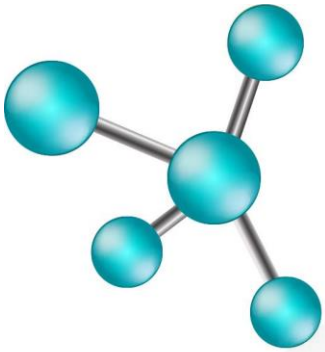

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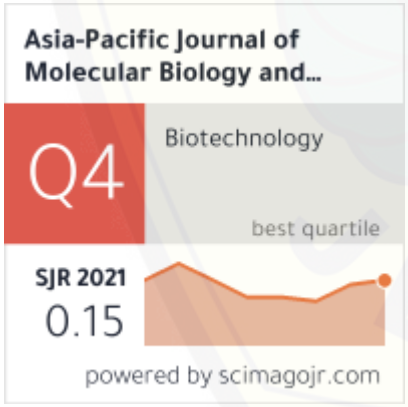

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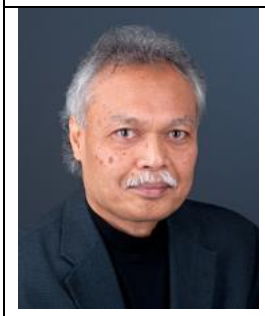	Dr. Teruna J. Siahaan is the Aya & Takeru Higuchi Distinguished Professor of Pharmaceutical Chemistry at The University of Kansas (KU) and a Fellow of the American Association of Pharmaceutical Scientists (AAPS). His research is focused on improving drug delivery to the brain and immune cells; he published 200 papers, obtained 12 patents, and edited two books. He serves as the Director of Global Health Education & Research Center, School of Pharmacy, KU; Co-Program Director of the T32 NIH Biotechnology Training Program, KU; and the Executive Board of Directors the Globalization Pharmaceutics Education Network (GPEN) Organization. He has received several honors and awards, including Self Faculty Scholar, KU; Pfizer Research Scholar Award; 2013 Mentor of the Year, KU; and 2014 PhRMA Foundation Award for Excellence in Pharmaceutics.
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	Professor Mohd Ali Hassan obtained his degree in Chemical Engineering at The University of Leeds, United Kingdom and PhD in Environmental Biotechnology from University of Okayama, Japan. He has more than 30 years of teaching and research experience. In 2002 he was promoted to the post of Professor at University Putra Malaysia (UPM) in the field of Environmental Biotechnology. He has worked extensively on international biomass, bioenergy and zero-emission projects throughout his career, particularly with Japan and Korea, in collaboration with the palm oil industry in Malaysia. He has succeeded in obtaining R&D funding from local and international agencies and companies. He managed to set up the Serdang Biomass Town and Biorefinery in UPM campus, since 2012. He heads an international SATREPS project on biomass and zero emission project at the palm oil mill funded by JICA-JST (Japan) and Ministry of Higher Education Malaysia. He was Dean, Faculty of Biotechnology, UPM from 2007-2014. Now he heads the Industry and Community Relations Unit at The Faculty of Biotechnology and Biomolecular Sciences UPM. He has published more than 180 journal papers, has several patents, book chapters, national and international awards. He has completed 10 research projects, with 2 commercialised products, and has supervised and graduated many postgraduate and undergraduate students. He was awarded the Top Research Scientist of Malaysia by the Academy of Sciences Malaysia in 2013, and Fellow of the Academy of Sciences Malaysia in 2016. He has also received the Research Exchange Award from the Korean Society for Biotechnology and Bioengineering, the Malaysia Research Star Award 2017 and the Malaysian Microbiology Award 2017. Currently he is Vice President of Asia Federation of Biotechnology, President of Asia Federation of Biotechnology Malaysia Chapter and a Committee Member of Biomass Asia Association. His current h index (Scopus) is 31, with more than 3000 citations.
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	Margaret Duffy is a Research Fellow of the Kay Kendall Leukaemia Fund, pursuing research into haematological malignancies with a focus on multiple myeloma. Margaret achieved a 1st class honours degree in Biomedical Science from the National University of Ireland Galway, before completing a PhD at the University of Glasgow. Her PhD studies centred around viral:host interactions and improving the stability of adenoviral vectors in blood. Her subsequent postdoctoral research was focused on developing adenoviruses as tools for gene therapy, vaccination and oncolytic applications. Margaret also spent time working as a Marie Curie IAPP researcher at a biotech company in the Netherlands, specialising in adenovirus vectorisation and production. Now based at the Department of Oncology University

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Internal transcribed spacer 2 (ITS2) based molecular identification of malaria vectors from Bangsring Banyuwangi-Indonesia

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Abstract. Since the malaria outbreak in 2011, the breeding place of *Anopheles* in Bangsring Village on Banyuwangi District has been monitored by District Public Health Office as part of a vector surveillance program. Morphological identification is still a standard tool to observe *Anopheles* occurrence and diversity, but the presence of cryptic species made it unreliable. In this study, a molecular approach called DNA barcoding technique was used to assist the morphology-based techniques to identify *Anopheles* species found in Bangsring. The internal transcribed spacer 2 (ITS2) sequence was used as molecular marker. Based on the morphological features, we were able to identify *Anopheles* (*An.*) *vagus, An. subpictus, An. sundaicus and An. aconitus*. ITS2 sequences from the four identified species were then analyzed simultaneously with eighteen reference sequences from NCBI which had a high similarity of 98-100%. The NJ phylogenetic tree formed three major clades, where the two clades as monophyletic clades were *An. vagus* and *An. aconitus*. Another clade was formed as polyphyletic clade containing *An. subpictus* and *An. sundaicus*. Although *An. subpictus* and *An. sundaicus* were placed in the same clade, seven nucleotide differences were observed in their ITS2 sequence. The intra-specific variation of those two species was 0.08 and 0.49%, respectively, while the interspecific variation was 1.39%. Interspecific variation which was higher than the mean intra-specific variation might indicate that *An. sundaicus* and *An. subpictus* were a distantly species. However, the value of interspecific variation lower than 3% might also indicate that those species were classified as a complex species. All ITS2 sequences from morphologically identified species had similar results with molecular-based techniques. This result showed that molecular identification using the ITS2 sequence was reliable in supporting morphological identification among closely related anopheline mosquitoes and gave further information about their evolutionary divergence.

