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The structural peculiarities of karyotypes in species of *Propsilocerus akamusi* sibling group (Diptera: Chironomidae)

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Abstract. First results of the study of karyotype structure, centromeric and pericentromeric heterochromatin organization in the sibling species *P. akamusi* Tokunaga and *P. jacuticus* Zvereva are presented. Karyotypes of these species have an unusual structure: the centromeric region of each chromosome has 3 large heterochromatic bands of similar morphology. To identify true centromeric bands, C-banding of polytene chromosomes of salivary glands had been performed. Only the central of three heterochromatic bands in each chromosome in both species showed clear C-bands and could be identified as centromeric bands. Staining of adjacent heterochromatic bands was weak. Microdissection of centromeric and adjacent heterochromatic bands of chromosomes II and III of *P. akamusi* was performed, and DNA-probes were generated by DOP-PCR. DNA-probes were hybridized to polytene chromosomes of *P. akamusi* using the fluorescent *in situ* hybridization technique (FISH). All probes showed high homology to the site from which they were originated, and low or no homology to other centromeric and adjacent heterochromatic bands both within the same chromosome and the other chromosomes. No signals were detected outside of the centromeric regions. Centromeric DNA probe from chromosome II showed low homology to centromeric regions of chromosomes III, meaning that the centromeric regions of *P. akamusi* have complex structure, and composition of centromeric DNA differs in different chromosomes. No signals had been observed from hybridization of *P. akamusi* DNA-probes with chromosomes of *P. jacuticus*. So, it is possible to suggest that centromeric DNA is strongly divergent between *Propsilocerus* siblings.

Key words: *Propsilocerus akamusi*, *Propsilocerus jacuticus*, centromere, heterochromatin, karyotype.

Introduction

At present, the study of karyotype and chromosomal polymorphism in *Propsilocerus* Kieffer, 1923 is in its infancy. The first data on the chromosome number of *P. akamusi* Tokunaga, 1938 (= *Tokunagayusurica akamusi*) from a Japanese population appeared in 1980 (Yanagida, Masuda, 1980). It was shown that this species has the haploid chromosome number n = 3, a typical number for Orthocladiinae (Michailova, 1989). Later, the polytene chromosome banding pattern was studied in *P. akamusi*, *P. jacuticus* Zvereva, 1950, and *P. paradoxus* (Lundström, 1915) (Kiknadze et al., 2004, 2005; Petrova et al., 2003). All species have n = 3, but their karyotypes were clearly differentiated in banding sequences. The *P.*

akamusi group of sibling species, which includes *P. akamusi*, *P. jacuticus*, and *P. taihuensis* (Wen, Zhou, Rong), was described by morphological characters (Saether, Wang, 1996). Further karyological data supported the taxonomic conclusion (Kiknadze et al., 2005). The banding patterns in all species of *P. akamusi* group have a high level of homology. Their karyotypes differ by a few fixed inversions. The presence of huge heterochromatic bands in the centromeric regions of all three chromosomes of karyotypes is a typical character of *P. akamusi* group.

The paper represents a detailed cytogenetic analysis of banding sequences divergence in sibling species, *P. akamusi* and *P. jacuticus*. The additional investigations using differential C-staining, microdissection and FISH-technique were carried out to understand the molecular organization of centromere region. Chromosome polymorphism was not found in both species.

Material and methods

Thirty forth-instar larvae of *P. akamusi* were collected in the Chaobai River, Tianjin City, China, 04.IX.2003. Twenty five larvae of *P jacuticus* were collected in the Ob River, Novosibirsk Prov. near Akademgorodok, Russia, 12.V.2004 and one hundred larvae – in the Yaya River, Kemerovo Prov. near village Yaya, Russia, 20.V.2004.

The material was fixed in 3 : 1 mixture of absolute ethanol and glacial acetic acid. Isolated salivary glands were squashed for polytene chromosome preparations. The larval salivary gland nuclei with highest level of polyteny were used for the study of polytene chromosome banding sequences and for the preparation of chromosome map. A routine aceto-orcein method of squashes was used (Keyl, Keyl, 1959; Kiknadze et al., 1991). The mapping of polytene chromosome banding sequences was done according to a conventional one (Petrova et al. , 2003).

The C-banding technique was performed according to Sigareva (1981). The squashes were put in saturated solution of $Ba(OH)_2$ at room temperature for 15 min, rinsed four times in distilled water, incubated in 2xSSC at 60º C for 2 hr, rinsed twice in distilled water and air-dried. Giemsa solution (4%) in standard phosphate buffer with pH 6.5-7.0 was used to stain air-dried squashes. The slides were mounted in Canada balsam.

