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Development of Exon-Primed Intron-Crossing (EPIC) PCR primers for the malaria vector *Anopheles pseudopunctipennis* (Diptera: Culicidae)

Développement d'amorces PCR Exon-Primed Intron-Crossing (EPIC) pour le vecteur de paludisme Anopheles pseudopunctipennis *(Diptera : Culicidae)*

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ABSTRACT

Using the *Anopheles gambiae* Giles genome as a template, we designed, screened and identified 14 novel Exon-Primed Intron-Crossing (EPIC) PCR primer pairs for *Anopheles pseudopunctipennis* Theobald 1901, a major vector of human *Plasmodium* sp. in South America. These primers were designed to target the conserved regions flanking consecutive exons of different genes and enabled the amplification of 17 loci of which nine were polymorphic. Polymorphisms at these loci ranged from two to four alleles. Intron length polymorphism analysis is a useful tool, which will allow the study of the population structure of this mosquito species, which remains poorly understood.

RÉSUMÉ

Utilisant le génome d'*Anopheles gambiae* Giles comme modèle, nous avons identifié 14 nouvelles Exon-Primed Intron-Crossing (EPIC) paires d'amorces PCR pour *Anopheles pseudopunctipennis* Theobald 1901, un vecteur majeur de *Plasmodium* sp. humains en Amérique du Sud. Ces amorces ont été conçues afin de cibler les régions conservées flanquant les exons consécutifs de différents gènes et ont permis l'amplification de 17 loci dont neuf étaient polymorphes. Le polymorphisme de ces loci varie de deux à quatre allèles. L'analyse du polymorphisme de longueur d'intron est un outil utile qui permettra l'étude de la structure de la population de cette espèce de moustique, qui demeure mal comprise.

1. Introduction

The mosquito *Anopheles pseudopunctipennis* Theobald 1901 (Diptera: Culicidae) is a major vector of human *Plasmodium* sp. in South America. It is a difficult species to characterize as demonstrated by its variable behavior, habits and ecological needs [1], its inconsistencies as a malaria vector in its wide distribution range [2] and the several morphological subspecies described [3]. Cross-mating experiments and cytogenetic studies pointed out evidence that these mosquitoes are comprised of a species complex [4,5]. Although the genetic variability and population structure of *An. pseudopunctipennis* has been studied biochemically, many aspects remain poorly known

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[6]. This species lacks specific molecular tools, and because of its high level of variability, more data are urgently needed to better understand how the population genetic structure of this mosquito is related to malaria transmission. Such data will help to better target vector control strategies by Public Health authorities.

Variability of non-coding sequences, particularly intron sequences, is a valuable marker of population variation and subdivision, and can be assayed by PCR amplification using conserved exon primers. Intron-targeted PCR was pioneered by Lessa [7]. This approach, called Exon-Primed Intron-Crossing (EPIC)-PCR [8,9], has been shown to identify substantial variability, mainly from intron length polymorphism. Introns constitute suitable markers for analyzing population structure within a species [10–15] as well as for reconstructing relationships among closely related species [16–18]. EPIC have been also used in gene mapping [19–23], and phylogeography [24–28] where they are the most widely used nuclear markers for such studies [29]. EPIC-PCR has several advantages in populations genetic studies:

- by using primers from heterologous genes, cloning and sequencing steps can be avoided [30,31];
- cross-species amplification is easier than when primers are designed from highly conserved exon sequences;
- for the same reason, within species, PCR artefacts such as null alleles are expected to be less frequent.

Further advantages are that intron systems do not require previous knowledge of the genome to be analyzed; they are generally polymorphic and sometimes hypervariable; are expected to be codominant and selectively neutral; are easily amplifiable by PCR; can be revealed on simple agarose or acrylamide gels; and can be obtained at low cost [32]. Moreover, having both the exon and intron fragments, EPIC can be useful for examining genetic variation at the intraspecific and interspecific level simultaneously, a feature that is particularly useful when studying species complexes [27]. EPIC markers are becoming more popular for use in population genetic studies in insects [13–15,33,34] and do not require assumptions about a particular model of evolution that is often required for microsatellites [14]. In the present article, primers for EPIC amplification of intron sequences for *An. pseudopunctipennis* are designed, of which several pairs amplify (length)-polymorphic loci that can be used in population genetic studies of this malaria vector.

