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REDESIGNING PRIMER OF *CYTOCHROME OXIDASE SUBUNIT* 1 (*CO1*) GENE FOR SPECIFIC IDENTIFICATION OF MALARIA VECTOR *Anopheles* sp.

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Abstract

Malaria remains a serious global health issue. Due to the absence of a malaria vaccine, vector control is deemed one of the most effective preventive strategies. *Anopheles* sp., with 20 species, is a vector of malaria in endemic areas across Indonesia. Identifying the vector of *Anopheles* sp. helps determine an effective and efficient vector control strategy. It is also important to determine disease epidemiology because different species have distinctive characteristics as malaria vectors. Several siblings and cryptic species are difficult to identify based on their morphological characteristics. These limitations have prompted the development of methods for species identification through DNA barcoding techniques. One of the molecular markers widely used in DNA barcoding is the *Cytochrome Oxidase Subunit 1* (CO1) gene derived from mitochondrial DNA. This study aimed to redesign the CO1 primer with a database from *Anopheles* sp. for a more precise and accurate interpretation of *Anopheles* sp. The design of CO1 primer was developed through a bioinformatics approach using the *Anopheles* sp. in NCBI database system. The redesigned primer was applied to the genomic DNA sample of *Anopheles* sp. obtained from the landing collection which had been morphologically identified in advance. The molecular identification on the new CO1 primer was done by CO1 sequence amplification, purification of PCR results, sequencing, and data analysis. The redesigned CO1 primer was named sra-CO1 with a forward sequence of 5' CCCGGAGCATTTATTGGGGA 3' and a reverse sequence of 5' AACCTGTTCCTGCCCCATTT 3' with a product length of 247 bp. The sra-CO1 primer has been successfully used in the molecular identification of *Anopheles vagus* and *Anopheles vagus limosus*, with the accession number NCBI *Anopheles vagus* MK628547.1. These results prove that the sra-CO1 primer can be used in the molecular identification of the genus *Anopheles* sp.

INTRODUCTION

Malaria is an infectious disease caused by the *Plasmodium* sp. transmitted by female *Anopheles* sp. during blood-feeding to humans as hosts [1]. The genus *Anopheles* sp. is the world's largest member of the order Diptera family Culicidae with 80 species including malaria vectors [1–3]. *Anopheles* sp. has diverse morphological characteristics and behaviours, including feeding locations (indoor or outdoor), biting behaviour (predominantly in humans, animals, or both), geographic distribution, and different vectorial abilities (vector or non-vector) [4]. This complexity calls forth a well-rounded control strategy as the precursor to the effective prevention of malaria cases [2].

Malaria prevails in some areas of Indonesia, and some of these areas become malaria-endemic [5]. One of these areas is Bangsring village, Wongsorejo district of Banyuwangi regency, where an extraordinary incident involving malaria once took place. Malaria prevention so far has been dominated by vector control programs, the example

is nullifying adult mosquitoes and larvae through fogging. However, this has not been efficient. Controlling vectors through fogging can result in vector resistance to insecticides [6]. Understanding the bionomic and vectorial capacity of *Anopheles* sp. lends credence to overcoming this potential resistance, so species identification needs to be done before vector control measures [7]. For effective prevention, vector controls need to be more focused, strategic, and sustainable.

The identification of *Anopheles* sp. is an important step to determine the appropriate and efficient vector control strategy, which can be based on morphological and molecular characteristics [4]. Morphological identification, however, is lengthy and difficult, especially for incomplete or damaged specimens [1]. Currently, molecular identification is more widely used to determine the complex species of *Anopheles* sp. consisting of sibling and cryptic species [8, 9]. DNA barcoding technique is an identification method using standardized short molecular markers based on DNA sequence [2]. The technique can describe new species, identify invasive species, distinguish closely related species, and reveal complex species, as a solution to the limited number of morphological taxonomists, extensive identification time, and challenges to identifying intact or damaged organisms [10].

Molecular markers derived from mitochondrial DNA have many advantages because they are maternally inherited, a high number of DNA copies in animal cells, conservative regions with genetic variation can separate closely related species, differentiate intraspecific and interspecific species, low recombination, and be easily amplified [10, 11]. The CO1 gene in mitochondrial DNA is widely used in studying population genetics, biogeography, speciation, and organism systematics [12]. Animal evolution studies generally use CO1 as the best molecular marker [11, 13]. The CO1 gene marker of 650 bp in length is proven 100% successful in insect DNA barcoding [12, 14]. Because it deals with a coding region, CO1 gene often undergoes insertions and deletions compared to other genes. It tends to have short sequences with conservative natures, but can differentiate between complex species [13, 15].

CO1 molecular markers requires specific primers for successful CO1 DNA sequence amplification [16]. The previous study used “Folmer Region” CO1 primers (LCO1490F and HCO2198R) [17], yet several issues still surfaced, such as 1) low reproducibility [2, 12]; 2) identification limited to only three species, namely *Anopheles vagus* (~686 bp), *Anopheles indefinitus* (~733 bp) and *Anopheles subpictus* (~704 bp); and 3) limited data on CO1 *Anopheles* sp. on NCBI. Aiming to address these downsides, CO1 primer was redesigned to obtain specific primers for the identification of *Anopheles* sp. using a molecular approach. This step aided in determining an effective and efficient strategy to control malaria vector.

