ORIGINAL PAPER

doi: 10.5455/medarh.2021.75.418-423
MED ARCH. 2021 DEC; 75(6): 418-423
RECEIVED: NOV 02, 2021
ACCEPTED: DEC 11, 2021

Redesigning Primer of ITS2 (Internal Transcribed Spacer 2) for Specific Molecular Characterization of Malaria Vectors Anopheles Species

Kartika Senjarini[1], Muhammad Khalid Abdullah[1], Nuril Azizah[1], Miatin Alvin Septianasari[1], Ahmad Tosin[1], Rike Oktarianti[1], Syubbanul Wathon[1], Tri Yudani Mardining Raras[2]

[1]Biology Department, Faculty of Mathematics and Natural Sciences, University of Jember, Jember, East Java, Indonesia

[2]Master Program in Biomedical Sciences; Faculty of Medicine, University of Brawijaya, Jl. Veteran Malang, East Java, Indonesia

Corresponding author: Dr. rer. nat. Kartika Senjarini. Biology Department, Faculty of Mathematics and Natural Sciences, University of Jember, Jl. Kalimantan 37 Jember 68121, East Java-Indonesia. Tel.: +62-81-358-346388; Fax.: +62-331-330225. E-mail: senjarini@unej.ac.id. ORCID ID: http//www.orcid.org: 0000-0001-7041-1719.

ABSTRACT

Background: *Anopheles* mosquitoes are vectors of malaria, which is a serious health issue in Indonesia. Thus, vector control is an important approach taken to overcome this disease. The first and most important step in vector control is vector identification. As some *Anopheles* species share similar morphological features, molecular identification helps make the process more accurate by using specific DNA sequences as molecular markers such as Internal Transcribed Spacer 2 (ITS2). Many of the available ITS2 primers are universally designed for insects and, as such, are typically less specific for identifying certain genera, such as *Anopheles* sp. Therefore, redesigning a specific ITS2 primer is needed for specific *Anopheles* identification. **Objective:** Our objective was to redesign a specific PCR primer for *Anopheles species*. **Methods:** The redesigned primer, named *sma-ITS2*, was then tested using mosquito samples from the *Anopheles* genus and other genera. Each mosquito was identified morphologically and their genomes were extracted. DNA samples were then amplified using the redesigned primer. **Results:** The *sma-ITS2* primer pair was capable of amplifying ITS2 sequences from all of the *Anopheles* samples and unable to amplify any of the non-*Anopheles* samples, suggesting that it is specific to *Anopheles* only. ll *Anopheles* samples were also able to be identified, only *An. indefinitus* were not able to be separated from its complex species, *An. vagus*. **Conclusion:** The *sma-ITS2* primer pair was able to identify intra-species of *Anopheles*, but its efficiency in making differentiations within a species complex should be evaluated further.

Keywords: Primer, ITS2, Anopheles, Vector, Malaria.

1. BACKGROUND

Malaria is a life-threatening disease in many tropical countries, and there was an estimated 229 million cases of the disease in 2019 (1). Most cases occur in children under five years age (2). Malaria is caused by *Plasmodium sp. (3)* which is transmitted to humans via the bite of an infected female *Anopheles* mosquito (4). Vector control can reduce malaria transmission by reducing the vector population and the amount of contact between vector and host. Vector control for malaria is commonly conducted both biologically and chemically. For instance, indoor residual spraying and insecticide-treated bed nets are examples of chemically-based, vector control methods (5, 6). However, there are numerous reports of vector resistance to insecticides (7). Furthermore, different species or subspecies within the *Anopheles* species complex may exhibit different behaviors related to malaria transmission, which could complicate the proper vector control methods needed. For example, *Anopheles gambiae* is species complex composed of distinct species, some of which are human feeders while others are not. *An. gambiae* is known as anthropophilic and endophagic, while the other type are more exophagic and exophilic (8, 9). Therefore, an understanding of the basic bionomic information and vectorial capacity of *Anopheles* is important, and *Anopheles* species identification is necessary for the vector control of malaria (7).

