Polymorphisms detected by random PCR distinguish between different chromosomal forms of *Anopheles gambiae*

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**ABSTRACT** We have applied PCR amplification using random primers to distinguish between incipient species of the malaria vector *Anopheles gambiae*. Individuals belonging to three chromosomally characterized West African forms of this mosquito, which are important epidemiologically as they differ in vectorial capacity, were sampled both from laboratory stocks and from wild populations collected in three localities. The techniques used allowed for the unambiguous classification of the mosquitoes, providing a tool for rapid and efficient diagnosis, which previously relied on cytological examination of polytene chromosomes.

The fact that most *Anopheles* mosquitoes belong to different species complexes with members that are often hard, or even impossible, to distinguish morphologically makes their population biology difficult to study and represents a major difficulty in malaria entomology. Correct analysis of the distribution of specific malaria vectors is one of the prerequisites for meaningful epidemiological studies and for the planning and monitoring of successful malaria control or eradication programs, particularly those based on antivector measures (for review, see ref. 1). Vectorial capacity can vary tremendously even between members of the same species complex, as best exemplified by the fact that the anthropophilic species *Anopheles arabiensis* is a very efficient malaria vector, while its often sympatric sibling species *Anopheles quadriannulatus* does not represent a health problem due to its preference for feeding on animals (2). The situation is further complicated by the fact that relevant biological differences are detected not only between species but also between incipient taxa within the same species. For example, three chromosomal forms of *Anopheles gambiae* in the strict sense (s.s.) that carry different strain-specific combinations of inversions and differ in vectorial capacity are found in Mali and are given the names Savanna, Mopti, and Bamako (3). These forms appear to be partially or fully isolated reproductively in nature: in sympatric areas hybrids between Savanna and the other two forms have been observed at frequencies lower than expected, but no individuals carrying heterozygous complements of the Mopti and Bamako inversions are seen in nature, even though the two forms produce viable progeny under laboratory conditions (4, 5). The larval stages of the Mopti form of *A. gambiae* s.s. occur mainly in man-made habitats, such as irrigated areas, even in the dry season, while the Savanna and Bamako forms have a tendency to breed in more natural sites, exploiting rain-dependent pools for larval development. This affects their spatial and seasonal distribution, since the Mopti form will breed throughout the year and can therefore displace the other forms in irrigated areas, leading to changes in the patterns of malaria entomology (6). The efficient and rapid identification of these three “strains” is therefore of utmost importance for epidemiological studies in this part of Africa. Since the three forms can, at present, be differentiated only cytologically, by the combinations of their specific chromosomal inversions, it becomes obvious that the study of their biology and vectorial competence would greatly benefit from the development of a more convenient method for distinguishing between these populations or incipient species.

The problems in identifying the members of mosquito populations have led to extensive efforts to develop techniques that would allow for fast and reliable diagnostic procedures, in particular among species belonging to the same complex. These techniques include, in addition to the cytological examination of polytene chromosomes, other “traditional” tools such as genetic compatibility (7), isozyme analysis (8), and immunological procedures (9), and also methods based on recombinant DNA technology (10–12). Although the latter tools have proven useful, one major drawback of techniques based on DNA hybridization or sequence determination of specific genes is that they are time consuming. In addition, they require sophisticated equipment that is often only found in specialized laboratories. Some of these problems have recently been solved by Crampton and his collaborators (13–15), who have improved a technique based on the hybridization of repetitive oligonucleotide probes, specific to the individual sibling species, allowing for the rapid identification of members of the *A. gambiae* species complex. In another development, mosquitoes within a species complex have been distinguished by using PCR-based identification of either rRNA-encoding (rDNA) sequences (16) or anonymous DNA segments by using random amplified polymorphic DNA (RAPD) markers (for review, see ref. 17). While the former technique can be developed to yield unambiguous information, the latter has the advantage that it is extremely fast and it also provides molecular markers that can be mapped by recombination (18, 19). Furthermore, the genomic fingerprints obtained through RAPD-PCR can also be used in a variety of ways in molecular ecological applications (20). In attempts to differentiate between mosquito species such fingerprints were shown to distinguish between *A. gambiae* and *A. arabiensis* (21) and also between members of species complexes of another disease vector, *Aedes aegypti* (22, 23).