MATERIALS AND METHODS

Landing Collection and Morphological Identification of *Anopheles* sp.

Mosquito catching was carried out at 18.00–00.00 (GMT +7) in Bangsring Village, Banyuwangi District, East Java Province, Indonesia (8°04’48.1”S 114°25’01.1”E). Morphological identification was performed using a stereo microscope and the morphological identification book *Anopheles* sp. [18, 19]. The identification also determined the sex of the mosquito based on antennae shape, ornamentation of the proboscis and palps. The genus identification based on ornamentation at the base of the mosquito's wings, and abdomen color.

CO1 Primer Design

The DNA sequences were from the Local Database Sequences CO1 *Anopheles* sp. in previous studies and BLAST CO1 sequences from NCBI. Primer design was developed using online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer design analysis focused on primer length, melting temperature, G-C content, the sequence at the 3' end, primer specificity, primer degeneration, complementary sequence from primary, secondary structure, and product length.

The Isolation and Visualization of *Anopheles* sp.

Genomic DNA was isolated using a modified salting-out extraction method. Genome extraction was carried out by adding mosquito samples with 400 µL of homogenizing buffer (Tris-Cl 10mM, EDTA 2mM, NaCl 0.4M). The samples were then homogenized using a micropistil, mixed with 40 µL SDS 20%, and 8 µL of 20 mg/ml Proteinase-K, which were then incubated at 65º C for 1 hour. The suspension was then mixed with 300 µL of 6M NaCl and homogenized and centrifuged at 10,000 rpm for 30 minutes at 4º C. The supernatant was transferred to a new microtube and mixed with isopropanol and stored in a freezer at -20ºC for one hour. The suspension was then centrifuged at 10,000 rpm for 20 minutes at 4º C. Afterward, the supernatant was discarded. The pellet was then washed with 70% ethanol and dried in a desiccator, rehydrated with 50 µL of ddH2O (suspended), and mixed with 3 µL of RNAse.

The visualization of genomic DNA isolation was done by electrophoresis at 100 volts for 40 minutes using agarose gel at 1% concentration and the addition of 0.5 µL of Ethidium Bromide. The agarose gel was immersed in 350-mL 1x TAE buffer. As much as 5 µL DNA sample isolated from the genome was taken and mixed with 1 µL loading dye. The sample was then inserted into the agarose gel well, and then 5 µL of the 10,000 bp Geneaid DNA ladder was inserted into another gel well. The agarose gel was visualized using a UV-transilluminator to identify DNA bands.

The Amplification of CO1 Sequence

The CO1 sequence was amplified in vitro using a PCR machine with the developed CO1 primer design. The PCR underwent minor modifications of the annealing temperature of the reference (Table 1). The amplification results were proven by electrophoresis using a 1 kb DNA marker (Geneaid, Taiwan).

Table 1. PCR conditions after minor modifications [21]

	Temperature	Duration	Description
Predenaturation	94°C	5 minutes	-
Denaturation	94°C	40 seconds	
Annealing	54°C	1 minute	
Extension	72°C	1.5 minutes	
Denaturation	94°C	40 seconds	35 cycles
Annealing	54°C	1 minute	
Extension	72°C	1.5 minutes	
Final Extension	72°C	5 minutes	

PCR Result Purification

The DNA bands on the agarose gel were cut using a sterile cutter and then purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega, USA). The DNA purification aimed to separate DNA from agarose gel by using column chromatography so that in the column (stationary phase) the sample would be separated. The purification also used wash buffer (mobile phase) to dissolve the sample so that the DNA sample was aggregated at the bottom of microtube. This kit consisted of an SV minicolumn and a collection microtube, membrane binding solution, membrane washing solution, and nuclease-free water. The membrane binding solution dissolved the agarose gel and attached the DNA to SV minicolumn. The membrane-washing solution dissolved all components attached to the SV membrane. Nuclease-free water helped dissolve DNA bound to the SV membrane.

Sequencing and Data Analysis

The purified DNA was sequenced with the aid of 1st Base Singapore using the Sanger method. The sequencing data was edited using the Bioedit software. The DNA was aligned with the aid of ClustalX2 software. Afterward, the phylogenetic tree of all sequences was reconstructed using MEGA 6 with the Neighbor-Joining bootstrap 1000 method.

RESULTS AND DISCUSSION

The landing collection in Bangsring village obtained 235 adult mosquitoes during times of mosquito collection. The morphological identification showed several *Anopheles* sp. species, including *Anopheles vagus* (55), *Anopheles vagus vagus* (55), and *Anopheles vagus limosus* (125) as the most dominating species. These were members of the *Pyretophorus* Series species complex of the *Subpictus* group. Adult mosquitoes from these species are difficult to distinguish due to morphological similarity [20].

