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Cytogenetic analysis of *Anopheles ovengensis* revealed high structural divergence of chromosomes in the *Anopheles nili* group

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Abstract

Cytogenetic analysis is an informative classical approach to understanding the relationships among members in a group of closely related species of mosquitoes. *Anopheles ovengensis* is a recently discovered species of the *An. nili* group and is one of the important malaria vectors in the African equatorial forest. This study characterized polytene chromosomes of *An. ovengensis* and compared them with polytene chromosomes of *An. nili*. Using fluorescent *in situ* hybridization and chromosome banding pattern comparison we have established correspondence between chromosomal arms of *An. ovengensis* and *An. nili*. Analysis of chromosome morphology in the two species revealed a limited similarity in the banding patterns. The most extensive reorganization occurs in pericentromeric and intercalary heterochromatin. Chromosomes of *An. ovengensis* are joined together by a diffuse chromocenter and they have two large regions of intercalary heterochromatin in arms 2L and 3R. In contrast, the chromocenter and intercalary heterochromatin are not seen in *An. nili* chromosomes. Comparative analysis of the arm association suggests the occurrence of a whole-arm translocation between the two members of the group. The observed, substantial reorganizations of chromosome structure implies either a rapid rate of chromosome evolution in the *An. nili* group, or that the two species belong to different taxonomic groups within subgenus Cellia.

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Contributions

I.V.S. designed research; M.V.S., A.P., A.X., and I.V.S. performed cytogenetic research; C.N. and C.A-N. conducted field work and mosquito identification under supervision of P. A-A. and F.S., M.V.S., A.P. and I.V.S. analyzed data; I.V.S. and M.V.S. wrote the paper which was critically revised by A.P., C.A-N., and F.S.

Conflict of interests

The authors declare no conflict of interest.

Keywords

malaria mosquito; *Anopheles ovengensis*; *Anopheles nili*; polytene chromosomes; fluorescent *in situ* hybridization

1. Introduction

Malaria has a devastating impact on public health and welfare on the African continent. It is now becoming clear that the end point of all efforts to reduce the current malaria impact must be the eventual elimination of the disease (Enayati and Hemingway, 2010). Vector control is seen as a cornerstone of the malaria control strategy. Because of economic and practical reasons, vector control mainly relies on the use of synthetic insecticides (Takken and Knols, 2009). However, this strategy is inefficient if all major vector species are not targeted. Many malaria vectors belong to species complexes or groups, and members within these complexes or groups can vary significantly in their vectorial capacity. Moreover, species can be further subdivided into populations adapted to different environments. Some malaria control initiatives have failed because they targeted the wrong species or population (Coluzzi, 1992; Van Bortel et al., 2001). Understanding and targeting the heterogeneity and complexity of all major vector species and populations is necessary for effective vector control and malaria elimination (Enayati and Hemingway, 2010). *Anopheles gambiae, An. arabiensis, An. funestus, An. moucheti,* and *An. nili* are the major malaria vectors in sub-Saharan Africa because they are anthropophilic and susceptible to *Plasmodium falciparum* (Fontenille and Simard, 2004). Most studies of African malaria vectors have involved the *An. gambiae* complex and, to a lesser extent, the *An. funestus* group, in part, because molecular and cytogenetic tools for characterizing population structure, ecological adaptation, and taxonomic status of species are available for these species. Similar tools for the *An. nili* group have been lacking until recently (Berthomieu et al., 2003; Kengne et al., 2003; Peery et al., 2011; Sharakhova et al., 2011). This represented a critical barrier to progress in the field of vector biology and control because members of the *An. nili* group contribute substantially to malaria transmission in African humid savannah and forested areas. The recent findings of circulation of *P. falciparum* along with other *Plasmodium* species in great apes and monkeys (Duval et al., 2010; Prugnolle et al., 2010; Prugnolle et al., 2011) raise concerns about pathogen transfer between humans and primates and highlight the need to improve our knowledge of forest malaria vectors.

Although correct species identification is crucial for successful vector control, the taxonomic status of members of the *An. nili* group remains unclear. Because of this knowledge gap, the distribution, behavior, adaptation, and role in malaria transmission that can be attributed to each member of this group is also largely unknown (Fontenille and Simard, 2004). Analysis of sequence variation in the ribosomal DNA second internal transcribed spacer (ITS2) and D3 28S region allowed identification of four species within the *An. nili* group (namely, *An. nili s.s., An. somalicus, An. carnevalei,* and *An. ovengensis*) (Kengne et al., 2003). A comprehensive study in Cameroon confirmed that *An. nili s.s.* (hereafter *An. nili*) is the major malaria vector of the group and is widespread in humid savannah and degraded forest environments (Antonio-Nkondjio et al., 2006). This study has emphasized the exophagic behavior of *An. ovengensis* and *An. carnevalei.* It has also demonstrated that *An. ovengensis* is abundant in deep intact forests of Central Africa, where it substantially contributes to malaria transmission (Antonio-Nkondjio et al., 2006). In Equatorial Guinea, sporozoite rates in *An. ovengensis* can reach 4.1% (n=74), which is higher than that of *An. gambiae* in the same area (3.3%, n=603) (Ridl et al., 2008), confirming a major yet overlooked role for *An. ovengensis* in malaria epidemiology in these settings.

A recent study used a combination of nuclear (microsatellite and ribosomal DNA) and mitochondrial DNA markers to explore the levels of genetic polymorphism and divergence among species of the *An. nili* group in the savannah and forested areas of Cameroon (Ndo et al. PLoS ONE, in press). The study detected a large number of fixed mutations between *An. nili* and *An. ovengensis*, as well as among other members of the group. The genetic distance has been estimated 4 to 8 fold higher than that commonly reported among cryptic *Anopheles* species. This high genetic divergence within the *An. nili* group suggests that its members might belong to different species groups. The aim of our study was to perform the first cytogenetic analysis of *An. ovengensis* and to compare structural organization of polytene chromosomes in *An. ovengensis* and *An. nili*.

