Genetica (2010) 138:775–786
DOI 10.1007/s10709-010-9458-8

Neo-sex chromosome diversity in Neotropical melanopline grasshoppers (Melanoplinae, Acrididae)

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Received: 3 August 2009 / Accepted: 9 April 2010 / Published online: 22 April 2010

Abstract We report the results of a study on the neo-sex chromosome systems of six Neotropical Melanoplinae species for contributing to a better understanding of their origin and behaviour of these systems. Our analyses included detailed descriptions of the structure and behaviour of the sex chromosome configurations in male and female meiosis of species belonging to the genera *Ronderosia*, *Dichromatos* and *Atrachelacris*. Three species, *R. forcipatus*, *R. malloi* and *A. unicolor*, showed typical Robertsonian fusion-derived neo sex-chromosomes. However, the male metaphase I orientation of *R. bergi* sex pair indicated that more than one rearrangement was involved in its origin. The two species of *Dichromatos* presented a multiple neo-$X_1X_2Y/X_1X_1X_2X_2$ sex system, with two Robertsonian fusions involved in their genesis. Observations of female meiosis, confirmed the nature of the sex-chromosomes analyzed. Our results also showed different degrees of homology divergence between the neo-sex chromosomes and emphasize the plasticity of the chromosome complement of the Neotropical Melanoplinae to establish Robertsonian fusions and generate novel sex-chromosome systems. We also discuss karyotypic diversity within this group in terms of the centromeric drive theory of chromosomal evolution.

Keywords Grasshoppers · Karyotypic evolution · Melanoplines · Neo-sex chromosomes

Dedicated in fond memory of Prof. Alejo Mesa, pioneer in the study of Neotropical chromosomal diversity of grasshoppers.

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Introduction

Neo-sex chromosomes occur in a wide range of plant and animal species (Barlow and Wiens 1976; Hewitt 1979; Schmid et al. 1992; Smith and Virkki 1978; White 1973). These sex systems usually result from the centric (Robertsonian, Rb) fusion or reciprocal translocation between one of the sex chromosomes (X or Y) and one or more autosomes (A) (Bidau and Martí 2001; Hewitt 1979; King 1993; Mesa et al. 2001; White 1973; White et al. 1967). The comparative analysis of neo-XY systems of related species also indicates that these chromosomes evolve towards progressive genetic differentiation between the two new sex chromosomes (Charlesworth and Wall 1999; Grützner et al. 2004; Sáez 1963). Various models have been proposed to explain the possible adaptive value of these systems and their subsequent evolution (Charlesworth and Wall 1999).

Because of their relatively recent evolutionary origin, neo-X and neo-Y chromosomes are very useful model systems to investigate how the Y chromosome stopped to recombine extensively and became a "degenerate" element (Charlesworth 1996). Thus for example, neo-sex chromosomes of *Drosophila miranda* have provided a unique

opportunity to analyze, at the molecular level, the evolutionary process of Y chromosome degeneration (Steinemann and Steinemann 1998). Likewise, the experimental approach in the *Drosophila nasuta nasuta-albomicans* complex, in which a novel chromosomal race produced by laboratory hybridization, allowed the study of the process of transition of an autosome to a Y sex chromosome from the moment of its commitment to the male genome (Tanuja et al. 1999).

Many principles of the evolution of sex chromosomes can be applied to the neo-chromosomes that evolve from Rb or reciprocal translocation between a sex chromosome and an autosome. Sex chromosome-autosome translocations often occur in more than one genus within a family, as is evident in bats and gazelles (Ashley 2002). When the new sex-chromosome mechanism is established, the homology shared by the neo-X and the neo-Y due to its autosomal past, begins to gradually disappear. Essentially, chromosome rearrangements like Rb fusions that involve an original X and an autosome, could affect the recombination rate in the paracentromeric autosomal region creating tight linkage between the sex chromosome and genes that are favorable in the heterogametic sex (Charlesworth et al. 2005).

In spite of the apparent karyotypic stability of Acridoidea with the vast majority of species exhibiting a modal karyotype (2n = 23♂/24♀) with acro-telocentric chromosomes and an XO/XX sex chromosome mechanism (FN = 23/24), Neotropical Melanoplines present a large number of species with derived karyotypes mainly due to the occurrence of Rb translocations (Bidau 1990, Bidau and Marti 1995, Colombo et al. 2005; Martí and Bidau 1995; Mesa et al. 1982). This taxonomic group becomes even more interesting in this respect if we consider only the Dichroplini tribe because, up to now, more than 75% of the known species have incorporated neo-XY systems (Carbonell and Mesa 2006; Mesa et al. 1982). An example of the extreme divergence from the ancestral 2n and FN is the case of *Dichroplus silveiraguidoi* (2n = 8, XY, FN = 13/14) (Cardoso et al. 1974; Sáez and Pérez-Mosquera 1977). Rb fusions between X chromosomes and autosomes have occurred many times in the evolutionary history of Acridoidea and, especially in Neotropical Melanoplinae which exhibit an amazing diversity of neo-XY chromosomes (Bidau and Martí 2001; Colombo et al. 2005; Mesa et al. 1982; Mesa et al. 2001). Moreover in some cases, a second Rb translocation event occurred between the neo-Y and another autosome giving rise to a neo-X_1X_2Y complex sex-chromosome system. Only a few species within this group have been described with this sex-determining mechanism (Mesa 1962b; Mesa et al. 1982).

A number of studies confirm the tendency of Melanoplines to incorporate recurrently these chromosomal rearrangements (Carbonell and Mesa 2006; Cardoso and Dutra 1979; Díaz and Sáez 1968; Martí and Bidau 2001; Mesa 1962a, 1962b; Mesa et al. 1982; Sáez and Pérez-Mosquera 1977). Until now, the review of Mesa et al. (1982) is the most inclusive from the cytogenetic point of view, providing a description of the chromosome number, morphology, sex determination mechanism and occurrence of polymorphisms of the Neotropical Acridofauna. In this paper, we analyse the origin, differentiation and meiotic behaviour of novel chromosomal sex-determining mechanisms in related Melanopline species with the aim of contributing to a better understanding of the origin and behaviour of these systems.

Materials and Methods

Male and female adult grasshoppers were sampled from twenty localities, in six argentine provinces from 2005 to 2008 (Table 1, Fig. 1).

