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CELLULAR AND MOLECULAR BIOLOGY

Behaviour, feeding and cytogenetic features of the wingless blood-sucking ectoparasite *Cyanolicimex patagonicus* (Heteroptera: Cimicidae)

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Abstract: *Cyanolicimex* (Haematosiphoninae) includes a single species, *C. patagonicus*, which is found in the largest known colony of its avian host *Cyanoliseus patagonus* (Psittacidae) located in Patagonia (Argentina). Relationships between *Cyanolicimex* and other genera of Haematosiphoninae are still unclear because this genus shares some characters with other South American genera and possesses some similarities with *Hesperocimex* from the Neoarctic region. The aim of the present study was to provide additional data of *C. patagonicus* so as to better understand its relationships with other South American species. We examined some biological features of *C. patagonicus* in the field and we performed a cytogenetic analysis. We observed in the field that *C. patagonicus* does not live inside the hollow nests of *Cyanoliseus patagonus*. The cytogenetic analysis showed that the male karyotype is $2n=31=28A+X_1X_2Y$ and revealed an achiasmate male meiosis and of the collochore type. Our results together with available cytogenetic data in other cimicids, allow proposing the possible chromosomal rearrangements involved in the chromosomal evolution of *C. patagonicus* and also contribute to better understand the evolutionary divergence at the chromosomal level within Haematosiphoninae. Based on the whole evidence, we propose to place in four groups the species of Haematosiphoninae cytogenetically hitherto studied.

Key words: Achiasmate male meiosis, behavioural habits, bird bugs, chromosomal rearrangements, evolutionary trends, holocentric chromosomes.

INTRODUCTION

The Cimicidae (Hemiptera, Heteroptera) are wingless blood-sucking ectoparasites of warm-blooded animals, including birds and bats as primary hosts and humans as secondary hosts, which require transport by their hosts for dispersal (Usinger 1966 , Henry 2009 , Resh & Gardé 2009 , Potter 2011, Di Iorio 2012) . In Argentina, the subfamily Haematosiphoninae includes four species of blood-sucking insects specializing in avian hosts: *Acanthocrios furnarii* (Cordero and Vogelsang, 1928), *Ornithocoris*

toledoi Pinto, 1927, *Psitticimex uritui* (Lent and Abalos, 1946), and *Cyanolicimex patagonicus* Carpintero, Di Iorio, Masello and Turienzo, 2010. The relationships between *Cyanolicimex* Di Iorio, Masello & Turienzo, 2010 and other genera are still unclear because this genus shares some characters with other South American genera and possesses some similarities with *Hesperocimex* List, 1925 from the Neoarctic region. The characters shared with other South American genera include the absence of the apical tufts of hair in the middle tibia of the females (*Ornithocoris* Pinto,

1927), the antennal segment 2 longer than the anterior interocular space (*Psitticimex* Usinger, 1966), the maximum width of the pronotum in the middle of its length (*Acanthocrios* Del Ponte and Riesel, 1945, and *Psitticimex*), the shape of the spermaledge extended anteriorly (*Psitticimex*), and one species of Psittacidae (Aves) as a host (*Psitticimex*) (Di Iorio et al. 2010) . Regarding the similarities with *Hesperocimex*, in *Hesperocimex*, the pronotum has very long bristles at the lateral margins, the posterolateral angles of the pronotum are rounded, and the apical tufts of hairs are absent in the front and middle tibiae of the females (Usinger 1966) .

As it is typical in Hemiptera, cimicids have holocentric chromosomes (Ueshima 1966 , 1979 , Manna 1984 , Mola & Papeschi 2006 , Papeschi & Bressa 2006 , Poggio et al. 2009 , 2014 , Grozeva et al. 2010 , 2011 , 2014, Sadílek et al. 2013) . These chromosomes have no primary constriction and, thus, no localized centromere (Schrader 1947 , Wolf 1996) , and their behaviour in mitosis is different from that in meiosis. Even during meiosis, autosomes, sex chromosomes, and m-chromosomes behave differently. During mitosis, holocentric chromosomes attach to the spindle fibres along all their length, and the sister chromatids segregate parallel to each other and perpendicular to the polar spindle at mitotic anaphase (Hughes-Schrader & Schrader 1961, Dernburg 2001, Mola & Papeschi 2006, Melters et al. 2012, Bureš et al. 2013). During meiosis, the kinetic activity is restricted to telomeric regions and the chromosomes can be regarded as telokinetic (Motzko & Ruthmann 1984, Mola & Papeschi 2006, Papeschi & Bressa 2006, Bureš et al. 2013). In Cimicidae, the 53 species cytogenetically studied so far exhibit a considerably wide range of diploid chromosome numbers that vary from 10 to 47, with a modal number of 31, and can have either a simple sex chromosome system (XY/XX, male/female) or a multiple sex chromosome system

($X_{2-20}Y/X_{2-20}X_{2-20}$, male/female). Among the great variety of multiple sex chromosome systems described so far, the prevailing one in these species is $X_1X_2Y/X_1X_1X_2X_2$ (Ryckman & Ueshima 1964, Ueshima 1966, 1979, Manna 1984, Grozeva & Nokkala 2002, Poggio et al. 2009, Grozeva et al. 2010, Kuznetsova et al. 2011, Sadílek et al. 2013).

