Sebagian data dari Hasil Penelitian Pendukung IDB dengan judul: "**POTENSI BAKTERI BERASOSIASI VEKTOR NYAMUK *Anopheles sp.* DALAM MENGHAMBAT SIKLUS HIDUP DAN TRANSMISI PATOGEN MALARIA",** telah dipublikasikan di Prosiding internasional terindeks Web of Science, yang baru terbit di 22 Desember 2022 yaitu di ***Advances in Biological Sciences Research*: Vol 27, pp. 354–362** sebagaimana terlampir.

Data lainnya masih dalam proses analisis untuk di publikasi di Jurnal terindeks Scopus.

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Advances in Biological Sciences Research

Asmoro Lelono · Muhammad Akbar Bahar · Syubanul Wathon · Kartika Senjarini · Asep Ginanjar Arip · Ramdhan Putrasetya · Beny Andika · Nadhea Ayu Sukma · Bambang Sugiharto *Editors*

Proceedings of the 4th International Conference on Life Sciences and Biotechnology (ICOLIB 2021) · Volume 27

OPEN ACCESS

Advances in Biological Sciences Research 27

Series Editor

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ISSN 2731-7846 ISSN 2468-5747 (electronic)
Advances in Biological Sciences Research
ISBN 978-94-6463-061-9 ISBN 978-94-6463-062-6 (eBook)
https://doi.org/10.2991/978-94-6463-062-6

This Atlantis Press imprint is published by the registered company Atlantis Press International B.V.,
part of Springer Nature
The registered company address is: Van Godewijckstraat 30 3311 GX Dordrecht Netherlands

Preface ICOLIB 2021

I am pleased to welcome all of the speakers and participants to the 2021 International Conference on Life Sciences and Biotechnology (4th ICOLIB), which is held from 15 to 16 November 2021, virtually on Zoom meeting. The conference is organized by the Department of Biology, Faculty of Mathematics and Natural Sciences, the University of Jember.

This year the conference's theme is "Towards Sustainable Development: Application of Biosciences to Improve Welfare and Quality of Life". Along with the theme, we have four conference topics; there are Applied Sciences (Agriculture, Biotechnology and Bioinformatics), Basic Sciences (Ecology, Zoology, Botany, and Microbiology), Biodiversity and Bio-conservation, and Health and Medicine (Pharmacy and Medical Sciences). This scientific event provides a platform for researchers, academics, professionals, industries, and policymakers to exchange ideas, share the recent advances and development in life sciences, and can be a valuable place for starting fruitful collaboration, especially in uncovering the potential of biodiversity at the molecular level to biosphere.

This year's conference is also the first time held online due to the global pandemic situation. However, it becomes a blessing in disguise, because the conference becomes accessible to a wider audience and participants from all over the world. The number of participants registered is 223, among them, 170 participants will present their research.

Most of the participants are from Indonesia but also we have participants from Malaysia, Czech Republic, China, Philippines, Pakistan, and India. Besides the participants, the online of this conference also allows us to invite speakers from Netherlands, Germany, USA, and Australia. There will be Prof. A.G.G (Ton) Groothuis from GELIFES Institute, Groningen University Netherlands, Prof Antonius Suwanto from IPB Indonesia, Prof Simon Griffith from Department of Biological Sciences at Macquarie University, Sydney, Dr. Jorge A. Santiago-Blay from the Department of Paleobiology MRC-121 National Museum of Natural History Smithsonian Institution, USA, Prof. Elvira Hoerandl from George-August, Goothingen University Germany, Dr. Kahar Muzakar from Biology Department, Jember University, and Dr. Christina Bauch from Instituto Universitario in Lisbon, Portugal and Groningen University, Netherlands.

The output of this conference will be published in the Atlantis Press Proceeding: Part of Nature in series of "Advances in Biological Sciences Research", the Journal of ILMU Dasar MIPA, and the Journal of Tropical Biodiversity and Biotechnology. Finally, I would like to acknowledge the Board of Jember University which supported this conference. And I also like to thank to Vanadia and DAAD as one of the main sponsors which make this conference possible.

I hope this conference will be fruitful for everyone. We look forward to seeing you all at the next ICOLIB conference.

