MOLECULAR KARYOTYPING OF THE 2LA INVERSION IN *ANOPHELES GAMBIAE*

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Abstract. The African malaria vector *Anopheles gambiae* is polymorphic for alternative arrangements on the left arm of chromosome 2 (2La and 2L+[a]) that are non-randomly distributed with respect to degree of aridity. Detailed studies on the ecological role of inversion 2La have been hindered by the technical demands of traditional karyotype analysis and by sex- and stage-specific limitations on the availability of polytene chromosomes favorable for analysis. Recent molecular characterization of both inversion breakpoints presented the opportunity to develop a polymerase chain reaction (PCR)-based method for karyotype analysis. Here we report the development of this molecular diagnostic assay and the results of extensive field validation. When tested on 765 *An. gambiae* specimens sampled across Africa, the molecular approach compared favorably with traditional cytologic methods, correctly scoring > 94% of these specimens. By providing ready access to the 2La karyotype, this tool lays groundwork for future studies of the ecological genomics of this medically important species.

INTRODUCTION

Chromosomal inversions promote adaptation in a diversity of eukaryotes from yeast to primates.[1,2] In particular, paracentric chromosomal inversions have played a major role in the recent adaptive radiation of the *Anopheles gambiae* mosquito complex. Six of the seven morphologically indistinguishable species in this complex can be identified by unique fixed inversions in its karyotype.[3] Furthermore, polymorphic inversions seem to be one of the main driving forces behind ecological partitioning in *An. gambiae s.s.*—the type species of the complex and the most proficient vector of malaria in the world.[4] By suppressing recombination in heterokaryotypes, polymorphic chromosomal inversions place captured genes on an independent evolutionary tract from homologous genes in the ancestral arrangement. Such chromosomal diversity has allowed *An. gambiae s.s.* (hereafter, *An. gambiae*) to quickly adapt to both natural and anthropogenic heterogeneities in the environment, presumably increasing malaria transmission across both space and time.[3]

On the left arm of chromosome 2 and subsuming roughly one half its length, inversion 2La is a critical component to the ongoing ecological differentiation in this medically important species. Recent cytologic and molecular studies of 2La, long considered the derived arrangement relative to an arbitrary standard, leave little doubt that 2La is the ancestral arrangement from which 2L+[a] arose.[5,6] *An. gambiae* remains highly polymorphic for the two arrangements, although they are non-randomly distributed temporally and spatially with respect to degree of humidity in East and West Africa.[7,8] This pattern is most apparent in West Africa, where strong north-

south clines in the frequency of the 2La inversion range from fixation in the arid northern Sahel to absence in the humid southern rainforests (Figure 1). At sites along the cline where neither arrangement is fixed, seasonal fluctuations occur in which 2La cycles from low to high frequency between wet and dry seasons. In addition, the frequency of 2La is correlated with microclimatic differences in humidity that impact mosquito behavior: this arrangement is more common in mosquitoes found resting indoors where a nocturnal saturation deficit exists.[8] Such population heterogeneity has important epidemiologic and ecological consequences. For example, indoor residual spraying of insecticides against *An. gambiae* is an approach that will not necessarily impact the population uniformly, as seen in the Garki malaria control project in Nigeria.[9] The adaptive flexibility provided by this chromosomal polymorphism has allowed *An. gambiae* to exploit a very broad range of climatic conditions, an important factor underlying the wide distribution and abundance of this species across Africa as well as its status as primary malaria vector.