In Haematosiphoninae, only nine species of six genera have been so far cytogenetically analysed. Ueshima (1966, 1979) proposed to divide these species into three groups based on their morphological and cytogenetic features. The first group includes only the genus *Ornithocoris*, which has the least specialized spermaledge and a male diploid number 2n= 10 (8A+XY, male). The second group includes the genera *Haematosiphon Champion, 1900*, *Psitticimex*, and *Synxenoderus* List, 1925, which have a more specialized spermaledge and a diploid number 2n= 31 (28A+X_1X_2Y, male), and *Acanthocrios* (= *Caminicimex*) Del Ponte & Riesel, 1945, with two different diploid numbers (2n= 12= 10A+XY/XX, 2n= 34= 32A+XY, male). The third group includes only the genus *Hesperocimex*, which has an atypical lateroventral spermaledge and possesses high chromosome numbers (2n= 40–42) with simple (XY, male) and multiple sex chromosome systems ($X_1X_2X_3$Y, male) (Ryckman & Ueshima 1964, Ueshima 1966, Poggio et al. 2009, 2014, Kuznetsova et al. 2011) (Table I). Regarding the species *Acanthocrios furnarii*, Poggio et al. (2009) placed it in the first group along with the genus *Ornithocoris*, because of the low number of chromosomes (2n= 12) and the simple sex chromosome system (XY/XX) present in the Argentinean specimens analysed by the authors.

The aim of the present study was to get a better understand of the relationships of *Cyanolicimex patagonicus* with the other South American species of Haematosiphoninae, especially with *Psitticimex*. To this end, we examined some biological features of *C. patagonicus* in

Table I. Chromosome numbers and their sex chromosome systems in Haematosiphoninae (Hemiptera: Cimicidae). The Cimicidae species were ordered in decreasing number of chromosomes on each biogeographic region (except those with unknown numbers).

Species	2n ♂	2n ♀	n ♂	References
Neoarctic region				
Hesperocimex coloradensis	42	44	19+$X_1X_2X_3$Y	Ryckman & Ueshima, 1964
Hesperocimex sonorensis	42	42	20+XY	Ryckman & Ueshima, 1964
Hesperocimex cochimiensis	40	40	19+XY	Ryckman & Ueshima, 1964
Haematosiphon inodorus	31	32	14+X_1X_2Y	Ueshima, 1966
Synxenoderus comosus	31	32	14+X_1X_2Y	Ueshima, 1966
Cimexopsis nyctalis	-	-	-	
Neotropical region				
Central America				
Alayocimex tachornis	-	-	-	
South America				
Acanthocrios furnarii	34	-	16+XY	Ueshima, 1966 (prob. erroneous)
Cyanolicimex patagonicus	31		14+X_1X_2Y	Present work
Psitticimex uritui	31	-	14+X_1X_2Y	Ueshima, 1966; Poggio *et al.*, 2009
Acanthocrios furnarii	12	12	5+XY	Poggio *et al.*, 2009
Ornithocoris toledoi	-	-	4+XY	Ueshima, 1966
Ornithocoris pallidus	10	-	4+XY	Ueshima, 1966

the field. We analysed the mitotic and meiotic chromosomes, the male meiotic development, and the meiotic behaviour of autosomes and sex chromosomes of *C. patagonicus*. We propose the possible chromosome rearrangements involved in the chromosomal evolution of *C. patagonicus* and the main evolutionary trends for Haematosiphoninae. Based on the whole evidence, we propose to place in four groups the species of Haematosiphoninae cytogenetically hitherto studied.

MATERIALS AND METHODS

Live nymphs (n = 50) and adults (n = 28) of *C. patagonicus* were collected in December 2013 and January 2014 from the same colony of *Cyanoliseus patagonus* (Vieillot, 1818) in Bajada de Picoto (41º 03' 42.65'' S, 62º 50' 44.77'' W) (Viedma, Río Negro, Argentina) (Fig. 1). All specimens are conserved in the O.R. Di Iorio collection [DIOC]. There is no an environmental authorization document to carry out work with the *Cyanoliseus patagonus* colonies.

The specimens were brought to the laboratory alive. Gonads were dissected in a physiological solution previously described for *Ephestia* (Glaser, 1917;, Lockwood, 1961), swollen for 10 min in a hypotonic solution (0.075 M KCl), and then fixed for 15-30 min in freshly prepared Carnoy fixative (ethanol:chloroform:acetic acid, 6:3:1). Cells were dissociated in a drop of 60% acetic acid with the help of tungsten needles and spread on the slide by using a heating plate at 45º C as described in Traut (1976). Then, the preparations were dehydrated in an ethanol series of 70, 80, and

Figure 1. Habitat of *Cyanolicimex patagonicus*. (a, b) General views of the place where the colonies of *Cyanoliseus patagonus* live and *C. patagonicus* was collected; the height of the cliffs is generally greater than 4 m. (c) Small crevice on the left, below the entrance of a hollow nest of *C. patagonus*, corresponding to the collection of December 26th 2013. (d) Detail of the second small crevice inhabited by *C. patagonicus*. (e) Another small crevice below an excavation in the wall of the sandstone cliff (probably initiated by *C. patagonus* but later abandoned), corresponding to the second collection on January 17th 2014. Bar= 47 cm.

96% (30 s each), air-dried, and stained with 5% Giemsa in phosphate buffer solution (pH 7.0–7.2) for 7 min at 25º C (water bath). For a better chromosome resolution, the slides were stained with 4′,6-diamidino-2-phenylindole (DAPI; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland). Briefly, 75 µl of 0.01 µg/ml DAPI in MacIlvaine's buffer pH 7 (0.1 M citric acid, 0.2 M Na_2HPO_4) was placed on each slide, covered with a coverslip and incubated for 20 min at room temperature. The slides were then rinsed with distilled water, MacIlvaine's buffer and distilled water, and air-dried. Afterwards, slides were mounted in antifade (20 µl) based on DABCO (Sigma Aldrich; for composition see Traut et al. 1999), covered with a 24x32 mm coverslip, and the coverslip sealed with colourless nail varnish.