Male meiotic preparations were performed by squashing testes follicles in ferric haematoxylin. Mitotic metaphase chromosomes from female gastric caeca were obtained after 0.05% colchicine injection. A portion of caecum was ruptured in 45% acetic acid for 12 min and the suspension was dropped onto a warm slide where the material was disrupted, air-dried and stained with phosphate buffered 5% Giemsa.

Female meiosis slide preparation followed the laboratory protocol (Henriques-Gil et al. 1987; Martí and Bidau 1995). Briefly, gravid females were kept in observation chambers with ample food, water and humid sand to stimulate oviposition which triggers the continuation of the arrested meiosis. Upon observation of oviposition behaviour, females were etherized, and eggs extracted in insect saline were fixed in 3 methanol: 1 glacial acetic acid and kept at 4°C. Cytological preparations were made after placing the egg in 70% acetic acid for 20 min. Then, the exochorion was removed and the micropilar end of the egg squashed in lacto-propionic orcein. Gentle heating of the preparation is useful for obtaining well-spread first metaphases.

Silver staining of kinetochores and chromatid cores were carried out according to the procedure of Rufas (1985). Briefly, air-dried male meiotic preparations were incubated in 2xSSC at 60° C for 10 min and stained with 50% NO_3Ag in dH2O (pH adjusted to 3.5 with formic acid). Microscopic observation of silver stained preparations employed bright field and Nomarski interference optics. C-banding procedure followed Sumner (1972).

Chiasmata were scored at metaphase I in both sexes and classified by inspection as proximal (P), interstitial (I) and distal (D), corresponding to their presence in the first (para-

Table 1 Province, locality, geographic coordinates (in decimal degrees), species, and number of male and female individuals (M/F) of grasshoppers of the genera *Ronderosia*, *Dichromatos* and *Atrachelacris* studied in this paper

Province	Locality	LAT (S)/LON (W)	Species	Number of individuals (M/F)
Misiones	1. San Pedro	26.63/54.12	*R. bergi*	5/5
			D. schrottkyi	1/0
	2. Moconá	27.21/54.03	*R. bergi*	1/0
			D. lilloanus	2/3
	3. Gobernador Roca	27.17/55.46	*R. bergi*	3/0
	4. Colonia Alberdi	27.36/55.24	*R. bergi*	7/5
	5. 25 de Mayo	27.38/54.74	*R. bergi*	4/3
	6. Posadas	27.42/55.96	*R. bergi*	11/5
			R. forcipatus	1/0
	7. Yaguaroundí	26.71/54.25	*R. bergi*	42/24
			D. lilloanus	30/35
			D. schrottkyi	2/1
Chaco	8. Presidente de la Plaza	27.00/59.85	*R. bergi*	1/0
	9. Isla del Cerrito	27.43/58.87	*R. forcipatus*	4/2
Córdoba	10. La Carlota	33.42/63.32	*R. bergi*	3/2
	11. Alpa Corral	32.70/64.70	*R. bergi*	13/7
			R. forcipatus	0/2
San Luis	12. 3.5 km W of Saladillo	33.20/65.85	*R. bergi*	1/1
Formosa	13. Palma Sola	25.23/58.09	*R. bergi*	3/2
			A. unicolor	7/4
	14. Riacho He–He	25.31/58.28	*R. bergi*	2/4
	15. 25 km E of Clorinda	25.49/57.83	*R. forcipatus*	1/2
Entre Ríos	16. 4 km N of La Paz	30.72/59.57	*R. bergi*	1/1
			A. unicolor	1/0
Salta	17. Aeropuerto Internacional	24.84/65.48	*R. bergi*	9/4
			R. malloi	9/5
	18. La Caldera	24.60/65.38	*R. bergi*	6/4
	19. Lesser	24.71/65.44	*R. malloi*	1/0
	20. San Lorenzo	24.75/65.48	*R. bergi*	11/5
			R. malloi	0/2

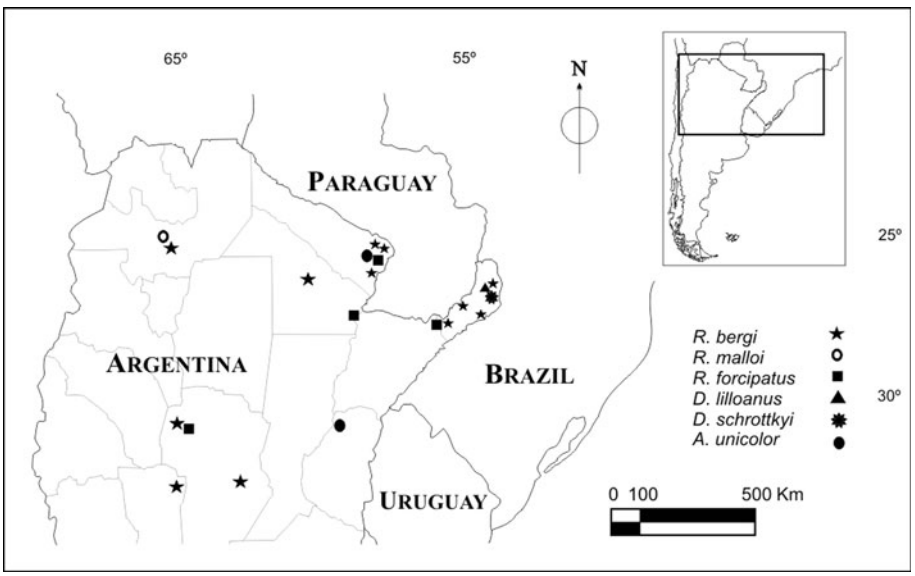


Fig. 1 Geographic distribution of Melanopline grasshoppers studied in this paper

centromeric), second, or third portion of the chromosome arm, respectively, when divided into three equal regions.

Using White's (1973) terminology the arms of the neo-X chromosome will be referred to as XR (the arm which shares homology with the neo-Y) and XL the arm derived from the original X chromosome. This nomenclature is strictly applicable to simple centric fusion-derived neo-XY systems (Bidau and Martí 2001). In the case of multiple $X_1X_2Y/X_1X_1X_2X_2$ sex chromosome determination systems, the neo-X_1 is formed by the XL and the XR arms. The neo-Y is now metacentric, formed by the YL limb while the other arm, the YR, shared homology with the neo-X_2.

Voucher specimens are deposited in the collection of the Laboratorio de Genética Evolutiva of the Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones (Appendix 1).

