In silico exploration of the potential barcode DNA in *Anopheles* sp., a malarian vector from Indonesia

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ABSTRACT

Malaria is an infectious disease caused by *Plasmodium* and transmitted to humans through the *Anopheles* mosquito vector. The large diversity of *Anopheles* in Indonesia, which consists of similar complex species, makes it difficult to identify species based on morphological characteristics. Therefore, it is necessary to have another approach to identifying species based on molecular characteristics using DNA molecular markers. This approach becomes important as a basis for vector-based malaria control efforts. This research aims to explore potential DNA barcodes based on in silico studies as a reference for DNA barcoding analysis of *Anopheles* species in Indonesia. The data sequences of CO1 and ITS2 from *Anopheles* sp. were collected on the NCBI database. The sequence alignment was performed using CLUSTALX2. The construction of a phylogenetic tree was carried out using MEGA XI. Data analysis was performed by observing the construction of phylogenetic trees, bootstrap scores, and genetic distance. The data sequence in NCBI shows that 10 species of the genus *Anopheles* as malaria vectors have been studied in Indonesia. Sequence alignment of the 10 species based on CO1 and ITS2 markers showed that ITS2 markers had higher genetic variation than CO1. Therefore, the ITS2 sequence has the potential to be a DNA barcode for the molecular identification of *Anopheles*.

Keywords: *Anopheles*, barcode, CO1, ITS2, malaria

INTRODUCTION

Malaria is a disease caused by a parasitic infection of *Plasmodium* transmitted by the *Anopheles* mosquito vector to humans as its host. The *Plasmodium* that infect humans and cause malaria worldwide consist of five species: *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi* (Sato, 2021). Malaria transmission occurs when female *Anopheles* mosquitoes, infected with *Plasmodium*, feed on human blood (Hang et al., 2021). Malaria still remains a global problem. In 2019, there were 229 million malaria cases reported worldwide, with 409,000 of them resulting in deaths (WHO, 2020). Malaria continues to be a focus of public health efforts in Indonesia, with a target to eliminate Malaria by 2030 (WHO, 2021). There are 39 districts or cities in eastern Indonesia, particularly in Papua, West Papua, and East Nusa Tenggara, that have the highest transmission rates (Debora et al., 2018).

Data from the Ministry of Health of the Republic of Indonesia (2020) showed that the annual parasite incidence in 2019 increased compared to 2018, from 0.84 to 0.93 per 1000 population. These facts indicate that some areas in Indonesia are still considered malaria-endemic regions. This is also related to the incomplete control program targeting the *Anopheles* population as the malaria vector in Indonesia.

The number of described *Anopheles* species worldwide is approximately 400 species, and about 70 of them are potential malaria vectors found in several countries (Sinka et al., 2012). Some confirmed *Anopheles* species as malaria vectors are also found in Indonesia. There are 26 *Anopheles* species declared as malaria vectors in Indonesia, including *An. aconitus, An. annularis, An. balabacensis, An. barbumbrosus, An. bancrofti, An. barbirostris, An. farauti, An flavirostris, An. kochi, An. koliensis, An. letifer, An. leucosphyrus,*

An. ludlowae, An. maculatus, An. minimus, An. nigerrimus, An. parangensis, An. peditaeniatus, An. punctulatus, An. sinensis, An. subpictus, An. sundaicus, An. tesselatus, An. umbrosus, dan *An. vagus* (Duver, 2017). These *Anopheles* species are distributed in several provinces in Indonesia, particularly in malaria-endemic areas. The presence of diverse *Anopheles* vectors has driven efforts for vector identification, which is a crucial step in determining vector control strategies. Vector control strategies are considered effective as malaria vaccines are currently unavailable.

Several *Anopheles* species found in malaria-endemic areas have the status of cryptic species and are included in species complexes. Cryptic species are species that have morphological similarities, making them very difficult to differentiate (Oliart et al., 2021). *Anopheles* species complexes exhibit differences in various aspects of their life behaviors, such as vectorial capacity, biting behavior (predominantly on humans, animals, or both), feeding location (indoors or outdoors), and different geographical distributions (Oshaghi et al., 2007). Species identification within *Anopheles* species complexes based on similar morphological characteristics often leads to species misidentification. Additionally, the loss or damage of key morphological identification features in specimen body parts can further complicate the identification process. Morphological identification alone is not sufficient to differentiate *Anopheles* species, especially within species complexes, thus requiring alternative approaches such as molecular species identification. The advancement of molecular biology and genetics technology enables the identification of *Anopheles* mosquitoes using DNA sequences as molecular markers. Commonly used DNA sequences for mosquito species identification are mitochondrial DNA Cytochrome Oxidase Subunit I (mtDNA COI) and ribosomal DNA Internal Transcribed Spacer 2 (rDNA ITS2) (Carter et al., 2019).