Microdissection of heterochromatic bands from centromeric regions of polytene chromosomes was carried out in the Centre of Microscopic Analysis of Institute of Cytology and Genetics under the inverted microscope Axiovert 10.

The generation of DNA probes from microdissected polytene chromosomes followed by DOP-PCR has been described previously (Rubtsov et al., 1996). The DNA fragments were labeled with biotin-11-dUTP or digoxigenin-11-dUTP over 15 additional PCR cycles.

Fluorescent *in situ* hybridization (FISH) was performed according to a standard protocol with salmon sperm DNA as a carrier DNA, as described by Rubtsov et al., 1996. Biotin- and digoxigenin-labelled probes were visualized with avidin-FITC and mouse antidigoxigenin antibodies conjugated to Cy3, respectively. Two-color FISH of microdissected probes on polytene chromosomes of *P. akamusi* and *P. jacuticus* was performed. Chromosomes were counterstained with 4,6-diamino-2-phenylindole (DAPI) and analyzed using an Axioskop 2 plus epifluorescent microscope (Carl Zeiss, Germany) equipped with CCD camera, CHROMA filter set and ISIS4 image-processing package (MetaSystems-Group, Inc., USA).

Results

Karyotype of *P. akamusi* (n=3)

Three polytene chromosomes of approximately equal lengths are present in salivary gland cells (Fig. 1). Chromosomes I and II are metacentric, and chromosome III is submetacentric. Petrova

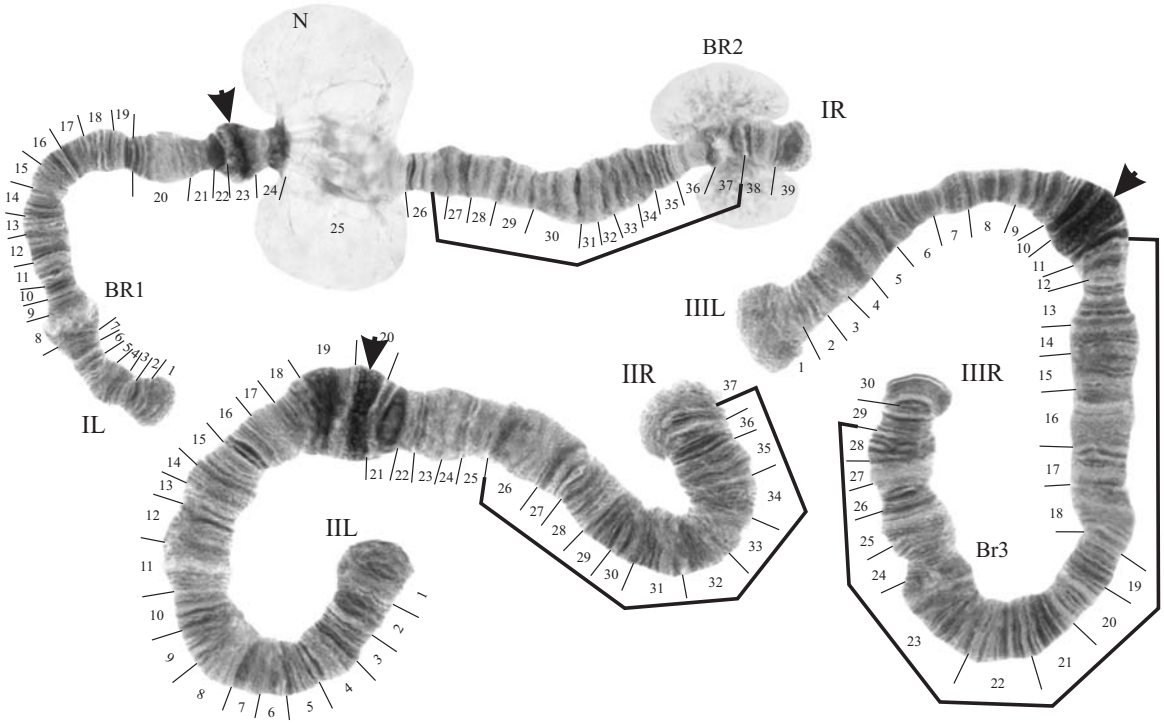

Fig. 1. Karyotype of *Propsilocerus akamusi*. IL, IR etc. - chromosome arms, BR - Balbiani Rings, N - nucleolus, centromeres are marked with arrows. Brakets show inversions, which differ karyotype of *P. akamusi* from *P. jacuticus*.

and coauthors (2003) designated chromosome arms in *P. akamusi* as short arm (S) and long arm (L). Because it is impossible to distinguish between short and long arms in metacentric chromosomes we have designated chromosome arms as left (L) and right (R) according to cytogenetic tradition (Bridges, 1935; Keyl, Keyl, 1959; Lefevre, 1976; Kiknadze et al., 1991), and we have traditionally begun mapping a chromosome with left arm.

