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Cytogenetic map for *Anopheles nili*: application for population genetics and comparative physical mapping

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Abstract

Anopheles nili is one of the major malaria vectors in Africa with a wide geographic distribution. However, the taxonomic and population genetic studies on this species are scarce. New research tools are urgently needed to genetically characterize this important malaria vector. In this study, a high-resolution cytogenetic map was developed for *An. nili* polytene chromosomes. Chromosomes were straightened and subdivided into 46 numbered divisions according to the banding pattern. Population analysis of *An. nili* females collected in Burkina Faso revealed the presence of two highly polymorphic inversions on the 2R chromosomal arm. A statistically significant departure from Hardy-Weinberg equilibrium due to a deficit in heterozygotes was detected for inversion 2Rb. To determine chromosome homologies and gene order conservation between *An. nili* and other major malaria vectors, PCR probes based on the *An. gambiae* coding sequences were mapped to *An. nili* chromosomes. Comparative mapping demonstrated that *An. nili* chromosomes have an *An. stephensi*-like arm association and that whole arm translocations and paracentric inversions were the major types of rearrangement in evolution of these mosquitoes. The minimum number of fixed inversions among *An. nili*, *An. gambiae*, and *An. stephensi* was calculated using the Multiple Genome Rearrangements (MGR), Genome Rearrangements In Man and Mouse (GRIMM), and Sorting Permutation by Reversals and block-INTERCHANGES (SPRING) programs. The data suggest that the *An. nili* is, at least, as diverged from *An. gambiae* as *An. stephensi*. We provide evidence that 2La/a arrangement of *An. gambiae* is present in outgroup species *An. nili* and *An. stephensi* confirming the ancestral status of the 2La inversion in the *An. gambiae* complex. Availability of the new polytene chromosome map, polymorphic inversions, and

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Appendix A. Supplementary data: Table S1. The chromosomal locations of the physically mapped DNA markers in *An. nili* and *An. stephensi* with the coordinates in the *An. gambiae* genome.

physically mapped DNA markers for *An. nili* will further stimulate population genetic, taxonomic, and genomic studies of this neglected malaria vector.

Keywords

malaria mosquito; *Anopheles nili*; *Anopheles gambiae*; *Anopheles stephensi*; physical mapping; polytene chromosomes; inversion; fluorescent *in situ* hybridization

1. Introduction

Malaria has a devastating impact on public health and welfare in the African continent. An important approach for reducing or eliminating malaria impact is vector control. However, vector control is inefficient if all major vector species are not targeted. Moreover, some malaria control initiatives fail because they target the wrong species or population (Coluzzi, 1992; Van Bortel et al., 2001). Mosquitoes of subgenus *Cellia*—*Anopheles gambiae*, *An. arabiensis*, *An. funestus*, *An. moucheti*, and *An. nili*—are the major malaria vectors because they are anthropophilic and susceptible to *Plasmodium falciparum* (Antonio-Nkondjio et al., 2006). These mosquitoes are responsible for >95% of the total malaria transmission in Africa (Mouchet et al., 2004). *Anopheles gambiae*, *An. arabiensis*, *An. funestus*, and *An. nili* have wide geographic distribution, spreading across most of West, Central, and East Africa whereas *An. moucheti* is restricted to forested areas of Central Africa (Hay et al., 2009). Among the widely distributed vectors, *An. gambiae*, *An. arabiensis*, and *An. funestus* have been intensively studied with the help of cytogenetic and molecular markers. *Anopheles nili* has received significantly less attention, in part, because available tools needed to precisely characterize species diversity and population structure are limited.

In contrast to *An. gambiae*, *An. arabiensis*, and *An. funestus*, *An. nili* inhabits dense vegetation or forest and breed in slow moving streams and large rivers (Ayala et al., 2009; Fontenille and Simard, 2004). This unique ecological adaptation explains the increased vectorial capacity of *An. nili* in some areas. For example, in forested areas of Cameroon, *An. nili* was found highly anthropophagous and responsible for 10.2% of malaria transmission (Antonio-Nkondjio et al., 2002; Antonio-Nkondjio et al., 2006). In coastal south-western Cameroon, *An. nili* accounted for 4.3% of the perennial malaria transmission (Bigoga et al., 2007). In a village in Eastern Senegal, *An. nili* was responsible for 56 infected bites per human per year (Dia et al., 2003). Sporozoite rates in *An. nili* can reach 3%, and the annual entomological inoculation rate can be over 100 (Carnevale et al., 1992).