Results

Neo-XY systems

Four of the analyzed species showed neo-XY sex-chromosome mechanisms and diploid numbers reduced with respect to the ancestral Cryptosacci karyotype. *Ronderosia malloi* (Liebermann, 1966) (Fig. 2) and *R. forcipatus* (Rehn, 1918) (Fig. 3) showed 2n = 20 ♂ (FN = 23)/20♀ (FN = 24) with one pair of large metacentric autosomes. *Atrachelacris unicolor* Giglio-Tos, 1894 (Fig. 4) and *Ronderosia bergi* (Stål, 1878) (Fig. 5) had 2n = 22 ♂ (FN = 23)/22 ♀ (FN = 24) with all autosomes telocentric.

The sex pair of these species is formed by a metacentric neo-X, product of the Rb fusion of the ancestral X and an autosome, while the homologue of the translocated autosome becomes the telocentric neo-Y. Despite the same

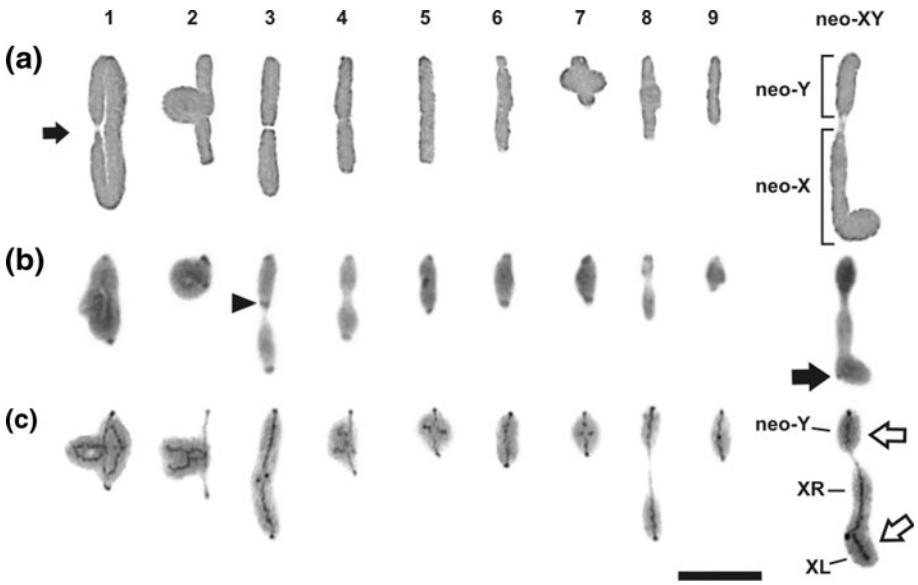


Fig. 2 *Ronderosia malloi* **a**, **b**, **c** Male metaphase I **(a)** The neo-X and neo-Y are indicated; *arrow* marks the metacentric autosomal bivalent (haematoxylin) **(b)** C-banding; *arrowhead* indicates a heterozygous distal heterochromatic block in L3 and the centromeric heterochromatic region of the neo-X which is indicated by an *arrow* **(c)** Silver impregnation; note the *zig-zag* morphology of the scaffolds of XL and neo-Y (*arrows*). *Bar* = 10 μm

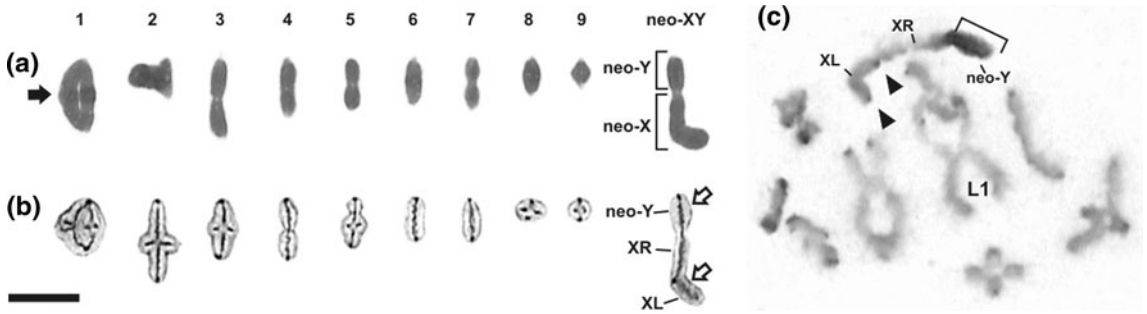


Fig. 3 *Ronderosia forcipatus* **a**, **b** Male metaphase I **(a)** Conventional staining; *arrow* indicates the metacentric autosomal bivalent; neo-X and neo-Y are shown by *brackets* **(b)** silver impregnation; arms of neo-X and neo-Y are indicated; *arrows* show the scaffolds of XL and the neo-Y **(c)** Diplotene; C-banding. Note homogeneously *darkly stained* neo-Y (*bracket*) and the centromeric and telomeric constitutive hetechromatin of the neo-X (*arrowheads*). *Bar* = 10 μm