Anopheles identification can be conducted both morphologically and molecularly. Morphological identification is typically accomplished by distin-

Primer	Sequence (5' → 3')	Length	Amplicon length	Tm	GC%	Self-complimentary	3' self complimentary
F1	AGGACACATGAACACCGACA	20	654	59.24	50.00	4.00	0.00
R1	TTGAGGCCTACTGGAATGTGG	21		59.72	52.38	6.00	0.00
F2	ACCGACACGTTGAACGCATA	20	654	60.32	50.00	7.00	3.00
R2	AGTCACACATCACTTGAGGC	20		57.82	50.00	4.00	2.00
F3	TGCAGGACACATGAACACCG	20	654	60.88	55.00	4.00	2.00
R3	AGGCCTACTGGAATGTGGTTT	21		59.29	47.62	6.00	0.00
F4	ACATGAACACCGACACGTTG	20	655	59.07	50.00	4.00	3.00
R4	CATCACTTGAGGCCTACTGGA	21		59.17	52.38	6.00	2.00
F5	CACATGAAACACCGACCGTT	20	654	59.07	50.00	4.00	2.00
R5	TCACTTGAGGCCTACTGGAA	20		57.98	50.00	6.00	1.00

Table 1. Five pair designed ITS2 primer. Note : Forward primer (F); Reverse primer (R); Temperature melting (Tm)

guishing *Anopheles* in terms of their antennae, palpus, proboscis, and the venation of the wings, legs, etc. (10, 11). However, low-quality specimens and the presence of a species complex within the *Anopheles* genera that have shared, indistinguishable morphological features has resulted in the misidentification of species (12). Given the limitations of morphological identification, it is desirable to undertake molecular identification to accurately identify specific vectors and associate them with specific behaviors to further support vector control (13).

Ribosomal DNA Internal Transcribed Spacer 2 (ITS2) is a conserved DNA sequence commonly used as a molecular marker to differentiate (14) and reconstruct the phylogeny of the *Anopheles* species complex (15) due to its high interspecific and low intraspecific variability (16). However, performance evaluations of commonly used ITS2 primers for *Anopheles* sequences have shown biased results. The available ITS2 primers are universally designed for insects and are unable to amplify the ITS2 sequence of *Anopheles* from different geographic locations.

2. OBJECTIVE

The objective of this study was to redesign a specific ITS2 primer for *Anopheles* sequences that would support molecular identification.

3. MATERIAL AND METHODS

Primer redesign

The local sequence collections of *Anopheles*, i.e., *An. vagus, An. subpictus, An. sundaicus, An. aconitus, An. maculipennis, An. daciae, An. barbirostris,* and *An. kochi* were initially aligned in the National Center for Biotechnology Information (NCBI) BLAST. Seven sequences with the highest query covers were selected from the BLAST results and used as a DNA template. Redesigning the new ITS2 primer was conducted by using an online primer-BLAST tool in NCBI. Each of the input sequence created a primer candidate. Qualifications used to select the best primer included sequence lengths of 18-24 base pairs (bp), the absence of self-complementarity (from which it is possible to form hairpin and dimer structures), a GC content of 40-60%, the absence of thymine at the 3' end of the primer sequence, and similar melting temperatures for the forward and reverse primers (17).

Mosquito sampling

Mosquitoes were collected from Bangsring village, Banyuwangi district, East Java province, Indonesia, which is a rural area close to the seashore. The global positioning system coordinates are 8°04'48.1"S 114°25'01.1"E. The larvae of *Anopheles* were collected at a nearby lagoon that was used as a breeding site for *Anopheles* mosquitoes. Adult mosquitoes were collected using an aspirator during feeding time (from 20.00 to 02.00 (UTC+7) and kept in a sealed cup. Adult mosquitoes were identified based on morphological characteristics using O'Connor's and Reid identification keys (10, 11). Larvae were brought to a field insectary and raised to adulthood.