Keywords: malaria, vector, *Anopheles* sp., ITS2, DNA barcoding

INTRODUCTION

In 2020, Indonesia was still categorized as the second malaria-endemic country in South-East Asia (WHO 2020). Indonesian Health Ministry reported increasing Annual Parasite Incidence (API) values in Indonesia from 0.84 in 2018 to 0.93 in 2019 (Kemenkes RI, 2019). Bangsring village is a part of Banyuwangi district located in East Java, which was identified as an endemic area

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after the malaria outbreak in 2011 (Dinas Kesehatan Kabupaten Banyuwangi 2019). Bangsring village is a typical coastal area, where the dominant anopheline mosquitoes breeding place is lagoon (brackish water). *An. sundaicus* is the most common *Anopheles* larvae found in Bangsring lagoon (Ndoen *et al.,* 2010). Several lagoons detected as *Anopheles* breeding places were monitored by District Public Health Office to prevent the risk of the re-emergence of malaria in Bangsring village, Banyuwangi, Indonesia.

Anopheles is a genus of mosquito that plays an essential role in malaria transmission (Molina-Cruz *et al.,* 2016). Compared to other genera, *Anopheles* is the most morphologically diverse, which approximately 100 *Anopheles* species reported as a malaria vector worldwide (Sinka *et al.,* 2011). The members of species complexes are quite difficult to distinguish morphologically (Bickford *et al.,* 2007). The highly morphological diversity also causes misidentification of *Anopheles* in malaria-endemic areas and causes bias in vector control data (Singh *et al.,* 2010). Researchers reported difficulties in distinguishing some *Anopheles* species with high morphological similarity. Those species are then grouped into species complexes such as *An. sundaicus* and *An. minimus* complexes (Surendran *et al.,* 2010; Blažejová et al. 2018; Chatpiyaphat et al. 2020). Malaria Annual Report in 2007 stated that there were about 13 species complexes in Southeast Asia, including Annularis, Barbirostris, Culifacies, Fluvialis, Leucosphyrus, Maculatus, Minimus, Phippinensis-Nivipes, Punctulatus, Sinensis, Subpictus, Sundaicus (WHO, 2007). Geographic isolation, pre-and post-mating barrier resulting in reproductive isolation are the main factors influencing the existence of species complexes (Sinka, 2013; Sum *et al.,* 2014; Lobo *et al.,* 2015; Barrón *et al.* 2019), and the presence of new species will occur if it takes over a long time (Fouet *et al.,* 2017).

The existence of *Anopheles* species complex is commonly detected by the polytene chromosome structure. The different banding pattern of X arm in the polytene chromosome was used to investigate and reveal the diversity of morphological features within *Anopheles* species complexes (Ramírez & Dessen, 2000). The cross-mating experiment is also an acceptable analysis to assess the existence of *Anopheles* species complexes (Jayatunga *et al.,* 2021). However, due to labor-intensive analysis in polytene chromosome structure, which is also limited to the certain mosquito life stage (instar IV larvae), the low success rate of the cross-mating experiment (Lanzaro & Lee, 2013; Zheng, 2020), DNA barcoding is nowadays widely used and becoming a more accessible tool in applying advanced molecular biotechnology to detect the existence of *Anopheles* species complexes (Beebe, 2018).

Using specific DNA sequences (DNA barcoding), organisms identity, and their genetic diversity can be analyzed (Meier *et al.,* 2008). This technique is considered more effective and efficient because these DNA sequences provide biological information that is easy to use to distinguish organismal entities and answer how a species' evolutionary process separated it from its ancestors (Beebe, 2018). The most commonly used DNA barcode for mosquito identification is internal transcribed spacer 2 (ITS2). ITS2 sequence is part of the ribosomal DNA in the nuclear genome (Zhang *et al.,* 2020; Senjarini *et al.,* 2021). This sequence is considered adequate to distinguish different species as well as members of the species complex (Barrón *et al.* 2019; Syafruddinid *et al.,* 2020; Senjarini *et al.,* 2021b). The most widely used method for eliminating and eradicating malaria cases worldwide is vector surveillance. The accuracy of the vector identification process is a crucial step in the pre- and post-vector control process in a malaria-endemic area. Therefore, the objective of this study was to do morphological and ITS2-based molecular characterization of potential malaria mosquito vectors from Bangsring village, Banyuwangi.

MATERIALS AND METHODS

Study site, mosquito collection, and morphological identification

This research was conducted from June 2020 – June 2021. Adult female mosquitoes were collected three times between August - December 2020 in Bangsring Village, Banyuwangi District, Indonesia (-8.1403261, 113.7163172), particularly in the coastal area. Four lagoons were detected as

the breeding site of *Anopheles* larvae. Adult female mosquitoes were collected using the human catch landing method. The collected mosquitoes were then identified using *Anopheles* morphological identification key (Reid, 1968) and were separated into different tubes.