Mitochondrial DNA, or mtDNA, is the genetic material inherited maternally and is conservative in nature, not undergoing recombination. This makes it widely used to track the evolutionary rate of an organism (Maulid et al., 2016). The high copy number of mtDNA facilitates DNA isolation from small or degraded specimens (Kusuma et al., 2021). Additionally, mtDNA contains variable regions that can differentiate closely related species (Wirdateti & Semiadi, 2017). The COI gene is part of the mtDNA and serves as a standardized molecular marker for animal identification. It has been extensively used in population studies, phylogeography, speciation, and systematics (Geller et al., 2013). The COI gene molecular marker is a short coding region (about 650 bp) that experiences a high rate of insertions, deletions, and nucleotide substitutions. Therefore, it can differentiate taxa within species complexes (Sari et al., 2021).

Mitochondrial DNA (mtDNA) contains genetic material that is distinct from nuclear DNA (nrDNA). Molecular markers from nuclear DNA, such as ITS, 18S, and 26S, are also widely used for organism identification and phylogenetic analysis. The Internal Transcribed Spacer (ITS) is commonly used for molecular systematic analysis at the species level due to its high variability (Hoggart et al., 2018). In eukaryotic organisms, ITS consists of two regions: ITS-1 and ITS-2. One of the regions frequently used for molecular identification of mosquitoes is the Internal Transcribed Spacer 2 (ITS2) sequence (Sum et al., 2014). The ITS2 sequence is a non-coding region located between the 5.8S ribosomal RNA (rRNA) small subunit coding gene and the 28S rRNA large subunit coding gene (Norris & Norris, 2015). The use of the ITS2 sequence as a molecular marker has advantages, as it is relatively short (around 700 bp) and has multiple copies in the nuclear genome, making it easily amplified using PCR methods (Ekasari et al., 2012). The ITS2 sequence can demonstrate close relationships between species, exhibit high viability at the interspecies level, be conserved at the intraspecies level, and differentiate species complexes (Fang et al., 2017).

The CO1 and ITS2 sequences have been

widely used as molecular markers for DNA barcoding of organisms. However, their specific potential for molecular identification of *Anopheles* species needs to be investigated. The study exploring the potential of CO1 and ITS2 sequences in *Anopheles* species aims to analyze both sequences in silico as appropriate molecular markers for DNA barcoding analysis of *Anopheles*, particularly for the *Anopheles* species found in Indonesia. The results of this research are important as a reference for the application of potential molecular markers for *Anopheles* identification, supporting the determination of effective and efficient strategies for malaria vector control.

METHOD
Sample collection
DNA sequences were collected from the GenBank (NCBI) database using the search feature based on nucleotide with the species name and desired gene specified (e.g., *Anopheles* CO1/ITS). The DNA sequences obtained were then selected based on inclusion and exclusion criteria. The inclusion criteria used to select *Anopheles* DNA sequences were CO1 and ITS2 sequences from *Anopheles* species found and studied in Indonesia. The exclusion criteria included CO1 and ITS2 sequences of *Anopheles* studied in various countries worldwide. The collected sequence data information included accession codes, nucleotide length, whether the sequences were partial or full, and the origin of the analyzed *Anopheles* species, indicating the name of the province or district. These data files were then stored in Microsoft Excel files. The nucleotide base sequence information was saved in Notepad files for further analysis to determine the gene or region for identifying *Anopheles* species through in silico approaches based on COI and ITS2 molecular markers from bioinformatics databases.

Sequence alignment
All DNA sequences stored in Notepad files were collected from NCBI and aligned using the CLUSTALX2 software. This was done to determine the similarities and differences in nucleotide base sequences among the sequences and to identify potential barcode sequences. Barcode potential sequences are those that are different and unique compared to others.

Construction of phylogenetic trees and genetic distances
The aligned DNA sequence data were then used to construct a phylogenetic tree using MEGA XI software with the Neighbour-Joining method and 1000x bootstrapping. The Neighbor-Joining (NJ) method is used to build a phylogenetic tree based on the distances between species (Saitou & Nei, 1987). The confidence values in the NJ phylogenetic tree are determined by the high bootstrap values, typically ranging from 100 to 1000 (Butet et al., 2019). The construction of the phylogenetic tree aims to determine the level of relatedness among *Anopheles* species based on their evolutionary rates. The obtained phylogenetic tree is then analyzed using the Kimura-2 parameter genetic distance method with a 1000x bootstrap. The NJ phylogenetic tree with Kimura's 2-parameter generally represents the topological relationships between taxa (Prasetya et al., 2011). The genetic distance analysis aims to assess the extent of nucleotide differences leading to genetic variation.