2. Materials and methods

2.1. Wild mosquito collection, preservation, and species identification

Anopheles ovengensis and *An. nili* adult females were collected by pyrethrum spraying and bednet traps in the villages of Nyabessan in Cameroon (2°80’N; 10°25’E) and Dinderesso (11°14'N; 4°23'W) in Burkina Faso, respectively. Specimens were identified in the field as members of the *An. nili* group by using morphological identification keys (Awono-Ambene et al., 2004; Gillies and Coetzee, 1987) and were further characterized by molecular assays as *An. ovengensis* and *An. nili* (Kengne et al., 2003). Semi-gravid females were dissected under a microscope, and their ovaries, at the appropriate stage, were preserved in Carnoy's fixative solution (3 methanol: 1 glacial acetic acid by volume). Tissues of both species were stored under identical conditions and were processed using the same protocol.

2.2. Chromosome preparation and imaging

Ovaries from half-gravid females stored in Carnoy's fixative solution were dissected in 50% propionic acid under a Leica MZ6 dissection microscope (Leica Microsystems GmbH, Wetzlar, Germany). A cover slip was placed on the follicles and tapped with a pencil to squash the cells. Preparations of semi-squashed nuclei were obtained by placing a coverslip on the follicles in a drop of 50% propionic acid followed by very gentle tapping. Hard tapping on the coverslip obtained preparations of fully squashed nuclei. The banding pattern of polytene chromosomes was examined using an Olympus CX-41 phase-contrast microscope (×1000) (Olympus America Inc., Melville, NY, USA). Slides with good chromosomal preparations were dipped in liquid nitrogen, then cover slips were removed and slides were dehydrated in 50%, 70%, 95%, and 100% ethanol. Chromosomes that showed a suitable level of polytenization were imaged by an Olympus BX-41 with an attached Olympus Q-Color 5 camera and Q-Imaging software (Olympus America Inc., Melville, NY, USA). The six best chromosomal slides were utilized to develop a preliminary map of *An. ovengensis*. Images of the chromosomes were combined, straightened, shaped, and cropped using Adobe® Photoshop. Chromosome preparations from 60 *An. ovengensis* females and from 100 *An. nili* females were analyzed for this study.

2.3. Fluorescence in situ hybridization

Primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) and gene sequences from the *An. gambiae* genome assembly. PCR products ranged from 400–600 bp in size. The genomic DNA of single *An. nili* mosquitoes was extracted using the Wizard SV Genomic Purification System (Promega Corporation, Madison, WI, USA) and was used as a template for PCR. PCR products were gel purified using the Geneclean kit (Qbiogene, Inc., Irvine, CA). The fluorescence *in situ* hybridization (FISH) procedure was conducted as previously described (Sharakhova et al., 2006). The DNA was labeled with Cy3-dUTP and Cy5-dUTP (GE Healthcare UK Ltd., Buckinghamshire, England) using Random Primers DNA Labeling System (Invitrogen Corporation, Carlsbad, CA, USA). DNA probes were

hybridized to the chromosomes of *An. ovengensis* at 39°C overnight in hybridization solution (Invitrogen Corporation, Carlsbad, CA, USA). Then the chromosomes were washed in 0.2XSSC (Saline-Sodium Citrate: 0.03M Sodium Chloride, 0.003M Sodium Citrate), counterstained with YOYO-1, and mounted in DABCO. Fluorescent signals were detected and recorded using a Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

3. Results

3.1. Structure of polytene chromosomes in An. ovengensis

We analyzed readable polytene chromosomes from the ovarian nurse cells of 44 *An. ovengensis* females obtained from a total of 60 chromosome preparations. The polytene chromosome complement consists of chromosome X and four autosomal arms. Pericentromeric regions of the polytene chromosomes in *An. ovengensis* form a diffuse chromocenter (Fig. 1A). Chromosome X can be easily distinguished from other chromosomes by having the shortest length. Arm 2R is the longest among the five arms and can be recognized by the presence of several distinct bands in the telomere. The major landmarks for arm 2L are a light telomere and a region of intercalary heterochromatin located approximately six bands apart from the centromere. Arm 3R has a large region of intercalary heterochromatin also located approximately six bands distally from the centromere. The regions of intercalary heterochromatin are indicated by asterisks in Fig. 1. Unlike arm 2L, arm 3R often displays asynapsis of homologous chromosomes in the region of intercalary heterochromatin and the chromosome can be easily broken in this region by squashing (Fig. 1B). Arm 3L has a lightly flared telomeric region and several large puffs. We detected no polymorphic inversions among our samples of *An. ovengensis*.

3.2. Chromosome arm associations in An. ovengensis

The assignment of chromosomal arms was done based on their relative length and associations. Because pericentromeric regions of the polytene chromosomes in *An. nili* do not form a chromocenter, it was easy to determine arm association in our previous study (Sharakhova et al., 2011). We found only one type of arm association in *An. nili*: 2R + 2L, 3R + 3L. In contrast, pericentromeric heterochromatin of the polytene chromosomes in *An. ovengensis* forms a diffuse chromocenter, which makes it more difficult to determine arm association. A chromocenter is a structure where heterochromatic pericentromeric regions of all polytene arms join together by ectopic contacts. In contrast, arms of mitotic chromosomes do not join with each other in a chromocenter, but they are associated as the following: 2R + 2L, 3R + 3L; the X chromosome is not associated with the autosomes. The organization of pericentromeric regions in a chromocenter is not uncommon feature of polytene chromosomes from ovarian nurse cell nuclei of mosquitoes. For example, the compact chromocenter has been observed in ovarian nurse cell nuclei of *An. funestus* (Sharakhov et al., 2001). When cell nuclei are squashed during chromosome preparation, the chromocenter loses its integrity and breaks up. Although this breakage may result in all possible arm associations, the real (not ectopic) arm associations are expected to be more frequent. To identify autosomal arm pairs with prevalent association we have analyzed 43 chromosomes spreads with a broken chromocenter in 10 squashed chromosome preparations (Fig. 1B). Although the analysis revealed all six possible variants of pair-wise associations in *An. ovengensis* (2R + 2L, 2R + 3R, 2R + 3L, 3R + 2L, 2L + 3L, and 3R + 3L) the associations 2R + 2L and 3R + 3L were more prevalent (Table 1). Pearson's χ^2 test indicates that the observed arm associations are significantly different than expected by random chance ($\chi^2 = 12.6$, d.f. = 5, $P < 0.026$).