Chromosome preparations were observed in a Leica DMLB microscope equipped with a Leica DFC350 FX CCD camera and the Leica IM50 version 4.0 software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). After examining all male chromosome preparations, black-and-white images were recorded, pseudocolored (light blue for DAPI) and processed with appropriate software. The total chromosome length measurements (TCL) were measured by means of the computer application MicroMeasure version 3.3 (Reeves 2001). The TCL of all bivalents and sex chromosomes was performed in selected cells at metaphase I (haploid set) (Table II).

RESULTS

Cyanolicimex patagonicus Carpintero, Di Iorio, Masello and Turienzo, 2010

= *Psitticimex uritui*, not Lent and Abalos, 1945: Aramburú (2012) (biology; reference), following Masello & Quillfeldt (2004): error of identification.

Material examined: ARGENTINA: Río Negro: Viedma, Bajada de Picoto (41º 03' 42.65'' S, 62º 50' 44.77'' W), December 26th 2013, H. Iuri leg., 50 nymphs and 21 adults collected in one crevice of the sandstone cliff were fixed for taxonomic determination [DIOC]; January 17th 2014, 7 adults collected in another crevice of the sandstone cliff were brought alive to the laboratory for meiotic and karyotype analyses (Fig. 1).

Data on behaviour and feeding of *Cyanolicimex patagonicus*

While *Cyanoliseus patagonus* parrots were roosting inside their hollow nests, nymphs

Table II. Total meiotic chromosome lengths (TCL) of *Cyanolicimex patagonicus* (mean ± SE) in the haploid set.

Chromosome Pair	TCL (μm)	Haploid set %
1	0.84 ± 0.01	8.31
2	0.77 ± 0.02	7.64
3	0.77 ± 0.02	7.62
4	0.71 ± 0.01	7.01
5	0.65 ± 0.02	6.36
6	0.64 ± 0.01	6.31
7	0.62 ± 0.01	6.13
8	0.60 ± 0.01	5.90
9	0.58 ± 0.01	5.72
10	0.55 ± 0.01	5.45
11	0.54 ± 0.01	5.33
12	0.52 ± 0.02	5.13
13	0.51 ± 0.01	5.03
14	0.48 ± 0.01	4.74
X_1	0.54 ± 0.01	5.31
X_2	0.51 ± 0.01	5.02
Y	0.30 ± 0.01	2.98
TCL	10.12 ± 0.14	100

and adults of all specimens of *C. patagonicus* were hidden inside crevices in the sandstone cliff, near the entrance of hollow nests (Fig. 1). In the afternoon, specimens of *C. patagonicus* were observed actively walking on the wall of the cliff, most probably searching for food, while the crevices were not occupied by *C. patagonus*. These new observations indicated that *C. patagonicus* does not live directly inside the hollow nests but in nesting shelters nearby. Nymphs and adults of *C. patagonicus* were observed falling to the sand at the base of the cliff and immediately burying themselves with rapid movements of the legs in a few seconds. This behaviour was also observed at the laboratory when live specimens were disturbed.

Cytogenetics of *Cyanolicimex patagonicus*

The observations made on chromosome behaviour during male meiosis indicated that *C. patagonicus* possesses holocentric chromosomes and a diploid chromosome number of 2n= 31, with a multiple sex chromosome system X_1X_2Y (Figs. 2, 4a). Autosomal bivalents showed a gradation of sizes, whereas the two X chromosomes are medium sized, with slight differences in size between them, and the Y chromosome is the smallest element of the metaphase I complement (Table II; Figs. 2, 3). Since no females were analysed, we proposed X_1X_2Y as the male sex chromosome system based on the multiple X chromosomes varying in number from two to 15 so far described in the species studied and their meiotic behaviour.

In the early meiotic prophase, the sex chromosomes were identified by being condensed and positively heteropyknotic (Fig. 4b, c). Pachytene was followed by a diffuse stage in which autosomes did not completely decondense, whereas the sex chromosomes decondensed to a lesser degree than autosomes; the positive heteropyknotic regions detected were scattered throughout the nucleus (Fig. 4d). No diplotene or diakinesis stages were

detected. As autosomal bivalents recondensed, it became evident that no chiasma was present. The homologous chromosomes lay side by side and the X and Y chromosomes were observed as univalents. From metaphase I onwards to the end of meiosis, one of the smallest autosomal bivalents and the Y sex chromosome showed negative heteropyknosis (Fig. 4e). At metaphase I, 14 autosomal bivalents were arranged in a circle and the three sex univalents were located among the bivalents (Fig. 4f). The three sex chromosomes were distinguished because they were composed of two chromatids. As metaphase I progressed, one of the homologs of some autosomal bivalents segregated precociously, leading the migration to the poles (Fig. 4e, f). At anaphase I, autosomal bivalents divided reductionally, whereas the three sex chromosomes segregated equationally (data not shown). Therefore, at telophase I, two nuclei with 17 chromosomes each ($14A+X_1X_2Y$) were observed (Fig. 4g). At metaphase II, the autosomes were arranged again in a circle, whereas the three sex chromosomes came close together and associated through the so-called "touch-and-go pairing", forming a pseudo-trivalent X_1X_2Y, which lay at the centre of the circle (Fig. 4h). The Y chromosome was oriented towards the spindle pole opposite to that of X_1 and X_2. At both metaphases I and II, the difference in size of both X chromosomes became quite evident, being X_1 slightly longer than X_2. At anaphase II, the autosomes divided equationally and the sex chromosomes underwent a reductional division (Fig. 4i). Thus, 16 chromosomes migrated to one of the poles ($14A+X_1X_2$) and 15 chromosomes to the opposite one ($14A+Y$).

