

## Short Report: Development of a Molecular Assay to Detect Predation on *Anopheles gambiae* Complex Larval Stages

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**Abstract.** We developed a molecular assay to detect predation on *Anopheles gambiae sensu lato* (s.l.) mosquitoes. This intergenic spacer ribosomal DNA polymerase chain reaction assay and restriction enzyme analysis uses *An. gambiae*-specific primers to detect mosquito DNA in the DNA extracts from whole invertebrate predators, which enables identification of species (*An. gambiae* s.s. versus *An. arabiensis*) and molecular forms (M versus S in *An. gambiae* s.s.). We show that *An. gambiae* s.l. DNA can be detected after ingestion by members of the families Lestidae (order Odonata) after four hours, Libellulidae (order Odonata) after six hours, and Notonectidae (order Hemiptera) after 24 hours. This method is an improvement over previously published methods because of ease of execution and increased time of detection after ingestion.

The *Anopheles gambiae* s.l. complex consists of seven morphologically identical mosquito species. *Anopheles gambiae sensu stricto* (s.s.), with its two recognized molecular forms M and S, and *A. arabiensis* are the main malaria vectors in sub-Saharan Africa. Control of these mosquitoes is essential for malaria prevention, but is limited by our incomplete understanding of their population biology. Among the various natural ecologic forces controlling these vector populations, predation on *An. gambiae* s.l. immature stages seems to be a major factor controlling population sizes because it contributes heavily (between 13.4% and 84.5%<sup>1</sup>) to overall larval mortality (between 92.6% and 97.1%<sup>2–4</sup>). However, relatively little research has been devoted to the effects of larval predation on mosquito population structure and ecology, possibly because of difficulties in identifying and quantifying the impact of the most common predators in the field.

To facilitate studies on *An. gambiae* s.l. larval predation, we have developed an assay that can be used to detect predation on larvae using DNA extracts from known or potential invertebrate predators. By feeding *An. gambiae* s.l. larvae to known predators and analyzing them at different times after ingestion, we determined the amount of time for which *An. gambiae* s.l. DNA can be detected in DNA extracts from the predators.

We collected potential *An. gambiae* s.l. predators from larval breeding habitats in Goundri and Monomtenga (12°30'N, 1°20'W and 12°06'N, 1°17'W, respectively), two rural villages in Burkina Faso. We identified three common families of invertebrates that fed readily on *An. gambiae* s.l. larvae: Notonectidae (Hemiptera), Libellulidae (Odonata), and Lestidae (Odonata). To determine detection limits of the assay, we starved predators for 24 hours before feeding them a single *An. gambiae* s.l. larva of known identity, and killed predators in ethanol immediately after ingestion or at various times after ingestion (1, 5, 6, 12, and 24 hours in Notonectidae; 0, 1, 4, 6, and 24 hours in Lestidae; and 0, 1, 6, 8, and 24 hours in Libellulidae). The larvae were obtained from single ovipositions of field-captured female mosquitoes. The specific iden-

tity and molecular form status of adult females were determined according to Scott and others<sup>5</sup> and Favia and others.<sup>6</sup> Because no M/S hybrids were ever reported in these villages (Costantini C and others, unpublished data), taxonomic identification of the mother allowed accurate identification of her offspring.

To determine the best method of predator DNA extraction, we collected Libellulidae and Lestidae from the Mill River in Hamden, Connecticut (41°25'N, 7°25'W), and fed them single laboratory-raised *An. gambiae* s.s. Legs and heads were removed from predators before DNA extraction. We compared three different DNA extraction methods for whole predators: Qiagen DNEasy (Qiagen, Valencia, CA), Easy-DNA (Invitrogen, Carlsbad, CA), and a modified version of the protocol used by Paskewitz and Collins.<sup>7</sup>

The modified protocol of Paskewitz and Collins<sup>7</sup> showed the most consistent results in terms of ability to detect larval mosquito DNA after ingestion. Using this protocol, we extracted DNA from the preserved predators from Burkina Faso. Each predator was rinsed in sterile water, ground in 200  $\mu$ L of grinding buffer (0.5 M EDTA, 1 M Tris, pH 7.5, 5 M NaCl, 10% sodium dodecyl sulfate), placed in a 65°C water bath for 30 minutes, vortexed with 28  $\mu$ L of 8 M potassium acetate, placed on ice for 30 minutes, and centrifuged at 14,000 rpm for 15 minutes. DNA was precipitated from the supernatant with 400  $\mu$ L of 100% ethanol and 30 minutes on ice. After a final centrifugation (14,000 rpm for 15 minutes) and rinse with 40  $\mu$ L of 70% ethanol (centrifuged at 14,000 rpm for 5 minutes), samples were dried, resuspended in 300  $\mu$ L of sterile water (150  $\mu$ L for Notonectidae because of their smaller mass), and incubated at 65°C for 15 minutes.