It was very easy to identify each chromosome in *P. akamusi* karyotype (Fig. 1).

Chromosome I has clear markers: nucleolus (region 25) near the centromeric region, small Balbiani ring (BR1) on left arm (region 8), large BR2 (region 37) on right arm, small constriction near left arm end (region 3), and many puffs. The centromeric region is located at the centre of chromosome I and it has a very unusual shape. Three heavily stained huge heterochromatic bands are located in this region (22-24). A real centromeric band can not be identified without special methods (differential staining, *in situ* hybridization of centromeric DNA). However, we have suggested that one of these bands (indicated with arrows on Fig. 1), could be a centromeric band according to heavy stained dense vacuolated structure.

Chromosome II (Fig. 1) has no large puffs as BR and nucleolus, and has a marker as the thicker region 26 with more heavily stained bands on both sides of the region. The centromeric region also displays three large heavy stained heterochromatic bands (region 19-21).

Chromosome III (Fig.1) can be easily identified because it is submetacentric. The short arm is characterized by a clear-cut constriction in region 6-7, and the long arm – by two constrictions in

regions 24 and 26 and by a heavily staining of telomeric band. This telomeric band is denser and more heavily stained than telomeric bands in all other arms. Centromeric region is located in region 10-11, and possesses three large heterochromatic bands as well as do centromeric regions of chromosomes I and II.

Karyotype of *P. jacuticus* (n=3)

The haploid karyotype includes three chromosomes as in that of *P. akamusi* (Figs 2 and 3). Chromosomes I and II are metacentric; chromosome III is submetacentric. The comparison of polytene chromosomes banding sequences in both species demonstrates their clear-cut homology: arms IL, IIL, IIIL are identical. However *P. jacuticus* differs by the fixed paracentric inversions in arms IR, IIR, IIIR.

Chromosome I (Fig. 2). The banding sequence of arm IL is completely homologous with IL of *P. akamusi*. The simple inversion 26b-37, which changes standard banding sequence in arm IR, is found in *P. akamusi*. As a result of inversion, large BR2 situated at the end of arm IR in *P. akamusi* is transferred closer to nucleolus at the centre of chromosome I (Figs 2, 3). Three large heterochromatic bands are present in the centromeric region 22-24.

Chromosome II (Fig. 2). The banding sequence of IIL is homologous with *P. akamusi* IIL. The arm IIR has simple inversion 27-37a. Three large centromeric bands are located at centromeric region 19-21 (Figs 2, 3).

Chromosome III (Fig. 2). The arm IIIL is homologous with *P. akamusi* IIL. The arm IIR differs from that of *P. akamusi* by a complex paracentric inversion 12-28. The centromeric region 10-11 contains three large heterochromatic bands (Figs 2, 3).

