Folia biologica (Kraków), vol. **52** (2004), No 3-4

Holocentric Chromosomes of Psocids (Insecta, Psocoptera) Analysed by C-banding, Silver Impregnation and Sequence Specific Fluorochromes CMA_3 and DAPI*

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Accepted September 6, 2004

GOLUB N. V., NOKKALA S., KUZNETSOVA V. G. 2004. Holocentric chromosomes of psocids (Insecta, Psocoptera) analysed by C-banding, silver impregnation and sequence specific fluorochromes CMA_3 and DAPI. Folia biol. (Kraków) **52**: 143-149.

The pattern of nucleolus attachment and C-heterochromatin distribution and molecular composition in the karyotypes of psocid species *Psococerastis gibbosa* (2n = 16+X), *Blaste conspurcata* (2n = 16+X) and *Amphipsocus japonicus* (2n = 14+neo-XY) were studied by C-banding, silver impregnation and sequence specific fluorochromes CMA_3 and DAPI. Every species was found to have a single nucleolus in male meiosis. In *P. gibbosa* the nucleolus is attached to an autosomal bivalent; in *B. conspurcata* to the X-chromosome; in *A. japonicus* to the neo-XY bivalent. The species show a rather small amount of constitutive heterochromatin, C-blocks demonstrating telomeric localization with rare exceptions. *P. gibbosa* is characterized by a polymorphism for C-blocks occurrence and distribution. In the autosomes of this species, C-heterochromatin consists of AT-rich DNA except for the nucleolus organizing region, which is also GC-rich; the X-chromosome shows both AT- and GC-rich clusters. In *A. japonicus* and *B. conspurcata*, C-heterochromatin of the autosomes and sex chromosomes consists of both GC-rich and AT-rich DNA clusters, which are largely co-localized.

Key words: Psocoptera, karyotypes, heterochromatin, nucleolus, CMA_3- and DAPI-positive sites.

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Considerable attention is presently focused on applying modern cytogenetic techniques to study holocentric chromosomes. $AgNO_3$-banding, C-banding, sequence specific fluorochrome staining, and fluorescence *in situ* hybridization (FISH) are used most extensively. By now, the majority of higher insect groups characterized by this chromosome type have been studied to a variable extent, among them Heteroptera (CAMACHO *et al*. 1985; REBAGLIATI *et al.* 2003; GROZEVA & NOKKALA 2003; GROZEVA *et al.* 2004), Aphidoidea (BLACKMAN 1990; KUZNETSOVA & GANDRABUR 1991; MANICARDI *et al.* 1996; MANDRIOLI *et al.* 1999; BIZZARO *et al.* 1999; HALES *et al.* 2000), Psylloidea (KUZNETSOVA *et al.* 1997; MARYAŃSKA-NADACHOWSKA *et al.* 2001), Coccoidea (FERRARO *et al.* 1998; NECHAYEVA *et al.* 2004), Auchenorrhyncha (PEREPELOV *et al.* 2002; KUZNETSOVA *et al.* 2003), Psocoptera (GOLUB & NOKKALA 2001), Odonata (PRASAD & THOMAS 1992; PEREPELOV *et al.* 1998, 2001) and Lepidoptera (REGO & MAREC 2003). Despite a large amount of publications, very little is yet known about the molecular composition of holocentric chromosomes.

The order Psocoptera is characterized by holocentric chromosomes. All cytogenetic studies on psocids with the exception of GOLUB and NOKKALA (2001) have been so far carried out using

*Supported by the Russian Academy of Sciences, Academy of Finland, by a scholarship of the Centre for International Mobility CIMO, and the Russian Foundation for Basic Research (No. 02-04-48649).

conventional staining techniques. GOLUB and NOKKALA (2001) reported the first data on C-heterochromatin occurrence and location in the karyotype of *Amphipsocus japonicus* (Amphipsocidae). In order to characterize psocid chromosomes in more details, C-banding, $AgNO_3$-banding, and sequence specific fluorochrome staining (CMA_3- and DAPI- banding) was used on spread meiotic chromosomes of *Amphipsocus japonicus*, *Psococerastis gibbosa* and *Blaste conspurcata*.