3.3. Chromosome arm homology among An. ovengensis, *An. nili and An. gambiae*

The appearance of *An. ovengensis* chromosomes is quite different from that of *An. nili* or *An. gambiae* chromosomes. The sex chromosome X, as the shortest, is obviously homologous among the three species. We established autosomal arm homology by successful FISH of three DNA probes derived from *An. gambiae* genes. Probe AGAP001763 was from arm 2R of *An. gambiae*, probes AGAP007010 and AGAP007031 were from arm 2L of *An. gambiae.* We mapped AGAP001763 to arm 2R while AGAP007010 and AGAP007031 mapped to arm 3L in *An. ovengensis* (Fig. 2). In our previous study, we labeled 25 gene fragments derived from arms 2R and 2L in *An. gambiae* and mapped them to chromosomes 2R and 3L in *An. nili*, including AGAP007010 and AGAP007031 (Sharakhova et al., 2011). Thus, we demonstrated that the 2R arms are homologous among the three species and the 2L arm of *An. gambiae* corresponds to the 3L arms in *An. ovengensis* and *An. nili.* We also established homology between polytene chromosomes 3R of *An. ovengensis* and 2L of *An. nili* based on the similarity in the banding pattern. The apparently identical banding pattern starts in subtelomeric regions and extends to subdivision 22C on 2L on the *An. nili* map (Sharakhova et al., 2011) (Fig. 3). For comparative purposes we adopted the system of autosomal arm notation proposed by (Green and Hunt, 1980). Accordingly, the autosomal arms in *An. gambiae* are named as the following: 2R = 2, 2L = 4, 3R = 3, and 3L = 5. We propose to name the sex chromosome X as element 1 in agreement with nomenclature of *Anopheles* autosomes (Green and Hunt, 1980). Likewise, H. J. Muller named chromosomal elements of *Drosophila* as A (chromosome X) and B, C, D, E, F (autosomes) (Muller, 1940).

Therefore, elements 1 and 2 correspond to the chromosomes X and 2R in *An. ovengensis, An. nili* and *An. gambiae.* Element 3 corresponds to arm 3L in *An. ovengensis* and *An. nili* (Fig. 2, 3) but to arm 2L in An. gambiae (Sharakhova et al., 2011). In contrast, element 4 has the same notation in *An. nili* and *An. gambiae* (arm 3R) but a different notation in *An. ovengensis* (arm 2L). Finally, element 5 corresponds to arm 3R in *An. ovengensis*, arm 2L in *An. nili* and arm 3L in *An. gambiae*. Thus, our data suggest a whole-arm translocation between chromosome elements 2 and 3 in *An. nili* and *An. ovengensis*.

3.4. Structural divergence of chromosomes in the An. nili group

Our analysis of chromosomes stained with a fluorescent dye, YOYO-1, revealed remarkable difference in pericentromeric heterochromatin between *An. ovengensis* and *An. nili* (Fig. 4). Based on chromosome morphology, we identified two types of heterochromatin: dark compact, and light diffuse. There was more heterochromatin in *An. ovengensis* chromosomes than in *An. nili* chromosomes. *Anopheles ovengensis* has large blocks of dark compact heterochromatin in pericentomeric regions of all chromosomes. While only the X chromosome has a large block of dark compact heterochromatin in *An. nili.* The two species were also different with respect to the morphology of the pericentromeric regions of autosomes (Fig. 3). The pericentromeric regions of chromosome elements 2, 3 and 4 were typically asynaptic in *An. nili* but not in *An. ovengensis.* In addition, chromosome arms 2L and 3R of *An. ovengensis* displayed large regions of light diffuse intercalary heterochromatin. This type of heterochromatin (both pericentomeric and intercalary) forms attachments to the nuclear periphery (Fig. 1, 4). Because the chromosomal location of nuclear envelope contacts differs between *An. ovengensis* and *An. nili* and because the pericentromeric regions of different arms stay closer to each other in *An. ovengensis* than in *An. nili,* the spatial organization of chromosomes is expected to be different between the two species. The regions of intercalary heterochromatin and the occurrence of asynapsis were consistent between nuclei and between specimens.

The euchromatic portion of polytene chromosomes was also found to be significantly divergent between *An. ovengensis* and *An. nili* (Fig. 3). We determined only limited similarity in banding patterns between all homologous chromosome elements of the two species except in element 5. The likely reason for this poor homology between the two maps is a large number of fixed chromosomal inversions between *An. ovengensis* and *An. nili.* However, the number and locations of the fixed inversions could not be precisely determined because of the limited similarity of the banding pattern. Extensive physical mapping on chromosomes in both species can be performed to identify the fixed inversions.