2. Materials and methods

2.1. Mosquitoes

In October 2006, females *An. pseudopunctipennis* were captured by one of us (F.L.) from a natural population in Mataral (S 18.6024, W 65.1444, altitude 1500 m), a small village situated in the dry inter-Andean valleys in the centre of Bolivia, using the human bait collection technique outside houses. In the field, collected insects were chloroform killed and stored over desiccant (silica gel) in small vials. In the laboratory, mosquitoes were identified using [35] and kept at –20 °C in their individual vials with silica gel until DNA extraction.

For the various EPIC-PCR (see paragraph below), positive controls consisted in females *An. gambiae* from a laboratory strain and were provided by our main laboratory at IRD-Montpellier (France). These mosquitoes were stored using the same conditions as for *An. pseudopunctipennis* until processing.

2.2. Selection of introns and design of primers

Primers for *An. pseudopunctipennis* were designed from the conserved regions of consecutive exons of different genes from the closely related species *Anopheles gambiae* Giles. Exon sequences were downloaded in 2005 at http://www.anobase.com in Excel format from the *An. gambiae* genome database. Gene candidates that were dispersed amongst the *An. gambiae* genome were selected, and close genes on the same chromosome were avoided. Gene candidates with a higher percentage of similarity with genes from Diptera *Apis mellifera* and *Drosophila melanogaster*, were first selected to enhance the chance of similarity to genes from *An. pseudopucntipennis*.

Intron lengths ranged from 100 to 500 bp. Primers pairs were designed to target the flanking exon sequences taking into account their stability (in terms of CG content and ending with CG or GC), their size (18–20 pb), their close annealing temperatures, and the low probability of primer-dimer formation during the PCR. Possible primers adjacent to the intron sequences were discarded. Fifty-four primer pairs were initially designed and screened by PCR using *An. pseudopunctipennis* DNA as a template (Table 1).

2.3. DNA extraction and amplification

An. pseudopunctipennis and *An. gambiae* DNA extractions were carried out on mosquito legs using a slightly modified cetyltrimethylammonium bromide (CTAB)-based protocol from Edwards [36]. The protocol was as followed: mosquito legs were homogenized in 200 µl lysis CTAB solution (100 mmol/l Tris HCl pH 8.0; 10 mmol/l EDTA pH 8.0; 1.4 mol/l NaCl and CTAB 2%) in 1.5 ml Eppendorf microcentrifuge tubes. Incubation was carried out at 65 °C for 15 min; the resulting extract was washed with 200 µl chloroform and centrifugated for 5 min at 12 000 rpm. The supernatant was precipitated in 200 µl isopropanol and centrifugated again at 12 000 rpm for 15 min. The pellet was washed with 200 µl 70% ethanol, centrifugated at 12 000 rpm for 5 min, dried at 37 °C for one hour and suspended in 100 µl nuclease-free H_2O.

DNA amplifications were carried out immediately after extraction in volumes of 25 µl (1 × *Taq* buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs (Eurogentec, Angers, France), 0.5 UI *Taq* polymerase (Quiagen, Courtaboeuf, France), 20–25 ng of DNA template, and depending of the locus 0.04 µM or 0.4 µM of each primer (Eurogentec, Angers, France) (Table 2). The optimum annealing temperatures for each primer pair are listed in Table 2. PCR were performed on a Perkin Elmer DNA Thermal Cycler 480 (US Instrument Division, Norwalk, CT, USA) and conditions were: 1 min at 94 °C, followed by 36 cycles of 30 s at 94 °C, 30 s at