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KOMUNIKASI SINGKAT

The effect of DMSO on ITS2 amplification in the molecular identification of *Anopheles farauti* Laveran (Diptera: Culicidae), from a colony established in the laboratory

Pengaruh DMSO terhadap amplifikasi ITS2 dalam identifikasi molekular *Anopheles farauti* Laveran (Diptera: Culicidae), dari koloni yang dikembangbiakan di laboratorium

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ABSTRACT

Sibling species identification is very important in the understanding of malaria epidemiology. Morphological criteria are usually used in the identification of anopheline species, but this fails when sibling or cryptic species occur. Analysis by PCR-RFLP of rDNA ITS2 is currently the most reliable and sensitive method for distinguishing between members of the *Anopheles punctulatus* group. The objective of this study was to investigate the effect of DMSO concentration on ITS2 amplification of *An. farauti* from the colony maintained at BATAN Jakarta using PCR-RFLP based on the rDNA ITS2. The results showed that the addition of 6 % and 7 % DMSO produced ITS2 amplification products in the size 750 bp. DMSO could be used in PCR to relieve secondary structures when amplifying high GC templates. Molecular identification of *An. farauti* is found to be *Anopheles farauti sensu stricto.*

Key words: molecular identification, sibling species, PCR-RFLP, rDNA ITS2, secondary structure

ABSTRAK

Identifikasi spesies sibling sangat penting untuk mempelajari epidemiologi malaria. Ciri morfologi secara umum digunakan dalam identifikasi spesies *Anopheles*, namun tidak sesuai ketika digunakan untuk spesies sibling dan spesies kriptik. Analisis dengan menggunakan PCR-RFLP dari ITS2 rDNA pada saat ini merupakan metode yang paling cocok dan sensitif untuk membedakan spesies *Anopheles punctulatus* group. Tujuan penelitian ini adalah untuk mengetahui pengaruh konsentrasi DMSO terhadap amplifikasi ITS2 dalam mengidentifikasi *An. farauti* dari koloni di BATAN Jakarta dengan PCR-RFLP berdasarkan ITS2 rDNA. Hasil penelitian menunjukkan bahwa penambahan DMSO dengan konsentrasi 6% dan 7% menghasilkan produk amplifikasi ITS2 dengan ukuran 750 pb. DMSO dapat digunakan dalam PCR untuk mencegah terbentuknya struktur sekunder

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ketika mengamplifikasi *template* dengan kandungan GC yang tinggi. Identifikasi molekular *An. farauti* dengan menggunakan PCR-RFLP merupakan *Anopheles farauti sensu stricto*.

Kata kunci: identifikasi molekular, spesies sibling, PCR-RFLP, ITS2 rDNA, struktur sekunder

INTRODUCTION

The main malaria vectors in the southwest Pacific islands of Moluccas, Papua, Papua New Guinea, the Bismarck Archipelago, the Solomon and Vanuatu are the members of the *Anopheles punctulatus* group. The species belong to this group originally are *An. farauti*, *An. koliensis* and *An. punctulatus*. These species could be differentiated morphologically based on the pale scales of the proboscis. The proboscis of *An. farauti* entirely dark scaled except for a narrow light ring at extreme apex. *An. koliensis* with a ventral patch of white scales on the apical third of the proboscis and variable size, and *An. punctulatus* with the apical half of the proboscis almost entirely white scaled (Rozebbom & Knigh 1946 cit Beebe et al. 2000a). Crossmating, cytogenetic, DNA probe and allozyme analyses now identified 12 species within *Anopheles punctulatus* group: *Anopheles farauti* 1–7 (*An. farauti* complex), *An. punctulatus*, *An. koliensis*, *An. clowi*, *An. rennellensis* and *An.* species near *punctulatus* (Beebe et al. 2000a; Beebe et al. 2000b; Schmidt et al. 2003).

Identification of *An. punctulatus* group commonly based on the proboscis morphology and sector spot on the costa is proving to be quite unreliable in differentiating them, making field studies on their biology and behavior of each of the members of the group difficult. Analysis by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) is currently the most reliable and sensitive method for distinguishing between members of the group. It is based on specific banding pattern for each of the sibling species (Beebe & Saul 1995; Benet et al. 2004).

The ITS2 region of *An. punctulatus* group has rapid evolution compared with the coding region. This conserved sequence especially 3' to the 5.8 rDNA and 5' to the 28S rDNA can pair and form stable stem loops. Due to this strong secondary structure, 10% dimethyl sulfoxide (DMSO) was used in the amplification reaction to eliminate the DNA secondary structure (Beebe & Saul 1995). But there is limited discussion on DMSO function and no information is given about how to run this experiment.

