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A standard photomap of ovarian nurse cell chromosomes for the European malaria vector *Anopheles atroparvus*

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Abstract

Anopheles atroparvus is one of the main malaria vectors of the Maculipennis group in Europe. Cytogenetic analysis based on salivary gland chromosomes has been used for taxonomic and population genetic studies of mosquitoes from this group. However, a high-resolution cytogenetic map that could be useful for physical genome mapping in *An. atroparvus* is still lacking. In the present study, a high-quality photomap for the polytene chromosomes from ovarian nurse cells of *An. atroparvus* was developed. Using fluorescent *in situ* hybridization, 10 genes from the 5 largest genomic supercontigs on the polytene chromosome were localized and 28% of the genome were anchored to the cytogenetic map. The study established chromosome arm homology between *An. atroparvus* and the major African malaria vector *An. gambiae* suggesting a whole-arm translocation between autosomes of these two species. The standard photomap for ovarian nurse cell chromosomes of *An. atroparvus* constructed will be useful for routine physical mapping. This map will assist in developing a fine-scale chromosome-based genome assembly for this species and will also facilitate comparative and evolutionary genomics studies in the genus *Anopheles*.

Keywords

malaria mosquito; cytogenetic map; genome assembly

Introduction

The malaria mosquito *Anopheles atroparvus* Van Thiel (Diptera, Culicidae) belongs to the subfamily Anophelinae, genus *Anopheles*, Maculipennis group and Maculipennis subgroup (Harbach, 2004). Species from the Maculipennis group are widely distributed throughout Eurasia and North America. The group comprises 13 palearctic members of which *An. messeae* Fall, *An. sacharovi* Favre, *An. labranhiae* Fall and *An. atroparvus* are currently

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No competing interests have been declared.

considered as dominant vector species in Europe and Middle East (Sinka *et al.*, 2010). The distribution of *An. atroparvus* in Europe ranges from Britain to Ukraine and the Caucasus region in Russia (Gornostaeva & Danilov, 2002; Sinka *et al.*, 2010). Although malaria was eradicated in Europe in the mid 20th century, it has recently re-emerged in several European countries: Azerbaijan, Georgia, Kyrgyzstan, Tajikistan, Turkey and Uzbekistan (WHO, 2009). The global climate change raises concerns about a malaria re-establishment in Southern Europe (Sainz-Elipe *et al.*, 2010) and revived interest in European malaria mosquitoes including *An. atroparvus*.

Due to its epidemiological importance and the availability of a well-established colony, *An. atroparvus* was one of the 16 species of *Anopheles* chosen for the genome sequencing project (Neafsey *et al.*, 2013; Neafsey *et al.*, 2014). The goal of this sequencing project was to understand the genetic basis of the difference in vectorial capacity between members of genus Anopheles and to establish a platform for comparative genomic analyses. The assembly of the *An. atroparvus* genome is currently available for analysis through VectorBase (http://www.vectorbase.org). The *An. atroparvus* genome assembly is 224 Mb in size and is represented by 1371 supercontigs. However, the development of a highly finished genome assembly for *An. atroparvus*, suitable for comparative genomic analyses, requires further anchoring, ordering and orienting the genomic supercontigs on chromosomes.

Two cytogenetic maps for the salivary gland polytene chromosomes of *An. atroparvus* have been developed: a drawn map (Kitzmiller *et al.*, 1967) and a photomap (Stegnii & Kabanova, 1978). These maps have been used to establish a standard chromosome nomenclature for the Maculipennis subgroup and to reconstruct a chromosome phylogeny between the Palearctic members of Macilipennis group (Stegnii, 1981). The comparison of the chromosome banding patterns of different species discovered the presence of fixed overlapping inversions. These analyses also identified homosequential species, which have the same banding pattern of chromosomes but differ from each other by the amount and morphology of heterochromatin (Sharakhova *et al.*, 1997). According to the chromosome phylogeny, homosequentional species *An. atroparvus* and *An. labranhiae* are basal members of the Maculipennis subgroup (Stegnii, 1981). This observation was confirmed by molecular phylogenetic analysis of enzymes (Bullini & Coluzzi, 1982) and the internal transcribe ribosomal spacer 2 (ITS2) (Marinucci *et al.*, 1999; Kampen, 2005). However, an additional ITS2 comparison of mosquito samples from multiple populations in Iran revealed some contradictions with the original phylogeny (Djadid *et al.*, 2007) and urged further validation. Molecular phylogenies usually reflect a high degree of genetic similarities related to the ancestral polymorphism and introgression, and they often contradict each other (Besansky *et al.*, 1994; Besansky *et al.*, 2003; White *et al.*, 2011). The development of a robust species phylogeny requires a combination of the chromosome inversion data (Kamali *et al.*, 2012) and molecular data (O'Loughlin *et al.*, 2014) with known location on the physical genome map. The availability of the genomic sequences for the species of Anopheles provides an opportunity to perform whole-genome phylogenetic analyses. However, the absence of high-quality cytogenetic photomaps for some of the species remains an impediment to physical genome mapping.