Asmoro Lelono
Chairman of 4th ICOLIB 2021

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AQ1 All of the articles in this proceedings volume have been presented at the International Conference on Life Science and Biotechnology (ICOLIB) on November 15–16, 2021 at Jember University Indonesia. These articles have been peer-reviewed by the members of the Scientific Committee of ICOLIB and approved by the Editor-in-Chief, who affirms AQ2 that this document is a truthful description of the conference's review process.

1 Review Procedure

The reviews were double-blind. Each submission was examined by two reviewer(s) independently. The conference submission management system was easy chair.

We divided the submission of the ICOLIB participant into two categories, the first is abstract and the second the full manuscript submission. The submissions of the abstract were first screened for generic quality, relatedness to the main topic and suitableness by the editorial team. Based on this initial screening, all of the abstracts would be classified by the main theme, i.e. Applied Sciences (Agriculture, Biotechnology & Bioinformatics), Basic Sciences (Ecology, Zoology, Botany, and Microbiology), Biodiversity & Bioconservation, Health & Medicine (Pharmacy & Medical Sciences). All of the selected abstracts would be presented by their author during the ICOLIB conferences. The committee gave an option to the authors to publish their manuscripts or just presented their work in the ICOLIB conference.

All of the complete manuscripts then follow the review process, the first step was to evaluate the relatedness to the proceedings series "Advances in Biological Sciences Research", scientific quality, novelty and contribution to the science. The second step is checking the similarity using Turnitin to evaluate the textual overlap and detect the possible sign of plagiarism. The third step was to send for peer review by matching each

B. Sugiharto—Editors-in-Chief of the ICOLIB.

A. Lelono et al. (Eds.): ICOLIB 2021, ABSR 27, pp. 1–3, 2023.
https://doi.org/10.2991/978-94-6463-062-6_1

paper's topic with the reviewers' expertise, taking into account any competing interests. However, in some case, we also sent the manuscript to the third reviewer to consider another opinion if the first two reviewers has an opposite decision. A paper could only be considered for acceptance if it had received favourable comments and suggestions from the two reviewers. The recommendations then sent back to the author to address the reviewer's comment. The acceptance or rejection of a revised manuscript was final. In the final steps, all of the manuscripts were adjusted in their layout and some of the technical editing for the pre-print version. This preprint document would be sent to the author for clarification. They also should be sent a statement of the novelty and originality of the study.

2 Quality Criteria

Reviewers were instructed to assess the quality of submissions solely based on the academic merit of their content along the following dimensions. The editorial gave a rubric for a guideline which contains some important aspect related to the quality of the manuscript such as:

1. Pertinence of the article's content to the scope and themes of the conference;
2. Clear demonstration of originality, novelty, and timeliness of the research;
3. Soundness of the methods, analyses, and results;
4. Adherence to the ethical standards and codes of conduct relevant to the research field;
5. Clarity, style, cohesion, and accuracy in language and other modes of expression, including figures and tables.

We have a policy that each manuscript should be reviewed by two reviewers and each reviewer only reviews two manuscripts. The consequences of this policy is that we contact more reviewers, in total we ask 61 reviewer for completing the review process.

3 Key Metrics

Total submissions	118
Number of articles sent for peer review	67
Number of accepted articles	61.
Acceptance rate	51.5%
Number of reviewers	61

4 Competing Interests

Neither the Editor-in-Chief nor any member of the Scientific Committee declares any competing interest.

Immunogenic Proteins from Salivary Gland of Potential Malaria Vector *An. vagus* and *An. sundaicus*

Ika Wahyuni, Rike Oktarianti, Syubbanul Wathon, Lailly Nur Uswatul Hasanah, and Kartika Senjarini(✉)

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Abstract. The salivary gland proteins of the *Anopheles* (An.) female mosquito play vital roles in the transmission of Plasmodium into the human host. A comprehensive understanding of *Anopheles* identity and its related salivary proteins is a key for vector-based malaria control. This research aims to analyse the protein profile and immunogenicity of Anopheles salivary gland proteins from 2 potential *Anopheles* vectors, *An. vagus* and *An. sundaicus*. Female *Anopheles* are collected from Bangsring village, Banyuwangi Regency by direct-landing collection. Each species are identified by morphological features and internal transcribed spacer 2 (ITS2). Immunogenic proteins were determined by Western Blotting with IgG from people living in the endemic area as primary antibodies. Immunoblotting results showed the presence of 3 immunogenic protein bands with a molecular weight of 34, 46, and 66 kDa in both species. Furthermore, the 99 kDa protein band was present merely in *An. sundaicus*. Moreover, the result showed that two distinct species have different profiles and immunogenic proteins.