RESULTS AND DISCUSSION
Anopheles species complexes that can form sibling species have similarities in morphological characteristics but differ genetically and ecologically in geographic distribution, biology, blood-feeding behavior, host preference, insecticide resistance, and vectorial capacity (Oshaghi et al., 2007). One important aspect of assessing the potential of *Anopheles* mosquitoes as vectors in a particular region is to examine their vectorial capacity. Vectorial capacity refers to the number of individuals effectively capable of transmitting malaria parasites during a blood-feeding process by *Anopheles* mosquitoes per unit of time (12 hours to one full night) from a single source of infection, which is a human infected with malaria (Duarsa, 2008). Factors influencing

the vectorial capacity of *Anopheles* species include the density calculation index, human blood index, gonotrophic cycle, and mosquito lifespan, or duration of infective days (Astuti et al., 2016). Sibling species have different vectorial capacities, which can have implications for control measures, highlighting the need for identification processes (Wahyuni et al., 2018).

Species identification based solely on morphological characteristics is not sufficient. The limitations of morphology-based identification for characterizing *Anopheles* species often lead to identification errors due to the presence of cryptic species. Therefore, molecular identification using a genomic approach called DNA barcoding is necessary. This method can effectively and efficiently identify species by utilizing short DNA sequences called molecular markers (Philips et al., 2018). The molecular markers commonly used for *Anopheles* identification are the CO1 and ITS2 sequences, as they can identify *Anopheles* complexes (Wilai et al., 2020). Based on the advantages of these two sequences, this study compares them to determine potential molecular markers for identifying *Anopheles* species from Indonesia.

The collection of sequences from the NCBI database includes 10 CO1 sequences and 7 ITS2 sequences from several *Anopheles* species that serve as malaria vectors in Indonesia (Table 1). The sequences of several *Anopheles* species belong to two subgenera, *Cellia* and *Anopheles*. These subgenera consist of several species that belong to species complexes, such as *An. subpictus, An. sundaicus,* and *An. vagus,* which are classified under the *Pyretophorus* series; *An. balabacensis, An. kochi, An. farauti* and *An. leucosphyrus,* which are classified under the *Neomyzomyia* series; as well as *An. nigerrimus,* which is classified under the *Myzorhynchus* series (Harbach, 2013). Additionally, outgroup species, namely *Armigeres flavus* and *Armigeres subalbatus*, were used to strengthen the analysis results. Jannah & Rahayu (2019) also mentioned that outgroup species are needed to establish the root or starting point of phylogenetic tree construction.

Based on the alignment results of the DNA sequence samples presented in Figure 1, it can be observed that the majority of nucleotide base sequences of *Anopheles* COI sequences exhibit high homology.

Table 1. *Anopheles* CO1 and ITS2 sequence data at NCBI

Species Name	CO1				ITS2			
	Accession	Length (bp)	Ref	Origin	Accession	Length (bp)	Ref	Origin
Anopheles balabacensis	DQ897941.1	250[P]	Sallum et al., 2007	Salaman, South Kalimantan	KC508611.	549[P]	Widyastuti et al., 2016	Kedondong atas, West Lombok
Anopheles kochi	MK893421.1	658[P]	Jatuwattana et al., 2020	Pucak, South Sulawesi	MT740910.	491[P]	Davidson et al., 2020	Karama, West Sulawesi
Anopheles aconitus	MT753033.1	650[P]	Davidson et al., 2020	Karama, West Sulawesi	OM974187.	515[F]	Hasanah et al., 2022	Bangsring, Banyuwangi
Anopheles subpictus	MT066058.1	658[P]	Wilai et al., 2020	Makassar, South Sulawesi	OM974190.	595[F]	Hasanah et al., 2022	Bangsring, Banyuwangi
Anopheles vagus	MT753036.1	667[P]	Davidson et al., 2020	Karama, West Sulawesi	OM974188.	657[F]	Hasanah et al., 2022	Bangsring, Banyuwangi
Anopheles sundaicus	GQ395880.1	442[P]	Zarowiecki et al., 2014	Tapanuli, North Sumatera	MW603837.	620[P]	Senjarini et al., 2021	Bangsring, Banyuwangi
Anopheles nigerrimus	MT753039.1	652[P]	Davidson et al., 2020	Karama, West Sulawesi	MT740906.	591[F]	Davidson et al., 2020	Karama, West Sulawesi
Anopheles leucosphyrus	DQ897939.1	250[P]	Sallum et al., 2007	Bukit Baru, North Sumatera				

Anopheles farauti	DQ420424.1	258[P]	Foley et al., 2007	Seram, Maluku
Anopheles karwari	MT753034.1	630[P]	Davidson et al., 2020	Karama, West Sulawesi

This indicates that *Anopheles* CO1 sequences have a low mutation rate, resulting in low interspecies variation. CO1 is mitochondrial DNA that is maternally inherited and has relatively stable and conservative characteristics, leading to a low mutation rate and low genetic variation (Da Fonseska et al., 2008; Dailami et al., 2016).

