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Neo-sex Chromosomes in the *Maculipennis* Species Group (*Dichroplus*: Acrididae, Melanoplinae): The Cases of *D. maculipennis* and *D. vittigerum*

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South American melanopline grasshoppers display a disproportionate number of derived karyotypes, including many cases of neo-sex chromosome systems. This is especially true of the genus *Dichroplus* and its *Maculipennis* species group. We analyzed the karyotype and neo-sex chromosomes in mitosis and meiosis of *Dichroplus maculipennis* and *D. vittigerum* from Argentina using conventional and fluorescent cytogenetic protocols in order to elucidate the behavior and origin of these neo-XY systems in relation to the current phylogeny of this group. Our results showed that *D. maculipennis* (2n = 22♂/22♀; neoXY/neoXX) and *D. vittigerum*, whose karyotype is described here for the first time (2n = 18♂/18♀; neoXY/neoXX), show highly evolved neo-XY systems, although with significant differences between them. Furthermore, both species differ for two autosomal fixed Robertsonian fusions present in *D. vittigerum*. Analysis of karyotypic character state optimization strongly suggests the independent origin and evolution of neo-sex systems within this species group.

Key words: *Dichroplus maculipennis*, *Dichroplus vittigerum*, grasshopper, karyotype character state optimization, Melanoplinae, sex chromosomes

INTRODUCTION

The origin, nature, genetics, and evolution of sex chromosomes has long been a topic of great relevance in biology, and their meiotic properties have been under study since the beginnings of cytogenetics (McClung, 1902; Stevens, 1905; White, 1973; Bachtrog, 2006; Kaiser and Bachtrog, 2010; Bachtrog et al., 2011, 2014). In this respect, some groups of organisms have proven to be excellent models for analyses of meiotic behavior. One such group is constituted by grasshoppers of the Acridoidea superfamily, especially the Acrididae, due to their large size, low number of chromosomes, and the clarity of their meiotic process (Hewitt, 1979; John, 1990; Bidau and Martí, 2010). Although it has been traditionally thought that this family is rather karyotypically conservative, this is so only in diploid number and gross morphology of chromosomes (Hewitt, 1979). Furthermore, many cases of karyotypic rearrangements that modify the number and structure of chromosomes have been reported, and these frequently involve sex chromosomes (White, 1973; Castillo et al., 2010a, b, 2014). Although the ancestral chromosomal sex-determining system of the Acrididae (and all Orthopteroid orders; Blackman, 1995) is known to be X0 male/XX female, the sex chromosomes have undergone evolutionary structural changes in many independent lineages, with the consequent production of multiple sex chromosome systems that impose selective pressures on the meiotic system required for their establishment in natural populations (Veltsos et al., 2008; Castillo et al., 2010b; Bidau et al., 2011; Warchalowska-Śliwa et al., 2011, 2015). These pressures involve modifications that ensure proper meiotic segregation of sex chromosomes, which in turn undergo further molecular and structural modifications in a process called “Y-chromosome degeneration” (Veltsos et al., 2008; Bidau et al., 2011).

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The South American Melanoplinae are an ideal acridoid group for the study of the evolution of sex chromosomes due to the disproportionately high number of cases in which neo-sex chromosome systems have arisen in different lineages (Mesa et al., 1982; Castillo et al., 2010a, b). One tribe, the Dichroplini is especially interesting in this respect because of its high chromosomal diversity in both autosomal and sex chromosome systems (Bidau and Martí, 2010; Castillo et al., 2010a, b, 2014). Within this tribe, the genus *Dichroplus* Stål, which includes 24 species, has received special attention from the cytogenetic point of view (e.g., Bidau and Martí, 2001).

Dichroplus maculipennis is a widespread grasshopper (common name, *tucura*) belonging to the *Maculipennis* subgroup of the homonymous species group (Cigliano and Otte, 2003). The subgroup also includes *D. conspersus* Bruner, 1900, *D. robustulus* (Stål, 1878), and *D. vittatus* Bruner, 1900 (Cigliano and Otte, 2003; Eades et al., 2015). *D. vittigerum* (Blanchard, 1851) is a closely related species within the *Maculipennis* group and the sole representative of the *Vittigerum* subgroup (Eades et al., 2015).

Dichroplus maculipennis has a wide but seemingly disjunct distribution in Chile, Argentina, Uruguay and southern Brazil (see fig. 11 in Cigliano and Otte, 2003) which overlaps widely with that of *D. vittigerum* (Eades et al., 2015). Both species are important components of orthopteran diversity in the Argentine pampas and Argentine and Chilean Patagonia, and *D. maculipennis* is a major pest in several areas (Sánchez and de Wysiecki, 2008; Mariottini et al., 2011, 2012, 2013).

