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Characterization of Heterochromatin in the Salivary Gland Chromosomes of *Chironomus riparius* Meigen and *Chironomus piger* Strenzke (Diptera, Chironomidae) by Differential and Fluorochrome Staining

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The constitutive heterochromatin of two homosequential sibling species, *Chironomus riparius* and *Chironomus piger*, was studied. The salivary gland chromosomes of both species were analyzed using three staining methods: orcein and C band staining combined with DAPI and CMA_3 fluorochrome staining. Both species have the chromosome set 2n = 8, with the same banding pattern and chromosome arm combinations: AB, CD, EF, G, but they differed in number and distribution of heterochromatic bands, AT-rich sequences (DAPI+) and GC-rich sequences (CMA_3+). In the polytene chromosomes of *C. piger*, C-bands were found in centromeres only. They contain two types of repetitive DNA sequences: DAPI+ (very weak) and CMA_3+ sequences. However, the polytene chromosomes of *C. riparius* have many interstitial heterochromatic bands in addition to the centromeric heterochromatin. Some of these bands contain both AT-rich and GC-rich sequences, while others are either AT-rich (DAPI+) or GC-rich (CMA_3+). Therefore, these closely related species differ both in the content and localization of constitutive heterochromatin. The species-specific organization of the constitutive heterochromatin can be used as an additional cytogenetic marker for species differentiation.

Key words: Chironomidae, *C. riparius, C. piger*, salivary gland chromosomes, C-banding, fluorochrome staining.

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Dipteran polytene chromosomes contribute a great deal towards assessing phylogenetic relationships among closely related species as well as to understanding the role of chromosome rearrangements involved in species differentiation. They are an excellent model for studying chromosome structure, function and genomic organization of the species (ZHIMULEV *et al*. 2004).

Chironomus riparius Meigen and *Chironomus piger* Strenzke are two sibling, homosequential species belonging to the *thummi* cytocomplex (KEYL 1962) with the same chromosome set (2n = 8) and banding pattern along their salivary gland chromosomes (KEYL & STRENZKE 1956). However, bands along the polytene chromosomes can be recognized by their thickness (KEYL 1962, 1965). In *C. riparius* they have higher DNA content due to duplication events during their evolution. Moreover, it was revealed that the *C. riparius* genome has 30% more DNA than the *C. piger* genome and the latter is considered a phylogenetically older species (KEYL 1965). In addition, the genome differences are correlated to differences in the concentrations of some transposable elements (Cla) which are six times more frequent in the *C. riparius* genome (SCHMIDT 1984). In *C. piger* polytene chromosomes, these transposable elements are restricted to the centromere regions

only, while in the *C. riparius* genome, in addition to the centromere regions, they have been detected in more than 200 sites of the polytene chromosomes (SCHAEFER & SCHMIDT 1981; SCHMIDT 1984). The higher concentrations of transposable elements were considered a consequence of an evolutionary amplification process (SCHMIDT 1984). Both species are also distinguished by the number and localization of repetitive DNA clusters (Alu and Hinf) which can provide valuable cytogenetic markers for both species (BOVERO *et al*. 2002; MICHAILOVA *et al*. 2009). *C. riparius* and *C. piger* polytene chromosomes have striking differences in the number of C-bands which in the *C. piger* genome are localized in centromere regions only while *C. riparius* possesses numerous C-bands including centromere regions (HÄGELE 1977). However, no information exists about the sites of localization of the C-bands and the exact organization of the constitutive heterochromatin detected by these bands. WARCHAŁOWSKA-ŚLIWA *et al*. (2013a, b) showed the significance of fluorochrome DAPI/CMA_3 staining for the development of a new approach of evaluation of heterochromatin organization and for revealing the differences among species with similar cytogenetic characteristics. LEEMANN and RUCH (1984) underlined the high sensitivity of dipteran polytene chromosomes for the fluorochrome method and showed its advantage in avoiding different drastic procedures that might alter the chromatin structure in the polytene chromosomes.

In the present study we characterize and compare the heterochromatin in closely related sibling species with an aim to: (1) localize the sites of C heterochromatin in polytene chromosomes of *C. riparius* and *C. piger* by a comparative analysis with chromosome maps done by HÄGELE (1970) and KIKNADZE *et al*. (1991); (2) characterize the heterochromatin of both species by applying DAPI/CMA_3 double staining and to show evolutionary diversification of sequences associated with heterochromatin.

Material and Methods

Fourth larva stage of *C. riparius* (23 individuals) and *C. piger* (25 individuals) collected near Sofia were fixed in ethanol-glacial acetic acid (3:1). Unstained gland polytene chromosomes of both species were squashed in 45% acetic acid. Constitutive heterochromatin (C-bands) in the salivary gland chromosomes was detected by a modified method (MICHAILOVA 1987). The heterochromatin in mitotic chromosomes was not analyzed due to their small size. In order to reveal the molecular composition of heterochromatin CG-specific chromomycin A_3 (CMA_3) and AT-specific 4'-6-diamidino-2-phenylindole (DAPI), some slides were stained according to the methods of SCHWEIZER (1976) and DONLON and MAGENIS (1983). After staining with fluorochromes the slides were mounted in anti-fade medium containing 1% n-propylgallate in 10M phosphatase buffer solution with 70% glicerol, pH 7.0. The chromosomes were analyzed using a Nikon gEclipse E400 fitted with a CCD DS-U1 camera, a set of standard filters and an NIS-Elements BR 3.0 image-analyzing system (Nikon Eclipse 400). Images were processed and arranged with Adobe Photoshop.