Material and Methods

Adult males of *Blaste conspurcata* (Rambur) (Psocidae) were collected from *Betula verrucosa* during July 2003 in the St. Petersburg area (Russia). Adult males of *Psococerastis gibbosa* (Sulzer) (Psocidae) were collected from natural populations inhabiting foliage and coniferous trees during July – August 1997 in the St. Petersburg area and during August – September 2003 in Kustavi (Finland). Adult males of *Amphipsocus japonicus* (Enderlein) (Amphipsocidae) were collected from natural populations inhabiting coniferous trees during August 2000 in Ussuriyski Krai, Gornotaezhnoe (Russia). All specimens were fixed in an alcohol / acetic acid mixture (3 : 1) and kept refrigerated at +4°C. Testes were dissected from abdomens and squashed on slides in a drop of 45% acetic acid. Coverslips were removed by the dry-ice technique (CONGER & FAIRCHILD 1953). Afterwards the slides were dehydrated in a fresh fixative solution and air-dried.

Ag-staining was performed according to the following techniques: for *P. gibbosa* and *A. japonicus* slides were incubated in borate buffer (pH 9.0-9.5) for 20-30 min at room temperature, subsequently incubated with 50% $AgNO_3$ and 1% gelatine developer during 3-6 min at 65°C, air-dried and mounted in Entellan. Slides of *B. conspurcata* were rinsed in borate buffer pH 9.5 for 20 min at room temperature, incubated with 50% $AgNO_3$ for 60 min at 65°C, thoroughly rinsed in water, treated by 5% formaline during 5 min at room temperature, and incubated with 50% $AgNO_3$ with a few drops of 5% formaline developer.

For C-banding the technique of SUMNER (1972) with modifications was used. In brief, slides were treated in 0.2 N HCl at room temperature for 20 min, rinsed in distilled water and transferred into saturated solution of $Ba(OH)_2$ x $8H_2O$ at room temperature for 4-6 min. After this, slides were rinsed in distilled water and treated by a 2xSSC solution with a small amount of EDTA added at 60°C for 1 h. Then slides were rinsed in distilled water and Sorensen's phosphate buffer (pH 6.8), and stained with 5% Giemsa solution.

For fluorochrome staining the preparations were pre-treated by C-banding (as described above). Then slides were stained with the GC-specific fluorochrome chromomycin A_3 (CMA_3) according to SCHWEIZER (1976) and with AT-specific fluorochrome DAPI (4'- 6 - diamidino-2-phenylindole) following the technique of DONLON and MAGENIS (1983) with small modifications. In brief, after 2xSSC treatment slides were subsequently rinsed in distilled water and in 10 M McIlvaine buffer (pH 4.9). Afterwards slides were immersed in Methyl Green solution in McIlvaine buffer (pH 4.9) for 6 min, and rinsed in McIlvaine buffer first at pH 4.9 and then at pH 7.0. After this, slides were stained with DAPI at a final concentration of 0.4 µg/ml in 10 mM McIlvaine buffer (pH 7.0) for 5 min, rinsed in buffer and stained with CMA_3 at a final concentration of 5 µg/ml in 10 mM McIlvaine buffer at pH 7.0 (25 µl of 96% ethanol was added to 500 µl of final staining solution) for 30 min. After staining, slides were rinsed in distilled water and mounted in the anti-fade medium (70% glycerol, 10 mM McIlvaine buffer at pH 7.0, 1% N-propyl gallate).

C- and $AgNO_3$-banded slides were analyzed using an Olympus BX 51 light microscope with an Olympus C-35 AD-4 camera; fluorochrome labeled slides were studied using a fluorescence microscope Dialux 22.

Results

Blaste conspurcata Rambur (Psocidae), 2n=16+X

The meiotic complement of males comprised eight pairs of autosomes and a univalent X chromosome (Fig. 1a) corresponding to earlier observations of this species (GOLUB *et al*. 1996; GOLUB 1999).

