O. macropterum	Doctor Manuel Belgrano, Jujuy, ARG.	24.082972/ 5.282278	2/0	6241 6242	C $S_{9\text{-}10d}$	$M_{3\text{-}4c}$ $M_{7\text{-}8c}$ S_{10c}	C $S_{9\text{-}10}$	M_{3c} M_{4c}	M_{3d} M_{4i} M_{7p}	L_{1d}	-	T
	Ledesma, Jujuy, ARG.	23.762389/ 64.851194	0/4	6259–6262								
O. virens	Iguazú, Misiones ARG.	25.987361/ 54.647444	3/2	6059-6061 6032-6033	C L_{2i} M_3 $M_{5\text{-}6i}$ M_{8i} $S_{9\text{-}10i}$ $S11_d$	L_{2c} $S_{9\text{-}10}$	M_{4i} $S_{9\text{-}10c}$	L_{2p} $S_{9\text{-}10p}$	L_{2p} M_{5p} M_{8p} M_{3d} S_{10p}	L_{1d}	$S_{9\text{-}10p}$	T
	Guaraní, Misiones ARG.	27.293778/ 54.203694	4/2	1862-1865 1847-1848								
	Gral. Manuel Belgrano, Misiones ARG.	26.174833/ 54.073528	2/5	5670-5671 5647-5651								
	Candelaria, Misiones, ARG.	27.42329/ 55.47848	1/0	9159								
Cl. bimaculata	San Patricio, Misiones, PY.	26.891333/ 56.863611	0/1	3057	C	C	-	M_{4p} X_d M_{8d}	L_{2p} $M_{8\text{-}9p}$ M_{3d}	L_{1d}	L_{2p}	T
	Paraguarí, Paraguarí, PY.	25.617028/ 57.127722	0/5	3120-3121 3953-3954 6345								
	Concepción, Corrientes, ARG.	28.141700/ 58.294500	0/6	9092-9096 9102								
	San Miguel, Corrientes, ARG.	27.979640/ 57.593271	2/0	9113, 9119								
P. signata	Candelaria, Misiones ARG.	27.336667/ 55.561111	0/10	0051 0093-0100 1658	C M_{4i}	L_{2p} X_{Lp}	C M_{4i} X_{Rp}	X_{Lp}	L_{2p} $M_{4\text{-}5p}$	L_{1d}	L_{2p}	T
	Capital, Misiones ARG.	27.705311/ 55.796900	1/2	1337 1351 1352								
	Apóstoles, Misiones ARG.	27.775214/ 55.591856	0/1	6532								

C = centromeric, i = interstitial, d = distal, p = proximal, T = telomeric.

Histone 3 partial gene (*H3*) (Colgan *et al.*, 1998), ribosomal DNA (rDNA) 5S and 18S (Cabral-de-Mello *et al.*, 2010) and small nuclear DNA (snDNA) U2 for Major Spliceosome complex (Bueno *et al.*, 2013). Telomeric probes were obtained using self-complementary primers $(TTAGG)_5$ and $(CCTAA)_5$ through PCR without DNA template, as described by IJdo *et al.* (1991). The labelling of the probes was carried out by PCR with digoxigenin-11-dUTP (Roche, Switzerland). Alternatively, the 18S rDNA probe was also labelled with Nick-Translation with biotin-14-dATP (Invitrogen, Life Technologies, USA), following the manufacturer's instructions. Single Fluorescence In Situ Hybridization experiments of the five probes were performed according to Cabral-de-Mello *et al.* (2010). The probes labelled with digoxigenin-11-dUTP were detected using anti-digoxigenin-rhodamine (Roche, Switzerland), whereas the probe labelled with biotin-14-dATP was identified using streptavidin Alexafluor 488 conjugate (Invitrogen, Life Technologies, USA). Chromosome preparations were counterstained using DAPI diluted in VECTASHIELD mounting media (Vector Laboratories, Burlingame, USA).

Photographs and karyotype construction

The chromosome preparations were photographed using a BX61 microscope (Life Science Solutions, Olympus, Japan) equipped with fluorescent and conventional lamps, and appropriate filters coupled to a DP70 cooled digital camera (Life Science Solutions, Olympus, Japan). Photographs were processed with image-processing software (Corel Draw 2017 v.19.0 and Adobe Photoshop CS6 v.13.0). Karyotypes were made using information about chromosome relative length and centromeric index (CI), obtained by measuring mitotic chromosomes at metaphase with DRAWID v.0.26 (Kirov *et al.*, 2017). Chromosomes were classified in three arbitrary size groups (L = large, M = medium-sized and S = small) and their morphology was determined following the nomenclature proposed by Levan (1964). The neo-XY sexual mechanism was described according to the terminology proposed by White (1940). Neo-X chromosome arms were referred to X_R, as the arm that shares ancestral homology with the neo-Y and X_L as the arm derived from the original X chromosome.

RESULTS

Karyotypes and heterochromatin

The diploid number of 2n = 23♂/24♀ was observed in four species: *Calcitrena maculosa* (Fig. 1A), *Ommexecha macropterum* (Fig. 1B), *O. virens* (Fig. 1C) and *Clarazella bimaculata* (Fig. 1D), with a simple sex chromosome system X0♂/XX♀. A reduced diploid number of 2n = 22♂/22♀ and a neo-XY♂/neo-XX♀ sex chromosome system was noticed in *Pachyossa signata* (Fig. 1E). Concerning chromosome size, two large autosomes were observed in all species (Fig. 1A–E); seven medium autosomes were observed in *Ca. maculosa* (Fig. 1A), *Cl. bimaculata* (Fig. 1D) and *P. signata* (Fig. 1E), whereas six medium autosomes were noticed in the two *Ommexecha* species; three small autosomes were observed in *Ommexecha* species (Fig. 1B, C), two in *Ca. maculosa* (Fig. 1A) and *Cl. bimaculata* (Fig. 1D), and one in *P. signata* (Fig. 1E). The X chromosome of the four species with X0♂/XX♀ sex system was medium-sized (Fig. 1A–D). In *P. signata*, the neo-X was similar in size to pair L_1 and the neo-Y was one of the smallest chromosomes of the complement (relative length = 1.2%, s = 0.1, the 's' means 'standard error (ES)'), similar in size to X_R (relative length = 1.3%, s = 0.2) (Fig. 1E).

Most autosomes showed acrocentric morphology, with the exception of the pair L_1 that was submetacentric in *Calcitrena maculosa*, *Ommexecha macropterum*, *O. virens*, and the pairs S_9 and S_{10} that were metacentric in *O. virens* (Fig. 1A–C in red). The X chromosomes were acrocentric (Fig. 1A–D), the neo-X chromosome was subtelocentric and the neo-Y was acrocentric in *Pachyossa* signata (Fig. 1E). The analysis of male metaphase I (Fig. 1F–J) allowed determination of the number of autosomal bivalents in all species, the presence of X chromosomes as univalent (Fig. 1F, G) and the presence of a heteromorphic sex bivalent (neo-XY) in *P. signata* (Fig. 1J). In the male metaphase I of the last species, we observed the neo-sex bivalent in a 'L' configuration, resulting from a distal chiasma between neo-X_R and neo-Y (Fig. 1J, inset). The variability in chromosome morphology caused variable FN, as follows: FN = 23♂/24♀ in *Clarazella bimaculata* (all acrocentric) and *P. signata* (acrocentric autosomes, subtelocentric neo-X and acrocentric neo-Y); FN = 25♂/26♀ in species with one bi-armed chromosome pair, i.e. *Ca. maculosa* and *O. macropterum*; and FN = 29♂/30♀ in *O. virens*, harbouring three bi-armed autosomal pairs.