DISCUSSION

Behaviour and feeding of *Cyanolicimex patagonicus* and comparison with other cimicid species

In the present study, all specimens of *C. patagonicus* were found hidden in the crevices during the day and some of them were seen walking on the wall of the cliff in the afternoon, probably in search for food in hollow nests with nestlings and/or adult parrots. During the nestling period, the breeding pairs of *Cyanoliseus patagonus* always fed the nestlings after arrival at the nests in the evening and before departure early in the morning and spent the night in the nest with their nestlings (Masello et al. 2006). Thus, both nestlings and adults can be food sources for *C. patagonicus*. Based on the pattern of forage throughout the day of *Cyanoliseus patagonus*, we can infer that *C. patagonicus* has a nocturnal feeding activity when the birds are roosting inside the hollow nests. These particular features in the biology of *C. patagonicus* are closely related to the biology of its avian host.

Burrowing parrots do not use any nesting material but, rather, deposit their eggs on the sandy bottom of the nest chamber (Mey et al. 2002). Thus, the hollow nests of *Cyanoliseus patagonus* provide no shelter for cimicid bugs, unlike the shelters offered by the tenant bird nests superimposed on the grass bed of *Furnarius rufus* (Gmelin, 1788) [Aves: Furnariidae] for *Acanthocrios furnarii* (Turienzo & Di Iorio 2010), or by the large and massive rod nests of *Myiopsitta monachus* (Boddaert, 1783) [Aves: Psittacidae] for *Psitticimex uritui* (Turienzo & Di Iorio 2011). Furthermore, the hiding behaviour observed in nymphs and adults of *C. patagonicus* is similar to that of *P. uritui*, which promptly hides under nest debris when the nests are disassembled, and different from that of *A. furnarii*, which remains in a death-feigning posture (Turienzo & Di Iorio 2010).
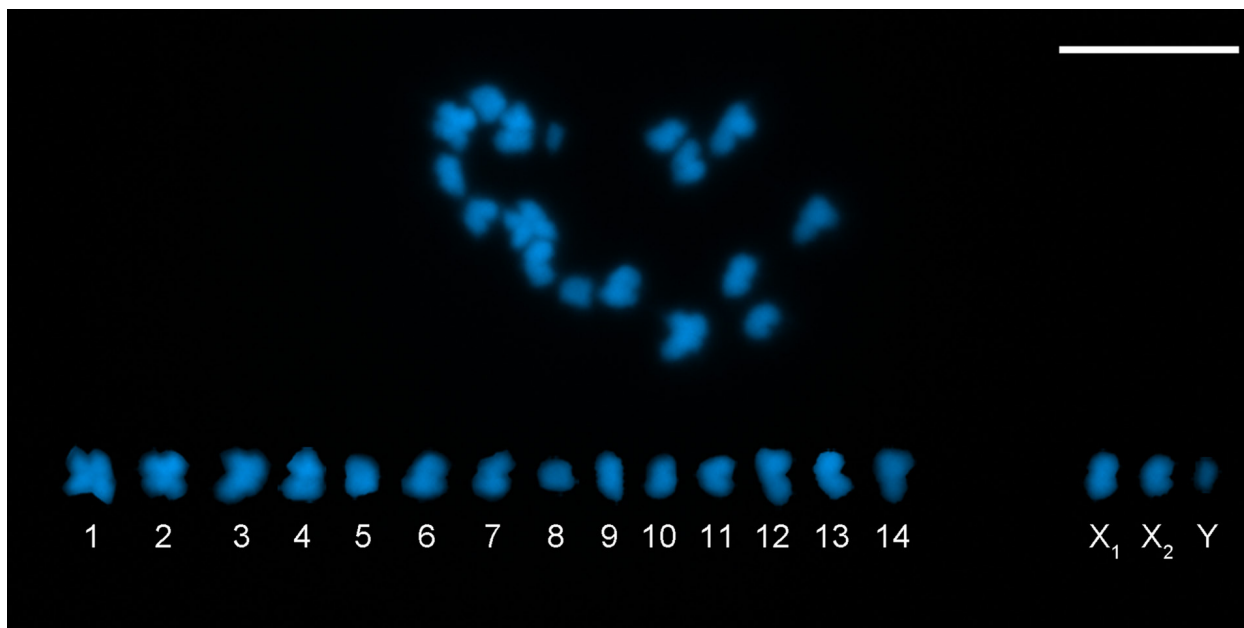
Figure 2. Male meiotic karyotype of *Cyanolicimex patagonicus*. 2n= 31= 28A+X_1X_2Y, n= 17= 14II+ X_1X_2Y. Chromosomes are stained with DAPI. Bar= 10 μm.
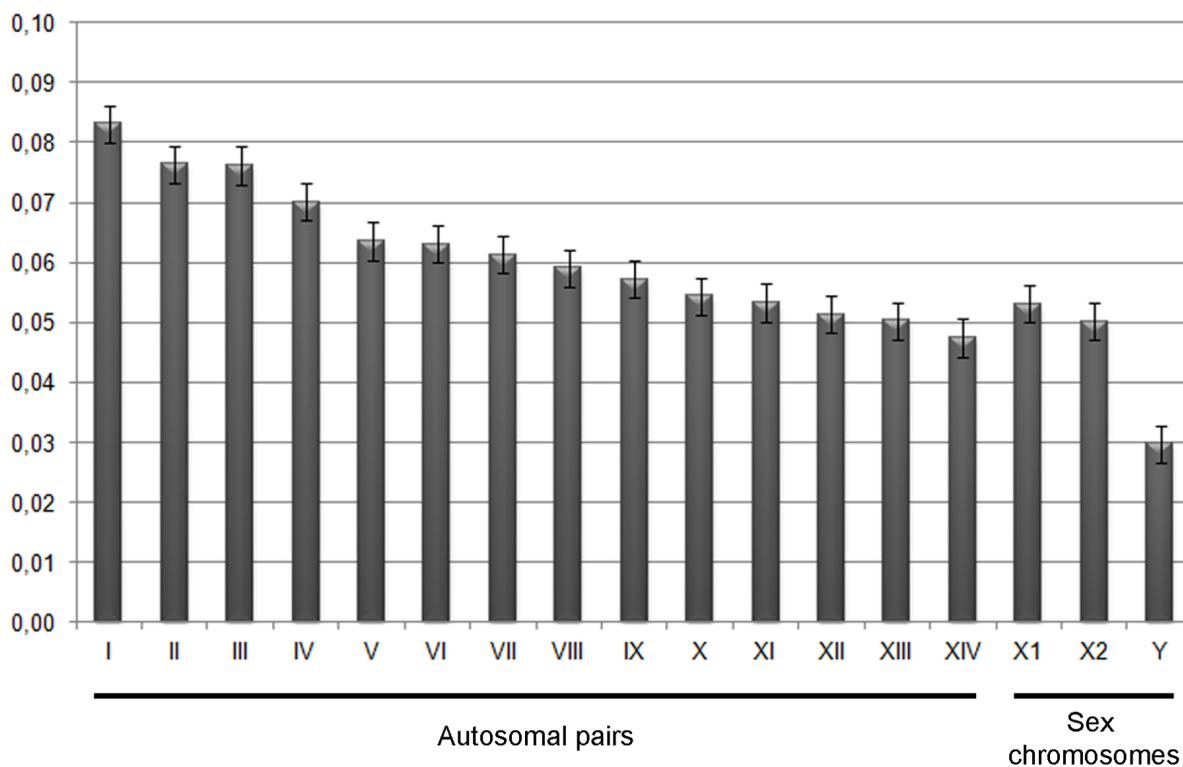