DNA extraction, PCR, and sequencing

Genomic DNA from single body of mosquito of each species was extracted using the salting-out extraction method using several solutions such as a homogenizing buffer (Tris-Cl 10 mM, EDTA 2 mM, NaCl 0,4 M), SDS 20%, 20 mg/ml proteinase-K (Thermo Fisher, USA), NaCl 6 M, isopropanol (Sigma, Germany), ethanol 70% (Aljanabi *et al.,* 1997). The extracted DNA genome was then eluted in nuclease-free water and visualized in 0.8% agarose (Nacalai, USA) in TAE buffer (242 g Tris, 57.1 ml acetic acid glacial, 0.5M EDTA) and ethidium bromide staining (Bio-Rad, USA). The successfully extracted DNA genome was then used as a PCR template. The paired ITS2 universal primer (forward 5'TGT GAA CTG CAG GAC ACA T 3' and reverse 5' TAT GCT TAA ATT CAG GGG GT 3') was used to amplify the ITS2 sequence. These primers were designed by Beebe & Saul (1995). Each reaction was conducted in 25 µl of total volume, which contained 12.5 µl of 2X PCR Mastermix (GoTaq, USA), 0.25 µM of each primer (final concentration), 10.25 µl nuclease-free water, and 1 µl of DNA template. PCR conditions were as follows: 95 °C for 5 min, 5 cycles of 94 °C for 30 s, 45 °C for 40 s, and 72 °C for 45 s, followed by 30 cycles of 94 °C for 30 s, 51 °C for 40 s and 72 °C for 45 s. This process was ended with the final extension step of 72 °C for 10 min. We performed PCR reactions for 25 individuals from both *An. vagus* and *An. sundaicus*, 24 individuals *An. subpictus* and 4 individuals *An. aconitus*. Through PCR, we expected the amplicon size was 700 bp for *An. vagus*, 600 bp for *An. sundaicus*, 650 bp for *An. subpictus* and 500 bp for *An. aconitus*. PCR product was then visualized in 1.5% agarose gel stained with ethidium bromide. PCR products showing clear positive bands were purified using Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) following the manufacturer's protocol. The purified PCR product was then sequenced using the Sanger method conducted in First Base Singapore.

DNA sequence analysis

The chromatograms of the ITS2 sequences were manually edited using BioEdit Software. Contig sequences were aligned using ClustalW in BioEdit software. The contig sequence of each identified species was then confirmed by comparing publicly available sequence data in NCBI using Basic Local Alignment Search Tool (BLAST). Some ITS2 sequences which have high similarity with ITS2 sequences in this study were collected and then analyzed as sequence references, including *An. aconitus* (Acc. Nr. MN203097, AY547361, GQ500119, AF230463, KY000680), *An. subpictus* (GQ870327, MT068436, KJ437450, MW078487), *An. sundaicus* (GQ284825, AY768541, MW078490, MT623070), and *An. vagus* (MN148590, HQ873039, MT623075, FJ654649, EU919718). Two closely related species of *An. aconitus*, namely *An. flavirostris* (AY943666) and *An. minimus* (KP298408) and a closely related species of the *Pyretophorus* series, *An. indefinitus* (GQ870332) were also used to construct phylogenetic tree, while *An. barbirostris* (AB435992) was selected as the outgroup. The nucleotide composition of all ITS2 sequences was analyzed using MEGA 11 software. The ITS2 sequence analysis, such as the interspecific variation of ITS2 sequences were analyzed using the Kimura-2 Parameter model. The phylogenetic tree was constructed using the Neighbour-Joining (NJ) model with Kimura-2 Parameter distance and statistically supported by bootstrapping of 1000 following the research conducted by Zomuanpuii *et al.* (2013).

RESULTS

Morphological characteristics

According to the morphological identification, the collected mosquitoes from Bangsring village belong to four species, including *An. subpictus, An. sundaicus, An. vagus* and *An. aconitus,* which was divided into two series of subgenus *Cellia*, i.e. *Pyretophorus*, and *Myzomyia*. The total of collected mosquitoes were 493 individuals during three times of mosquito collections. *An. vagus* was the most dominating species (240) and followed by *An. sundaicus* (225), *An. subpictus* (24), but only 4 individuals of *An. aconitus* that were collected

during the sampling period. Three species were classified into *Pyretophorus* series, including *An. subpictus, An. sundaicus* and *An. vagus,* while *An. aconitus* was classified into *Myzomyia* series. Species belonging to the *Pyretophorus* series were quite difficult to differentiate because the differences between those species were slight and complicated by geographical variation (Reid, 1968). *An. aconitus* was easy to be distinguished from the other three species and belonged to a different series.

As shown in Figure 1A, *An. vagus* was characterized by the presence of a pale band on its proboscis, which was not detected in the other two species belonging to *Pyretophorus* series (Figure 1D for *An. sundaicus* and 1G for *An. subpictus*). Based on their morphological characters, *An. sundaicus* and *An. subpictus* were not easy to be distinguished. The palpi characters of those two species were slightly different. The apical pale band of *An. sundaicus* were longer than *An. subpictus*. Speckled leg and a pale fringe spot between vein 5.2 and 6 of the wing venation were the keys to differentiating them. *An. sundaicus* had speckled leg (Figure 1F), while it was absent in *An. vagus* and *An. subpictus* (Figure 1C and 1I, respectively). The pale fringe spot between vein 5.2 and 6 of the wing venation was present in *An. vagus* and *An. subpictus* (Figure 1B and 1H, respectively), while it was not detected in *An. sundaicus* (Figure 1E).

Compared to the other three species in this study, *An. aconitus* was the easiest species to be identified. It had a unique character on its proboscis with the presence of a pale band like in *An. vagus*. However, the pale band of *An. aconitus* on its proboscis was broader, almost half of proboscis length compared to *An. vagus* (Figure 1J). The subapical and apical pale bands on its palpi showed the same length. Leg of *An. aconitus* was not speckled (Figure 1L).