Calcitrena maculosa, Clarazella bimaculata, *Ommexecha macropterum* and *Pachyossa signata* showed C-positive heterochromatic blocks in pericentromeric areas (Fig. 2; Table 1). Additionally, *Ca. maculosa* showed extra C-positive blocks in the interstitial regions of M_7 pair (Fig. 2A) and *O. macropterum* presented distal C-positive blocks in S_9, S_{11} pairs (Fig. 2B). In *O. virens,* the distribution of heterochromatin deviated from the centromeric pattern, since C-positive blocks were also present in interstitial regions of ten chromosomes and in the distal regions of S_{11} pair (Fig. 2C; Table 1). No extra C-positive blocks were noticed in *Cl. bimaculata*

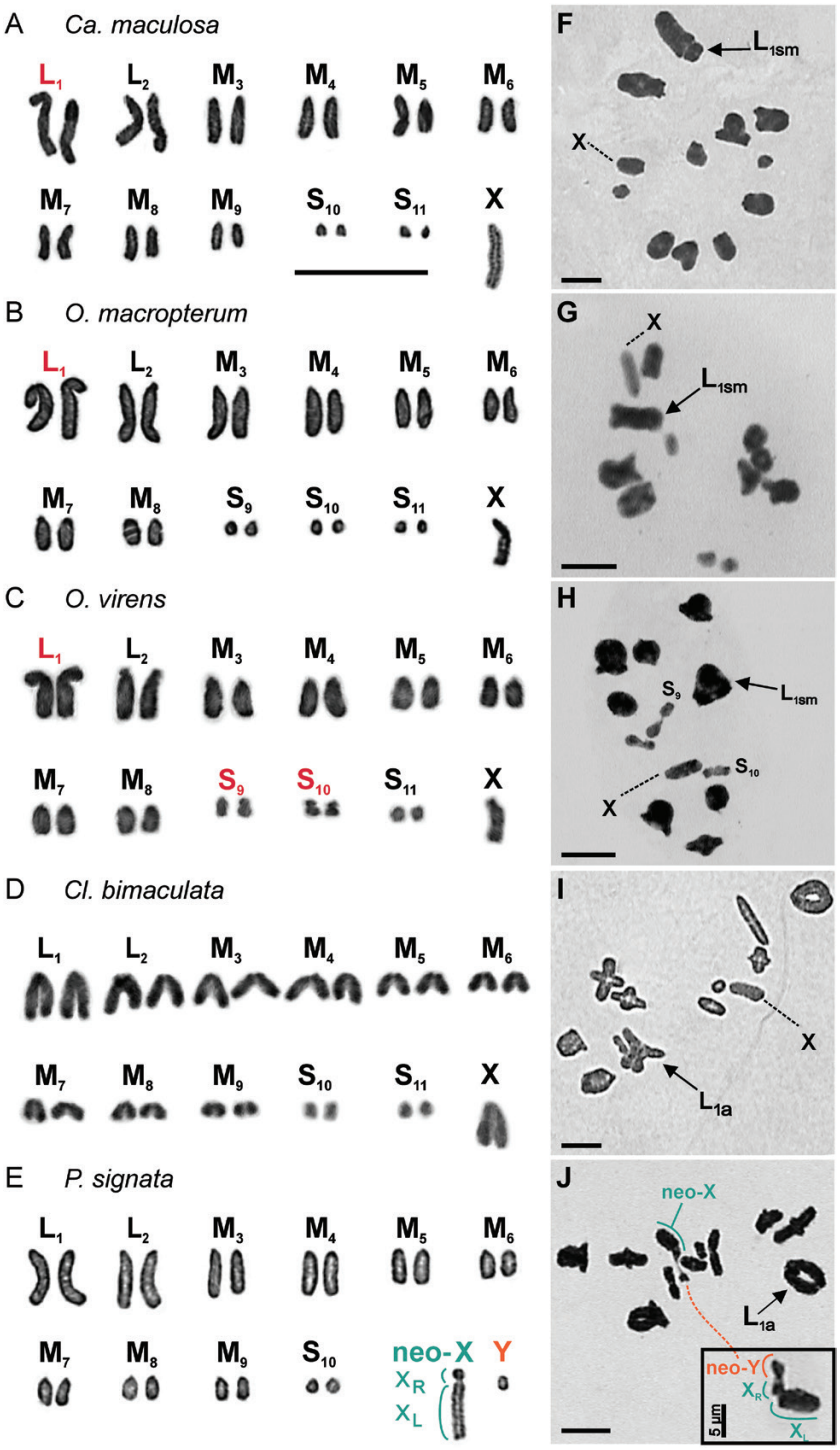

Figure 1. Conventional analysis of mitotic and meiotic chromosomes of five Ommexechinae species. The karyotypes (A–E) and metaphase I of males (F–J) are shown for *Ca. maculosa* (A, F), *O. macropterum* (B, G), *O. virens* (C, H), *Cl. bimaculata* (D, I) and *P. signata* (E, J). Male karyotypes were obtained from spermatogonial cells (A–C, E) or from cells in anaphase I (D). Chromosomes are arranged in decreasing order size, and bi-armed autosomes are indicated in red. Male metaphase I shows (F–J) the first pair of autosomes indicated with arrows and the sex chromosomes with dotted lines. The structure of *P. signata* neo-X chromosome is indicated with light-blue colour and the neo-Y with orange (E, J). Inset (J) highlight *P. signata* neo-XY. Bar = 10µm.