Comparative chromosome analysis revealed striking differences among Palearctic members of the Maculipennis group in the amount and morphology of pericentromeric heterochromatin and in spatial organization of the chromatin inside the nucleus (Sharakhova *et al.*, 1997; Stegniy, 1993). This observation led to a conclusion that reorganization of the three-dimensional chromatin structure is important for speciation in mosquitoes (Stegnii, 2006; Artemov *et al.*, 2010). Microdissection and sequencing of heterochromatin from the centromeric area of chromosome 2 in *An. atroparvus* demonstrated the presence of genes and repetitive elements in this region (Grushko *et al.*, 2004). Interspecific fluorescent *in situ* hybridization (FISH) of DNA clones from the microdissected region revealed rapid evolution of heterochromatic sequences among members of the Maculipennis group (Grushko *et al.*, 2009). Availability of the high-quality cytogenetic photomap for *An. atroparvus*, a basal species of the Maculipennis group, will stimulate further investigation of heterochromatin evolution and speciation in this group of mosquitoes.

The present study aims to construct the first cytogenetic photomap for the polytene chromosomes from ovarian nurse cells of *An. atroparvus*. The map will use phase-contrast digital images of unstained chromosomes.

Materials and methods

Mosquito strain and ovary preservations

Anopheles atroparvus mosquitoes were obtained from a laboratory colony hosted in the Tomsk State University, Russia. Adult females were fed on guinea pigs. Approximately 24 h post blood feeding, ovaries were pulled out and fixed in Carnoy's solution (3 ethanol : 1 glacial acetic acid by volume). Ovaries were preserved in fixative solution from 24 h up to one month at −20°C.

Chromosome preparation

For one preparation of ovarian nurse cell chromosomes, a single ovary from one pair was taken. Ovaries were held for 5 min, maturated and squashed in a drop of 50% propionic acid. The quality of the preparation was checked under an AxioImager A1 microscope (Carl Zeiss, OPTEC Company. Siberian Office, Novosibirsk, RF). High-quality preparations were then frozen in liquid nitrogen. Preparations were dehydrated in a series of ethanol (50%, 70%, 90% and 100%) and air dried.

Chromosome map development

Chromosome images were observed using an AxioImager A1 microscope with an attached CCD camera MRc5 in phase contrast using AxioVision rel. 4.7.1 software (OPTEC Company. Siberian Office, Novosibirsk, RF). Images were combined, straightened, shaped and cropped using AdobePhotoshop CS2 (George *et al.*, 2010; Artemov & Stegnii, 2011). Chromosome nomenclature was adopted from previously published chromosome maps for salivary glands of *An. atroparvus* (Stegnii & Kabanova, 1978) and ovarian nurse cell chromosomes of *An. messeae* (Stegnii & Sharakhova, 1991).

Fluorescent in situ hybridization

For the probe preparation, gene-specific primers were designed to amplify unique exon sequences from the beginning and end of each of 5 supercontigs using the Primer3 software (v.0.4.0) (Rozen & Skaletsky, 2000). Primer design was based on gene annotations from *An. atroparvus* genome assembly AatrE1 (Table 3) available at VectorBase (http://www.vectorbase.org). PCR was performed using 2X Immomix DNA polymerase (Bioline USA Inc., MA, USA) and a standard Immomix amplification protocol. Amplified fragments were labeled by nick-translation with Cy3 and Cy5 fluorescent dyes (GE Health Care, UK Ltd., Buckinghamshire, UK). FISH was performed using previously described standard protocol (Sharakhova *et al.*, 2006).