Adult mosquitoes of the *Anopheles vagus* have unspotted femur and leg tibia. On the palpus, the length of the subapical pale band is 1/3 of the apical pale band. Meanwhile, to distinguish member of *Subpictus* Complex between the sub-species *Anopheles vagus vagus* and *Anopheles vagus limosus* is based on characteristics of the proboscis and prehumeral parts of the wing [21]. The morphological identification results showed that *Anopheles vagus* had a dark prehumeral wing on all parts of the ring and there was a pale band on the proboscis. *Anopheles vagus vagus* had pale bands on the apical palps on the dorsal, lateral, and circular sides. These pale bands were also found on the prehumeral part of the wings. *Anopheles vagus limosus* had a proboscis with dark parts and dark prehumeral parts of the wings. The morphological characteristics of *Anopheles vagus vagus* and *Anopheles vagus limosus* species are shown in Figure 1.

Multiple studies have demonstrated different taxonomic positions between *Anopheles vagus vagus* and *Anopheles vagus limosus*. Based on molecular characteristics, *Anopheles vagus limosus* belongs to a subspecies of *Anopheles vagus* [21]. However, another study classifies *Anopheles vagus* and *Anopheles limosus* into different species [22]. The two species of *Anopheles* sp. assumedly have undergone speciation as these have similar genetic characteristics, despite different morphological characteristics [23].

Speciation or the formation of new species occurs when a population encounters several supporting factors. The speciation in *Anopheles vagus* and *Anopheles limosus* is associated with several driving factors, such as high diversity, interbreeding ability, preference for habitat conditions, and being host specific. A species of mosquito cannot mate with other species (interbreeding) because of

pre- and post-mating barriers. Mosquitoes tend to select the ecological conditions of their habitat, such as the preference for fresh or brackish water around the coast for brooding and larval development [15]. Specific host characteristics also minimize intraspecies competition for blood-feeding on the host, as evidenced by the blood-sucking behaviour of each species [24]. Continuous behavioural changes can determine the location of genes on chromosomes, thereby triggering speciation. However, speciation factors can also enable different species of *Anopheles* sp. to coexist in the same habitat [15, 25].

There are 460 species of *Anopheles* sp. worldwide, and approximately 100 species are malaria vectors [26]. In Indonesia, 29 species of *Anopheles* sp. have been identified, with 20 of them confirmed malaria vectors [27]. *Anopheles* has extensive morphological diversity [28], and several species of this genus are complex species. Most of the *Anopheles* mosquitoes involved in malaria transmission in South and Southeast Asian countries have been identified as species complexes, such as *Annularis, Barbirostris, Culifacies, Fluvialis, Leucosphyrus, Maculatus, Minimus, Phippinensis-Nivipes, Punctulatus, Sinensis, Subpictus,* and *Sundaicus* [29].

Researchers hypothesize that species complexes result from geographic and reproductive isolation [30], and, over a long period, a new species will occur [31]. Species belonging to the species complex are morphologically complicated to distinguish [32]. Several studies have reported that members of species complex have different vectorial capacities, biting activities, and habitat [33, 34]. This has led to numerous misidentifications of *Anopheles* in malaria-endemic areas, generating a biased vector control [35]. This is because species complex can have different vectorial capacities, biting preferences, and even habitats [36].