In C-banded preparations the X-chromosome and the eight bivalents displayed blocks of constitutive heterochromatin (Figs 1a-c, 3). The largest blocks were present in the X-chromosome and in one autosomal bivalent. C-blocks were confined to the telomeric regions of the chromosomes, however some bivalents showed also several tiny interstitial blocks.

In the studied population one male showed a pair of B-chromosomes. In this male B-chromosomes were easily distinguished from the other chromosomes since they carried no heterochromatic segments (Fig. 1b-c).

After $AgNO_3$-staining, a single nucleolus attached to the X-chromosome was found in every cell (Fig. 1d).

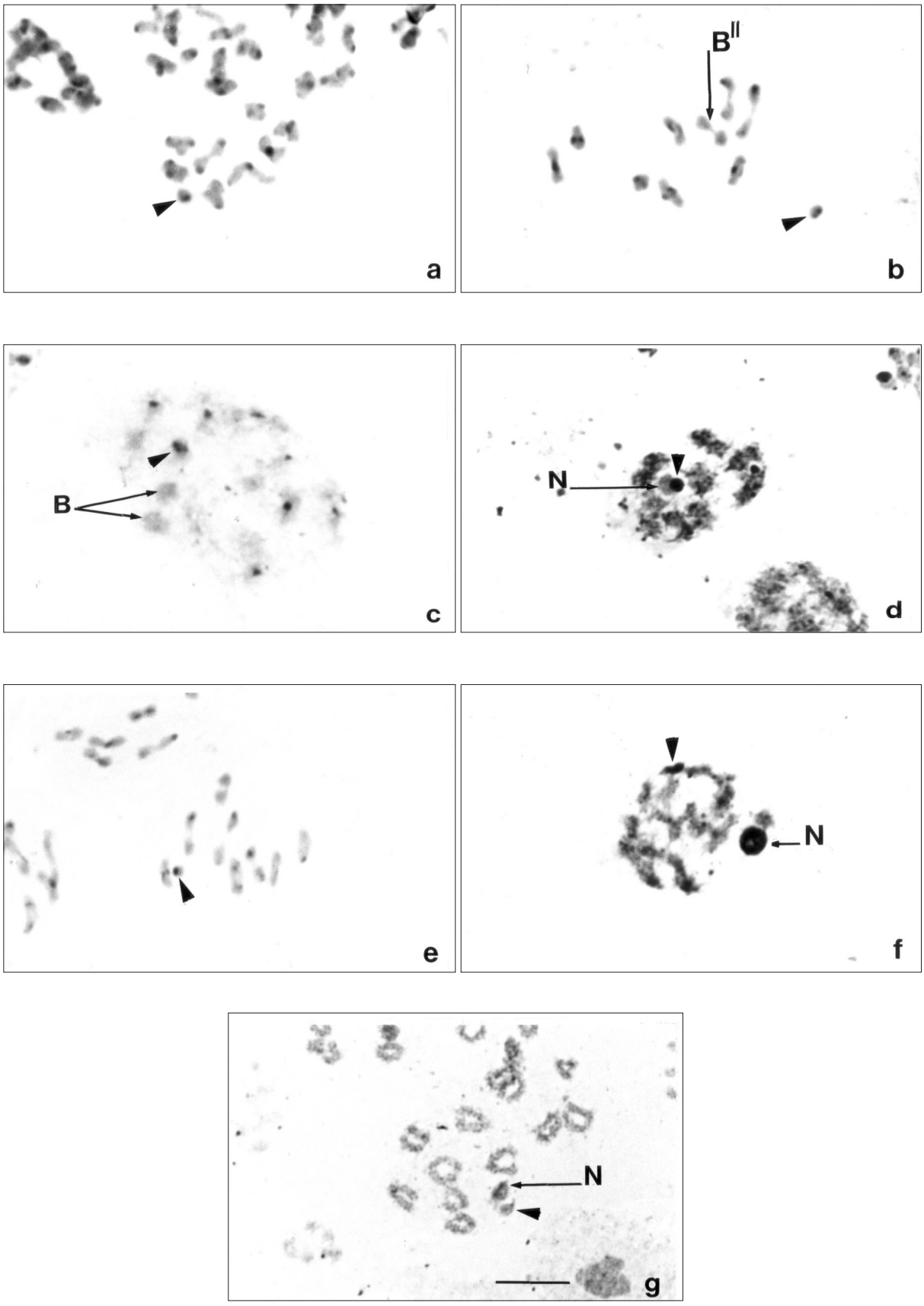

Fig. 1 a-g. Differential staining of psocid meiotic chromosomes. a-d – *Blaste conspurcata*: a-c – C-banding in an individual with standard complement n=8+X (a), and in an individual with B-chromosomes (b,c), d – $AgNO_3$- banding of prophase cell; e-f – *Psococerastis gibbosa*: e – C-banding, f – $AgNO_3$- banding of prophase cell; g – *Amphipsocus japonicus* $AgNO_3$-banding of prophase cell. X-chromosome (a-f) and neo-XY bivalent (g) are marked by arrowheads, B – B-chromosomes, N – nucleolus remnants. Bar = 5 μm.