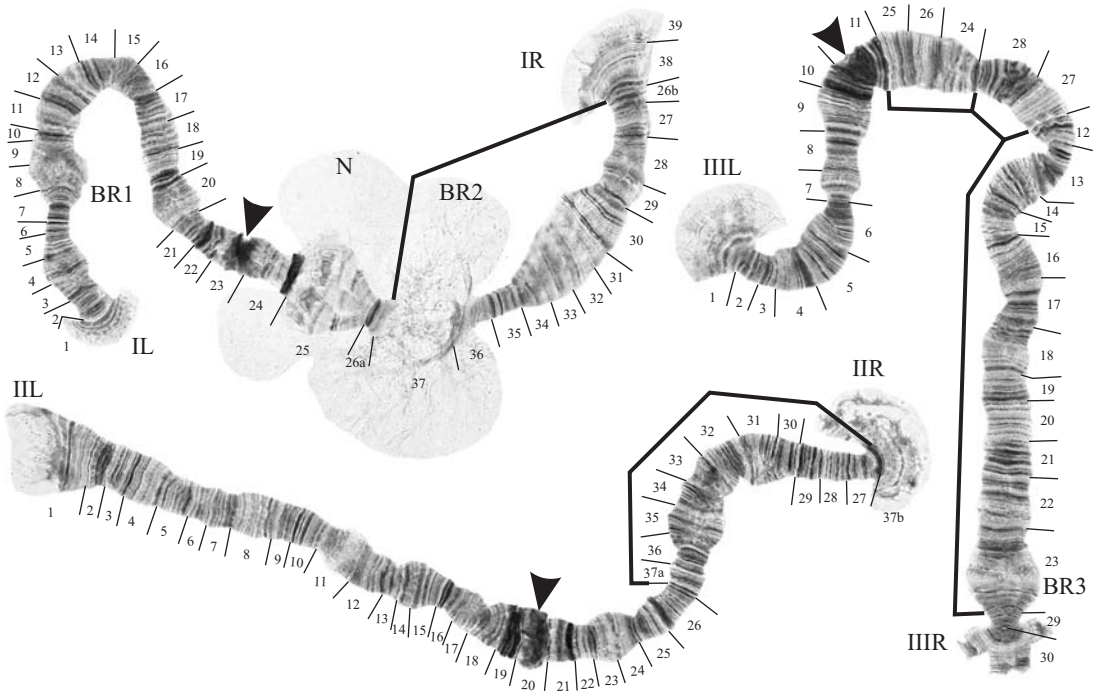

Fig. 2. Karyotype of *Propsilocerus jacuticus*. Abbreviations are as in Fig. 1.

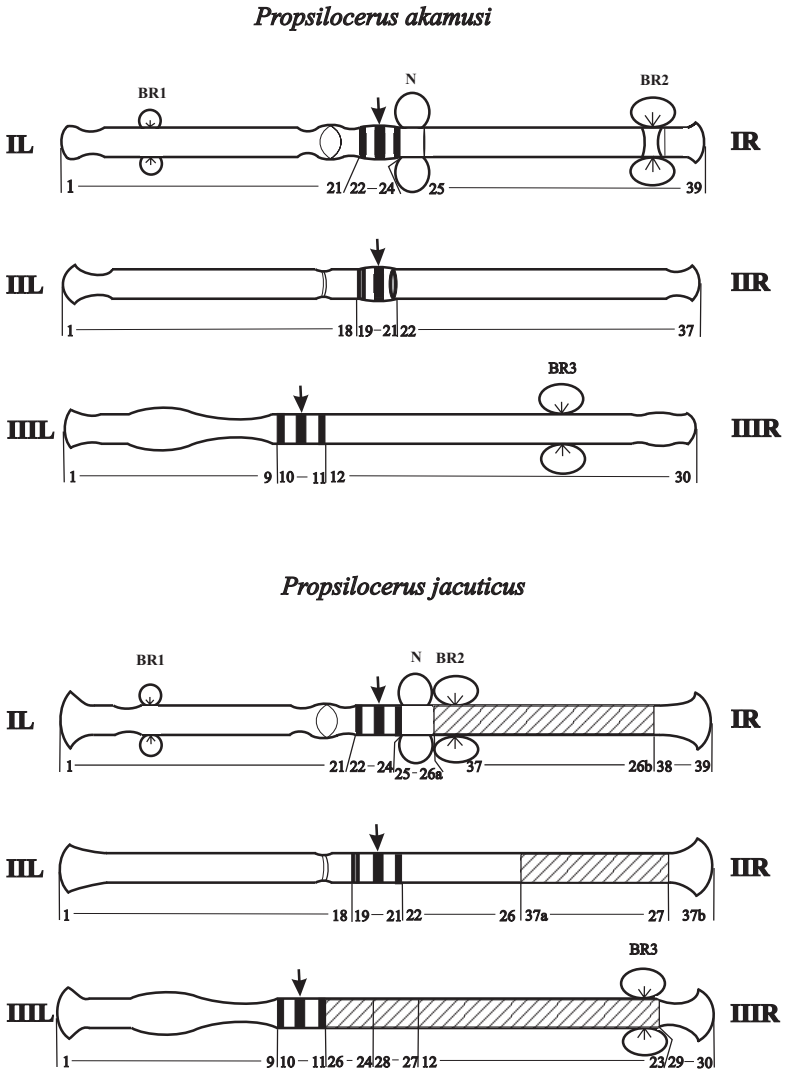

Fig. 3. Comparison of karyotypes of *Propsilocerus akamusi* and *P. jacuticus*.

Localization of centromeric bands revealed by C-banding technique

The C-banding technique was used to identify centromeric bands among three large heterochromatic bands in centromeric regions of polytene chromosomes of both species. Only one band in each chromosome of *P. akamusi* and *P. jacuticus* showed intensive C-positive reaction (Fig. 4, a, b). Other large bands were stained very weakly. Thus, it can be concluded that in each chromosome of *P. akamusi* and *P. jacuticus* only central heterochromatic band is true centromeric one.