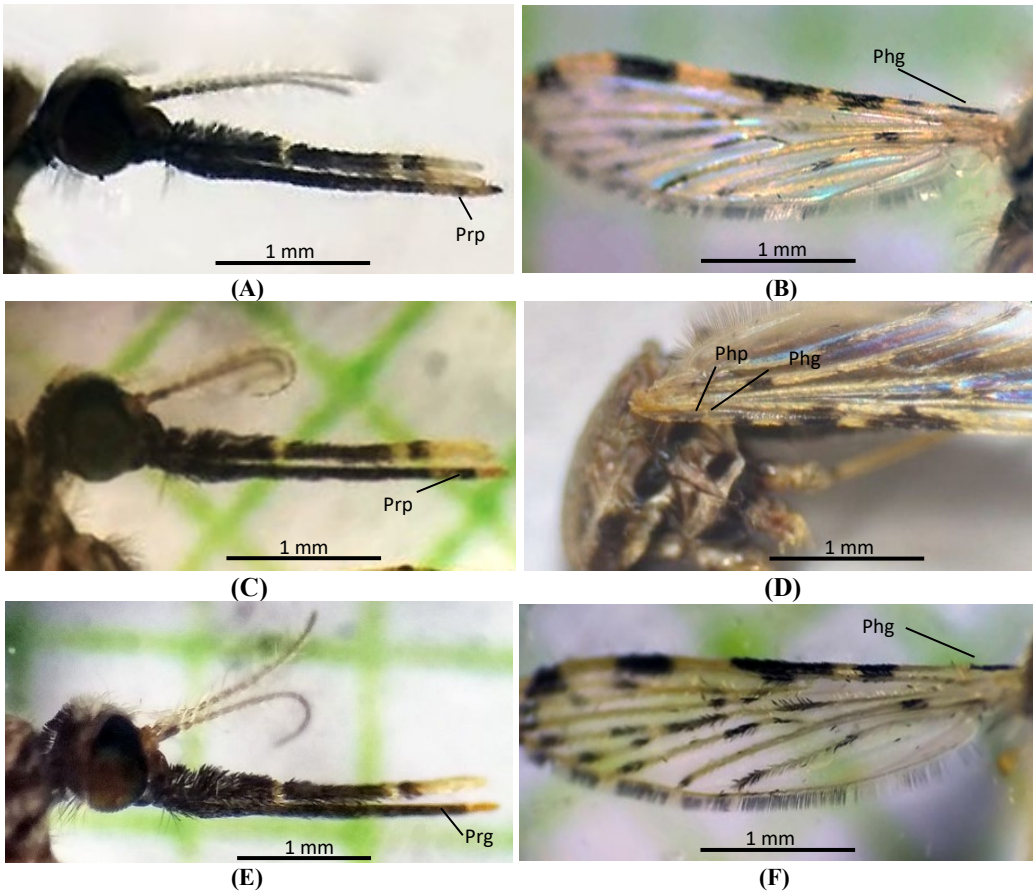

Figure 1. Differences in morphological characters of *Anopheles vagus vagus* and *Anopheles vagus limosus*: (A) Pale proboscis (Prp) *Anopheles vagus*; (B) Dark prehumeral wing (Phg) *Anopheles vagus*; (C) Pale proboscis (Prp) *Anopheles vagus vagus*; (D) Pale prehumeral (Php) and dark prehumeral (Phg) *Anopheles vagus vagus*; (E) Dark proboscis (Prg) *Anopheles vagus limosus*; (F) Dark prehumeral wing (Phg) *Anopheles vagus limosus.* (Nikon SMZ745 stereo microscope 50X magnification, Samsung A50 camera).

The morphological identification of *Anopheles* sp. in this study needs further confirmation due to the following issues: (1) the difficulty to differentiate pale and dark bands on the mosquito proboscis; (2) the morphological similarities among the mosquitoes found; and (3) the absence of parts of the hair on the mosquito's body such as on the legs, palps, proboscis, and wings [1, 21]. These issues demonstrate that *Anopheles* sp., being a species complex (sibling or cryptic), cannot be identified solely based on morphological characteristics. As a corollary, molecular identification is necessary.

The identification results based on morphological characteristics were confirmed by molecular identification through DNA barcoding using the redesigned CO1 primer. The results of redesigning CO1 primer for the molecular identification of *Anopheles* sp. marked a primer with a sequence of 5' forward primer CCCGGAGCATTTATTGGGGA 3' and reverse primer 5' AACCTGTTCCTGCCCCATTT 3'. The selected primer has a sequence length of 20 bp with the amplification product length being 247 bp. The melting temperature of the forward primer sequence is 59.82° C and the reverse primer sequence is 59.81° C. The value of the primer pair GC content is 50-55%, the self-complementary number is 0.0 and the self-complementary number is 0.00. The primer from this design is referred to as “sra-CO1 primers” (Table 2).

The sra-CO1 primer sequence is 20 bp in length, which corresponds to the standard primer criteria, 12-30 bp [16]. Short primer sequences are less specific because they are thought to bind to a non-target DNA template at an inaccurate annealing temperature. In contrast, primer sequences of more than 30 bp lead to the formation of secondary hybridization where primers attach to one another and the expected amplicon become unfeasible. Standard primer ranging from 12 to 30 bp allows optimum annealing temperature (50°C) [16].

The amplification product of sra-CO1 primer pair has 247 bp, which is shorter than the full-length region of CO1 gene (~600-700 bp). The shorter the amplification product, the more accurate it is to determine the varied sequences between closely related species. Nucleotide base variations help differentiate between species (CO1 barcode region) [37].

The primer sequence should be unique so that it only attaches to the region of the target sequence to be amplified. The primer sequence should not contain more than four repetitions of nitrogen bases, especially at the 3' end [38]. The forward primer sequence of sra-CO1 has four repetitions of Guanine, at the 16-19th sequence at the 3' end. The reverse primer sequence of sra-CO1 has four repetitions of Cytosine, at the 13-16th sequence at the 3' end. GC clamp sequence (the Guanine and Cytosine at the 3' end) is essential for primary design because the strong bond between Guanine and Cytosine stabilize hybridization. The forward and reverse primer sequences of sra-CO1 have 3 nitrogen bases of Guanine and Cytosine at the 3' end. The last five nitrogen bases at the 3' end should contain ≤ 3 nitrogen bases of Guanine and Cytosine so that the primer specifically attaches to the DNA template [39].

The optimal melting temperature is at <60°C because it is related to the annealing temperature in PCR analysis. The difference between the melting temperature and annealing temperature needs to be maintained at roughly 5°C to prevent the formation of a secondary structure in the primer pair [40]. PCR analysis generally uses annealing temperatures in the range of 52°-58°C [41]. When the annealing temperature exceeds 58°C, the primer pair is likely to stick to the non-target site. The annealing temperature difference between the forward and reverse sra-CO1 primers is 0.01°C, under which amplification can run effectively and the two primers achieve optimum performance at almost the same temperature. In addition, annealing temperatures at 52°-58°C are optimal for primer lengths of 18-20 bp [40]. This corresponds to the length of the sra-CO1 primer sequence is 20 bp in length.