Keywords: Immunogenic proteins · salivary gland · vector · Anopheles · Malaria

1 Introduction

Malaria is a vector-borne disease caused by *Plasmodium* (P.), which is transmitted by female *Anopheles* as a vector. *Plasmodiums* that cause illness in humans are *P. vivax, P. falciparum, P. ovale, and P. Malariae* [1]. Plasmodium is transmitted to the human host while the female Anopheles bites the human. Mosquito requires blood protein for nutrition and egg development [2]. The transmission of *Plasmodium* is due to the role of salivary gland proteins that mediate the blood meal process of mosquitoes into the human host.

Mosquito saliva contains immunomodulatory and antihaemostasis factors [3]-[5]. Immunogenic proteins can modulate the immune system, and against various agents that have a significant role in non-specific and specific immune responses [2]. Immunomodulatory proteins found in Hematophagous Arthropods are the D7 family, SGS (salivary

A. Lelono et al. (Eds.): ICOLIB 2021, ABSR 27, pp. 354–362, 2023.
https://doi.org/10.2991/978-94-6463-062-6_35

gland surface), and saglin [6, 7]. D7 family plays its role as a vasodilator, antihaemostatic, and immunomodulator [8] in the Culicidae family [2].

Antihaemostasis proteins facilitate blood-feeding by preventing the haemostasis process. Based on the function, there is three groups of antihaemostasis (1) vasodilator (2) anti-platelet aggregation, and (3) anticoagulant [2]. Vasodilator protein potentially inhibits vasoconstriction and enlarges the artery [2]. Several proteins are known as vasodilatory factors, such as sialokins, adenosine, and maxadilan [2]. Anti-platelet aggregation inhibits some aggregation factors like ADP and thrombin. One of the anti-platelet aggregations is apyrase which plays a role as anti-platelet by catabolizing ADP released and inhibit platelet activation [2]. Apyrase was found in *Aedes aegypty* [9], *Anopheles* [2], and Hemiptera [10]. This indicates that various types and activities of salivary protein are related to the vector species (genus or species-specific).

Some research demonstrates that the saliva of different species or genus has distinct secreted protein and immunogenicity activities [11, 12]. Taxonomical level influences the similarity of salivary protein of *An. albimanus, An. arabiensis, An. stephensi* and *An. gambiae*. The number of homologous proteins among the *Anopheles* species was the highest among the closely related anopheline species (among *An. gambiae* and *An. arabiensis*) and decreased with increasing phylogenetic distance (among species from the Subgenus *Cellia* [11].

Anopheles is an Arthropod with a high level of diversity. Morphological appearance is insufficient to distinguish the species, especially those closely related or cryptic species [13]. A molecular-based analysis is an approach to understanding the diversity of high organisms using one or more molecular markers. ITS2 (internal transcribed spacer 2) is a widely used molecular marker to determine *Anopheles* species [14]-[16]. It has a vast conservation area, is easy to amplify with PCR, has high variability, and is available in many online databases [14, 17]. A comprehensive understanding of salivary gland protein profile and immunogenicity related to vector identity is a crucial step to intervene in the transmission of disease from vectors to human hosts. It could be the development of biomarkers and vector-based vaccines. The hypothesis of the study is genetically different species of *Anopheles* have different immunogenic proteins. Therefore, this study aimed to determine the profile and immunogenicity of Anopheles mosquito proteins related to their genetic diversity analysis using ITS2 molecular markers.

