

USING *sma-its2* PRIMER FOR ITS2 (*Internal Transcribed Spacer-2*)- BASED MOLECULAR CHARACTERIZATION OF *Anopheles minimus* FROM KULONPROGO, YOGYAKARTA - INDONESIA

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ABSTRACT

Malaria is a public health threat caused by the Plasmodium infection transmitted by the *Anopheles* mosquito. Malaria vector control is highly dependent on the ability to determine mosquito species' vectorial and bionomic capacity. Species identification based on morphological characteristics as well as DNA-barcoding approaches is a very important step in determining vectorial capacity. Our research group has redesigned a new ITS2 primer, namely, *sma-its2*, which is specifically able to identify the *Anopheles* (*An.*) mosquito vector but cannot identify other mosquito vectors. This study wanted to test this primer's specificity further for identification of other *Anopheles* mosquitoes. We used *An. minimus* collected from Kulonprogo, Yogyakarta – Indonesia. The methods used in this research are as follows: landing collection, morphological identification, isolation of genomic DNA, PCR (Polymerase Chain Reaction), PCR product purification, sequencing, and data analysis. *An. vagus* from Bangsring, Banyuwangi – Indonesia, which had previously been identified using the same primary, was used as a positive control. The results of the morphological analysis showed that both species were in accordance with the vector identification key used in this study. The molecular analysis showed that the *sma-its2* primer could amplify the ITS2 sequence of *An. vagus* and *An. minimus*, producing 650 – 700 bp. However, further analysis of the ITS2 sequences of both species, resulted in the same species, namely *An. vagus*, with a different accession number in GenBank. This showed that the *sma-its2* primer can be used to identify *An. vagus* but cannot be used to identify *An. minimus*. Analysis of the primer position in the ITS2 sequences showed the presence of 3 nucleotides in the forward *sma-its2* primer that was not recognized by the *An. minimus* sequences and thus, hinder the successful identification of these species.

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1. INTRODUCTION

Malaria is a disease caused by infection with *Plasmodium sp.* and transmitted by the *Anopheles* mosquito as a vector. Due to the absence of an adequate vaccine and a large number of malaria pathogens resistant to malaria drugs, vector control is a very important strategy to reduce malaria cases. Malaria vector control should be specific. Thus, the method's selection should be based on vectorial capacity, behavior, and genetic characteristics (Setyaningsih et al., 2018). Species identification is an essential step in determining the vectorial capacity of disease-transmitting arthropods. This stage can be done using two approaches, namely morphologically and molecularly. Morphological identification is a method of identifying organisms based on the similarity of morphological characteristics (Batovska et al., 2016). However, this method requires a complete sample from a specimen of the organism (Jannah & Rahayu, 2019), it cannot distinguish sibling species (Batovska et al., 2016) and the difficulty increases when there are several species that have similar morphological characteristics (Luca et al., 2016). Another problem is that the parameters used can be subjective because it depends on the experience of the identifier, such as the difference in the length of the sub-apical dark band and the sub-apical pale band.

(Batovska et al., 2016) . Therefore, this approach must be accompanied by a more accurate identification method to support the results of morphological identification, i.e. molecular identification.

Molecular identification has a higher level of accuracy. This is due to the identification results that are able to distinguish sibling species. This identification method does not require a complete specimen because the identification of the organism is carried out based on the results of an analysis of the molecules from the sample. DNA molecules i.e. its sequences, are often used as molecular markers (Batovska et al., 2016) . Molecular markers commonly used in mosquito identification include cytochrome c oxidase subunit1 (CO1), CO2, second and third domains (D2 and D3) of 28S ribosomal DNA, and internal transcribed spacer 2 (ITS2) (Filali et al., 2018) . From these markers, the one that is commonly used to identify and study the evolutionary level of mosquitoes are ITS2 (Batovska et al., 2017) ; Senjarini et al., 2021a) .

Many researchers have designed ITS2 primers. However, the existing primers are generally universal for insect vectors (Soltanbeiglu et al., 2020 ; Suwannamit, 2021) and may not be able to be used to identify species from different locations as well as possible inconsistency of the PCR products (Rubio et al., 2016) . It can also provide biased results for the identification of *Anopheles* mosquitoes (Syafuruddin et al., 2020). That is why our research group has redesigned the ITS2 primer to specifically identify the *Anopheles* sp. i.e *sma-its2* (Senjarini, et al., 2021b) . The primers consisted of a forward primer (*sma-its2F*) composed of 20 nucleotides (5' AGG ACA CAT GAA CAC CGA CA 3') and a reverse primer (*sma-its2R*) composed of 21 nucleotides (5' TTG AGG CCT ACT GGA ATG TGG 3'). These primers have been shown to be able to identify *Anopheles vagus* and *Anopheles sundaicus* species, but cannot identify other mosquito vector such as *Aedes aegypti*, *Aedes albopictus*, *Culex sp* and *Armigeres sp*. Therefore, the objective of this study is to analyze *sma-its2* primer further to identify other *Anopheles* species, namely *An. minimus* and *An. vagus* as a positive control.