The percentage of GC content is related to the melting temperature of primer pairs. Standard GC content in the primer ranges between 40% and 60%. Any GC content over 50% causes the melting temperature to exceed 50°C, potentially causing the primer to form hairpins. Guanine and Cytosine have 3 hydrogen bonds which are stronger and more stable than those between Adenine and Thymine. As such, releasing these bonds requires a high temperature of more than 60°C [39]. The GC content in the sra-CO1 forward primer is 55% while that in the sra-CO1 reverse primer is 50%. These sra-CO1 primer pairs correspond to a good primer [41].

The sra-CO1 primer was tested with NCBI BLAST for its universality in the molecular identification of *Anopheles* sp. The results showed that 9 *Anopheles* sp. of the top 100 species ranked by BLAST sequences. The results from the top 100 species displayed more of the genus *Anopheles* indicating a higher primer universality. This showed that the presumed sra-CO1 primer could be used to identify the molecular characteristics of *Anopheles vagus*, *Anopheles squamosus, Anopheles annularis, Anopheles rupifes,* and *Anopheles subpictus* (Table 3).

Table 2. Five pair designed CO1 primer using NCBI BLAST Primer. Note: forward primer (F); reverse primer (R); temperature melting (Tm); Red box: sra-CO1 primer

Primer	Sequence (5' → 3')	Length (bp)	Amplicon Length (bp)	Tm (℃)	GC (%)	Self Complementary	Self 3' Complementary
F1	ACCCCGGAGCATTTATTGGG	20	153	60.11	55.00	4.00	0.00
R1	ATGCTATATCGGGGGCTCCT	20		59.96	55.00	4.00	2.00
F2	GGTCACCCCGGAGCATTTAT	20	161	59.82	55.00	5.00	2.00
R2	GGGAATGCTATATCGGGGGC	20		60.11	60.00	4.00	2.00
F3	CCCGGAGCATTTATTGGGGA	20	247	59.82	55.00	4.00	0.00
R3	AACCTGTTCCTGCCCCATTT	20		59.81	50.00	4.00	0.00
F4	AAAATGGGGCAGGAACAGGT	20	74	59.81	50.00	2.00	2.00
R4	AACTGAAGCTCCAGCGTGTG	20		60.88	55.00	8.00	2.00
F5	CTGAACTAGGTCACCCCGGA	20	262	60.61	60.00	8.00	2.00
R5	CAACCTGTTCCTGCCCCATT	20		60.54	55.00	4.00	1.00

Table 3. The prediction of primer universality of sra-CO1 in *Anopheles* sp. NCBI (another genus not listed)

No.	Description	Locate (bp)	Query cover	E Value	Ident (%)	Accession
1.	*Anopheles vagus* voucher CDRL02 cytochrome oxidase subunit 1 gene; partial cds; mitochondrial; ~678 bp; from India	*Forward* (93-112)	100%	0.43	100%	MK628547.1
2.	*Anopheles squamosus* voucher NMNH2017-044-F03 cytochrome oxidase subunit 1 (CO1) gene; partial cds; mitochondrial; ~623 bp; from India	*Forward* (48-64)	85%	27	100%	MK586126.1
3.	*Anopheles annularis* isolate G1 cytochrome oxidase subunit 1 (CO1) gene; partial cds; mitochondrial; ~564 bp; from India	*Forward* (25-41)	85%	27	100%	MK586126.1
4.	*Anopheles rupifes* voucher NMNH2017-028-F03 cytochrome oxidase subunit 1 (CO1) gene; partial cds; mitochondrial; ~594 bp; from Bangladesh	*Reverse* (283-264)	100%	0.43	100%	MK586031.1
5.	*Anopheles subpictus* voucher ST05 cytochrome oxidase subunit 1 (CO1) gene; partial cds; mitochondrial; ~ 678 bp; from India	*Reverse* (340-321)	100%	0.43	100%	MK603828.1

The CO1 gene amplification by using sra-CO1 primer resulted in a sequence length of 247 bp. The amplification product was tested for molecular identification of *Anopheles* sp. through the DNA barcoding approach. The DNA barcoding method is carried out using mini-barcoding with a product that is shorter than the full-length barcode region. Mini-barcoding offers more accurate and efficient results in identifying interspecies and intraspecies differences, determining specific barcode regions, identifying closely related species, determining conservative and varied sequences, and analyzing degraded DNA [42–44]. The amplification results of CO1 sequences by using sra-CO1 primer is 247 bp in length that fulfill the mini-barcode length criteria of around 100-400 bp [45].

The application of CO1 primers for molecular identification of *Anopheles* sp. in several studies have shown that the primers AnophF/HCO2198R and Uni-Minibar-JVF/Uni-minibar-JVR are mini-barcoding primers with amplification product lengths of 275 bp and 173 bp, respectively [46]. However, these primers cannot differentiate between *Anopheles gambiae* and *Anopheles arabiensis*. This corroborates that the two primers can be used to identify the genera *Aedes, Mansonia,* and *Culex* through molecular identification, but not genus *Anopheles*. Compared to the other two CO1 primers, the sra-CO1 primer is better used for molecular identification in the genus *Anopheles*.