2 Materials and Methods

2.1 Morphological and Molecular Identification

The specimen of mosquitoes is collected from their natural habitat in Bangsring village, near the coastal area of Bali Bay, and in rural areas of Bangsring village, Banyuwangi. The sampling location is located between latitudes 8°04' S and longitudes 114°25' E. The adult females were collected by direct landing collection using a hand-held aspirator and torchlight from the cowshed from 6 p.m.– 5 a.m. The Anopheles were identified based on the book of determination key of insects [18].

Total DNA was extracted using the salting-out method proposed by Aljanabi and Ichiar (1997) and was used as a DNA template for amplification. The ITS2 sequence was amplified using the primers ITS2-F (5'TGT GAA CTG CAG GAC ACA TG 3') and

ITS2-R (5'ATG CTT AAA TTT AGG GGG TA 3') and following reaction mix (50 μl): 1.25 μl primer-F, 1.25 μl primer-R, 21,5 μl sterile ddH2O,25 μl 2x PCR master mix and 1 μl DNA template. The following thermocycler parameters: 94ºC for 5 min, 4 cycles at 94ºC for 40 s, 94ºC for 1 min and 72ºC for 1.5 min, 29 cycles at 94ºC for 40 s, 51ºC for 1 min and 72ºC for 5 min then stand by at 16ºC. The PCR product will be showed a single band around 500 bp. Then PCR result was sequenced at the sequencing facility of 1st Base Singapore.

2.2 Ethical Consideration and Blood Sera Collection

Human blood sera collections were approved by KEPK (The Ethical Committee of Medical Research Faculty of Dentistry Universitas Jember) No. 1034/UN25.8/KEPK/DL/2020, approved on October 19th 2020. Sera samples were collected from healthy adult residents living in Bangsring village, Banyuwangi who were exposed by Anopheles. The blood sample was taken from the bronchial vein in the upper arm. 3 mL blood was taken and placed in a vacutainer without heparin. Then, it was kept for 15 to 45 min. The upper transparent layer was taken and centrifuged at 27ºC, 3200 rpm for 10 min. The supernatant was then kept at -20ºC. Infant Sera was used as a negative control.

2.3 Salivary Gland Protein Extraction and SDS-PAGE Analysis

The salivary gland (SG) was isolated by microdissection under a stereomicroscope and was pooled into microtube containing 10 μl PMSF on PBS for 10 pairs of SG. The SG was used as a sample for protein analysis and stored at -20ºC. The dissected SG was mixed in 1:1 lysis buffer (Tris-HCL 1 M pH 6.8, glycerol, 0,1% Bromophenol blue,10% SDS, and sterile H_2O). The mixture was boiled for 4 min. Then, 20 mL sample was loaded and separated onto 12% separating gel (30% acrylamide/bis-acrylamide, 1.5 M Tris-HCL pH 8.8, 10% SDS, sterile H_2O, 10% APS and TEMED) and 4% separating gel (acrylamide/bis-acrylamide, 1.5 M Tris-HCL pH 6.8, 10% SDS, sterile H_2O, 10% APS and TEMED). Molecular weight (MW) pre-stained protein marker (Blue Eye) was loaded onto the gel. The SDS-PAGE was run for 70 min, 150 V, in 1x electrode buffer pH 8.3. The gel was stained for 2 h with Coomassie Brilliant Blue and de-stained for 30 min three times.

2.4 Western Blotting

The SDS-PAGE gel was transferred to polyvinylidene difluoride (PVDF) membrane using semidry blotting (Biorad) at 110 mA for 1 h. The membrane was washed with Tris Buffer Saline (TBS) three times, then it was soaked with 5% skimmed milk on TBS for 1 h. Subsequently, the membrane was washed again with TBS three times and incubated with human sera at dilution 1: 250 (v/v) in skimmed milk on TBS overnight at 4ºC. Blots were washed with TBS three times then were incubated with goat anti-human IgG labeled alkaline phosphatase at dilution 1:5000 (v/v) for 2 h at room temperature. The PVDF membrane was washed again with TBS three times. Color development was done with NBT-BCIP Phosphatase substrate for 1 min.

