

## Cytogenetic divergence of genomes in *Chironomus plumosus* group (Diptera: Chironomidae)

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**Abstract.** The phylogenetic relationships and karyotype differentiation of 14 sibling species of *Chironomus plumosus* group were studied using comparative analysis of polytene chromosome banding sequences. Revision of banding sequence pools has been made for all species. Main and alternative banding sequences of 6 chromosomal arms (A, B, C, D, E, and F) of each species were used for the analysis. In total 133 banding sequences were analysed. Phylogenetic trees for each arm were constructed on the basis of break point numbers obtained from the pairwise comparison of banding sequences in the arm. Analysis of divergence of banding sequences in individual chromosomal arms has shown that different arms make an uneven contribution to differentiation of the *Ch. plumosus* group. Considering the level of divergence and the input of each chromosomal arm into karyotype differentiation of the *Ch. plumosus* group, the arms could be arranged as:  $E < F < A \leq B < D (\leq G) \leq C$ . To analyse the phylogenetic relationships of species of *Ch. plumosus* group integral phylogenetic NJ trees were constructed on the basis of combined data on separate chromosomal arms. It was shown that *Chironomus* sp. K, *Ch. agilis*, and *Ch. sp. prope agilis* are the most divergent species in the *Ch. plumosus* group. Among the other species *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J also show high level of divergence, as they form separate subcluster. To evaluate how the number of arms used for tree construction influences its topology, 3-arm (A, E, F), 5-arm (A, C, D, E, F) and 6-arm (A, B, C, D, E, F) trees were constructed. The study shows that the combination of data on 5 out of 7 chromosomal arms (about 70% of the genome) provides rather reliable results.

**Key word:** *Chironomus*, *Ch. plumosus* group, divergence, evolution, chromosome, karyotype, banding sequence, phylogenetic tree.

### INTRODUCTION

The study of cytogenetic divergence of genomes in groups of sibling species is of great interest for the understanding of chromosomal evolution patterns and evaluation of the role of chromosomal rearrangements in speciation, as such species are in the beginning of their divergence. Usually, karyotypes of sibling species differ by only

a few chromosomal rearrangements (first of all paracentric inversions). This allows to determinate exact positions of inversion break points and patterns of change of gene orders.

It is well known that the formation of sibling-species groups is a common phenomenon in the genus *Chironomus*. Such species have minor or no differences in morphology, but can be easily



distinguished by their karyotypes (Keyl, 1962; Martin, 1979; Wülker, 1980; Kiknadze et al., 1991, 1996). Due to the presence of polytene chromosomes with highly conserved banding patterns, it is possible to recognize homologous regions of chromosomes in karyotypes of all *Chironomus* species. It is also important that a unified mapping system of polytene chromosomes has been created for this genus (Keyl, 1962; Devai et al., 1989), which allows the reconstruction of the ways of divergence of chromosomal banding sequences during speciation.

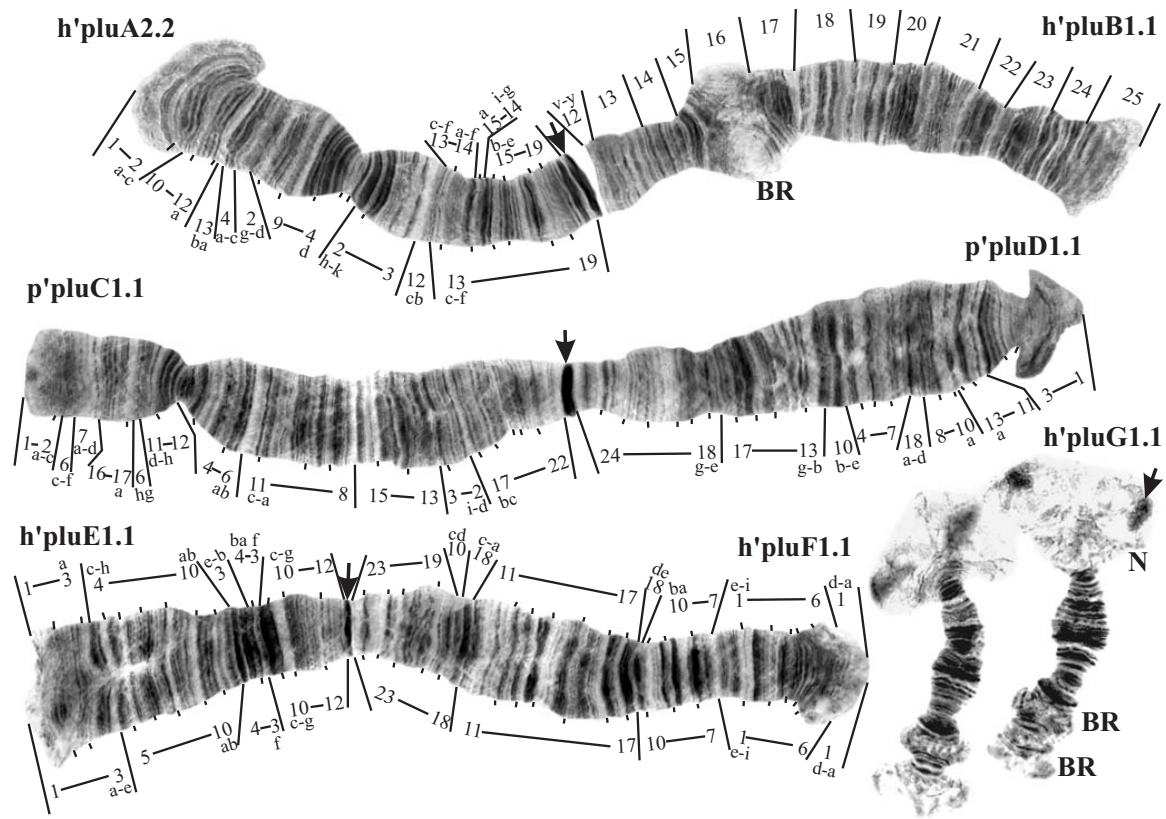
Earlier, the analysis of karyotype divergence in the genus *Chironomus* was performed based on the data for 3 out of 7 chromosomal arms – A, E, and F (Keyl, 1962; Martin et al., 1974; Martin, 1979; Wülker, 1980; Wülker et al., 1989; Shobanov, Zotov, 2001; Shobanov, 2002) that represents about 40% of the genome. Recently, mapping of arms C and D was developed and the analysis of banding sequence divergence in 5 out of 7 chromosomal arms (about 70% of the genome) became possible (Kiknadze et al., 2004a, b; Gunderina et al., 2005a, b). It was demonstrated that phylogenetic trees, constructed by the Neighbour-joining (NJ) method on the basis of the number of break points between different inversion banding sequences, show a high correlation with taxonomical phylogenies, based on morphological or genetic data (Kiknadze et al., 2003). Comparisons of 3- and 5-arm based phylogenetic trees have shown that addition of data on 2 chromosomal arms essentially improves the reliability of species clusterization (Kiknadze et al., 2004b; Gunderina et al., 2005b).