Results

Cytogenetic map for Anopheles atroparvus

Similar to other mosquitoes (Rai, 1963), *An. atroparvus* has 3 pairs of chromosomes (Kitzmiller *et al.*, 1967). Polytene chromosome complement from the nurse cells is represented by the smallest sex chromosome X, intermediate chromosome 2 with arms almost equal in length and the largest chromosome 3, which has 3R arm slightly longer than 3L arm (Table 1, Figure 1). Arm 3R is the longest among the others. Chromosomes of ovarian nurse cells do not form a chromocentre, and pericentromeric areas of each chromosome form independent attachments to the nuclear envelope (Sharakhova *et al.*, 1999; Stegniy, 1993). A photomap for the polytene chromosomes was constructed from ovarian nurse cells of *An. atroparvus* (Figure 2). Chromosomes were straightened using AdobePhotoshop and divided into 33 numbered divisions and 118 lettered subdivisions. The division borders and nomenclature were adopted from previously developed salivary gland chromosome map of *An. atroparvus* (Stegnii & Kabanova, 1978) and another map for the polytene chromosome from ovarian nurse cells of *An. messeae* (Stegnii & Sharakhova, 1991).

There are easily identifiable robust landmarks for the *An. atroparvus* chromosome arms. The sex chromosome X has a slightly flared light telomere and a large flared light centromeric end terminated by three diffuse bands in subdivisions 4C-5B. Some of these bands often become invisible because of their diffuse morphology and granulated mesh-like structure. Arms of chromosome 2 can be easily distinguished from each other by the morphology of their telomeres. The telomere end of 2R is dark and consists of series of distinct bands in subdivisions 6A-B. In contrast, the telomere end of the 2L arm was flared, light and had a dark thin band in region 21C. Arms of chromosome 2 are connected to each by diffuse-light granulated areas in regions 14BC-15BC, which has two dark diffuse bands on 2L arm in subdivision 15C. Two sets of wide dark bands in regions 10A-B can be considered as an additional landmark for the 2R arm. Arms of chromosome 3 are usually not connected to each other in squashed preparations due to the strong attachment to the nuclear envelope. Pericentromeric region of the 3R arm had a dark band in subdivision 32C. The homologous chromosomes are often unpaired in subdivision 32D. An additional landmark for the 3R arm was a set of three dark bands in the middle of the arm in regions 25A-C. Arm 3L was characterized by a long asynaptic area of homologous in pericentromeric subdivisions 33A-

C. Region 35B with two adjacent dark bands--dot-like and half-circle-like, “a bird eye,”--can be considered as additional landmark for the 3L arm (Stegnii & Kabanova, 1978) (Figure 2).

Physical mapping of the *An. atroparvus* genome

The utility of a cytogenetic map developed by the present study for *An. atroparvus* was validated by the physical mapping of 10 PCR-amplified DNA probes labeled with Cy3 and Cy5 dUTPs. For the mapping, the four largest supercontigs from autosomes and additional supercontigs were selected from the sex chromosome X (Table 2). Supercontigs were chosen based on the arm homology to *An. gambiae* established using Basic Local Alignment Search Tool (BLAST) in the VectorBase (https://www.vectorbase.org). One probe from the start of the supercontigs and another probe from the end of the supercontigs were labeled using red and blue fluorescent dyes, respectively (Figure 3). The PCR probes were designed to amplify the unique sequences from gene exons (Table 2). All probes produced clear unique signals and were successfully mapped to the chromosomes based on the banding patterns (Figure 3). Probes from the same genomic supercontigs were found on the same chromosome arm indicating no mistakes in sequence assembly. The largest genomic supercontig KI421882 of 20 Mb was mapped to the 2R arm in regions 6B-9B. This supercontig covered almost 9 lettered subdivisions on the 2R chromosome arm. The smallest supercontig KI421898 of 4 Mb was placed to the chromosome X in divisions 4A-4B. A good quality *An. atroparvus* genome assembly allowed assignment and orientation of 63 Mb equal to 28% of the genome to the chromosomes by physical mapping of 5 genomic supercontigs.

Arm homology between *An. atroparvus* and *An. gambiae*

The arm homology between *An. atroparvus* and *An. gambiae* was established using a BLAST tool in the VectorBase (https://www.vectorbase.org). The genomic supercontig KI421883 from the longest autosome 3R arm of *An. atroparvus* was found homologous to the longest arm 2R in *An. gambiae*. Supercontig KI421882 from 2R arm of *An. atroparvus* was mapped *in silico* to the 3R arm of *An. gambiae*. Supercontigs from 2L (KI421886) and 3L (KI421887) arms of *An. atroparvus* were found in 2L and 3L of *An. gambiae*, respectively. Probes from the supercontig KI421898 homologous to the X chromosome in *An. gambiae* hybridized also to the X chromosome in *An. atroparvus*. These results suggest a whole-arm translocation between autosomes of these two species. Thus, *An. atroparvus* has arm association typical to that found in two other Asian species from genus *Anopheles, An. stephensi* (Sharakhova *et al.*, 2010) and *An. sinensis* (Lianga *et al.*, 2014) (Figure 4).