Figure 3. Male ideogram of *Cyanolicimex patagonicus*. 2n= 31= 28A+X_1X_2Y. The TCL (Y axis) and autosomal pairs and sex chromosomes were put in the x- and y-axes.
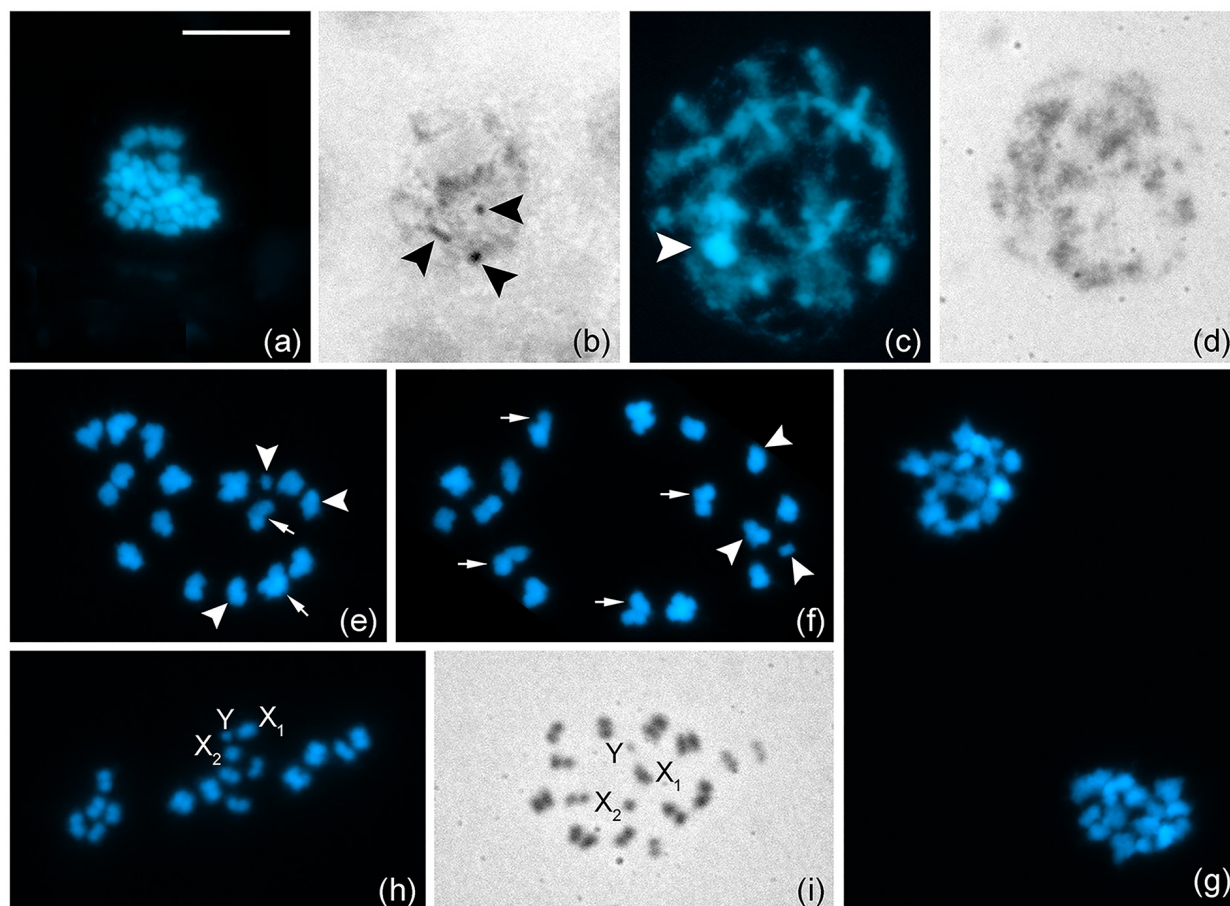
Figure 4. Mitotic and meiotic chromosomes of *Cyanolicimex patagonicus*. (a) Spermatogonial metaphase. (b) Early meiotic prophase. (c) Pachytene. (d) Diffuse stage. (e) Metaphase I, equatorial view. (f) Metaphase I, polar view. (g) Telophase I. (h) Metaphase II, equatorial view. (i) Metaphase II, polar view. Arrowheads: sex chromosomes. Arrows: one of the homologues of some autosomal bivalents segregating precociously. (a, c, e-h) Chromosomes are stained with DAPI. (b, d, i) Chromosomes are stained with 5 % Giemsa. Bar= 10 μm.