ITS2 sequences analysis

We expected to yield amplicon from all PCR reactions as follows i.e. 700 bp for *An. vagus*, 600 bp for *An. sundaicus*, 650 bp for *An. subpictus* and 500 bp for *An. aconitus*. After sequencing, the length of ITS2 sequence amplicons of four morphologically identified anopheline species varied from 701 bp in *An. vagus,* 524 bp in *An. sundaicus,* 595 bp in *An. subpictus* and 515 bp in *An. aconitus*. Original sequences of the ITS2 region have been deposited on the GeneBank (NCBI) database under the following accession numbers: OM974187 for *An. aconitus*, OM974188 for *An. vagus*, OM974189 for *An. sundaicus,* and OM974190 for *An. subpictus*. Contig sequences of four *Anopheles* species from Bangsring were then compared with other sequences using the BLAST search tool. The results showed those sequences were also identified as *An. vagus*, *An. sundaicus, An. subpictus, and An. aconitus*.

Molecular characteristics based on the ITS2 sequence were analyzed, including GC content and molecular distance among them. Based on 492 site positions, the GC content of *An. vagus, An. sundaicus, An. subpictus* and *An. aconitus* in this study were 58%, 58%, 57.5% and 55.7% respectively (Table 1). ITS2 of *An. vagus* had the longest sequence and the highest GC content. The molecular distances within (intraspecific variation) and between (interspecific variation) species were analyzed using the Kimura-2 parameter model. The intraspecific variation of the four species found in this study compared to the reference sequences from the NCBI had a value ranged of 0.08-0.95% (Table 2). Intraspecific variation of *An. aconitus* was the highest, while *An. sundaicus* was the lowest. Interspecific variation was also analyzed to estimate evolutionary divergence between species. The interspecific variation of the four species and closely related species ranged from 0.81 to 82.18% (Table 3). The value of the closest molecular distance was between *An. subpictus* and *An. indefinitus*, while the furthest is between *An. subpictus* and *An. aconitus*. Low values of interspecific variation were also observed between *An. sundaicus* and *An. subpictus* by 1.39%. For deeper investigation, we aligned the ITS2 sequence *of An. sundaicus* and *An. subpictus* from Bangsring separately from other sequences. Seven nucleotide differences were observed including one indel (insertion-deletion), 3 transversion and 3 transition (Figure 2).

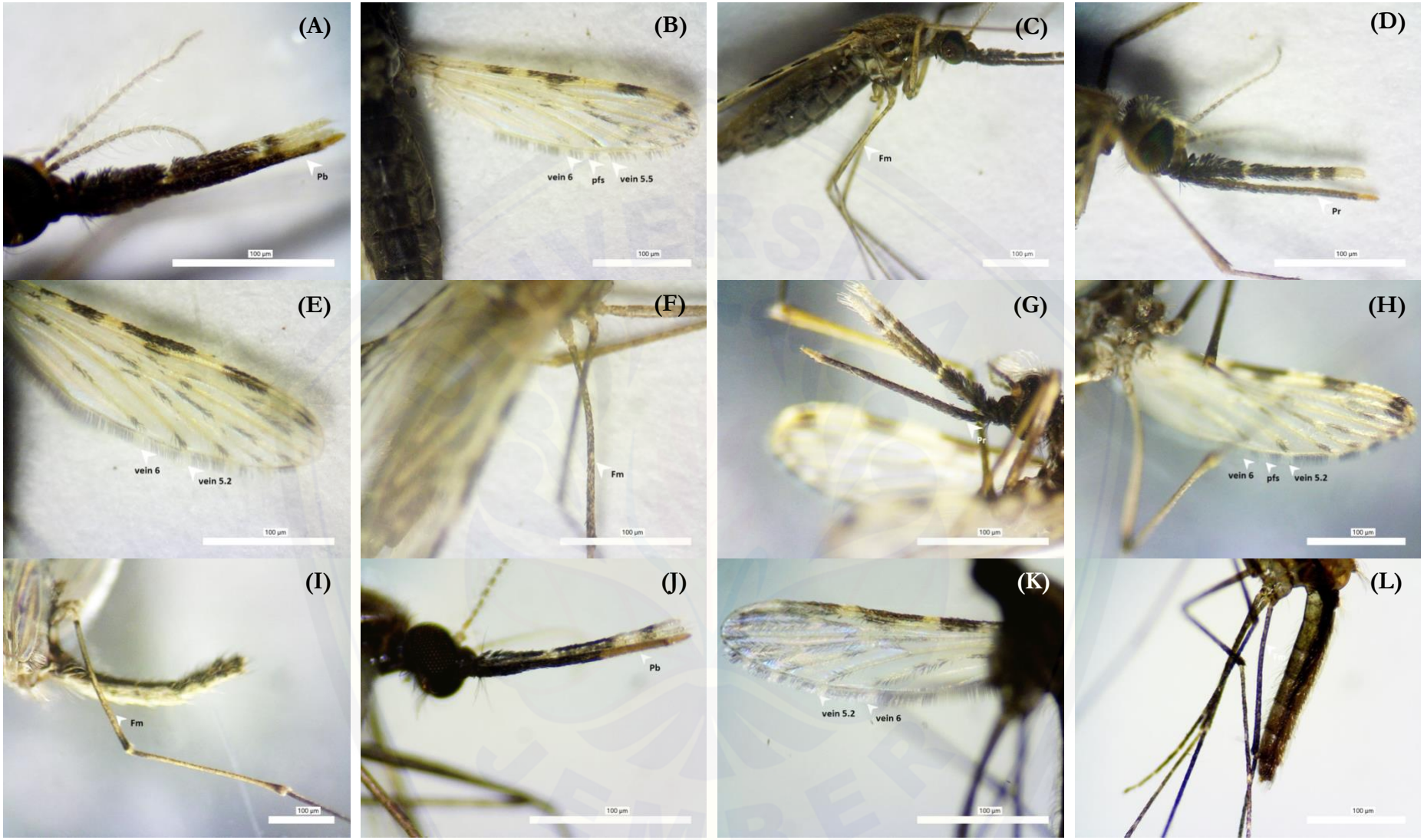

Figure 1. Morphological characteristics of *An. vagus* (A-C), *An. sundaicus* (D-F), *An. subpictus* (G-I), and *An. aconitus* (J-L); *An. vagus* and *An. aconitus* have a pale band on its proboscis (A and D, respectively), while being absent in *An. sundaicus* and *An. subpictus* (D and G, respectively; *An. vagus* and *An. subpictus* have a pale fringe spot on the wing margin between veins 5.2 and 6 (B and H, respectively), while there is an absence in *An. sundaicus* and *An. aconitus* (E and K, respectively). *An. sundaicus* is the only species with speckled leg (F), while others are fully dark (C, I, and L). Pale band (pb); proboscis (pr); pale fringe spot (pfs); femora (fm).