In all these studies, only the main banding sequences, i.e. banding sequences, have the highest frequencies in species populations and are most important for divergence of species karyotypes, were used. However, it is well known that many *Chironomus* species are highly polymorphic and the banding sequence pools of some species (i.e. the total pool of banding sequences found in all

populations of the species) can include up to 60 banding sequences (Kiknadze et al., 2004b). In such cases, the banding sequence pool includes fluctuating banding sequences (alternative, rare, and unique) in addition to main banding sequences (Gunderina et al., 1999; Golygina, 1999; Gunderina, 2001). Thus, it is important to evaluate the influence of fluctuating banding sequences on phylogenetic constructions when they are added to phylogenetic analysis. To conduct this study, the *Ch. plumosus* group was chosen, as it is the largest and the best studied group in *Chironomus*. At present, the *Ch. plumosus* group includes 14 species from different Holarctic regions: *Ch. agilis* Shobanov, Djomin, 1988, *Ch. sp. prope agilis* (= *Ch. agilis* 2 sensu Kiknadze et al., 1991), *Ch. balatonicus* Devai, Wülker, Scholl, 1983, *Ch. bonus* Shilova, Dzhvarsheishvili, 1974, *Ch. borokensis* Kerkis et al., 1988, *Ch. entis* Shobanov, 1989, *Ch. muratensis* Ryser, Scholl, Wülker, 1983, *Ch. nudiventris* Ryser, Scholl, Wülker, 1983, *Ch. plumosus* (Linnaeus, 1758), *Ch. sinicus* Kiknadze et al., 2005, *Chironomus* sp. J sensu Kiknadze et al., 1991, *Chironomus* sp. K sensu Golygina, Ueno, 2005, *Ch. suwai* Golygina, Martin, 2003, *Ch. usenicus* Loginova, Belyanina, 1994; three of them – *Ch. sinicus* from China and *Ch. suwai* and *Chironomus* sp. K from Japan – were described only recently (Golygina et al., 2003, Golygina, Ueno, 2005; Kiknadze et al., 2005) and were not included in phylogenetic analysis. It is significant that 6 out of 7 chromosomal arms, used in this study – A, B, C, D, E, and F – represent 90% of the genome.

## MATERIAL AND METHODS

Data on chromosomal polymorphism in all sibling species of the *Ch. plumosus* group from the Holarctic region were used. Most species have 4 chromosomes: two metacentric (AB, CD), one submetacentric (EF), and the short telocentric (G) (Fig. 1). Thus all species have 7 chromosomal



**Fig. 1.** Karyotype of *Chironomus plumosus*. h'pluA2.2, h'pluB1.1 etc. - genotypic combinations of banding sequences, BR - Balbiani ring, N - nucleolus; arrows indicate centromeric bands. Mapping in Keyl-Devai mapping system is shown below arms (arms A, E, and F are mapped according to Keyl (1962), arms C and D – according to Butler et al. (1999)). Our revised version of the mapping of arms A, E and F in Keyl-Devai mapping system is shown above the arms. Arm B is mapped in the Maximova-Shobanov mapping system according to Shobanov (1994a).

arms. Karyotypes of species differ mainly by paracentric inversions in different chromosomal arms, but other chromosomal rearrangements, such as changes in centromeric heterochromatin size (*Ch. borokensis*, *Ch. sp. prope agilis*, *Chironomus sp. K*) or tandem fusion of chromosomes (*Ch. nudiventris*), also occurred during evolution of the *Ch. plumosus* group (Kiknadze et al., 1991, 1996).

Most of banding sequences used in this study have been described previously (Dyomin, Schobanov, 1990; Kiknadze et al., 1991, 2000, 2004b; Michailova, Petrova, 1991; Shobanov,

1994b; Golygina, 1999; Butler et al., 1999; Golygina, Kiknadze, 2001). Each banding sequence in each chromosomal arm is numbered according to the order of its discovery, and these numbers prefixed by an abbreviation of the species name (for example, agi – for *Ch. agilis*, bal – for *Ch. balatonicus* etc.), with a further prefix p' for Palearctic sequences, n' for Nearctic sequences, or h' for Holarctic sequences (e.g. p'agiA1, n'entC3, h'pluE1, etc.).

Mapping of banding sequences in arms A, C, D, E, and F was performed according to the Keyl-Devai mapping system (Keyl, 1962; Devai et al.,

1989). This system and that of Maximova-Shobanov (Maximova, 1976; Shobanov, 1994) were used for mapping of arm B. Revision of mapping of banding sequences in *Ch. plumosus* group will be published elsewhere.

The calculation of break point numbers between banding sequence pairs, estimation of similarity of banding sequences, and construction of phylogenetic trees was done as described previously (Kiknadze et al., 2003; Gunderina et al., 2005a).

Banding sequences of each species were classified into four categories (Gunderina et al., 1999; Golygina, 1999):

1. **Main banding sequences** occur in each population and have the highest frequency in all or most populations. The number of main banding sequences is the same in all species and is equal to the number of chromosomal arms (7). They could be found in homo- or heterozygous states.

2. **Alternative banding sequences** have a wide range of occurrence. They could be found in both homo- and heterozygous states and become dominant in some populations.

3. **Rare banding sequences** could be found in only some populations, in the heterozygous state only, and had low frequencies. Different rare sequences could be recorded in different populations.

4. **Unique banding sequences** were found only once in one of the species populations.

The main and alternative banding sequences of *Ch. plumosus* group species were chosen for this study. The combined banding sequence pool of *Ch. plumosus* group contains 22 main and alternative banding sequences of arm A, 20 those of arm B, 18 – of arm C, 22 – of arm D, 18 – of arm E, and 17 – of arm F.

Main banding sequences of *Chironomus piger* were used as outgroups for construction of phylogenetic trees, as they have been chosen as a standard for the genus *Chironomus* in Keyl-Devai mapping system.

## RESULTS

Revision of banding sequence pools has been made for all 14 sibling species of the *Ch. plumosus* group. The data on the number of banding sequences found in the banding sequence pools of different species are presented in Figure 2. The highest number of banding sequences has been found in the best studied species of the group – *Ch. plumosus* (58), *Ch. entis* (54), and *Ch. balatonicus* (54). The fourth position is occupied by *Ch. muratensis* (26 banding sequences). Lesser numbers of banding sequences have been found in *Ch. borokensis* (19), *Ch. nudiventris* (19), *Ch. usenicus* (16), and *Chironomus* sp. J (15). Other species have 7 to 11 banding sequences in their banding sequence pools.

