Bed bug cytogenetics: karyotype, sex chromosome system, FISH mapping of 18S rDNA, and male meiosis in *Cimex lectularius* Linnaeus, 1758 (Heteroptera: Cimicidae)

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Abstract. Bugs (Insecta: Heteroptera) are frequently used as examples of unusual cytogenetic characters, and the family Cimicidae is one of most interest in this respect. We have performed a cytogenetic study of the common bed bug *Cimex lectularius* Linnaeus, 1758 using both classical (Schiff-Giemsa and AgNO₃-staining) and molecular cytogenetic techniques (base-specific DAPI/CMA₃ fluorochromes and FISH with an 18S rDNA probe). Males originated from a wild population of *C. lectularius* were found to have 2n = 26 + X₁X₂Y, holokinetic chromosomes, 18S rRNA genes located on the X₁ and Y chromosomes; achiasmate male meiosis of a collochore type; MI and MII plates nonradial and radial respectively.

Key words: holokinetic chromosomes, karyotype, multiple sex chromosomes, achiasmate collochore meiosis, FISH with an 18S rDNA probe, *Cimex lectularius*.

INTRODUCTION

The bed bug genus *Cimex* Linnaeus, 1758 is a relatively small group of highly specialized hematophagous ectoparasites, with 17 species distributed primarily across the Holarctic and associated with humans, bats, and birds (Schuh, Slater, 1995; Simov et al., 2006). Till now, the chromosomal complement and different aspects of chromosome behavior during male meiosis have been studied in 14 *Cimex* species (Ueshima, 1963, 1966; Grozeva, Nokkala, 2002; for other references see Ueshima, 1979), including the common bed bug *C. lectularius* Linnaeus 1758 (Slack, 1938, 1939a, b; Darlington, 1939; Ueshima, 1966). As is the case with other Heteroptera, *C. lectularius* displays holokinetic chromosomes, i.e. chromosomes having, instead of localized centromere, a kinetochore plate spread along their whole or almost whole length. Among several peculiarities of *Cimex* cytogenetics, multiple sex chromosome systems are the most conspicuous. Although, within Cimicidae, multiple sex chromosomes are not unique to the genus *Cimex* (some species in at least nine other Cimicidae genera have multiple X chromosomes; see Ueshima, 1979), the number of X is, by far, greatest in *C. lectularius*. In the latter, the number of autosomes is consistently 26, whereas the number of X chromosomes varies from two (X₁X₂Y) to 15 (X₁X₂Y + 13 extra Xs) in different populations and sometimes between males of one population. Autosomal bivalents show normal a sequence.
of meiotic divisions, with homologous chromosomes segregating in the first round of meiosis, and sister chromatids separating in the second. This order is however reversed in sex chromosomes, and this is true even where 15 Xs exist. Sex chromosomes always undergo post-reductional meiosis, i.e. the equational separation at anaphase I and the reductive segregation at anaphase II, the sex chromosome behavior typical of the Heteroptera. Autosomal bivalents are suggested to be chiasmate, with the single chiasma being formed in every bivalent (Ueshima, 1966, 1979).

All the published cytogenetic studies on *C. lectularius* have used a standard chromosome technique. In the present study, a wild North-Western Russian population of *C. lectularius* was analyzed using the standard Schiff-Giemsa technique, AgNO₃-staining, fluorochrome DAPI/CMA₃-banding, and fluorescent *in situ* hybridization (FISH) with an 18S rDNA probe. As a result, the karyotype, sex chromosome system, the distribution and nucleotide sequences of C-heterochromatin regions, chromosomal location of 18S rRNA genes, and meiosis in males are reported here.

**MATERIAL AND METHODS**

**Insects**

Adults and nymphs of *Cimex lectularius* were collected in 2009 from a wild population (St. Petersburg). A total of 6 males were studied. Specimens were fixed alive in 96% ethanol: glacial acetic acid (3:1) and stored in fixative at 4°C until further use.

**Preparations**

The gonads were dissected out and squashed in a drop of 45% acetic acid. The cover slip was removed by the dry ice method. Slides were dehydrated in fresh fixative and air dried. The preparations were first analyzed with a phase contrast microscope at 400x. The best 28 chromosome preparations were used for different staining techniques.