The present work focuses on developing a methodology that would be helpful in differentiating between the Savanna, Mopti, and Bamako forms of *A. gambiae*. We used two different techniques that are both based on the PCR amplification of nonspecific sequences. One of them uses RAPD loci to differentiate between the two chromosomal forms, while the second one uses a slightly modified procedure based on consensus tRNA gene primers (24). We

**Abbreviation:** RAPD, random amplified polymorphic DNA.

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demonstrate that both methods can be used to distinguish members of the *A. gambiae* complex and, most importantly, also between sympatric chromosomal forms of *A. gambiae* s.s., including mosquitoes collected in the field.

**MATERIALS AND METHODS**

**Strains.** The following laboratory strains of *A. gambiae* were used in this analysis; their inversion genotype (4) is given in parentheses: GASUA (Xag, 2R, 2La, 3R, 3L), a strain extracted in 1989 from a Forest-Savanna 2Rd and 2La polymorphic *A. gambiae* colony, established in 1986 from adult females collected in the village of Suakoko (Liberia) (this strain is used as the standard reference for *A. gambiae* s.s.); GASEL-M (Xag, 2Rbc/u, 2La, 3R, 3L), a Mopti strain established in 1987 from adult females collected in the village of Selenkenyi (Mali); GAMOR-B (Xag, 2Rjcu, b/+; 2La, 3R, 3L), a Bamako strain of *A. gambiae* s.s., established in 1991 from adult females collected in the village of Moribabougou (Mali); GAKUI-M (Xag, 2Rbc/u, 2La, 3R, 3L), a Mopti strain established in 1991 from adult females collected in the village of Kuiti (Burkina Faso); GAMOR-M (Xag, 2Rbc/u, 2La, 3R, 3L), a Mopti strain established in 1991 from adult females collected in the village of Moribabougou (Mali); ARMAD (Xbcd, 2Rb, 2La, 3R, 3L), an *A. arabiensis* strain established in 1992 from adult females collected in the village of Mopti (Mali); and ARZAG (Xbcd, 2Ra/+; b/+; c/+; d/+; 2La, 3Ra/+; 3L), an *A. arabiensis* strain established in 1985 from adult females collected in Zagtouli (Burkina Faso). Field collections were performed in the villages Selenkenyi, Balanbani, and Moribabougou in Mali.

**Primers.** For the tRNA gene amplification the following primers were used: T1, 5'-ATCCCATAGTCGCTGGTTTC-3'; T2, 5'-CGATAGCTCAGTTGGTAGAG-3'; T3, 5'-TCGTGGCCGAGTGGTTAA-3'; and T4, 5'-TCGTGCCAAGTGGTTA-3'. The decameric RAPD primers were purchased from Operon Technologies (Alameda, CA). Only the results obtained with primer L02 (5'-TGGGGCTCAA-3') are shown here.

**DNA Extraction.** Single mosquitoes from laboratory strain (stored in ethanol) were dried and then soaked in 100 μl of extraction buffer (1% SDS/25 mM NaCl/25 mM EDTA) for 10 min. Field-collected mosquitoes were stored in Carnoy’s fixative (ethanol/chloroform/glacial acetic acid, 6:3:1 vol/vol) rather than ethanol before further processing. After rehydration, all mosquitoes were homogenized with a polypropylene pestle and placed (after the addition of 200 μl of extraction buffer) at 65°C for 10 min. Following the addition of 200 μl of 3 M potassium acetate (pH 7.2) the homogenates were placed on ice for 30 min and then centrifuged for 10 min at 15,000 rpm in a table-top microcentrifuge (Eppendorf). The supernatants were extracted twice with phenol/chloroform and precipitated with the addition of 2 vol of ethanol, and the pellets were resuspended in 50 μl of distilled water. One microliter of this DNA solution was used for each PCR-amplification reaction.

**PCR Amplification.** Amplification using the tRNA gene primers. Reaction mixtures of 50 μl were prepared, using 1.25 units of *Taq* DNA polymerase, 0.2 mM each dNTP, 1 μM primer, and 1 μl of DNA solution in the buffer recommended by Stratagene. The reaction was cycled 40 times through the following scheme: 45 sec at 94°C, 45 sec at 50°C, and 1.2 min at 72°C. Amplification using the RAPD primers. The assays consisted of 25 μl of a solution containing 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.015% gelatin, 200 μM each dATP, dCTP, dGTP, and dTTP, 15 ng of a single RAPD primer, 0.5 unit of *Taq* DNA polymerase, and 1 μl of DNA solution. Amplification proceeded through 45 cycles (1 min at 94°C, 1 min at 36°C, 2 min at 72°C). In most experiments *Taq* DNA polymerase from Stratagene was used. The amplification products were separated electrophoretically on a 1.4% agarose gel and visualized with UV light.