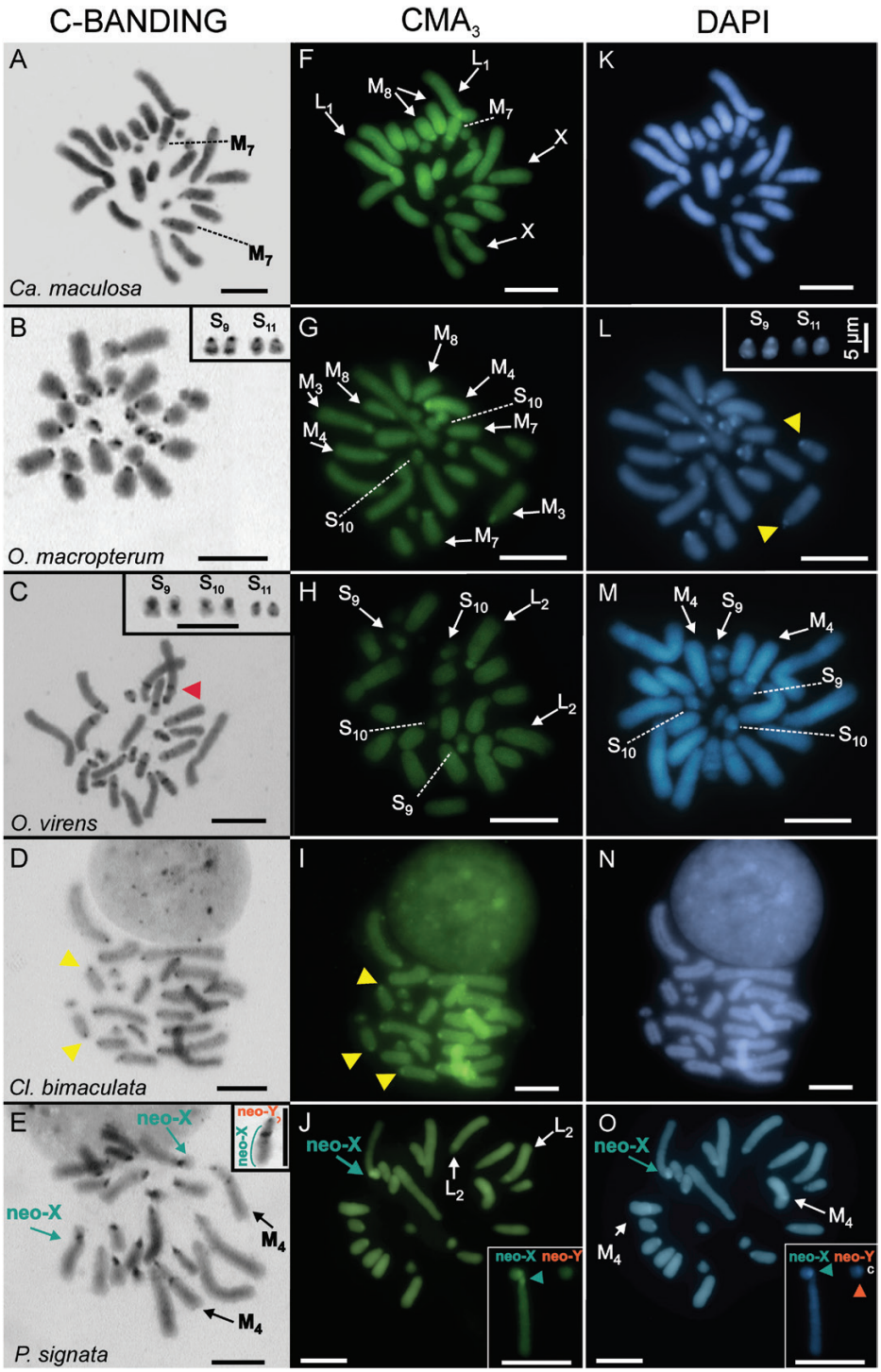

Figure 2. Distribution of heterochromatin and chromatin base-richness in five Ommexechinae species. C banding (A–E), CMA3 (F–J) and DAPI (K–O) staining were performed in *Ca. maculosa* (A, F, K), *O. macropterum* (B, G, L), *O. virens* (C, H, M), *Cl. bimaculata* (D, I, N) and *P. signata* (E, J, O). Pictures were obtained from female somatic mitosis, in exception to CMA3 and DAPI for *P. signata* (J, O), in which spermatogonial mitosis is presented, and also in (E, inset), in which C-banding pattern is shown for neo-sex bivalent at metaphase I. Autosomes with positive signals for each technique are indicated with black and white arrows and dotted lines. Yellow arrowheads exemplify the centromeric positive pattern for different banding techniques: C-positive (D), CMA_3 positive (I), and DAPI positive (L). The red arrowhead exemplifies

(Fig. 2D). *Pachyossa signata* showed extra C-positive heterochromatic blocks at interstitial position in M_4 pair (Fig. 2E).

Chromatin affinity to fluorochromes dyes differed between species, resulting in two general banding patterns (Fig. 2F–J; Table 1): species showing CMA_3 and DAPI positive bands (CMA_3+, DAPI+) and species showing only both CMA_3 positive and DAPI negative (CMA_3+/DAPI–) bands. *Clarazella bimaculata* showed CMA_3+/DAPI– bands in the centromeric regions of all chromosomes (Fig. 2I), whereas *Calcitrena maculosa* showed CMA_3+/DAPI– bands in the centromeric regions of five autosomal pairs (Table 1) and in the interstitial region of one M_7 chromosome (Fig. 2F). *Ommexecha macropterum* showed CMA_3+ bands in the centromeric regions of four M pairs (Table 1) and in S_{10}, and also DAPI+ bands in the centromeric region of all chromosomes, and in the distal region of S_9 and S_{11}. *Pachyossa signata* showed CMA_3+ bands located interstitially in L_2, and DAPI+ bands were found in the centromeric regions of all autosomes and in the interstitial region of M_4 pair. The pericentromeric region of the neo-X was CMA_3+/DAPI–, whereas pericentromeric regions of neo-Y and neo-X_R were DAPI+. *Ommexecha virens* showed CMA_3+/DAPI– in S_9 and S_{10} 'p' arms, CMA_3+ bands in the centromeric region of L_2, DAPI+ bands in the interstitial region of M_4 and in the centromeric region of S_9 and S_{10}.

Mapping of repetitive DNAs

The chromosomal mapping of 18S and 5S rDNA showed variability in loci number and chromosomal location (Table 1; Fig. 3). FISH experiments with 18S rDNA probe reveal two clusters of rDNA genes in *Calcitrena maculosa*, one located on the X chromosome and the other on the autosomal bivalent M_8 at metaphase I (Fig. 3A). In mitotic metaphases of *Ommexecha macropterum*, two pairs of homologous chromosomes (M_3 and M_4) showed hybridization signals for the 18S rDNA probe (Fig. 3B). In *O. virens*, three rDNA clusters were identified, located on the autosomal bivalents L_2, S_9 and S_{10} at metaphase I (Fig. 3C), as well as in *Clarazella bimaculata*, in which M_4 M_8 and X showed hybridization signals (Fig. 3D). In *Pachyossa signata*, hybridization signal was observed only in the neo-X chromosome (Fig. 3D). On the other hand, FISH with the 5S probe revealed clusters in three chromosomal bivalents in *Ca. maculosa* (L_2, M_3 and M_4) and *P. signata* (L_2, M_4 and M_5) at metaphases I, and in three autosomal pairs in *O. macropterum* (M_3, M_4 and M_7) at mitotic metaphase (Fig. 3F, G, J). Eight mitotic chromosomes showed hybridization signals in *O. virens*, including pairs M_3, M_5, S_{10} and only one chromosome of the pairs L_2 and M_8 (Fig. 3H). In *Cl. bimaculata*, four hybridization signals were observed in L_2, M_3, M_8 and M_9 bivalents at metaphase I (Fig. 3I).

In all species studied, a single cluster of U2 snDNA was restricted to the distal end of the long arms of either acrocentric or submetacentric L_1 pairs (Fig. 3K–O). At metaphase I, a single cluster of *H3* was observed in the proximal region of an autosomal bivalent in *Cl. bimaculata* (L_2), *Ca. maculosa* (M_7) and *P. signata* (L_2), whereas two clusters were observed in *O. virens* (proximal in S_9 and S_{10}) (Fig. 3P–S). The telomere probe identified only the actual telomeres (i.e. located at the chromosome ends) across all analysed species (Fig. 4). All data for the chromosomal location of mapped repetitive DNAs are represented in the ideograms of Figure 5.

DISCUSSION

Chromosomal inversions and neo-sex chromosomes are distinctive features in Ommexechinae

Our analyses in Ommexechinae highlight the occurrence of deviations from the standard karyotype described for most modern grasshoppers, i.e. 2n = 23/24, X0/XX and acrocentric chromosomes (Mesa *et al.*, 1982). The karyotypes analysed of *Ca. maculosa*, *Cl. bimaculata*, *O. macropterum* and *P. signata* were coincident with previous descriptions (Mesa *et al.*, 1982). On the contrary, in *O. virens* we noticed the occurrence of S_9 and S_{10} with metacentric morphology, in contrast with previous observations of acrocentric morphology, without changes in chromosome number (Mesa, 1963; Carvalho *et al.*, 2011; Souza *et al.*, 2015).

Based on our data, and the available chromosomal descriptions (Mesa, 1963; Mesa & Ferreira, 1977; Mesa *et al.*, 1982; Carvalho *et al.*, 2011), the main hallmark in Ommexechinae karyotype evolution is the occurrence of pericentric inversions, driving changes in chromosome morphology and FN, without affecting the diploid number of the species. Pericentric inversions were observed not only in autosomes of the studied species (e.g. in L_1 of *Calcitrena maculosa*, *Ommexecha macropterum* and *O. viren*, and S_9 and S_{10} pairs of *O. virens*), but also in the X chromosome of *Descampsacris serrulatum* Thunberg, 1824 and the neo-Y of *Spathalium audouini* Blanchard, 1836

additional interstitial C positive blocks (C). Neo-sex chromosomes and their positive signals are indicated green (neo-X) and orange (neo-Y). Insets show in detail the banding patterns of chromosomes of S group in *O. macropterum* (B) and *O. virens* (C), and *P. signata* neo-XY (E, J, O). C = centromere, bar = 10μm except indicated otherwise.