**Standard staining**

To study the number and behavior of chromosomes, the preparations were stained following the Schiff-Giemsa method developed by Grozeva, Nokkala (1996). The preparations were first subjected to hydrolysis in 1 N HCl at room temperature for 20 min, then in 1 N HCl at 60°C for 8 min, and stained in Schiff’s reagent for 20 min. After rinsing thoroughly in distilled water, the preparations were additionally stained in 4% Giemsa in Sorensen’s buffer, pH 6.8 for 20 min, rinsed with distilled water, air-dried, and mounted in Entellan.

**AgNO₃ -staining**

To check the localization of nucleolar organizer regions (NORs), the 1-step method with a protective colloidal developer (Howell, Black, 1980) was followed.

**Fluorochrome banding**

To reveal the base composition of C-heterochromatin, staining by GC-specific chromomycin A₃ (CMA₃) and AT-specific 4-6-diamidino-2-phenylindole (DAPI) were used according to Schweizer (1976), and Donlon, Magenis (1983) respectively, with some modifications. C-banding pretreatment was first carried out using 0.2 N HCl at room temperature for 30 min, followed by 7-8 min treatment in saturated Ba(OH)₂ at room temperature and then an incubation in 2x SSC at 60°C for 1 h. The preparations (without Giemsa) were stained first with CMA₃ (2,5 μg/ml) for 25 min and then with DAPI (0.4 μg/ml) for 5 min. After staining, the preparations were rinsed in the McIlvaine buffer, pH 7 and mounted in an antifade medium (700 μl of glycerol, 300 μl of 10 mM McIlvaine buffer, pH 7, and 10 mg of N-propyl gallate).
Fluorescence in situ hybridization (FISH)
DNA isolation, PCR amplification, probe generation

Genomic DNA from a male of *Pyrrhocoris apterus* L., 1758 (Heteroptera, Pyrrhocoridae) was isolated using a Chelex-100 extracted method. FISH was carried out on *C. lectularius* chromosomes using an 18S rDNA gene probe. The target 18S rRNA gene was PCR amplified from the genomic DNA of *P. apterus* using primers: 18S_R 5'-CGATACGCGAATGGCTCAAT-3', 18S_F 5'-ACAAGGGGCACGGACGTAATCAAC-3', and labeled by PCR with Biotin.

**FISH**

*In situ* hybridization was performed as described by Schwarzacher, Heslop-Harrison (2000) with modifications. Chromosome preparations were dehydrated through 70/80/96% ethanol at RT and treated with 100 μg/ml RNaseA (Sigma) for 60 min at 37°C in humid chamber; washed three times in 2x SSC (5 min each) at RT; dehydrated through 70/80/96% ethanol at RT; incubated in 5 mg/ml pepsin in 0.01 N HCl for 15 min at 37°C; washed sequentially in 1x PBS, in PBSx1/0.05M MgCl₂ for 5 min each, in 1% PFA in PBSx1/0.05M MgCl₂ for 10 min, in 1x PBS for 5 min, in PBSx1/0.05M MgCl₂ for 5 min at RT each; dehydrated through 70/80/96% ethanol at RT or at ice cold and finally dried. After pretreatment hybridization mixture containing about 100 ng of labeled probe, 50% formamide, 2×SSC, 10% (w/v) dextran sulfate, 1% (w/v) Tween-20 and 10 μg salmon-sperm DNA was added on preparations. Slides were mounted using glass coverslip and rubber cement. The slides were denatured for 5 min at 75°C. Then the chromosome slides were incubated for 42–44 h at 37°C. Following hybridization, the slides were washed in 2x SSC for 3 min at 45°C, then in 50% formamide in 2x SSC for 10 min at 45°C, twice in 2x SSC (10 min each), twice in 0.2x SSC (10 min each) at 45°C, blocked in 1.5% (w/v) BSA/4x SSC/0.1% Tween-20 for 30 min at 37°C in a humid chamber. 18S rRNA gene probes were detected with 5 μg/ml Avidin-Alexa Fluor 488 (Invitrogen). The detection reaction was performed in 1.5 % BSA/ 4x SSC/ 0.1% Tween-20 for 1 h at 37°C. Slides were washed three times in 4x SSC/ 0.02% Tween-20 (10 min each) at 45°C and dehydrated through 70/80/96% ethanol at RT. Chromosome preparations were mounted in an mounting-antifade (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip.