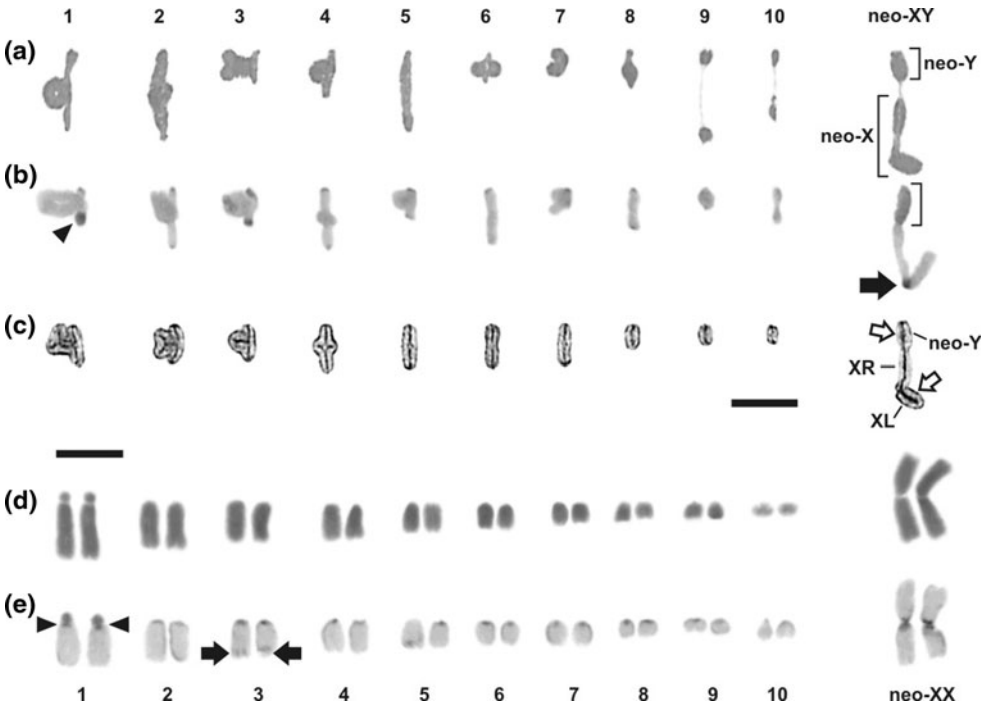


Fig. 4 *Atrachelacris unicolor* **a**, **b**, **c** Male metaphase I **(a)** neo-X and neo-Y chromosomes are indicated **(b)** C-banding; note C-positive centromeric region in neo-X (*arrow*); the neo-Y is homogenously darkly stained (*bracket*); *arrowhead* indicates a C-positive block of heterochromatin in L1 **(c)** silver impregnation, showing neo-X arms and the neo-Y; the chromatid cores are indicated with *arrows*. Female mitotic metaphase **(d)** Giemsa staining; **(e)** C-banding, *arrows* indicate terminal C-positive heterochomatin; this female was homozygous for a proximal C-block in L1 (*arrowhead*) *Bar* = 10 μm

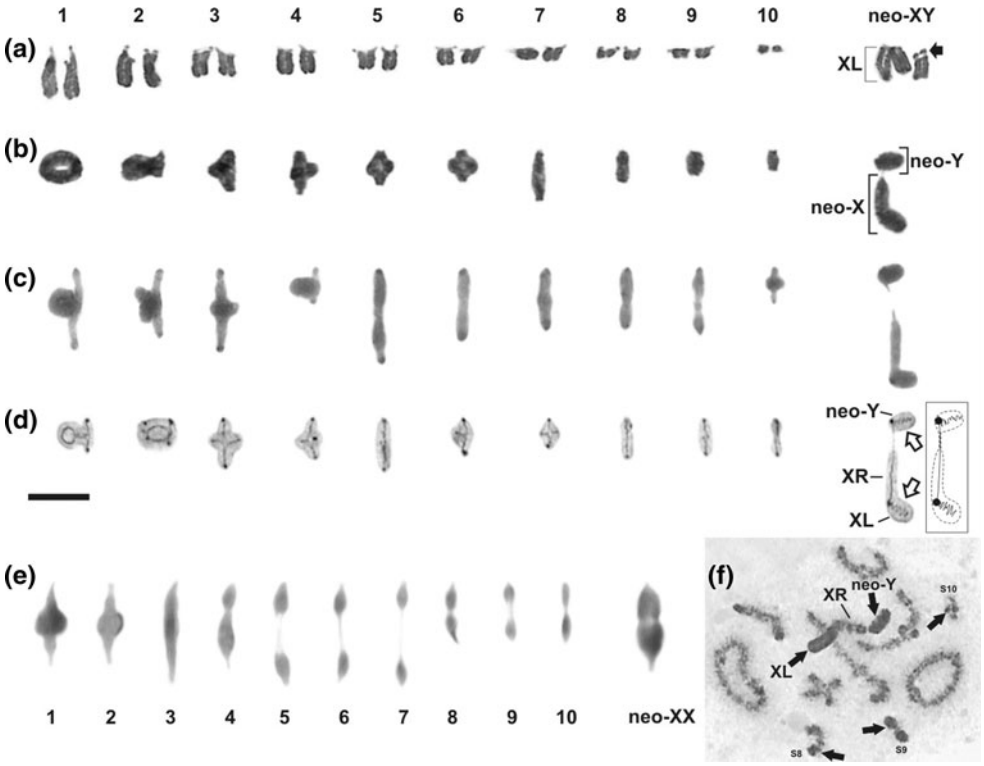


Fig. 5 *Ronderosia bergi* (**a**) Spermatogonial metaphase. Brackets indicate the negatively heteropycnotic XL arm; *arrow* points to the short arm of the neo-Y (haematoxylin) **b**, **c**, **d** Male metaphase I. (**b**) Conventional staining, neo-X and neo-Y are indicated (*brackets*) **c**) C-banding (**d**) silver impregnation of kinetochores and chromatid cores (*scaffold*), the neo-X and neo-Y are showed; arrows indicate the *zig-zag* morphology of the scaffolds of the XL and neo-Y (**e**) Female metaphase I; note strong distal distribution of chiasmata **(f)** Male diplotene; *arrows* indicate positively heteropycnotic regions *Bar* = 10 μm

origin proposed for the neo-sex chromosome system of the four species, the neo-sex pair of *R. malloi*, *R. forcipatus* and *A. unicolor* involved the largest pair of autosomes, while the history of the *R. bergi* neo-sex system is different because the second largest pair participated in its formation.

The centromeres of the neo-X and neo-Y of *R. malloi*, *R. forcipatus* and *A. unicolor* at metaphase I, are distantly localized from the pairing region, thus in metaphase I the sex pair adopts an L-shaped configuration (Figs. 2 a, b, c; 3 a, b; 4 a, b, c). In these three cases both members of the sex pair are associated in a strictly terminal fashion from

prophase I to metaphase I. Differently, in *R. bergi* the sex-bivalent assumed a characteristic C-shape at metaphase I due to the position of both centromeres. So during this phase the sex bivalent oriented with the neo-Y parallel to the XL arm of the neo-X, at right angle with XR (Figs. 5 b, c, d).

In all species, XL showed the typical positive heteropycnosis of the Acridoid X chromosome during diplotene. The neo-Y also showed the same differential condensation pattern while the XR presented euchromatic characteristics in the four species. At diplotene of *R. forcipatus* the centromeric and telomeric regions of the positively heteropycnotic neo-X were evidenced by C-banding, while the neo-Y showed homogeneus dark staining (Fig. 3 c). The centromeric region of the neo-X of *R. malloi* was C-positive and the neo-Y homogeneously banded (Fig. 2 b). During anaphase I of this species the neo-chromosomes segregated to opposite poles, in all analyzed cells ($n = 29$).