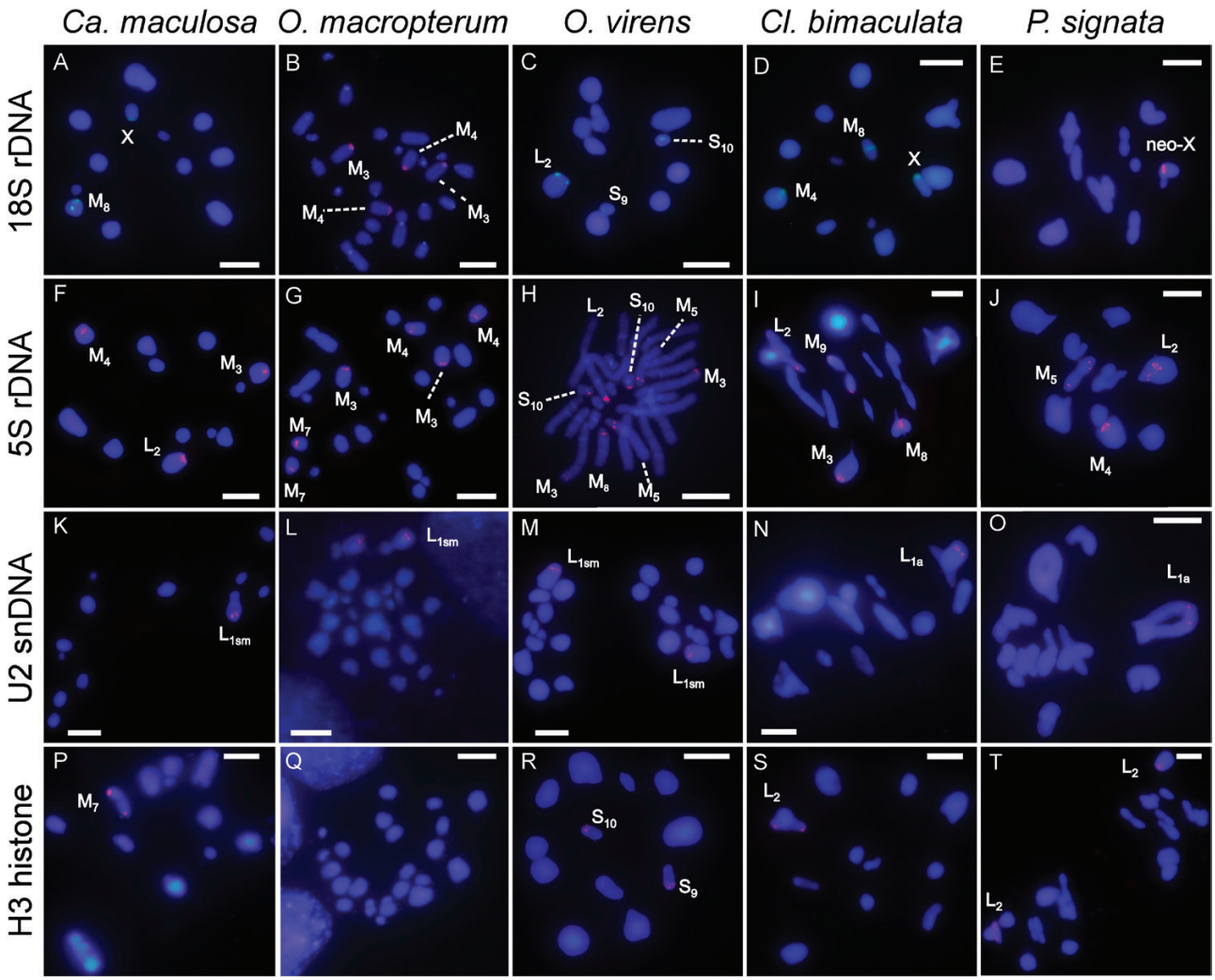

Figure 3. Fluorescence *in situ* hybridization of multigene families in mitotic and meiotic chromosomes of five Ommexechinae species, counterstained with DAPI (blue). Chromosomes with positive signals for 18S rDNA (A–E), 5S rDNA (F–J), U2 snDNA (K–O) and *H3* (P–T) probes are indicated in white for *Ca. maculosa* (A, F, K, P), *O. macropterum* (B, G, L, Q), *O. virens* (C, H, M, R), *Cl. bimaculata* (D, I, N, S) and *P. signata* (E, J, O T). Meiotic cells are presented in most species, except for *O. macropterum* (B, G, L) and *O. virens* (H), in which mitotic cells are presented. *H3* FISH experiment was unsuccessful in *O. macropterum*, due to lack of material (Q). Bar = 10μm.

(Mesa, 1963; Mesa *et al.*, 1990; Carvalho *et al.*, 2011). A high frequency of pericentric inversions, as noticed in Ommexechidae, was also observed in other specific lineages of grasshoppers, such as the Morabini (Eumastacoidea: Morabidae) and Trimerotropini (Acridoidea: Acrididae) (White, 1949; White *et al.*, 1967; Hewitt, 1979). The high frequency of such chromosome rearrangements, i.e. pericentric inversions, could be the result of the propensity of specific groups to the recurrent fixation of them, as proposed by White (1973) under the term karyotype orthoselection.

Centric fusions are also frequent in Ommexechinae, as was inferred from the observation of reduction in the diploid number and changes in both chromosome size and morphology. In this group, all described centric fusions involved the so-called ancestral X chromosome and different autosomes, resulting in the formation of neo-XY/XX sex chromosome determination systems (SCDS), with bi-armed neo-X chromosomes. These SCDS are found in *c.* 25% of Ommexechinae species with available cytogenetic data (Supporting Information, Table S1).

The independent origin of neo-sex chromosomes was reported in several acridid grasshoppers (see: Colombo *et al.*, 2005; Warchałowska-Śliwa *et al.*, 2011; Castillo *et al.*, 2016; Jetybayev *et al.*, 2017a). The neo-sex chromosomes of other Ommexechinae (*Spathalium audouini* Blanchard, 1836, *S. helios* Rehn, 1918 and

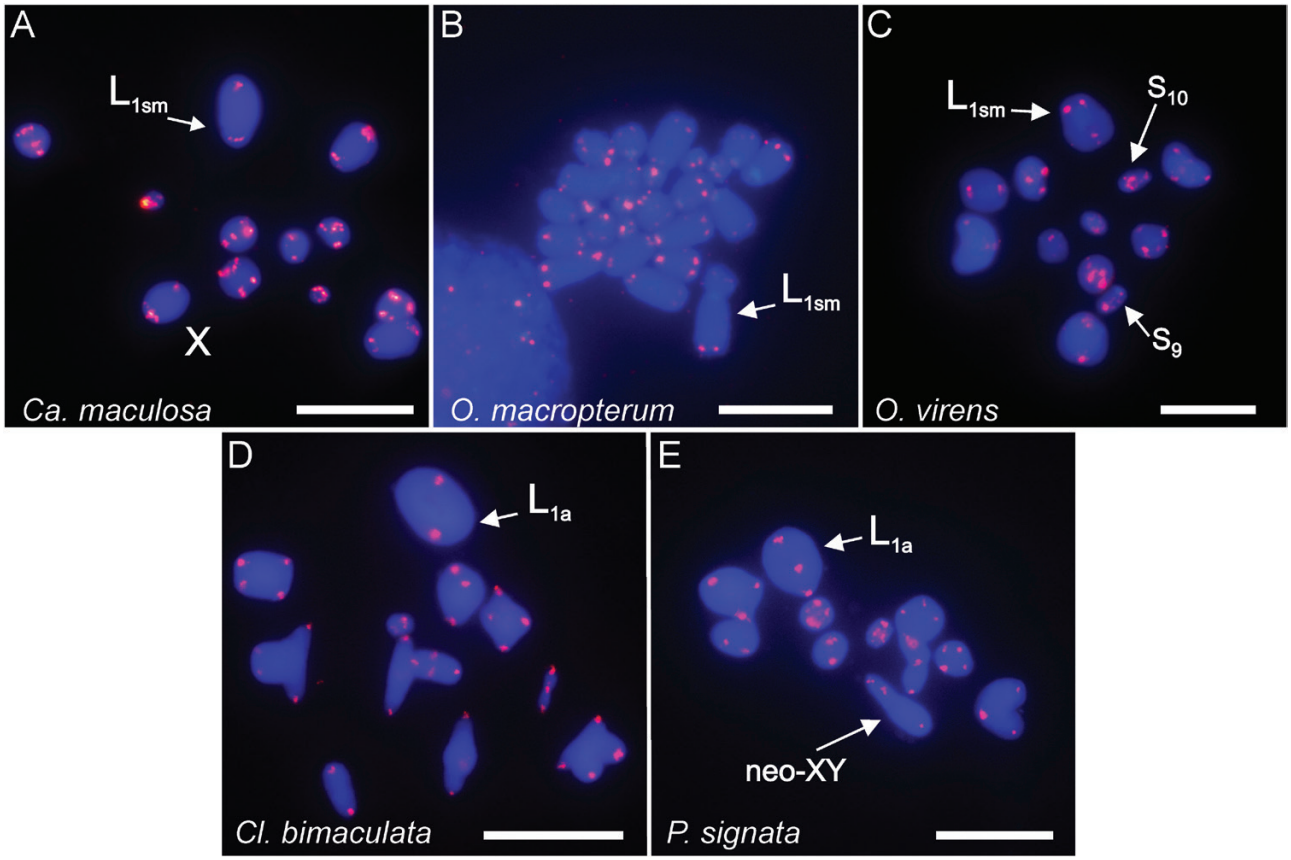

Figure 4. Fluorescence *in situ* hybridization of the (TTAGG)n telomere probe (red signals) in meiotic chromosomes of five Ommexechinae species, counterstained with DAPI (blue). Rearranged chromosomes with are indicated for *Ca. maculosa* (A), *O. macropterum* (B), *O. virens* (C), *Cl. bimaculata* (D), *P. signata* (E). Bar = 10μm.