**Microscopy and imaging**

Chromosome preparations were analyzed under a Leica DM 4000B microscope (Leica Microsystems Wetzlar GmbH, Germany) with a 100x objective. Fluorescence images were taken with a Leica DFC 350 FX camera using Leica Application Suite 2.8.1 software with an Image Overlay module. The preparations were stored partly at the Institute of Biodiversity and Ecosystem Research, BAS in Sofia and partly at the Zoological Institute, RAS in St Petersburg.

**RESULTS**

We analyzed meiosis in males of *C. lectularius* using the standard chromosome technique, AgNO₃-staining, fluorochrome DAPI/CMA₃ staining and 18S rDNA FISH technique. Based primarily upon the behavior of chromosomes in the consecutive stages of meiosis, the chromosome complement in males was expressed as a formula 2n = 29 (26A + X₁X₂Y). Although females were not studied, sex chromosome mechanism was referred to a X₃X₄Y system (rather than XY₃Y₄) due to the Ueshima’s (1966) observations of several populations of *C. lectularius* in which females showed 4Xs (X₁X₂X₃X₄) while males 2Xs...
Bed bug cytogenetics: karyotype, sex chromosome system, FISH, male meiosis

The analysis of the chromosomes after FISH with an 18S rDNA probe as well after CMA3-staining evidenced the presence of the 18S rDNA loci on the X1 and Y chromosomes. This marker allowed the precise identification of the X1 and Y chromosomes to be made in mitotic cells (Figs 1-3) and at different stages of meiosis (Figs 4-9). The location of the NORs on the sex chromosomes was confirmed by the AgNO3-staining (Fig. 10).

The mitotic chromosomes gradually decrease in size and lack visible constrictions since they are holokinetic. Consequently, neither homologous chromosomes nor sex chromosomes are possible to identify in the routinely stained preparations (not shown). The X1 and Y chromosomes can be however easily detected both in metaphase and prometaphase cells after FISH with the 18S DNA probe (Figs 1-3). The majority of the mitotic cells show two chromosomes, most likely X1 and Y, with clusters of rRNA genes (Figs 1, 2); however, hybridization signals are well over two in some prometaphase cells (Fig. 3).

Among the meiotic stages, those referred to the prophase and early metaphase I (MI) are of chief interest for characterisation of male meiosis in C. lectularius. During prophase the chromosomes gradually condense from leptotene to pachytene, and no diplotene or diakinesis stages are observed. In the course of condensation, the homologues of bivalents open out, while remaining connected with each other, at least, at one site by tenacious thread-like structures (Figs 4, 5). By the metaphase I (MI), bivalents are completely condensed and consist of parallel-aligned chromosomes with occasionally one or both ends diverging, and no signs of the existence of chiasmata are seen during this stage (Figs 6-9). Taken together these observations are indicative of achiasmate meiosis, the connecting threads between homologues representing the colochores previously described in several Miridae species (Nokkala, Nokkala, 1986) and recently also in C. emarginatus Simov, Ivanova, Schunger, 2006 (as Cimex sp. in Grozeva, Nokkala, 2002).

At MI, 13 autosomal bivalents and 3 sex chromosome univalents are observed (Figs 7-9). MI plates are nonradial with X1, X2, and Y chromosomes located among the bivalents. The sex chromosomes are usually recognized at this stage because they are composed of two chromatids (contrary to 4 in autosomal bivalents). The autosomal bivalents are of gradually decreasing size with neither very large nor very small entities as do the autosomes at the mitotic metaphases. The sex chromosomes are similar in size to the larger half-bivalents, the X1 being clearly longer than the X2 while of similar size with the Y. Sex chromosomes, X1 and Y at least, tend to be located close to each other or even connected by ends probably due to the presence of