The correlation between the frequency of the 2La inversion of *An. gambiae* and degree of humidity has been known for nearly 30 years. However, progress at understanding this phenomenon more deeply at the genetic and molecular level has been stalled for lack of key tools. The *An. gambiae* genome project overcame one major obstacle to genetic analysis.[10] A significant remaining barrier to studying inversions in *An. gambiae* is the requirement for karyotyped specimens: those whose chromosomal banding pattern has been read from polytene chromosomes by a skilled cytogeneticist with the aid of a microscope. Polytene chromosomes favorable for interpretation of the banding pattern are limited to one tissue and developmental stage of one sex: the large nurse cells within the ovaries of half-gravid females. Such a constraint increases the time, effort, and expense needed for fresh sample collection while precluding the use of any previous collections that were inadequately preserved for cytogenetics, of the wrong
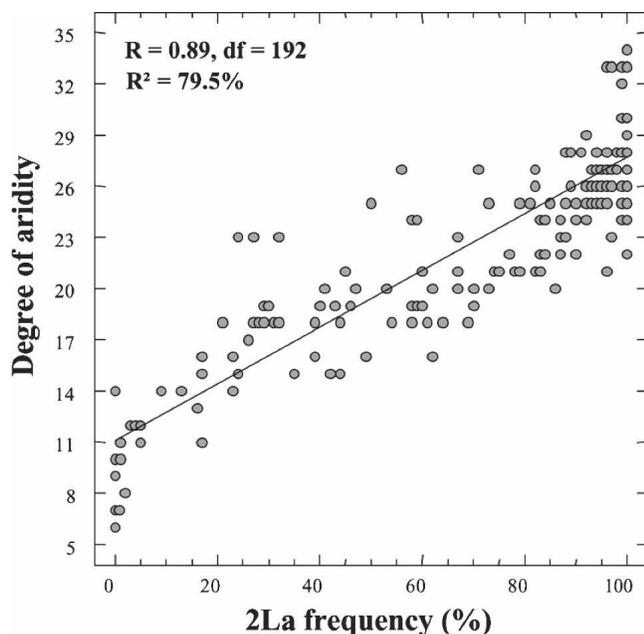
R = 0.89, df = 192
R² = 79.5%

FIGURE 1. Scatter diagram of the relationship between degree of aridity and the frequency of the 2La arrangement in West African populations of *An. gambiae*, based on ~24,000 mosquitoes sampled from 194 sites (modified from Coluzzi 1992[22]). The solid line is the fitted linear regression.

sex, or the incorrect developmental stage. This constraint also effectively limits study of the ecological genomics of inversions to half-gravid females, unless researchers invest considerable effort in rearing immatures to adulthood and blood-feeding surviving females—often recalcitrant to taking blood meals—to stimulate oogenesis. Moreover, karyotype analysis is labor intensive and requires uncommon expertise. In combination, these factors have removed the study of inversions in *An. gambiae* beyond the reach of most laboratories, and they explain lack of progress in this area.

The recent molecular cloning and sequence characterization of the 2La breakpoints delimited this rearrangement with a high degree of precision relative to previous cytogenetic estimates.[6] Importantly, these data also provide the basis for a DNA-based strategy to determine the 2La karyotype of both sexes and all developmental stages, overcoming the major limitations to traditional karyotype analysis. Here we report a simple polymerase chain reaction (PCR) assay for molecular karyotyping of the 2La inversion. To our knowledge, it is the first extensively validated molecular assay for determining chromosomal arrangements in any organism.

MATERIALS AND METHODS

Sample collection and preparation. *Anopheles gambiae s.l.* were collected from the following 57 sites from nine African countries—The Gambia: Ballingho (15°37′ W, 13°28′ N), Bambali (15°19′ W, 13°28′ N), Bassé (13°13′ N, 14°15′ W), Kaur (13°41′ N, 15°18′ W), McCarthy Island (13°31′ N, 14°46′ W); Senegal: Kedougou (12°36′ N, 12°14′ W); Mali: Bancoumana (12°20′ N, 8°20′ W), Banambani (12°48′ N, 08°03′ W), Douna (13°13′ N, 05°55′ W), Fanzana (13°20′ N, 06°13′ W), Kela (11°88′ N, 8°45′ W), Moribabougou (12°41′ N, 07°57′ W), N'Gabakoro (12°43′ N, 07°01′ W); Burkina Faso:

Monomtenga (12°06′ N, 01°17′ W); Cameroon: Batao (9°57′ N, 14°09′ E), Bidzar (9°54′ N, 14°07′ E), Bodova (9°59′ N, 14°10′ E), Boussibelika (2°44′ N, 9°52′ E), Campo (2°23′ N, 9°50′ E), Carrefour Poli (8°32′ N, 13°32′ E), Djiffere (13°56′ N, 16°46′ W), Dombé (2°57′ N, 9°55′ E), Doujouf (10°34′ N, 14°17′ E), Eboundja (2°49′ N, 9°54′ E), Gouna (8°31′ N, 13°34′ E), Grand Batanga (2°52′ N, 9°53′ E), Karba (10°03′ N, 14°09′ E), Kossoum (10°04′ N, 14°11′ E), Lam (10°04′ N, 14°08′ E), Lendi (2°55′ N, 9°56′ E), Lolabé (2°40′ N, 9°51′ E), Massila (10°11′ N, 14°09′ E), Mfou (3°44′ N, 11°38′ E), Morongo (10°06′ N, 14°12′ E), Mounda (10°22′ N, 14°14′ E), Moussourtouk (10°20′ N, 14°14′ E), Nlende Dibé (2°46′ N, 9°53′ E), Ouro Gadji (8°31′ N, 13°36′ E), Sanguere Ngal (9°13′ N, 13°30′ E), Tibati (6°28′ N, 12°37′ E), Tongo (8°55′ N, 13°31′ E); Angola: Cavaco (13°26′ E, 12°33′ S), Cabinda (5°32′ S, 12°11′ E), Namibe (2°09′ E, 15°10′ S), Nazare and Mateba (Luanda) (13°23′ E, 8°45′ S); Uganda: Entebbe (0°04′ N, 32°28′ E), Pallisa (1°12′ N, 33°43′ E), Bugala Island (0°40′ S, 32°20′ E); Kenya: Ahero (0°10′ S, 34°55′ E), Rota (0° 08′ S, 34°36′ E); Magaoni (4°11′ N, 39°26′ E), Mtepeni (3°38′ S, 39°45′ E), Loboi-Baringo (0°43′ N, 36°19′ E), Mwea (0°44′ S, 36°79′ E), Vanga (4°39′ S, 39°13′ E); and Zimbabwe: Mana Pools National Park (16°09′ S, 29°26′ E).

Indoor resting adult mosquitoes were collected by pyrethrum spray catch or manual aspiration. *An. gambiae s.l.* were identified morphologically.[11] Ovaries of half-gravid specimens were dissected and fixed in Carnoy's solution (3:1 ethanol:glacial acetic acid). Karyotyping was performed according to standard protocol.[12] If possible, excess ovarian tissue and slides were retained for re-examination in case of discrepancy.

Across the participating institutions, DNA was extracted from individual mosquitoes by one of four methods: Qiagen (Valencia, CA) DNeasy Extraction Kit, Gentra (Gentra Systems, Inc., Minneapolis, MN) "Puregene" Kit, and Promega (Promega Corp., Madison, WI) Wizard SV 96 system following manufacturer's protocols, or a standard non-commercial protocol.[13] Quality and quantity of extracted DNA was assessed spectrophotometrically for a subset of the samples using the NanoDrop-5000 (NanoDrop Technologies, Wilmington, DE). The DNeasy Kit produced the highest yield and best quality DNA of the four methods, but all provided reliably amplifiable DNA. Species-level identification of *An. gambiae s.l.* was performed using the rDNA-based PCR assay of Scott and others.[14]

Primer design and PCR. Prospective primer pairs for the amplification of both arrangements were obtained by the input of SUA (2La) or PEST (2L+ᵃ) breakpoint sequences into Primer3.[15] Conservation of the intended target site was inferred by examination of aligned sequences (ClustalX) from three strains of *An. gambiae* (SUA, Bamako, and PEST), *An. arabiensis*, and *An. merus* (GenBank accession no. DQ230889–DQ230901 from Sharakhov and others).[6] Before broader application, robustness of prospective primer combinations was tested on laboratory colonies of known karyotype and a subset of field specimens.

PCR reactions were carried out in a 25-μL reaction that included 200 μmol/L each dNTP, 2.5 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 5 pmol of each primer, 2.5 U Taq polymerase, and 1–50 ng of template DNA. Thermocycler conditions were 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45

seconds; a final elongation at 72°C for 10 minutes; and a 4°C hold. The resulting products were analyzed on 1.5–2% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Molecular karyotyping strategy. There are two approaches for designing a PCR assay to karyotype inversions. The first relies on available fixed nucleotide differences (substitutions or insertion/deletions) between arrangements as markers for each arrangement.[16] This strategy avoids complex breakpoint regions and derives its success from reduced recombination near the breakpoints of inversion heterozygotes. However, non-zero levels of crossing-over or gene conversion in heterozygotes can eventually decay the association between the molecular marker and the arrangement. This risk is emphasized by the recent report of gene conversion inferred in a coding region abutting one breakpoint of In(3R)Payne in *Drosophila melanogaster.*[17] The second strategy, which we used, uses the rearrangement itself as the basis for discrimination. Primer pairs whose PCR products physically cross the breakpoints will only amplify from one of the two alternative arrangements.[18] Although complexities associated with many breakpoints (e.g., duplications and large insertions of repetitive DNA) can complicate design of this type of assay, it eliminates the risk of breakdown between molecular marker and arrangement and minimizes the possibility of misdiagnosis.