Microdissection and FISH analysis of centromeric DNA

Centromeric band of chromosome II (band 2-2 in Fig. 5) in *P. akamusi* was microdissected. This band was chosen for dissection because the distance between heterochromatic bands in cen-

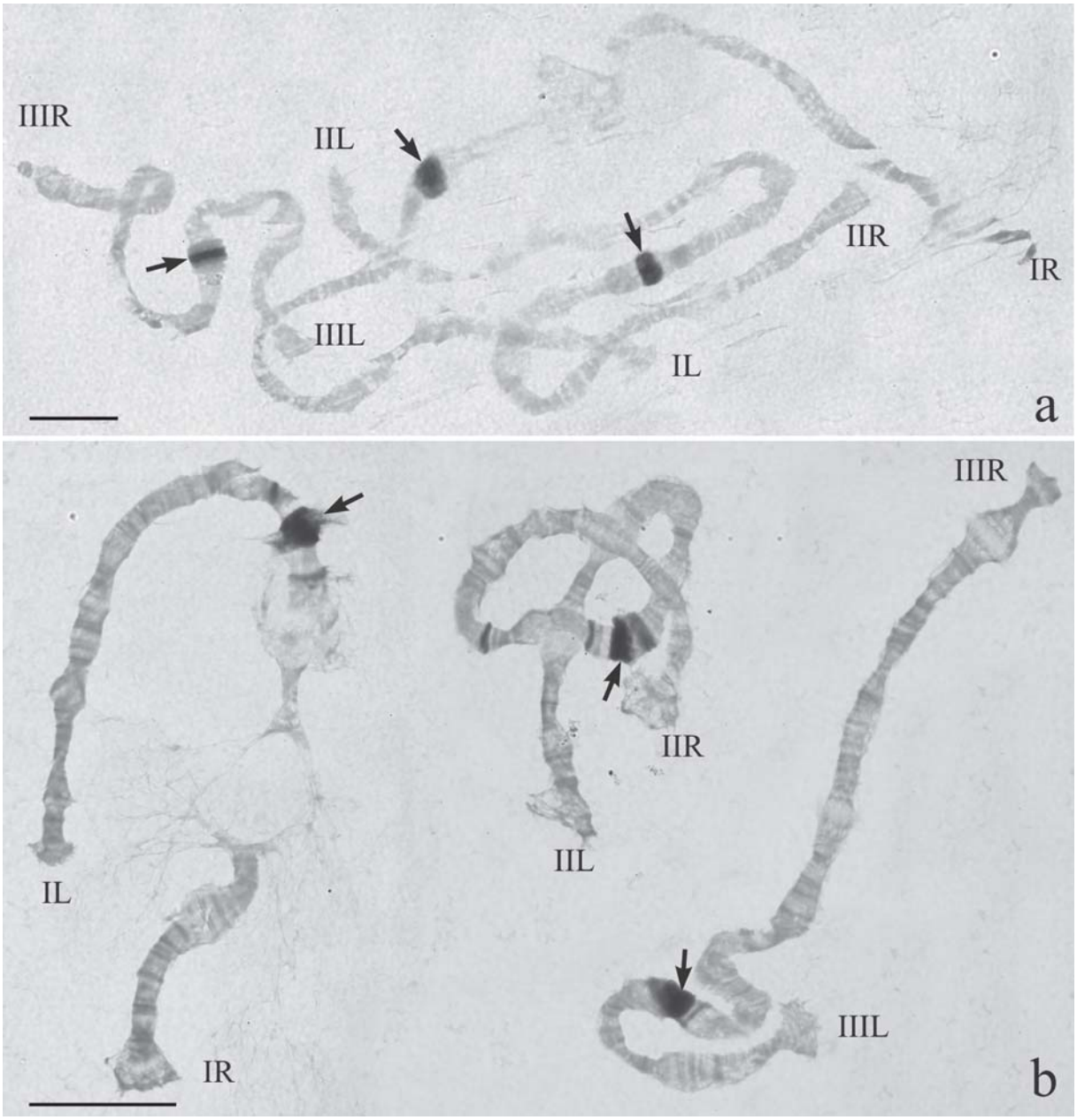

Fig. 4, a, b. C-banding of polytene chromosomes. **a** - *Propsilocerus akamusi*. **b** - *P. jacuticus*. Centromeres are marked with arrows. Bar = 25 μm.