2. RESEARCH METHOD

Landing Collection of *Anopheles sp.*

Landing collection was carried out to collect female *Anopheles* mosquito species. This stage was carried out in the cowshed of the residents of Hargowilis Village, Kokap 1 District, Kulon Progo, Yogyakarta. Sampling was conducted according to the active time of mosquitoes (19.00 – 04.00 WIB) by using an aspirator.

Morphological identification of *Anopheles* spp.

Morphological identification was carried out directly in the sampling area. This process begins by freezing the mosquitoes in a -20°C freezer for \pm 30 seconds, then placing the mosquitoes on a glass object on a millimeter paper using a stereo microscope. Observations were made on the parts of the mosquito's body that were the key for identification i.e. the legs, wings, palpus, and proboscis. The morphological based identification was determined based on the books of Anopheline Mosquitoes of Malaya and Borneo and Illustrated Key of *Anopheles* (Diptera: Culicidae) (Reid, 1968 ; O'connor & Soepanto, 2013) .

Anopheles minimus genome DNA isolation

Mosquitoes that have been identified morphologically are then identified molecularly. The identification method begins with the isolation of genomic DNA. This stage was carried out by extracting DNA from whole body of *Anopheles* using the salting-out extraction method, i.e., separating DNA using salt and organic solvents (Dai et al., 2014) . Genomic DNA extraction was done in a 1.5 ml tube first, by adding 100 μ l of homogenizing buffer made from 10 mM Tris-Cl, 2 mM EDTA, and 0.4 M NaCl, which then homogenized using a micropistile until the solution becomes quite murky. After the solution was sufficiently homogeneous, 10 μ l of 20% SDS and 2 μ l of proteinase K were added, and was then incubated for 2 hours in a thermoshaker at 65°C. Incubated DNA samples were added with 75 μ l of 6M NaCl, then homogenized and centrifuged at 10,000 rpm 4°C for 30 minutes. The supernatant formed from the results of centrifugation was transferred to a new 1.5 ml tube, then isopropanol was added to as much as the volume of the supernatant and incubated in a freezer at 20°C for 1 hour. After incubation, the sample was centrifuged again at 10,000 rpm 4°C for 20 minutes. The supernatant formed from the results of centrifugation was discarded so that a pellet containing mosquito DNA was obtained. The pellets were then washed with 75 μ l of 70% ethanol, then dried using vacuum dry and rehydrated with ddH₂O. The results of genomic DNA isolation were visualized by electrophoresis method using agarose gel with a concentration of 0.5% and TAE buffer solution as agarose solvent and running buffer. Electrophoresis was carried out at a voltage of 100 Volts for 50 minutes. The agarose gel was visualized using a UV Transilluminator to determine the success of the isolation of genomic DNA labeled with Ethidium Bromide (EtBr).

ITS2 Sequence Amplification using Primer SMA-ITS2

The isolated genomic DNA was then used as a template in the amplification process by Polymerase Chain Reaction (PCR), using the conditions as described in Senjarini et al., (2021b) and using the primer of *sma-its2*. After the ITS2 sequence amplification process, DNA samples were electrophoresed to determine whether or not

the amplification process was successful, which was then followed by purification of the PCR product. PCR products showing single band DNA were purified using a DNA purification kit (Jena Bioscience, Germany) according to the manual in the kit.

Sequencing and Phylogenetic Analysis

The purified DNA is then sequenced through the 1stBase service in Singapore. The sequencing chromatograms were then visualized, edited and a consensus sequence was made using the BioEdit application. The consensus sequences were then BLASTed for species confirmation based on the similarity of the nucleotide base arrangement in GeneBank. The ITS2 sequences from other *Anopheles* that have a reasonably high level of similarity with the sample in this study were also collected. The sample sequences and the sequences that have been collected are then aligned using the Clustal W software (Munawar et al., 2020). By comparing ITS2 sequences from other *Anopheles*, a phylogenetic tree can be constructed using the *Neighbor Joining method* with the Kimura two-parameter model and a bootstrap value of 1000 on *MEGA -X software*. Phylogenetic tree construction was carried out to determine the relationship between species based on the evolution of the *ITS2 sequences* (Sawabe et al., 2021).