Assay design. For amplification across the 2La distal breakpoint (from region 1 to region 2; Figure 2), primers 23A2 and 27A2 were chosen among other pairs tested because of robust and specific amplification of the expected 492-bp product (Figure 2; Table 1). Both primers are located in noncoding intergenic regions of unique sequence. Long segments of repetitive DNA, including the duplication of > 700 bp from the 2La proximal breakpoint, challenged primer design across the 2L+[a] breakpoints (Figure 2). Attempts to cross entire or partial repetitive regions were unsuccessful because of unreliable

Primer	Target	Sequence 5′–3′
23A2	Universal	CTCGAAGGGACAGCGAATTA
27A2	2La	ACACATGCTCCTTGTGAACG
DPCross5	2L+[a]	GGTATTTCTGGTCACTCTGTTGG

amplification, multiple amplification products differing in length from the expected size, and/or excessive length. Ultimately the 2L+[a] proximal breakpoint (region 2 to region 4; Figure 2) was specifically targeted by designing a 23-bp primer, DPCross5 (Table 1), whose first 18 bp at the 5′-end correspond to repetitive DNA inserted at the breakpoint but whose last 5 bp at the 3′-end anneal in the unique flanking DNA of region 2. Incorporation of the last 5 bp of region 2 before the breakpoint prevented primer-extension from multiple (repetitive) sites, whereas inclusion of the first 18 bp of repetitive DNA at the breakpoint precluded DPCross5 from annealing to 2La chromosomes. The primer pair DPCross5 and 23A2 specifically targets the 2L+[a] proximal breakpoint, amplifying a 207-bp product (Figure 2).

Before full-scale validation, all three primers (23A2, 27A2, and DPCross5) were combined into a single PCR reaction and tested on artificial heterozygotes (1:1 mixture of DNA from 2La/a and 2L+[a]/+[a] karyotypes determined cytologically). Both primer pairs amplified well even with < 1 ng of template, suggesting that competition between the products was negligible despite sharing of primer 23A2 and that the three-primer cocktail can be used in a single PCR reaction to identify karyotype.

Validation and performance. Wide-scale validation of the PCR assay was performed on karyotyped specimens collected from across much of the range of *An. gambiae,* including samples from West, Central, and East Africa (Figure 3; Table
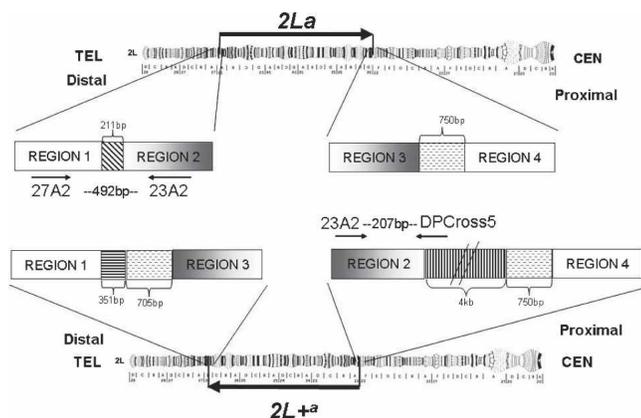

FIGURE 2. Schematic representation of the PCR diagnostic strategy for karyotyping 2La and 2L+[a] chromosomes (not to scale). Boxes labeled as regions 1–4 on each arrangement are homologous single copy sequences flanking the breakpoints. Hatched boxes represent repetitive DNA. Stippled box represents sequence present once at the proximal breakpoint of the 2La arrangement and duplicated at both breakpoints of the 2L+[a] arrangement. Orientation and target of the PCR primers are indicated by arrows labeled 23A2, 27A2, and DPCross5. TEL, telomere; CEN, centromere.
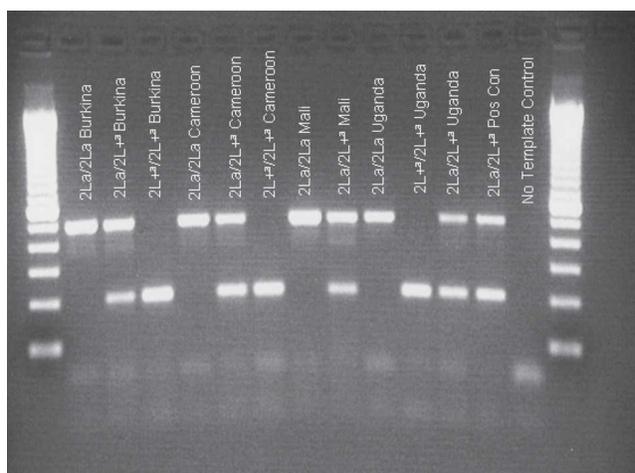