For localizing the C, GC-rich and AT-rich bands, we used standard chromosome maps done by HÄGELE (1970) and KIKNADZE *et al*. (1991). In the text large letters and numbers correspond to those of the standard map done by HÄGELE (1970). Due to difficulties in identifying the exact sites of bright AT and GC rich bands, these bands are shown in the region where they were found, according to the map of HÄGELE (1970). We numbered C-bands conditionally for precise comparative analysis between the localization of C-bands and AT-rich and GC-rich regions.

Results

Cytogenetic characteristics of *Chironomus riparius* and *Chironomus piger*

Both species have the chromosome set 2n = 8, with the same banding patterns and chromosome arm combinations AB CD EF G (Figs 1a-d and 2a-d). Chromosomes AB, CD are metacentric whereas EF is submetacentric and chromosome G is acrocentric. Chromosome G of both species has three Balbiani rings (BRa, BRb, BRc) but BRa is expressed in few cells of the salivary glands only, the nucleolar organizer region (NOR) is located near the telomere region of chromosome G in both species. Very often this telomere displays a "dark knob" which might occur in condensed or decondensed state.

Characterization of heterochromatin by C-banding technique

The polytene chromosome band patterns are different after applying this method: the chromosomes are coloured slightly blue-lilac, with the exception of single thin or heavy stained C-bands.

In *C. piger*, C-bands occur in the centromere regions of chromosomes AB, CD, EF and G (Figs 3d, h and 4d, h) (Table 1). In chromosome AB (Fig. 3d) two bands located close to each other

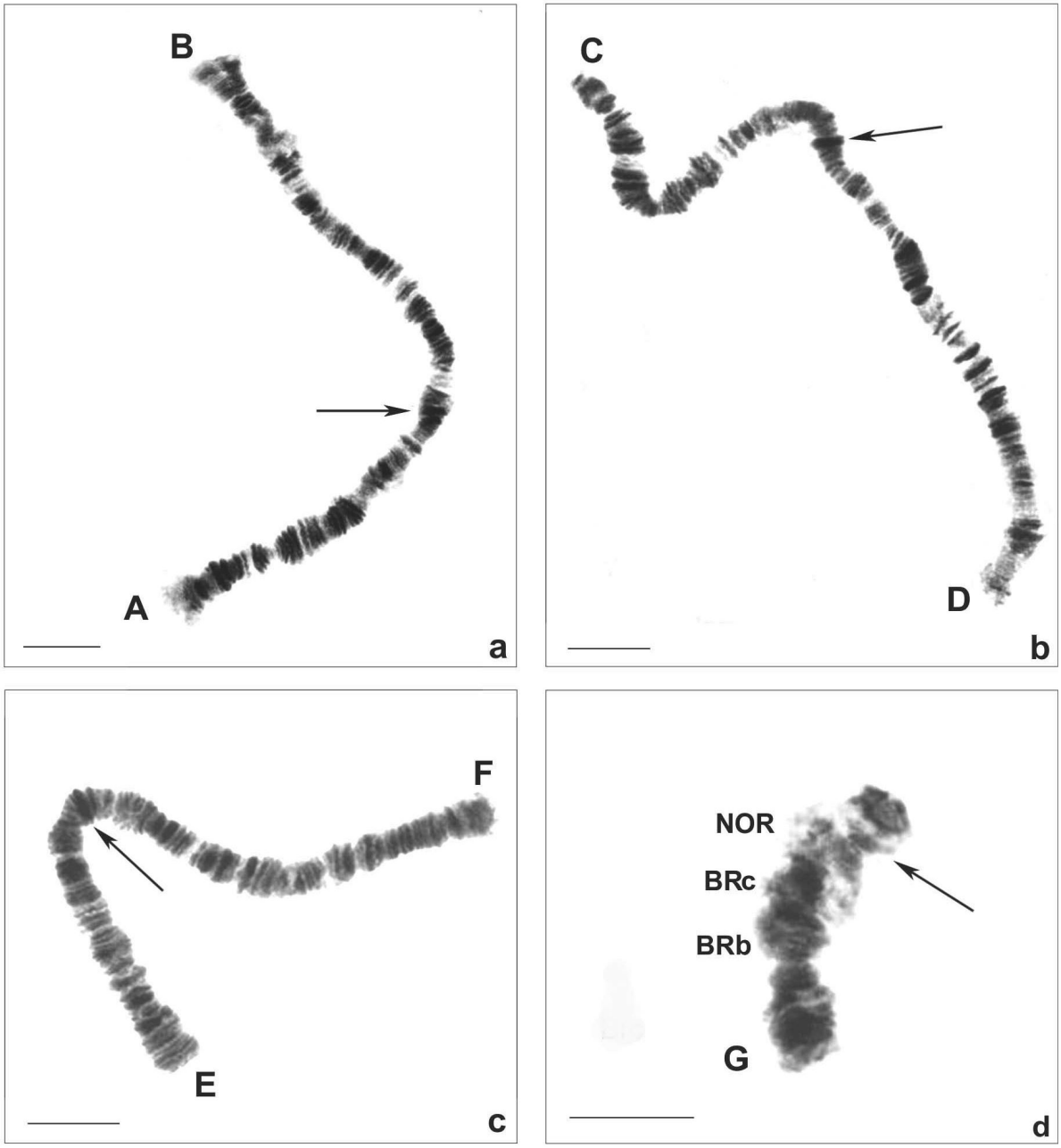

Fig. 1. Acet orcein staining of the polytene chromosomes of *Chironomus piger*: AB (a), CD (b), EF (c) and G (d); arrows indicate the centromere region. Chromosome G with active nucleolar organizer region (NOR) and two Balbiani rings – BRb, BRc (d). Bar = 10 μm.