Molecular identification

To confirm the availability of the redesigned primer, *Anopheles* sequences were amplified using the redesigned primer. The genome was extracted by incubating the bodies of 5 adult *Anopheles* specimens in 1.5 ml Eppendorf tubes in 400 µL of homogenizing buffer (Tris-CL 10 mM, EDTA 2 mM, NaCl 0.4 M) and homogenized using a micropestle. Then, 40 µL of 20% SDS and 8 µL of 20 mg/ml Proteinase-K was added to the tubes, which were then incubated at 65 °C for 1 hour. Following incubation, 300 µL of homogenized 6 M NaCl was added and the suspension centrifuged for 30 minutes at 4 °C and 10,000 rpm. The supernatant was then transferred to a new tube. Isopropanol was added and the supernatant was stored in a freezer at -20 °C for 1 hour, after which it was centrifuged for 20 minutes at 4 °C and 10,000 rpm. The supernatant was removed, and the pellets containing DNA were washed using 70% ethanol. These were dried using a desiccator and rehydrated with ddH2O. The extracted genomes were then amplified using the new, redesigned primer. A 25 µL PCR cocktail containing 0.5 pmol PCR master mix (12.5 µL), 9 µL of aquabidest, 1.25 µL of each forward and reverse primers, and 1 µL of DNA template was used. The amplification was performed at 95 °C for 5 minutes, followed by 35 cycles of amplification, denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 45 seconds, and a final extension at 72 °C for 7 minutes. The extracted genomes were visualized via electrophoresis on 8% agarose for 30 minutes at 100 V. The electrophoresis process was conducted in TAE buffer (Tris, glacial acetic acid, and EDTA). The amplicons were purified

Primer sequence (5' → 3')	Code	PCR condition	Sample used	Reference
AGGACACATGAACACCGACA	sma-ITS2F	Predenaturation: 95 °C; 5" Denaturation: 95 °C; 30" Annealing: 54 °C; 30" Extension: 72 °C; 45" Final extension: 72 °C; 7' 35 cycles	*An. vagus vagus* *An. vagus limosus* *An. sundaicus* *An. indefinitus*	This study
TTGAGGCCTACTGGAATGTGG	sma-ITS2R			
TGTGAACTGCAGGACACAT	ITS2A	Predenaturation: 95 °C; 1' Denaturation: 95 °C; 30" Annealing: 51 °C; 30" Extension: 72 °C; 1' Final extension: 72 °C; 10' 35 cycles	*An. barbirostris* *An. campestri* *An. dissidens* *An. saeungae* *An. wejchoochotei*	(17)t
TATGCTTAAATTCAGGGGGT	ITS2B			
TGTGAACTGCAGGACACAT	ITS2a	Predenaturation: 94 °C; 3' Denaturation: 94 °C; 30" Annealing: 55 °C; 30" Extension: 72 °C; 1' Final extension: 72 °C; 10' 35 cycles	*An. gambiae* *An. quadrimaculatus* *An. punctulatus* *An. maculipennis* *An. funestus*	(18)
TATGCTTAAATTCAGGGGGT	ITS2b			
TGTGAACTGCAGGACACAT	ITS2A	Predenaturation: 94 °C; 2' Denaturation: 94 °C; 30" Annealing: 50 °C; 30" Extension: 72 °C; 40" Final extension: 72 °C; 10' 40 cycles	*An. minimus* *An. aconitus* *An. varuna* *An. pampanai*	(19)
TATGCTTAAATTCAGGGGGT	ITS2B			
TGTGAACTGCAGGACA	ITS2A	Predenaturation: 94 °C; 5' Denaturation: 94 °C; 1' Annealing: 51 °C; 1' Extension: 72 °C; 2' Final extension: 72 °C; 10' 35 cycles	*An. cracens* *An. maculatus* *An. karwari* *An. barbirostris* *An. sinensis*	(20)
TATGCTTAAATTCAGGGGGT	ITS2B			

Table 2. The comparison of primer sequences and PCR settings between sma-ITS2 and previously designed primers.

using the Wizard SV Gel Kit and PCR Clean-Up System (Promega Corp., Madison, WI, USA). The purified amplicons were then sequenced using the Sanger method at 1st Base in Singapore.

Sequence analysis

The sequences were processed using Bio-Edit software (Nucleics, Sydney, Australia) to edit illegible sections at the beginning and end of the sequences. Consensus sequences were then generated. Sequence alignment was conducted using ClustalX2 software (Informer Technologies Inc., Los Angeles, CA, USA). The consensus sequences were aligned using BLAST in NCBI to confirm their identities. These data were then used to construct a neighbor-joining phylogenetic tree with 1000 bootstrap values using MEGA X.