Chromosome complement and male meiosis of *Cyanolicimex patagonicus*

Considering the cytogenetic features, Haematosiphoninae constitute an interesting subfamily because the nine species studied so far show achiasmate male meiosis and a wide karyological diversity with regard to the diploid number of autosomes (which ranges from 8 to 40) and sex chromosome systems (five species with XY/XX, three with $X_1X_2Y/X_1X_1X_2X_2$ and one with $X_1X_2X_3Y/X_1X_1X_2X_2X_3X_3$) (Table I) (Ryckman & Ueshima 1964, Ueshima 1979, Poggio et al. 2009). Three patterns of achiasmate meiosis have been described in seven heteropteran families

belonging to the infraorders Leptopodomorpha (Saldidae), Cimicomorpha (Anthocoridae, Cimicidae, Nabidae, Microphysidae, and Miridae) and Nepomorpha (Micronectidae) (Ituarte & Papeschi 2004, reviewed in Kuznetsova et al. 2011). The most frequent pattern, i.e. alignment type, is observed in Saldidae, Microphysidae, Anthocoridae, Micronectidae and Corixidae, in which the homologous chromosomes are aligned and joined along their entire lengths from prophase I to metaphase I, and segregate reductionally (Nokkala & Nokkala 1983, 1984, Kuznetsova & Maryanska-Nadachowska 2000, Nokkala & Grozeva 2000, Ituarte & Papeschi
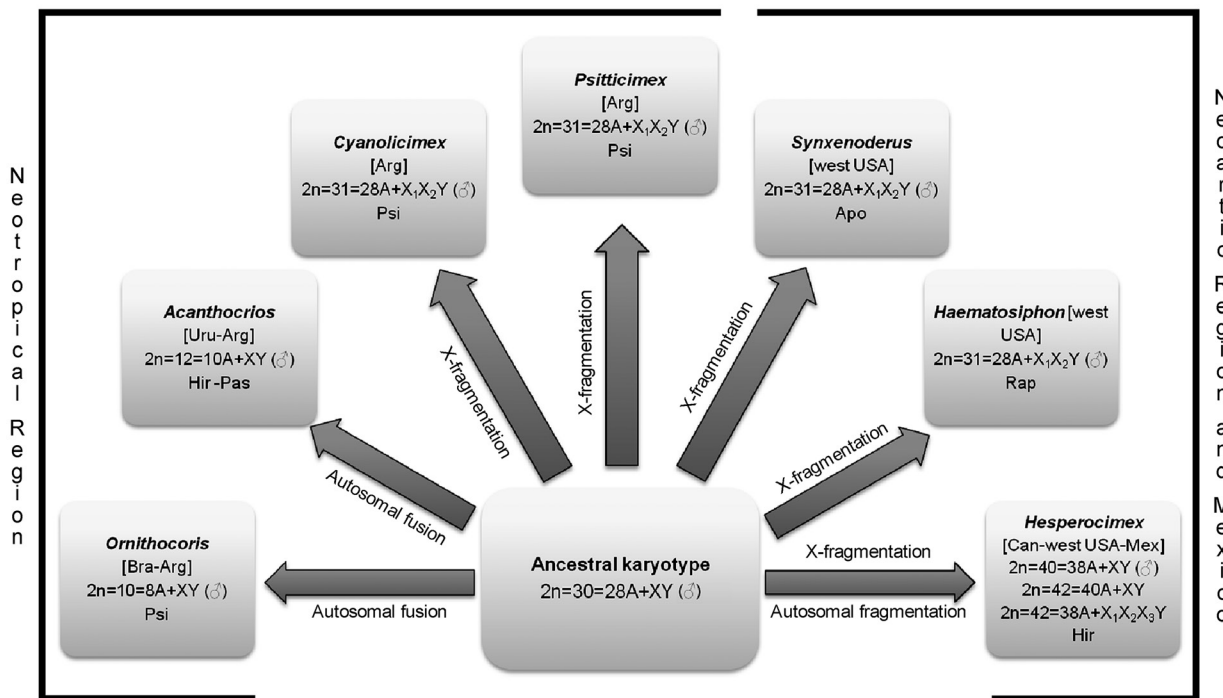
Figure 5. Schematic interpretation of karyotype evolution hypothesis proposed to species of Haematosiphoninae. Avian hosts are Apo, Apodidae; Hir, Hirundinidae; Pas, Passeriformes (Icteridae, Passeridae, Thraupidae, Troglodytidae); Psi, Psittacidae; and Rap, raptorial birds (Accipitridae, Cathartidae, Falconidae, Strigidae, Tytonidae). Bra, Brazil; Arg, Argentina; Uru, Uruguay; Can, Canada; Mex, Mexico; USA, United States of America.