Additionally, nucleotide differences can also be a sign of some genetic variations. This suggests that *Anopheles* CO1 sequences have the potential to be used as species markers (barcodes) for *Anopheles* in Indonesia. According to Jannah & Rahayu (2019), an important criterion for determining whether a sequence can be used as a molecular marker or DNA barcode is the presence of nucleotide base characters that are "unique" or automorphic, meaning they are only found in specific species and can be used to differentiate them from other species. The alignment results of *Anopheles* CO1 sequences can be seen in Figure 1.
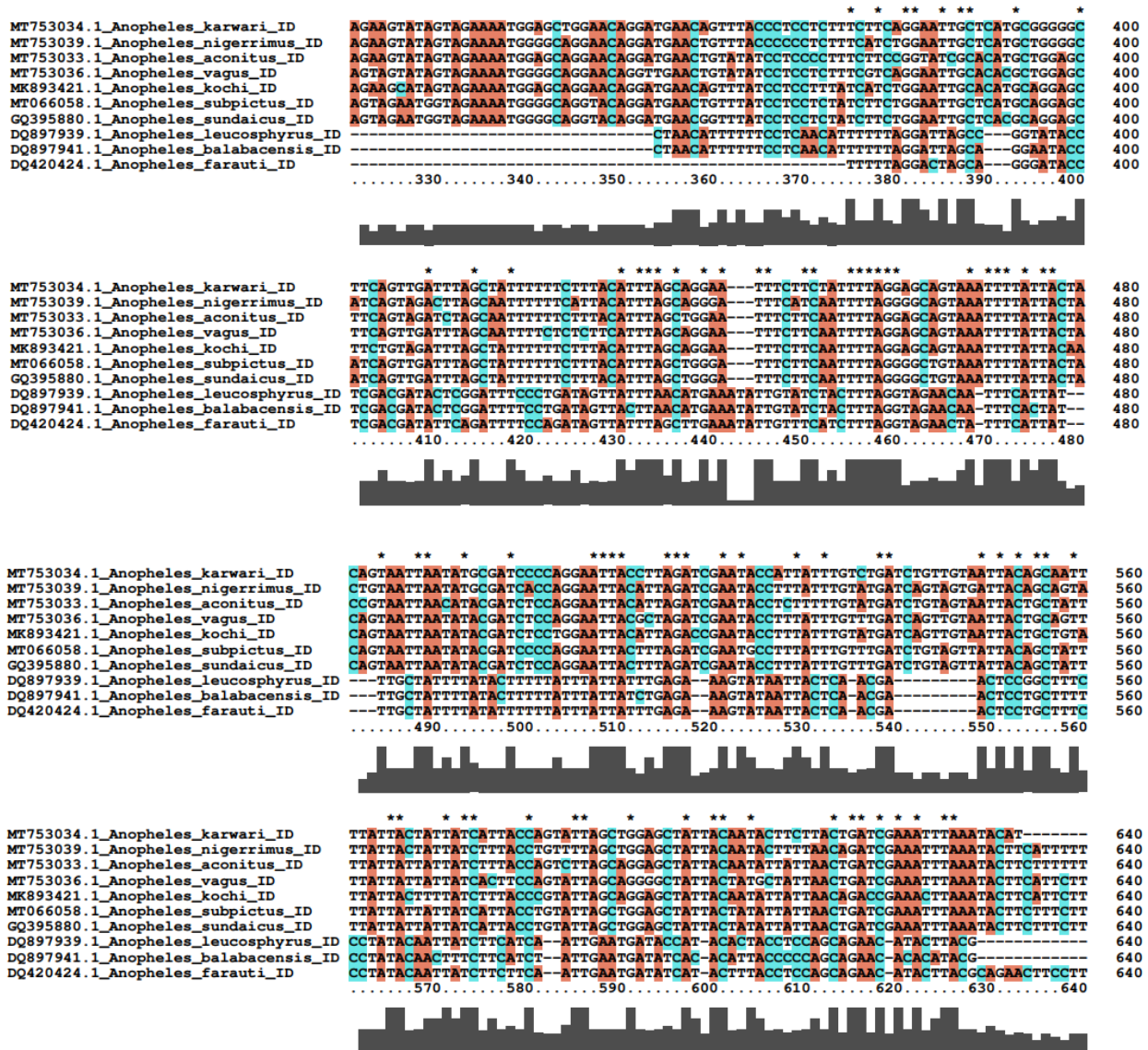

Figure 1.   Alignment of *Anopheles* CO1 sequences, the sign (*) indicates homology, gaps indicate deletions or insertions. Each color indicates a specific nitrogenous base.