The taxonomic and population complexity is a common feature of the principal vectors of malaria in Africa (Krzywinski and Besansky, 2003). *Anopheles nili* is assumed to represent the larger taxonomic units, namely, the *An. nili* group. Most recently, a new member of the *An. nili* group, *An. ovengensis*, was described based on morphology and bionomics (Awono-Ambene et al., 2004). The distribution, behavior, adaptation, and role in malaria transmission that can be attributed to each member of these groups is largely unknown (Fontenille and Simard, 2004). Distinction between members of the *An. nili* group is difficult because the morphological differences are subtle. Only three studies described species composition and population structure of *An. nili* using genetic tools (Awono-Ambene et al., 2006; Kengne et al., 2003; Ndo et al., 2010).

A study of sequence variations of the ribosomal DNA second internal transcribed spacer (ITS2) and D3 28S region between morphological forms has revealed four genetic patterns corresponding to typical *An. nili* s.s., *An. carnevalei*, *An. somalicus*, and *An. ovengensis* (Kengne et al., 2003). Genetic distances between ITS2 consensus haplotypes for each of

these four species were in the range 0.11–0.25. This value is much higher than expected between populations within the same species and is similar to those observed within the *An. funestus* group (Hackett et al., 2000; Koekemoer et al., 2002). A multilocus enzyme analysis of the genetic variability and population structure has detected species-specific alleles and large differences in shared allele frequencies among three malaria vector species of the *An. nili* group in Cameroon: *An. nili*, *An. carnevalei*, and *An. ovengensis* (Awono-Ambene et al., 2006). These results have provided further support for recent taxonomic classification within the *An. nili* group. Finally, the most recent population genetics study used microsatellite markers and mtDNA genes of *An. nili* (Ndo et al., 2010). It has been demonstrated that the high genetic homogeneity of *An. nili s.s.* is typical for West and Central Africa but the *An. nili* marginal population in the Democratic Republic of Congo is genetically isolated.

The commonly used markers for inferring anopheline taxonomic and population status include chromosomal inversions. Differential adaptations of mosquitoes to various environments are often associated with dramatic changes in composition and frequency of polymorphic inversions. *An. gambiae s.s.* is highly polymorphic for inversions on chromosome 2 (Pombi et al., 2008). These are nonrandomly distributed temporally and spatially with respect to degree of aridity (Coluzzi et al., 2002; Coluzzi et al., 1979; Powell et al., 1999; Simard et al., 2009; Toure et al., 1998). Moreover, frequencies of these inversions are higher indoors where the nocturnal saturation deficit is higher than it is outdoors (Bryan et al., 1982). Such population heterogeneity has important epidemiological and ecological consequences. Polytene chromosome analysis has revealed a complex population structure within *An. gambiae s.s.* and *An. funestus*. Highly significant departures from the Hardy-Weinberg equilibrium have been recorded for specific polymorphic inversions, with the alternative homokaryotypes (standard and inverted) significantly more frequent than expected (Costantini et al., 1999; Toure et al., 1998). The only one cytogenetic study of *An. nili* included 68 females from the M'Poka village area (3°55'S, 14°29'E) in the Democratic Republic of Congo (Miles et al., 1984). This study developed an incomplete map for *An. nili* but did not detect any inversion. The cytogenetic map did not divide polytene chromosomes by regions, and thus, possible inversion breakpoints and physically mapped markers could not be graphically defined.

Physical and genetic maps have been used for localizing loci responsible for epidemiologically important phenotypes in malaria mosquitoes. For example, integrated genetic and physical maps of genes and microsatellites for *An. funestus* (Sharakhov et al., 2004; Sharakhov et al., 2002; Wondji et al., 2005) have been used to localize Single Nucleotide Polymorphisms (SNPs) and to identify a quantitative trait locus (QTL) conferring pyrethroid resistance in this vector (Wondji et al., 2007a; Wondji et al., 2007b). Wild *An. gambiae* mosquitoes from Mali were genotyped with 25 physically mapped microsatellite markers spaced throughout the genome (Niare et al., 2002; Zheng et al., 1996). This enabled researchers to perform a genome-wide scan with ~9-centimorgan (cM) resolution and to identify the *P. falciparum* resistance island in a small region located in the proximal part of the 2L chromosomal arm (Riehle et al., 2006). The success of using the *An. gambiae* genome (Holt et al., 2002) for gene mapping of less studied malaria mosquito species, like *An. nili*, will depend on degree of gene order conservation.