Ronderosia malloi and *R. forcipatus* also underwent an autosomal rearrangement. Their autosomal complement is composed of 8 telocentric and a metacentric pair (L1) produced by a Rb fusion of pairs 3 and 5 of the ancestral karyotype (Figs. 2 a, 3 a). In *R. malloi* the metacentric bivalent usually exhibited one chiasma per arm (83.7%), but on occasions the long arm showed bichiasmate configurations at metaphase I (16.3%). During diplotene the short arm of L1 of *R. forcipatus*, was weakly stained with the C-banding technique, while the long arm was C-negative (Fig. 3 c). At metaphase I the metacentric bivalent usually formed one chiasma per arm (73.3%) but sometimes the long arm showed a bichiasmate configuration (26.7%).

The karyotype of *A. unicolor* males consist of ten pairs of telocentric autosomes grouped in three large, four medium-sized, and three small autosomal bivalents (Table 2), plus a heteromorphic sex pair. During the first metaphase the neo-Y was homogenously darkly C-banded and the centromeric region of the neo-X was evidenced (Fig. 4 b). At the same phase, C-banding revealed centromeric heterochromatin in all autosomes, except the S10 pair which showed no evident C-positive bands. Moreover, in all analyzed cells pair L1 was heterozygous for a proximal C-positive band (Fig. 4 b). Mitotic metaphases of females presented 20 telocentric autosomes and two metacentric sex-chromosomes (neo-XX) which displayed C-positive signals in the centromeric region (Fig. 4 e). At male anaphase I, the sex chromosomes segregated to opposite poles in 96.67% of the cells, while in only 3.33% both migrated to the same pole ($n = 30$).

Ronderosia bergi presents 10 telocentric autosomes bivalents (Fig. 5 b, c, d), three of which (S8-S10) show proximal heteropycnotic segments at diplotene (Fig. 5 f). C-banding evidenced centromeric constitutive heterochromatin in the autosomes (Fig. 5 c). The distribution of C-bands was shared by almost all analyzed individuals except those from Riacho He–He where S8 showed an interstitial C-positive segment in heterozygous condition (3.44%). Also, S10 was polymorphic for a proximal heterochromatic block in males from Yaguaroundí (6.45%). During anaphase I the sex bivalent segregated to opposite poles in all (100%) the cells analyzed ($n = 30$). In spermatogonial metaphase both arms of the neo-X showed equivalent lengths. Furthermore, XL was negatively heteropycnotic and, at the same phase the neo-Y was distinguished by a conspicuous short arm (Fig. 5 a). Female meiosis at metaphase I displayed 10 telocentric bivalents plus a metacentric bivalent, the neo-XX sex pair (Fig. 5 e).

Neo-XY chromosome scaffold: after silver impregnation, kinetochores and the fibrous network of non-histone proteins (scaffolds) were observed in metaphase I. These chromatid cores allowed a precise identification of chiasma sites. The neo-X of *R. bergi* showed a contrasting interchromatidic core structure between both arms: XL showed a *zig-zag* structure of cohesiveness like that of the neo-Y, while XR presented a typical autosomal scaffold configuration (Fig. 5 d). The same situation was observed in the neo-sex chromosomes of *R. malloi* and *R. forcipatus* (Fig. 2 c; 3 b). Although neo-sex scaffolds and kinetochores of *A. unicolor* were evident with this procedure, no special morphology in the interchromatidic core structure of the XL or the neo-Y was observed (Fig. 4 c).

Neo-X1X2Y systems

The diploid set of *Dichromatos lilloanus* (Liebermann, 1948) (Fig. 6) and *D. schrottkyi* (Rehn, 1918) (Fig. 7) is $2n = 21$ (FN = 23) in males and $2n = 22$ (FN = 24) in females with a neo-$X_1X_2Y/X_1X_1X_2X_2$ complex sex-determining system. Metaphase I spermatocytes of both species showed 9 telocentric bivalents and a sex-trivalent. The difference in size of the bivalents was not large enough to classify them in large, medium and small classes. The first pair was the largest, and the rest of the bivalents decreased gradually in size (Figs. 6 a, b, c; 7 a, b, c).

The sex trivalent of males is formed by the submetacentric neo-X_1, the telocentric neo-X_2 and the metacentric neo-Y. The XR arm of the neo-X_1 always forms a distal association with the YL limb of the neo-Y. The other arm of the neo-Y, YR, is associated distally with the neo-X_2 at metaphase I. The trivalent was always disjunctionally (convergently) orientated at metaphase I, the neo-X_1 and neo-X_2 centromeres pointing to the same spindle pole and the neo-Y centromere towards the opposite one. All analyzed anaphase I cells ($n = 29$) of *D. lilloanus* and 97.57% ($n = 41$) of *D. schrottkyi* showed disjunctional segregation. At metaphase I, constitutive heterochromatin of the

Table 2 Species of the genera *Ronderosia*, *Atrachelacris* and *Dichromatos* studied indicating the chromosomal sex determination mechanism (CSDM), male and female chromosome number M/F 2n, male and female fundamental number (M/F FN), autosomal (*A*) size distribution indicating metacentric (*m*) and telocentric (*t*) bivalents, male mean autosomal chiasma frequency (A chiasmata ± SD) and percent distribution of proximal (*P*), interstitial (*I*) and distal (*D*) chiasmata, type and number of chiasmata between NeoX (or NeoXs) and NeoY chromosomes, sex pair or trivalent configuration at metaphase I(*MI*)

CSDM	Species	M/F 2n	M/F FN	A size distribution	A chiasmata ± SD (%P, I, D)	NeoX(s)-NeoY chiasmata	NeoX(s)-Y configuration at MI	Pycnosis					
								XR	XL	Neo-Y	YL	YR	X_2
Neo-XY/XX system	*R. malloi*	20 (18 + XY)/20(18 + XX)	23/24	L1 (m):L2:L3:M4-M7:S8:S9 (t)	10.31 ± 0.50 (8,17,75)	D	L-shaped	I	+	+			
	R. forcipatus	20 (18 + XY)/20(18 + XX)	23/24	L1 (m):L2:L3:M4-M7:S8:S9 (t)	10.30 ± 0.65 (7,29,64)	D	L-shaped	I	+	+			
	R. bergi	22 (20 + XY)/22(20 + XX)	23/24	L1:L2:M3-M7:S8-S10 (t)	11.97 ± 1.13 (24,18,58)	P	C-shaped	I	+	+			
	A. unicolor	22 (20 + XY)/22(20 + XX)	23/24	L1:L2:L3:M4-M7:S8:S9 (t)	11.60 ± 0.60 (23,7,70)	D	L-shaped	I	+	+			
Neo-$X_1X_2Y/X_1X_1X_2X_2$ system	*D. lilloanus*	21(18 + X1X2Y)/22 (18 + X1X1X2X2)	23/24	M1-M7:S8:S9 (t)	9.52 ± 072 (21,13,66)	DD	Convergent	I	+		I	I	I
	D. schrottkyi	21(18 + X1X2Y)/22 (18 + X1X1X2X2)	23/24	M1-M7:S8:S9 (t)	9.45 ± 0.51 (7,7,86)	DD	Convergent	I	+		I	I	I