Despite the considerable cytogenetic interest that the Dichroplini have caused, *D. maculipennis* and *D. vittigerum* have been rather neglected from this angle especially considering the wealth of such studies in other species of the genus and the *Maculipennis* species group regarding population cytogenetics, and the structure and behavior of neo-sex chromosomes (Bidau, 1990, 1991; Castillo et al., 2010a, b; Bidau et al., 2011; Miño et al., 2011). Considering this, the aim of this paper focuses on shedding light on the neo-sex chromosome systems of *D. maculipennis*, analyzing the chromosome morphology, structure and meiotic behavior of males and females, and those of the closely related species *D. vittigerum* reported here for the first time. We also propose for the first time an explanatory hypothesis of the origin of the neo-sex chromosomes and clarify some issues concerning the chromosome number, originally mentioned in Mesa et al. (1982). Additionally, we re-evaluate the *Maculipennis* species group sex chromosome evolution.

MATERIALS AND METHODS

Individuals of *D. maculipennis* (male and female adults) used in this study were collected from Isla Arce, Chubut province, Argentina (n = 11♂) (45.00 S–65.51 W; 10 m above sea level). We also used individuals belonging to the first laboratory generation [F1] of specimens originally collected in the southern of Pampas region (Laprida department, Buenos Aires province, (37.55 S–60.82 W), and maintained in a rearing room under controlled conditions (30°C, 14L: 10D, 40% RH) (n = 10♂/20♀). Eighteen adult males of *D. vittigerum* were collected in a *mallín* formation at Bariloche, Río Negro province, Argentina (41.14 S–71.31 W, 820 m.a.s.l). Voucher specimens of *D. maculipennis* and *D. vittigerum* are deposited in the collections of the the Laboratorio de Genética Evolutiva IBS, CONICET-UNaM, Posadas and INTA EEA Bariloche, respectively. Male meiotic preparations were performed by crushing testes follicles in ferric haematoxylin. Female meiosis slide preparation followed the laboratory protocol cited in Martí and Bidau (1995). Mitotic metaphase chromosomes from male and female gastric caeca were obtained following the procedure described by Castillo et al. (2011).

Silver staining of kinetochores and chromatid cores were performed according to the procedure of Rufas (1985). Briefly, air-dried male meiotic preparations were incubated in 2 × SSC at 60°C for 10 min and stained with 50% AgNO3 in dH2O (pH adjusted to 3.5 with formic acid). Microscopic observation of silver stained preparations involved bright field and Nomarski interference optics. C-banding was performed following the protocol of Sumner (1972), with modifications. Chromomycine A3 (CMA3) and DAPI (4′, 6-diamidino-2-fenylindole) staining was performed following the protocol described in Schweizer (1980).

Autosomes were classified in three arbitrary size groups as is standard practice for the description of acridoid karyotypes (Hewitt, 1979; John, 1983): L (large), M (medium-sized), and S (small) autosomes. We used the terminology proposed by White (1940a, b) to describe the chromosome arms of recently evolved neo-X and neo-Y chromosomes: the autosomal arm of neo-X, which shares homology with the neo-Y, is referred to as XR, while XL is the arm derived from the original X chromosome fused to an autosome. This nomenclature is strictly applicable to simple centric fusion-derived neo-XY systems. Measurements of chromosome lengths were performed on gut caeca mitotic metaphases using MicroMeasure v. 3.3 (Reeves, 2001).

Karyotype optimization

To re-evaluate the *maculipennis* species group chromosomal evolution onto the *Dichroplus* phylogeny and test the hypothesis proposed by Colombo et al., 2005, we mapped the character "autosomes involved in neo-sex chromosome formation" using the software TNT v1.1 (Goloboff et al., 2008).We consider autosomes from the standard karyotypes (2n = 22 + X0/XX) involved in neo-sex chromosome origin as the karyotypic character to map onto the phylogenetic hypothesis of *Dichroplus*.

Character states for the autosome from the standard 2n = 23 chromosome number involved in the origin of neo-sex chromosome, was arbitrarily coded as 0: X0; 1: L_1–L_3; 2: M_4–M_5; 3: X; 4: several sequential X-autosome fusions. L, M and S, refer to the size of the autosomes involved in the formation of the neo-sex system; in some cases, a specific autosome pair is proposed, based on relative lengths, in others it was estimated from published illustrations (Sáez and Pérez Mosquera, 1977; Castillo, 2010b; Bidau and Martí, 2001). However, using obvious and/or inferred rearrangements as a character is an oversimplification as many other transformations may take part along the evolutionary change of karyotypes, but it has the advantage of being a character reported in every cytogenetic study (Grant et al., 2006).