The number of main banding sequences is the same for all species (7) and is equal to the number of chromosomal arms (linkage groups) (Table 1 and Fig. 2). Alternative banding sequences can be found only in some species and not in all chromosomal arms. At the same time several alternative banding sequences can occur in one chromosomal arm (Table 1). In the *Ch. plumosus* group 8 species have alternative banding sequences, the highest number of these sequences (8) was found in *Ch. entis*. *Ch. plumosus* and *Ch. nudiventris* have 6 alternative banding sequences, *Ch. balatonicus* and *Ch. borokensis* – 5, *Ch. muratensis* and *Ch. usenicus* – two, and *Ch. agilis* – one alternative banding sequence.

As a total, 117 main and alternative banding sequences from 6 chromosomal arms (A, B, C, D, E, and F) were used for analysis of karyotype divergence within the *Ch. plumosus* group (Table 1). The seventh arm – G – is not mapped completely due to the presence of many complex chromosomal rearrangements.

**Arm A** has 22 main and alternative banding sequences in the combined banding sequence pool of *Ch. plumosus* group species (Table 1). Phylogenetic relationships of banding sequences in arm



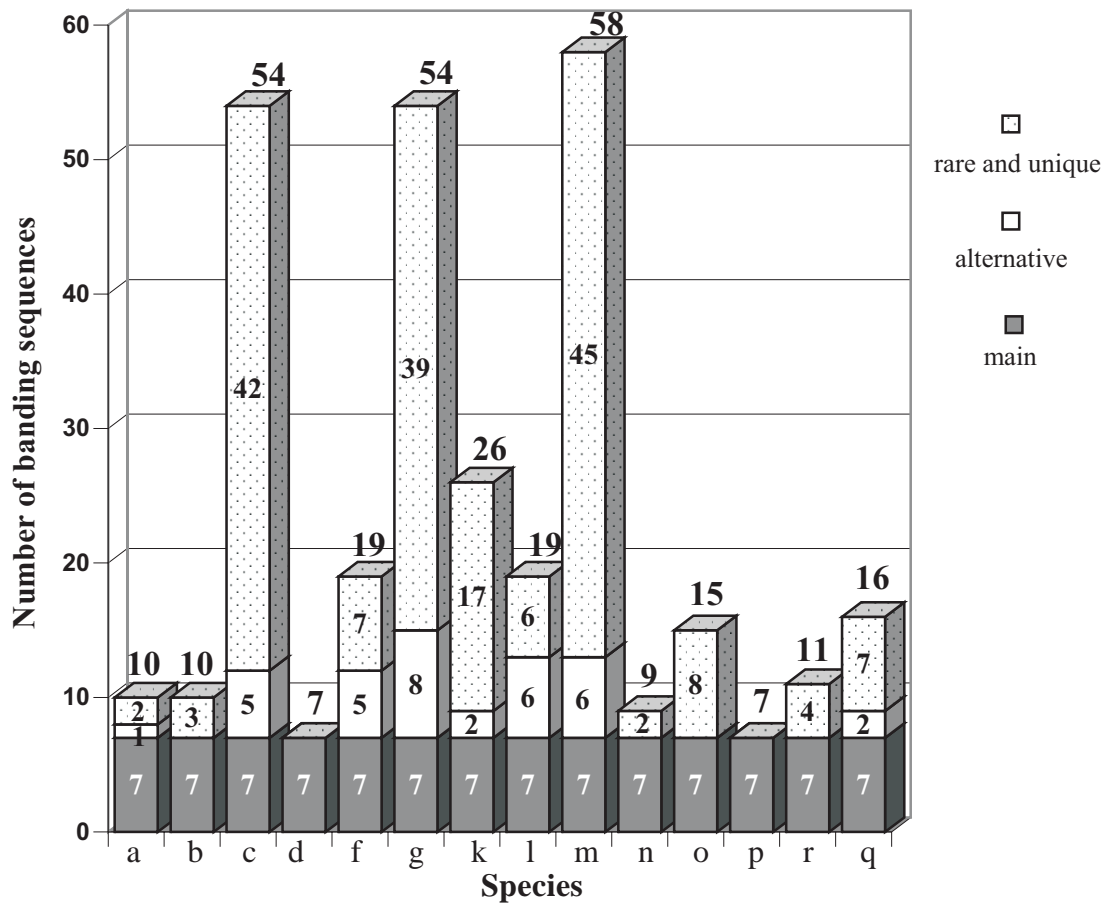
**Table 1.** Banding sequences, which have been used for phylogenetic analysis in the present paper.

\*Banding sequences are denoted by symbols, which include zoogeographic distribution (p' - Palearctic, n' - Nearctic, h' - Holarctic), symbol of species (agi - *C. agilis*, bal - *C. balatonicus* etc.), symbol of arm (A, B, C etc.) and serial number of inversion sequence in the arm.

Species	Category of banding seq.	Arm						
		A	B	C	D	E	F	G
<i>Ch. agilis</i>	main	p'agiA1*	p'agiB1	h'agiC1	p'agiD1	h'agiE1	p'agiF1	p'agiG1
	alternative		p'agiB2					
<i>Ch. sp. prope agilis</i>	main	p'ag2A1	p'ag2B1	p'ag2C1	p'ag2D1	h'ag2E1	p'ag2F1	p'ag2G1
<i>Ch. balatonicus</i>	main	p'balA1	p'balB1	p'balC1	p'balD2	p'balE1	p'balF1	h'balG1
	alternative	p'balA2		p'balC2	p'balD1 p'balD8			p'balG2
<i>Ch. bonus</i>	main	h'bonA1	h'bonB1	p'bonC1	p'bonD1	h'bonE1	h'bonF1	h'bonG1
<i>Ch. borokensis</i>	main	p'borA1	p'borB1	p'borC1	p'borD1	h'borE1	h'borF1	p'borG1
	alternative	p'borA2	h'borB2		h'borD2		p'borF2	p'borG2
<i>Ch. entis</i>	main	p'entA1	p'entB1	p'entC2	h'entD1	h'entE1	h'entF1	h'entG1
	alternative	p'entA2 h'entA4 h'entA11		p'entC1 n'entC3	n'entD4	h'entE2	n'entF4	
<i>Ch. muratensis</i>	main	p'murA1	h'murB1	p'murC1	p'murD2	h'murE1	h'murF1	p'murG1
	alternative		p'murB3		p'murD1			
<i>Ch. nudiventris</i>	main	p'nudA1	p'nudB1	p'nudC1	h'nudD1	h'nudE1	h'nudF1	p'nudG1
	alternative	h'nudA2	h'nudB3 p'nudB4		p'nudD2	h'nudE2	p'nudF2	
<i>Ch. plumosus</i>	main	h'pluA2	h'pluB1	p'pluC1	p'pluD1	h'pluE1	h'pluF1	h'pluG1
	alternative	p'pluA1 n'pluA9	h'pluB2	h'pluC2	h'pluD2	h'pluE2		
<i>Ch. sinicus</i>	main	p'sinA1	p'sinB1	p'sinC1	p'sinD1	h'sinE1	p'sinF1	p'sinG1
<i>Ch. sp. J</i>	main	h'spJA1	p'spJB1	p'spJC1	h'spJD1	h'spJE1	h'spJF1	p'spJG1
<i>Ch. sp. K</i>	main	p'spKA1	p'spKB1	p'spKC1	p'spKD1	h'spKE1	p'spKF1	p'spKG1
<i>Ch. suwai</i>	main	h'suwA1	p'suwB1	p'suwC1	h'suwD1	h'suwE1	p'suwF1	p'suwG1
<i>Ch. usenicus</i>	main	p'useA1	h'useB1	h'useC1	p'useD1	p'useE1	p'useF1	h'useG1
	alternative				h'useD2	h'useE3		