Figs 1-20. Different stages of male meiosis in C. lectularius after FISH with an 18S rDNA probe (1-9, 13, 18-20), AgNO3 (10), DAPI (11), CMA, (12), and Schiff-Giemsa (14-17). 1-3 - Spermatogonial metaphases. Arrows show X, and Y with signals, arrowheads point to additional signals in some plates; 4-6 - Prometaphase I. X, and Y with signals lie together (4) or separately (5, 6); 7-9 - Metaphase I. X, and Y with signals lie together or separately. Autosomal bivalents are condensed and consist of parallel-aligned chromosomes; 10 - Diffuse stage. Nucleolar proteins are localized on the sex chromatin body; 11-12 - A metaphase I plate. X, and Y with CMA, positive signals (12); 13 - Anaphase I. There are signals in both daughter cells; 14-17 - Metaphase II. Radial plates with sex chromosomes placed inside the ring formed by autosomes; 18-20 - Consecutive stages of sperm formation. Every sperm has a signal. Bar = 10μm.

Comp. Cytogenet., 2010 4(2)
telomere heterochromatin (Figs 7, 8). Among
the 26 MII cells examined, only two showed X1
and Y chromosomes lying separately (Fig. 9).

In two MII nuclei after DAPI and
CMA3-staining respectively, the autosomal
bivalents and one of the Xs (X2) display a
fairly homogenous CMA3/DAPI staining,
however at least two bivalents (arrowed)
show CMA3/DAPI positive, AT/GC rich
heterochromatic regions on the telomeres of
every homologue (Figs 11, 12). The other X
(X1) and the Y chromosome exhibit several
CMA3-bright bands, two-three on the X1 and
one-two on the Y, indicating they are GC-rich.
FISH experiments are in general agreement
with the results of CMA3-staining, i.e. the
18S rDNA loci are co-localized with some of
the CMA3-bands suggesting that these CMA3-
positive sites represent rDNA cistrons. X1
and Y chromosomes each show hybridization
signals indicating clusters of 18S rRNA genes
are located near the end of X1 and near the
end of Y chromosomes respectively (Figs 7-9).
Noteworthy is a size polymorphism for
the 18S rDNA clusters on X1 and Y, which is
occasionally observed even within the same
male (Figs 6-9).

The daughter metaphase II (MII) cells
each contain 16 chromosomes, including
13 autosomes, 2Xs, and the Y (Figs 14-17),
indicative of the reductional segregation of
autosomes and equational separation of sex
chromosomes at anaphase I (AI) (Fig. 13). At
MII, sex chromosomes undergo a characteristic
“touch-and-go” pairing, with 2Xs lying on one
side of the equatorial plate and the Y on the
other. In contrast to MI, metaphase II (MII)
plates are clearly radial, and sex chromosomes
appear here as a pseudotrivalent located inside
a ring formed by autosomes.

At anaphase II (AII), autosomes divide
equationally, but sex chromosomes undergo a
reductional division, i.e. both X chromosomes
moved to one pole and the Y to the other (the
so-called sex chromosome post-reduction) as
evidenced by the only 18S rDNA/FISH signal
in every daughter telophase II (TII) plate (Fig.
18) as well as in every spermatid and every
sperm (Figs 19, 20).

**DISCUSSION**

The common bed bug, *Cimex lectularius*,
is one of the most widely recognized insects
all over the world. Slack (1938) was the first to
study chromosome cytology of *C. lectularius*.
Shortly afterwards Darlington (1939) and
then Ueshima (1966, 1967, 1979) studied and
discussed the unique aspects of male meiosis
in this species. Based on the observations of
these authors, *C. lectularius* males have the
standard complement of 2n = 26 + X1Y,
the X chromosomes varying in number from
two (X1X2Y) to 15 (X1X2Y + 13 extra Xs)
in different populations while occasionally
between males of the same population and
even between cells of the same male. For
example, Ueshima (1966) has investigated
males and females in six populations originated
from USA (Berkeley, California; Columbus,
Ohio), Mexico (Monterey; La Piedad), Japan
(Nagasaki), France (Durtal), Egypt (Cairo),
and Czech Republic (Moravia) respectively.
The number of sex chromosomes was shown
to be stable within every population (2Xs in
Berkeley, La Piedad, Nagasaki, and Durtal;
6Xs in Cairo and Moravia) except for the Ohio
population, in which males had either 7Xs or
9Xs. Notice that the transmission of additional
sex chromosomes throughout meiosis was,
except in a very few cases, quite regular,
and they seemed not to be important for sex
determination.