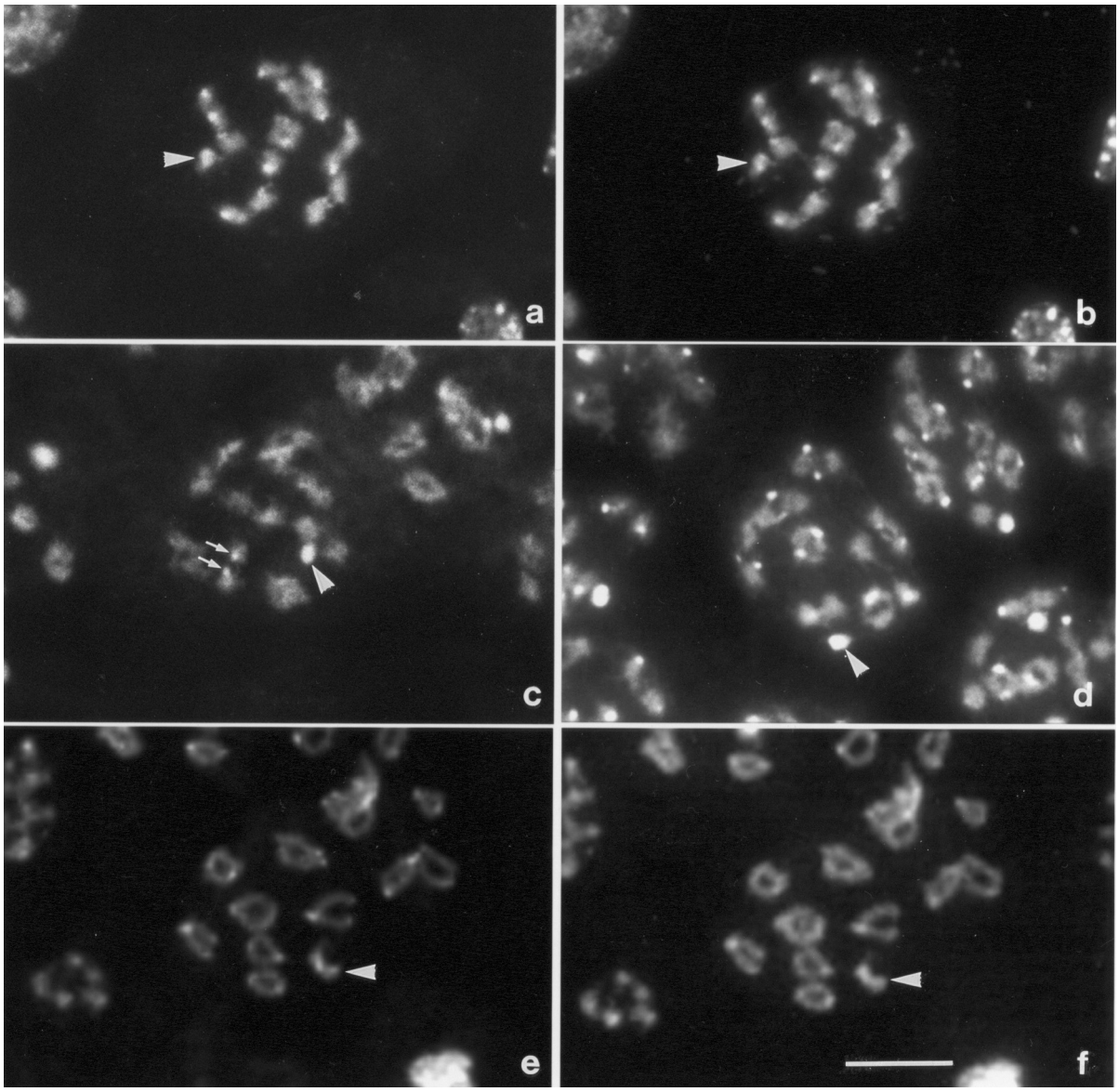

Fig. 2. Fluorescent CMA_3- and DAPI – staining of psocid meiotic chromosomes. a-b – *Blaste conspurcata*: a – CMA_3, b – DAPI; c-d – *Psococerastis gibbosa*: c – CMA_3, chromomycin – positive blocks are arrowed; d – DAPI; e-f – *Amphipsocus japonicus*: e – CMA_3, f – DAPI. X-chromosome (a-d) and neo-XY bivalent (e-f) are marked by arrowheads. Bar = 5 μm.

CMA_3- and DAPI-banding revealed bright signals in the telomeric regions of every autosomal bivalent and of the X-chromosome (Figs 2a-b). The comparison of two different banding patterns showed that CMA_3-positive and DAPI-positive regions of all chromosomes were co-localized in the majority of cases. B-chromosomes had no positive fluorescent signals.

Psococerastis gibbosa Sulzer (Psocidae), 2n=16+X

The meiotic complement of males comprised eight pairs of autosomes and a univalent X chromosome (Fig. 1e) corresponding to earlier observations of this species (GOLUB *et al.* 1996; GOLUB 1999).