4. Discussion

Cytogenetic studies of polytene chromosomes have been useful for understanding population genetics, taxonomy and systematics of various groups of malaria mosquitoes (Chandra et al., 2010; Coluzzi et al., 2002; Rafael et al., 2004; Somboon et al., 2008; Spillings et al., 2009). The major goal of this study was to conduct the cytogenetic analysis of a neglected malaria vector, *An. ovengensis*, and to evaluate karyotypic divergence in the *An. nili* group. Our comparative cytogenetic study of *An. nili* and *An. ovengensis* has yielded surprising results. First, we detected very few similarities in banding pattern between polytene chromosomes of *An. nili* and *An. ovengensis.* Usually, polytene chromosomes of species of the same group or complex have very similar banding patterns and differ only by few fixed inversions with no other apparent chromosomal differences (Coluzzi et al., 2002; Green and Hunt, 1980). Second, we discovered dramatic differences in the location and morphology of heterochromatic regions between *An. nili* and *An. ovengensis*. In addition to the differences in morphology of the pericentric heterochromatin, *An. ovengensis* has intercalary heterochromatin that could not be identified in *An. nili.* Finally, we determined the difference in arm association between *An. nili* and *An. ovengensis*. This finding indicates that a whole-arm translocation might have occurred during the evolution of the *An. nili* group. Whole-arm translocations and paracentric inversions are the common types of rearrangements in anopheline mosquitoes of subgenus Cellia (Green and Hunt, 1980; Sharakhov et al., 2001). Partial arm translocations and pericentric inversions have not been described in *Anopheles* even when species from different subgenera were compared (Cornel and Collins, 2000). Therefore, chromosomal arms are expected to preserve their integrity even across large evolutionary distances. However, whole-arm translocations have never been documented within a group of closely related species but is normally found among much more distantly related species that belong to different series (e.g., among *An. gambiae, An. funestus*, and *An. stephensi* (Xia et al., 2010) (Fig. 5). Therefore, these data point to a high chromosomal divergence between two species of the *An. nili* group.

Two hypotheses could explain the profound differences in the karyotypes of *An. nili* and *An. ovengensis.* First, members of the *An. nili* group could have unusually high rates of chromosome evolution. Second, *An. nili* and *An. ovengensis* could actually belong to different taxonomic groups within subgenus Cellia. Nonuniform speeds of chromosomal rearrangements have been documented in different organisms. Comparison of vertebrate genomes demonstrated a slow rate of chromosomal evolution in fish and birds and an accelerated rate of genome rearrangements in mammals (Ellegren, 2010; Murphy et al., 2005; Postlethwait et al., 2000). Within mammals, rodent lineages have undergone 3.2–3.5 chromosome rearrangements per million years (Myr) while primates have accumulated only 1.6 rearrangements per Myr since the two lineages diverged. Within carnivores, the rate of chromosome evolution in *Canidae* is much higher than in other lineages (Yang et al., 1999). Comparison of genomic sequences of 12 species of *Drosophila* revealed that inversions have been fixed at different rates in different lineages (Bhutkar et al., 2008). For example, 29 fixed inversions are located between *D. melanogaster* and *D. yakuba*. All but one of these

inversions occurred in the *D. yakuba* lineage (Ranz et al., 2007). Likewise, the distribution of polymorphic rearrangements varies dramatically among lineages. More than 500 polymorphic inversions are known for *D. melanogaster* while only 14 inversions have been described for its close relative, *D. simulans* (Aulard et al., 2004). Within the *An. gambiae* species complex, *An. gambiae s.s.* and *An. arabiensis* are highly polymorphic for chromosomal inversions while their sibling species *An. merus* is chromosomally monomorphic (Coluzzi et al., 2002). Two highly polymorphic inversions have been found in *An. nili* from Burkina Faso and one of them has been recorded at low frequency in *An. nili* from Cameroon (Peery et al., 2011; Sharakhova et al., 2011). The lack of inversion polymorphism in *An. ovengensis* reported here could be due to a limited sample size and distribution. Thus, the level of inversion polymorphism in the *An. nili* group remains to be investigated.

Variations in heterochromatin structure and location have been observed within the *An. maculipennis* subgroup (Sharakhova et al., 1997; Stegnii, 1987), albeit to a lesser extent than within the *An. nili* group. Evolutionary transformations of heterochromatin with respect to chromosomal location and structure have been demonstrated between *An. gambiae* and *An. funestus*, which belong to different series within subgenus Cellia (Sharakhov et al., 2002; Sharakhov et al., 2001). Another study has found a cluster of genes within the centric heterochromatin in *D. melanogaster* but within euchromatin in *D. ananassae, D. pseudoobscura*, and *D. virilis* (Yasuhara et al., 2005). The unique features of heterochromatic genes in *D. melanogaster* include the accumulation of transposable elements, increased AT richness, longer introns, and association with H3K9me2-enriched domains (a heterochromatin-specific histone modification) (Yasuhara et al., 2005; Yasuhara and Wakimoto, 2006, 2008). A study of the *An. gambiae* genome showed that heterochromatin accumulates genes important for regulation of gene expression and chromatin organization and harbors genes encoding for odorant receptors, cuticular proteins, and serin-type endopeptidases (Sharakhova et al., 2010). It is possible that changes in heterochromatin have been a mechanism of genetic reinforcement during speciation in the *An. nili* group. A number of studies have demonstrated direct associations between diffuse heterochromatin and the nuclear envelope in fruit flies and mosquitoes (Baricheva et al., 1996; Hochstrasser and Sedat, 1987; Sharakhov et al., 2001). Closely related species within the *An. maculipennis* and the *D. melanogaster* subgroups can be discriminated on the basis of the spatial localization and morphology of the chromosomal regions to which the nuclear envelope is attached in germ-line cells (Stegnii, 1987; Stegnii and Vasserlauf, 1994). The differences in organization of heterochromatin between *An. nili* and *An. ovengensis* suggest spatial reorganization of nuclear architecture during speciation in the *An. nili* group. Reorganizations of heterochromatin and nuclear architecture may lead to substantial changes in global gene expression patterns (Jost et al., 2012; Pezer et al., 2010; Van de Vosse et al., 2011).