Each DNA extract from the predators was subjected to two rounds of polymerase chain reaction (PCR) amplification of a 202-basepair region of the intergenic spacer (IGS) rDNA of *An. gambiae* s.l., followed by *Mse* I restriction enzyme analysis of the PCR products. Restriction with *Mse* I resulted in diagnostic fragments that distinguish between the M (68 basepairs and 134 basepairs) and S (202 basepairs) forms of *An. gambiae* s.s., and *An. arabiensis* (62 basepairs and 140 basepairs). The PCR assay is designed for the same IGS rDNA region and sites as used previously,<sup>8</sup> but using a different forward primer (783R: 5'-CGTTTCTCACATCAAGA-

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CAATCAAGTC-3'). This allowed the amplification of a smaller fragment (202 basepairs) than in the previous assay. Given that the prey DNA is fragmented by digestion in the predator, amplification of small DNA fragments should increase the ability to detect prey DNA over time. We used 2  $\mu$ L of a 1:10 dilution of predator DNA extract in a 15- $\mu$ L PCR. The PCR mixture and cycling conditions and the restriction enzyme analysis were carried out as described by Santolamazza and others.<sup>8</sup> It should be noted that because distinguishing *An. arabiensis* from *An. gambiae* M requires separating PCR fragments that differ by only six basepairs, it is necessary to use a high-percentage agarose gel and helpful to run control DNA in each lane for comparison. We were able to differentiate among bands using a 3% agarose gel.

After a single PCR amplification followed by digestion with *Mse* I (Figure 1), we detected *An. gambiae* s.l. DNA immediately after ingestion in Libellulidae at a success rate of 81% (21 samples) and in Lestidae at a success rate of 100% (16 samples). We detected larval DNA from second, third, and fourth instars in both Lestidae and Libellulidae. We did not feed any of the predators first-instar larvae. We obtained a positive result one hour after ingestion in Lestidae (1 sample) but did not detect mosquito DNA in Notonectidae (14 samples) with a single PCR. This may be due to Notonectidae feeding behavior; they inject digestive enzymes into their prey, ingest the liquefied material, and discard the rest of the body. This reduces the amount and quality of larval DNA in Notonectidae compared with Libellulidae and Lestidae, which ingest the entire larva.

To improve the detection limits of the assay by increasing the probability of having a visible PCR product from small and degraded prey DNA, we carried out a second PCR amplification using 2  $\mu$ L of the PCR products from the first

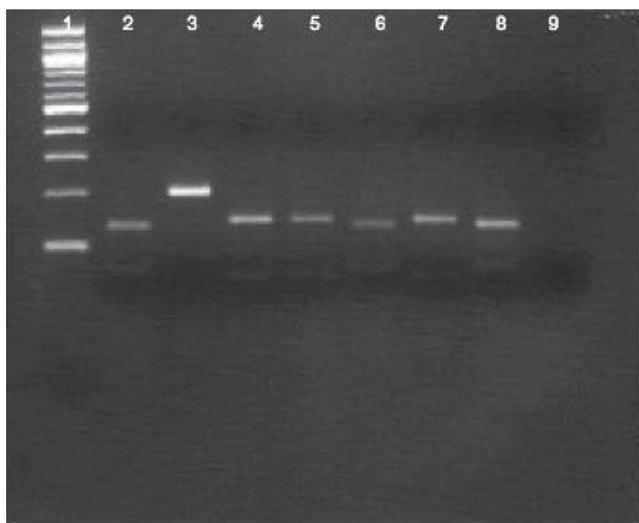


FIGURE 1. Polymerase chain reaction (PCR) used to detect DNA of members of the *Anopheles gambiae* s.l. complex in extracts from predators. Lane 1, 100-basepair ladder; lane 2, *An. gambiae* s.s. molecular form M; lane 3, *An. gambiae* s.s. molecular form S; lane 4, *An. Arabiensis*; lane 5, Libellulidae fed *An. arabiensis* (0 hours after ingestion); lane 6, Libellulidae fed *An. gambiae* s.s. form M (0 hours after ingestion); lane 7, Lestidae fed *An. arabiensis* (0 hours after ingestion); lane 8, Lestidae fed *An. gambiae* s.s. form M (0 hours after ingestion); lane 9, negative control. All PCR products digested with *Mse* I.