We consider this karyotypic character because the one used in Colombo et al. (2005), X-A fusions, does not account for other neo-sex chromosomes origins not explained by this kind of rearrangements. Moreover, the primary homology of the X-Autosome fusion character states, are difficult to establish because within the L, M or S group of chromosomes we cannot discriminate L1 from L2 or M5 from M6. For this reason, we relied on publications in which, based on relative chromosome length, the authors proposed the ancestral chromosome pair involved in the origin of the neo-sex determination system.

RESULTS

The karyotypes of *Dichroplus maculipennis* and *D. vittigerum*

All individuals of *D. maculipennis* analyzed by us share

a neo-XY/neo-XX sex chromosome determining system and a diploid number of 2n = 22♂/22♀. It comprises 10 pairs of telocentric autosomes: L1–L4, M5–M8 and S9–S10 plus a pair of neo-sex chromosomes (Fig. 1). S9 is the megameric bivalent.

Dichroplus vittigerum has 2n = 18♂/18♀. The karyotype consists of two large metacentric autosomal pairs, $L_{met}1$ and $L_{met}2$ produced by Robertsonian fusion, four medium-sized telocentric pairs (M3–M6) and two small telocentric pairs (S7–S8) (Fig. 2). The neo-sex chromosomes exhibited a metacentric neo-X and a telocentric neo-Y (Fig. 2).

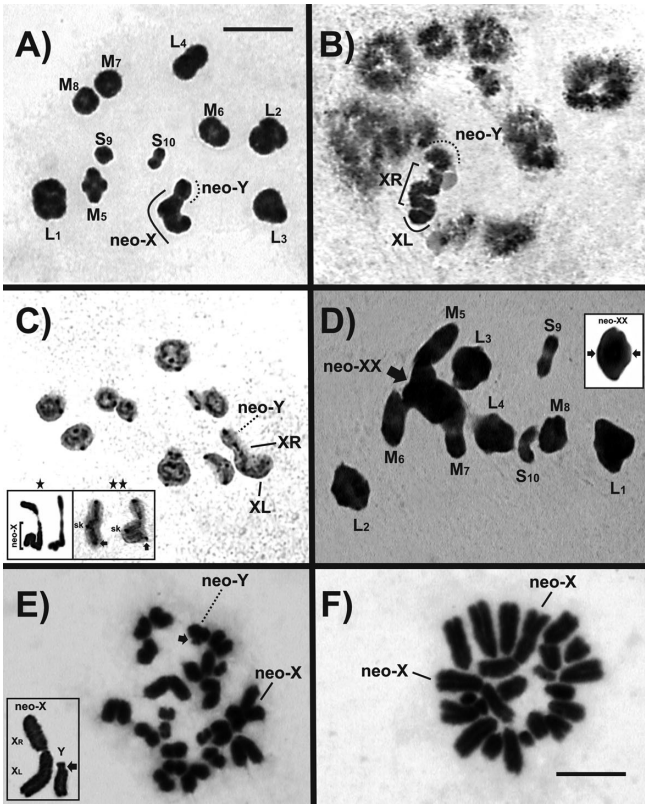

Fig. 1. Male and female meiosis of *Dichroplus maculipennis*. **(A)** Male metaphase I showing ten autosomal bivalents and the neo-X (curved line), neo-Y (dotted line) sex bivalent. **(B)** Typical male diffuse diplotene. XL (curved line) and XR (straight line) arms of the neo-X, and the neo-Y (dotted line) are indicated. **(C)** Silver-stained male metaphase I; arms of the neo-X with black lines and the neo-Y with dotted line are noted. Inset: different metaphase I orientations of the neo-XY bivalent. ★: in both sex bivalents (haematoxylin staining) the distal end of XL appears to show neocentric activity. ★★: both silver-stained sex-bivalents show dense silver deposits at the distal end of XL (arrows) in addition to those corresponding to the standard kinetochores (sk). **(D)** Female metaphase I showing ten autosomal bivalents and the neo-XX sex bivalent (black arrow). Inset: the sex bivalent showing a distal chiasma in each arm (black arrows). **(E)** Male mitotic metaphases showing the sex chromosomes neo-X (black line) and neo-Y (dotted line). Inset show the arms of the neo-X and the short arm of the neo-Y with an arrow. **(F)** Female mitotic metaphases showing the sex chromosomes (black lines). Bar = 10 μm.