FIGURE 3. Molecular karyotype analysis. Shown is an image of electrophoretically separated PCR products from the 2La diagnostic assay, flanked by molecular weight markers (100-bp ladder; Life Technologies, Gaithersburg, MD). Each lane is labeled with the specimen's geographic origin and its karyotype, determined independently by traditional cytogenetic methods. Expected (and observed) product sizes for the 2La and 2L+[a] arrangements are 492 and 207 bp, respectively. 2La/2L+[a] Pos Con, positive control.

2). PCR results were categorized as no-calls (no bands produced at either 492 or 207 bp), false-calls (bands produced inconsistent with karyotype), and correct-calls (bands produced consistent with karyotype). Overall, in 720 of 765 (94.1%) specimens tested, the diagnostic called the karyotype correctly. In only 3.3% of specimens did the diagnostic produce a call inconsistent with the karyotype. No-calls represented an even smaller fraction (2.6%) of the tested specimens.

Not surprisingly, heterokaryotypic specimens accounted for the majority (19/25, 76%) of the false-calls. In these 2La/2L+[a] specimens, if a mutation occurred at the target site of one of the arrangement-specific primers (27A2 and DPCross5) or at one of the two target sites (one on each arrangement) for 23A2, it would cause a null (non-amplifying) allele. Only a single PCR product would be produced, and the specimen would be incorrectly scored as a homozygote. Of the 19 cytogenetically determined heterokaryotypes that were false-calls by the molecular diagnostic, 12 (63%) were called as 2L+/2L+ homokaryotypes, whereas the other 7 (37%) were called as 2La/2La homokaryotypes. Assuming that none of the 19 false-calls involving heterokaryotypes can by explained by errors in reading or recording the cytogenetic data or other human error, it suggests a null allele rate of ~2% for this assay. Null alleles are well known from microsatellite-based surveys of *An. gambiae* populations,[19] and they presumably reflect a high rate of standing variation in this species. When the rate of microsatellite null alleles was inferred from the X chromosome in ~100 hemizygous *An. gambiae* males surveyed in Burkina Faso, it ranged from 0% to 31% across 17 loci (average, ~5%).[20] These data imply that no molecular diagnostic assay based on single copy nuclear sequence is likely to achieve a perfect performance, because it will be subject to the pitfalls of null alleles. Conservatively assuming that all false-calls (3.3%) and all no-calls (2.6%) recorded in this study are caused by null alleles (almost certainly an overestimate; see below), the overall error rate does not exceed previous estimates of null alleles in *An. gambiae*.

Homokaryotypes accounted for 16 of the 20 (80%) no-calls but only 6 of the 25 (24%) false calls. No-calls probably occurred because of DNA degradation or target site mutations on both chromosomes, with the former being more likely than the latter. (At an average null allele rate of 5%, only four no-calls from homokaryotypes would have been expected.) Given the strategic design of this molecular karyotyping assay, it is more difficult to explain the occurrence of six false-calls involving homokaryotypes. The most parsimonious explanation involves human error, including mixed-up specimens/DNA and/or errors in reading or recording the karyotype. In some cases, this hypothesis could be confirmed by re-checking remaining ovaries or slides.

Initially, we recorded a total of 33 false-calls. For 11 of the 33 specimens in question, additional chromosomes or original slides were available for re-examination, which was conducted blind (without knowledge of the original karyotype assessment). Chromosomal reassessment agreed with the molecular karyotype in 8 of 11 specimens, which were subsequently recorded as correct-calls. The original karyotype was confirmed in the remaining three specimens. In view of the fact that 8 of 11 (~73%) false-calls proved to be mistaken cytologic karyotypes on re-examination, it is probable that some of the remaining 22 false-calls that could not be re-examined also represent mistaken karyotypes. Regardless, these data show remarkable agreement between molecular and cytologic karyotyping. There is some error inherent in the use of either method; the reliability of molecular karyotyping seems comparable or superior to traditional cytogenetics.