A are shown in Fig. 3, a. Banding sequences of one species are marked by the same colour. Main banding sequences are typed in bold. Homologous banding sequences of different species are enclosed in boxes, hence if a box contains only one banding sequence it means that the sequence is species-specific, i.e. there are no homologous sequences in the sequence pools of other species. As shown in Figure 3a, only 3 species – *Ch. balatonicus*, *Ch. entis*, and *Ch. muratensis* – have species-specific main banding sequences. The main

sequence of *Ch. nudiventris* (p'nudA1) has no homologous banding sequences among the main and alternative sequences of the other species, but it is identical to one of the rear banding sequences of *Chironomus* sp. J and thus could not be considered as species-specific. Six species have alternative banding sequences in arm A (Table 2). Only two alternative sequences – p'balA2 and h'entA11 – are species-specific (Fig. 3, a). Most of the banding sequences fall into 4 groups with 3 or 4 homologous banding sequences in each (Fig.



**Fig. 2.** Volumes of banding sequence pools in different species of the *Ch. plumosus* group. Black areas mark main banding sequences, blank areas mark alternative banding sequences, and dotted areas mark rare and unique banding sequences. **a** - *Ch. agilis*. **b** - *Ch. sp. prope agilis*. **c** - *Ch. balatonicus*. **d** - *Ch. bonus*. **f** - *Ch. borokensis*. **g** - *Ch. entis*. **k** - *Ch. muratensis*. **l** - *Ch. nudiventris*. **m** - *Ch. plumosus*. **n** - *Ch. sinicus*. **o** - *Chironomus* sp. **j** - *Chironomus* sp. **k**. **r** - *Ch. suwai*. **q** - *Ch. usenicus*.

3, a). Any banding sequence in arm A could be derived from its closest neighbour by one inversion (number of inversion steps between banding sequences indicated by numbers near lines that connect neighbouring boxes). The most diverged p'balA2 and p'entA1 differed by 6 inversions.

Homologous p'agiA1, p'ag2A1, p'entA2, and p'spKA1 could be considered as the most ancient among banding sequences of the *Ch. plumosus* group in arm A as they are the closest to p'pigA1. The most evolutionary fruitful was ho-

mologous h'entA4, h'spJA1, and h'nudA2, which produced 4 other groups of banding sequences (Fig. 3, a).

Two major clusters could be seen on the phylogenetic tree (Fig. 3, b), constructed on the basis of break point numbers obtained from the pairwise comparison of banding sequences in arm A. The first cluster contains the main sequences of *Ch. agilis*, *Ch. sp. prope agilis*, *Chironomus* sp. K, and one alternative sequence of *Ch. entis*. It is necessary to note that this cluster also includes

**Table 2.** Comparison of main banding sequences among members of the *Ch. plumosus* group. <sup>1</sup>Species-specific banding sequences are printed in bold, <sup>2</sup>Fixed banding sequences are enclosed in boxes. <sup>3</sup>Cen.het. = centromeric heterochromatin. Designations of banding sequences are the same as in Table 1.