were observed. Often C bands appeared at telomeres in chromosomes AB (A1, G3), CD (A1, F2) and EF (A1, D3). In Figure 4h (point 2) a condensed "dark knob" at the telomere can be observed

In addition to centromere regions, many interstitial C bands located on both sides of the centromere regions can be found in polytene chromosomes of *C. riparius* (Figs 5d, h and 6d, h) (Table 2). Very rarely the telomeres of all chromosomes are C-bands and chromosome G has a well defined "dark knob" at the telomere (Fig. 6h) (point 2). Bands in arms A (regions A4, B2, C2, C4), D (regions C6, D4) and F (regions C2) are occasionally C-banded.

Characterization of heterochromatin by fluorochrome staining

After DAPI/CMA_3 double staining, some chromosome regions show either a positive (+) or negative (-) reaction with fluorochromes, depending on the base composition of the DNA molecule. In *C. piger* the centromere regions of all chromo-

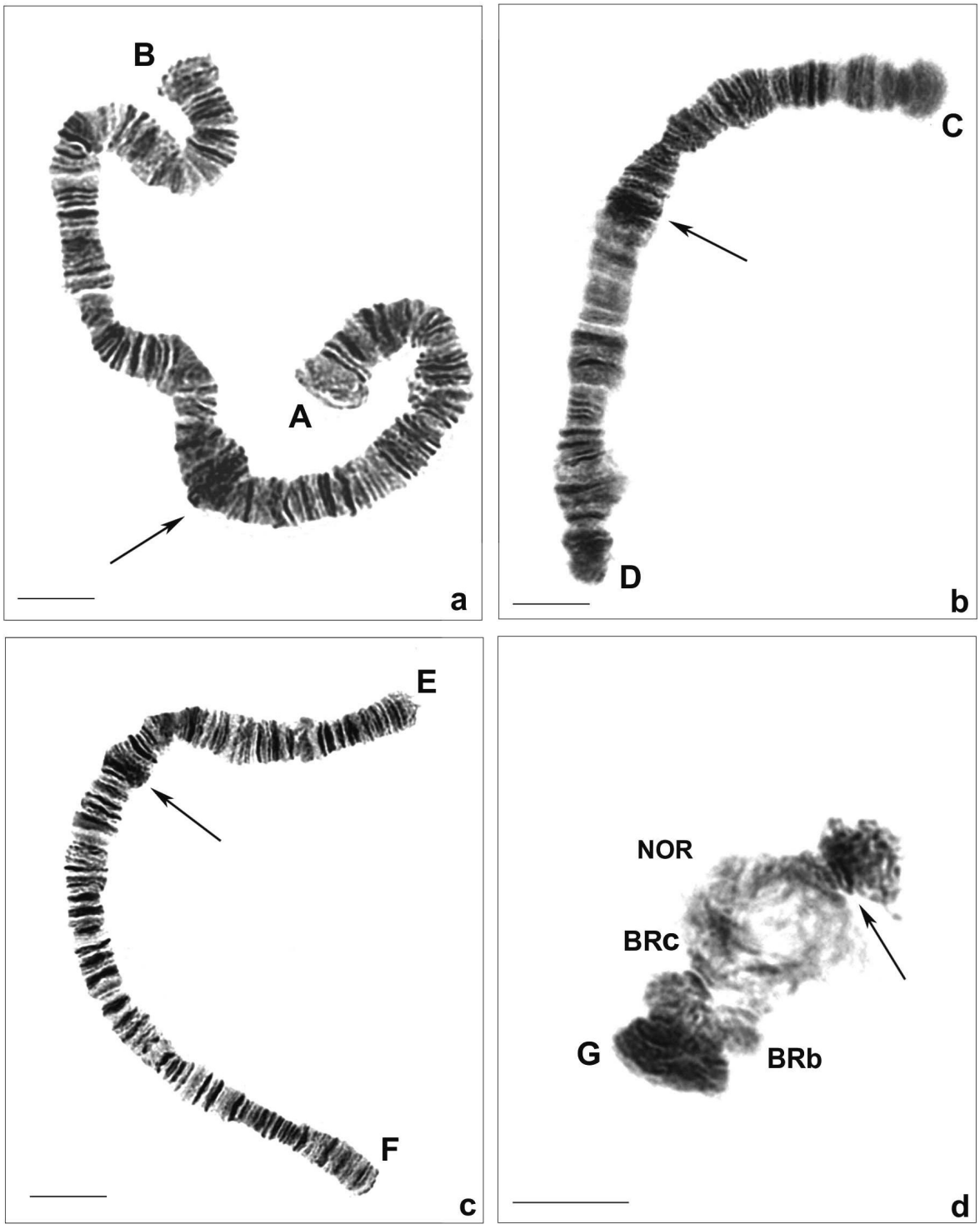

Fig. 2. Acet orcein staining of the polytene chromosomes of *Chironomus riparius*: AB (a), CD (b), EF (c) and G (d); arrows indicate the centromere region. Chromosome G with active nucleolar organizer region (NOR) and two Balbiani Rings – BRb, BRc (d). Bar = 10 μm.