Discussion

A standard cytogenetic photomap of the ovarian nurse cell polytene chromosomes for the dominant European malaria vector *An. atroparvus* was developed. This map is one from the series of cytogenetic maps constructed for species from the genus *Anopheles* during the last decade (Sharakhov *et al.*, 2001; Sharakhova *et al.*, 2006; George *et al.*, 2010; Sharakhova *et al.*, 2011; Lianga *et al.*, 2014). The original photomap for *An. atroparvus* was developed using chromosomes stained with orsein (Stegnii & Kabanova, 1978), and its utilization for

the physical mapping purposes was difficult. Herein, phase contrast images of unstained chromosomes taken with digital technology were used. This approach allowed a clear banding pattern of the chromosomes to be obtained. To make the pattern recognition and mapping convincing, chromosomes were completely straightened using AdobePhotoshop. Each of the 10 PCR-amplified probes of genes from the *An. atroparvus* genome assembly was assigned to a band or interband position on the chromosome map facilitating the precise physical mapping of 5 genomic supercontigs (Figures 2 and 3). Because of the good quality of the genome assembly for *An. atroparvus*, 28% of the genome was placed to the chromosomes. In the present study, a much higher efficiency of physical mapping was obtained using the already assembled genome compared with when physical mapping was performed before the genome assembly became available. For example, 88% of the *An. gambiae* genome was placed to chromosomes based on 2000 BAC clones *in situ* hybridizations (Holt *et al.*, 2002). Many of these clones hybridized to the same positions on the chromosomes.

Arm homology was demonstrated between *An. atroparvus* and *An. gambiae*; the major malaria vector in Africa. Similar to other studies, a whole-arm translocation between autosomes of these two species was found. This type of chromosome rearrangement is common in the genus *Anopheles* (Sharakhova *et al.*, 2013). To compare chromosome arrangements in different species, a system of autosome arm notation proposed by Green and Hunt was adopted (Green & Hunt, 1980). Accordingly, the autosome arms in *An. gambiae* were named as follows: 2R = 2, 2L = 4, 3R = 3 and 3L = 5. Three different arrangements of chromosome arm association are possible: 1) (2+3) (4+5); 2) (2+4) (3+5) and 3) (2+5) (3+4). The first type of arm association has been described for the African mosquitoes of the *An. gambiae* complex from the ubgenus Cellia (Figure 4A). The second arm association has been discovered in *An. funestus*, another African mosquito from the subgenus Cellia (Green & Hunt, 1980). Interestingly, the same arm association has also been found in the American mosquito *An. albimanus* from the subgenus *Nyssorynchus* (Figure 4B) (Cornel & Collins, 2000). The present study demonstrated that *An. atroparvus* has the third type of arm association (2+5) (3+4) (Figure 4C). The same arm association has been described in Asian malaria vectors: *An. stephensi* from the subgenus Cellia (Sharakhova *et al.*, 2006) and *An. sinensis* from the subgenus Anopheles (Lianga *et al.*, 2014), and also in an African mosquito *An. nili* from the subgenus Cellia (Sharakhova *et al.*, 2011). It is unclear if these rearrangements reflect phylogenetic relationships between the species or if they had happened multiple times in evolution of the genus *Anopheles*. Physical mapping of additional species may help to solve this problem.

Further application of the cytogenetic map constructed by this study for *An. atroparvus*, the dominant malaria vector in Europe, will help to develop a high-quality genome assembly for this species. The availability of the chromosome-based genome maps for multiple species of *Anopheles* will facilitate comparative genomic studies and will improve our understanding of phylogenetic relationship in this group of mosquitoes.