Physical genome maps provide a suitable basis for comparative genomics, which can be used for determining the extent of chromosome conservation and rearrangement across species. The whole-arm translocations have been visually determined based on the banding pattern even within subgenus *Cellia* (Green and Hunt, 1980). Comparison of the physical maps of *An. gambiae*, *An. funestus*, and *An. stephensi* has confirmed this observation (Sharakhov et al., 2002; Sharakhova et al., 2006; Xia et al., 2008). It has been demonstrated that paracentric inversions and whole-arm translocations are the major types of

rearrangements and that the 2R arm has the fastest rate of inversion fixation among autosomes in genus *Anopheles* (Cornel and Collins, 2000; Sharakhov et al., 2002; Xia et al., 2010). Physical mapping of an outgroup species, *An. stephensi*, identified the 2La/a inversion in this species supporting the ancestral state of this arrangement in the *An. gambiae* complex (Sharakhov et al., 2006; Xia et al., 2008). However, evidence of the presence of the 2La/a arrangement in other outgroup species is needed to rule out a possibility of the independent origin of the same inversion in *An. stephensi*.

In this study, a new high-resolution cytogenetic map was developed for the African malaria vector *An. nili*. The map consists of straightened images of polytene chromosomes from ovarian nurse cells with numbered divisions and lettered subdivisions. Two paracentric polymorphic inversions were observed for the first time in field specimens collected in Burkina Faso and are described. We also report the chromosomal location of 23 DNA probes and comparative physical mapping with *An. gambiae* and *An. stephensi*. The integrated map of polytene chromosomes, inversion breakpoints, and physical markers will facilitate population and genomics studies of this neglected malaria vector.

2. Materials and methods

2.1. Wild mosquito collection, preservation, and species identification

Anopheles nili adult females were collected by pyrethrum spraying and bednet traps in two localities of Cameroon: Mbebe (2°80'N, 10°25'E) and Magba (5°57'N, 11°13'E) both situated along different river systems and at different altitudes. *Anopheles nili* specimens were also collected in the village of Dinderesso (11°14'N; 4°23'W) in Burkina Faso. Specimens were identified in the field as members of the *An. nili* group by using morphological identification keys (Awono-Ambene et al., 2004; Brunhes et al., 1998; Gillies and Coetzee, 1987) and were further characterized by molecular assays as *An. nili* s.s. (Kengne et al., 2003). 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).

2.2. Chromosome preparation, imaging, and inversion scoring

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 slide was placed on the follicles and pressed to squash the cells. The banding pattern of polytene chromosomes was examined using an Olympus CX-41 phase-contrast microscope (x1000) (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 seven best chromosomal slides were utilized to develop a map. Images of the chromosomes were combined, straightened, shaped, and cropped using Adobe® Photoshop. Homozygous and heterozygous inversions were scored using the chromosomal map presented in this article. Alternative chromosomal arrangements were considered as different alleles of the same locus, and conformance to Hardy-Weinberg equilibrium was tested with Fisher's exact tests available in GENEPOP V4.0 (Rousset, 2008). A Fis value was computed as in Weir and Cockerham (1984).

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 *in situ* hybridization procedure was conducted as previously described (Sharakhova et al., 2006). The DNA was labeled with Cy3-AP3-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 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).