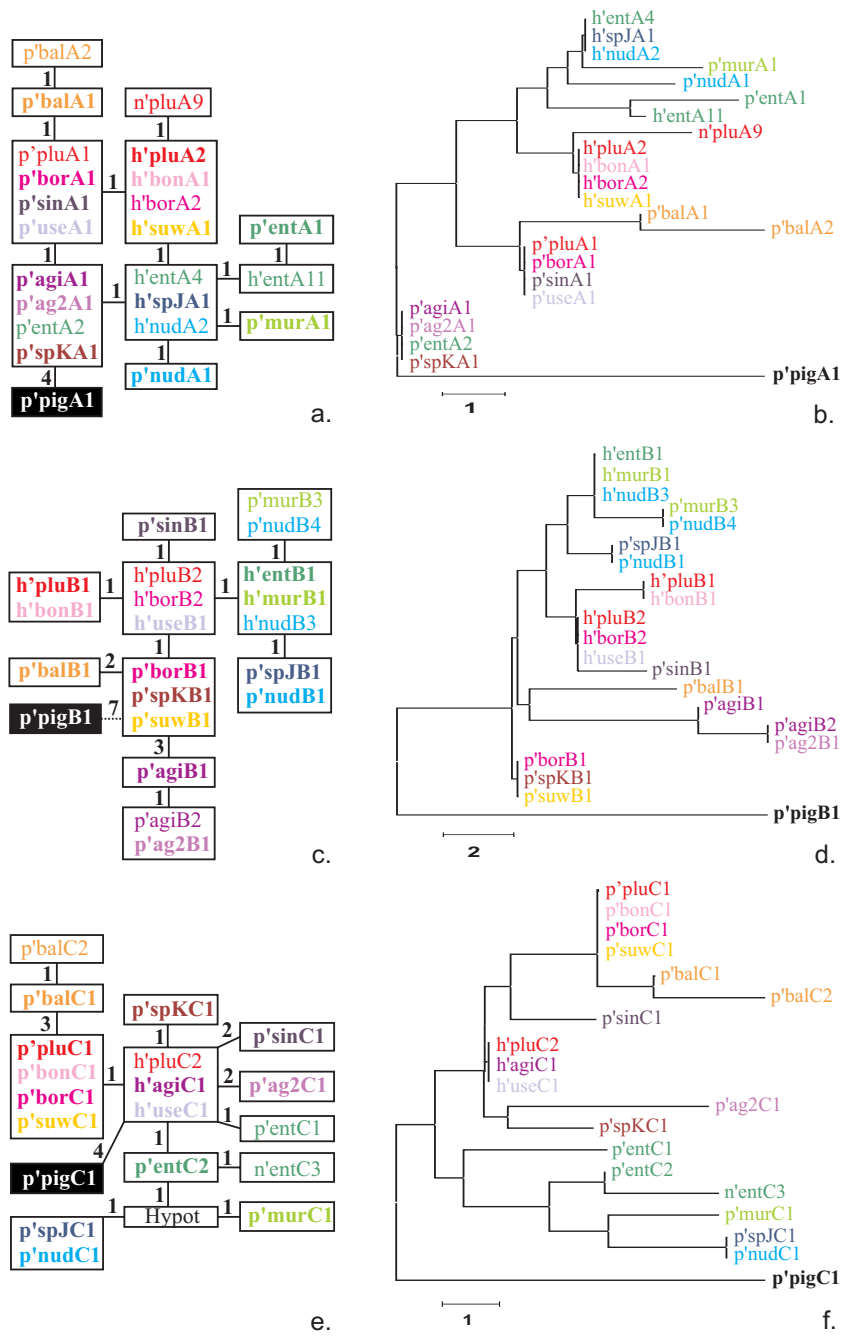
Species	Main banding sequence in arm							Other karyotype features
	A	B	C	D	E	F	G	
<i>Ch. agilis</i>	p'agiA1	<b>p'agiB1<sup>1</sup></b>	h'agiC1	p'agiD1	h'agiE1	p'agiF1	p'agiG1	
<i>Ch. sp. pr. agilis</i>	p'ag2A1	p'ag2B1	<b>p'ag2C1<sup>2</sup></b>	p'ag2D1	h'ag2E1	p'ag2F1	p'ag2G1	Large Cen.het. <sup>3</sup>
<i>Ch. balatonicus</i>	<b>p'balA1</b>	<b>p'balB1</b>	<b>p'balC1</b>	<b>p'balD2</b>	<b>p'balE1</b>	<b>p'balF1</b>	h'balG1	
<i>Ch. bonus</i>	h'bonA1	h'bonB1	p'bonC1	p'bonD1	h'bonE1	h'bonF1	h'bonG1	Fixed B-chromosome, homologs of arm G are tightly paired
<i>Ch. borokensis</i>	p'borA1	p'borB1	p'borC1	p'borD1	h'borE1	p'borF1	<b>p'borG1</b>	Large Cen.het.
<i>Ch. entis</i>	<b>p'entA1</b>	h'entB1	<b>p'entC2</b>	h'entD1	h'entE1	h'entF1	<b>h'entG1</b>	
<i>Ch. muratensis</i>	<b>p'murA1</b>	h'murB1	<b>p'murC1</b>	<b>p'murD2</b>	h'murE1	h'murF1	<b>p'murG1</b>	Additional nuclei in arm C
<i>Ch. nudiventris</i>	p'nudA1	p'nudB1	p'nudC1	h'nudD1	h'nudE1	h'nudF1	p'nudG1	Fusion of arms E and G (2n=6)
<i>Ch. plumosus</i>	h'pluA2	h'pluB1	p'pluC1	p'pluD1	h'pluE1	h'pluF1	h'pluG1	
<i>Ch. sinicus</i>	p'sinA1	<b>p'sinB1</b>	<b>p'sinC1</b>	<b>p'sinD1</b>	h'sinE1	<b>p'sinF1</b>	<b>p'sinG1</b>	
<i>Ch. sp. J</i>	h'spJA1	p'spJB1	p'spJC1	h'spJD1	h'spJE1	h'spJF1	p'spJG1	
<i>Ch. sp. K</i>	p'spKA1	p'spKB1	<b>p'spKC1</b>	<b>p'spKD1</b>	h'spKE1	p'spKF1	<b>p'spKG1</b>	Large Cen.het.
<i>Ch. suwai</i>	h'suwA1	p'suwB1	p'suwC1	h'suwD1	h'suwE1	p'suwF1	p'suwG1	
<i>Ch. usenicus</i>	p'useA1	h'useB1	h'useC1	p'useD1	<b>p'useE1</b>	p'useF1	h'useG1	Large Cen.het.

p'pigA1. This confirms that the above-mentioned banding sequences are the most ancient in the *Ch. plumosus* group. But it also indicates that divergence between the banding sequences of the *Ch. plumosus* group and p'pigA1 is not so strong and the latter is not to be used as the outgroup in phylogenetic trees.

The second cluster is much larger than the first one and consists of two subclusters. One subcluster contains all of the banding sequences of *Ch. balatonicus*, *Ch. sinicus*, *Ch. usenicus*, and some banding sequences of *Ch. borokensis* and *Ch. plumosus*. Two second-order subclusters could be seen in another subcluster: one consists of banding sequences of *Ch. bonus*, *Ch. borokensis*, *Ch. plumosus* and *Ch. suwai*, while the other includes those of *Ch. entis*, *Ch. muratensis*,

*Ch. nudiventris*, and *Chironomus* sp. J. It is important to note that the clustering of banding sequences on the phylogenetic tree is in good accordance with the phylogram structure.

**Arm B.** Twenty main and alternative banding sequences are known in arm B (Fig. 3c). As in arm A, only 3 species have species-specific main banding sequences (Fig. 3c and Table 2). All other banding sequences (both main and alternative) have homologous sequences in banding sequence pools of other species. The maximum number of species with homologous banding sequences in arm B is three. Most sequences in neighbouring blocks of the phylogram differ from each other by simple inversions, but p'balB1 and p'agiB1 could be derived from their closest neighbour p'borB1 by 2 and 3 inversion steps, respectively (Fig. 3c).



**Fig. 3, a-f.** Phylogenetic relationships of main and alternative banding sequences in arms A, B, and C of species of the *Ch. plumosus* group. **a, c, e** - phylograms of banding sequences in arms A, B, and C, respectively. Banding sequences of one species are marked by the same colour. Main banding sequences are typed in bold. Homologous banding sequences of different species are enclosed in boxes. Numbers near lines that connect neighbouring boxes indicate numbers of inversion steps between sequences in the boxes. **b, d, f** - phylogenetic trees of banding sequences in arms A, B, and C, respectively, constructed by NJ-method on the basis of break point numbers between pairs of banding sequences.



The maximum number of inversion steps between most divergent banding sequences is seven (for example, between p'ag2B1 and p'nudB1). The main banding sequences p'borB1, p'spKB1, and p'suwB1 appear to be the most ancient in arm B, as they are the closest to p'pigB1 (7 inversion steps) (Fig. 3, c).