Mosquito colonies maintained at The National Agency of Atomic Energy (BATAN) Jakarta were obtained from The Naval Medical Research Unit 2 (NAMRU 2) Jakarta. Especially for this *An. farauti* colony which was maintained at BATAN, the name was based on its morphological identification, therefore the exact name of this sibling species remains unclear. In the former study conducted by Benet et al. (2004) reported that specimens that were identified morphologically as *An. farauti* were consisted of *An. farauti sensu stricto*, *An. farauti* 4, *An. farauti* 2, *An. koliensis* and *An. punctulatus* by PCR-RFLP analysis, therefore is very important to identify the *An. farauti* colony from BATAN.

The aim of this study is to investigate the effect of DMSO concentration on ITS2 amplification in the identification of *An. farauti* complex species from the colony maintained at BATAN Jakarta using PCR-RFLP.

METHODOLOGY

Sample preparation

An. farauti was obtained from a colony maintained at BATAN Jakarta. Mosquitoes were preserved in vials and kept dry in tins containing silica gel for molecular identification using PCR-RFLP. The investigation was performed from July to September 2010 at the Laboratory of Genetic Engineering of Research Center for Biotechnology, University of Gadjah Mada Yogyakarta.

DNA extraction

The DNA was extracted by following the procedure described in Li et al. (2005). Each of mosquito specimens were individually homogenized in 50 µl of TE buffer consisting of 10 mM Tris-HCl, 1mM EDTA, pH 8. These

tubes were incubated at 100 °C for 10 minutes and centrifuged at 9,500 rpm for 10 minutes. Supernatant was transferred to new tube and stored at -20 °C for further use.

PCR for effect of DMSO on ITS2 amplification

DNA template of 3 µl was added directly to PCR mixture in final reaction volume of 25 µl that contained 50 mM KCl, 10 mM Tris-HCL, pH 8.3, 2.5 mM $MgCl_2$, 0.2 mM dNTP, various concentrations (4%, 6%, 7%, 8% and 10%) of DMSO, 1.25 unit of *Taq* polymerase and 0.6 µM of each primer. The internal transcribed spacer 2 (ITS2) was amplified using forward primer ITS2A, 5'-TGTGAACTGCAGGACACAT-3' and reverse primer ITS2B, 5'-TATAGCTTAAA TTCAGGGGGT-3' (Beebe & Saul 1995). A Perkin-Elmer type GeneAmp System 2400 thermocycler was used for all reactions with following parameters: an initial denaturation at 94 °C for five minutes, and then 35 cycles at 94 °C for one minute, 53 °C for one minute, and 72 °C for two minutes, followed by a final extention of 7 minutes at 72 °C. The ITS2 products were subjected to electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized on an ultraviolet transilluminator. The ITS2 amplification results with optimum DMSO (6%) then was used for subsequent enzyme reaction analysis.

Product digestion and visualization

The ITS2 products were subjected to electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized on an ultraviolet transilluminator. The PCR products were then digested with five units of *Msp* I (final volume 20 µl) and the resulting products resolved by electrophoresis on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized on an ultraviolet transilluminator. The patterns of bands were compared to known patterns for *An. punctulatus* group (Beebe & Saul 1995).

RESULTS AND DISCUSSION

Various concentration of DMSO (4%, 6%, 7%, 8% and 10%) were investigated to define conditions resulting consistent amplification of ITS2 product. Figure 1 shows that amplification of ITS2 had DMSO optima in 6% and 7%.

DNA from individual mosquitoes was used as a template for PCR amplification of the ITS2 after 35 cycles delivered products of approximately 750 bp (Figure 2 lane 3). Species-specific banding patterns were generated from an *Msp* I digestion using one-fifth of the PCR mixture and could be visualized on a 2% agarose gel containing ethidium bromide. All the above samples produced bands at ≈ 300, 190, and 150-bp. (Figure 2 lane 1-2).

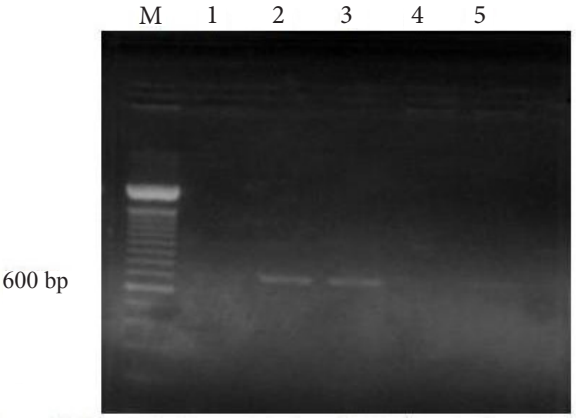

Figure 1. Determination of optimum DMSO concentration of ITS2 from *Anopheles farauti.* M: 100 bp marker; 1: 4% DMSO; 2: 6% DMSO; 3: 7% DMSO; 4: 8% DMSO; 5: 10% DMSO. bp= basepairs.