3. RESULT AND DISCUSSION

The landing collection managed to collect 46 *Anopheles* mosquitoes. Based on the morphological identification results, our samples comprised of 26 *An. vagus*, 12 *An. maculatus*, 4 *An. minimus*, 2 *An. aconitus*, 1 *An. annularis*, and 1 *An. barbirostris*. This research then focuses on sample of *An. vagus* and *An. minimus*. The morphological character of the mosquito that became the positive control, namely *An. vagus* were as follow: the costa and venation 1 on the wing having 4 or more white spots, at the tips of and between the wing venations 5.2 and 6 were white fringed. Proboscis length was approximately equal to the length of the palpus, femur and tibia were not blotched, the junction of the tibia with tarsus on hind legs was not white fringed. Furthermore, the 5th tarsus on the hind legs is black, and the palpus has a sub apical dark band with a length $\frac{1}{3}$ of the apical pale band. Meanwhile, the morphological character of *An. minimus* characterized by the presence of 4 or more pale spots on the ribs and venation of 1 wing, there was no white tuft at the end of the 6 wing venation, while at the end of the venation 1 to 5.2 there was a white tuft. Feet on *An. minimus* was not spotting. *An. minimus* also did not have a white bracelet at the junction of the *tibia* and *tarsus*, and the *tarsus* of the 5 hind legs was black. The palpus was approximately the same length as the proboscis and had an apical pale band on the palpus that was approximately the same length as the sub apical pale band, while the proboscis showed a broad or not completely black pale marks. Thus, the morphological characters of the two species can be clearly distinguished (Figure 1).

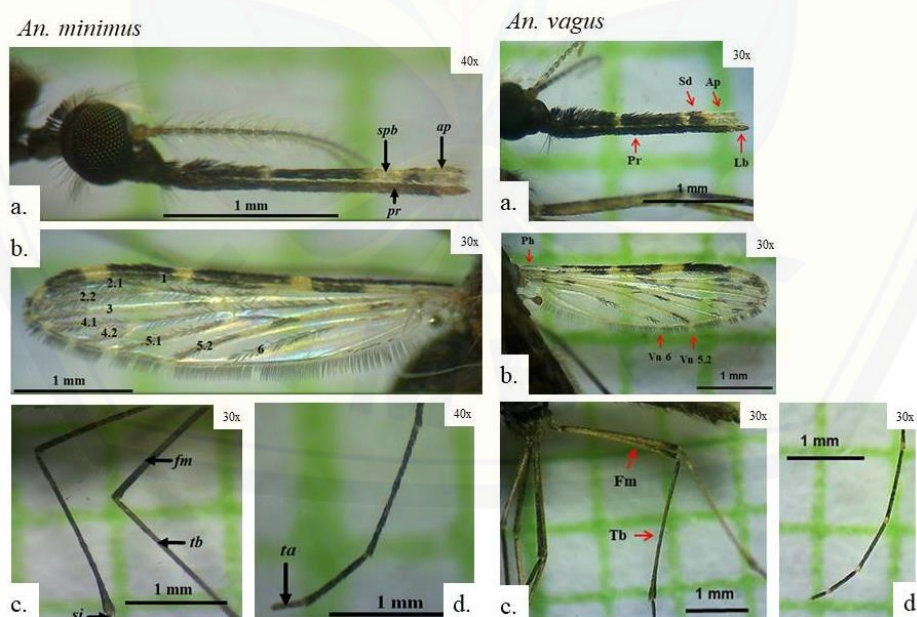


Figure 1. Morphological characteristics of *An. minimus* and *An. vagus*; a. apical pale band (ap), subapical pale band (sdb), subapical dark band (Sd) on the palpus, labellum (Lb) and proboscis (pr), b. white tuft at the tip of venation 5.2 (Vn 5.2), c. femur (fm), tibia (tb) and tarsi (si), d. 5th tarsus hind leg (ta) (Optilab Miconos, Indonesia).

The results of the isolation of the two species' genomic DNA showed bands with a molecular weight significantly above 10,000 bp (Figure 2a). This was in accordance with the literature, in which the genomic DNA sequence of *Anopheles* sp. has a size ranging from 250 Mb (Wang et al., 2019). This genome was then used as a template for PCR with the *sma-its2* primer. The PCR results showed that the ITS2 sequence was successfully amplified, resulting 650 – 700 bp for *An. minimus* and *An. vagus* (Figure 2b). This is in accordance with the approximate length of the ITS2 sequences for *Anopheles*, which ranges at 224 – 724 bp (Dritsou et al., 2014). This product is then purified for sequencing as shown in Figure 2c. The success of PCR in this study showed that the *sma-its2* primer was able to amplify the ITS2 sequence of both *Anopheles* samples.