2.4. Computational methods and analysis

The BLASTN and BLASTX algorithms were used to identify similar sequences in the *An. gambiae* genome, which is available at VectorBase (Lawson et al., 2009). The Multiple Genome Rearrangements (MGR) (Bourque and Pevzner, 2002), Genome Rearrangements In Man and Mouse (GRIMM) (Tesler, 2002), and Sorting Permutation by Reversals and block-INterchanGes (SPRING) (Lin et al., 2006) programs were utilized to calculate inversion distances among *An. gambiae*, *An. stephensi*, and *An. nili*. The MGR program is available at www.cs.ucsd.edu/groups/bioinformatics/MGR. The signed option of the MGR program was used. This program implements an algorithm that minimizes the sum of the rearrangements over all the edges of the phylogenetic tree (Bourque and Pevzner, 2002). The GRIMM program (unsigned option) was used to perform a pair-wise analysis of rearrangements (Tesler, 2002). GRIMM software uses the Hannenhalli and Pevzner algorithms for computing the minimum number of rearrangement events and for finding optimal scenarios for transforming one genome into another (<http://grimm.ucsd.edu/GRIMM/>). The SPRING program is available at <http://algorithm.cs.nthu.edu.tw/tools/SPRING/index.php>. SPRING computes both the breakpoint and rearrangement distances between any pair of two chromosomes (Lin et al., 2006). All these algorithms use gene order information to estimate rearrangement distances. MGR uses heuristic strategies to reconstruct a phylogenetic tree of input species. SPRING uses the Neighbor-Joining method to reconstruct a tree.

3. Results

3. 1. Polytene chromosome map for *An. nili*

Readable polytene chromosomes were obtained from the ovarian nurse cells of ~80% of *An. nili* females (Table 1). The polytene chromosome complement consisted of one short sex chromosome and four autosomal arms. The assignment of chromosomal arms was done based on their relative length and associations. The appearance of *An. nili* chromosomes is quite different from the appearance of *An. gambiae* chromosomes: they are shorter and more diffuse. Pericentromeric regions of the polytene chromosomes in *An. nili* do not form a chromocenter (Fig. 1). Therefore, it was easy to determine arm association. We found only one type of arm association (2R+2L, 3R+3L) that was used to identify left and right arms of the same chromosome. Chromosomes were divided into 46 regions on the basis of its banding patterns (Fig. 2).

Landmarks which can be used for arm recognition are the following. Similarly to other anopheline mosquitoes, the X chromosome is the shortest in the polytene complement. Telomeric end of the X chromosome is light and slightly flared. Pericentromeric area is always synaptic and forms three distinct dark bands in pericentromeric region 6BC. The most proximal band is significantly underreplicated and associates with the nucleolus.

Additional landmarks for the $\times$ chromosome include a large dark band in subdivision 5C and a puff in the middle of the chromosome (subdivision 3C) surrounded by two pairs of thin bands on both sides.

The 2R arm is the longest, and it has a flared striped telomeric end that contains 4 to 6 thin bands. The centromeric area of 2R is usually asynaptic and forms mesh-like heterochromatin structures. Additional landmarks for this arm are 3 large puffs in subdivisions 10A, 10C, and 11B, and 5 thin bands in subdivision 18B. The 2L arm is significantly shorter than 2R and has a light largely flared telomeric end. The pericentromeric end of 2L is usually narrow and dark due to underreplication. Three dark bands in subdivision 27C and a large puff in the middle of the arm (subdivision 23A) can be considered as additional landmarks for this arm.

In contrast to chromosome 2, chromosome 3 has arms of almost equal size; 3R is just slightly longer than 3L. The 3R arm can be recognized by the light, flared telomeric end and a puff in subdivision 29B. Bands of the telomeric region of 3R are thin and light. Telomeric area of 3L is also light and flared, but in contrast to 3R, it contains a dark dense band in the proximal part of subdivision 46C. The region following the telomere can be as puffy as the peritelomeric region of 3R. Pericentromeric areas of both arms are asynaptic. Asynapsis usually affects a longer pericentromeric area on 3R. An additional landmark for 3L is a large puffy area in region 44AC.

3.2. Identification and characterization of polymorphic inversions

Two highly polymorphic paracentric chromosomal inversions were found in Burkina Faso. Both inversions were on 2R: 2Rb and 2Rc. Inversion 2Rc was also observed in Cameroon, although at very low frequency, being recorded in only one specimen from Magba at the heterozygous state (Table 1). For convenience of mapping, the breakpoints of inversions were placed in the boundaries between regions 11C/12A (distal 2Rb), 14C/15A (proximal 2Rb), and 15C/16A (distal 2Rc), 17C/18A (proximal 2Rc). Two large bands in subdivision 13A and a light puff in subdivision 14B can serve as landmarks for discrimination of standard and inverted variants of inversion 2Rb (Fig. 3). In the case of the standard ($2R^{+b}/+^b$) chromosome, the light puff in subdivision 14B appears in the proximal part of the inversion, while in the case of homozygous inversion ($2Rb/b$), this region is near the distal part. The 2Rc inversion has a very good marker—a large dense band—located close to the proximal end of the inversion. This marker allows unambiguous identification of the alternative homozygotes $2Rc/c$ and $2R^{+c}/+^c$. To our knowledge, this is the first description of polymorphic inversions in *An. nili*. No inversion was found on chromosomes $\times$, 2L, 3R, and 3L.