tromeric region of the chromosome II is larger than in other chromosomes (Fig. 1). Heterochromatic bands 2-1 and 3-1 neighboring to centromeric bands in chromosomes II and III (Fig. 5) also were dissected to compare DNA characteristics of centromeric and noncentromeric bands. Correspondently DNA libraries of isolated bands had been designated as pak2-1, pak2-2 and pak3-1. The centromeric band from chromosome II was dissected twice, so DNA-libraries pak2-2[1] and

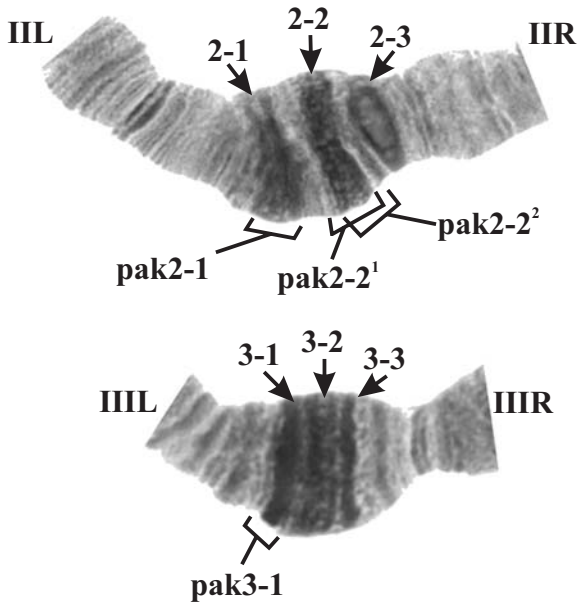

Fig. 5. Scheme of microdissection of heterochromatic bands in centromeric regions of chromosomes II and III of *Propsilocerus akamusi*.

pak2-2² originated from the same band.

The results of FISH analysis of DNA-probes obtained on polytene chromosomes of *P. akamusi* are shown in Fig. 6. The positive hybridization was observed in heterochromatic bands of centromeric regions only. No signals on the other parts of all chromosomes were detected.

As mentioned above, DNA from centromeric band 2-2 of chromosome II was obtained twice. Both DNA-probes were strongly hybridized to centromeric band 2-2 only (Fig. 7). Weaker signals could be seen also in centromeric bands of the chromosomes I and III. Very weak signals were observed some times in some of adjacent heterochromatic bands. This result means that centromeric DNA of the chromosome II has a low homology to centromeric DNA of the other chromosomes in *P. akamusi*. A homology was much lower between centromeric DNA and DNA of adjacent heterochromatic bands.

DNA-probe pak2-1 was strongly hybridized to the band 2-1 of chromosome II (Fig. 7, a). Weaker signals could be also detected in the centromeric band 2-2 of the same chromosome; hybridization was very weak in centromeric regions of other chromosomes.

The hybridization of DNA-probe pak3-1 from noncentromeric band of the chromosome III showed the similar pattern: strong signal was observed in band 3-1 and weak signals in centromeric and some of adjacent heterochromatic bands of all three chromosomes (Fig. 7, b).

Based on these results it is possible to conclude that DNA of the real centromeric band is strongly diverged between different chromosomes of *P. akamusi*. DNA of adjacent heterochromatic bands has a low homology with centromeric DNA, and is also diverged between different chromosomes.

FISH of all probes, obtained from *P. akamusi*, gave no distinct signals on the polytene chromosomes of *P. jacuticus*. That means that centromeric DNA and DNA of adjacent heterochromatic bands diverged greatly between these sibling species.

Discussion

Comparative study of the karyotypes of the sibling species, *P. akamusi* and *P. jacuticus*, demonstrates their clear-cut differentiation by fixed paracentric inversions in three chromosomal arms (IR, IIR, IIIR). All these inversions are large, and most parts of the arms are involved in rearrangements. The inversions in arms IR and IIR are simple, in the arm IIIR – complex. Such karyotype divergence is typical for siblings in Chironomidae (Gunderina, Kiknadze, 2000).