The molecular identification of *Anopheles* sp. began with the isolation of genomic DNA using the salting out method combined with isopropanol and enzymes [47]. DNA isolation employs method with organic solutions (phenol-chloroform), method with inorganic solutions (enzymes or detergents), spin column method, chelex method, CaCl density gradient method, and salting out method [48]. The isolation of mosquito DNA in this study employed salting out method combined with isopropanol and enzymes [47]. In addition to its promising results in DNA isolation, salting out method is deemed advantageous as it only requires easy-to-obtain, affordable, and non-toxic material [48]. Genomic DNA isolation has been successfully carried out as shown in the Figure 2(A).

The amplification of the CO1 gene by PCR showed that the sra-CO1 primer amplified the CO1 gene sequences encoding *Anopheles vagus,* and *Anopheles vagus limosus* from Bangsring. This was indicated by a single band of ~250 bp in each species (Figure 2 (B)). The DNA purification to obtain pure CO1 DNA using PCR Clean-Up System showed a single band of similar size to the PCR results (Figure 2 (C)).

The purified DNA was sequenced using the Sanger method from 1st Base Singapore to obtain the sequence of nucleotide bases. The sequencing data results were analyzed using Bioedit to gain a concensus as the basis for BLAST at NCBI. The identification results in *Anopheles vagus* and *Anopheles vagus limosus* referred to *Anopheles vagus* with the same accession number, MK628547.1 (Table 4) with an identical percentage of 98%.

The quality of CO1 sequences was analyzed using Bioedit software based on the peak chromatogram of the 4 nitrogen bases (A, T, G, C). The result of DNA sequencing were decent, as evidenced by clear nitrogen base peaks without buildup. The alignment of the CO1 sequence involving *Anopheles vagus* shows no gaps in nucleotide base sequences (Figure 3), implying a strong interspecies relationship. The gap in DNA sequences indicates high interspecific genetic variation or distant interspecies relationship [49].

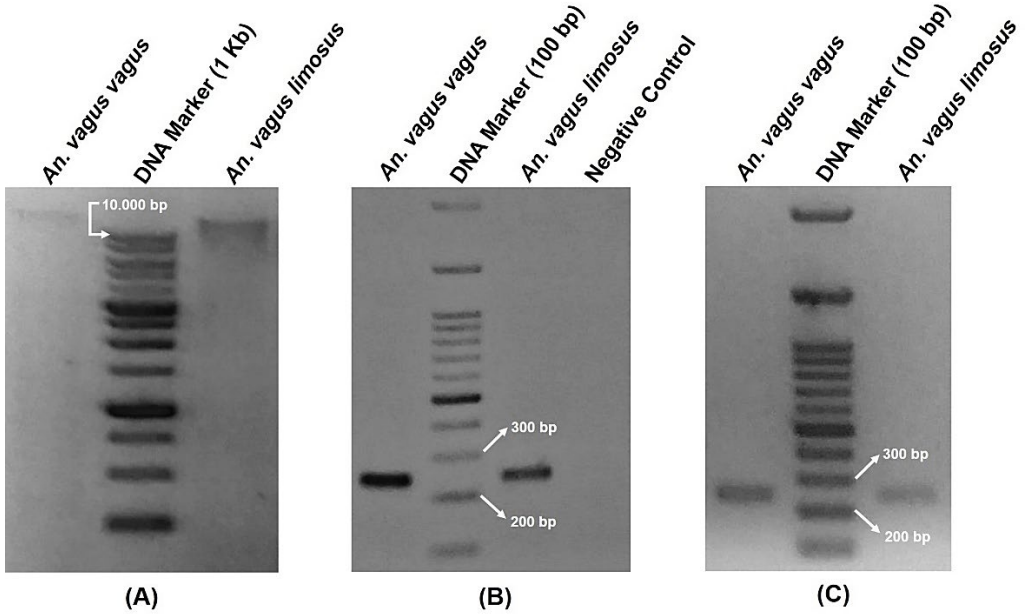

Figure 2. DNA visualization from *Anopheles vagus vagus* and *Anopheles vagus limosus*: (A) whole genome isolation; (B) using sra-CO1 sequences amplification; and (C) PCR product purification. (Samsung A50 camera).