The first cytogenetic study of *An. nili* included 4 specimens collected in the bushes around a cattle kraal near Popa Falls of Okavango River (18º03’ S, 21º39’ E) in Namibia and 68 specimens from the M’Poka village area (3º55’ S, 14º29’ E) in Congo. We investigated the correspondence between the first published *An. nili* cytogenetic map (Miles et al., 1984) and photomaps of *An. nili* (Sharakhova et al., 2011) and *An. ovengensis* (Fig. 3). Our analysis revealed that the originally published chromosomes of *An. nili* (Miles et al., 1984) were more similar to chromosomes of *An. ovengensis* than to the more recently published chromosomes of *An. nili* (Sharakhova et al., 2011). In addition to the similarity of the banding patterns, we noticed the same position of diffuse intercalary heterochromatin in chromosomes of *An. nili* (Miles et al., 1984) and in chromosomes of *An. ovengensis* (Fig. 3). Regions of intercalary heterochromatin appear as puffy areas separated from the centromeres by regions of euchromatin. We consider these regions of intercalary

heterochromatin as the landmarks for recognition of chromosome arms in *An. ovengensis*. Specifically, intercalary heterochromatin is seen on arms 4 and 5 of the *An. nili* map (Miles et al., 1984) as well as on arms 2L and 3R of *An. ovengensis*, which correspond to arms 3R and 2L of *An. nili*, respectively (Fig. 3). Intercalary heterochromatin has not been detected in chromosomes of *An. nili* in our studies (Fig. 3, 4) (Sharakhova et al., 2011). Importantly, the autosomal arms 2, 3, 4, and 5 of the *An. ovengensis* map correspond to the arms with the same names of the *An. nili* map (Miles et al., 1984). Distinction between members of the *An. nili* group is difficult because the morphological differences are subtle (Awono-Ambene et al., 2004; Gillies and Coetzee, 1987) and molecular diagnostics assays have not been available until 2003 (Kengne et al., 2003). A recent study has demonstrated that the equatorial forest might harbor many more species of the *An. nili* group than were previously described (Ndo et al. PLoS ONE, in press). Therefore, we believe that the first published *An. nili* chromosomes (Miles et al., 1984) belong an *An. ovengensis*-like species from the *An. nili* group.

Anopheles nili and *An. ovengensis* might belong to different taxonomic groups within subgenus Cellia. According to the accepted rates of molecular evolution of 2.2% per Myr for ITS2 (Schlotterer et al., 1994) and 2% per Myr for mtDNA (DeSalle et al., 1987), a recent study estimated the divergence time among members of the *An. nili* group at about 0.8 to 6 Myr or 0.2 to 3 Myr, respectively (Ndo et al. PLoS ONE, in press). In addition, ribosomal DNA haplotypes found in populations and species from the *An. nili* group differed from one another by a large number of fixed mutations and insertion/deletions leading to genetic distance estimates 4 to 8 fold higher than those commonly reported among cryptic *Anopheles* species (Collins and Paskewitz, 1996; Kengne et al., 2003). This high genetic divergence within the *An. nili* group suggests that its members may not belong to the same species group. Therefore, the diversity of *Anopheles* species in the African equatorial forest could be much higher than expected and needs to be thoroughly assessed to improve current vector control measures.

5. Conclusion

This cytogenetic study of *An. ovengensis* is one of the first steps toward detailed characterization of genome sequences for this important but neglected malaria vector. The observed, substantial reorganizations of chromosome structure between *An. ovengensis* and *An. nili* suggest either a rapid rate of chromosome evolution in the *An. nili* group, or that the two species belong to different taxonomic groups. These hypotheses can be tested by a combination of extensive physical mapping and whole-genome molecular analyses. Cytogenetic and physical mapping, coupled with advances in genome sequencing are the major approaches to understanding mosquito taxonomy, systematics, evolution, ecology, and population genetics. Future studies will highlight genetic features associated with ecological adaptation, population differentiation, and speciation of malaria vectors in equatorial forest. We hypothesize that other species, which currently belong to the *An. nili* group, could differ from each other by fixed chromosomal inversions and heterochromatin structure. Cytogenetic and physical mapping can identify fixed inversions and reorganizations of heterochromatin. Because of the high readability of polytene chromosomes in species of the *An. nili* group, this study will be both feasible and informative.

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Highlights

- We report on the first cytogenetic analysis of *Anopheles ovengensis*.
- We found a limited similarity in the banding patterns between species.
- We observed substantial reorganization of heterochromatin.
- Our comparative analysis suggests a whole-arm translocation in the *An. nili* group.