It is interesting that the karyotypes of both *Propsilocerus* species are monomorphic in natural populations in spite of their wide distribution in East Asia. For example, *P. akamusi* is very common midge in Siberia, Japan, and China (Saether, Wang, 1996; Wang, 2000a, b; Kiknadze et al., 2004). Usually, widely distributed chironomid spe-

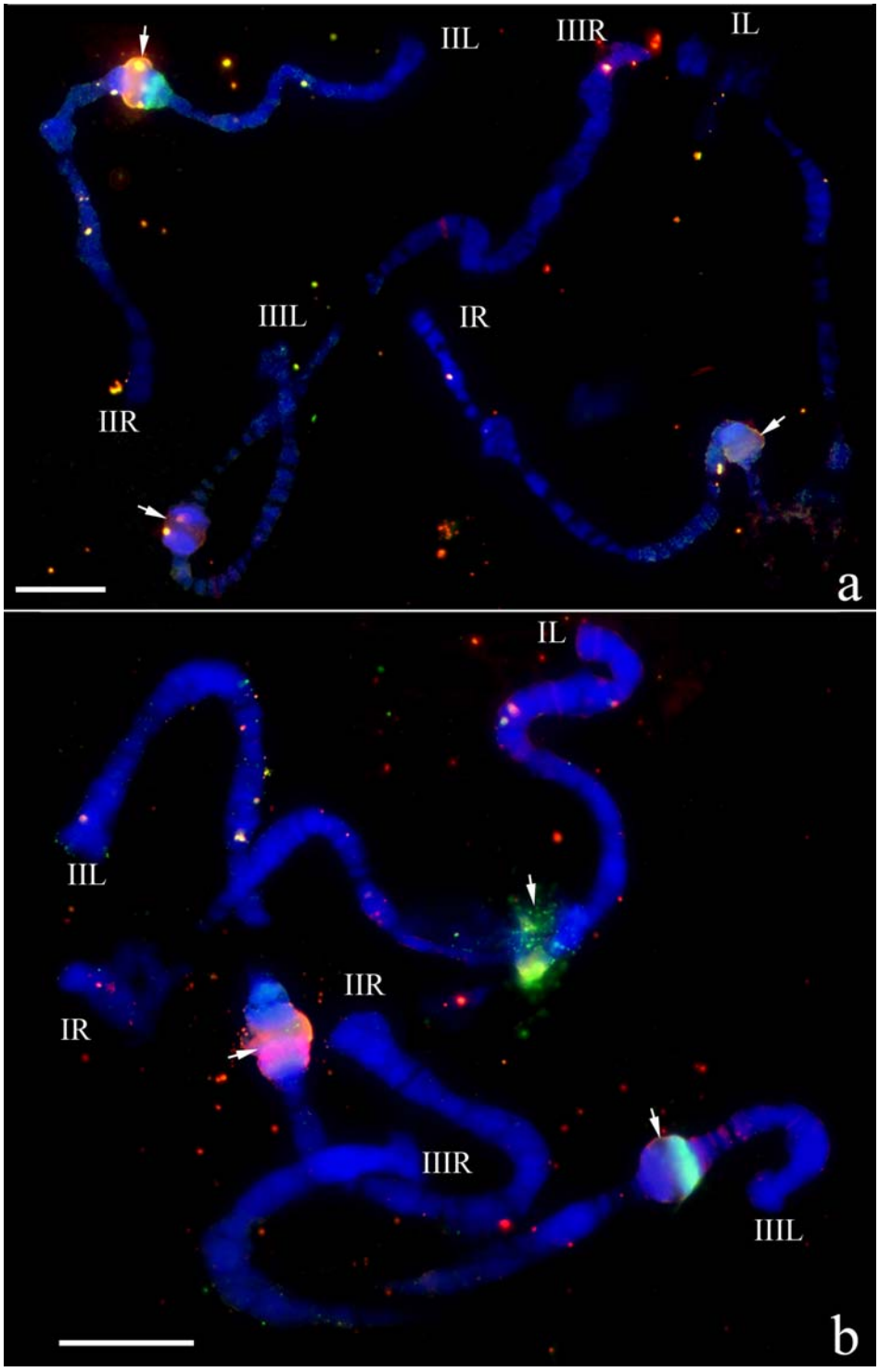

Fig. 6 a, b. FISH of DNA-probes with polytene chromosomes of *Propsilocerus akamusi*. **a** - DNA-probe pak2-1 - green signal (avidin-FITC) and DNA-probe pak 2-2^{2} - red signal (Cy3). **b** - DNA-probe pak2-2^{1} - red signal (Cy3), DNA-probe pak 3-1 - green signal (avidin-FITC). Centromeric bands are marked with arrows. Bar = 25 μm.