In Burkina Faso, frequencies of inverted and standard 2Rb variants were almost equal (Table 1). However, a statistically significant departure from Hardy-Weinberg proportions due to a deficit in heterozygotes (e.g., positive Fis value) was detected at this locus (single test level, $P=0.008$). Inversion 2Rc occurred at high frequency in the sample (Table 1), with no significant deviation from Hardy-Weinberg equilibrium. Altogether, these results suggest that chromosomal inversions (at least 2Rb) may be exposed to natural selection in *An. nili*, as formerly observed in other major African malaria vectors (Coluzzi et al., 1979; Coluzzi et al., 2002; Cohuet et al., 2005).

3.3. Chromosome arm homology and gene order conservation between *An. nili* and *An. gambiae*

Chromosomal arm homology was established by successful hybridization of two DNA probes, AGAP001983 and AGAP001984, to the 2R arm and 23 probes to the 3L arm of *An. nili* (Fig. 2, 4). These physical locations were compared with the positions of the

homologous sequences in the *An. gambiae* genome. We found that chromosomes X, 2R, and 3R are homologous between the two species. The 2L arm of *An. gambiae* corresponds to the 3L arm of *An. nili*. The 3L arm of *An. gambiae* corresponds to the 2L arm of *An. nili*. Mapping of DNA clones to the polytene chromosomes of *An. stephensi* identified the same pattern of arm homology with *An. gambiae* (Sharakhova et al., 2006). Thus, our analysis demonstrated that *An. nili* chromosomes have the *An. stephensi*-like type of arm association.

This study used physically mapped DNA markers on the 3L arm of *An. nili* (Table S1) to infer the phylogenetic status of the 2La inversion in the *An. gambiae* complex. Our previous study has found that the gene order across the 2La breakpoints is identical in the *An. stephensi* 3L chromosome (Xia et al., 2008). In *An. gambiae* PEST strain (standard 2L⁺/⁺), the distal breakpoint is located between AGAP005778 and AGAP005779, and the proximal breakpoint is located between AGAP007068 and AGAP007069 (Sharakhov et al., 2006). The distal and proximal breakpoints of the 2La inversion have been mapped to chromosomal arm 3L of *An. nili* in this study and to chromosomal arm 3L of *An. stephensi* in our previous study (Xia et al., 2010). Our results indicate that the gene orders that span both breakpoints of the 2La/a arrangement in *An. gambiae* are the same as in outgroup species *An. nili* and *An. stephensi* (Fig. 5). Independent analysis of the gene order in a new outgroup species confirmed the ancestral status of the 2La inversion in the *An. gambiae* complex. Using the cross-species mapping, we identified conserved syntenic blocks among *An. gambiae*, *An. stephensi*, and *An. nili*. Five conserved synteny blocks were found between *An. gambiae* and *An. nili*. The lengths of blocks varied between 0.3 and 0.8 Mb.

Three-way analysis of rearrangements can be very efficient in assigning major rearrangement events to one of three lineages and determining phylogenetic relations between the species. We have calculated inversion distances among *An. gambiae*, *An. nili*, and *An. stephensi* based on known locations of 23 DNA markers common to all three species. Given that the size of the *An. gambiae* 2L arm is 49 Mb, this comparison has been done at the ~2 Mb level of resolution. The following gene orders for these arms were analyzed by the MGR and SPRING programs:

>*An. nili* 3L

14 13 12 11 23 22 21 10 9 8 7 15 16 17 18 19 20 6 5 4 3 2 1

>*An. stephensi* 3L

21 22 23 20 19 18 15 16 17 10 9 8 7 6 5 4 3 2 1 14 13 12 11

>*An. gambiae* 2L

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Both the MGR program (signed option) and SPRING program (rearrangement distance matrix) estimated nine fixed inversions between *An. nili* and *An. stephensi*, 19 fixed inversions between *An. stephensi* and *An. gambiae*, and 19 fixed inversions between *An. gambiae* and *An. nili* (Table 2). The breakpoint distance matrix of the SPRING program identified nine fixed inversions between *An. nili* and *An. stephensi*, 18 fixed inversions between *An. stephensi* and *An. gambiae*, and 17 fixed inversions between *An. gambiae* and *An. nili* (Fig. 6, Table 2). The SPRING program generated a rooted tree based on an assumption that a species with a bigger sum of total distances to other species can be regarded as a root. We also used the GRIMM program without assuming directionality of the markers (unsigned option) to perform a pair-wise analysis of rearrangements (Tesler, 2002). The GRIMM program calculated five fixed inversions between *An. gambiae* and *An. nili*, five fixed inversions between *An. gambiae* and *An. stephensi*, and six fixed inversions between *An. nili* and *An. stephensi*.

4. Discussion

The major goal of this study was to develop a cytogenetic research tool and assess its effectiveness for population genetic and evolutionary genomic studies of a neglected malaria vector, *An. nili*. A noticeable feature of our cytogenetic map is that the polytene chromosomes were straightened and divided by regions according to the banding patterns, as was done for *An. gambiae* (Coluzzi et al., 2002) and *An. funestus* (Sharakhov et al., 2001). Therefore, we were able to locate inversion breakpoints on the map with respect to lettered subdivisions. We described landmarks for recognition of chromosomal arms and inversions. Interestingly, two polymorphic inversions were found on the 2R chromosomal arm of *An. nili*. This observation confirmed the propensity of the 2R arm to generating inversions and agreed with the fact that this arm is enriched with polymorphic inversions in *An. gambiae* and *An. funestus* (Pombi et al., 2008; Sharakhov et al., 2004). High frequency of the inversion in the Burkina Faso population make them useful markers for studying population structure and ecological adaptations of this important vector. Similarly, *An. gambiae* and *An. funestus* populations are much more polymorphic in a savanna (West Africa), than in a forest (Central Africa) environment suggesting a universal role of inversion polymorphism in local adaptation.

Various molecular markers can be successfully used for physical mapping in malaria mosquitoes. The first *An. gambiae* physical map included 46 clones microdissected from the different divisions of polytene chromosomes (Zheng et al., 1991). Later, microsatellite markers (Zheng et al., 1996), randomly amplified polymorphic DNA (RAPD) markers (Dimopoulos et al., 1996), cosmids, and cDNA markers (della Torre et al., 1996) were physically mapped to the *An. gambiae* chromosomes. The most detailed mosquito physical map has been developed for the *An. gambiae* genome project (Holt et al., 2002). In our study, we used PCR probes based on coding sequences found in the *An. gambiae* genome. Availability of physically mapped DNA markers for *An. nili* gave us a first-time opportunity to perform a comparative cytogenetic study with other major malaria vectors. The analysis demonstrated that chromosomes of *An. nili* have the same pattern of arm association as chromosomes of the Asian vector *An. stephensi*, which differ from the patterns of arm association found in other African malaria vectors, *An. funestus* and *An. gambiae* (Sharakhov et al., 2002). The same arm association found in the African and Asian malaria mosquitoes could be a consequence of the common ancestry or a result of convergent evolution. In any case, it is clear that whole-arm translocations happened repeatedly in evolution of the subgenus *Cellia*.

The success of using the *An. gambiae* reference genome for gene mapping of any malaria mosquito species will depend on degree of gene order conservation. The MGR, GRIMM, and SPRING analyses identified several fixed inversions among homologous chromosomes of *An. nili*, *An. stephensi*, and *An. gambiae*. This observation indicates that the gene order is not preserved between *An. nili* and *An. gambiae* and that paracentric inversions are common among different series of subgenus *Cellia*. The inversion distance data suggest that the *An. nili* is, at least, as diverged from *An. gambiae* as *An. stephensi*. Thus, the large-scale genomic information cannot be easily transferred from the better studied *An. gambiae* to the less studied *An. nili*. We demonstrated that the gene order is preserved between *An. gambiae* and *An. nili* at a small-scale resolution. The average length of conserved syntenic blocks between the 2L arm of *An. gambiae* and the 3L arm *An. nili* was 0.47 Mb. The lengths of conserved syntenic blocks of the 2R arm and the X chromosome are expected to be even smaller (Xia et al., 2010).