4. RESULTS

The sma-ITS2 primer

Five pairs of forward and reverse primers were generated (Table 1). Primer pair 1 was chosen for several advantageous characteristics. This primer pair's self-3-complementarity value was 0, thus it was impossible for a dimer structure to form, hence the DNA amplification process would not be disturbed. Primer pair 1 had the lowest self 3-complementarity value among the primer candidates, a suitable guanine-cytosine (GC) content value, and a low melting temperature difference between the forward and reverse primers. Primer pair 1 (forward primer) was named *sma-ITS2F* (5'AGG ACA CAT GAA CAC CGA CA 3') and the reverse primer was named *sma-ITS2R* (5' TTG AGG CCT ACT GGA ATG TGG 3'). The GC content of sma-ITS2F and sma-ITS2R was 50% and 52.38%, respectively.

The forward and reverse primers we chose had a melting temperature difference of 0.48 ^{o}C. The forward primer was also chosen based on the absence of thymine at the 3' end, which was present on the other 4 candidates.

A comparison between the ITS2 primer sequences according to different PCR conditions is listed in Table 2. All of the forward primers in Table 2 (including our redesigned primer) have the same nucleotide sequence part: AGGACA. The presence of the exact same nucleotide sequence indicates that it is universally present in *Anopheles*. Therefore, this particular sequence was considered essential for the ITS2 *Anopheles* primers. In addition, there was a sequence part found in all reverse primers: GCTTAAATT. This sequence was not found our redesigned primer nor in any of the other four pri-

mer candidates. There were small differences in the PCR settings for each primer that were made based on the different properties of each primer.

Morphological characteristics of malaria vector

Three species of *Anopheles* mosquitoes were identified: *An. vagus, An. sundaicus,* and *An. indefinitus*. *An. vagus* was identified and separated into two subspecies: *An. vagus vagus* and *An. vagus limosus* (Table 3). The characteristics of each identified individual matched manual guidebooks (10,11).

Molecular characteristics of malaria vector

The genomes from *Anopheles, Aedes, Culex,* and *Armigeres* samples were successfully extracted using the salting-out method. All samples had genome sizes above 10,000 bp. The PCR assay used on the *Anopheles* samples using the *sma-ITS2* primer demonstrated that amplification worked well. The electrophoresis assay of the PCR products showed single DNA bands for each sample, with sizes ranging from 550 bp to 660 bp. The estimated sizes of the DNA bands from *An. vagus vagus, An. vagus limosus, An. indefinitus,* and *An. sundaicus* were 630 bp, 660 bp, 645 bp, and 550 bp, respectively (Figure 1a). These DNA bands were expected to be the ITS2 sequences. The electrophoresis assay on the PCR products of *Ae. aegypti, Ae. albopictus, Culex* sp., and *Armigeres* sp. showed no DNA bands from these samples on the agarose gel. The only DNA band that did appear was from *An. vagus vagus* (the positive control) (Figure 1b).

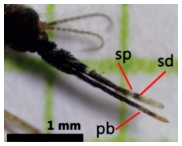
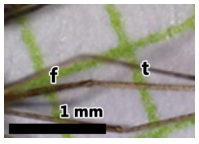
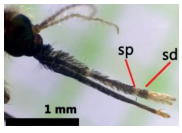
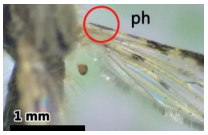
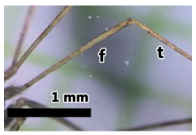
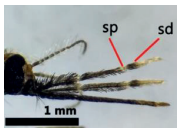
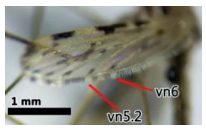
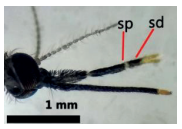
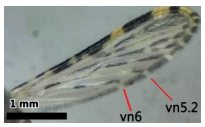
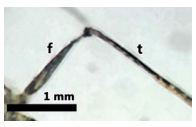
Species	Head	Wing	Leg
An. vagus vagus			
An. vagus limosus			
An. sundaicus			
An. indefinitus			

Table 3. Morphological characteristics of Anopheles sp. from Bangsring, Banyuwangi District. Note : pale band (pb); subapical dark band (sd); subapical pale band (sp); prehumeral (ph); femur (f); tibia (t)