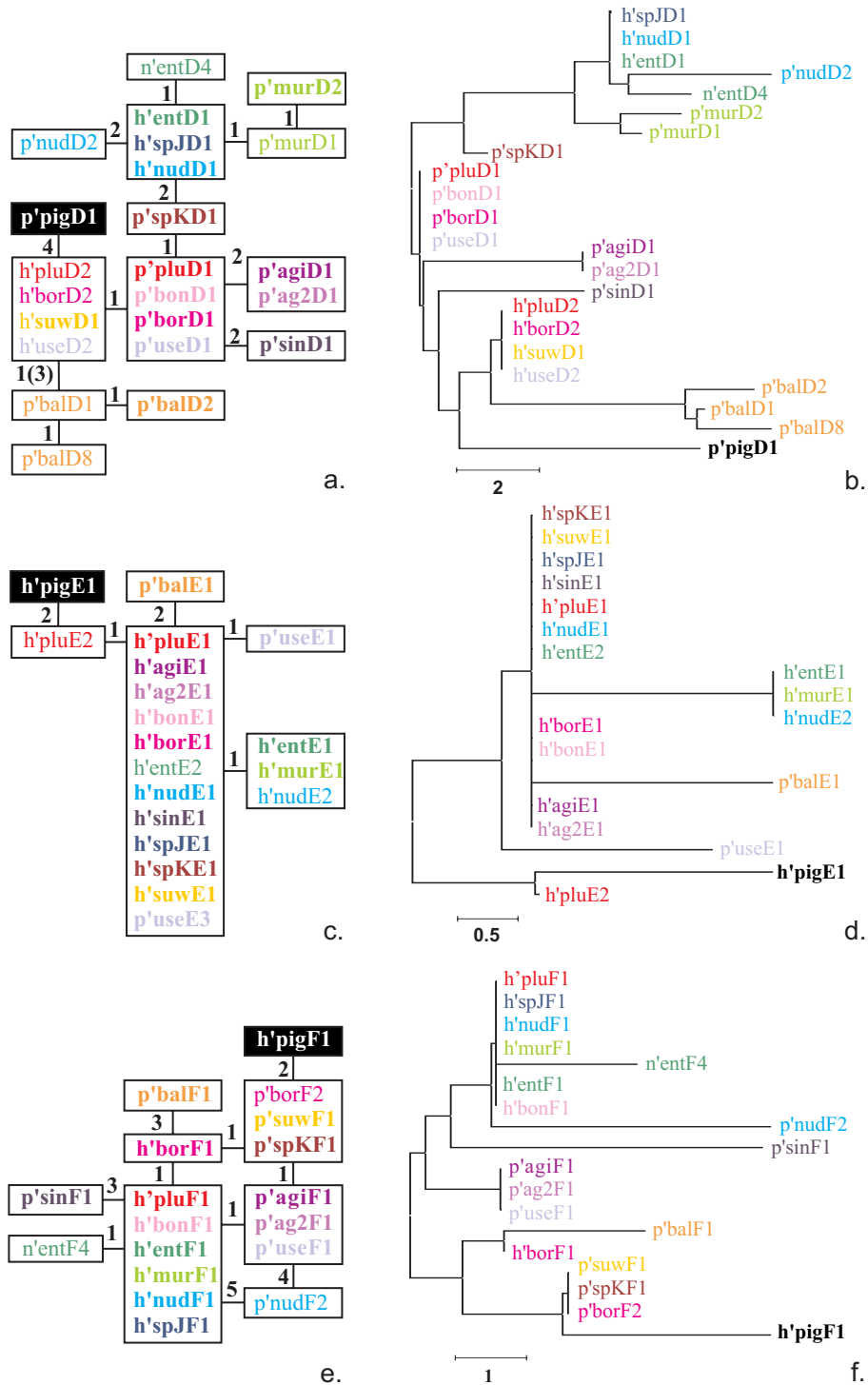
Two clusters could be seen on the phylogenetic tree (Fig. 3, d). The first cluster contains the main banding sequences of *Chironomus* sp. K, *Ch. suwai*, and *Ch. borokensis*. All other banding sequences, including the alternative p'borB2, fall into the second cluster. This cluster consists of several first- and second-order subclusters. As in arm A, banding sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J could be found in one subcluster. It is necessary to note that the banding sequences of *Ch. agilis* and *Ch. sp. prope agilis* also fall into one separate subcluster in arm B, which is not true for arm A. The clusterization of banding sequences on the phylogenetic tree is very consistent with the phylogram structure. On the phylogenetic tree (Fig. 3, d), p'pigB1 lies separately from the other banding sequences, i.e. the extent of divergence between the *Ch. plumosus* group and the *Ch. piger* banding sequences is much higher in arm B than in arm A and thus, p'pigB1 could be used as an outgroup.

**Arm C** has 18 main and alternative banding sequences (Fig. 3, e). In contrast to arms A and B, half of the banding sequences in arm C (6 main and 3 alternative) are species-specific (Fig. 3e, Table 2). The maximum number of homologous banding sequences is four. Although many of arm C sequences differ from their closest neighbours by simple inversions, there is considerable number of more divergent sequences (5 out of 18). Such sequences have 2 or even 3 inversion steps to their closest neighbours. The most divergent banding sequences differ by eight inversion steps (for example, p'murC1 and p'balC2). The banding sequence h'pluC2 and its homologs appear to

be the most ancient in arm C, as they are the closest to p'pigC1 (Fig. 3, e). This group of banding sequences was also highly productive during evolution of the *Ch. plumosus* group and has produced 6 other groups of banding sequences.

On the phylogenetic tree (Fig. 3, f), all banding sequences of the *Ch. plumosus* group fall into two major clusters. The first bifurcation occurs between the banding sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, *Chironomus* sp. J and the banding sequences of all other species of the *Ch. plumosus* group. Moreover, in contrast to the situation in arms A and B, the banding sequences of *Ch. entis* separate from those of *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J. One more interesting fact is that p'entC1 also appears in this cluster (Fig. 3, f) although its ancestor is h'pluC2 and, therefore, it was logical to expect that these banding sequences would cluster together. The second cluster is divided into three subclusters; main and alternative banding sequences of *Ch. plumosus* again could be found in different subclusters as it was in arms A and B. It could be pointed out that p'pigC1 lies on the separate branch of the phylogenetic tree and thus could be used as the outgroup.

**Arm D.** There are 22 main and alternative banding sequences in arm D (Fig. 4, a). The main sequences are species-specific in 4 species, and alternative sequences are such in 5 species (Fig. 4, a, Table 2). The maximum number of homologous banding sequences in arm D is four. Most of the banding sequences, which could be found in neighbouring blocks of the phylogram, differ by simple inversions, but there are four cases where the number of inversion steps between blocks is two (Fig. 4, a). The most divergent banding sequences differ by 8 inversions. The banding sequence h'pluD2 and its homologs in other species could be considered the most ancient in the *Ch. plumosus* group (4 inversion steps from p'pigD1) (Fig. 4, a). However, two other groups of banding sequences (homologous to p'pluD1



**Fig. 4, a-f.** Phylogenetic relationships of main and alternative banding sequences in arms D, E, and F of species of the *Ch. plumosus* group. **a, c, e** - phylograms of banding sequences in arms D, E, and F, respectively. Designations are the same as in Fig. 3. **b, d, f** - phylogenetic trees of banding sequences in arms D, E, and F, respectively.

and h'entD1) appeared to be most evolutionarily fruitful.

As can be seen on the phylogenetic tree (Fig. 4, b), all banding sequences of arm D are combined into two main clusters. As in arms A, B, and C, the banding sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J fall into one separate cluster. In arm D, however, they are combined with p'spKD1 because it is in fact the common ancestor for this group of sequences. The second cluster contains all other banding sequences of the *Ch. plumosus* group species and p'pigD1, which means that the latter could not be used as an outgroup in this arm (Fig. 4, b). This cluster in turn is divided into several subclusters of different orders. It is necessary to note that the main and alternative banding sequences of both *Ch. borokensis* and *Ch. plumosus* fall into two different subclusters, as observed in all previously described arms.