Neo-sex chromosome structure and meiotic behavior

The sex pair of *Dichroplus maculipennis* includes a large metacentric neo-X and an acrocentric neo-Y, whose length is comparable to the XR arm of the neo-X (see below). *Dichroplus vittigerum* shows a roughly similar sex-chromosome pair (Figs. 1, 2). However, it is notable that the neo-Y of *D. maculipennis* shows a conspicuous short arm, making it an acrocentric (Fig. 1E). In order to tentatively identify the autosome involved in the formation of the neo-XY system of *D. maculipennis* we measured the chromosomes and chromosome arms of male and female mitotic cells obtained from gut caeca using as controls, metaphase cells of a related species with a standard X0/XX karyotype, *D. fuscus*. In *D. maculipennis*, XR is readily distinguished from XL by its heterochromatic content which is also apparent in C-banded meioses (see below). Also, the Y chromosome is readily identified by a conspicuous short arm, absent in all other uni-armed chromosomes. Using this approach, the autosome involved in the Rb fusion with the X is probably the longest medium-sized autosome corresponding to M4 of the standard karyotype of *D. fuscus* (Supplementary Table S1 online). A similar analysis could not be performed in *D. vittigerum* due to lack of adequate mitotic material. However, judging from the meiotic figures, an M autosome was also involved although probably much shorter than the standard M4 (Fig. 2).

In *D. maculipennis*, the XL arm showed the typical allocyclic behavior of the acridoid X chromosome during late pachytene and diplotene; however, the neo-XY bivalent usually was visualized as a large positively heteropycnotic bulk within the typically diffuse prophase I stages of this species (Fig. 1B). We were able to differentiate one sex chromosome from the other at these phases due to the less condensed state of the XR and neo-Y; nevertheless, both elements showed different pycnotic properties as compared with the autosomal complement (Fig. 1B). The centromeric region of the positively heteropycnotic neo-X was evidenced by C-banding (Fig. 3A, B), while the neo-Y was distinguished from

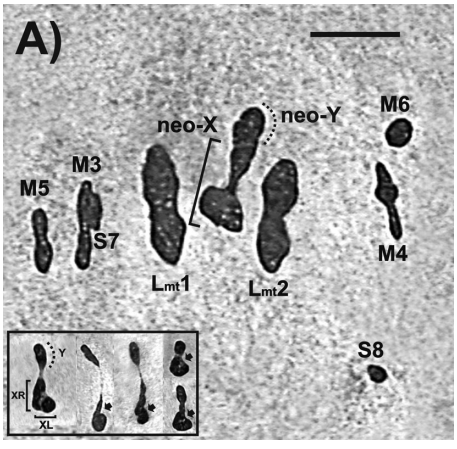

Fig. 2. **(A)** Male metaphase I of *Dichroplus vittigerum* showing two large metacentric autosomal bivalents (Lmt), six telocentric autosomal bivalents, and the neo-X (straight line) and neo-Y (dotted line). Inset: Arms of the neo-X are indicated with straight lines and the neo-Y with dotted line; different metaphase I configurations of the neo-XY bivalent are showed with red arrows. Bar = 10 μm.