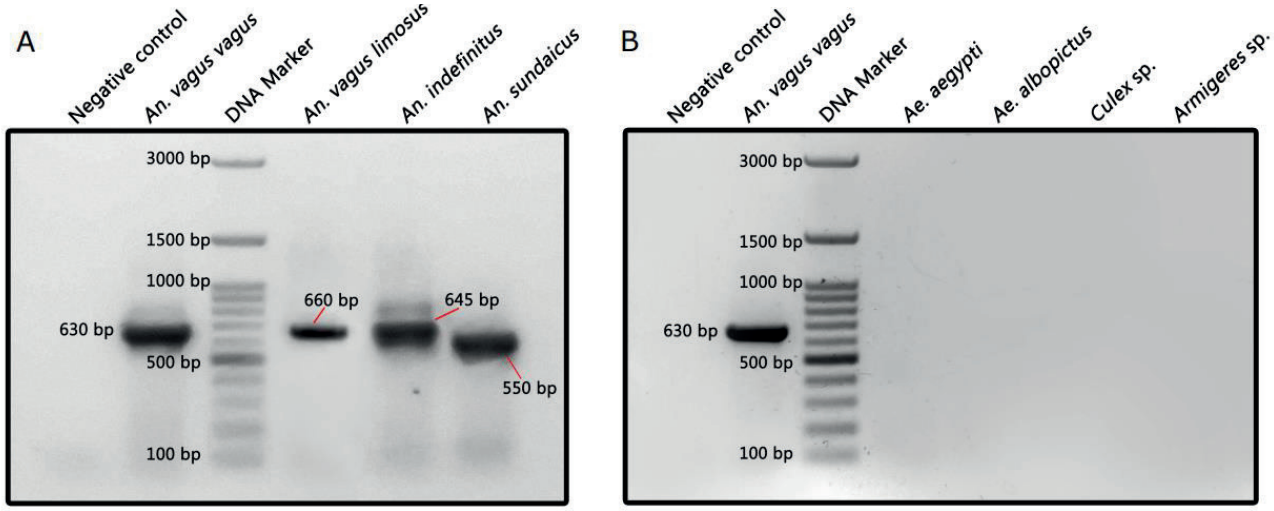


Figure 1. The PCR products of all samples using sma-ITS2 primer

The exact size of each sequence was known after sequencing and aligning the samples. The ITS2 sequence length of *An. vagus vagus, An. vagus limosus, An. indefinitus,* and *An. sundaicus* were 625 bp, 655 bp, 646 bp, and 551 bp, respectively. All ITS2 sequences in this study are still in the process of being submitted to NCBI. A species was considered to be accurately identified when the query sequence matched sequences from GenBank that had the same species name and the highest statistical match. The query and matching sequences are shown in Table 4. The *An. vagus vagus* query sequence showed the highest similarity, with two sequences from the same species: *An. vagus* FJ654649.1 and *An. vagus* MN203100.1. The *An. sundaicus* query sequence also showed high similarity with two GenBank sequences within the same species: *An. sundaicus* GQ284825.1 and *An. sundaicus* AY768541.1. A neighbor-joining tree demonstrated that the *An. sundaicus* sequence grouped in one clade with the other two *An. sundaicus* sequences from GenBank.

The *An. vagus limosus* query sequence showed the highest similarity with two sequences from the same species, but different subspecies: the first was *An. vagus limosus* MW319822.1 and the latter was *An. vagus vagus* MW314227.1. The *An. indefinitus* query sequence

Sample sequence	Description	Max. Score	Query Cover	Percent Identity	Accession Number
An. vagus vagus	An. vagus	1188	99%	0.0	FJ654649.1
	An. vagus	1168	99%	0.0	MN203100.1
An. vagus limosus	An. vagus limosus	1197	99%	0.0	MW319822.1
	An. vagus limosus	1199	99%	0.0	MW314227.1
An. sundaicus	An. sundaicus	1003	100%	0.0	GQ284825.1
	An. sundaicus	1003	100%	0.0	AY768541.1
An. indefinitus	An. vagus	1166	99%	0.0	MN203100.1
	An. vagus	1160	99%	0.0	FJ654649.1

Table 4. The query sequences obtained by sequencing and their matching GenBank sequences

showed the highest similarity with *An. vagus* MN203100.1 and FJ654649.1. This result indicates that the *An. sundaicus* sample was related to the other two *An. sundaicus* samples from GenBank as the same species. Meanwhile, the other samples: *An. vagus vagus, An. vagus limosus,* and *An. indefinitus* formed a single clade with the remainder of the GenBank sequences. Hence, the neighbor-joining tree failed to separate these samples into distinct species. In addition, there were no clear boundaries between the *An. vagus vagus* and *An. vagus limosus* GenBank sequences (Figure 2).