somes show bright fluorescence with DAPI+ and CMA_3+ staining (Figs 3a-c, e-g and 4a-c, e-g) (Table 1). However, double staining at the centromere regions of chromosome AB reveals a very weak DAPI+ signal (Fig 3c) (Table 1). In chromosome G very often AT-rich base pairs are not detected. However, if the centromere region of chromosome G is marked by DAPI+/CMA_3+, double-bands are observed and the condensed telomere heterochromatin is always stained intensively by DAPI+ and CMA_3+ bands (Fig. 4e-g). In this chromosome the active NOR is brightly

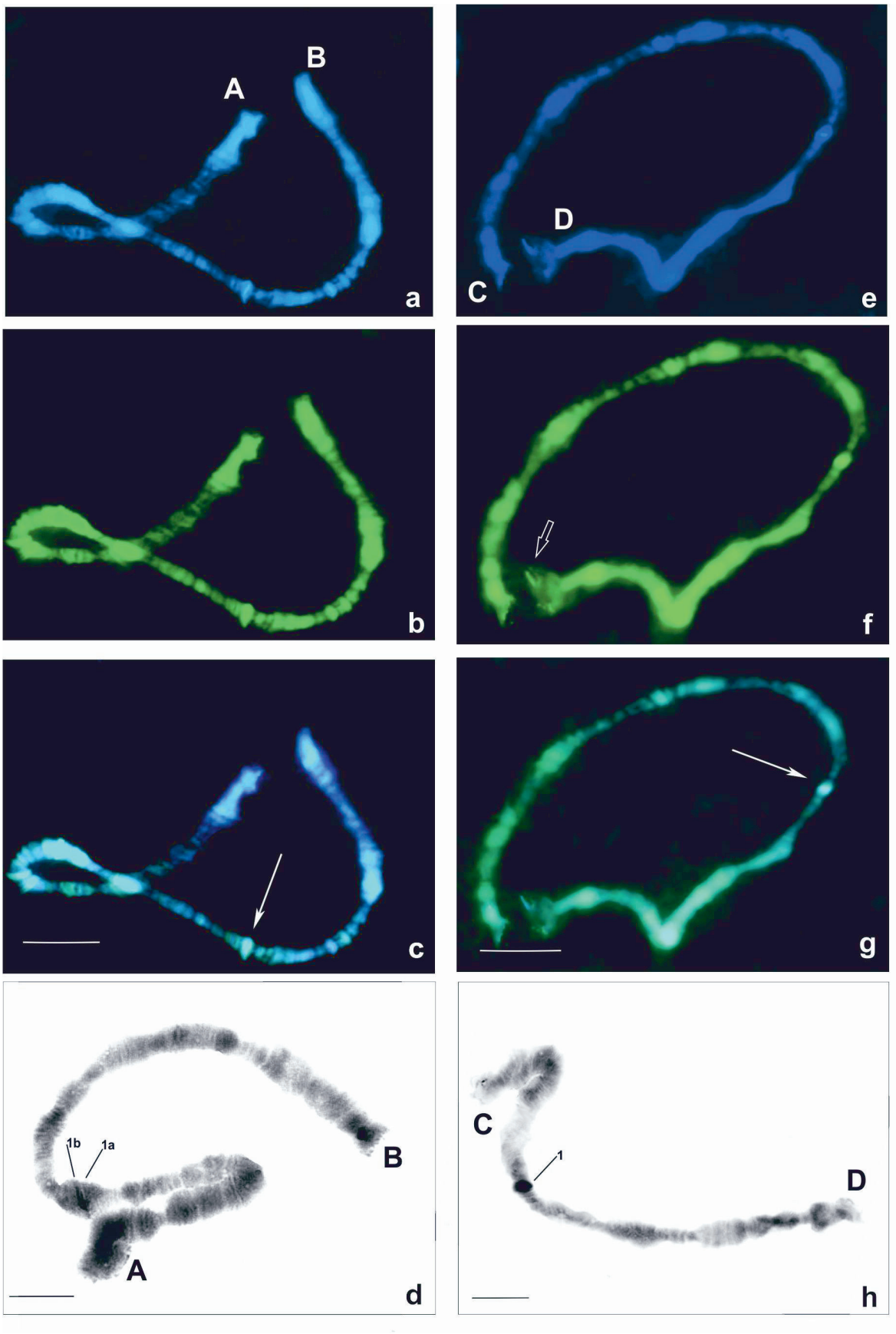

Fig. 3. Fluorochrome-stained heterochromatin and C-banding of chromosomes AB (a-d) and CD (e-h) of *Chironomus piger*; DAPI (blue) (a, e), CMA_3 (green) (b, f) and double DAPI/CMA_3 (c, g); small open arrow indicates localization of CMA_3+ base pairs (f); long arrows indicate sites rich in both AT and GC base pairs (c, g). Two physically close C-bands are marked by 1a, 1b (d) and 1 (h). Bar = 10 μm.