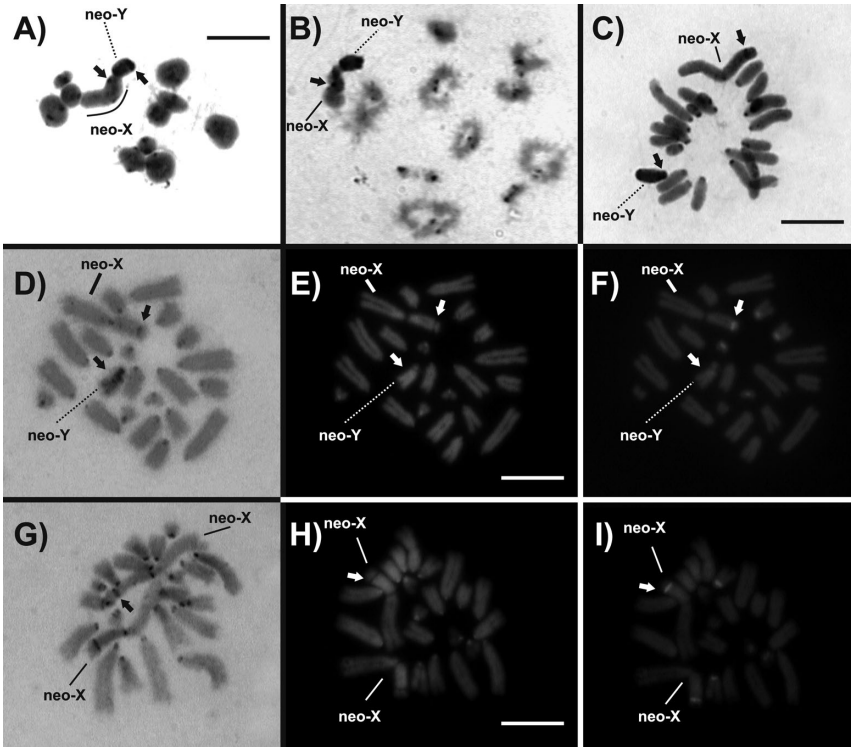

Fig. 3. *Dichroplus maculipennis*. **(A–D, G)** C-banding. **(A)** Male prometaphase I, the neo-X (curved line) and the neo-Y (dotted line) are indicated; centromeric and distal heterochromatic blocks and centromeric heterochromatin are signaled with black arrows in the neo-X and in the neo-Y respectively **(B)** Male diplotene; the neo-X (black line) and the neo-Y (dotted line) are shown; black arrow indicate the centromeric heterochratic block. **(C)** Spermatogonial metaphase; the distal heterochromatic blocks in the neo-X (black line) are shown and the centromeric heterochromatin of the neo-Y (dotted line). **(D–F)** Male mitotic metaphases from gastric caecum; **(D)** the distal heterochromatic blocks in the neo-X are signaled with a black arrow and the C+ heterochromatic pattern of the neo-Y (dotted line) is indicate with an arrow. In **(E)**, the neo-Y (dotted line) DAPI+ and a negative CMA_3 distal block of the neo-X (straight line) are shown with arrows; the negative CMA_3 pattern of the neo-Y (dotted line) and the positive CMA_3 block of the neo-X (straight line) are shown in **(F)**. **(G–I)** Female mitotic metaphases from gastric caecum; **(G)** the distal heterochromatic blocks in the neo-X are signaled with an arrow. In **(H)** and **(I)** the distal DAPI- and positive CMA_3 of the neo-X are shown with white arrows, respectively. Bar = 10 μm.

the autosomes in spermatogonial metaphases due to its homogeneous dark staining (Fig. 3C); the same pattern was observed in the neo-Y at metaphase I (Fig. 3A, B). Moreover, we used the heterochromatic pattern shown by the neo-Y in meiosis, as a cytological marker, to identify and confirm the sex chromosome in male gastric caeca metaphases (Fig. 3D). Besides, the distal third of XR exhibited a heterochromatic C- positive block observed in male metaphase I, and spermatogonial and somatic mitoses (Fig. 3A–F). This block is absent in the neo-Y chromosome. In female mitotic metaphase all chromosomes showed pericentromeric C-positive heterochromatin while the distal third of XR showed the C-positive block described above (Fig. 3G–I). This block was also evidenced with sequential DAPI/CMA3 banding in male and female gastric caecum cells (Fig. 3D–I).

The neo-XY centromeres of *D. maculipennis* were observed distantly localized from the pairing region and assume frequently (87%) an L-shaped configuration at first metaphase. However, additional configurations were distinguished in 470 cells analyzed. A significant proportion showed the distal end of XL with kinetochoric (neocentric) activity in addition to the standard centromere of the neo-X. Such activity could result in disjunctional or non-disjunctional orientation of the neo-XY bivalent (Fig. 1C, inset).

However, the final segregation behavior of the sex bivalent appears not to be seriously affected due to these configurations, since a low percentage (0.4%) of abnormal sperm formation occurs (n = 2024). A similar behavior of the neo-XY bivalent of *D. vittigerum* males was observed (Fig. 2, inset). The assumption of neo-centromeric activity was empirically supported by the observation of silver stained cells treated for kinetochore and scaffold visualization in which kinetochore-like structures other than the standard kinetochore, were observed in XL (Fig. 1C, inset). This technique also facilitated visualization of interchromatid core structure between both arms of the neo-X: XL showed a zig-zag structure of cohesiveness like that of the neo-Y, while XR presented a typical autosomal scaffold configuration (Fig. 1C). In female meiocytes, the neo-X-neo-X bivalent shows a regular autosomal-like appearance, with regular chiasma formation in both arms (Fig. 1D).