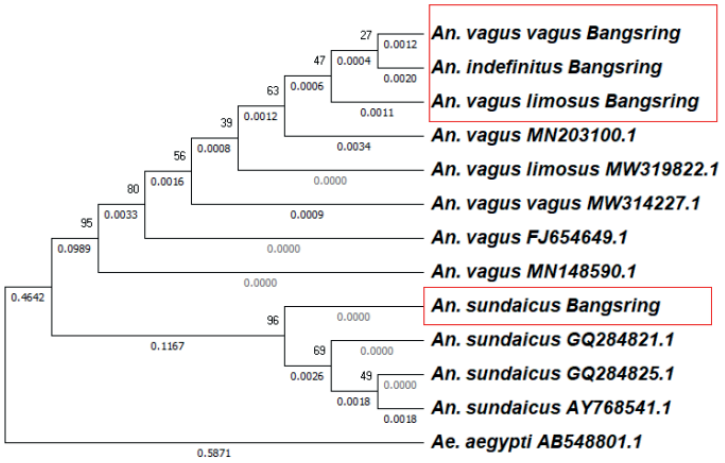


Figure 2. Neighbor-Joining Phylogenetic Tree of identified Anopheles by using primer sma-ITS2

5. DISCUSSION

The sma-ITS2 primer

If the self 3-complementarity value is higher than 0, one or more dimer structures could appear. There are 2 types of dimer structures: a self dimer and a cross dimer. Self dimers occur when a primer binds with another primer of the same type. Cross dimers occur when primer pairs (forward and reverse primers) bind to one another (21).

Typically, the GC content value for a functional primer is between 45% and 60% (22). The GC content value influences the melting and annealing temperature. A GC content value that is too high could result in a high DNA melting temperature, as there are 3 hydrogen bonds between guanine and cytosine nitrogen bases whose separation requires a high temperature (22). The optimum annealing temperature is usually 5 ^{o}C below the melting temperature; therefore, if the GC content value of the primer is too high, the target sequence will not amplify well. In contrast, if the GC content of the primer is too low, the melting and annealing temperatures will also be low. This can cause the primer to bind randomly and non-specifically to a target sequence. The presence of at least one thymine at the 3' end could have caused a mismatch or led to hairpin structures that would have disrupted the amplification process (21).

Molecular characteristics of malaria vector

A previous study (22) found that the ITS2 length of *Anopheles* ranged from 398 to 506 bp. This result indicates that the primer was able to amplify the ITS2 from *Anopheles* in this research but not capable of amplifying the ITS2 sequences of the non-*Anopheles* mosquitoes. The inability of *sma-ITS2* to amplify the ITS2 sequence of the non-*Anopheles* samples was due to the absence of a suitable sequence to bind to the primer. Based on the alignment results between the ITS2 sequence of *Anopheles* and the non-*Anopheles* samples from GenBank using ClustalX2 software (Informer Technologies, Inc., Los Angeles, CA, USA) a considerable difference in the nucleotide composition between ITS2 in *Anopheles* and non-*Anopheles* mosquitoes was observed. Furthermore, a substantial length difference in the ITS2 sequence between *Anopheles* and non-*Anopheles* mosquitoes is known to exist. This limits the primer's ability to initiate amplification of the sequences.

Our results suggests that the primer accurately identified the species of these two samples based on the ITS2 sequence. This has been also confirmed by the phylogenetic tree constructed based on this ITS2 sequences. Neighbor-joining is an algorithm used for the construction of phylogenetic trees using a method that bases the amount of difference between two sequences to construct a tree (23).