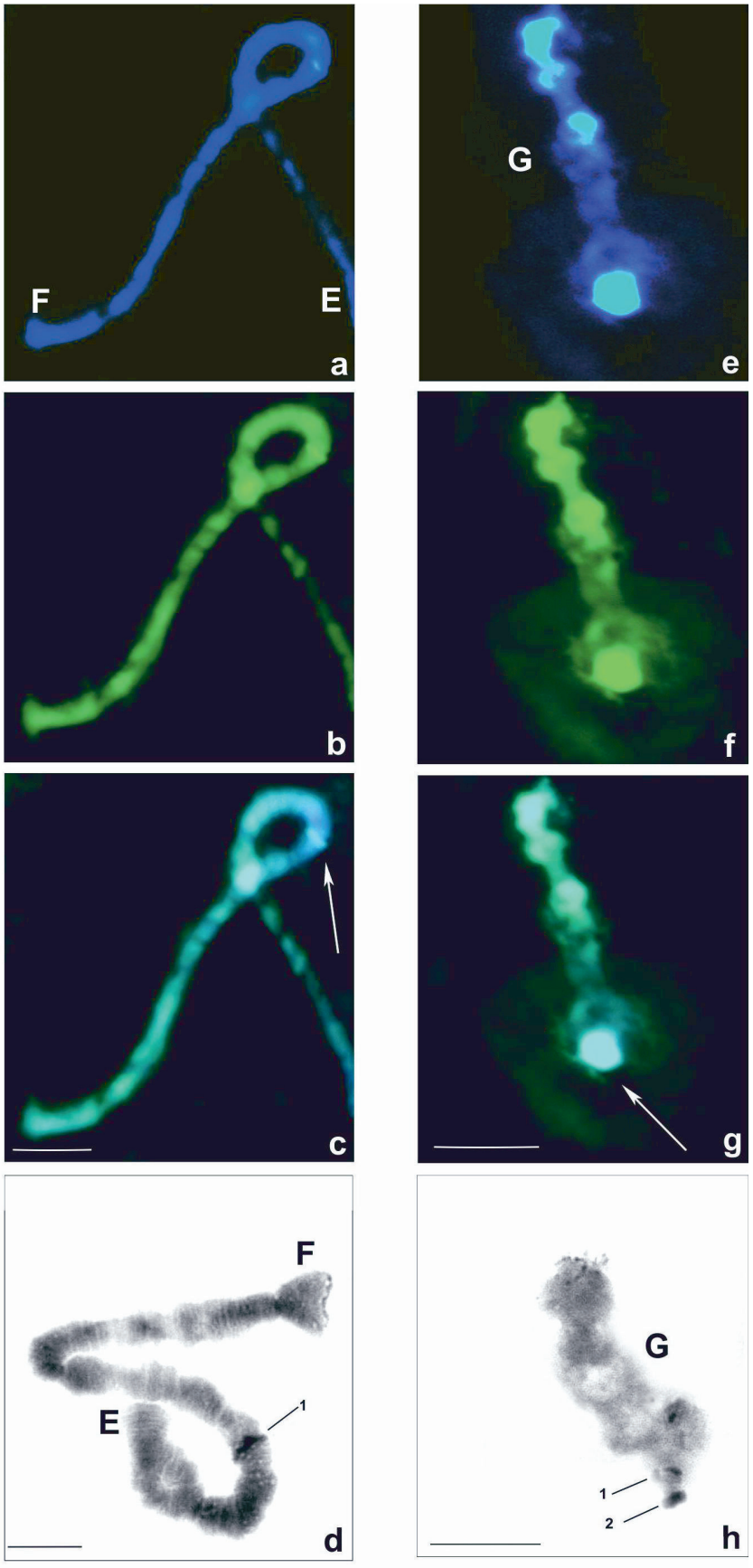

Fig. 4. Fluorochrome-stained heterochromatin and C-banding of chromosomes EF (a-d) and G (e-h) of *Chironomus piger*; DAPI (blue) (a, e), CMA_3 (green) (b, f) and double DAPI/CMA_3 (c, g). Active NOR is DAPI-/CMA_3+ (g); long arrows indicate the site rich in both AT and GC base pairs (c, g). Chromosomes with C-bands (d, h) are marked by 1 (d) and 1, 2 (h). Bar = 10 μm.

Table 1

Localization of constant C-bands and base-specific fluorochromes in polytene chromosomes of *C. piger*

Conditional numbers	Chromosome AB			Chromosome CD			Chromosome EF			Chromosome G		
	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)
1a, 1b	D2c, D2d	+	+	C3 (C3e-i)	+	+	B2 (B2k)	+	+	E1c	+	+
2										E2a	+	+

Letters and numbers correspond to that of the chromosome map done by HÄGELE (1970)

stained by CMA_3 (Fig. 4f). Sometimes the telomeres of all chromosomes show a bright CMA_3 signal as dot structures (e.g., Fig. 3f).