Karyotype character state optimization

Our reconstruction of the autosomes involved in the origin of neo-sex chromosomes (from the standard 2n = 23 X0/XX karyotype) as a character on the phylogenetic hypothesis of Colombo et al. (2005) is shown in Fig. 4. The character state optimization on the phylogeny evidences the X0/XX type as the plesiomorphic condition. Our results suggest that neo-sex chromosomes arose at least seven times within the considered group: in *D. vittigerum*, in *D. maculipennis* +*D. vittatus* (however, see Discussion for this particular case), in *D. silveiraguidoi* +*D. obscurus* (however, see Discussion for this particular case), in the *Atrachelacris–Ronderosia* clade, in *Scotussa daguerrei* and in *Leiotettix pulcher.* Three autopomorphies were recovered in the optimization (in *Dichroplus vittigerum*, *Scotussa daguerrei* and *Leiotettix pulcher*) being the character state 1 homoplastic. Aditionally, the fusion M4–M5 was recovered as a synapomorphy for the clade *Atrachelacris-Ronderosia* (Fig. 4).

DISCUSSION

Neo-sex chromosomes are of frequent occurrence in South American melanoplines, and in particular within the Dichroplini tribe in which Robertsonian fusions and other rearrangements occur at unusually high frequencies (Bidau, 1990; Castillo et al., 2010a, b). Neo-sex systems are usually, but not always, the result of centric fusion between an

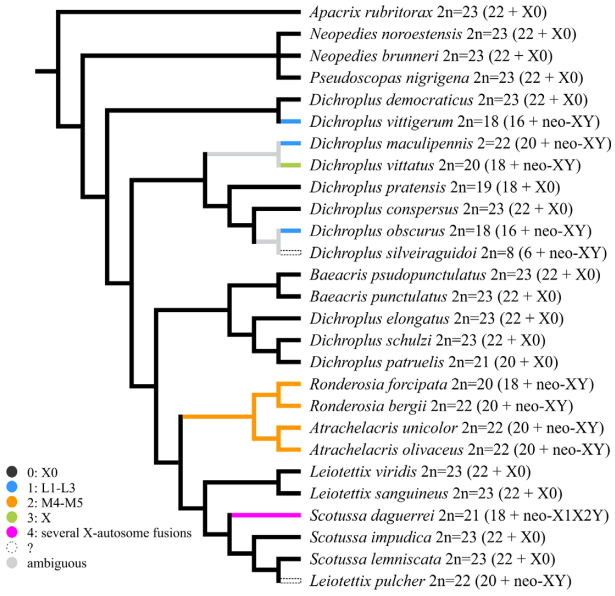

Fig. 4. Karyotype character state optimization suggesting the several independent origins of neo-sex chromosomes (see text for explanation).

autosome and the X chromosome with further possibilities of evolution toward a neo-X1X2Y system through the involvement of a second autosome (Bidau and Martí, 2001; Castillo et al., 2010a, b; Bidau et al., 2011).

Within this karyotypically diverse group, many cases of neo-sex chromosomes at several evolutionary stages are known, which make them ideal models for the study of sex chromosome evolution (Sáez, 1963; Hewitt, 1979; Castillo et al., 2010a, b; Bidau et al., 2011). This impressive neo-sex chromosome diversity resulted from recurrent independent rearrangements starting from the standard X0/XX system in the evolutionary history of several lineages (Bidau and Martí, 2001; Mesa et al., 2001; Castillo et al., 2010b; Castillo et al., 2014). In general, when simultaneous centromeric breakage of the X chromosome and an autosome and subsequent fusion occur, a neo-sex chromosome arises (Castillo et al., 2010b) although other initial rearrangements may occasionally be involved (Bidau and Martí, 2001). The result is a neo-X chromosome, while the homolog of the fused autosome is now called the neo-Y (White, 1940a, b, 1973). While the mechanism of the physical chromosome rearrangement *per se* is usually not difficult to understand, the evolutionary implications of this phenomenon require a more detailed interpretation (Mesa et al., 2001; Veltsos et al., 2008; Bidau et al., 2011).

The evolutionary instances of neo-sex chromosomes found in Dichroplini are represented by systems showing particular cytogenetic properties involving synaptic ability and the possibility of free recombination along the fused autosome (XR) and the neo-Y. These properties vary continuously from the conservation of complete homology, full synapsis and chiasma formation in recently emerged systems, to almost complete degeneration and loss of homology of the neo-Y in putative ancient systems. Degeneration involves further chromosomal rearrangements, gene loss, and accumulation of non-coding repetitive sequences (Bidau and Martí, 2001; Mesa et al., 2001; Castillo et al., 2014; Palacios-Gimenez et al., 2013; Palacios-Gimenez et al., 2015). However, complete loss of the neo-Y, transforming the system into a "neo-X0/XX" one, has never occurred as far as the available information indicates (Bidau et al., 2011). Furthermore, there are several species exhibiting different structural and genetic instances of neo-sex chromosome systems in which a single A-X centric fusion does not explain their structure and complex meiotic behavior (e.g., *Boliviacris noroestensis*, *Dichroplus vittatus*) (Bidau and Martí, 2001; Castillo et al., 2014).