Tetrixocephalus willemsei Gurney & Liebermann, 1963) were congruent with the observed pattern in numerous Acrididae, where centric fusion involved large- or medium-sized autosomes (Mesa, 1961; Mesa & Ferreira, 1977; Mesa *et al.*, 1990). The unusual neo-XY found in *P. signata* is a unique example among neotropical orthopterans, in which the autosome involved in the chromosome rearrangement with the X chromosome was the smallest chromosome of the complement (Mesa, 1964; this work). In Ommexechinae, most neo-sex chromosomes evolved independently through X-autosome fusions, with the exception of two *Spatalium* species that probably share the same origin for the neo-XY sex chromosome system (Supporting Information, Table S1) (Mesa *et al.*, 1990).

In *Pachyossa signata*, the constitutive heterochromatin was restricted to the centromeric regions in both neo-sex chromosomes, which showed pericentromeric CMA_3+ bands in both X_R and X_L arms, and a pericentromeric DAPI+ band in the neo-Y and the X_R arms. Additionally, chromosome length was similar between the neo-X_R and the neo-Y. Both results (length and chromosome banding patterns) indicate absence of heterochromatinization in the neo-sex chromosomes beyond the centromeres and absence of loss of genetic material in the neo-Y. Low accumulation of repetitive DNAs and heterochromatin (restricted to the proximal region of centromeres) in neo-sex chromosomes was also observed in other species of grasshoppers (Castillo *et al.*, 2010; Palacios-Gimenez *et al.*, 2013; Jetybayev *et al.*, 2017a), contrasting with the highly heterochromatic nature of the neo-Y in other species (Jetybayev *et al.*, 2017b; Ferretti *et al.*, 2020). These differences, together with the location of chiasmata, have been traditionally used to infer the stage of neo-XY differentiation. In *P. signata*, there is evidence of low differentiation between the neo-X and neo-Y chromosomes, in spite of the distal location of chiasmata and in contraposition to the canonical path of sex chromosome evolution, which includes heterochromatinization of the Y chromosome leading to morphologically distinct sex chromosomes [reviewed in Bachtrog (2013)].

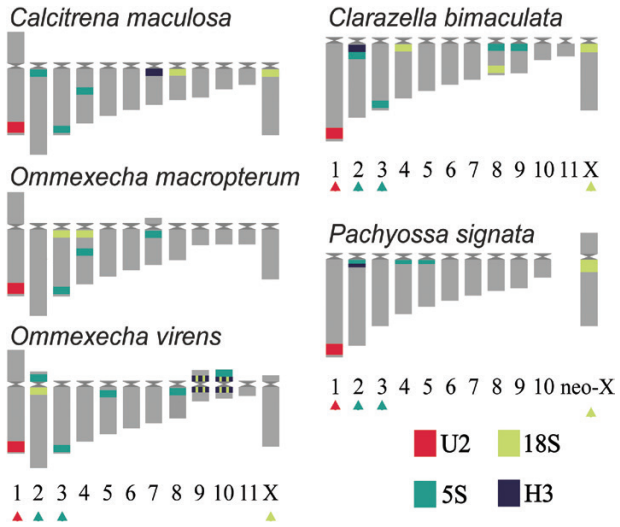

Figure 5. Schematic representation of multigene families chromosomal locations. Species names, probes and chromosome numbers are indicated in each figure. Coloured arrowheads show chromosomes that shared probe locations for at least three species and, therefore, homeology is suggested, as follows: L1 chromosomes of all species (U2 snDNA), L2 (5S rDNA location plus *H3* in some cases) of *P. signata*, *Cl. bimaculata*, *O. virens* and *Ca. maculosa*, M3 of *Cl. bimaculata*, *Ca. maculosa*, *O. virens* and *O. macropterum* (5S rDNA) and X chromosomes of *Cl. bimaculata, Ca. maculosa* together with XR of *P. signata* (18S rDNA).

Heterochromatin and repetitive DNA evidence plasticity on Ommexechinae chromosomes

The main pattern of centromeric constitutive heterochromatin (CH) combined to a reduced number of additional blocks might extend to the subfamily level, since it is coincident to what has been previously observed in *Ommexecha virens* and *Descampsacris serrulatum* (Mesa *et al.*, 1990; Carvalho *et al.*, 2011). The constraints underlying CH distribution might be similar to other Acridoidea groups, where the centromeric pattern was also observed (Santos *et al.*, 1983; John *et al.*, 1985; Cabrero & Camacho, 1986). The only different pattern was observed in *O. virens*, which showed an increased amount of CH in relation to other species and to previous studies (Carvalho *et al.*, 2011), distributed mainly in interstitial regions. Different mechanisms are involved the distribution of the repetitive components of CH, including the amplification or deletion by replication slippage, unequal exchange, among others (reviewed in: John, 1988; Palomeque & Lorite, 2008). The observed intraspecific variability of CH in *O. virens*, could be related to the differential impact of these mechanisms over the wide geographical distribution of the species in South America (Cigliano *et al.*, 2020).

Chromatin composition, inferred from fluorochrome banding patterns, was variable between species. The localization CMA_3+ or DAPI+ bands was always coincident with the localization of heterochromatic C-positive blocks, whereas no fluorescent positive bands were detected in some other C-positive blocks. Constitutive heterochromatin was enriched in GC base pairs in *Cl. bimaculata* and *Ca. maculosa*, whereas the CH was enriched in both CG and AT base pairs in the remaining species, contrasting with a previously described nucleotide composition of CH (CG-rich regions) in *Descampsacris serrulatum* and *O. virens* (Carvalho *et al.*, 2011). The observed heterogeneity in the base-richness of CH is also common in other modern grasshoppers (Acrididae and Romaleidae), which has been described within and between chromosomes of the same species, as well as between chromosomes of different species (John *et al.*, 1985; De França Rocha *et al.,* 2015). Some CG-rich regions were coincident with the presence of 18S rDNA in *Ca. maculosa* (X and M_8), *O. macropterum* (M_4, M_3), *O. virens* (L_2, S_9, S_{10}), *Cl. bimaculata* (M_8, M_4, X) and *P. signata* (neo-X), and other multigene families, such as *H3* and 5S rDNA in *P. signata* (L_2) and *Cl. bimaculata* (L_2, M_8, M_9). This indicates that CMA_3+ could not only correspond to the nucleolar organizer regions, but also to other multigene-family rich regions in Ommexechinae.

In *Ommexecha virens*, both S_9 and S_{10} pairs showed DAPI+ bands at centromeres and CMA_3+/DAPI– pericentromeric bands in their short arms, which were also coincident with the presence of different multigene-families in pericentromeric and distal regions (see below). In *O. macropterum*, chromosomes from the S group showed a similar centromeric DAPI+ pattern, with additional DAPI+ bands in the distal regions of S_9 and S_{11} pair, whereas S_{10} showed CMA_3+ bands near the centromeres and showed no correspondence with any multigene family. Although both species are congeneric, our results suggest morphological and structural divergence of these chromosomes, given by the banding patterns and the location of multigene-family sequences. *Ommexecha virens* also showed a population polymorphism for rDNAs and *H3*, evidencing plasticity of these DNA repeats in its genome and hindering their chromosome homology determination (see discussion below).