Three *Anopheles* species, namely *An. leucosphyrus*, *An. balabacensis*, and *An. farauti*, consistently exhibit several genetic variations indicated by nucleotide sequence differences when compared to other *Anopheles* species. The nucleotide differences are found at positions 390-392, 442-444, 469, 479-483, 518-519, 535, 540-548, 582-582, 599 and 619 in the sequence. Based on the complex species classification of *Anopheles*, these three species belong to the *Neomyzomyia* series (Harbach, 2013). The presence of these nucleotide variations can be interpreted as unique characters that can be used as molecular markers (barcodes) to identify *Anopheles* mosquitoes at the *Neomyzomyia* series level, particularly for molecular identification of the three mentioned species, *An. leucosphyrus*, *An. balabacensis*, and *An. farauti*.
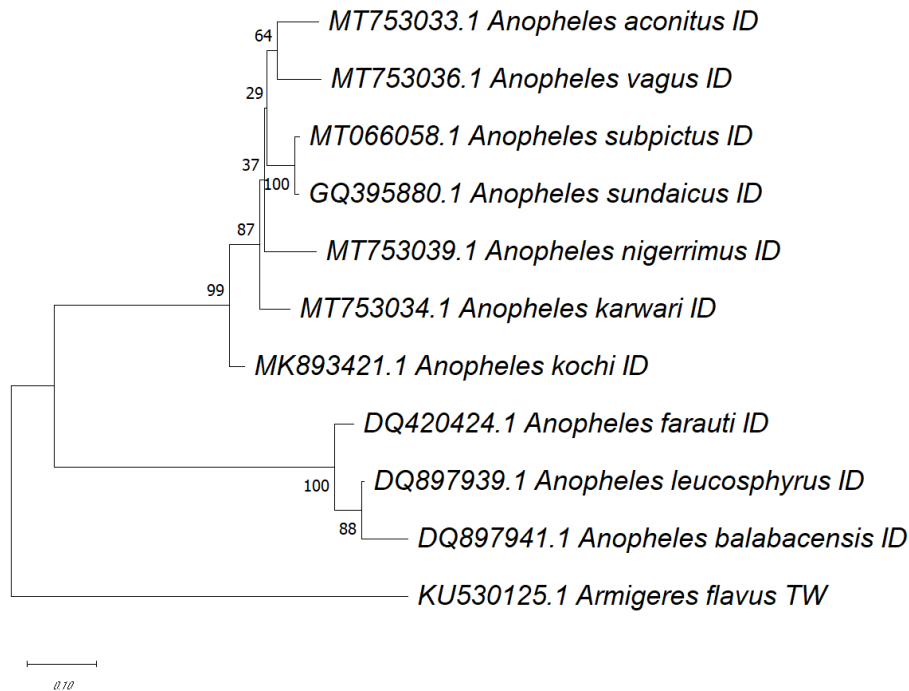

Figure 2. Phylogenetic tree based on *Anopheles* COI sequences using the Neighbor Joining method with bootstrap 1000x.

The phylogenetic tree consists of several branches accompanied by bootstrap values that indicate the relationship between species. Bootstrap represents the total repetition of the phylogenetic tree arrangement conducted randomly, considering the similarity of nucleotide sequence orders, which can be used as a reference to measure the validity of a phylogenetic tree result (Lemoine et al., 2018). If the bootstrap value is above 70%, the phylogenetic tree is considered accurate (Subari et al., 2021). A high bootstrap value can be interpreted as indicating the validity and stability of a phylogenetic tree (Saleky et al., 2020). The construction of the phylogenetic tree based on *Anopheles* CO1 sequences in Figure 2 shows the formation of several branches resulting from clustering using the Neighbor-Joining method based on genetic distances between similar parts of the nucleotide sequence (Rizal et al., 2020). The branches formed indicate closely related species or those belonging to the same series, such as *An. subpictus* and *An. sundaicus,* which are classified under the *Pyretophorus* series. Both species have morphological similarities in the palpus and proboscis (Sindhania et al., 2020). Additionally, *An sundaicus* and *An. subpictus* are closely related due to their preference for the same habitat (Cooper et al., 2010). Other species that form a branch are *An. farauti*, *An. leucosphyrus* and *An. balabacensis,* which belong to the *Neomyzomyia* series. This is consistent with the statement by Anzani et al. (2021) that closely related species will be grouped into the same

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branch. Furthermore, the construction of the phylogenetic tree also shows the formation of a specific clade containing *An. leucosphyrus*, *An. balabacensis*, and *An. farauti*. These three species form a separate clade because the sequence alignment reveals their long similarities and nucleotide sequence orders, which sets them apart from other species. This corresponds to the statement by Rizal et al., (2020) that the length similarity of sequences will affect the construction of the phylogenetic tree.