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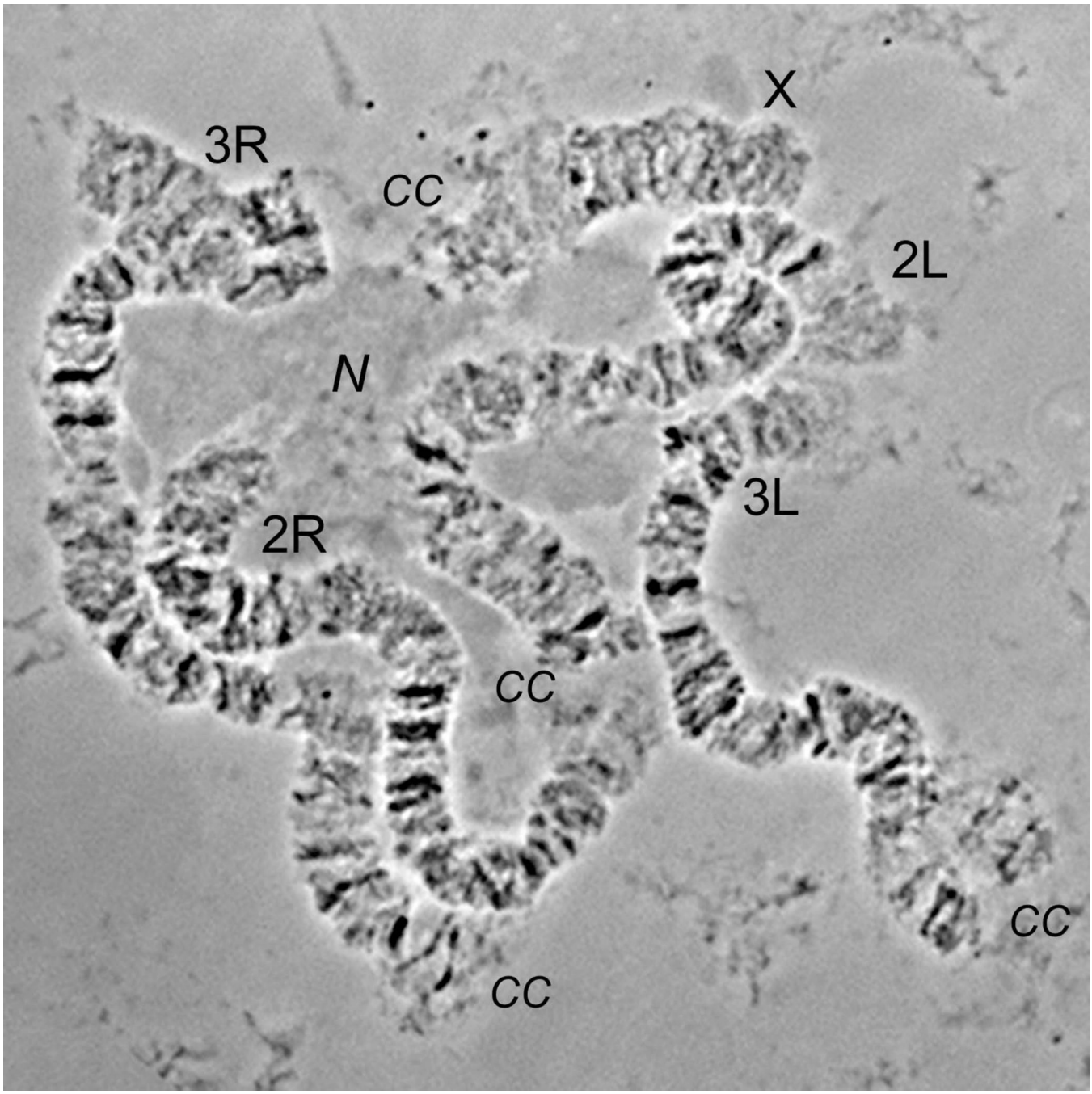

Figure 1. Chromosome complement of ovarian nurse cells of *An. atroparvus*

Chromosome arms are shown as X, 2R, 2L, 3R, 3L. Pericentromeric regions and nucleolus are indicated as *CC* and *N* respectively.