The table also provides information about the condensation patterns of the Neo-chromosomes arms at diplotene visualized with conventional staining technique as positive heteropycnosis (+) or isopycnosis (I)

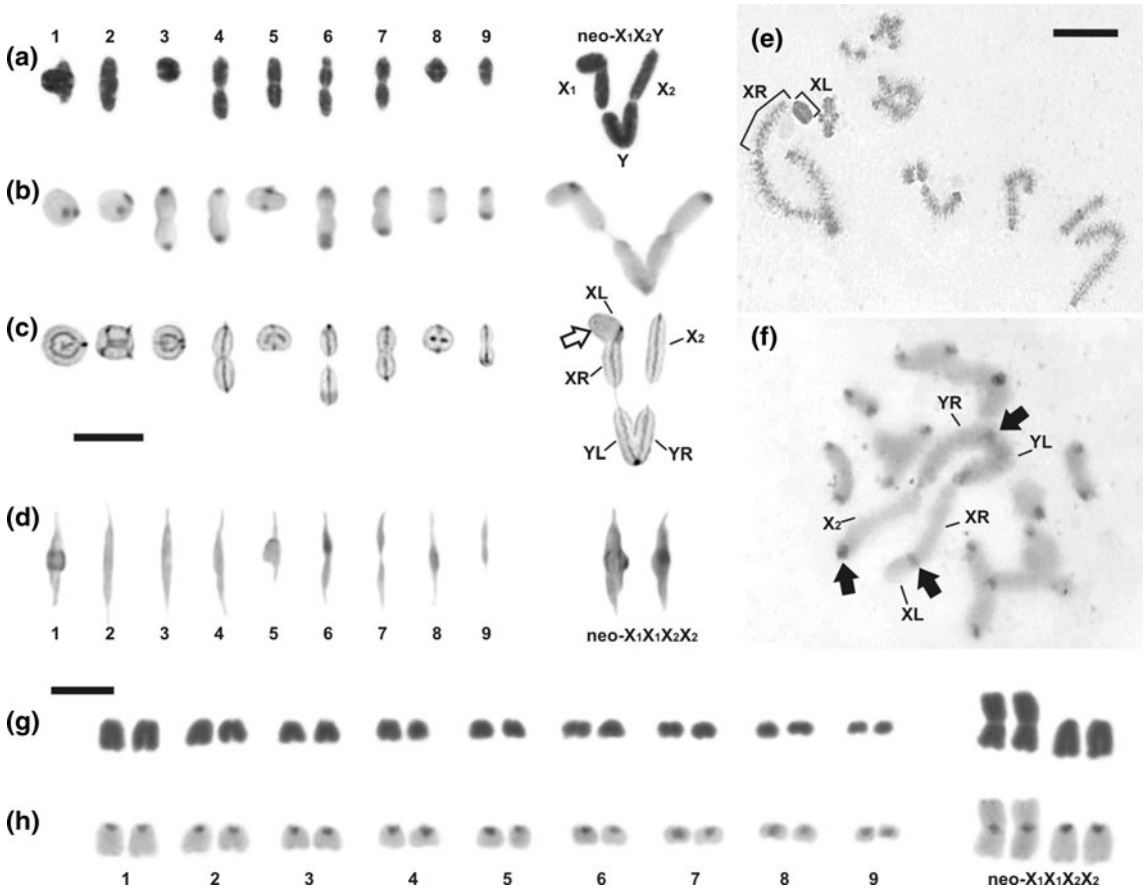


Fig. 6 *Dichromatos lilloanus* **a**, **b**, **c** Male metaphase I **(a)** Haematoxylin; the neo-X1X2Y trivalent is indicated **(b)** C-banding **(c)** Silver impregnation; neo-sex chromosome arms are indicated; arrow shows the absence of the scaffold in XL **(d)** Female meiotic metaphase I (orcein); note strong distal distribution of chiasmata **(e**, **f)** Male diplotene **(e)** XL (positively heteropycnotic) and XR are indicated **(f)** C-banding; *arrows* indicate C-positive centromeric regions on the sex chromosomes **(g**, **h)** Female mitotic metaphase **(g)** Karyotype with Giemsa staining **(h)** C-banding. *Bar* = 10 μm

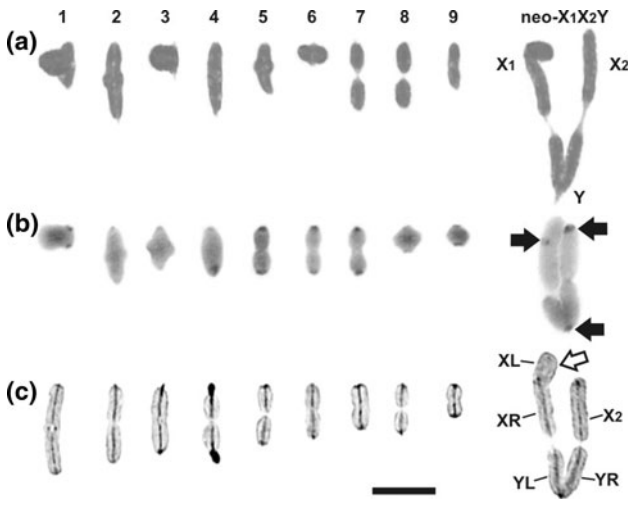


Fig. 7 *Dichromatos schrottkyi* **a**, **b**, **c** Male metaphase I **(a)** Haematoxylin; the neo-sex trivalent is indicated **b)** C-banding; *arrows* show centromeric heterochromatin of the sex chromosomes **(c)** Silver impregnation indicating neo-sex chromosomes arms and the absence of the scaffold of the XL arm (*arrow*). *Bar* = 10 μm

neo-X_1, neo-X_2 and neo-Y was evident with C-banding at the centromeric regions of both species (Fig. 6 b; 7 b).