Table 2. Genetic range of *Anopheles* CO1 sequences

Species Group	An. leucosphyrus	An. balabacensis	An. kochi	An. farauti	An. aconitus	An. subpictus	An. vagus	An. sundaicus	An. karwari	An. nigerrimus
An. leucosphyrus		7,17%	74,21%	8,80%	80,88%	83,83%	82,02%	81,19%	72,65%	77,58%
An. balabacensis	0,0717		78,66%	11,86%	87,79%	89,43%	89,04%	86,57%	79,62%	86,57%
An. kochi	0,7421	0,7866		74,05%	12,80%	13,23%	14,13%	12,80%	14,12%	13,68%
An. farauti	0,0880	0,1186	0,7405		80,19%	81,78%	77,47%	78,65%	76,00%	84,56%
An. aconitus	0,8088	0,8779	0,1280	0,8019		11,93%	12,39%	11,93%	14,12%	15,08%
An. subpictus	0,8383	0,8943	0,1323	0,8178	0,1193		13,24%	1,49%	10,66%	12,36%
An. vagus	0,8202	0,8904	0,1413	0,7747	0,1239	0,1324		12,36%	12,82%	16,40%
An. sundaicus	0,8119	0,8657	0,1280	0,7865	0,1193	0,0149	0,1236		11,10%	12,80%
An. karwari	0,7265	0,7962	0,1412	0,7600	0,1412	0,1066	0,1282	0,1110		11,94%
An. nigerrimus	0,7758	0,8657	0,1368	0,8456	0,1508	0,1236	0,1640	0,1280	0,1194	

Based on the genetic distance values of CO1 sequences among *Anopheles* species in Table 2, the smallest value is 1.49% between *An. subpictus* and *An. sundaicus*, while the largest value is 89.43% between *An. balabacensis* and *An. subpictus*. The percentage of genetic distance represents the status of the same or different species (Sahara et al., 2018), grouping, the proximity of each individual within a population, and nucleotide differences (Abinawanto et al., 2021). According to Weeraratne et al. (2018), a range of genetic distance values below 3% is interpreted as the same species, while species are considered different if the genetic distance value is above 3%. Based on this, the genetic distance of CO1 sequences among *Anopheles* species indicates that each sample represents a different species. Furthermore, the high genetic distance in each sample also represents the significant nucleotide differences among *Anopheles* species.

The alignment results of the ITS2 marker sequences in Figure 3 show the presence of unique sequences that have the potential to be barcodes, particularly in species such as *An. nigerrimus*, *An. aconitus, An. kochi, An. balabacensis*, and *An. vagus*, as they differ from other species. This is evident in the sequence positions 69-71, 100-103, 110-113, 209-213, 289, 298, 319-321, 346, and 382 in *An. nigerrimus*; positions 110-113, 158-162, 298, 319-321, 346, and 411 in *An. balabacensis*; positions 179-188 and 247-252 in *An. aconitus*; and positions 222-223, 234, and 325-328 in *An. kochi*. The alignment results of the ITS2 marker sequences demonstrate greater genetic variation among *Anopheles* species compared to the alignment of the CO1 marker. This suggests that ITS2 has a faster mutation rate than the CO1 sequences (Batovska et al., 2017). The ITS2 sequence is a nuclear locus that undergoes frequent cross-overs, resulting in recombinants with significant genetic variation (Perwitasari et al., 2020). Based on this, the abundant genetic variation in the ITS2 sequences has the potential to detect the emergence of new taxa (Fang et al., 2017). The use of the ITS2 molecular marker in several studies has shown its ability to differentiate members of several cryptic species from the *Anopheles* complex found in the Asian region, even at the group level (Brosseau et al., 2019; Hempolchom et al., 2013).