An. vagus vagus, An. vagus limosus, and *An. indefinitus* are, along with several other species, members of the *An. subpictus* complex. A species complex is a group of closely related organisms that are similar in appearance and often have unclear boundaries between them. Members of the *An. subpictus* complex have exhibited different bio-ecological traits such as susceptibility to common insecticides, salinity tolerance, and vectorial capacity (24). The nucleotide substitution rates among

the sequences within this clade were very low (or none), indicating that there was a very small amount of nucleotide substitution within the ITS2 sequences of this species. Based on the alignment results, there were only two nucleotides that differed between the sequences belonging to *An. indefinitus* and *An. vagus vagus* and *An. vagus limosus*. Meanwhile, *An. vagus vagus* and *An. vagus limosus* had no nucleotide differences between their sequences. Furthermore, there was only one ITS2 sequence for *An. indefinitus* registered in GenBank, which had accession number GQ870332 and was from the Philippines. When this sequence was tested with Basic Local Alignment Search Tool (BLAST), it showed the highest similarity to the *An. vagus* GQ480823.1 sequence from East Timor. Given these results, further analysis based on the morphological and molecular characteristics of *An. indefinitus* is needed.

Previous studies have shown that a redesigned ITS2 primer can distinguish between closely related *Anopheles* mosquitoes, including species complexes (16-18, 25). A redesigned primer has also identified several species - previously described as distinct species - as a single species (20). This is similar to the case of *An. vagus* and *An. indefinitus* identification using our primer. One solution that has been proposed to solve this issue is to use other molecular markers such as Cytochrome C Oxidase 1 to compare the identification results between molecular markers (12). Further analysis could be then conducted based on those results.

- **Acknowledgment**: This research has been financially supported by "Hibah Penelitian Pendukung IDB Universitas Jember".
- **Patient Consent Form**: None.
- **Author's Contribution**: K.S. contributed to the conception, design of the study, data analysis and manuscript preparation. M.K.A., N.A., M.A.S. and A.T. performed data acquisition and experimental laboratory works. M.K.A., N.A., R.O., S.W. and T.Y.M.R. were involved in article drafting. All authors have approved the final version of the manuscript.
- **Conflicts of interest**: All authors declare no conflicts of interest.
- **Financial support and sponsorship**: None.