Table 4. The results of BLAST NCBI *Anopheles vagus vagus* and *Anopheles vagus limosus*

DNA Sample	Description	Max score	Query cover	E. Value	Ident (%)	Accession
Anopheles vagus vagus	*Anopheles vagus* voucher CDRL02; 678 bp; from India	431	99%	8e-117	97.98	MK628547.1
	Anopheles vagus voucher MT0U12 ; 594 bp; from Bangladesh	425	99%	4e-115	97.58	MK189164.1
	Anopheles vagus voucher *Viet_E-8*; 649 bp; from North Vietnam	420	99%	2e-113	97.18	MH425444.1
Anopheles vagus limosus	*Anopheles vagus* voucher CDRL02; 678 bp; from India	435	99%	6e-118	98.38	MK628547.1
	Anopheles vagus voucher MT0U12; 594 bp; from Bangladesh	429	99%	3e-116	97.98	MK189164.1
	Anopheles vagus voucher Viet_E-8`; 649 bp; from North Vietnam	424	99%	1e-114	97.57	MH425444.1

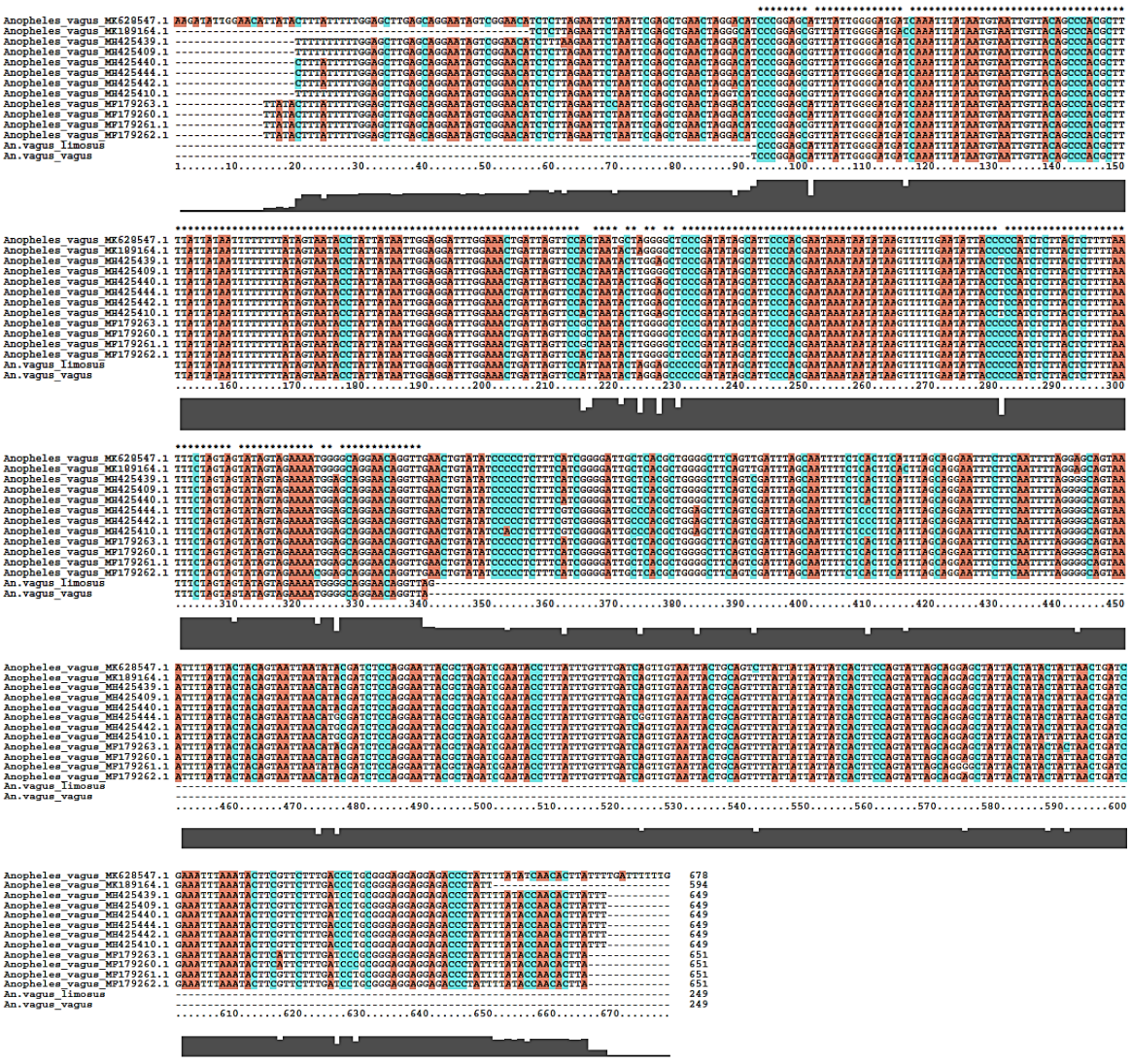

Figure 3. The alignment results of the *Anopheles* sp. CO1 sequences.

The amplification of the CO1 sequence of *Anopheles* sp. was marked at 247 bp, and this cohered with the molecular identification because the BLAST results confirmed similar species, *Anopheles vagus*. This demonstrates the effectiveness of mini-barcoding sequence for the molecular identification of *Anopheles* sp. The DNA sequence product with a length of 247 bp is parallel to the nucleotide base of *Anopheles vagus* MK628547.1. This shows that the sra-CO1 primer can aid species identification of *Anopheles* sp., but can't distinguish at the subspecies level, namelly *Anopheles vagus vagus* and *Anopheles vagus limosus*. Another study shows that *Anopheles vagus* with different morphologies are still categorized as one species due to indifferent DNA sequences, as confirmed in the BLAST analysis on NCBI sequences [21].