The construction of the phylogenetic tree based on ITS2 sequences shows bootstrap values ranging from 32% to 100% (Figure 4). The branches indicate the formation of a clade between *An. subpictus* and *An. sundaicus*, indicating their close relationship. These two species also form a clade with *An. vagus*. This is because these three species are sibling species within the *Pyretophorus* series. *An. balabancensis* and *An. kochi* also belong to the same clade. According to (Harbach, 2013) *An. kochi* and *An. balabancensis* belong to the *Neomyzomyia* series (Foley et al., 1998).
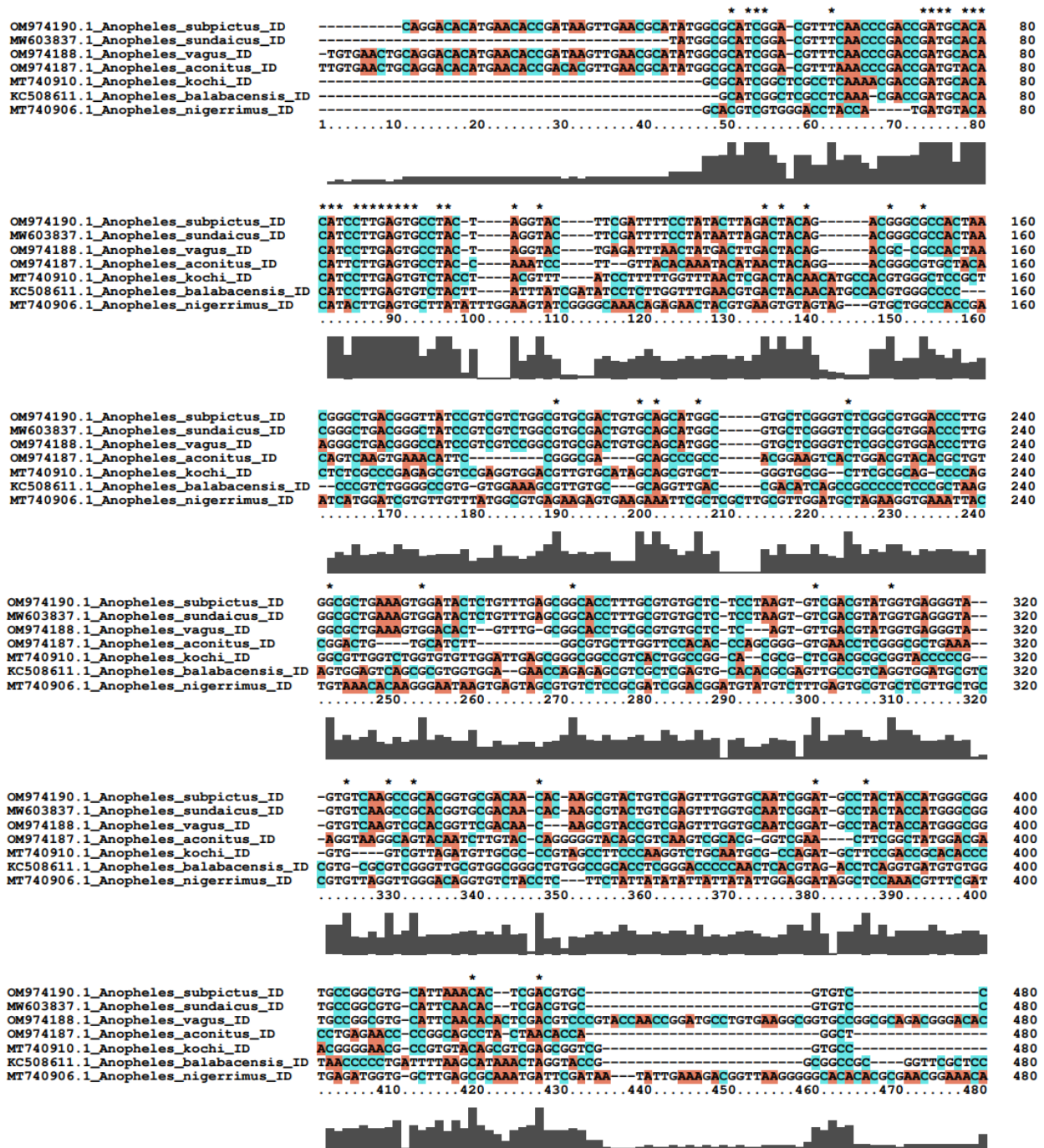
Figure 3.   Alignment of *Anopheles* ITS2 sequences, the sign (*) indicates homology, gaps indicate deletions or insertions. Each color indicates a specific nitrogenous base.

The data on genetic distance values between *Anopheles* species based on ITS2 sequences in Table 3 show that the smallest value is 1.61% between *An. subpictus* and *An. sundaicus*, while the largest value is 193.65% between *An. vagus* and *An. nigerrimus*. Based on this, the genetic distance value of ITS2 sequences between *An. subpictus* and *An. sundaicus* is less than 3%, indicating that these two species are likely sibling species. According to references describing the identification key of *Anopheles* (Harbach, 2013), they are sibling species within the *Pyretophorus* series. Furthermore, the genetic distance in other ITS2 sequence samples shows very large values, indicating that they represent different species. A comparison between the ITS2 and CO1 sequences reveals that each analyzed *Anopheles* sample shows higher genetic distance values in the ITS2