**Arm E.** There are 18 main and alternative banding sequences in arm E (Fig. 4, c). The main banding sequences of 10 out of 14 species are homologous to h'pluE1. Only *Ch. balatonicus* and *Ch. usenicus* have species-specific main banding sequences, which differ from the banding sequences of the main block by complex and simple inversion, respectively (Fig. 4, c, Table 2). The maximum number of inversion steps between banding sequences in arm E is three.

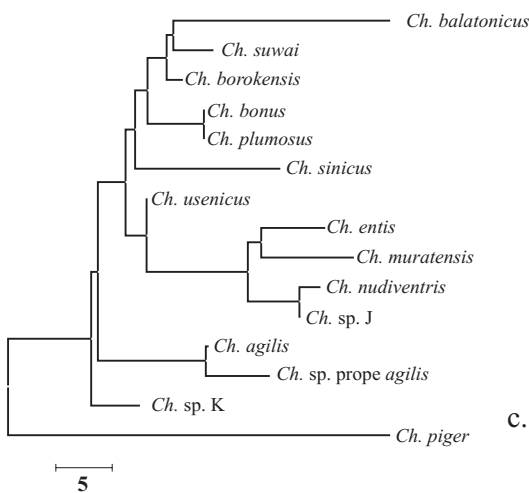
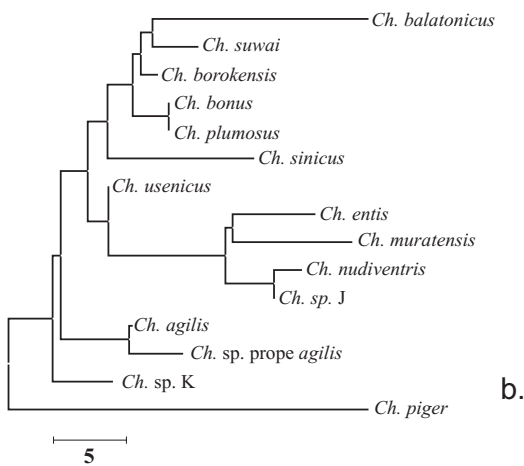
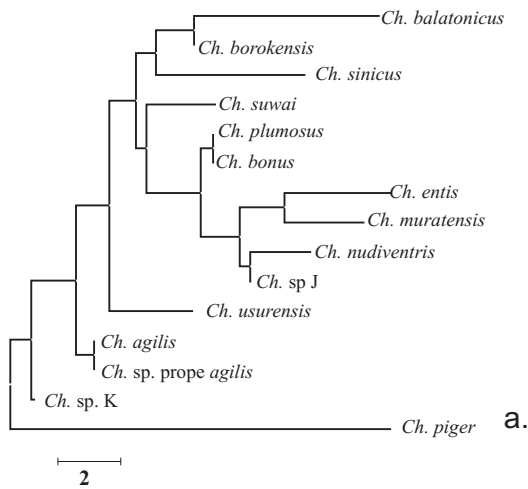
As could be expected from the phylogram, virtually all banding sequences fall into one cluster on the phylogenetic tree (Fig. 4, d), with p'useE1 lying as a separate branch. Only one banding sequence – h'pluE2 – is not included in this cluster and forms the second cluster on the tree with h'pigE1.

**Arm F** has 17 main and alternative banding sequences (Fig. 4, e). Species-specific main banding sequences had been found only in *Ch. balatonicus*, *Ch. borokensis*, and *Ch. sinicus*, but two of these sequences – p'balF1 and p'sinF1 – are complex inversions and differ from their ancestors

by 3 inversion steps (Fig. 4e, Table 2). Most of the banding sequences fall into three blocks. The largest contains 6 main sequences. It is also necessary to note that the alternative p'nudF2 in fact is closer to the main banding sequence of *Ch. agilis* p'agiF1 (4 inversion steps) than to the main banding sequence of *Ch. nudiventris* h'nudF1 (5 inversions) (Fig. 4, e). Such a number of inversions between main and alternative sequences of the same species is very unusual and has not been found in any other arm. Arm F also is the only chromosomal arm where the distance between the banding sequence of *Ch. piger* and its closest neighbour from the *Ch. plumosus* group (p'borF2 and homologous p'suwF1 and p'spKF1) is smaller than distance between some of the neighbouring banding sequences inside the *Ch. plumosus* group. For example, h'pigF1 and p'borF2 differ only by 2 inversions, while h'pluF1 and p'sinF1 – by 3, and h'nudF1 and p'nudF2 – by 5 inversions (Fig. 4, e). The maximum number of inversion steps between banding sequences in arm F is eight (between p'sinF1 and p'nudF2).

Two main clusters could be seen on the phylogenetic tree of arm F (Fig. 4, f). The first one contains banding sequences of *Ch. balatonicus*, *Ch. borokensis*, *Chironomus* sp. K, *Ch. suwai*, and the banding sequence h'pigF1 (which indicates that it cannot be used as an outgroup). The second cluster separates into two subclusters: one with the banding sequences of *Ch. agilis*, *Ch. sp. prope agilis*, and *Ch. usenicus*, and the other with the banding sequences of *Ch. bonus*, *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, *Ch. plumosus*, and *Chironomus* sp. J. Except for arm E, which has very low divergence between banding sequences, this is the only other arm where sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J do not separate from the banding sequences of other species.

So far, arm G has not been fully mapped, so it was impossible to establish exact phylogenetic relationships of the banding sequences in this arm.



It can be seen that there is a great similarity in the banding sequences of *Ch. plumosus*, *Ch. balatonicus*, *Ch. bonus*, *Ch. borokensis*, *Ch. suwai*, and *Ch. usenicus*. In fact, the main banding sequences of the first three species are identical, and the banding sequences of the others differ from them by one or two inversion steps. A comparison of all banding sequences in this arm shows that 5 species have species-specific main banding sequences (Table 2).

In addition to the analysis of banding sequence divergence in each chromosomal arm, a comparison of divergence of different species has been performed. As shown in Table 2, nine out of 14 species of the *Ch. plumosus* group have at least one arm with species-specific main banding sequences, and in 6 of these species such sequences are fixed, i.e. none of arm's banding sequences is homologous to any other banding sequence of any other species. The number of fixed species-specific banding sequences varies from one for *Ch. sp. prope agilis* to six for *Ch. balatonicus* (Table 2). It is important to note that species-specific banding sequences in chromosomes II (CD) and III (EF) of *Ch. balatonicus* are formed by complex pericentric inversions while such inversions were not recorded in other species of the *Ch. plumosus* group.