The CO1 sequences of *Anopheles vagus* and *Anopheles vagus limosus* were reconstructed by a phylogenetic tree to determine their relationship using the Neighbor-Joining method with bootstrap 1000 (Figure 4). The phylogenetic tree reconstruction showed that *Anopheles vagus vagus* and *Anopheles vagus limosus* formed a monophyletic branch with *Anopheles* sp. MH392206.1 from the Congo with a bootstrap value of 80%. *Anopheles vagus vagus* is more closely related to *Anopheles vagus* MK685257.1 from China, MF179259.1 from China, MH425411.1 from North Vietnam, MH425439.1 from North Vietnam, MK628547.1 from India, and MK189164.1 from Bangladesh. *Anopheles vagus vagus* and *Anopheles vagus limosus* were in different clades because of different nucleotide bases during alignment. This can generate new clades so that specific primers are needed to distinguish the molecular characteristics of associated subspecies.

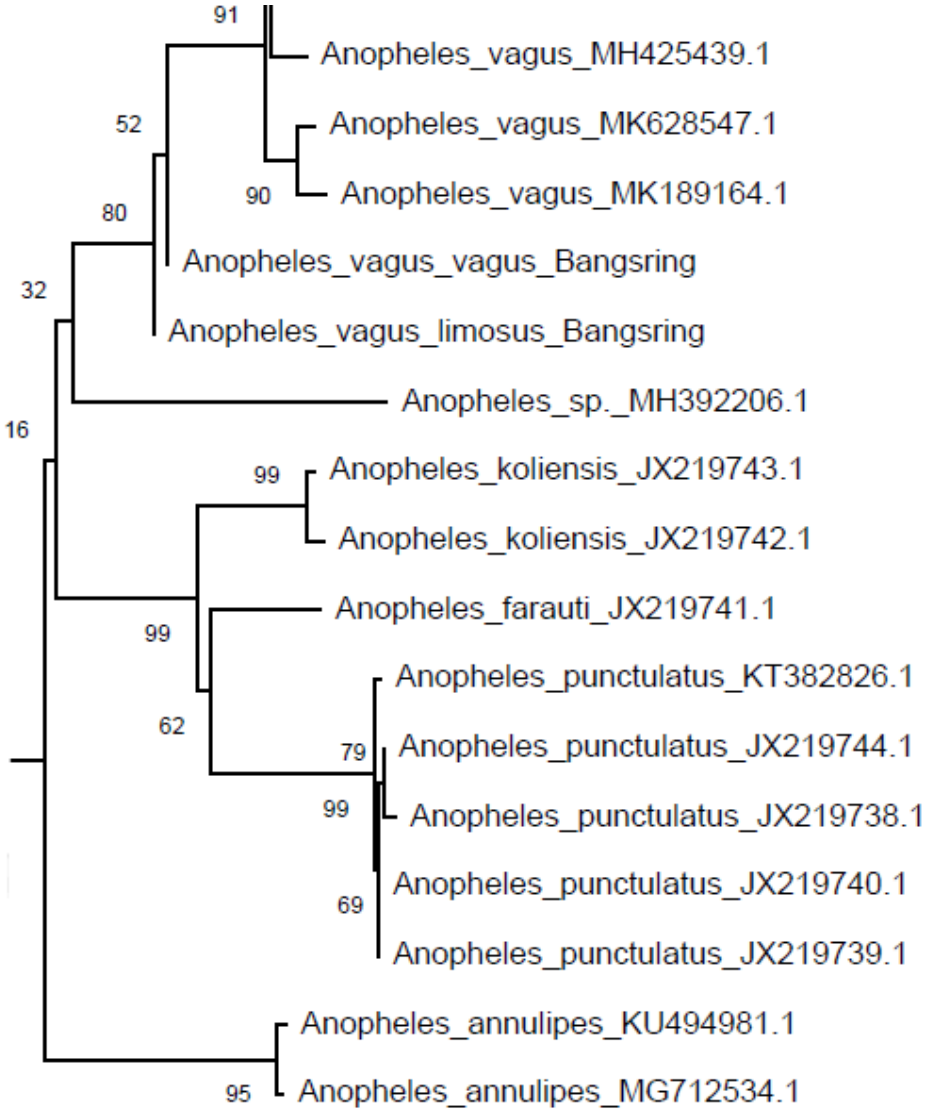

Figure 4. Neighbor-joining phylogenetic tree of *Anopheles* sp. based on CO1 sequences.

In conclusion, the primer design for CO1 coding gene in this study has a sequence of forward 5' CCCGGAGCATTTATTGGGGA 3' and reverse 5' AACCTGTTCCTGCCCCATTT 3' named sra-CO1. The PCR analysis of CO1 gene sequences using sra-CO1 primers shows that *Anopheles vagus vagus* and *Anopheles vagus limosus* refer to the same Accession Number, MK628547.1. This shows that the sra-CO1 primer can successfully identify the molecular characteristics at the species level rather than subspecies level. The results of the phylogenetic tree show that *Anopheles vagus vagus* is closer to *Anopheles vagus* than *Anopheles vagus limosus*.

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CONFLICT OF INTEREST

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