The sixth autosomic pair of *D. lilloanus* was polymorphic for an interstitial C-positive block (8% heterozygotes) (Fig. 6 b). *Dichromatos schrottkyi* showed centromeric C-bands, although pairs 1, 4 and 8 showed weak staining. In addition, pair 4 presented a heteromorphic C-positive segment in the paracentromeric region in one male (Fig. 7 b). Silver impregnation of *D. lilloanus* autosomes revealed uni- and bichiasmate configurations in pairs 1–3 with predominance of P/D chiasmata whereas the remaining bivalents presented usually one distal chiasma (Fig. 6 c).

Female meiotic cells of *D. lilloanus* showed 9 telocentric autosomal bivalents, and two sex bivalents: a metacentric neo-X_1X_1 and a telocentric neo-X_2X_2. The telocentric sex bivalent was larger enough to be distinguished at metaphase I from the autosomes (Fig. 6 d). Female mitotic metaphases of *D. lilloanus* showed 18 telocentric chromosomes plus a metacentric pair (neo-X_1), and a telocentric pair (neo-X_2) (Fig. 6 g). The centromeric

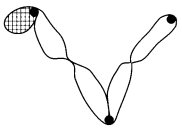
Specie	2n M/F	A/A fusion	Configuration at Metaphase I	neo-XY origin and behavior
Ronderosia forcipatus	20/20			X-A Rb fusion: 1st pair Distal XR-NeoY chiasmata
Ronderosia malloi	20/20	Rb 3-5		
Atrachelacris unicolor	22/22	No	L-shape	
Ronderosia bergi	22/22	No	 C-shape	X-A Rb fusion: 2nd pair NeoY pericentric inversion Proximal XR-NeoY chiasmata
Dichromatos lilloanus	21/21	No		X-A Rb fusion: 2nd pair NeoY-A Rb fusion: 1st pair Distal NeoY-X1X2 chiasmata
Dichromatos schrottkyi	21/21	No	Convergent	

Fig. 8 Summary of the chromosomal characteristics *of Ronderosia forcipatus, R. malloi, R. bergi, Atrachelacris unicolor, Dichromatos lilloanus* and *D. schrottkyi.* Male and female chromosome numbers (2n) of each species are indicated as well as the autosomal fusions (A/A), configuration of the sex bivalent during the metaphase I and the origin of neo-sex systems. Black circles represent centromeric regions; the XL arm of the neo-X chromosome is shaded

region of all chromosomes was C-banded and additional weakly C-positive signals were detected in the distal region of both neo-X_1 and neo-X_2 chromosomes (Fig. 6 h).

Neo-X_1X_2Y sex chromosomes scaffold: at metaphase I, the neo-sex trivalent of the two species presented a typical scaffold configuration with the kinetochores appearing as unique round structures and the chromatid cores associated all along their length. The chromatid axes of the XL in both cases were normally absent and the neo-Y scaffold showed a *zig zag* structure of cohesiveness in sister chromatid cores (Fig. 6 c; 7 c).

Chiasma frequency and distribution

Autosomal male chiasma frequency was relatively low in all species with the lowest values for both *Dichromatos* species (Table 2) in which most cells showed the minimum number of 9 chiasmata. Although mean chiasma frequency of both species was not significantly different and both showed a tendency to distal distribution of chiasmata (Table 2), this distribution was highly significantly different ($\chi 2 = 33.78$, $P < 0.001$) with *D. schcrottkyi* showing the strongest distal localization. In the case of *R. forcipatus* and *R. malloi*, both species also show non significantly different autosomal chiasma frequencies (Table 2). However, chiasma distribution (distally biased, Table 2) is significantly different with *R. malloi* having more distal autosomal chiasmata per cell ($\chi 2 = 20.63$, $P < 0.001$). *Atrachelacris unicolor* and *R. bergi* have the highest autosomal chiasma frequencies but although the former shows strong distal localization, the latter is the specie with the highest frequency of $P + I$ chiasmata (42%) (Table 2).

Sex chromosomes of all species invariably showed extremely distal chiasmata among originally homologous regions (Table 2).

Although in this work an extensive study of female chiasma frequency was not performed, it is clear that at least for two species, females show a strong tendency to low chiasma frequencies and distal localization (92% D chiasmata in *R. bergi*) (Figs. 5e, 6d).

Discussion

We analyzed the chromosomal characteristics including male and female karyotypes, meiosis and sex-chromosomes of six Melanopline grasshopper species showing

neo-sex chromosomes in order to contribute to the knowledge about the origin, behaviour and evolutionary history of neo-sex systems.

Populations of *R. bergi* were cytogenetically analyzed at different points of its geographic differences in chromosome complement (Table 1) to test if the wide variation in pigmentation could be correlated to structural variation in the chromosome complement. All individuals of the studied populations showed the karyotypic number described by Mesa (1962a) and the characteristic "C-shape" disposition of the sex pair at metaphase I (Fig. 8), which provides evidence of the complex origin of the system, involving a pericentric inversion (or centric shift) in the neo-Y (Cardoso and Dutra 1979; Díaz and Sáez 1968; Hewitt 1979; Mesa 1962a; White, 1973; this work). Thus no correlation was found between pigmentation diversity and karyotype. The complexity of the evolutionary history of *R. bergi* neo-sex chromosomes is clear when we compare it with the related species, *Dichroplus obscurus*. The latter, presents the sex pair with an "L-shape" configuration at metaphase I (Bidau and Martí 2001). We observed the same orientation in the sex bivalents of *R. forcipatus*, *R. malloi* and *A. unicolor* (Fig. 8). However the same length of the XR arm and the neo-Y in *D. obscurus* indicates that the system could be more recent in evolutionary time. On the other hand, the advanced state of the neo-sex system of *R. forcipatus, R. malloi* and *A. unicolor* is suggested by the marked length difference between the XR and neo-Y. Conversely, in *Leiotettix sanguineus* for example, the synapsed region between sex chromosomes is throughout the XR arm length, indicating that the neo-Y still conserves the homology with the latter (Mesa et al. 2001; Mesa and de Mesa 1967).