2004, Kuznetsova et al. 2004, Grozeva et al. 2008, Stoianova et al. 2015). The second achiasmate meiotic pattern, so-called collochore type, has been described in Miridae and Cimicidae species (Nokkala & Nokkala 1986, Grozeva & Nokkala 2002, Grozeva 2003, Grozeva & Simov 2008, 2009, Poggio et al. 2009, Grozeva et al. 2010, 2011, Jauset et al. 2015). Lastly, an intermediate model between the meiosis alignment type and the meiosis collochore type in *Arachnocoris trinitatus* Bergroth, 1916 (the only representative of the Arachnocorini tribe) has been observed in Nabidae *sensu stricto* (Kuznetsova et al. 2007, Kuznetsova & Grozeva 2008). In our study, the cytogenetic analysis carried out in *C. patagonicus* clearly demonstrates that male meiosis is achiasmate and of the collochore type. Therefore, no diplotene or diakinesis was observed during male meiosis I of *C. patagonicus.* At metaphase I, the homologous

chromosomes lay side by side connected to each other through their medial region by tenacious threads, the so-called collochores, which are points of close contact between homologous chromosomes that fulfil the function of chiasmata in achiasmate meiosis without being the result of genetic crossing-over and exchange of chromatid segments. Another distinctive behavioural feature observed in *C. patagonicus* was the repulsion of the terminal regions in only one end of each pair of autosomes. In contrast, in *A. furnarii* and *P. uritui* this repulsion is observed at both ends of each pair (Poggio et al. 2009), and in *Cimex lectularius* (Linnaeus, 1758) at one or both ends (Grozeva et al. 2010). Based on the cytogenetic studies carried out up to the present, the achiasmate male meiosis of collochore type should be considered as a cytogenetic feature shared by all the members

of Cimicidae (Grozeva & Nokkala 2002, Poggio et al. 2009, Kuznetsova et al. 2011).

The findings of achiasmate male meiosis in three different heteropteran infraorders (Nepomorpha, Leptopodomorpha, and Cimicomorpha), as well as the molecular phylogeny for Cimicomorpha based on combined analysis of *16S rDNA*, *18S rDNA*, *28S rDNA* and *mitochondrial cytochrome oxidase* subunit I (COI) sequence data and 73 morphological characters (Schuh et al. 2009), support that this type of meiosis would have originated more than once during evolution as the result of independent events in Heteroptera (Kuznetsova et al. 2011). The independent occurrence of achiasmate meiosis in distant groups points to multiple origins for this kind of meiosis. Moreover, the presence of achiasmate meiosis even within a given group might sometimes be polyphyletic (John 1990). On the other hand, taking into account the molecular phylogeny obtained by Schuh et al. (2009), Kuznetsova et al. (2011) proposed a monophyletic origin for the achiasmate meiosis in Cimicomorpha.

A genetic consequence of the achiasmate nature of male meiosis in *C. patagonicus* is the absence of intra-chromosomal recombination, which implies that the allelic combinations present in each chromosome of the heterogametic sex will remain together and unchanged (except for mutation events) from one generation to the other. Hence, achiasmate male meiosis keeps together particular combinations of alleles, which are probably co-adapted and function as supergenes. Although we have no information about the chiasma frequency in the females of this species, we can assume that it follows the general pattern of Heteroptera species, which is one chiasma per bivalent. On the other hand, *C. patagonicus* possesses the second highest diploid chromosome number among Haematosiphoninae species of the

Neotropical region, a fact that brings about a concomitant increase in the likelihood of new allelic combinations because of the independent chromosome assortment (inter-chromosomal recombination). Therefore, the main sources of genetic variation in *C. patagonicus* will be the intra-chromosomal recombination in females and the inter-chromosomal recombination in both sexes.

Chromosomal evolution and comparative cytogenetics of Haematosiphoninae

Karyotypes are species-specific features that have evolved through natural selection and can contribute to elucidate the possible evolutionary processes involved in chromosome diversity and the putative role of chromosomal changes (i.e. chromosomal rearrangements, differences in number) in the speciation process (Papeschi & Bressa 2006, Bressa et al. 2009, Poggio et al. 2009, 2013, 2014, Chirino et al. 2013, 2017, Chirino & Bressa 2014). Most hypotheses on karyotype evolution in Heteroptera include both autosomal and sex chromosomes fusions and fragmentations (Ueshima 1979, Manna 1984, Thomas 1987, Papeschi 1994, 1996, Pérez et al. 2004, Papeschi & Bressa 2006, Chirino et al. 2017). Fused holocentric chromosomes cannot give rise to dicentric chromosomes because of the extended kinetochore. Although chromosome fragmentations usually result in the deletion of acentric fragments in monocentric chromosomes, such deleterious effects are largely prevented in species with holocentric chromosomes. Any chromosome fragment exhibits a part of the kinetochore plate and can attach to spindle fibres at cell divisions (Papeschi & Bressa 2006, Hipp et al. 2010, Bureš et al. 2013). Therefore, fusion and fragmentation rearrangements are conventionally accepted as the commonest mechanisms of chromosomal evolution in holocentric systems since both rearrangements have less constraints