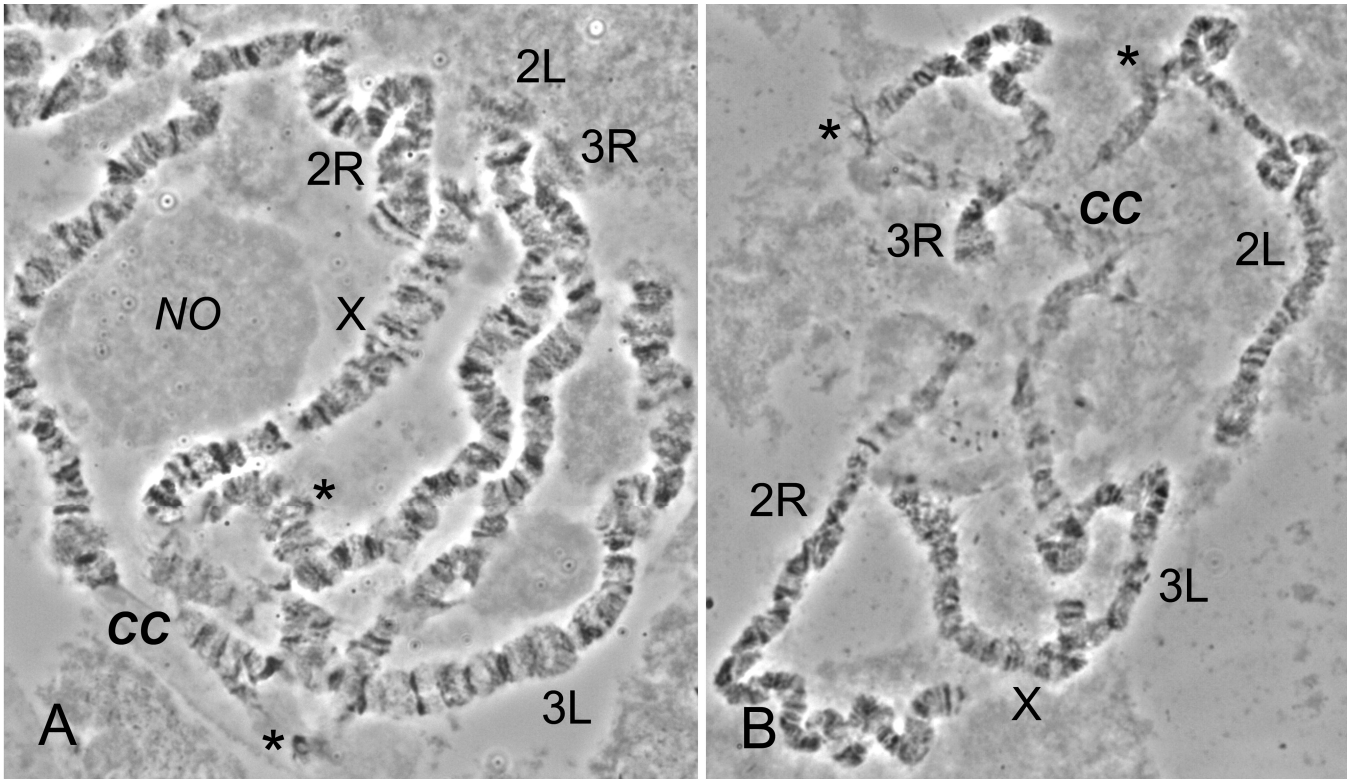

Fig. 1.

Polytene chromosomes from ovarian nurse cells of *An. ovengensis*. Semi-squashed (A) and fully squashed (B) unstained nuclei are imaged with a phase-contrast microscopy. The chromocenter located at the nuclear periphery, *CC*, and nucleolus, *NO*, are shown in (A). The spread-out chromocenter is shown in (B). Regions of intercalary heterochromatin are marked by asterisks.

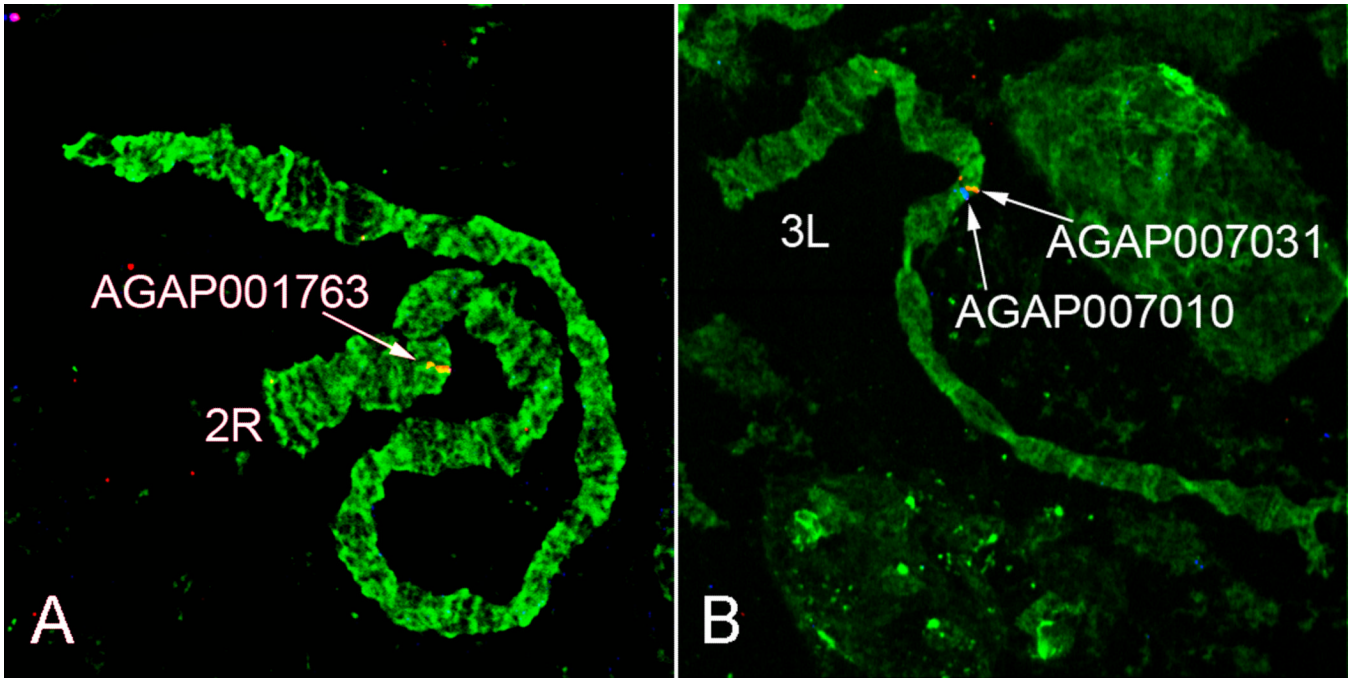

Fig. 2.
FISH of DNA probes homologous to the *An. gambiae* gene sequences with polytene chromosomes 2R (A) and 3L (B) of *An. ovengensis*. Probes were labeled with Cy3, red, and Cy5, blue, fluorochromes. Arrows indicate signals of hybridization.