Multiple (above two) X chromosomes have
been described in both natural populations and
laboratory stocks of *C. lectularius*. According
to Darlington (1939) the average number of Xs is higher in wild populations than in laboratory cultures, however a closer look at the presently available data on this species is called for.

The most common sex chromosome mechanism in the Heteroptera is XX/XY system, and the multiple sex chromosome systems are suggested to have originated from the original XY system (Ueshima, 1979). In the Cimicidae, a total of 45 species have been hitherto cytologically studied and both putative ancestral XY and a great number of derived multiple systems, among which X,Y clearly prevails, were found (Ueshima, 1966, 1979 and references therein; Manna, 1984; Grozeva, Nokkala, 2002; Poggio et al., 2009). This is also true for the genus *Cimex* in which as many as 14 of the 17 described species have been studied (Ueshima, 1963, 1966, 1967; Grozeva, Nokkala, 2002; for other references see Ueshima, 1979). The origin of multiple systems in the Heteroptera is usually ascribed to simple transverse dissociation (fission) of the original X chromosome (Schrader, 1947; Ueshima, 1966, 1979), the process which is facilitated by the holokinetic nature of the bugs’ chromosomes. The distinguishing features of multiple sex chromosome systems formed by dissociation are that the newly originated sex chromosomes are smaller than the original ones, and there is no accompanying change in the number of autosomes in a derived complement compared to that with XY sex chromosome system. Ueshima (1966) has discovered XY, X,Y, and X, X,Y systems in 7 species of the *Cimex pilosellus* (Horváth, 1910) complex and argued for the dissociation hypothesis on the basis that as the number of X chromosomes increases, their size decreases. However this problem clearly calls for further investigation using modern cytological techniques. It is noteworthy that the application of C-banding to study the chromosomes of several Triatominae (Reduviidae) species, led Panzera et al. (2010) to the conclusion that chromosomal rearrangements other than dissociations might have been involved in the formation of the multiple sex chromosome systems in the Heteroptera.

As is typical in the Heteroptera, chromosomes of *C. lectularius* are holokinetic, i.e. without localized centromere. In males studied herein meiosis follows a standard for the Heteroptera pattern as previously described in other populations of this species (Slack, 1939b; Darlington, 1939; Ueshima, 1966, 1979). The sex chromosomes behave as univalents during the first round of meiosis and undergo equational separation at anaphase I. At metaphase II, the Xs and Y appear associated end-to-end to form a pseudotrivalent, which is located inside the ring of autosomes. During anaphase II, Xs and Y chromosomes undergo reductional division segregating to opposite poles. This reversed order of sex chromosome behavior during meiotic divisions is characteristic of the Heteroptera, referred to as inverted meiosis or sex chromosome post-reduction (Hughes-Schrader, Schrader, 1961).

At present, the cytogenetic studies of the Heteroptera are mainly focused on C-heterochromatin and nucleolus organizer regions (NORs), whereas molecular cytogenetic techniques such as immunofluorescence ones and different modifications of FISH (fluorescent *in situ* hybridization), including chromosome painting, BAC-FISH technique, and GISH/FISH mapping of genes, are not yet used in the Heteroptera. By now, some of these techniques have been applied to some economically important holokinetic insects (aphids *Acyrthosiphon pisum* (Harris, 1776); cabbage moth *Mamestra brassica* (Linnaeus, 1758); silkworm *Bombyx mori* (Linnaeus, 1758)) and have provided useful insight into
the understanding of their genome constitution (Mandrioli et al. 2003; Yoshido et al., 2005; Mandrioli, Borsatti, 2007; Marec et al., 2010).