C-bands at centromere regions in *C. riparius* on all chromosomes show bright fluorescence with DAPI+ (AT-rich) and CMA_3+ (GC-rich) fluorochromes. The dark, heavy interstitial C-bands in chromosome AB (1, 2, 3, 4, 6) (Fig. 5d), chromosome CD (1, 4, 5, 6, 7) (Fig. 5h) and chromosome EF (4, 5, 8, 9, 10, 11) (Fig. 6d) have bright distinct DAPI+ and CMA_3+ bands (Figs 5a-c, e-g and 6a-c, e-g) (Table 2). The "dark knob" at the telomere of chromosome G consists of DAPI+ and CMA_3+ (Fig. 6e-g). Some other C bands are GC-rich, detected by bright CMA_3+ fluorescence, while others show bright fluorescence with DAPI+ (Table 2) (Figs 5a-c, e-g and 6a-c, e-g). It is worth underlying that the active NOR (Fig. 7a-c) and all telomeres have bright CMA_3 bands and very often the telomeres have grain structures (Figs 5b, f and 6b, f). In some cells, in arm A, the intercalary band (A4) shows bright DAPI+ and CMA_3+ fluorescence (Fig. 5c), while other intercalary bands in arm A (C2 and C4) are GC-rich only (Fig. 5b). An additional intercalary band in arm D (C6) is rich in GC base pairs, while those in region D4 show bright fluorescence with DAPI+ and CMA_3+ (Fig. 5g).The band in region C5 is rich in AT base pairs (Fig. 5e). In most cells of arm F, the band in region C2 occurs in a heterozygous state and is rich in AT and GC base pairs (Fig. 6 c).

Discussion

The combination of DAPI/CMA_3 double staining approach has provided new information about the base composition of DNA in the polytene chromosomes of two homosequential species. Along the polytene chromosomes of these species, most bands contain some DNA sequences in which GC base pairs are closely interspersed among AT base pairs. It is known that centromeres in the polytene chromosomes are associated with constitutive heterochromatin (HÄGELE 1977). In both studied species the centromeres occur as C-bands and contain two types of repetitive DNA sequences – A+T-rich (DAPI+) and G+C-rich (CMA_3+) sequences. However, the data obtained from centromere regions of *C. piger* polytene chromosomes show very weak DAPI+ in chromosomes AB, EF and G or often the very weak DAPI+ is not seen. Also, SCHMIDT (1980) detected that AT-rich satellite II DNA sequences (with 21% GC content) isolated from some chironomid (*Glyptotendipes barbipes* Staeger) genomes hybridized with centromere regions of four chromosomes of *C. riparius* and with some additional bands with higher DNA content. However, the same author showed that *C. piger* polytene chromosomes hybridized with the same satellite DNA at the centromere regions only, with the exception of chromosome CD on which a hybridization signal was not detected. This study shows that closely related species differ not only in the content of the constitutive heterochromatin but also in its organization. The polytene chromosomes of *C. riparius* have many interstitial bands which are known to have greater DNA content than their homologous *C. piger* bands (KEYL 1965; HÄGELE 1977). In fact, some of these bands contain AT and GC sequences. Others contain either AT-rich (DAPI+) or GC-rich (CMA_3+) base pairs (see Table 2). The results reveal heterogeneity in the composition of *C. riparius* heterochromatin, a sign detected in other insects as well (WARCHAŁOWSKA-ŚLIWA *et al*. 2013a, b; CRISTIANO *et al*. 2014). Moreover, it is interesting to note that these interstitial heterochromatin regions are sites of some somatic breakpoint aberrations induced by stress agents in the environment (MICHAILOVA *et al*. 2007; ILKOVA *et al*. 2013). These data are in good agreement with the idea of a significant role of repetitive DNA sequences in promoting chromosome rearrangements (REDI *et al*. 2001).

Using base-specific fluorochrome staining techniques, we found that the polytene chromosome