After C-banding, the X-chromosome and 7 of 8 bivalents displayed blocks of constitutive heterochromatin (Fig. 1e). In 6 bivalents C-blocks were located in telomeric regions, while in one bivalent no telomeric blocks were found, however, one intercalary block was observed in each homologue (Fig. 3). The intercalary blocks were smaller as compared to the telomeric ones; the X-chromosome showed the largest C-block. In the first meiotic prophase telomeric blocks could be observed either on the "chiasmatic" or on the "kinetic" chromosomal ends. In the majority of cells, up to 4 autosomal bivalents displayed a heterochromatic block only in one of the homologous chromosomes.

After $AgNO_3$-staining, a single nucleolus attached to one of the largest autosomal bivalents

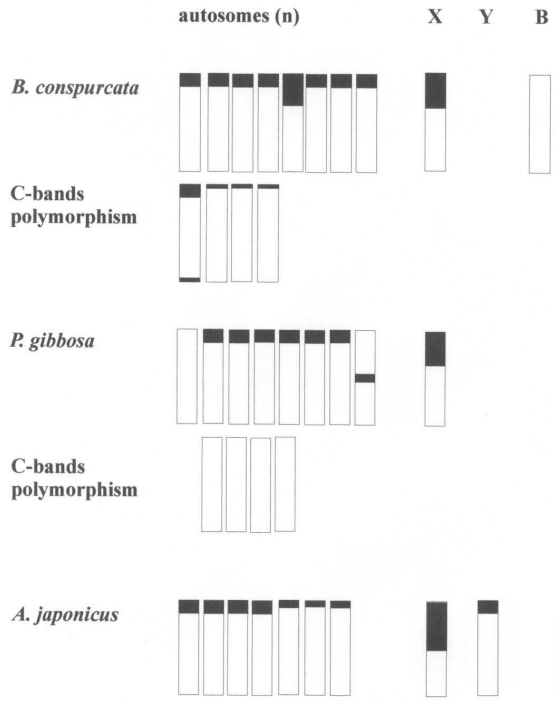

Fig. 3. Ideograms illustrating C-banding patterns in *B. conspurcata, P. gibbosa* and *A. japonicus*.

was revealed in every cell; the X-chromosome was strongly impregnated as compared to the autosomes (Fig. 1f).