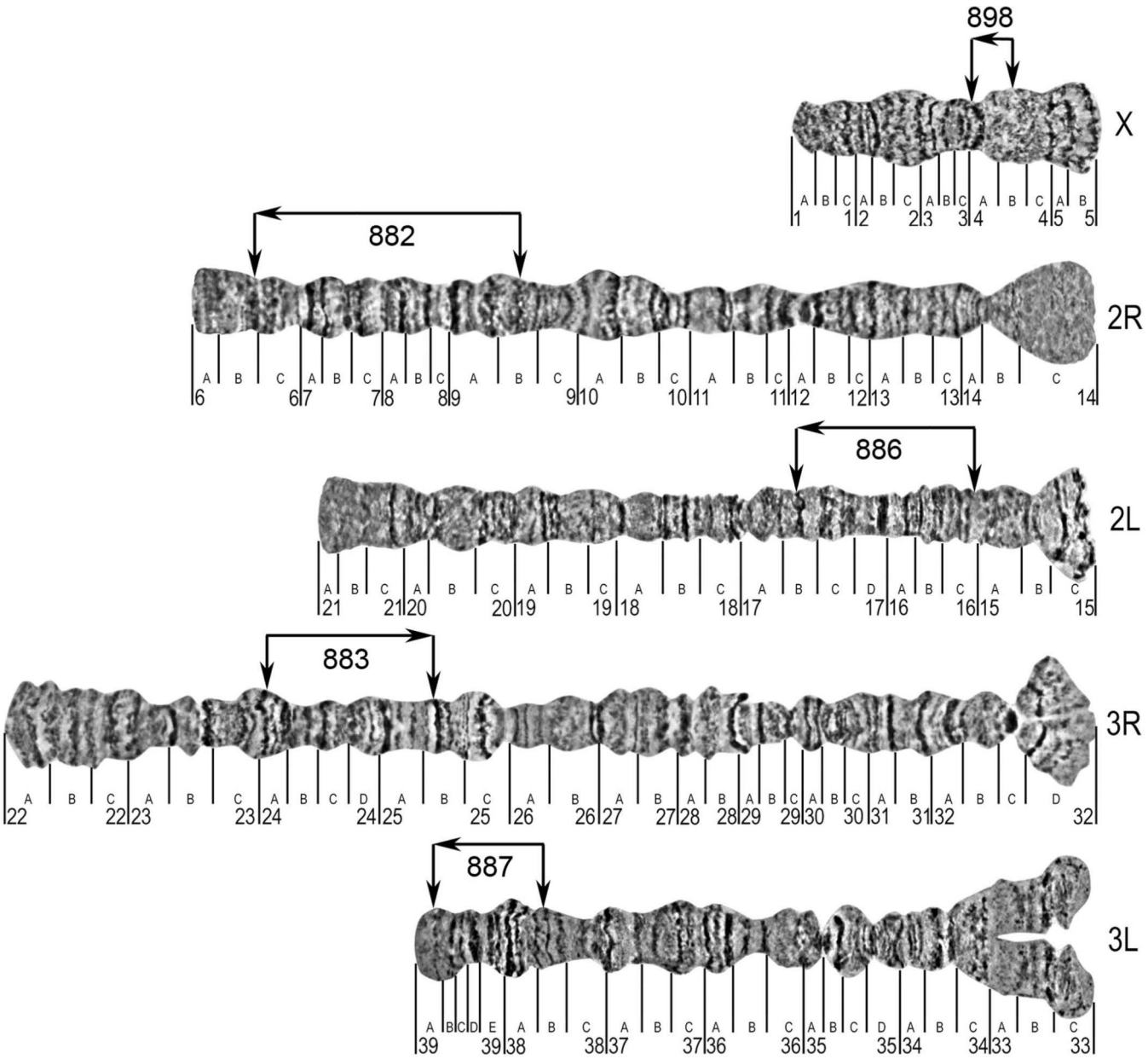

Figure 2. A standard cytogenetic photomap of ovarian nurse cell chromosomes of *An. atroparvus*

The lines below the chromosomes indicate the boundaries of numbered and lettered divisions and subdivisions respectively. The locations of DNA probes from the start and the end of the genomic supercontigs are indicated by vertical arrows above the chromosomes. The orientations of the genomic supercontigs are shown by horizontal arrows above the chromosomes.