The dispersion of repetitive DNAs, mainly rDNA clusters, highlights differentiation between Ommexechinae karyotypes as their distribution was highly variable, even in closely related species, as in species of *Ommexecha*. This dynamism is evident for both rDNA clusters (18S rDNA and 5S rDNA), with a higher degree for 5S rDNA. This is a common pattern for these sequences that are highly dynamic in the

karyotypes of grasshoppers (Cabrero & Camacho, 2008; Cabral-de-Mello *et al.,* 2011b; Castillo *et al.*, 2017; Ferretti *et al.*, 2019) and also in other insects (Nguyen *et al.*, 2010; Cabral-de-Mello *et al.*, 2011b; Bardella *et al.*, 2016; Pita *et al.*, 2016; Salanitro *et al.*, 2017; Anjos *et al.,* 2019). This dynamic has been attributed to transposition and ectopic recombination events (Ferretti *et al.*, 2019; references therein).

The intrinsic dynamism (multiplication and movement) of rDNA clusters might explain the high intraspecific and interspecific variability in number of loci, chromosomes (autosomes and/or sex chromosomes) and chromosomal position in Ommexechinae karyotypes. In *O. virens*, the number of 18S rDNA clusters varied in individuals from different populations, from one (this work) to three (Carvalho *et al.*, 2011; Souza *et al.*, 2015), with a shared cluster located on the proximal region of the L_2 pair. Meanwhile, four (Souza *et al.*, 2015) to five (this work) clusters were observed for 5S rDNA.

Contrasting with rDNAs, the *H3* and U2 snRNA genes were less variable. The presence of *H3* at proximal position in a single autosomal pair in three species was coincident with the hypothesis of the existence of a single ancestral cluster for grasshoppers, that is maintained by purifying selection (Cabrero *et al.*, 2009). *Ommexecha virens* showed an additional cluster of *H3* (S_{10}) in comparison to the other three species studied and to a previous chromosomal mapping in other populations of the same species, where only one cluster was revealed in pair S_9 (Souza *et al.*, 2015). In our analysis, the occurrence of *H3* co-located with rRNA genes and C-positive heterochromatic blocks in chromosomes S_9 and S_{10} might indicate the accumulation of some repetitive DNA sequences in these chromosomes, which could be related to their morphological differentiation, besides the putative pericentric inversion. The U2 number and location of the U2 snRNA gene is highly stable for the five species, in comparison to other sequences, occurring only in pair L_1 at the subterminal position.

The detection of $(TTAGG)_n$ telomeric repeats only in the chromosome ends could be explained by several mechanisms that could have acted on the rearranged bi-armed chromosomes (L_1 *Ca. maculosa*, *O. macropterum* and *O. virens;* S_9 and S_{10} in *O. virens,* neo-X in *P. signata*), such as the lack of inclusion of telomeric sequences in the rearrangements, the deletion of interstitial sequences or their degeneration by substitution (Slijepcevic, 1998; Bolzán & Bianchi, 2006; Lin & Yan, 2008).

Besides the restriction of the U2 snRNA gene to the pair L_1, the remaining mapped multigene families were also coincident in chromosomal location in the analysed species, suggesting a putative similarity or homology between four chromosome pairs in Ommexechinae (Fig. 5 arrowheads). In this sense, the chromosomal mapping of 18S rDNA highlighted similarities between the X chromosomes of *Cl. bimaculata*, *Ca. maculosa* and *P. signata* X_R arm. The localization of 5S rDNA in the proximal region of L_2 and the distal region of M_3 chromosomes was shared by *Ca. maculosa*, *O. virens* and *Cl. bimaculata*. The homology between L_2 chromosomes of *Cl. bimaculata* and *P. signata* could be reinforced based on the close localization of *H3* and 5S probes in these species. Finally, we also determined the homology between the morphologically differentiated L_1 (both acrocentric and submetacentric), based on the conserved location of the U2 snRNA gene in the subterminal position. This location differs from the frequent interstitial U2 snDNA location described for L_1 pairs of other orthopterans lineages (Bueno *et al.*, 2013; Palacios-Gimenez *et al.*, 2013; Castillo *et al.*, 2017), and led us to a better understanding of the existing hypothesis of chromosomal evolution of the group.

Revisiting Mesa's hypothesis about the evolution of the L_1 chromosome in Ommexechidae

The more suitable explanation to the observed differences in L_1 morphology across ommexechids would be the occurrence of an ancestral pericentric inversion involving the standard acrocentric L_1 pair. This statement consists of Mesa's hypothesis, proposed throughout his career to explain the curious pattern observed in Ommexechidae (Mesa, 1963; Mesa & Ferreira, 1977; Mesa *et al.*, 1982).

According to Mesa, the rearranged chromosome would have been fixed in an Ommexechidae ancestor and later inherited by the extant species. Among the 23 species of Ommexechinae studied, the morphology of pair L_1 was described in 15 species, and the biarmed pair L_1 was present in 13 of them, i.e. 86% (Supporting Information, Table S1). This high frequency led to the idea that the rearrangement occurred at least in a common ancestor of the subfamily. It is interesting to note that established rearrangements do not prevent later speciation events or exclude the possibility of later chromosomal rearrangements (Guerrero *et al.*, 2012). Bearing this in mind, deviations from the submetacentric morphology could be explained by the later occurrence and fixation of independent pericentric inversions. Accordingly, the acrocentric L_1 pair in *P. signata* and *Cl. bimaculata*, would be the result of the establishment of a second pericentric inversion, which would have restored the acrocentric chromosome morphology, and would have originated independently in each lineage (Mesa *et al.*, 1982).

Until now, there was no evidence beyond morphological information that could allow a more

in-depth discussion of Mesa's hypotheses about the homology of the L_1 chromosome. In spite of the morphological differences, the location of U2 snDNA restricted to L_1 chromosomes is a good indication that L_1 are homologous across lineages of Ommexechinae. The analysis of chromosomal mapping of the U2 snDNA gene also highlighted a distinctiveness: Ommexechinae acrocentric L_1 pairs are structurally different from some Acridoidea acrocentric L_1 pairs, because they differ in U2 snDNA location. Based on this information, we proposed a series of events that could be involved in the evolution of the L_1 chromosome in Ommexechinae, causing the observed variations in morphology and repositioning of the U2 snRNA gene, in comparison to other modern grasshoppers (Fig. 6).

First, the 'ancestral' acrocentric L_1 went through a large pericentric inversion involving the interstitial region, which included U2 snDNA cluster and turned the acrocentric morphology to the submetacentric one in Ommexechidae (Fig. 6A1–2) and produced the displacement of snDNA U2 to a distal location. The rearranged chromosome reached fixation and it was inherited by extant species, remaining unchanged in some lineages (as in *O. virens*, *O. macropterum* and *Ca. maculosa*). Then, a novel pericentric inversion, involving the entire short arm and part of the large arm of L_1, produced a reversion to the 'ancestral' acrocentric state without modifying the U2 distal location, as observed in *P. signata* and *Cl. bimaculata* (Fig. 6A2–3).

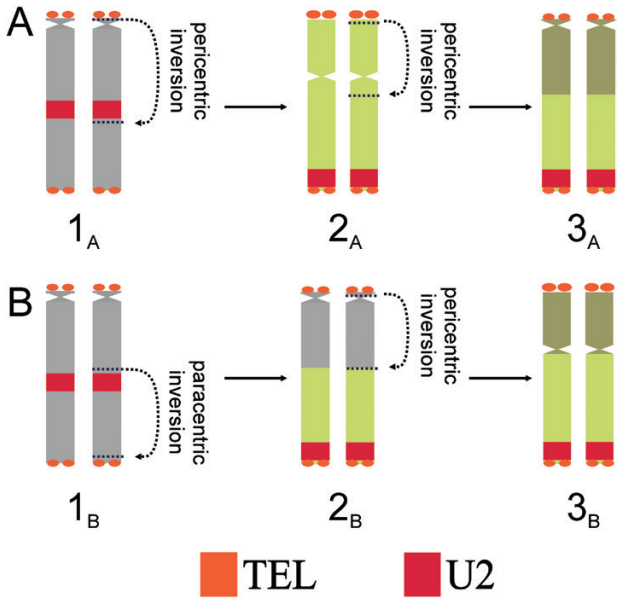

Figure 6. Schematic representation of two pathways in Ommexechinae L1 autosome evolution. To explain the variable L1 morphology and the distal U2 snDNA location, at least two different inversions must be considered. In the first hypothesis (A), two pericentric inversions are considered, the first one (A1–2) modifying L1 morphology (from acrocentric to submetacentric) and U2 snDNA location (from interstitial to distal), and the second one (A2–3) restoring the acrocentric morphology in *P. signata* and *Cl. bimaculata*. The alternative hypothesis involves two different kinds of inversions (B). First, a paracentric inversion changes U2 location without affecting morphology (B1–2) and, then, a pericentric one only changes L1 morphology (B2–3). Both hypotheses are further explained in the paragraph.