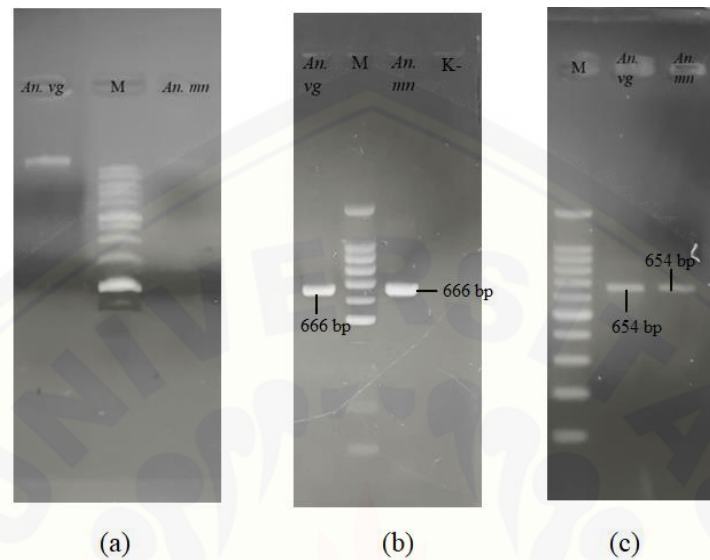


Figure 2 . Electrophoresis results from *An. vagus* (*An. vg*) and *An. minimus* (*An. mn*): (a.) whole-genome isolation, (b.) using *sma-its2* primer for ITS2 sequences amplification, (c.) PCR product purification. (Samsung A50 camera).

Analysis of the ITS2 sequences from the sequencing results can be seen in Table 1 and 2. Based on the Table 1, the ITS2 sequence of *An. vagus* Bangsring has a similarity level of 99.05 – 99.84% with *An. vagus* MN203100, FJG54649, and MN148590. Meanwhile, Table 2 showed that *An. minimus* has a similarity level of 99.21% – 100% with *An. vagus* MN148590, FJG54649, and MN203100. The level of similarity is known from the percent of identity value. Other parameters that show the similarity of species in the BLAST results are query cover, maximum score, and expected value. Query cover is the percentage of sample sequence length covered by the sequence in GeneBank. The maximum score is the highest score from the sample sequence that matches the sequence in GeneBank, while the expected value is the probability value that different results will be obtained when the sample sequence is compared again with the sequence in GeneBank. Based on this definition, it can be concluded that the lower the E. value, the higher the homology level, and vice versa (Fathiya et al., 2018).

Table 1. Results of ITS2 Sequence BLAST in *Anopheles vagus*

Description	Max. Score	Query Cover	E. value	Per. identity.	Accession
<i>Anopheles vagus</i> voucher 1841-AN4 internal transcribed spacer 2, partial sequence	1158	99%	0.0	99.84%	MN203100.1
<i>Anopheles vagus</i> 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1157	100%	0.0	99.68%	FJG54649.1
<i>Anopheles vagus</i> voucher AAV5 internal transcribed spacer 2 and large subunit ribosomal RNA gene, partial sequence	1129	100%	0.0	99.05%	MN148590.1

Table 2. Results of *ITS2 Sequence BLAST* on *Anopheles minimus*

Description	Max. Score	Query Cover	E. value	Per. identity.	Accession
<i>Anopheles vagus</i> voucher 1841-AN4 internal transcribed spacer 2, partial sequence	1166	100%	0.0	100%	MN203100.1
<i>Anopheles vagus</i> 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1149	98%	0.0	99.84%	FJG54649.1
<i>Anopheles vagus</i> voucher AAV5 internal transcribed spacer 2 and large subunit ribosomal RNA gene, partial sequence	1134	100%	0.0	99.21%	MN148590.1