After fluorochrome staining, terminally located DAPI-positive signals in 6 autosomal bivalents, sub-median signals in one autosomal bivalent and bright signals in the X-chromosome were observed (Fig. 2d). In some bivalents the signals were present in one of the homologues. CMA_3-positive signals were found only in one autosomal bivalent and in the X-chromosome (Fig. 2c).

Amphipsocus japonicus End. (Amphipsocidae), 2n=14+neo-XY

The meiotic complement of males comprised seven pairs of autosomes and sex chromosomes, which are of the neo-XY type (Fig. 1g) corresponding to earlier observations of this species (GOLUB & NOKKALA 2001).

In the $AgNO_3$-stained karyotype, a single nucleolus associated with the neo-XY bivalent was revealed in every cell (Fig. 1g).

After fluorochrome staining, CMA_3- and DAPI-positive signals were observed in the telomeric regions of every autosomal bivalent and of the sex chromosomes (Figs 2e-f, 3). The blocks were largely co-localized in the chromosomes. In the autosomal bivalents DAPI-blocks were smaller as compared to CMA-blocks; in contrast CMA-blocks in sex chromosomes were smaller than DAPI-blocks.

Discussion

C-banding, AgNOR-banding and sequence specific fluorochromes DAPI and CMA_3 were used to study holocentric chromosomes of three psocid species, *Amphipsocus japonicus* (Amphipsocidae), *Psococerastis gibbosa* and *Blaste conspurcata* (Psocidae). All the techniques, excepting for C-banding recently used in a cytogenetic study of *A. japonicus* (GOLUB & NOKKALA 2001), were applied for the first time to psocid chromosomes. Important information was provided on the pattern of nucleolus attachment and distribution and molecular composition of constitutive heterochromatin in Psocoptera karyotypes. Using $AgNO_3$-staining, a single nucleolus was found in all three studied species, however the location of the NOR was different. In *A. japonicus* and *B. conspurcata* the nucleolus is attached to the sex chromosomes, while in *P. gibbosa* – to one of the largest autosomal bivalents. Further investigations are needed in order to infer the ancestral pattern of NOR attachment in Psocoptera: whether the NOR rRNA-sequences

were transmitted to sex chromosomes from the autosomes or vice versa in the evolution of the group.

In the closest relatives of Psocoptera in Aphidoidea, the nucleolus is housed in the sex chromosomes of all studied species (KUZNETSOVA & GANDRABUR 1991; MANICARDI & GAUTAM 1994). Such a pattern probably represents an autapomorphy of the group within Sternorrhyncha, since in the three representatives of Coccoidea from the same homopteran subdivision so far studied have NOR's associated with autosomes (FERRARO *et al*. 1998; COOK 2001; NECHAYEVA *et al*. 2004). In Psylloidea and Auchenorrhyncha the nucleolus is likewise housed in the autosomes, however only a few species have been studied in these groups (MARYAŃSKA-NADACHOWSKA *et al.* 1992; KUZNETSOVA *et al.* 2003). In Heteroptera, as in Psocoptera, the nucleolus may be attached both to autosomes and to the sex chromosomes in different species (CAMACHO *et al*. 1985; REBAGLIATI *et al*. 2003; GROZEVA *et al*. 2004).

In all three psocid species, the amount of constitutive heterochromatin revealed by C-banding is relatively small, and is consistent with the general characteristics of holocentric chromosomes (BLACKMAN 1985). Only a few deviating examples are known, among which the aphid tribe Tramini is the most distinguished (BLACKMAN 1990). Some specialised chromosomes, such as the sex chromosomes, tend to show the greatest amount of C-heterochromatin (CAMACHO *et al*. 1985; PAPESCHI 1988; MANICARDI & GAUTAM 1994; GROZEVA *et al.* 2004) and this seems to be true for psocopterans as evidenced by the species studied (GOLUB & NOKKALA 2001; present paper).