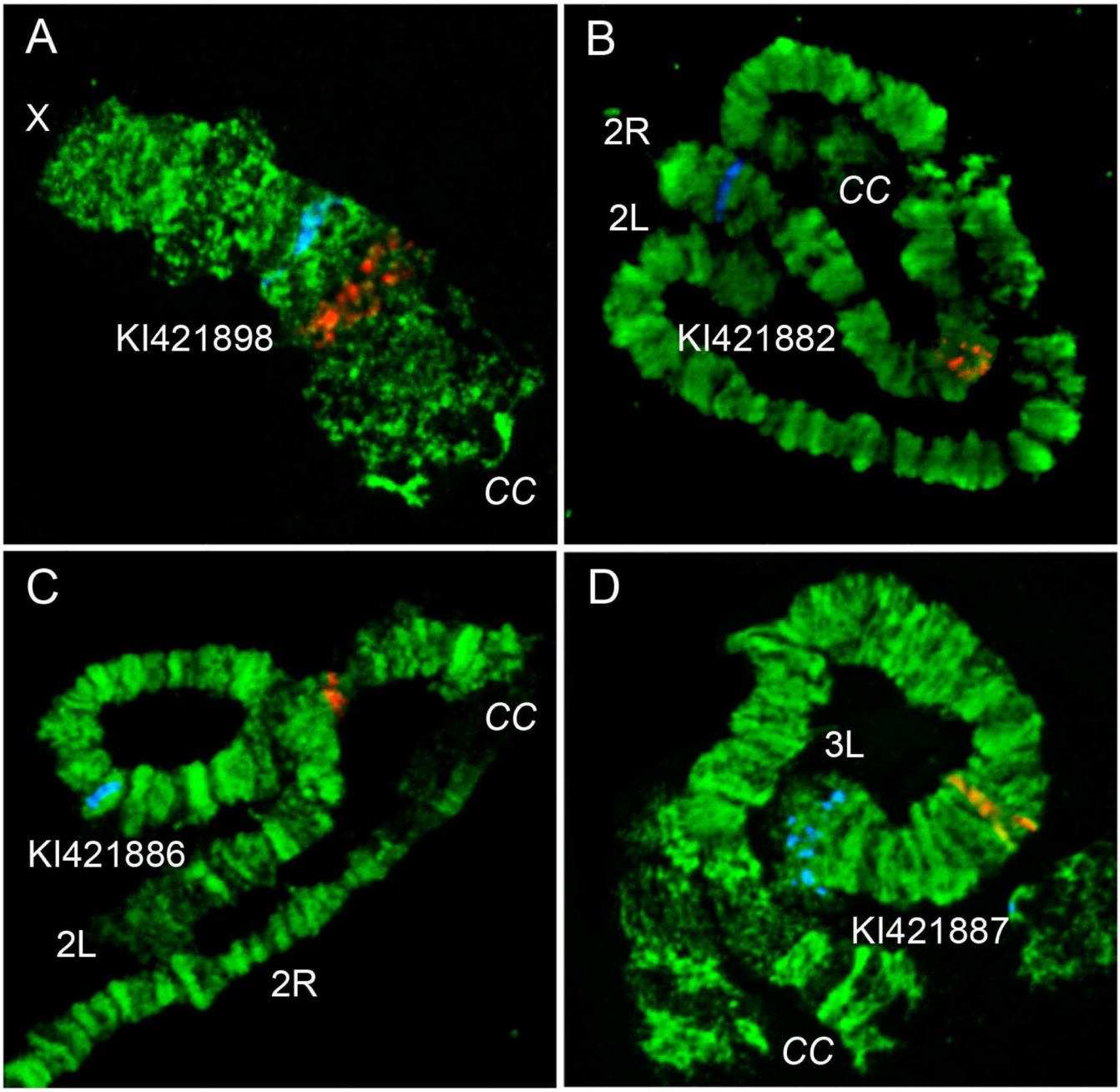

Figure 3. Examples of FISH on chromosomes

The position of red and blue signal demonstrate the beginning and the end of the genomic supercontigs KI421898 (A), KI421882 (B), KI421886 (C) and KI421887 (D) on chromosomes X, 2R, 2L and 3L, respectively.