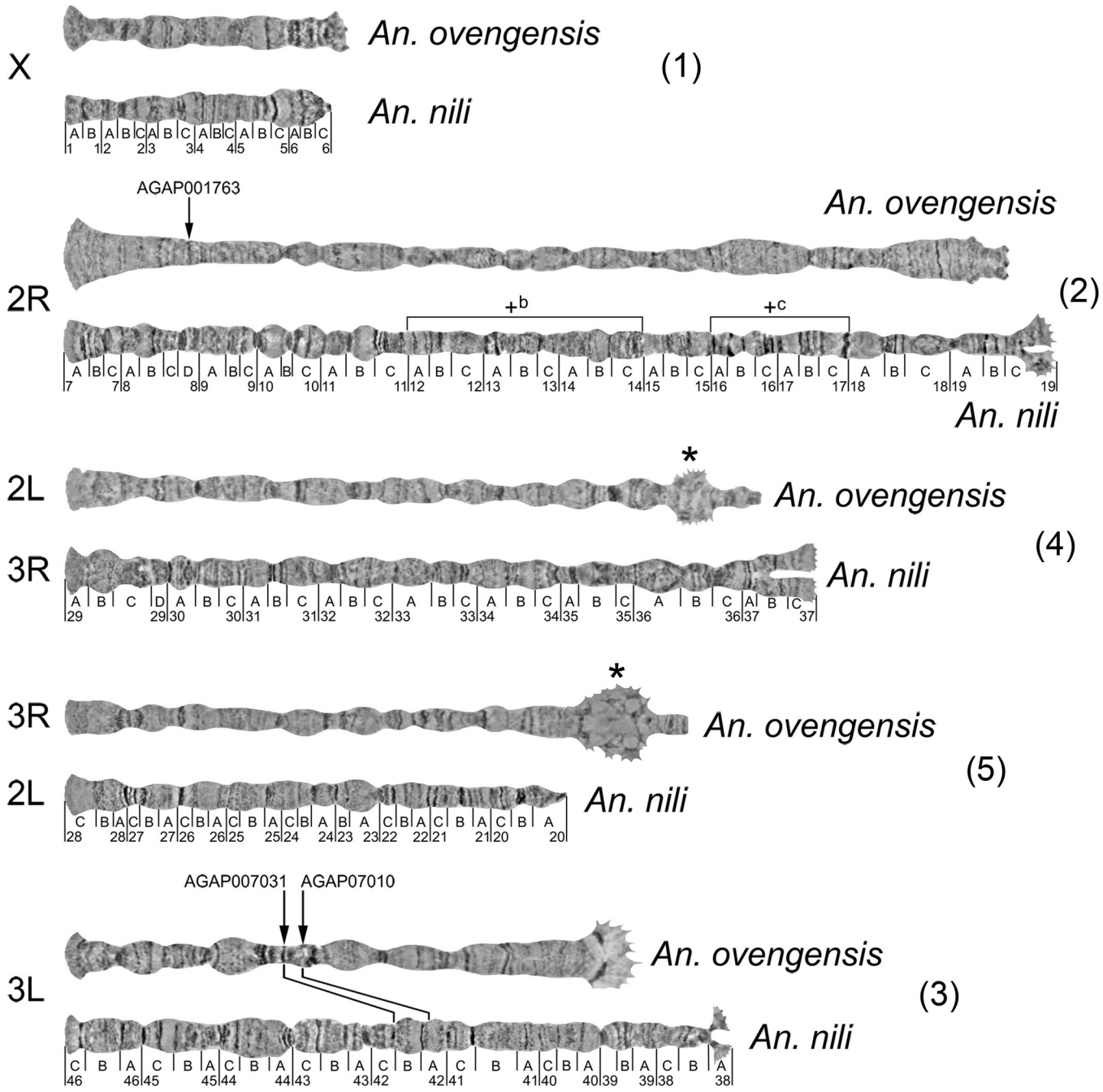

Fig. 3.

Comparison of the banding pattern of polytene chromosomes between *An. ovengensis* and *An. nili*. Chromosomal arm homology between the species is shown by numbers in parentheses. Arrows indicate localization of DNA probes homologous to the *An. gambiae* gene sequences on chromosomes of *An. ovengensis.* Intercalary heterochromatin in *An. ovengensis* is shown by asterisks. Lines between chromosomes 3L connect homologous markers mapped in both species. Polymorphic inversions in *An. nili* are shown by brackets above the 2R arm. Pericentromeric regions of chromosomes are oriented to the right. Unstained chromosome images were taken with a phase-contrast microscopy. The chromosomes are ordered according to the arm notation in *An. ovengensis.*

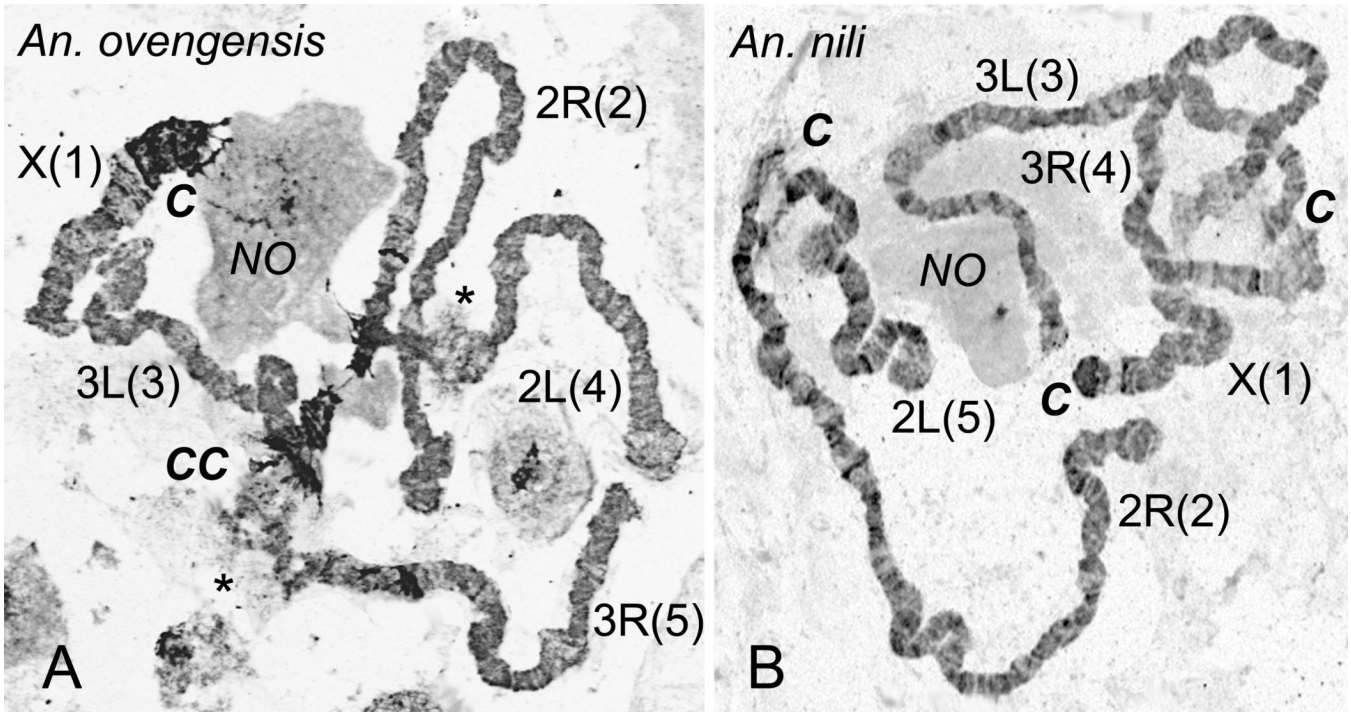

Fig. 4.