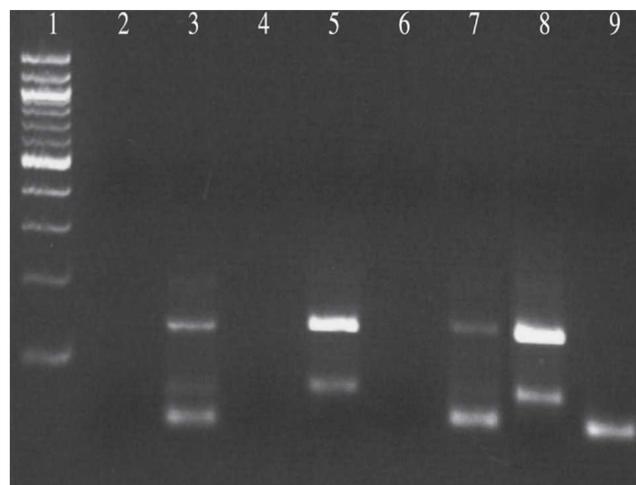


FIGURE 2. Two sequential polymerase chain reactions (PCRs) used to detect *Anopheles gambiae* s.s. DNA after ingestion by predators. Lane 1, 100-basepair ladder; lane 2, Libellulidae fed *An. gambiae* s.s. M form (6 hours after ingestion); lane 3, Libellulidae fed *An. gambiae* s.s. M form (6 hours after ingestion), second PCR; lane 4, Notonectidae fed *An. gambiae* s.s. M form (24 hours after ingestion); lane 5, Notonectidae fed *An. gambiae* s.s. M form (24 hours after ingestion), second PCR; lane 6, Lestidae fed *An. gambiae* s.s. M form (4 hours after ingestion); lane 7, Lestidae fed *An. gambiae* s.s. M form (4 hours after ingestion), second PCR; lane 8, *An. gambiae* s.s. M form, second PCR; lane 9, negative control, second PCR. All PCR products digested with *Mse* I.

amplification as templates, followed by digestion with *Mse* I (same conditions as for the first PCR and restriction analysis reactions). After a second round of PCR, we detected *An. gambiae* s.l. DNA immediately after ingestion in all three predator taxa, after 4 hours of digestion in Lestidae, 6 hours in Libellulidae, and 24 hours in Notonectidae (Figure 2). These increased detection times in Lestidae and Libellulidae and the ability to detect *Anopheles* DNA in Notonectidae demonstrate the increased sensitivity obtained by performing two sequential PCRs.

Although encouraging, these results need to be considered with caution because PCR amplification was not consistent when using the same DNA samples in replicate PCR amplifications over a period of one year. We suspect that this was due to degradation over time of the DNA extracts, which were kept in water at  $-20^{\circ}\text{C}$ . Unavailability of additional predator samples and exhaustion of DNA extracts prevented further optimization of the protocol.

This PCR-based assay is more sensitive and less expensive and time-consuming than the precipitin test,<sup>1,3</sup> which is unable to distinguish between species and molecular forms within the *An. gambiae* complex and requires antibody production. This new PCR-based test also represents an improvement over a similar PCR method of Morales and others,<sup>9</sup> which reported positive prey detection in a single predator (Libellulidae) one hour after ingestion. In contrast, our assay detects larval DNA up to six hours after ingestion by Libellulidae predators. The use of a single PCR amplification rather than two and the targeting of a larger IGS rDNA region than in our study (390 basepairs versus 202 basepairs) could be responsible for the reduced sensitivity of their method compared with ours.

This assay successfully detects larval DNA immediately af-

ter ingestion in all predator DNA extracts but prey DNA detection varies over time and for different predators, ranging from 4 hours to 24 hours after ingestion. This variation in DNA prey detection appears to be related to the predator digestion type (external versus internal), which in turn affects the amount and quality of prey DNA. This PCR-based assay will be useful for future studies of *An. gambiae* larval ecology, particularly in identifying larval predators in natural breeding sites, and determining whether predation pressure differs among M and S *An. gambiae* s.s. forms and *An. arabiensis*. An assay similar to the one described here could also be developed to detect predation on adult mosquitoes.

Further refinements of this method should include optimization of DNA extractions and storage conditions. Another modification of this general protocol that could facilitate field predation studies would include an optimization of prey DNA detection at intervals tailored to the predators' feeding rates. Detection of prey DNA over an extended period of time can actually bias estimates of the feeding rates. Because this assay can only detect the presence or absence of *Anopheles* larval DNA, multiple feedings cannot be discerned when ingestion intervals of the predator are shorter than the prey DNA detection time. Although we detected larval DNA in the second through the fourth instars immediately after digestion, we did not test the effect of larval instar on detection after digestion. This would be a useful next step in the development of this assay. In addition, we did not address whether larval DNA can be detected in a predator that consumes another predator that has fed on *An. gambiae*.

Received September 19, 2006. Accepted for publication May 8, 2007.

**Acknowledgments:** We thank Antoine Sanou and the staff at the Centre National de Recherche et de Formation sur le Paludisme for assistance with field collections and laboratory work in Burkina Faso, and Dr. L. Zheng (Yale University) for providing larval G3 *An. gambiae*.

**Financial support:** This study was supported by a Yale Institute for Biosphere Studies Field Ecology Pilot Grant and the Department of Ecology and Evolutionary Biology Chair's Discretionary Fund to Erika Schielke, and National Institutes of Health grant R01 AI46018 to Jeffrey Powell.

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