However, another alternative cannot be excluded (Fig. 6B): a paracentric inversion occurred in the ancestral acrocentric L_1 pair and displaced the U2 gene to a distal position, maintaining the morphology unchanged, as seen in in *Cl. bimaculata* and *P. signata* (Fig. 6B1–2). Following, a smaller pericentric inversion changed the acrocentric morphology to a submetacentric one and was fixed and inherited by the remaining species (Fig. 6B2–3). In both alternative pathways, at least two different ancient inversions are necessary to explain the observed pattern in morphology and U2 gene location.

To know the extent of Mesa's hypothesis, and to determine whether the proposed ancestral L_1 inversion occurred in an Ommexechidae or an Ommexechinae ancestor, the morphology of the first pair should be interpreted, including species from the other subfamilies and under a robust phylogenetic hypothesis. The reason for this is the existence of well-documented, homoplasic CRs in orthopterans, for example, in sex chromosomes (e.g. Colombo *et al.*, 2005; Warchałowska-Śliwa *et al.*, 2011; Castillo *et al.*, 2016; Jetybayev *et al.*, 2017a). In this sense, the submetacentric L_1 present in species of the Aucacridinae subfamily (Ommexechidae) could be the result of an independent pericentric inversion event. Nevertheless, L_1 chromosomes of ommexechids provide an exciting opportunity to deepen our understanding of chromosomal plasticity among grasshoppers, an area that could be better understood with actual genomic technologies.

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REFERENCES

Anjos A, **Ruiz-Ruano FJ**, **Camacho JPM**, **Loreto V**, **Cabrero J**, **De Souza MJ**, **Cabral-de-Mello DC. 2015.** U1 snDNA clusters in grasshoppers: chromosomal dynamics and genomic organization. *Heredity* **114:** 207–219.

Anjos A, **Paladini A**, **Evangelista O**, **Cabral-de-Mello DC. 2019.** Insights into chromosomal evolution of Cicadomorpha using fluorochrome staining and mapping 18S rRNA and H3 histone genes. *Journal of Zoological Systematic Research* **57:** 314–322.

Bachtrog D. 2013. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nature Reviews Genetics* **14:** 113–124.

Bardella VB, **Pita S**, **Vanzela ALL**, **Galvão C**, **Panzera F. 2016.** Heterochromatin base pair composition and diversification in holocentric chromosomes of kissing bugs (Hemiptera, Reduviidae). *Memórias do Instituto Oswaldo Cruz* **111:** 614–624.

Bolzán AD, **Bianchi MS. 2006.** Telomeres, interstitial telomeric repeat sequences, and chromosomal aberrations. *Mutation Research/Reviews in Mutation Research* **612:** 189–214.

Bueno D, **Palacios-Gimenez OM**, **Cabral-de-Mello DC. 2013.** Chromosomal mapping of repetitive DNAs in the grasshopper *Abracris flavolineata* reveal possible ancestry of the B chromosome and H3 histone spreading. *PLoS One* **8:** e66532.

Cabral-de-Mello DC, **Moura RC**, **Martins C. 2010.** Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. *Heredity* **104:** 393–400.

Cabral-de-Mello DC, **Cabrero J**, **López-León MD**, **Camacho JPM. 2011a.** Evolutionary dynamics of 5S rDNA location in acridid grasshoppers and its relationship with H3 histone gene and 45S rDNA location. *Genetica* **139:** 921–931.

Cabral-de-Mello DC, **Oliveira SG**, **De Moura RC**, **Martins C. 2011b.** Chromosomal organization of the 18S and 5S rRNAs and histone H3 genes in *Scarabaeinae coleopterans*: insights into the evolutionary dynamics of multigene families and heterochromatin. *BMC Genetics* **12:** 88.

Cabrero J, **Camacho JPM. 1986.** Cytogenetic studies in gomphocerine grasshoppers. I. Comparative analysis of chromosome C-banding pattern. *Heredity* **56:** 365.

Cabrero J, **Camacho JPM. 2008.** Location and expression of ribosomal RNA genes in grasshoppers: abundance of silent and cryptic loci. *Chromosome Research* **16:** 595–607.

Cabrero J, **López-León MD**, **Teruel M**, **Camacho JPM. 2009.** Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. *Chromosome Research* **17:** 397–404.

Carvalho DB, **Rocha MF**, **Loreto V**, **Silva AE**, **Souza MJ. 2011.** *Ommexecha virens* (Thunberg, 1824) and *Descampsacris serrulatum* (Serville, 1831) (Orthoptera, Ommexechidae): karyotypes, constitutive heterochromatin and nucleolar organizing regions. *Comparative Cytogenetics* **5:** 123–132.

Castillo ER, **Marti DA**, **Bidau CJ. 2010.** Sex and neo-sex chromosomes in Orthoptera: a review. *Journal of Orthoptera Research* **19:** 213–231.

Castillo ER, **Taffarel A**, **Martí DA. 2011.** Una técnica alternativa para el cariotipado mitótico en saltamontes: bandeo C y fluorescente en *Adimantus ornatissimus* (Orthoptera: Acrididae). *Revista de Ciencia y Tecnología* **16:** 30–34.

Castillo ERD, **Taffarel A**, **Mariottini Y**, **Fernández-Arhex V**, **Martí DA**, **Bidau CJ. 2016.** Neo-sex chromosomes in the Maculipennis species group (Dichroplus: Acrididae, Melanoplinae): the cases of *D. maculipennis* and *D. vittigerum*. *Zoological Science* **33:** 303–310.

Castillo ERD, **Taffarel A**, **Maronna MM**, **Cigliano MM**, **Palacios-Gimenez OM**, **Cabral-de-Mello DC**, **Martí DA. 2017.** Phylogeny and chromosomal diversification in the *Dichroplus elongatus* species group (Orthoptera, Melanoplinae). *PLoS One* **12:** e0172352.

Cigliano MM, **Braun H**, **Eades DC**, **Otte D. 2020.** *Ommexechidae. Orthoptera species file, v. 5.0/5.0*. Available at http://Orthoptera.SpeciesFile.org (accessed 3 August 2020).

Colgan DJ, **Mclauchlan A**, **Wilson GD**, **Livingston SP**, **Edgecombe GD**, **Macaranas J**, **Cassis G**, **Gray MR. 1998.** Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. *Australian Journal of Zoology* **46:** 419–437.

Colombo P, **Cigliano MM**, **Sequeira AS**, **Lange CE**, **Vilardi JC**, **Confalonieri VA. 2005.** Phylogenetic relationships in *Dichroplus* Stål (Orthoptera: Acrididae: Melanoplinae) inferred from molecular and morphological data: testing karyotype diversification. *Cladistics* **21:** 375–389.

De França Rocha M, **Pine MB**, **Dos Santos Oliveira EFA**, **Loreto V**, **Gallo RB**, **Da Silva CR**, **Campos de Domenico F**, **Da Rosa R. 2015.** Spreading of heterochromatin and karyotype differentiation in two *Tropidacris* Scudder, 1869 species (Orthoptera, Romaleidae). *Comparative Cytogenetics* **9:** 435.

Faria R, **Navarro A. 2010.** Chromosomal speciation revisited: rearranging theory with pieces of evidence. *Trends in Ecology & Evolution* **25:** 660–669.