sequence. This indicates that the nucleotide differences in the ITS2 sequences of *Anopheles* are relatively high, resulting in increased genetic variation (Muhajirah et al., 2021). However, the results also show that the genetic distance between *An. subpictus* and *An. sundaicus* is higher in CO1. This is because *An. sundaicus* and *An. subpictus* have a very close relationship due to sharing the same habitat (Cooper et al., 2010). Both species have morphological characteristics and belong to the *Pyretophorus* series, subgenus *Cellia*. They exhibit morphological similarities, particularly in the palpus and proboscis regions (Sindhania et al., 2020)*.
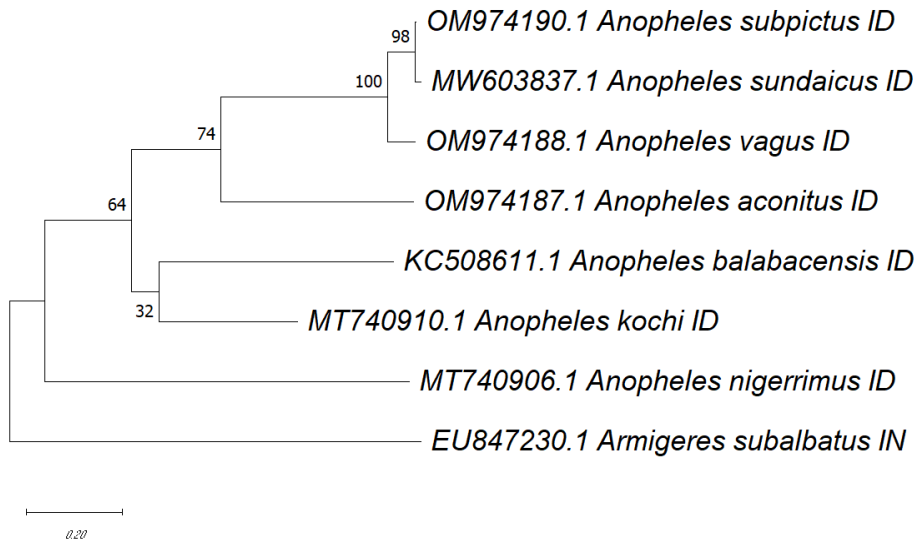

Figure 4. Phylogenetic tree based on the Anopheles ITS2 sequence using the Neighbor Joining method with bootstrap 1000x.

The high genetic variation in ITS2 can occur because it is a non-coding region that has a higher mutation rate compared to coding regions. In the ITS region, genetic changes such as mutations frequently occur, leading to different genetic variations among species (Hartati et al., 2021). On the other hand, genes with low variation tend to be conservative due to a slow evolutionary rate, resulting in the conservation of nucleotide sites in subsequent generations (Bachry et al., 2020).

Based on these genetic distance results, it can be inferred that ITS2 sequences have higher potential as barcodes for *Anopheles* species due to their high genetic variation. A high genetic distance indicates a greater evolutionary divergence or rate between sequence pairs. Conversely, low genetic distance indicates a closer relationship (Windasari et al., 2022). Additionally, CO1 is also recommended as a molecular marker for identifying *Anopheles* species because CO1 is a maternal mitochondrial DNA that can track the evolutionary rate of an organism (Beebe, 2018).

Table 3. Genetic range of *Anopheles* ITS2 sequences

Species Group	An. balabacensis	An. kochi	An. aconitus	An. subpictus	An. vagus	An. sundaicus	An. nigerrimus
An. balabacensis		78,29%	123,76%	93,17%	96,55%	91,66%	155,06%
An. kochi	0,7829		99,54%	102,85%	97,39%	103,67%	113,28%
An. aconitus	1,2376	0,9954		83,61%	79,33%	84,70%	141,22%
An. subpictus	0,9317	1,0285	0,8361		12,61%	1,61%	182,55%
An. vagus	0,9655	0,9739	0,7933	0,1261		12,13%	193,65%
An. sundaicus	0,9166	1,0367	0,8470	0,0161	0,1213		184,06%
An. nigerrimus	1,5506	1,1328	1,4122	1,8255	1,9365	1,8406	

Genetic diversity and environmental factors also influence species variation due to the interaction between genes and the environment. Individuals with the same genotype may not necessarily have the same phenotypic characteristics. Climate and weather, air humidity, light intensity, wind speed, environmental temperature, and altitude in a

living organism's habitat are just a few examples of the environmental factors that can affect phenotypic traits (morphology) (Handayani & Ismadi, 2017).

CONCLUSION

The ITS2 sequence used as a molecular marker for species identification in *Anopheles* exhibits higher genetic variation compared to CO1. This is because the ITS2 sequence is located in the nuclear genome and undergoes frequent recombination, resulting in genetic recombination. ITS2 sequences also have a faster mutation rate compared to CO1 sequences, leading to greater genetic variation. On the other hand, CO1 sequences represent mitochondrial DNA, which is maternally inherited and therefore more conservative. ITS2 has been proven effective in distinguishing cryptic species within the *Anopheles* complex group. Therefore, the ITS2 marker has the potential to serve as a barcode for *Anopheles* species from Indonesia.

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