Five species, *Ch. bonus*, *Ch. nudiventris*, *Chironomus sp. J*, *Ch. plumosus*, and *Ch. suwai*, have no species-specific main banding sequences. In these cases, other types of chromosomal polymorphisms, such as a reduction in chromosome number (*Ch. nudiventris* – *Chironomus sp. J*), changes in the size of centromeric heterochromatin (*Ch. suwai* – *Ch. borokensis*), the presence of additional chromosomes, and the extent of homologous pairing (*Ch. bonus* – *Ch. plumosus*) become the main characteristics that alter species

**Fig. 5, a-c.** Integral phylogenetic trees of *Ch. plumosus*-group species, constructed by NJ-method. **a** - AEF phylogenetic tree. **b** - ACDEF phylogenetic tree. **c** - ABCDEF phylogenetic tree.

karyotypes.

To obtain a complete picture of the phylogenetic relationship of the *Ch. plumosus* group of species, integral phylogenetic trees were constructed on the basis of combined data on separate chromosomal arms. To evaluate how the number of arms used for phylogenetic tree construction influences its topology, 3-arm (A, E, F), 5-arm (A, C, D, E, F) and 6-arm (A, B, C, D, E, F) trees were constructed (Fig. 5). As can be seen in this figure, 3- and 5-arm trees (Fig. 5, a, b) differ by both the length of the branches and the clusterization of some species. For example, in the case of the 3-arm phylogenetic tree, *Ch. plumosus*, *Ch. bonus*, and *Ch. suwai* fall into one cluster with *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J, whereas in the 5-arm tree they combine with *Ch. balatonicus*, *Ch. borokensis*, and *Ch. sinicus*. However, comparisons of 5- and 6-arm trees show no significant differences in branch lengths or tree topology (Fig. 5, b, c). It is important to note that even on the 3-arm phylogenetic tree, *Ch. piger* is well-separated from the *Ch. plumosus* group, which means that it can be used as an outgroup.

On the 6-arm phylogenetic tree (Fig. 5, c), most of the *Ch. plumosus* group falls into one large cluster, with the exception of *Chironomus* sp. K, *Ch. agilis*, and *Ch. sp. prope agilis*. This cluster in turn separates into 2 subclusters: one with *Ch. balatonicus*, *Ch. bonus*, *Ch. borokensis*, *Ch. plumosus*, *Ch. suwai* and *Ch. sinicus*, the other with *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, *Chironomus* sp. J, and *Ch. usenicus*.

## DISCUSSION

Summarizing all data on the divergence of banding sequences in individual chromosomal arms, it can be concluded that different arms make an uneven contribution to differentiation of the *Ch. plumosus* group. The lowest banding sequence divergence occurs in arm E. Only 2 species have species-specific main banding sequences,

and just one of them - p'balE1 - is fixed in the species karyotype (i.e. this species has no banding sequences in arm E homologous to any banding sequence of the other species). Most of the main and alternative banding sequences in arm E are homologous.

Arm F plays the most important role in karyotype differentiation: 2 species have fixed species-specific main banding sequences, whereas a lesser number of species have homologous banding sequences (6 as compared to 12 in arm E), and some sequences differ from their ancestors by complex rearrangements.

The level of sequence divergence in arm A is slightly higher than in arm F. There are 3 species-specific main banding sequences (although only two of them are fixed) and no more than 4 species have homologous banding sequences.

The divergence in arm B appeared to be only slightly higher than in arm A. As in arm A, 3 species have species-specific main banding sequences (with only two of them fixed), but not more than 3 species (as compared to 4 in arm A) can share the same banding sequence (Fig. 3c, Table 2).

Arm G plays a considerable role in karyotype divergence: 5 main banding sequences are species-specific, with 4 of them fixed.

One of the most polymorphic arms is arm D. Although not so many species have fixed species-specific main banding sequences (4), there are many banding sequences formed by complex inversions (with 2 or 3 inversion steps to their closest neighbour on the phylogram). Finally, arm C could be considered as the most divergent arm in the *Ch. plumosus* group. Six out of 14 species have fixed species-specific main banding sequences and most of these sequences were formed by complex inversions (with 2 or 3 inversion steps to their closest known neighbour).

Thus, considering the level of divergence and the contribution of each chromosomal arm into karyotype differentiation of the *Ch. plumosus*



group, the arms can be arranged as:  $E < F < A \leq B < D (\leq G) \leq C$ .

Comparison of chromosomal divergence in the genus *Chironomus* shows that the low degree of divergence in arms A, E, and F and high divergence of arms C and D is typical for the genus as a whole (Gunderina et al., 2005b). This allows us to use the banding sequences of arms A, E, and F to reveal the phylogenetic relationships between larger taxa than sibling groups, such as cytocomplexes. However, data on 3 arms are not enough for reliable phylogenetic analysis of sibling species, so it is necessary to use data for other arms. Our study indicates that the combination of data on 5 out of 7 chromosomal arms provides rather reliable results.

On the basis of data obtained, we can conclude that two species – *Ch. plumosus* and *Ch. borokensis* – are the closest to an ancestral form of the *Ch. plumosus* group because their main and alternative banding sequences can be considered as ancestors of most of the banding sequences of the other species. The discussion about the ancestral species of the *Ch. plumosus* group has been continued for many years (Michailova, Fisher, 1986; Petrova et al., 1986; Kiknadze et al., 1989; Shobanov, 1989, 2000, 2005). Previously, it was concluded that the ancestral species of the group is *Ch. plumosus*, based on a comparative analysis of karyotype and larval morphology of the species (Michailova, Fisher, 1986; Petrova et al., 1986; Kiknadze et al., 1989; Shobanov, 1989). Later, Shobanov (2000, 2005) suggested that *Ch. borokensis* could also be considered as an ancestral species. Our data suggest that neither of these two species, in essence, can be considered as the real ancestral one because the banding sequence pools of both species lack some banding sequences that should be present in the banding sequence pool of the ancestral form. It could be assumed that the banding sequence pool of the ancestral form should have some banding sequences, which nowadays exist in the band-

ing sequence pools of *Ch. plumosus* or *Ch. borokensis*, and some banding sequences of *Ch. agilis* and *Ch. entis* in arm A. As for the size of the centromeric regions of this ancestor, it is still an open question. It is possible that the answer will be obtained after conducting detailed molecular analysis of centromeric and genomic DNA in the *Ch. plumosus* group.

Concerning the phylogenetic relationships of the other species of the *Ch. plumosus* group, it is possible to note a clear separation of species *Ch. agilis*, *Ch. sp. prope agilis* on the one hand and *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J on the other hand. Such a pattern could also be seen in phylogenetic trees constructed on the basis of isozyme (Filippova et al., 1989; Gunderina, 2001) and globin (Shobanov, 1990) analysis, confirming the high level of divergence of the species *Ch. agilis*, *Ch. sp. prope agilis*, *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, *Chironomus* sp. J from the other species of the *Ch. plumosus* group.

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