Ferretti ABSM, **Ruiz-Ruano FJ**, **Milani D**, **Loreto V**, **Martí DA**, **Ramos E**, **Martins C**, **Cabral-de-Mello DC. 2019.** How dynamic could be the 45S rDNA cistron? An intriguing variability in a grasshopper species revealed by integration of chromosomal and genomic data. *Chromosoma* **128:** 165–175.

Ferretti AB, **Milani D**, **Palacios-Gimenez OM**, **Ruiz-Ruano FJ**, **Cabral-de-Mello DC. 2020.** High dynamism

for neo-sex chromosomes: satellite DNAs reveal complex evolution in a grasshopper. *Heredity* **125:** 124–137.

Guerrero RF, **Rousset F**, **Kirkpatrick M. 2012.** Coalescent patterns for chromosomal inversions in divergent populations. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 430–438.

Hewitt GM. 1979. Insecta 1: Orthoptera. Grasshoppers and crickets. In: John B, Bauer H, Kayano H, Levan A, White M, eds. *Animal cytogenetics 3*. Berlin: Gebrüder Borntraeger.

IJdo JW, **Wells RA**, **Baldini A**, **Reeders ST. 1991.** Improved telomere detection using a telomere repeat probe (TTAGGG) n generated by PCR. *Nucleic Acids Research* **19:** 4780.

Jetybayev IY, **Bugrov AG**, **Ünal M**, **Buleu OG**, **Rubtsov NB. 2017a.** Molecular cytogenetic analysis reveals the existence of two independent neo-XY sex chromosome systems in Anatolian Pamphagidae grasshoppers. *BMC Evolutionary Biology* **17:** 1–12.

Jetybayev IY, **Bugrov AG**, **Buleu, OG**, **Bogomolov AG**, **Rubtsov NB. 2017b.** Origin and evolution of the neo-sex chromosomes in Pamphagidae grasshoppers through chromosome fusion and following heteromorphization. *Genes* **8:** 323.

John B. 1988. The biology of heterochromatin. In: Verma RS, ed. *Heterochromatin Molecular and Structural Aspects*. Cambridge: Cambridge University Press, 1–47.

John B, **King M**, **Schweizer D**, **Mendelak M. 1985.** Equilocality of heterochromatin distribution and heterochromatin heterogeneity in acridid grasshoppers. *Chromosoma* **91:** 185–200.

Kirkpatrick M. 2010. How and why chromosome inversions evolve. *PLoS Biology* **8:** e1000501.

Kirov I, **Khrustaleva L**, **Van Laere K**, **Soloviev A**, **Meeus S**, **Romanov D**, **Fesenko I. 2017.** DRAWID: user-friendly java software for chromosome measurements and idiogram drawing. *Comparative Cytogenetics* **11:** 747–757.

Leavitt JR, **Hiat KD**, **Whiting MF**, **Song H. 2013.** Searching for the optimal data partitioning strategy in mitochondrial phylogenomics: a phylogeny of Acridoidea (Insecta: Orthoptera: Caelifera) as a case study. *Molecular Phylogenetics and Evolution* **67:** 494–508.

Levan A, **Fredga K**, **Sandberg AA. 1964.** Nomenclature for centromeric position on chromosomes. *Hereditas* **52:** 201–220.

Lin KW, **Yan J. 2008.** Endings in the middle: current knowledge of interstitial telomeric sequences. *Mutation Research/Reviews in Mutation Research* **658:** 95–110.

Mesa A. 1961. Morfologia falica y cariologia de *Neuquenina fictor* (Rehn) (Orthoptera-Acridoidea). *Comunicación Zoologica del Museo de Historia Natural de Montevideo* **5:** 1–11.

Mesa A. 1963. Acerca de la cariología de Ommexechidae (Orthoptera-Acridoidea). *Revista de la Sociedad Uruguaya de Entomología* **5:** 37–43.

Mesa A. 1964. Los cromosomas de *Pachyossa sp.* (Orthoptera, Ommexechidae). *Revista de la Sociedad Uruguaya de Entomología* **6:** 49–54.

Mesa A, **Ferreira A. 1977.** Cytological studies in family Ommexechidae (Orthoptera-Acridoidea). *Acrida* **6:** 261–271.

Mesa A, **Ferreira A**, **Carbonell CS. 1982.** Cariología de los acridoideos neotropicales: estado actual de su conocimiento y nuevas contribuciones. *Annales de la Société Entomologique de France* **18:** 507–526.

Mesa A, **Fontanetti CS**, **Costa H. 1990.** The karyotype of the grasshopper *Spathalium helios* Rehn 1918 (Orthoptera, Acridoidea, Ommexechidae). *Revista Brasileira de Genética* **13:** 705–710.

Nguyen P, **Sahara K**, **Yoshido A**, **Marec F. 2010.** Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). *Genetica* **138:** 343–354.

Núñez O. 1968. An acetic-haematoxylin squash method for small chromosomes. *Caryologia* **21:** 115–119.

Palacios-Gimenez OM, **Castillo ER**, **Martí DA**, **Cabral-de-Mello DC. 2013.** Tracking the evolution of sex chromosome systems in Melanoplinae grasshoppers through chromosomal mapping of repetitive DNA sequences. *BMC Evolutionary Biology* **13:** 167.

Palomeque T, **Lorite P. 2008.** Satellite DNA in insects: a review. *Heredity* **100:** 564–573.

Pita S, **Lorite P**, **Nattero J**, **Galvão C**, **Alevi KCC**, **Teves SC**, **Azeredo-Oliveira MTV**, **Panzera F. 2016.** New arrangements on several species subcomplexes of *Triatoma* genus based on the chromosomal position of ribosomal genes (Hemiptera – Triatominae). *Infection, Genetics and Evolution* **43:** 225–231.

Salanitro LB, **Massaccesi AC**, **Urbisaglia S**, **Bressa MJ**, **Chirino MG. 2017.** A karyotype comparison between two species of bordered plant bugs (Hemiptera, Heteroptera, Largidae) by conventional chromosome staining, C-banding and rDNA-FISH. *Comparative Cytogenetics* **11:** 239–248.

Sambrook J, **Russel DW. 2001.** *Molecular cloning. A laboratory manual*, *3rd edn*. New York: Cold Spring Harbor Laboratory Press.

Santos JL, **Arana P**, **Giráldez R. 1983.** Chromosome C-banding patterns in Spanish Acridoidea. *Genetica* **61:** 65–74.

Schweizer D. 1980. Simultaneous fluorescent staining of R bands and specific heterochromatic regions (DA-DAPI bands) in human chromosomes. *Cytogenetics and Genome Research* **27:** 190–193.

Slijepcevic P. 1998. Telomeres and mechanisms of Robertsonian fusion. *Chromosoma* **107:** 136–140.

Souza TE, **Silva-Neto LC**, **Santos JF**, **Loreto V**, **Rieger TT. 2015.** A small B chromosome in the grasshopper *Ommexecha virens* (Ommexechidae). *Genetics and Molecular Research* **14:** 17505–17510.

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. *Experimental Cell Research* **75:** 304–306.

Warchałowska-Śliwa E, **Maryańska-Nadachowska A**, **Grzywacz B**, **Karamysheva T**, **Lehmann AW**, **Lehmann GU**, **Heller KG. 2011.** Changes in the numbers of chromosomes and sex determination system in bushcrickets of the genus *Odontura* (Orthoptera: Tettigoniidae: Phaneropterinae). *European Journal of Entomology* **108:** 183–195.

White MJD. 1940. The origin and evolution of multiple sex-chromosome mechanisms. *Journal of Genetics* **40:** 303–333.

White MJD. 1949. A cytological survey of wild populations of *Trimerotropis* and *Circotettix* (Orthoptera, Acrididae). I. The chromosomes of twelve species. *Genetics* **34:** 537.

White MJD. 1973. Animal cytology and evolution, *3rd edn*. Cambridge: Cambridge University Press.

White MJD, **Blackith RE**, **Blackith RM**, **Cheney J. 1967.** Cytogenetics of the *viatica* group morabine grasshoppers. I. The coastal species. *Australian Journal of Zoology* **15:** 263–302.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Available cytogenetic information for Ommexechidae grasshoppers.