**RESULTS**

To develop a set of primers suitable to differentiate between the Mopti and Bamako forms of *A. gambiae* s.s., we designed oligonucleotides based on a recently developed strategy for fingerprinting bacterial strains (24). This approach uses consensus sequences derived from the best alignment of tRNA genes, and it relies upon the fact that these genes usually occur clustered in multiple copies in the genome of most organisms. As no mosquito tRNA sequences were available in the nucleic acid data bases at the time, we chose to align dipteran tRNA genes to obtain the desired consensus sequences. The primers designed, based on the best alignment of 61 *Drosophila* tRNA genes, are shown in Materials and Methods. A subsequent search of the nucleic acids data bases with the primer sequences revealed that primers T1, T2, and T4 are colinear with *Drosophila* genes encoding tyrosine tRNAs, while T3 has a perfect match with serine tRNAs. If one allows for mismatches, then more tRNA-encoding genes are found to share extensive similarity with the primers. All four primers used in this analysis have the same 5'-to-3' orientation with respect to the corresponding tRNA gene.

In a first series of reactions we amplified genomic DNA from individual mosquitoes belonging to different laboratory strains. These strains had been kept in the laboratory for several years under mass mating conditions. All four primers, when used alone (or in combination, results not shown), produce fingerprints consisting of a multitude of bands of different size and intensity. Fig. 1, as an example, shows the patterns obtained when primers T2 and T4 are used to amplify DNA from four *A. gambiae* strains: GASUL-M, GAMOR-B, and GASUA, and the *A. arabiensis* strain ARMAD and ARZAG (from left to right), using the tRNA gene-based primers T2 (A) and T4 (B). The same individuals were used in the two reactions. The symbols x and o mark amplification products that are common to all five strains but differ quantitatively.
the same three individuals from each of the A. gambiae s.s. strains GASUA, GASEL-M, and GAMOR-B and the A. arabiensis strains ARMAD and ARZAG. Clear differences can be observed among the patterns obtained for the five different strains, while the fingerprints produced within strains are invariant. For reasons that we do not understand, some primers also reproducibly amplified the genomic DNAs of one strain better (or worse) than that of another, although the same DNA extraction protocols were followed throughout. This was also detected for the RAPD-PCR amplifications (see below). The differences observed are both quantitative and qualitative. Some bands are apparently common to all five strains, while some of the fragments are specific to only one of the five. For example, the two bands in Fig. 1A labeled with a "x" and an "o" can be detected in all five strains, but they clearly differ in intensity. In contrast to the three A. gambiae s.s. strains, in the case of the two samples of A. arabiensis, for the most part only quantitative variation can be observed when the tRNA-based primers are used, since most bands are common to both ARZAG and ARMAD. It is also noteworthy, that in spite of the fact that the T2 and T4 primers not only are colinear with Drosophila tyrosine tRNA genes but also overlap with each other, the fingerprinting patterns obtained differ markedly between the two primers, demonstrating that they do not necessarily amplify the same set of tRNA genes.

The amplification achieved with primer T3 is shown in Fig. 2. Comparing the patterns obtained, one can detect obvious differences between the Forrest-Savanna, Mopti, and Bamako laboratory strains, although, again, some bands are common to both strains.

In addition to the tRNA-derived primers, we also tested whether RAPD-PCR could be used for the purpose of identifying the different strains of A. gambiae, and in particular the Bamako and Mopti chromosomal forms. Fig. 3 shows the amplification achieved when the decanucleotide L02 is used to amplify DNA from the same individuals tested with the tRNA-derived primers shown in Fig. 2. Here again, the three A. gambiae s.s. and the two A. arabiensis strains can readily be distinguished. No intrasinus variation is apparent when this primer is used, while the quantitative and qualitative differences in the fingerprints are enough to classify the individuals as belonging to one or another strain.