C-blocks are locate in telomeres in all autosomes of *B. conspurcata* and *A. japonicus*, and in all but one autosomal pairs of *P. gibbosa*. This pattern has been repeatedly described in different groups and is a characteristic property of holocentric chromosomes. In monocentric chromosomes, heterochromatic regions are typically confined to the centromeres and are much less common in telomeric regions (SCHWEIZER & LOIDL 1987). An intercalary location of C-bands, as in the one autosomal pair of *P. gibbosa*, seldom occurs in holocentric chromosomes. Chromosomes in bivalents of the studied psocid species contained telomeric blocks both at their "chiasmatic" and "kinetic" ends providing clear evidence for the absence of terminal chiasmata in Psocoptera bivalents.

In species with holocentric chromosomes without centromeres, individual chromosomes can be detected only by size differences, when present, or based on markers in some cytogenetic techniques. In *B. conspurcata* one of the larger autosomal pairs is easy to distinguish because of a large block of heterochromatin which always occurs at the chiasmatic ends of the chromosomes. In *P. gibbosa* two autosomal pairs are easily distinguishable, since they exceptionally do not possess C-blocks or an intercalary C-block.

P. gibbosa and *B. conspurcata* are polymorphic for size and number of C-blocks. Polymorphism for the occurrence of C-blocks and their distribution has been repeatedly reported in different insect groups, including those with holocentric chromosomes (PANZERA *et al*. 1992; MANICARDI & GAUTAM 1994; GROZEVA & NOKKALA 2003). However, in *P. gibbosa* polymorphism for C-bands is more impressive than in all other species. In this species up to 4 bivalents possessed C-blocks only in one homologue, which suggests a high level of polymorphism in the studied populations.

For detecting different classes of C-heterochromatin sequence specific fluorochromes CMA_3 and DAPI were applied, possessing affinity to GC-rich DNA and to AT-rich DNA, respectively. In *P. gibbosa* C-heterochromatin was found to consist mostly of AT-rich sequences. CMA_3-positive signals were observed, demonstrating GC-clusters only in the X-chromosome and in one of the autosomal bivalents. However, a different pattern appeared in *B. conspurcata* and *A. japonicus*. In these species CMA_3- and DAPI-positive signals were observed in the same loci in the chromosomes suggesting that C-bands are both GC-rich and AT-rich. However, in autosomal bivalents of *A. japonicus* DAPI-blocks were smaller as compared with CMA_3-blocks, whereas in *B. conspurcata* the opposite situation occurred. This is probably associated with a different content of AT-rich and GC-rich DNA in C-bands of this species.

It is known that CMA_3-bright segments in the chromosomes correspond to the clusters of rRNA (NORs) in the majority of insects, including those with such as Auchenorrhyncha (KUZNETSOVA *et al.* 2003), Aphidoidea (MANICARDI *et al.* 1996; MANDRIOLI *et al*. 1999; BIZZARO *et al.* 1999), Coccoidea (NECHAYEVA *et al.* 2004), and Heteroptera (GROZEVA *et al*. 2004). In *P. gibbosa* the observed CMA_3-bright blocks in the autosomal bivalent correspond to the NOR as evidenced by $AgNO_3$-staining. However, in *B. conspurcata* and *A. japonicus* bright segments produced by CMA_3 appeared in all autosomal bivalents and in the sex chromosomes, whereas in every species a single nucleolus attached to the sex chromosomes was revealed after $AgNO_3$-staining. Thus, only the bands in the sex chromosomes are indicative of NORs, while other bands are GC-rich regions other than NORs. This finding confirms the previous obser-

vations that not all chromomycin positive segments are the sites of rRNA cistrons (SCHWEIZER *et al*. 1983; NECHAYEVA *et al*. 2004).

Acknowledgement

We thank Dr. Michail MANDELSHTAM for collecting *B. conspurcata* used in our study.

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