Differences in organization of polytene chromosomes between *An. ovengensis* and *An. nili.* A) Chromosomes of *An. ovengensis* form a chromocenter, *CC*, and display a large amount of heterochromatin—dark and granular areas of chromosomes. Asterisks indicate regions of intercalary heterochromatin. B) Pericentromeric regions, *C*, of *An. nili* do not form a chromocenter and display a small amount of heterochromatin. Chromosomal arm homologous between the species are shown with numbers in parentheses. *NO*—nucleolus. The preparations were stained with YOYO-1. Chromosome images were taken with a fluorescent microscope and converted into grayscale inverted images.

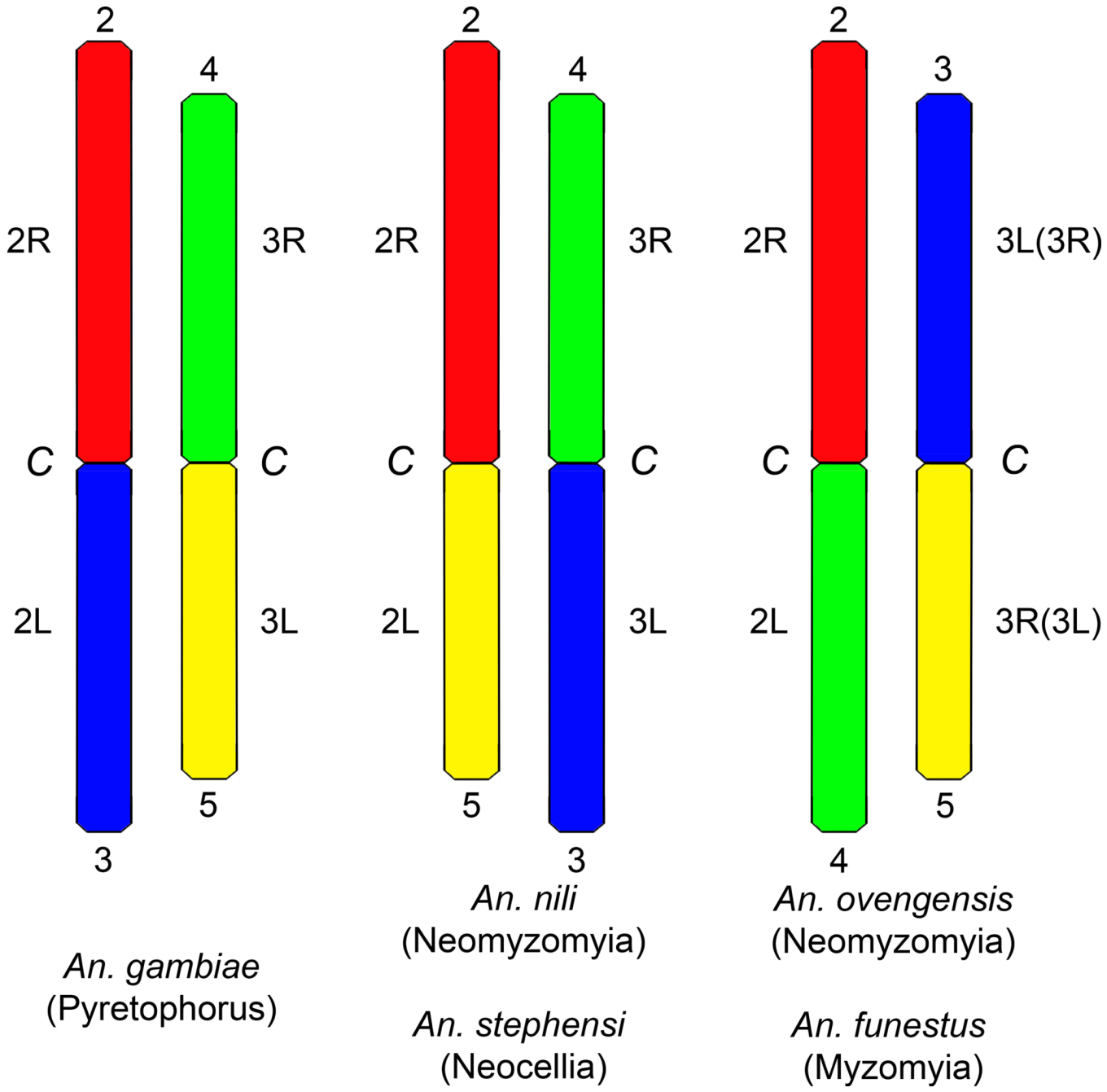

Fig. 5.

A scheme showing proposed chromosomal arm homology and arm translocations among members of subgenus Cellia. Homologous arms are indicated by the numbers above and below chromosomes. Names of series are shown in parentheses below the species names. Notations for 3R and 3L arms of *An. funestus* are in parentheses. *C*—pericentromeric regions.

Table 1

Frequencies of chromosome arm associations in ovarian nurse cells of *An. ovengensis.*

	Arm association type						
	2R+3L	**2R+2L**	**2R+3R**	**3L+2L**	**3R+3L**	**3R+2L**	**Total**
Number of cases	3	13	6	4	12	5	43
Frequency (%)	7.0	30.2	14.0	9.3	27.9	11.6	100