Besides the results presented above, we compared the profiles obtained from the three additional Mopti laboratory strains (GASEL-M, GAKUI-M, and GAMOR-M) to one another, using both RAPD and tRNA-based primers, and no differences could be detected. We should mention, though, that only a limited number of individuals were tested from the latter two strains, as these died before completion of the analysis.

Since the mosquitoes initially analyzed were from inbred laboratory cultures, we decided to test, in a further series of experiments, whether strain-specific diagnostic polymorphisms can also be detected in mosquitoes collected in the field. For this we concentrated on the examination of mosquitoes belonging to the two chromosomal forms Mopti and Bamako (24 and 23 specimens, respectively). The collections were taken in three localities in Mali, located less than 100 km from one another. The mosquitoes were stored in Carnoy's fixative and, prior to the extraction of the genomic DNA, the ovaries were removed and the karyotypes of the mosquitoes were determined. The results of this analysis are shown in Table 1. While Mopti individuals (with a total of five different inversion complements) were found only among the Selekenyi collection, Bamako mosquitoes homozygous or heterozygous for two inversion complements were found in all the other populations.

Table 1. Chromosome arm 2R karyotype determined for the field isolates from the three localities in Mali

<table>
<thead>
<tr>
<th>Chromosomal form</th>
<th>2R karyotype</th>
<th>Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bamako</td>
<td>jbcu/jbcu</td>
<td>Sel 50, 58, 60</td>
</tr>
<tr>
<td></td>
<td>jbcu/jcu</td>
<td>Sel 7, 30, 42, 43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bal 267, 273</td>
</tr>
<tr>
<td></td>
<td>jcu/jcu</td>
<td>Mor 105, 175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sel 5, 27, 33, 44, 53, 66, 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bal 244, 259</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mor 24, 127, 130</td>
</tr>
<tr>
<td>Mopti</td>
<td>bc/bc</td>
<td>Sel 68, 69, 79</td>
</tr>
<tr>
<td></td>
<td>bc/u or bcu/+</td>
<td>Sel 2, 9, 23, 29, 45, 47, 48, 55, 56</td>
</tr>
<tr>
<td></td>
<td>bc/+</td>
<td>Sel 18, 24, 34, 38</td>
</tr>
<tr>
<td></td>
<td>u/+</td>
<td>Sel 22</td>
</tr>
<tr>
<td></td>
<td>u/u</td>
<td>Sel 1, 15, 17, 32, 39, 61, 70</td>
</tr>
</tbody>
</table>

The genotypes were determined by microscopic examination of the polytene chromosomes prepared from half-gravid females. Sel, Ban, and Mor refer to the collection sites Selekenyi, Balanbani, and Moribabougou, respectively, while the numbers refer to the code of the individual mosquito analyzed.
three localities. Fig. 4 shows the results of the PCR amplifications of the individuals with both the T3 tRNA-based primer and the L02 decanucleotide primer. Both primers can clearly differentiate between the two chromosomal forms, although the patterns are fairly similar. In both cases diagnostic amplification products can be observed. Interestingly, no noticeable differences can be observed among individuals from each form, although the Mopti mosquitoes shown here represent five different genotypes and the Bamako individuals are isolates from the three different villages. On the other hand, it is interesting that there were extensive differences between the patterns of the field-collected and the laboratory mosquitoes when the same primers were used (see Figs. 2 and 4A for primer T3 and Figs. 3 and 4B for L02).

We looked into the potential reasons for the fingerprinting pattern differences. A summary of this analysis is presented in Fig. 5. An important reason for irreproducibility may be found in the source of the thermostable DNA polymerase used in the experiment. Fig. 5A (in which DNA from the laboratory strain GASEL-M was used for the amplification with primer T4) shows that three different polymerases yield different patterns. The second reason for the differences is that field-collected mosquitoes were stored (for shipment reasons) in Carnoy’s fixative, while the DNA from laboratory strains was extracted from mosquitoes that had been stored in ethanol. In Fig. 5A, two of the polymerases were tested for the fingerprinting of the laboratory strain GASEL-M, but the mosquitoes had been stored for 24 hr in Carnoy’s fixative prior to DNA isolation. (B) Mosquitoes were stored directly or after 24 hr storage in Carnoy’s fixative before amplification (-C). Lanes Ga and GaC, GASEL-M; lanes SeIC, MorC, and BalC; three field-collected Mopti mosquitoes from the villages Selenkenyi, Morigabougou, and Balanbani, respectively; lane NT, no DNA template.