The alignment results supported the BLAST results, which showed almost no difference in the nucleotide base arrangement of the *Anopheles* sequences. There are many differences in nucleotides composition between ITS2 sequences of *An. minimus* in this study with *An. minimus* in GeneBank. This indicates that the ITS2 sequence obtained with the *sma-its2* primer successfully identified the positive control, *An. vagus*, but was not successfully used to identify *An. minimus*. When compared morphologically, the two species had very different morphological characters and did not allow errors in the morphological identification that were carried out previously. Further phylogenetic tree analysis was carried out based on the ITS2 sequence generated from *An. minimus* and *An. vagus* compared to *Anopheles* species with the highest homology level from BLAST result. The phylogenetic tree produced 2 clades (Figure 3). Samples of *An. minimus*, *An. vagus* Bangsring, *An. vagus* MT740902, and *An. vagus* FJ654649, *An. subpictus*, and *An. sundaicus* belongs to 1 clade, while *An. minimus* whose sequences were collected from GeneBank belongs to the same clade as *An. harrisoni* and *An. aconitus*. These results indicate that the sequencing results of *An. minimus* had a low level of accuracy or reliability because they are not in accordance with the literature in which *An. minimus* and *An. vagus* should be in different clades. *An. minimus* is a member of the Myzomia series, while *An. vagus* is a member of the Pyretophorus series (Zomuanpuui et al., 2013).

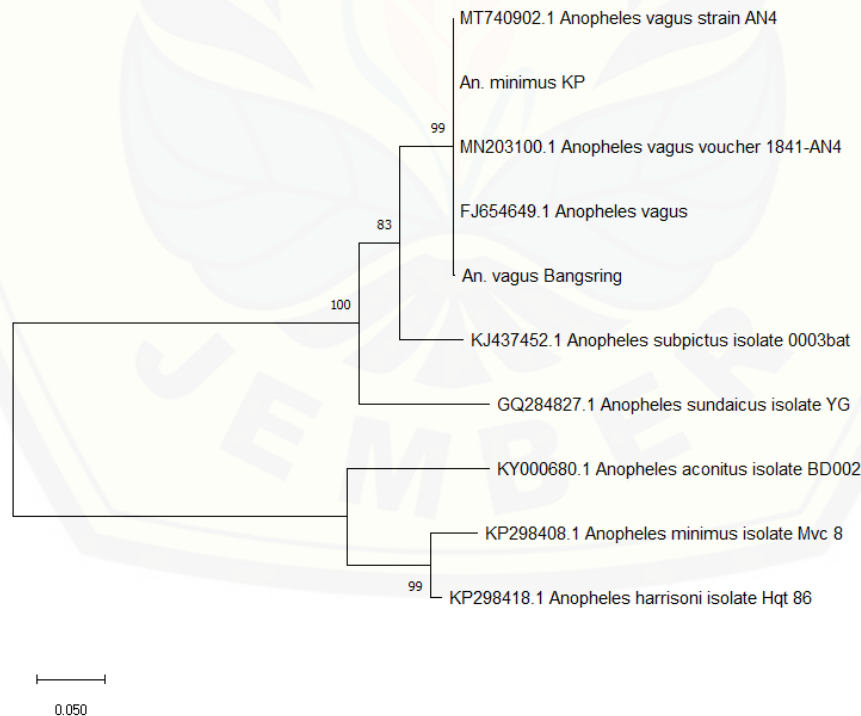


Figure 3. Results of phylogenetic tree construction from *Anopheles* species in this study: *An. minimus* KP, *An. vagus* Bangsring, and other *Anopheles* species from GeneBank.

To further ensure the *sma-its2* primer ability for identification of *An. minimus*, primer annealing analysis was carried out into the ITS2 sequence of *An. minimus* from GeneBank and also from our sample using *SnapGene* software. All nucleotides of *sma-its2F* primer and 12 nucleotides of *sma-its2R* primer were able to anneal into the

ITS2 sequences of *An. minimus* from GeneBank (Figure 4). Thus, the *sma-its2* primer should be able to be used to amplify the ITS2 sequences and identify *An. minimus*. However, when both primers were remapped on the *An. minimus* sequences of this study, only *sma-its2F* was able to anneal after removing 3 nucleotides from the 3'-end of this primer (Figure 4). Non-attachment of the 3'-end primer will cause an inefficient amplification process (Wages, 2020). Thus, it can be concluded that the *sma-its2* primer cannot be used to molecular identify *An. minimus* in this study.



Figure 4. The *sma-its2* primer annealing mapping on ITS2 sequences (a.) *An. minimus* (FN646403.1) from NCBI and (b.) *An. minimus* in this study.

4. CONCLUSION

This study showed that the *sma-its2* primer was succeeded in identifying *An. vagus*. This primer was also able to amplify ITS2 sequences of *An. minimus*, but the ITS2 sequences produced by this process could not be used further to identify *An. minimus*. The results of the morphological characterization of the two species showed very different characters and did not allow errors in morphological identification.

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