Table 1. Nucleotide composition of ITS2 sequences for each species.

Species name	T (U)	C	A	G	%GC	Total nucleotide for alignment analysis
An. vagus	22.8	25.4	19.2	32.6	58,0	448
An. sundaicus	23.5	27.3	18.6	30.7	58,0	469
An. subpictus	23.9	26.7	18.6	30.8	57,5	468
An. aconitus	20.3	29.3	24.0	26.4	55,7	458

Note: Total of nucleotides presented in this table were after alignment analysis, out of 492 site positions.
All frequencies are given in %, Thymine (T); Uracil (U); Cytosine (C); Adenine (A); Guanine (G).

Table 2. Estimates of average evolutionary divergence over sequence pairs within groups (intraspecific variation).

Species group	Mean distance	Distance (%)
An. vagus	0.0027	0.27%
An. subpictus	0.0049	0.49%
An. sundaicus	0.0008	0.08%
An. aconitus	0.0095	0.95%

Note: The number of base substitutions per site from averaging over all sequence pairs within each group is shown. Analyses were conducted using the Kimura 2-parameter model. This analysis involved 25 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Table 3. Estimates of evolutionary divergence over sequence pairs between groups (interspecific variation).

Species group	*An. vagus*	*An. subpictus*	*An. sundaicus*	*An. indefinitus*	*An. aconitus*	*An. flavirostris*	*An. minimus*
An. vagus		18.98%	18.59%	18.37%	78.27%	75.28%	79.55%
An. subpictus	0,1898		1,39%	0.81%	82.18%	76,77%	77.65%
An. sundaicus	0,1859	0,0139		1.19%	81.37%	76.15%	77.98%
An. indefinitus	0,1837	0,0081	0,0119		80.78%	75.48%	77.29%
An. aconitus	0,7827	0,8218	0,8137	0,8078		17.07%	20.39%
An. flavirostris	0,7528	0,7677	0,7615	0,7548	0,1707		9.40%
An. minimus	0,7955	0,7765	0,7798	0,7729	0,2039	0,0940	

Note: The number of base substitutions per site from averaging all sequence pairs between groups is shown. Analyses were conducted using the Kimura 2-parameter model. This analysis involved 25 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. The yellow column shows the given molecular distance in percent.

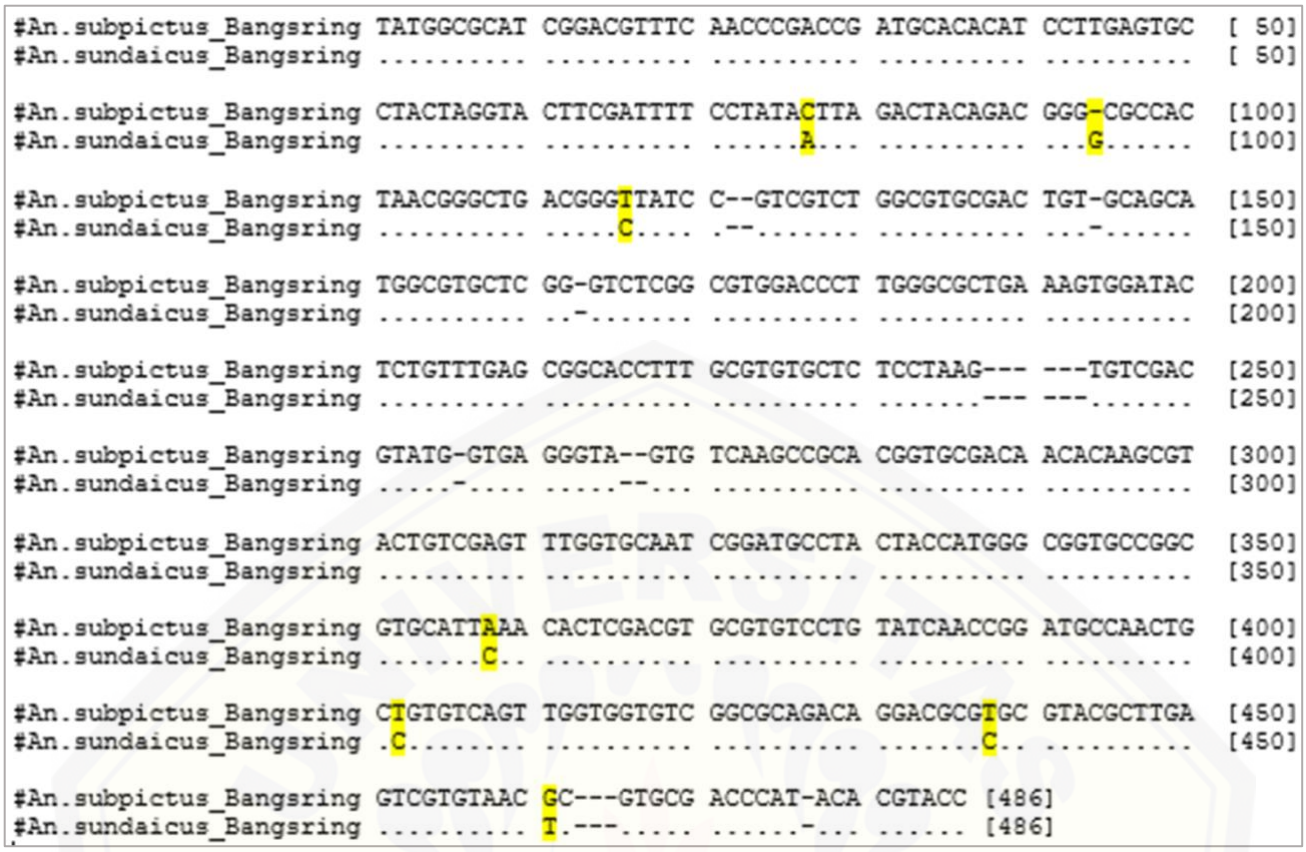

Figure 2. ITS2 sequence pairwise alignment of *An. subpictus* and *An. sundaicus* isolate from Bangsring, Banyuwangi, Indonesia.