In Carbonell et al. (2006) the distribution of *R. forcipatus* does not include the location where we found it, so this is probably a new record for the species (Castillo, 2008). The observed discrepancies with the first cytogenetic description of the species (Colombo et al. 2005), are due to a presence of a Rb metacentric produced by the centric fusion of autosomes 3 and 5 of the ancestral karyotype (Fig. 8). In spite of this, both reports agree on the sex determination system. As in *R. malloi* and *A. unicolor*, the XR arm of the neo-X has marked differences in length with respect to the neo-Y. Also, the high level of heterochromatinization of the neo-Y and the extreme polarization of the single chiasma in terminal position between neo-Y and XR, could be a strategy for the convergent orientation and normal segregation of the sex bivalent; also, indicating an advanced state of the system.

Based on the cytogenetic information provided by Mesa et al. (1982), multiple $X_1X_2Y/X_1X_1X_2X_2$- sex-chromosome systems have been described in a few species of grasshoppers, all of them South American Melanoplines with restricted geographic distributions (Hewitt 1979; Mesa 1962b; Mesa and de Mesa 1967; White 1973). Mesa (1962b) and Mesa et al. (1982) described the male chromosomal complement of the two species of *Dichromatos* analyzed in this paper, under the genus *Eurotettix*, but the present work is the first to present a more complete cytogenetic study. In *D. lilloanus* the complex sex-mechanism arose from a neo-XY system where a second centric fusion between the neo-Y and another autosome took place (Fig. 8) (Hewitt 1979; Mesa 1962b; White 1973). We also observed a small degree of divergence (changes in the morphology, behaviour and variations in the chromatin condensation patterns) between the XR and YL arm and this would indicate a more recent origin of the neo-system of this species than that of *D. schrottkyi*.

The same origin proposed for the multiple system of *D. lilloanus* is used to explain the sex-determining mechanism of *D. schrottkyi.* Based on the relative lengths of the XR and YL arms, which are significantly different, we propose an advanced state of divergence of these elements which would indicate that the first fusion process is ancient. Furthermore, little divergence between the YR and the X_2 was evident, indicating the second fusion as a recent event. Moreover, judging by the association patterns of the sex trivalent in male meiosis the multiple sex system may be advanced. Otherwise the association between the sex chromosomes should be throughout the sex chromosomes length, with formation of chiasmata in the proximal and interstitial regions indicating homology and a recent origin, as in *Leiotettix politus* (Mesa and de Mesa 1967).

Melanoplinae is a very large subfamily of Acrididae widely distributed in the Americas and Eurasia. It includes at least 144 genera and almost 1,200 species (Eades and Otte 2009). There is at present no evidence for the origin of the extraordinary karyotypic diversification of the South American Melanoplinae as compared with Melanoplines from the rest of the world, which are chromosomally much more conservative (Hewitt 1979; Mesa et al. 1982). South American Melanoplinae are characterized by a wide array of derived karyotypes, most of them involving Rb fusions both between autosomes, and between autosomes and sex-chromosomes. A possible explanation for this disproportionate frequency of Rb karyotypes could be found in the centromeric drive theory (Pardo-Manuel de Villena and Sapienza 2001), originally proposed for mammals but also of great explanatory power in other large taxonomic groups (e.g. fishes; Bidau, Martínez and Molina, personal communication), which suggests that establishment of biarmed or, alternatively, uniarmed chromosomes within an evolutionary lineage, reflects a bias of female meiosis, favouring either less or more centromeres passed to the

oocyte nucleus, respectively. This bias would result from the essential asymmetry of the female meiotic spindle thus, when heterozygous Rb configurations occur, the partner bearing less centromeres will attach more frequently to the spindle pole that is less efficient in capturing centromeres thus favouring metacentric chromosomes. The direction of this bias however, may change frequently over evolutionary time (Pardo-Manuel de Villena and Sapienza 2001).

Grasshopper female spindles are extremely asymmetric and, for example, drive for B-chromosomes have been demonstrated during female meiosis (Hewitt 1976, 1979). Recently, Bidau and Martí (2004) obtained indirect evidence of the operation of drive for metacentric chromosomes in the Melanopline *Dichroplus pratensis*. One factor that might lead an egg pole to switch from capturing more centromeres to capturing fewer centromeres could be selection to reduce the burden of B chromosomes which are very frequent in grasshoppers (Bidau and Martí 2004; Palestis et al. 2004; Talbert et al. 2009). The reversion of the process could result from selection towards more interchromosomal and intrachromosomal recombination in marginal populations which could help adaptation to and colonization of, new environments (Bidau and Martí 2002). Thus, lineages that tend to fix neo-XY systems (and autosomal Rb fusions) would predictably have lower incidences of B-chromosomes (usually telocentric).

Thus, it is possible that the tendency for fixation of centric fusions in Neotropical Melanoplinae is and inherent tendency of metacentric Rb chromosomes, including the neo-sex chromosomes, to migrate to the oocyte pole preferentially. It is interesting that five of the species herein examined show strong distal localization of chiasmata which could favour, aiding disjunction and later differentiation, the establishment of new Rb fusions, including those involving autosomes and sex-chromosomes. Besides, Rb fusions tend to automatically eliminate P chiasmata in grasshoppers (Bidau and Martí 2002). It is also worth noting that the only species showing a high frequency of $P + I$ chiasmata, *R. bergi*, is the only one forming a proximal chiasma between the neo-sex chromosomes. This could also reinforce the hypothesis of the South American origin of Melanoplinae (Amédégnato et al. 2003); high karyotypic diversity would be a consequence of an ancient origin and drive mechanisms which could have been inverted during expansion out of South America, favouring acrocentric karyotypes.

Extensive cytogenetic studies involving Neotropical Melanoplines, and those particularly centered on structure and male and female meiotic behaviour of neo-sex chromosomes could be useful models for the study of the origin, differentiation and meiotic behaviour of newly evolved chromosomal sex-determining mechanisms.

Acknowledgments The authors are very grateful to María Marta Cigliano for introducing us to the fascinating world of *Ronderosia* and allies. We also wish to especially acknowledge Prof. Pavel Borodin (Novosibirsk, Russia) for his critical reading of a previous version of the manuscript. This research was partially supported by FONCyT PICTO 37035 BID 1728/OC-AR to DAM. DAM acknowledges the continuous support of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Misiones. ERC is PhD candidate at Universidad Nacional de Córdoba (Doctorado en Ciencias Biológicas) and is very grateful to all people in our laboratory and to CEDIT for postgraduate scholarship. CJB acknowledges the support of CNPq (grant 480596/2007-7), FAPERJ (grant APQ1 3225/2007) and IOC/FIOCRUZ, all institutions from Brazil.

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