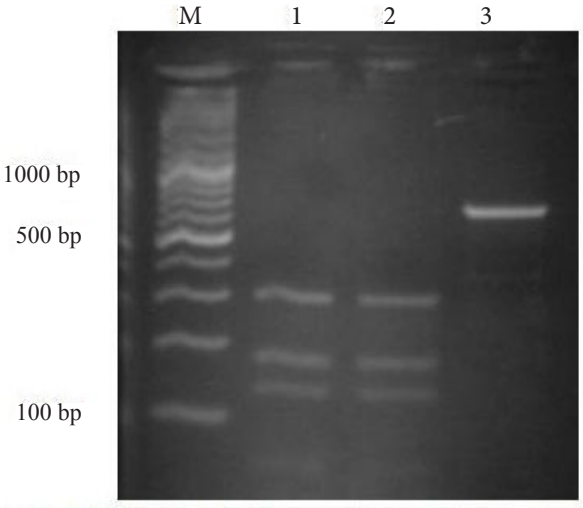

Figure 2. Polymerase chain reaction-restriction fragment length polymorphism pattern for internal transcribed spacer 2 of *Anopheles farauti* from BATAN separated on a 2% agarose gel. M: 100 bp marker; 1-2: *An. farauti sensu stricto*; 3: Undigested ITS2 amplification product. 6% DMSO was used in the PCR reaction. bp= basepairs.

Molecular identification of *An. farauti* from the colony maintained at BATAN Jakarta using PCR-RFLP is *An. farauti sensu stricto* (formerly *An. farauti* No.1), confirming the PCR-RFLP result by Beebe & Saul (1995).

Amplification of ITS2 occurred at 6 % and 7% of DMSO. The PCR products of ITS2 approximately 750 bp size. The PCR product of ITS2 also was detected at 10% DMSO with faint band. This result showed that the apparent inhibitory effect of increasing DMSO concentrations in PCR is presumably due to the inactivation of *Tag* DNA polymerase (Gelfand 1989), so that more enzymes were required. In this study, 6% DMSO can be used to amplify PCR products ITS2 required 1.25 U *Tag* polymerase, but another study by Beebe & Saul (1995) showed that used 10% DMSO for ITS2 amplification was required more *Tag* polymerase enzyme concentration (2.5 U).

DMSO is an organosulfur compound with a high polarity and high dielectric constant, that is used in PCR to facilitate strand separation of double helix DNA by altering its melting characteristics, especially acts by disrupting inter and intrastrand re-annealing and then to disrupt secondary structure formation in the DNA template (Simon et al. 2009; Jansen et al. 2010). This is particularly useful in templates with high GC content, because the increased hydrogen bond strength increases the difficulty of denaturing the template and causes intermolecular secondary structures to form more readily, which can compete with primer annealing (Chakrabarti & Schutt 2001). Thus the addition of DMSO can greatly improve yields and specificities of PCR priming reactions.

The effect of DMSO that we observed in this study may be related in part to the destabilizing influence of DMSO on dsDNA. The presence of a high GC ratio would stabilize dsDNA in both PCR products and intramolecular secondary structures, and could inhibit the PCR. Moreover Bower et al. (2009) reported that the GC ratio ITS2 from *An. punctulatus* group varied between 64-71.3%. The 5' end of ITS2 usually has multiple tandem copies of G-C rich repeat (63.5-73%), 109-188 bp that folds into a conserved stem structure.

The ITS2 from several anophelines, e.g. sibling species of the members of *An. maculipennis* complex, *An. funestus* group and *An. minimus* group could be amplified without addition of DMSO in PCR (Djadid et al. 2007; Garros et al. 2004; Van Bortel et al. 2000), because the ITS2 from these species has slightly low GC content, e.g. the ITS2 of *An. maculipennis* complex has a GC content of 49.33-54.76% (Djadid et al. 2007). Therefore, the finding in this experiment may be applied by researcher who will do research on the *An. punctulatus* group.

PCR-RFLP analysis based on the ITS2 region of the rDNA is successfully applied to mosquito colony from BATAN, although that technique has been modified by Suyono et al. (2011). Molecular identification result is *An. farauti sensu stricto* (formerly *An. farauti* 1) which belongs to *An. farauti* complex included in *An. punctulatus* group. Sibling species within *An. farauti* complex are very similar and can only be rediably differentiated by allozyme and DNA-based technology (Folley et al. 1993 cit Mueller et al. 2002; Beebe & Saul 1995). At present there is no reliable way of morphollogically identifying any of the members of *An. punctulatus* group and identification using proboscis morphology should be approached with great caution (Beebe et al. 2000a).

DMSO concentration is an important factor in the PCR reaction to amplify ITS2 of *An. punctulatus* group. DMSO could be used in PCR to relieve secondary structures when amplifying high GC templates. Molecular identification of *An. farauti* from the colony maintained at BATAN Jakarta using PCR-RFLP of ITS2 revealed to be *Anopheles farauti sensu stricto* (formerly *An. farauti* 1).

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