Three major clades were formed in the NJ phylogenetic tree (Figure 3). Two clades formed as monophyletic including *An. vagus* and *An. aconitus*. While the others were formed as polyphyletic clades composed by *An. sundaicus*, *An. subpictus*. and *An. indefinitus* (as a closely related species). Clades A and B were composed of members of the *Pyretophorus* series, while C was composed of members of the *Myzomyia* series. The three major clades were supported with a high bootstrap value of 96-100%.

Clade A was heterogeneously composed by *An. sundaicus, An. subpictus* and *An. indefinitus* with varied internal branches from 18 - 93% bootstrap values (Figure 4). This clade was then named as Sundaicus-Subpictus Complex. *An. sundaicus* from Bangsring, Banyuwangi had high similarity to *An. sundaicus* GQ284825, MT623070, and AY768541 from Indonesia (unspecified location), Malaysia, South-East Asia country (unspecified location), respectively. *An. sundaicus* from Bangsring, Banyuwangi differed to *An. sundaicus* GQ284825, MT623070, and AY768541 by the presence of one deletion. *An. sundaicus* MW078490 from India was separated from other *An. sundaicus* and placed together with the group *An. subpictus*. *An. indefinitus* (GQ870332) from Philippine as reference sequence was also placed together with *An. subpictus* group and highly similar to *An. subpictus* (MW078487) from Sri Lanka. *An. subpictus* from Bangsring, Banyuwangi was highly similar to *An. subpictus* GQ870327, MT068436, and MW078487 from Flores, Sulawesi, and India, respectively. *An. subpictus* from Bangsring, Banyuwangi differed to *An. subpictus* GQ870327, MT068436, and MW078487 by the presence of two deletions and one ambiguous nucleotide in the reference sequences.

Clade B members included *An. vagus* from Bangsring-Banyuwangi, India, China, Malaysia, and Indonesia (unspecified location). The ITS2 sequence of *An. vagus* from Bangsring-Banyuwangi was closely related to *An. vagus* FJ654649 from Indonesia (unspecified location), which was supported by the high bootstrap values of 93%. Those sequences were differed only by the one ambiguous nucleotide in the ITS2 sequence of *An. vagus* FJ654649.

Clade C was composed of *An. aconitus* from Bangsring-Banyuwangi, East Timor, Indonesia (unspecific location), Thailand, Srilanka, and South-East Asia (unspecific location). ITS2 sequences of *An. aconitus* from Bangsring, Banyuwangi was similar to *An. aconitus* MN203097, GQ500119 from other Indonesia (unspecified location) and East Timor, respectively. *An. minimus* (KP298408) from Vietnam and *An. flavirostris* (AY943666) Philippine as reference sequences were shown to be closely related to *An. aconitus*.