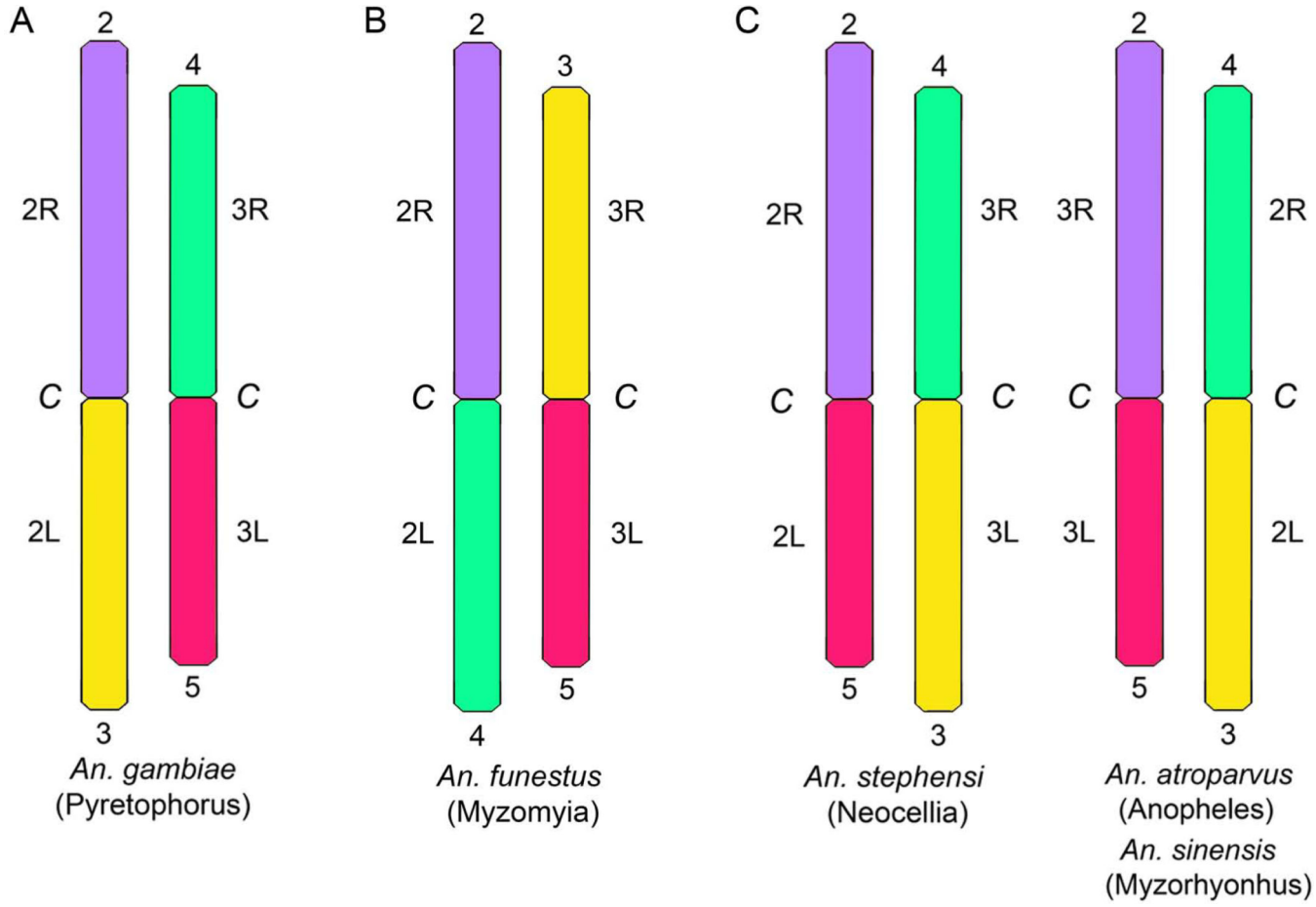

Figure 4. Chromosome arm homology between *An. gambiae* (A), *An. funestus* (B) and *An. stephensi, An. atroparvus, An. sinensis* (C)

Homologous arms are indicated by the numbers above and below chromosomes. Notations for chromosome arms are shown on sides of the chromosomes. *C* - pericentromeric regions. Names of series are shown in parentheses below the species names.

Table 1

The measurements of *An. atroparvus* ovarian nurse cells polytene chromosomes

Chromosome	X	2	3
Average length (μm)	210	1135	1200
Relative length (%)	8.3	44.4	47.3
Centromere position (%)	Not applicable	46	38.4

Table 2

List of supercontigs/genes mapped to the chromosome positions

Supercontig accession#	Supercontig size (bp)	Probe/gene	Gene start-end positions	Chromosome location
KI421882	20238125	AATE008453	66762-76445	2R:9B
		AATE010796	16318761-16325046	2R:6B
KI421883	16623535	AATE005670	1516240-1518054	3R:24A
		AATE008545	13695270-13697405	3R:25B
KI421886	11808074	AATE001693	35088-46700	2L:16C
		AATE008759	11195642-11201764	2L:17B
KI421887	10405585	AATE011741	966864-974650	3L:38B
		AATE006315	9676291-9681308	3L:39A
KI421898	4025992	AATE018661	2294-6247	X:4B
		AATE018218	3538365-3539771	X:4A

Table 3

List of primer sequences used for PCR amplification of DNA probes

Supercontig accession #	Gene accession #	Forward primer	Reverse primer
KI421882	AATE008453	GTCGAATGCGCAGTCTGTAA	GCCAGGATCACTCCAATGTT
	AATE010796	CGCGTACATGTCATCGAAAC	TGAATTCGCTGTACAACACG
KI421883	AATE005670	GAAGAACCAAACCACCCAGA	TACTGGAACAGTGGCTCGTG
	AATE008545	GCTACAAGATGCAGGGCTTC	TGGTCGAGCTGGAAGAAGTT
KI421886	AATE001693	GTGTCATCCCACGAGGACTT	AGCGCTCCTCGTACTGTGTT
	AATE008759	CTCCAAGGTGGACAAATCGT	CGACCGTGATGTAGATGTGC
KI421887	AATE011741	GAGATTACGCGGACGAAGAG	GATCTTGGTGGGTGTTTGCT
	AATE006315	CCGAGAGTGAGGAGAAAACG	TTGCTGATCTAGCCCGTACC
KI421898	AATE018661	GCGAGGAGCTGATACAGACC	TTCTTCTTGCGCTCCTTGAT
	AATE018218	GTTCCCGGACGAACAGTTTA	CAGCTTGAAGTGGTGGATGA