than in monocentric ones. On the other hand, discussions on karyotype evolution in Heteroptera use the concept of modal numbers in family, tribe or genera levels to propose the ancestral one for the analysed group (Ueshima 1979, Manna 1984, Papeschi & Bressa 2006). Within the family Cimicidae, the previous cytogenetic studies revealed that the most frequent chromosome complement is 2n= 28 with the simple system XY/XX. Therefore, it is safe to assume that this chromosome number could be the ancestral complement of the family (Ueshima 1979, Manna 1984). Our results, together with previous cytogenetic data on Haematosiphoninae and the ancestral diploid number proposed for Cimicidae, led us to propose the main evolutionary trends for the subfamily Haematosiphoninae (Fig. 5). From this ancestral karyotype, autosome fusions led to a reduction in the diploid number to 2n= 10 and 2n= 12, as that observed in *Ornithocoris* and *Acanthocrios* (Neotropical region), respectively. This evolutionary trend is supported by the existence of an inverse relationship between the chromosome size and the chromosome number (Papeschi 1988, Chirino & Bressa 2014, Chirino et al. 2017). The possibility of their occurrence is supported by the fact that the autosomal fusions have been found in heterozygous condition in natural populations of *Belostoma plebejum* (Stål, 1858) (Belostomatidae) (Papeschi 1994), *Triatoma infestans* (Klug, 1834) (Poggio et al. 2013) and *Mepraia gajardoi* Frías, Henry and González, 1998 (Pérez et al. 2004) (Reduviidae). A fragmentation of the X chromosome in the ancestral karyotype resulted in multiple X chromosomes, X_1 and X_2, and led to a karyotype with 2n= 31 chromosomes, as that observed in *Psitticimex* and *Cyanolicimex* (Neotropical region) and *Synxenoderus* and *Haematosiphon* (Neoarctic region). It is generally accepted that multiple sex chromosome systems in Heteroptera are the result of fragmentation(s) of the X and/or Y chromosome(s) from an

ancestral simple system (Chirino et al. 2013 and see references cited therein). The main facts that support this hypothesis are the holocentric nature of heteropteran chromosomes and the achiasmate male meiosis of sex chromosomes (Ueshima 1979, Manna 1984, Thomas 1987). In the origin of a derived multiple sexual system, the increase in the number of sex chromosomes is not accompanied by a reduction in the number of autosomes, and the two X chromosomes are slightly different in size, making it unlikely to have arisen from aneuploidy, i.e. non-disjunction (Ueshima 1979, Papeschi 1994, 1996, Franco et al. 2006, Papeschi & Bressa 2006). Lastly, autosome fragmentation led to an increase in the number of autosomes to 2n= 40 and 2n= 42, as that observed in *Hesperocimex cochimiensis* Ryckman & Ueshima, 1963 and *H. sonorensis* Ryckman, 1958, respectively (Neoarctic region). In both *Hesperocimex* species, the single X chromosome represents a reversal from a multiple to a simple sex chromosome system. In *H. coloradensis* List, 1925, a further X chromosome fragmentation originated a derived $X_1X_2X_3Y$ multiple sex chromosome system (Neoarctic region).

In a recent study, Roth et al. (2019) constructed a molecular phylogeny of Cimicidae based on DNA sequences of four genes (*cytochrome c oxidase subunit I*, *16S rRNA*, the D3 region of *28S rRNA* and two segments of *18S rRNA*). The consensus tree showed: i) the monophyly of the Haematosiphoninae subfamily, ii) *Cyanolicimex patagonicus* placed at the base of Haematosiphoninae, and as a sister genus of the other species studied (*Synxenoderus comosus* List, 1925, *Psitticimex uritui*, *Haematosiphon inodorus* (Duges, 1892), *Cimexopis nyctalis* List, 1925, *Acanthocrios furnarii*. and *Ornithocoris pallidus* Usinger, 1959); iii) *A. furnarii* and *O. pallidus* grouped together in a cluster and *C. nyctalis* as its closest genus, and iv) *P. uritui* and *H. inodorus* grouped together in

a cluster and *S. comosus* as its closest genus. In the present study, we propose to place in four groups the species of Haematosiphoninae based on the morphological and behavioural traits, the primary birds' hosts, the biogeographical distribution, and the cytogenetic features,: i) *Ornithocoris toledoi*, *O. pallidus*, and *A. furnarii* (Neotropical Region); ii) *P. uritui* and *C. patagonicus* (Neotropical Region), iii) *S. comosus* and *H. inodorus* (Neoarctic Region); and iv) *Hesperocimex cochimiensis*, *H. sonorensis* and *H. coloradensis* (Neoarctic Region). According to this clustering, we allow proposing *Cyanolicimex* as the closest genus to *Psitticimex* from the remaining South American genera. Considering the molecular phylogeny (Roth et al. 2019) together with our results, we also cannot exclude to place *C. patagonicus*, *S. comosus*, *P. uritui*, and *H. inodorus* in one group if we do not take into account their geographical distribution. Thus, all these species of Haematosiphoninae would be placed in three instead of four groups. Future cytogenetic and molecular studies should include the genera *Alayocimex* Hernandez Triana & De la Cruz, 1994 and *Cimexopis List, 1925*, and *Alayocimex* and *Hesperocimex*, respectively. These studies will provide complementary evidence to infer the chromosomal evolution and interspecific relationships in the subfamily Haematosiphoninae.

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