In the case of *Dichroplus*, neo-sex systems are mainly grouped within the *Maculipennis* species group which includes at least five species with neo-XY sex systems: *D. obscurus*, *D. vittatus*, *D. silveiraguidoi*, *D. vittigerum* and *D. maculipennis* (Sáez, 1957; Mesa, 1971; Cosen and Sáez, 1974; Lafuente and Guerra, 1977; Mesa et al., 1982; Bidau and Martí, 2001). It is known that neo-sex pair formation has involved different autosomes in different species (Castillo et al., 2010b). Furthermore, mechanisms other than simple X-autosome fusion have been involved in some species (e.g., *D. vittatus*, Bidau and Martí, 2001). Based on our results, we propose a centric fusion between the ancestral M4 pair of the ancestral 2n = 23/24 karyotype, with the X chromosome as the first mutational event involved in the origin of the neo-X of *D. maculipennis*. A shorter M element was probably involved in the case of *D. vittigerum*. Besides, the neo-Y of *D. maculipennis* showed a conspicuous short arm absent in all other rod-shaped chromosomes possibly due to a pericentric inversion (Fig. 1E).

Regarding male meiosis, we identified comparable configurations and behaviors in *D. maculipennis* and *D. vittigerum*, and although we observed that a typical "L" shape configuration at metaphase I occurred frequently, two other orientations were also identified. These atypical orientations are the result of neo-centric activity in the XL arm. Although the origin of this activity is unknown in *D. maculipennis* and *D. vittigerum*, it has been reported and thoroughly analyzed in another species of the *Maculipennis* group, *D. vittatus*, which has a neo-XY system of very complex origin (Bidau and Martí, 2001; see below). In *D. vittatus*, neocentric activity was explained as a result of rearrangements that involved the neo-X chromosome; however, the very irregular male meiotic behavior seen in this species does not seem to impair male fertility and the neo-XY system is fixed across the wide distribution area of the species (Bidau and Martí, 2001). The same probably applies to both species studied here but the recurrent occurrence of neocentromeres in neo-X chromosomes deserves further study as to their origin.

The characteristics of the neo-sex systems of *D. maculipennis* and *D. vittigerum* suggest an advanced stage in the evolution of sex chromosomes. Particularly in *D. maculipennis*, the sex chromosomes do not show interstitial chiasmata in males but only a terminal end-to-end association suggesting synaptic impairment and lack of homology. The neo-Y shows a pericentric inversion, is positively heteropycnotic at first prophase, and shows homogeneous staining when C-banded. Also, an interstitial heterochromatic block is present in XR, but absent in its former homolog. In *D. vittigerum*, XR and neo-Y also show no evi-

dence of synapsis and recombination, while showing an invariable terminal attachment between each other.

An important problem regarding the abundance of neo-sex systems in the Dichroplini in general and *Dichroplus* in particular is that of their independent or common evolutionary origin. In this regard, Colombo et al. (2005) proposed a phylogenetic hypothesis for *Dichroplus* in which the monophyly of the *Maculipennis* group is moderately well-supported. Within this approach *D. maculipennis* is grouped with *D. vittatus*. Our results and the available cytogenetic data allow the comparison of both neo-sex chromosome systems, supporting an independent origin (this work; see also Bidau and Martí, 2001). While a X-X centric fusion in a female produced a metacentric iso-chromosome, which then underwent a tandem fusion with an S autosome and posterior pericentric inversion in the formation of the neo-XY of *D. vittatus* (Bidau and Martí, 2001), the neo-X in *D. maculipennis* was consequence of a centric fusion between the ancestral X chromosome and an autosome from the standard M group (M4), the most usual form of neo-XY systems generation in grasshoppers. Despite their seemingly close relationship (Fig. 4) both species have followed independent evolutionary pathways with respect to their sex chromosomes. Within this same scenario, *D. vittigerum* is grouped with an X0 species, *D. democraticus* again suggesting an independent formation of the neo-sex system.

Furthermore, although the karyotypes of *D. vittigerum* and *D. obscurus* are superficially similar (presence of two autosomal fixed fusions and a neo-XY system) the neo-Y of the latter species is undoubtedly derived from a different autosome (one from the L group) and the species is most closely related to *D. silveiraguidoi*, the grasshopper with the most rearranged karyotype (2n = 6 + XY/XX; Sáez, 1956) known to date.