The physically mapped DNA markers on the 3L arm were used to demonstrate the presence of the 2La inversion in outgroup species *An. nili* (this study) and *An. stephensi* (Xia et al.,

2010; Xia et al., 2008) and to confirm the ancestral status of the 2La inversion determined previously from the breakpoint structure (Sharakhov et al., 2006). The physical mapping evidence of the presence of the 2La/a arrangement in at least two outgroup species ruled out a possibility of the independent origin of the same inversion in multiple lineages. Moreover, this evidence is consistent with earlier cytological identification of the 2La inversion in two members of the *An. subpictus* complex, i.e., outside of the *An. gambiae* complex (Ayala and Coluzzi, 2005).

The new cytogenetic map for *An. nili* will be one of the major tools for physical mapping and assembly of the genome sequences. Cytogenetic and physical mappings 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 this neglected malaria vector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Polytene chromosomes from ovarian nurse cells of *An. nili* from Cameroon. The centromeric regions (c) and nucleolus (n) are shown.

Figure 2.

A cytogenetic map of *An. nili* showing the location of two polymorphic inversions on the 2R chromosomal arm and physically mapped DNA markers.

Figure 3. Polymorphic inversions on the 2R arm of *An. nili* from Burkina Faso. The standard (A), inverted (B), and double heterozygote (C) polymorphic inversions 2Rb and 2Rc are shown.

Figure 4.

Fluorescent *in situ* hybridization of two DNA probes labeled with Cy3 with euchromatic regions on the 3L chromosomal arm: AGAP006965 (A) and AGAP006958 (B). Right panels show the banding pattern of the chromosomes. Left panels show fluorescence due to hybridization. Arrows indicate signals of hybridization.

Figure 5.

The gene orders comparison among *An. gambiae*, *An. nili*, and *An. stephensi* near the breakpoints of the 2La inversion of *An. gambiae* (shown by stars). Arrows indicate orientation of conserved syntenic blocks. Lines between chromosomes connect homologous markers and conserved syntenic blocks between species. Shaded divisions on the *An. gambiae* chromosomes denote the genomic coordinates in the 2La/a arrangement.

Figure 6.

(A) An unrooted tree of *An. gambiae*, *An. nili*, and *An. stephensi* recovered by MGR program. The number of rearrangements that occurred on each edge is shown. A3 is a putative ancestral species. (B) A rooted SPRING phylogenetic tree corresponding to the breakpoint distance matrix. (C) A rooted SPRING phylogenetic tree corresponding to the rearrangement distance matrix. The numbers of fixed inversions between *An. nili* and *An. gambiae*/*An. stephensi* are shown in parentheses.

Table 1Chromosome readability and polymorphism in *An. nili* populations from Burkina Faso and Cameroon.

	Burkina Faso	Cameroon	
	Dinderesso (11°14'N;4°23'W)	Mbebe (2°80'N, 10°25'E)	Magba (5°57'N, 11°13'E)
Number of preparations analyzed	71	60	87
Number of readable preparations (%)	56 (79)	49 (82)	78 (90)
Inversion 2Rb			
Inversion frequency	0.464	0	0
Fis ^a	+ 0.362	-	-
P(HW) ^b	0.008	-	-
Inversion 2Rc			
Inversion frequency	0.759	0	0.006
Fis ^a	+0.082	-	-
P(HW) ^b	0.713	-	-

^aFis was computed as in (Weir and Cockerham, 1984).^bExact test for conformance to Hardy-Weinberg equilibrium, as implemented in GENEPOP.

Table 2

Pairwise distance matrix of the input genomes obtained from MGR and computed rearrangement and breakpoint distance matrix obtained from SPRING.

Rearrangement distance matrix obtained from MGR and SPRING			
	<i>An. gambiae</i> 2L	<i>An. nili</i> 3L	<i>An. stephensi</i> 3L
<i>An. gambiae</i> 2L	0	19	19
<i>An. nili</i> 3L	19	0	9
<i>An. stephensi</i> 3L	19	9	0

Breakpoint distance matrix obtained from SPRING			
	<i>An. gambiae</i> 2L	<i>An. nili</i> 3L	<i>An. stephensi</i> 3L
<i>An. gambiae</i> 2L	0	17	18
<i>An. nili</i> 3L	17	0	9
<i>An. stephensi</i> 3L	18	9	0