Other members of the *An. gambiae* complex are not polymorphic for the 2La inversion. *An. arabiensis* and *An. merus* are fixed for the ancestral 2La arrangement, whereas *An. melas*, *An. quadriannulatus,* and *An. bwambae* are all fixed for the derived 2L+[a] arrangement. Although practical application of the 2La diagnostic assay is not foreseen in these species, it was of interest to determine whether sequence conservation was sufficient for the assay to yield the expected products. *An. bwambae* was not available for testing, but wild-caught specimens of all other species gave results consistent

TABLE 2
Performance of the 2La molecular karyotyping PCR assay in field collections of *An. gambiae s.s.*

| | Country samples | | | | | | | | |
	Gambia	Senegal	Mali	Burkina Faso	Cameroon	Angola	Uganda	Kenya	Overall
Proportion scored*									
2La/a	24/24 (100%)	24/24 (100%)	241/243 (99.2%)	45/45 (100%)	46/48 (95.8%)	2/2 (100%)	NA	7/7 (100%)	389/393 (99.0%)
2L+[a]/+[a]	N/A	N/A	N/A	1/1 (100%)	152/158 (96.2%)	30/34 (88.2%)	3/4 (75%)	27/28 (96.4%)	213/225 (94.7%)
2La+[a]	19/19 (100%)	14/14 (100%)	10/10 (100%)	6/6 (100%)	40/42 (95.2%)	37/38 (97.4%)	4/4 (100%)	13/14 (92.9%)	143/147 (97.3%)
Total	43/43 (100%)	38/38 (100%)	251/253 (99.2%)	52/52 (100%)	238/248 (95%)	69/74 (93.2%)	7/8 (87.5%)	47/49 (95.9%)	745/765 (97.4%)
Proportion congruent†									
2La/a	24/24 (100%)	24/24 (100%)	239/241 (99.2%)	45/45 (100%)	46/46 (100%)	2/2 (100%)	N/A	7/7 (100%)	387/389 (99.5%)
2L+[a]/+[a]	N/A	N/A	N/A	1/1 (100%)	150/152 (98.7%)	29/30 (96.7%)	3/3 (100%)	26/27 (96.3%)	209/213 (98.1%)
2La/+[a]	14/19 (73.7%)	14/14 (100%)	9/10 (90%)	4/6 (66.7%)	36/40 (90%)	31/37 (83.8%)	3/4 (75%)	13/13 (100%)	124/143 (86.7%)
Total	38/43 (89.4%)	38/38 (100%)	248/251 (98.8%)	50/52 (96.2%)	232/238 (97.5%)	62/69 (89.9%)	6/7 (85.7%)	46/47 (97.9%)	720/745 (96.6%)

* Proportion of specimens that amplified by PCR.
† Proportion of specimens whose molecular karotype matched the karotype as determined by traditional cytogenetics.
N/A, not available.

with known karyotype. Of 83 *An. arabiensis* tested from Senegal, Burkina Faso, Cameroon, Uganda, Kenya, and Zimbabwe, 82 (99%) produced the expected 2La band of 492 bp, and one specimen failed to amplify. Similarly, all eight *An. merus* tested from Kenya produced the expected 2La band. For the alternative arrangement, all nine *An. quadriannulatus* tested from Zimbabwe and all *An. melas* from Senegal that amplified (three of four) produced the expected 2L+[a] band at 207 bp.

Conclusion and prospects. We developed and extensively field-validated a PCR assay that can be implemented for high-throughput karyotyping of the 2La inversion from any developmental stage of *An. gambiae*. Its overall accuracy (~94%) compares favorably with traditional cytologic methods. This simple yet powerful tool is a prerequisite for in-depth behavioral and ecological studies of the 2La inversion in immatures and males. Together with the complete genome sequence of *An. gambiae*, this tool provides an entry point to the ecological genomics of 2La, and it will revitalize efforts begun much earlier[21] to understand the relationship between 2La and factors affecting malaria transmission intensity and vectorial capacity.

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