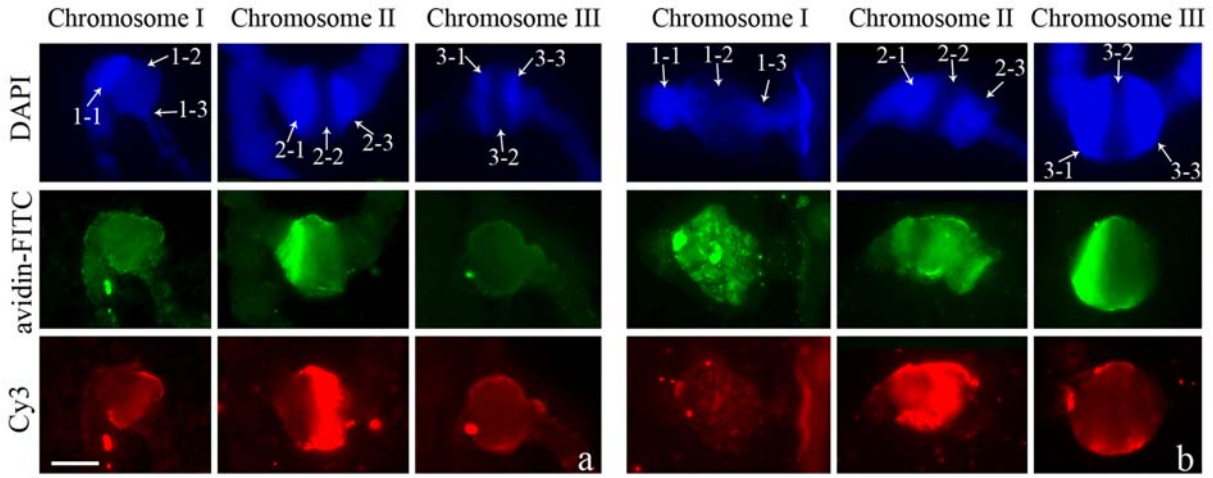

Fig. 7, a, b. FISH of DNA-probes with polytene chromosomes of *Propsilocerus akamusi* – close-up of centromeric regions. **a** - DNA-probes pak2-1 (avidin-FITC) and pak 2-2^2 (Cy3). **b** - DNA-probe pak2-2^1 (Cy3) and pak 3-1 (avidin-FITC). Numbers with arrows mark heterochromatic bands. Bar = 10 μm.

cies are highly chromosomally polymorphic, and chromosomal polymorphism in these species is used as cytogenetic marker of adaptation of populations to different environments (Keyl, 1962; Michailova, 1989; Kiknadze et al., 1991, 2004). Possibly, chromosomal mechanism of adaptation of *P. akamusi* and *P. jacuticus* is replaced by genetic mechanisms.

One of the most interesting characters of karyotype structure of *P. akamusi* and *P. jacuticus* in comparison with *Chironomus* spp. is an unusual organization of centromeric regions. The centromeric regions in each of four *Chironomus* polytene chromosomes are identified as single dense bands with intensive C-staining (Keyl, 1962; Sigareva, 1985). The size of centromeric bands is species-specific; AT-rich tandem DNA repeats are presented in centromeric bands; centromeric bands of all chromosomes in karyotype contain the same major DNA repeat (Schmidt, Keyl, 1981; Hankeln et al., 1989, 1994; Rovira et al., 1993; Siirin et al., 2003). The homology of centromeric SauDNA was determined also among the most of sibling-species in *Ch. plumosus* group (Kiknadze et al., 1991; Hankeln et al., 1994; Siirin et al., 2003).

Another situation was observed in studied species of *Propsilocerus*. Three huge heterochromatic bands were found in the centromeric regions of all three chromosomes in *P. akamusi* and *P. jacuticus*. The same picture was observed in the third member of *P. akamusi* group – *P. taihuensis* (our unpublished data). We have found that only central of three heterochromatic bands in each of chromosome demonstrates intensive C-staining, thus, these central bands can be considered as true centromeric bands. FISH of microdissected centromeric band of *P. akamusi* had shown that probably different major centromeric DNA repeats were presented in each chromosome. No homology between centromeric DNA of *P. akamusi* and *P. jacuticus* was detected by FISH. So it is possible to conclude that centromeric DNA characteristics are more complex in sibling species of *P. akamusi* group than in genus *Chironomus*. It is necessary to note that the genus *Propsilocerus* includes six species: *P. akamusi*, *P. jacuticus*, and *P. taihuensis* form one cluster on evolutionary dendrogram, whereas *P. paradoxus* and *P.*

lacustris Kieffer, 1923 form another cluster; *P. sinicus* Saether et Wang, 1996 differs from both clusters (Saether, Wang, 1996). Huge heterochromatic bands in centromeric regions were observed in species of *P. akamusi* group only. We suppose that a divergence in centromeric regions was one of the important factors of speciation in *Propsilocerus*.

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