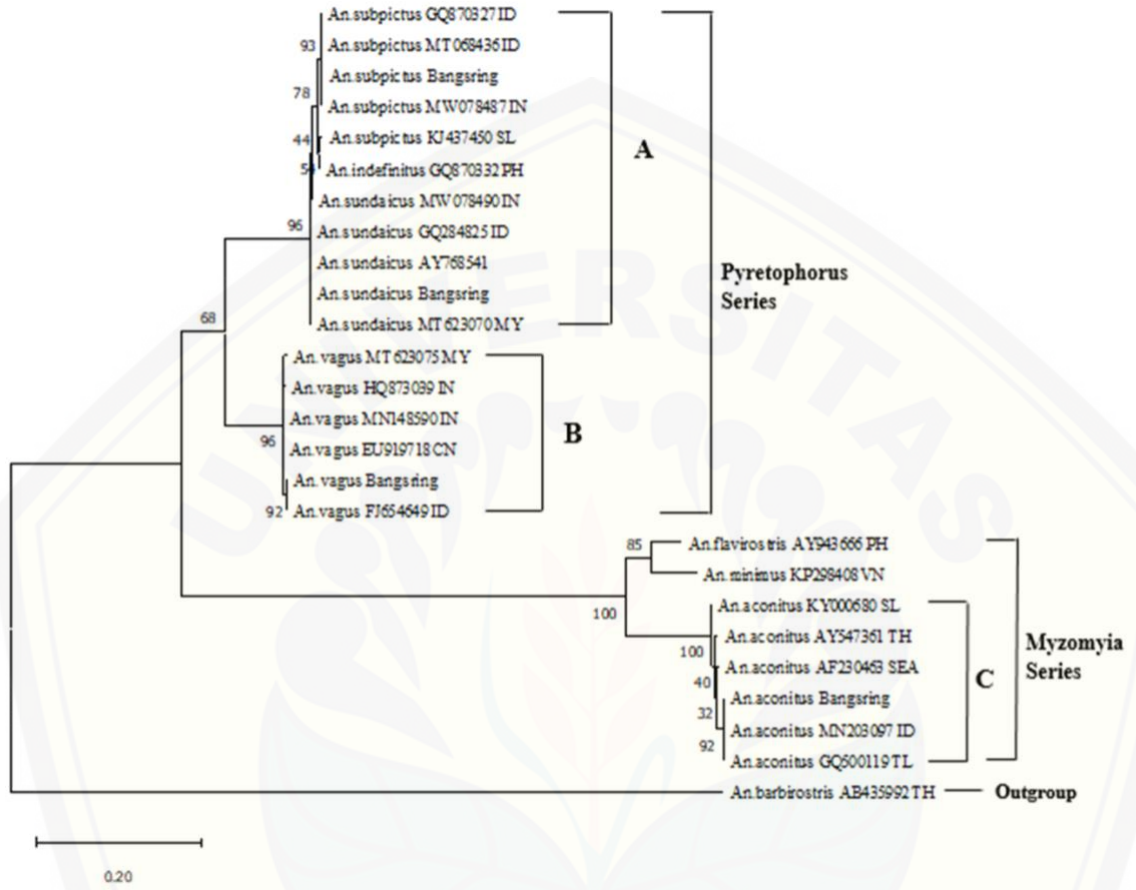

Figure 3. NJ phylogenetic tree based on ITS2 sequences. Four *Anopheles* species from Bangsring Banyuwangi marked by the addition of "Bangsring". The phylogenetic tree was constructed in MEGA11 using the Kimura-2 Parameter model and statistically supported by a boostrapping of 1000.

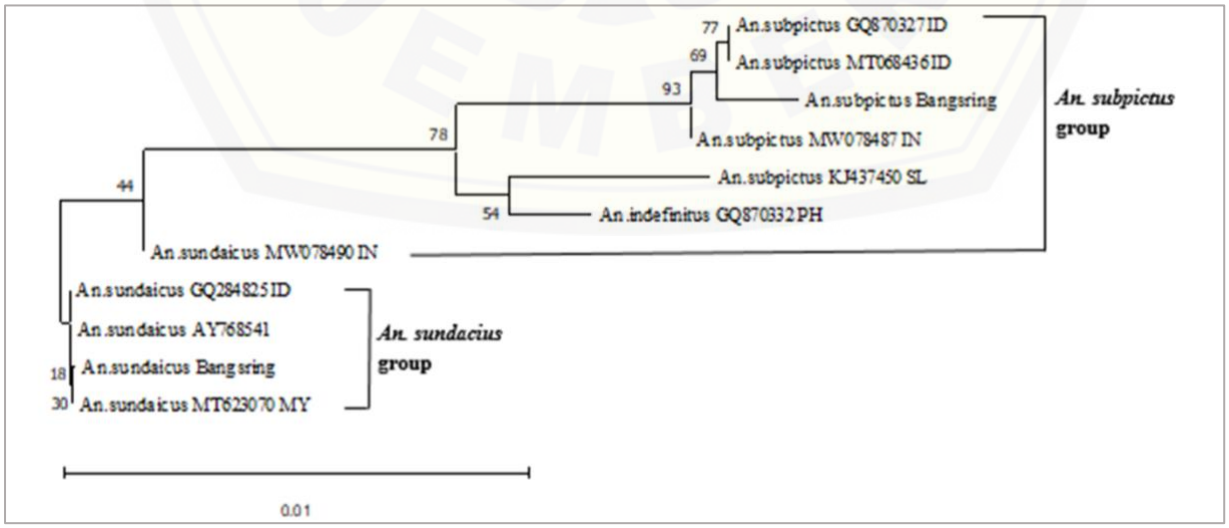

Figure 4. Sub-tree of NJ phylogenetic tree of Sundaicus-Subpictus Complex (Clade A).

DISCUSSION

In this study, we succeeded in identifying four anopheline species from Bangsring Banyuwangi based on their morphological characters such as palpi, proboscis, and wing ornament. *An. aconitus* (the member of the *Myzomyia* series) was easily distinguished and separated from *An. sundaicus*, *An. subpictus* and *An. vagus* (the members of the *Pyretophorus* series). Members of the *Pyrophorus* series have a relatively broad pale band on their foreleg tarsi, whereas *Myzomyia* is completely dark or with a narrow pale band (Reid, 1966; O'connor & Soepanto, 2013).

Morphological-based identification is easy to determine the distinctive species but quite difficult for closely related species (Surendran *et al.,* 2013). However, since this method is relatively simple, inexpensive, and easy to do in the field, this approach is still commonly used to identify anopheline species (Erlank *et al.,* 2018). Due to morphological variation within and between species (species complexes), final identification using this approach needs additional tools to overcome this problem (Gómez & Correa, 2017). This is because many researchers report that members of the *Pyretophorus* series such as *An. sundaicus* and *An. subpictus* being a complex species and its morphological features often lead to misidentification (Jude *et al.,* 2014; Of *et al.,* 2012; Syafruddinid *et al.,* 2020). This also occur in *An. aconitus* which shares many morphological features with other *Myzomyia* series such as *An. minimus* (Reid, 1968). To prevent mis-identification of *An. aconitus* found in Bangsring Banyuwangi, we also used molecular techniques.

An. vagus and *An. aconitus* were easy to be distinguished morphologically by the length of their pale band on proboscis. *An. vagus* was relatively easy to be distinguished from the other two members of *Pyretophorus* because of the presence of this pale band on the proboscis. This is in contrast to other studies mentioning the difficulties in the morphological characterization of the *Pyretophorus* series member (Cooper *et al.,* 2010; Surendran *et al.,* 2010). The short subapical pale and dark band on its palpi was also facilitating a quite easy identification.