Within this context, the cytogenetic evidence presented here allows to reevaluate the hypothesis of a neo-XY common ancestor for the *D. maculipennis-D. vittatus* group, proposed by Colombo et al. (2005). Our reconstruction of the neo-sex chromosome character suggests that independent X-autosome centric fusions arose five times within the *Maculipennis* species group: in *D. vittigerum*, in *D. maculipennis*, in *D. silveiraguidoi*, in *D. obscurus*, and in *D. vittatus* (Fig. 4). Hence, the hypothesis of a single X-autosome fusion taking place *prior* to the speciation process in this group is not plausible considering the evidence presented here. This means that instead of a single neo-XY ancestor (derived from an X0 species by a simple centric fusion), each lineage leading to each neo-XY species underwent independent rearrangements producing completely different neo-sex systems. However we agree that a single event of X-A centric fusion could have occurred in the ancestors of the *Ronderosia-Atrachelacris* group (Fig. 4). This evidence indicates that neo-sex chromosome formation in the ancestor did not deter this group from undergoing speciation several times (Colombo et al., 2005) clearly contradicting the hypothesis of Mesa et al. (2001) which proposed that an X-autosome fusion would condemn a species to extinction. Besides, *Ronderosia* is probably a relatively recent clade compared with other genera of the Paranense-Pampeano assemblage (Castillo et al., unpublished data). A different picture is evidenced in species of the *Maculipennis* group, where chromosome rearrangements leading to neo-XY systems probably occurred alongside the speciation process (whether or not triggered the speciation process). Also, the antiquity of systems such as those of *D. maculipennis* and *D. vittatus* can be inferred from the fact that despite a degree of abnormal male meiotic behavior, especially in *D. vitattus* (Bidau and Martí, 2001), the neo-XY systems have been successfully established in these species of wide geographic distribution. Indeed, *Dichroplus* species show remarkable homeostasis regarding the abnormal behavior of Robertsonian configurations (Bidau, 1990, 1991).

The cytogenetic evidence suggests that the replacement of X0-XX by XY-XX was favored many times in the evolutionary history of Neotropical Dichroplini. The high frequency of neo-sex systems, and their independent origins point to a higher incidence of chromosomal rearrangement within this group, which is reinforced by the high incidence of autosomal rearrangements. If this is the case, are centric fusions (or other rearrangements involved in neo-sex chromosome formation) random chromosome restructurings due to events of non-homologous recombination? Is it possible that X-A translocations occur randomly because different autosomes are involved in neo-sex chromosome formation? However, it is also reasonable to assume that larger autosomes could be more readily selected for than smaller ones due to intrinsic properties of the resultant meiotic configurations (see Castillo et al., 2010a). This is also supported by the almost inexistence of autosome-autosome fusion involving S chromosomes (Bidau, 1990). Differently from other insect groups, little is known about the intimate mechanism of sex-determination in Orthoptera and most derives from extrapolation of theoretical and empirical data of other biological systems (White, 1973; Pannell and Pujol, 2009; Kaiser and Bachtrog, 2010; Bidau et al., 2011). Thus, the main question in this respect is: are neo-sex chromosomes in Orthoptera subject to the classical path of sex chromosome evolution? All the cytogenetic evidence currently available points to a different path, for whose elucidation new molecular evidence is necessary, especially relating to neo-Y chromosome degeneration and the mapping of sex-determining genes in the new sex chromosomes in this particular group. Recently, the molecular mapping of C0t-1 DNA fraction evidenced in melanopline species (i.e., *Eurotettix minor*, *Dichromatos lilloanus*, and *D. schrottkyi*) points to a relatively restricted spreading of this repetitive DNA in neo-sex chromosomes, which contrasts with the repetitive DNA accumulation expected after recombination restriction (Palacios-Gimenez et al., 2013). However, *R. bergii* neo-Y shows a marked dispersion of this repetitive DNA fraction throughout the long arm of the neo-Y chromosome (Palacios-Gimenez et al., 2015). Research also suggests different accumulation/diversification patterns of repetitive DNAs of neo-Y chromosomes in this closely related species; such empirical data could be evidence for the loss of selection pressure in chromosomal regions in which recombination is abolished, leading to a high rate of genetic diversification (Palacios-Gimenez et al., 2013; Castillo et al., 2014; Palacios-Gimenez et al., 2015).

Finally, despite descriptive studies of neo-XY chromosomes in Melanoplinae nothing is known about their evolutionary meaning or their roles in sex determination, which

remains a mystery in Orthoptera. The description and understanding of neo-sex chromosome structure, meiotic behavior, and their origin in species of Orthoptera is a not much explored field and actually deserves more attention than it has received (Bidau et al., 2011).

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