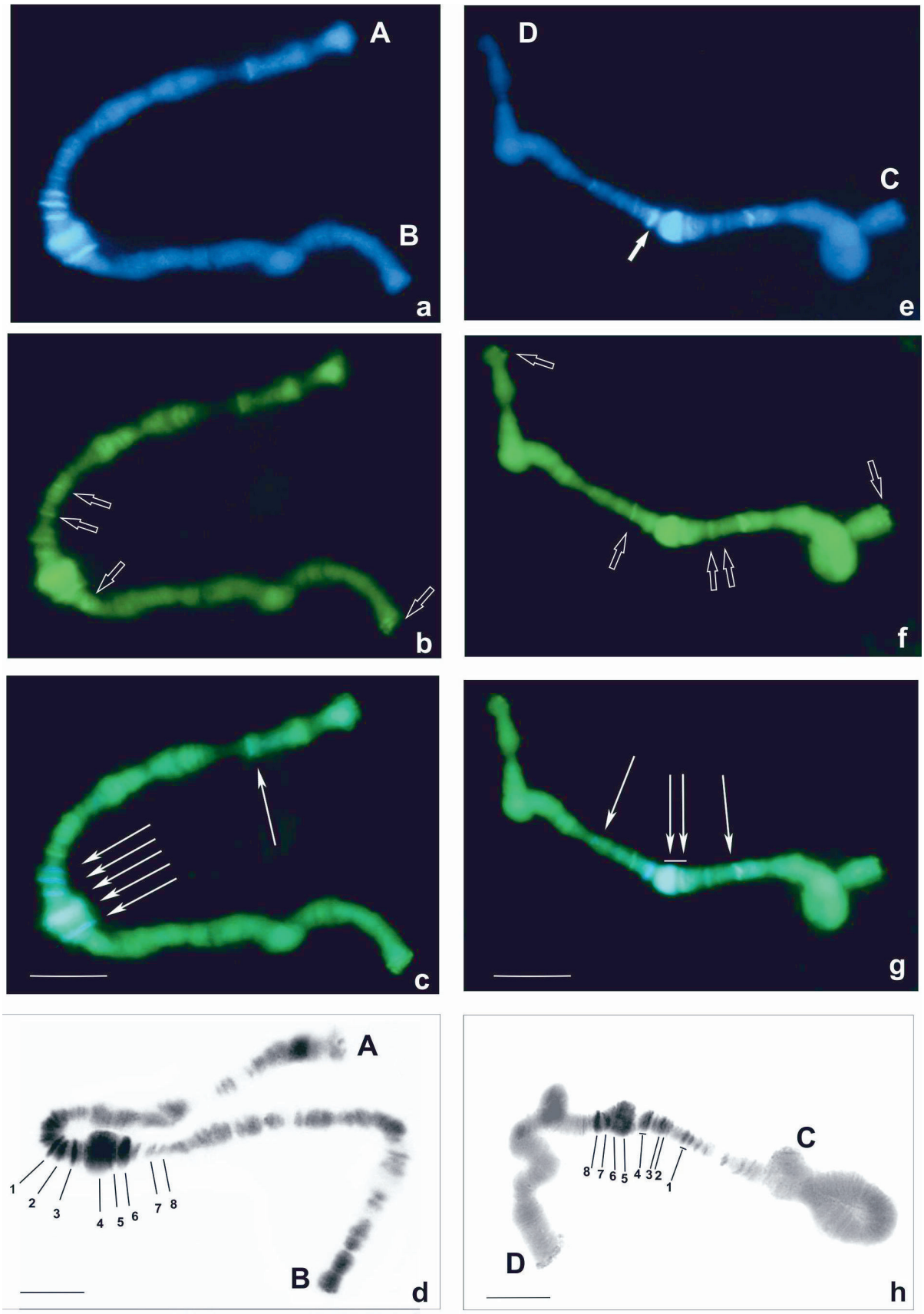

Fig. 5. Fluorochrome-stained heterochromatin and C-banding of chromosomes AB (a-d) and CD (e-h) of *Chironomus riparius*; DAPI (blue) (a, e), CMA_3 (green) (b, f) and double DAPI/CMA_3 (c, g). Small open arrows indicate localization of CMA_3+ bands (b, f) and small solid arrow marks a DAPI+ band (e); long arrows indicate a site rich in both AT and GC base pairs (c, g). Chromosomes with C bands indicated by numbers (d, h). Bar = 10 μm.