On the other hand, the presence of pale yellowish scales on the femora is the most prominent character to distinguish and separate *An. sundaicus* from *An. subpictus* (Reid, 1966; Sindhania *et al.,* 2020). The presence of pale yellowish scales area on femora is the unique character to determine *An. sundaicus*. However, a lousy handling process will make those yellowish scales damage and often caused misidentification to *An. subpictus*. Therefore, molecular identification is needed to further confirm its relationship. Many studies had reported that *An. sundaicus* and *An. subpictus* are closely related species as evidenced by morphological and molecular similarities (Surendran *et al.,* 2010; Jayatunga *et al.,* 2018; Sindhania *et al.*, 2020). Based on their wing variations, another report differentiated *An. aconitus* collected from Central Java, Indonesia, into type A-D (Boewono & Nalim, 1991). Compared to those results, *An. aconitus* collected from Bangsring village, Banyuwangi district, Indonesia was similar to *An. aconitus* type A, in which the costal of the wing containing four pale spots including sector, subcostal, preapical and apical (Figure 1B). The foreleg tarsi of *An. aconitus* in this study was observed to have narrow pale bands. This character distinguishes the *Myzomyia* series from *Pyrophorus* (Reid, 1966; O'connor & Soepanto, 2013).

The use of morphological-based identification alone in determining the entity of anopheline mosquitoes can cause several problems, such as the fact that morphological features such as scales, hairs, wing fringe are easily damaged and cause identification errors (Weeraratne *et al.,* 2018). The relative lengths of pale and dark bands on the palpi, proboscis, and wing ornamentation overlap among closely related species (Reid, 1966; Lorenz *et al.,* 2012; Gómez *et al.,* 2013), and some of the closely related species are reported to be misidentified (Cooper *et al.,* 2010; Surendran *et al.,* 2010). Environmental factors such as temperature and sunlight intensity also encourage morphological plasticity in some parts of the *Anopheles* body, which are essential for species discrimination (Hidalgo *et al.,* 2015). Another reported case is the finding that cold temperatures can trigger hyper-melanic in anopheline mosquitoes by increasing melanin productivity and encouraging the identification of closely related species (Singh *et al.,* 2010). Therefore, to support the morphology data, the molecular-

based identification using the ITS2 sequence (DNA Barcoding) was used in this study.

Based on morphological and cytogenic polymorphism, four sibling species of *An. subpictus* (professionally named as form A-D) from the Southeast coast of Peninsular India had been discovered by Suguna *et al.* (1994). But unfortunately, this founding was not supported by the molecular evidence, which will cause controversy later. For instance, the study conducted by Surendran *et al.* (2010) provided molecular evidence of *An. subpictus* collected from Sri Lanka, which was morphologically similar to *An. subpictus* B that was closely related to *An. sundaicus*. Despite the morphological complexity of the two species, here we provide morphological and molecular evidence that *An. sundaicus* and *An. subpictus* from Bangsring, Banyuwangi, was a distinct species by the presence of seven nucleotide differences between them. The difficulties in differentiating *An. sundaicus* and *An. subpictus* also occurred in Sri Lanka specimens studied by Surendran *et al.* (2010). They concluded that morphological identification of *An. subpictus* species complex was not suitable and led to some misidentification in using the molecular-based method.

Two monophyletic clades were observed in the constructed phylogenetic tree. There were *An. vagus* and *An. aconitus*, supported by high bootstrap values of 96 and 100%, respectively. *An. subpictus* and *An. sundaicus* were close together and form a polyphyletic clade. Separation of *An. vagus* to *An. sundaicus* and *An. subpictus* was also supported by high interspecific distance values of 18.59 and 18.98%, respectively. In the other hand, the imperfect separation (polyphyletic clade) of *An. sundaicus* and *An. subpictus* may be due to the small interspecific distance between them which was only 1.39%.

Both *An. sundaicus* and *An. subpictus* had a slight intra-specific variation of 0.08 and 0.49%, respectively. Based on that result, we proposed that *An. sundaicus* and *An. subpictus* from Bangsring Banyuwangi was a distinct species because the inter-specific distance was higher than intra-specific variation. Furthermore, the ITS2 sequences of those two species are almost identical, where the GC content present difference is only 0.5%. The slight difference in GC content indicates that the two species are closely related. These results suggest that *An. sundaicus* and *An. subpictus* found in the coastal area of Bangsring Village, Banyuwangi was categorized as Sundaicus-Subpictus complex. Sindhania *et al.* (2020) also found that *An. sundaicus* D and *An. subpictus* B and C from the Indian subcontinent had 1.1% and 1.5% molecular distances, respectively. However, slight differences in ITS2 sequences between *An. subpictus* and *An. sundaicus* (Figure 5) may also indicate that the species is a distinct species. GC content in the ITS2 sequence is associated with secondary structures that might impact biological processes, such as mating incompatibility (Zhang *et al.,* 2020; Jayatunga *et al.,* 2021).

Same as *An. vagus*, *An. aconitus* was also found in monophyletic clade. The low intra-specific variation within *An. aconitus* from Bangsring and another region was observed. The results of morphological identification of *An. aconitus* were relevant to molecular identification, which resulted in the distinct separation of this species from *Pyretophorus* and other members of the *Myzomyia* series. The addition of *An. minimus* and *An. flavirostris* on the phylogenetic tree aims to determine the relationship between *Myzomyia* series. *An. aconitus* was completely separated from *An. minimus* and *An. flavirostris* with a high bootstrap value of 100%. These results provide information that although *An. aconitus* and *An. minimus* share some morphological features, but their ITS2 was different.

CONCLUSION

This present study showed that ITS2 sequence was able to identify four morphologically identified *Anopheles* species from Bangsring Banyuwangi, Indonesia. A phylogenetic tree based on the ITS2 sequence was successfully constructed and two monophyletic clades were formed, namely clade *An. vagus* and *An. aconitus*, while *An. sundaicus* and *An. subpictus* share in the same clade and form a polyphyletic clade. Though *An. sundaicus* and *An. subpictus* were the most closely related species, we have shown that they are distinct species morphologically as well as molecularly. These results indicated that those

species in Bangsring population are likely to be sympatric and belong to *An. subpictus* complexes.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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