The base-specific fluorochromes DAPI and CMA₃ are currently widely applied in cytogenetic studies of the Heteroptera to get information about the distribution of the AT and GC repeats along the chromosomes. In most heteropteran species the only pair of NOR-bearing chromosomes has been detected by different techniques such as fluorochrome banding, Ag-NOR staining or occasionally FISH with an rDNA probe. The location of NORs is however variable. In different species, the NORs are revealed on autosomes or on sex chromosomes (see reviews by Papeschi and Bressa, 2006a, b) but occasionally they occur on both sex chromosomes and autosomes of a species (Morielle-Souza, Azeredo-Oliveira, 2007; Bressa et al., 2008).

In our study of C. lectularius, the two CMA₃-positive GC-rich bands were discovered at the telomeres of sex chromosomes (X₁ and Y). NORs are known to be largely GC-rich in the Heteroptera (Grozeva et al., 2004; Papeschi, Bressa, 2006a, b; Kuznetsova et al., 2007), and CMA₃+/DAPI- regions of C. lectularius were therefore interpreted as the sites of NORs, and FISH with the 18S rDNA probe confirmed this assessment. As expected, FISH/rDNA signals were observed in every spermatid, confirming thus the location of NORs on one of the X chromosomes (X₁) and on the Y chromosome, which underwent a reductional division at anaphase II.

Ueshima (1966, 1979) has argued for chiasmate meiosis in C. lectularius with one chiasma being formed in every bivalent. However, our study did not support this generalization suggesting the occurrence of achiasmate meiosis in C. lectularius, and this pattern seems to be characteristic of the family Cimicidae as a whole (Grozeva, Nokkala, 2002; Poggio et al., 2009). Strong support for this suggestion is known to come from the absence of diplotene and diakinesis stages (Nokkala, Nokkala, 1986) and such is the case in C. lectularius. According to our observations, males of C. lectularius follow achiasmate meiosis of the specific collochore type. In this meiosis there are no chiasmata; after synopsis, the opening-out of bivalents takes place, and tenacious threads, the so-called collochores, are formed to hold homologous chromosomes together in the absence of chiasmata. The collochore meiosis was first described in Drosophila melanogaster Meigen, 1830 (Cooper, 1964), thereafter in four Miridae species (Nokkala, Nokkala, 1986) and quite recently in Cimex emarginatus (as Cimex sp. in Grozeva, Nokkala, 2002) and some other Cimicidae species (Poggio et al., 2009). One further type of achiasmate meiosis known in the Heteroptera is the alignment type. In this meiosis, homologous chromosomes are held together along all their length during prophase, this alignment remaining unchanged till the beginning of anaphase I (Nokkala, Nokkala, 1983, 1984; Nokkala, Grozeva, 2000; Grozeva et al., 2008). Furthermore, a pattern intermediate between collochore and alignment meioses has been quite recently described in Arachnocoris trinitatus Bergroth, 1916 from the Nabidae (Kuznetsova et al., 2007; Kuznetsova, Grozeva, 2008), the cimicomorphan family with primarily meiosis of the alignment type (Nokkala, Nokkala, 1984; Kuznetsova, Maryańska-Nadachowska, 2000; Kuznetsova et al., 2004).

The form of metaphase I and metaphase II plates in C. lectularius is noteworthy, and this is the pattern which seems to be species-specific in the Heteroptera (Ueshima, 1979). In this species, the metaphase I plates were found to be nonradial but metaphase II plates radial.
It is interesting to note in this connection that in another Cimicidae species, *Psiticipimex uritui* Lent, Abalos, 1946, both MI and MII plates seem to be radial (Poggio et al., 2009: Figs 2b, c).

**CONCLUSIONS**

The results of the present study support the following assertions: (1) *Cimex lectularius* displays holokinetic chromosomes; (2) male karyotype includes 2n = 26 + X1X2Y; (3) 18S rRNA genes are located on the X1 and Y chromosomes; (4) males have achiasmic meiosis of the collochore type; (5) sex chromosomes undergo post-reductional meiosis, i.e. the equational separation at AI and the reductive segregation at AII; (6) MI plates are radial and MII plates are nonradial.

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