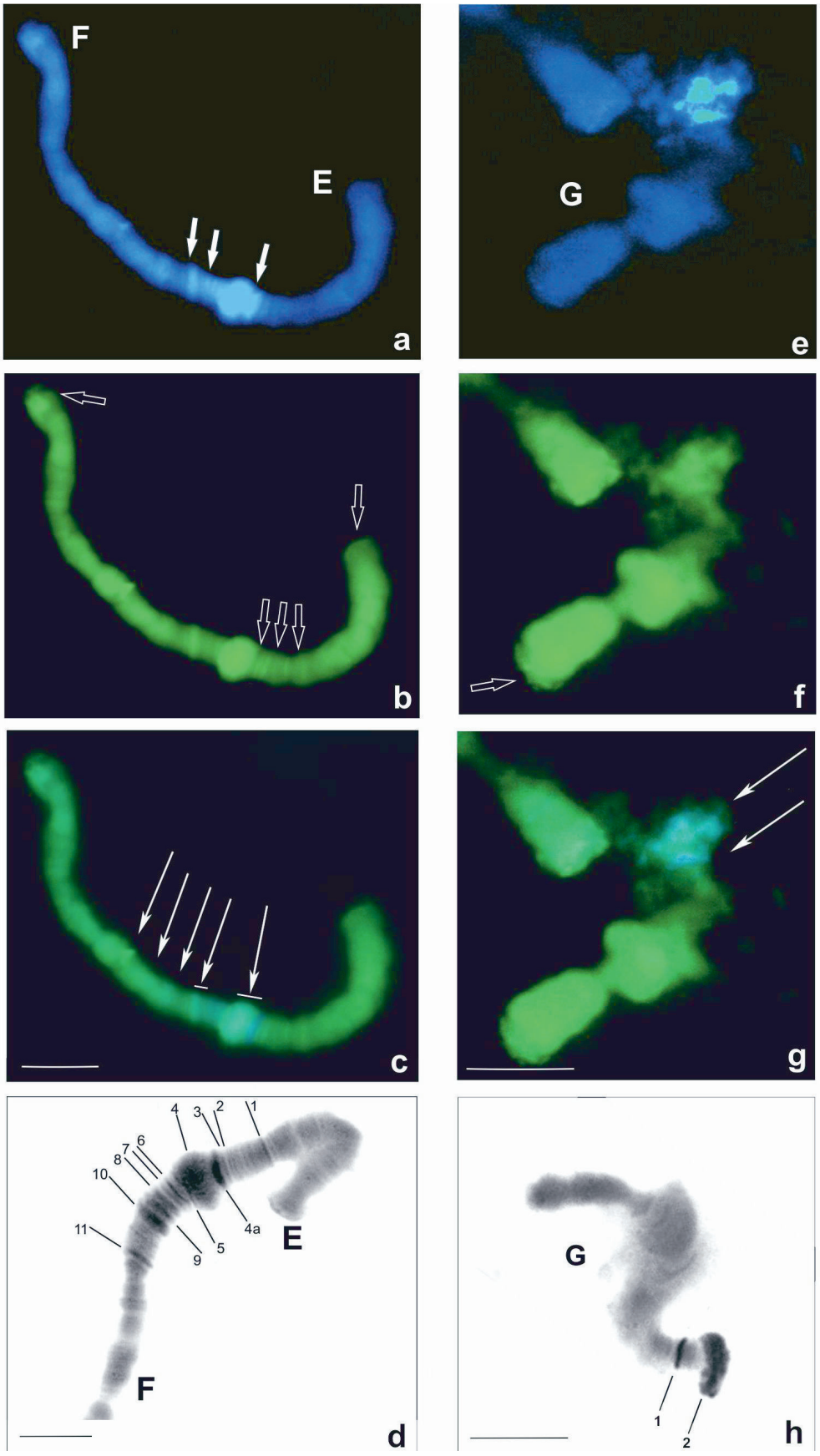

Fig. 6. Fluorochrome-stained heterochromatin and C-banding of chromosomes EF (a-d) and G (e-h) of *Chironomus riparius*; DAPI (blue) (a, e), CMA_3 (green) (b, f) and double DAPI/CMA_3 (c, g). Small open arrows indicate localization of CMA_3+ bands (b) and small solid arrow marks a DAPI+ band (a); long arrows indicate a site rich in both AT and GC base pairs (c, g). Chromosomes with C bands indicated by numbers (d, h). Bar = 10 μm.

Table 2

Localization of constant C-bands and base-specific fluorochromes in polytene chromosomes of *C. riparius*

Conditional numbers	Chromosome AB			Chromosome CD			Chromosome EF			Chromosome G		
	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)	C-bands (regions)	AT-rich regions (DAPI)	GC-rich region (CMA_3)
1	D1	+	+	C1	+	+	B1	-	+	E1c	+	+
2	D1	+	+	C1	-	+	B1	-	+	E2a	+	+
3	D1	+	+	C2	-	+	B1	-	+			
4a							B2	+	-			
4	D2cd (centromere)	+	+	C2	+	+	B2ij (centromere)	+	+			
5	D3	-	+	C3egh (centromere)	+	+	B2 (m/n)	+	+			
6	D3	+	+	C4	+	+	B2	+	-			
7	E1	-	+	C4	+	+	B3	+	-			
8	E1	-	+	C5	+	-	B3 (1)	+	+			
9							B3 (2)	+	+			
10							B3 (3)	+	+			
11							B4	+	+			

Letters and numbers correspond to that of the chromosome map done by HÄGELE (1970)

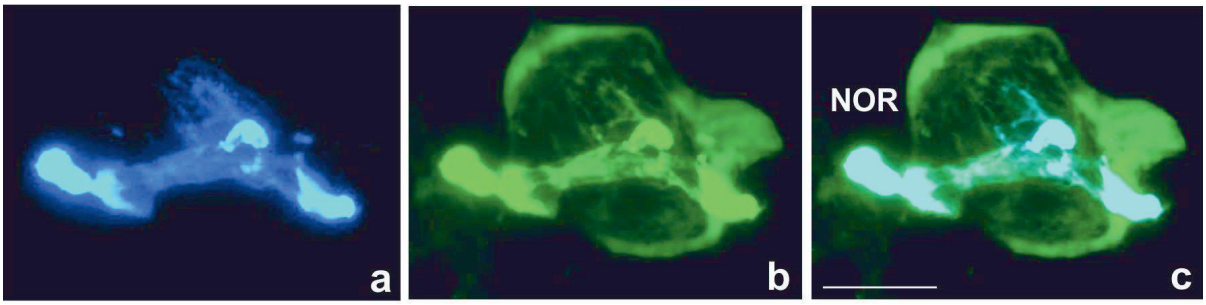

Fig. 7. Fluorochrome-stained heterochromatin of chromosome G of *Chironomus riparius*; DAPI (blue) (a), CMA_3 (green) (b) and double DAPI/CMA_3 (c). Active nucleolar organizer region (NOR) is DAPI-/CMA_3+. Bar = 10 μm.

bands of both sibling species are more brightly fluorescent after staining with CMA_3 fluorochrome. A similar phenomenon was also found in some *Drosophila* and *Sciara* species (EASTMAN *et al.* 1980). Especially interesting are the telomeres of the polytene chromosomes and the active NORs of both species containing clusters of GC base pairs without closely interspersed AT base pairs. Some studies of Orthoptera (WARCHAŁOWSKA-ŚLIWA *et al*. 2013a b) and Hymenoptera (CRISTIANO *et al*. 2014) also demonstrated NOR sites coincident with DAPI-/CMA_3+ staining. Therefore, this technique is very useful for marking the regions in chromosomes rich in GC or AT base pairs. For instance, in both species, these are the NOR sites, telomeres and some interstitial C-bands of *C. riparius* polytene chromosomes with GC compositions only.

The results reveal that despite being closely related, both species differ considerably in their DAPI and CMA_3 bright regions, and therefore, in the type and amount of heterochromatin present in their genome.

In conclusion, it is worth underlying the species-specific organization of the constitutive heterochromatin which can be used as an additional cytogenetic marker for species differentiation. The results suggest that repetitive DNA, the main component of heterochromatin, evolves in different ways in the studies species. However, this idea needs further evaluation by molecular analysis.

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