REFERENCES

1. WHO. World malaria report 2020- WHO. 2020. 1–115 p.
2. Roberts D, Matthews G. Risk factors of malaria in children under the age of five years old in Uganda. Malar J. 2016;15(1):1–11. doi: 10.1186/s12936-016-1290-x
3. Autino B, Noris A, Russo R, Castelli F. Epidemiology of malaria in endemic areas. Mediterr J Hematol Infect Dis. 2012;4(1). doi: 10.4084/MJHID.2012.060
4. Aly ASI, Vaughan AM, Kappe SHI. Malaria Parasite Development in the Mosquito and Infection of the Mammalian Host. Annu Rev Microbiol [Internet]. 2011;23(1):1–7. doi: 10.1038/jid.2014.371
5. Lindblade KA, Eisele TP, Gimnig JE, Alaii JA, Odhiambo F, Ter Kuile FO, et al. Sustainability of reductions in malaria transmission and infant mortality in Western Kenya with use of insecticide-treated bednets 4 to 6 years of follow-up. J Am Med Assoc. 2004;291(21):2571–80. doi: 10.1001/jama.291.21.2571
6. St. Laurent B, Supratman S, Asih PBS, Bretz D, Mueller J, Miller HC, et al. Behaviour and molecular identification of Anopheles malaria vectors in Jayapura district, Papua province, Indonesia. Malar J. 2016;15(1):1–8. doi: 10.4269/ajtmh.15-0562
7. Musrifah. Pengendalian Kimia dan Resistensi Vektor Anopheles Dewasa pada Kawasan Endemis Malaria di Dunia. J Fak Kesehat Masy. 2017;11(1):46–51. doi: 10.12928/kesmas.v11i1.5855
8. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, et al. The dominant anopheles vectors of human malaria in the Asia-Pacific region: Occurrence data, distribution maps and bionomic précis. Parasites and Vectors. 2011;4(1). doi: 10.1186/1756-3305-4-89
9. Killeen GF, Seyoum A, Sikaala C, Zomboko AS, Gimnig JE, Govella NJ, et al. Eliminating malaria vectors. Parasites and Vectors. 2013;6(1):1–10. doi: 10.1186/1756-3305-6-172
10. Reid JA. A note on Anopheles subpictus Grassi and A. indefinitus Ludlow (Diptera: Culicidae). J Med Entomol. 1966;3(3):327–31. doi: 10.1093/jmedent/3.3-4.327
11. O'connor CT, Soepanto A. Kunci bergambar nyamuk Anopheles dewasa di Indonesia dan bergambar jentik Anopheles di Indonesia. 2013. 1–31 p.
12. Weeraratne TC, Surendran SN, Reimer LJ, Wondji CS, Perera MDB, Walton C, et al. Molecular characterization of Anopheline (Diptera: Culicidae) mosquitoes from eight geographical locations of Sri Lanka. Malar J. 2017;16(1):1–14. doi: 10.1186/s12936-017-1876-y
13. Laurent BS, Cooke M, Krishnankutty SM, Asih P, Mueller JD, Kahindi S, et al. Molecular characterization reveals diverse and unknown malaria vectors in the western Kenyan highlands. Am J Trop Med Hyg. 2016;94(2):327–35. doi: 10.4269/ajtmh.15-0562
14. Kumar NP, Rajavel AR, Natarajan R, Jambulingam P. DNA barcodes can distinguish species of indian mosquitoes (Diptera: Culicidae). J Med Entomol. 2007;44(1):1–7. doi: 10.1603/0022-2585(2007)44[1:DBCDSO]2.0.CO;2
15. Mohanty A, Swain S, Kar SK, Hazra RK. Analysis of the phylogenetic relationship of Anopheles species, subgenus Cellia (Diptera: Culicidae) and using it to define the relationship of morphologically similar species. Infect Genet Evol. 2009;9(6):1204–24. doi: 10.1016/j.meegid.2009.06.021
16. Fang Y, Shi WQ, Zhang Y. Molecular phylogeny of Anopheles hyrcanus group members based on ITS2 rDNA. Parasites and Vectors. 2017;10(1):1–11. doi: 10.1186/s13071-017-2351-x
17. Brosseau L, Udom C, Sukkanon C, Chareonviriyaphap T, Bangs MJ, Saeung A, et al. A multiplex PCR assay for the identification of five species of the Anopheles barbirostris complex in Thailand. Parasites and Vectors [Internet]. 2019;12(1):1–10. doi:10.1186/s13071-019-3494-8
18. Kengne P, Ambene PA. Molecular identification of the Anopheles nili group of African malaria vectors. Med Vet Entomol. 2003;17:67–74. doi: 10.1046/j.1365-2915.2003.00411.x.
19. Laurent B, Burton TA, Zubaidah S, Miller HC, Asih PB, Baharuddin A, et al. Host attraction and biting behaviour of Anopheles mosquitoes in South Halmahera, Indonesia. Malar J. 2017;16(1):1–9. doi: 10.4269/ajtmh.15-0562
20. Sum JS, Lee WC, Amir A, Braima KA, Jeffery J, Abdul-Aziz NM, et al. Phylogenetic study of six species of Anopheles mosquitoes in Peninsular Malaysia based on inter-transcribed spacer region 2 (ITS2) of ribosomal DNA. Parasites and Vectors. 2014;7(1):1–8. doi: 10.1186/1756-3305-7-309
21. Eling KS D, Kurniawan R, Muhimmah I. Karakteristik Primer pada Polymerase Chain Reaction(PCR) untuk Sekuensing DNA: Mini Review. Semin Inform Medis 2014. 2014;93–102. Available from: http://snimed.fit.uii.ac.id/
22. Elsalam KAA. Bioinformatic tools and guideline for PCR primer design. African J Biotechnol. 2003;2(5):91–5. doi: 10.5897/AJB2003.000-1019
23. Weeraratne TC, Surendran SN, Parakrama Karunaratne SHP. DNA barcoding of morphologically characterized mosquitoes belonging to the subfamily Culicinae from Sri Lanka. Parasit Vectors. 2018;11(1):266. doi: 10.1186/s12936-017-1876-y
24. Surendran SN, Sarma DK, Jude PJ, Kemppainen P, Kanthakumaran N, Gajapathy K, et al. Molecular characterization and identification of members of the Anopheles subpictus complex in Sri Lanka. Malar J. 2013;12(1):1–11. doi: 10.1186/1475-2875-12-304
25. Garros C, Koekemoer LL, Coetzee M, Coosemans M, Manguin S. A single multiplex assay to identify major malaria vectors within the African Anopheles funestus and the oriental An. minimus groups. Am J Trop Med Hyg. 2004;70(6):583–90. doi: 10.4269/ajtmh.2004.70.583