Fig. 5. Reproducibility of the technique. Different sources of commercially available thermostable DNA polymerases were tested with primer T4 (A), and the storage of the mosquitoes in Carnoy’s fixative was investigated for the consistency of the fingerprint pattern produced by the PCR amplification using primer T4 or T3. (A) DNA was extracted from GASEL-M mosquitoes and three different thermostable polymerases (V, Vent from New England Biolabs; B, Boehringer; and S, Stratagene) were used for the amplification (DNA extracted from two mosquitoes was amplified). In the case of the lanes marked with C, the mosquitoes were stored in Carnoy’s fixative prior to DNA isolation. (B) Mosquitoes were used directly or after 24-hr storage in Carnoy’s fixative before amplification (-C). Lanes Ga and GaC, GASEL-M; lanes SeIC, MorC, and BalC; three field-collected Mopti mosquitoes from the villages Selenkenyi, Morigabougou, and Balanbani, respectively; lane NT, no DNA template.

Fig. 4. Amplification of genomic DNA isolated from field-collected mosquitoes, using the tRNA gene-based primer T3 (A) and RAPD-PCR with primer L02 (B). Five Mopti and six Bamako individuals are shown. The labels on top of the lanes refer to the individuals presented in Table 1. M, molecular weight marker.

DISCUSSION

We have used PCR amplification with random primers to distinguish between different chromosomal forms of A. gambiae and have shown that this approach can be used for the identification of mosquitoes collected in the field. On a purely technical basis, this report shows that both kinds of primers used in this analysis can be of value for this purpose. Although it is not readily apparent from the results presented here, the protocol based on the primers derived from tRNA gene consensus sequences was, in our hands, more efficient than the RAPD-PCR method. The reason for this is that all four primers used in this analysis yielded reproducible results and were immediately successful in differentiating between the strains under investigation. In contrast, of 60 decanucleotide primers analyzed, most were found not to be of use in the diagnosis of the chromosomal forms for different reasons.
These reasons included, among others, the failure of about 50% of the primers to detect variation between the strains and, most importantly, the frequent irreproducibility of the results (25). Thus, should RAPD-PCR be chosen as the method for similar investigations, we stress the point that several primers may have to be extensively screened before optimal ones can be identified.

Perhaps the reason that tRNA gene-based primers seem to give better results may be that these are not random, as they will clearly amplify clustered genes whose sequences were used to design them. For example, the two marked bands in Fig. 1A that are seen in all strains examined could represent a tRNA locus that would differ only in the copy number of individual genes. Moreover, our results indicate that not only random primers but also primers recognizing only specific gene arrangements can be useful in the characterization of different chromosomal forms of *A. gambiae*, similarly to what was described for bacteria (24). Indeed, even the two overlapping primers T2 and T4 yielded different and useful amplification patterns. Whether these differences are due to the additional amplification of non-tRNA genes cannot be determined.

RAPD-PCR has recently been used to distinguish between sibling species of disease vector mosquitoes (21–23). We show that this technique, and, in particular, the related tRNA-based primer method, can be extended to differentiate between populations of *A. gambiae* s.s., for which there are indications that they represent incipient species (4). A recent report showing that two biotypes of the whitefly *Bemisia tabaci* represent two different species, and which was based, among others, on the results of PCR amplification with random primers (26, 27), raised a controversy as to whether the results of such an analysis can be used *prima facie* for answering such a question (28–30). Our data clearly support the hypothesis of separate taxonomic units, although they do not offer unambiguous clues as to whether the Savanna, Mopti, and Bamako populations do indeed represent different species. On the other hand, the possibility to distinguish between them without the need for specialized cytological examination offers a most important improvement for the epidemiological analysis of malaria vectors. In our hands, the main problem that has to be taken care of is that of the irreproducibility of the results in the cases where different enzyme sources are used for the amplification assays, or when the mosquitoes are stored in different media. In spite of this, we have seen that when the same conditions are used, all Mopti specimens can be consistently differentiated from the Bamako ones.

In conclusion, our results show that the differences observed in the comparison of the different chromosomal forms are not restricted to the level of the chromosome linearity, but rather they can also be detected on the level of DNA sequences, where polymorphisms between them are really abundant.

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