Table 1. ITS2 sequence homologies of collected *An. vagus* and *An. sundaicus* from Bangsring with selected sequence from GenBank

Species	Description	Max score	Total score	Query cover	E value	Ident	Accession
An. vagus	*Anopheles vagus* 5,8S ribosomal RNA gene, partial sequence, internal transcribed spscer 2, complete sequence and 28S ribosomal RNA…….	1179	1179	99%	0,0	99,69%	FJ654649.1
	Anopheles vagus genes for 5,8S ribosomal RNA, ITS1, 28S ribosomal RNA, partial sequence, strain: VN08	1151	1151	99%	0,0	99,07%	AB731658.1
An. sundaicus	*Anopheles sundaicus* internal transcribed spacer 2 and 28S ribosomal RNA gene…	953	953	96%	0,0	97,35%	AY768541.1
	Anopheles sundaicus isolate MA102–39 5,8S ribosomal RNA..gene..	950	950	96%	0,0	97,17%	AF369560.1

Information: Max score: maximum score; E value: expect value; Ident: identity.

3 Results and Discussion

In total, 584 individual mosquitoes were collected during landing collection. Morphological identification showed Anopheles population in Bangsring village was mainly dominated by *An. vagus* and *An. sundaicus* with a percentage of 75.43% and 23.38%, respectively. Both species are Cellia subgenus which has three or more pale bands on the palpus. Moreover, *An. vagus* has 3–5 times apical pale band (Ap) than the subapical dark band (Sd) while *An. sundaicus* has 1/3 length of Sd compared with the Ap. The hind leg. *An. vagus* has a non-speckled hind leg, generally white or black hair in femur and tibia, *An. sundaicus* has speckled femur and tibia on its hind leg [18].

The ITS2 sequence was used as an approach for species confirmation. The PCR-ITS2 yielded a different product size from each species, 498 bp for *An. vagus* and 486 bp

for *An. sundaicus* (not included on the journal). Based on the sequence alignment and comparison on BLAST online software, ITS2 sequence of *An. vagus* from Bangsring has 99,67% similarity with *An. vagus* FJ654649.1 (Table 1), an identified species from East Java [19]. *An. vagus* is widely known as the secondary malarian vector in Sukabumi [20] and Banten [5] and has been approved to be a vector of *Plasmodium vivax* [21]. The ITS2 sequence of collected *An. sundaicus* showed 97,35% similarity with AY768541.1, *An. sundaicus* from Indonesia which has been known as the primary vector of malaria in various regions of Indonesia [22, 23].

The taxonomic level has an impact on protein similarity. The closer of phylogenetic relationships having likeness in protein profiles and antigenic activities [11]. Some proteins could be a ubiquitous salivary protein that can be found in the salivary gland of other blood-sucking insects [18], could function as housekeeping and secreted protein for blood feeding [11]. The profile of SG proteins is analysed using SDS-PAGE analysis. The salivary gland protein extract (SGE) of *An. vagus* showed 14 dominant bands of protein with molecular weight of 14, 15, 16, 31, 34, 41, 43, 46, 54, 64, 66, 75, 99 and 230 kDA. Whereas, *An. sundaicus* showed 15 dominant protein bands with a molecular weight of 14, 15, 16, 31, 34, 41, 46, 48, 54, 64, 66, 75, 99, 230, and 262 (Fig. 1). Most of the protein are appeared in both *An. vagus* and *An. sundaicus.* These results are supported the previous research mentioned that different species have different protein profiles. The difference of the two species is minor as *An. vagus* and *An. sundaicus* are members of subgenus *Cellia*, causing them to have high salivary protein similarity. Earlier research shows that *An. gambiae* and *An. arabiensis* (*An. gambiae sensu lato*) which were identified using COII molecular marker have 73% of salivary gland protein similarity [11].

The results of western blotting showed that specific proteins of 34, 46 and 66 kDA appeared on both species (Fig. 2). Previous studies revealed these proteins are ubiquitous proteins found in Culicidae and hematophagous Arthropoda [1]. The putative protein of 34 kDa was related to the D7 protein family. D7 protein has been reported to act as an immunomodulator, vasodilator, and anticoagulant in *Ae. aegypthi* [22, 23] and *Anopheles* species [1]. Whereas protein of 46 kDA is commonly known as coagulant and anti-inflammation protein, serpin. The putative protein of 66 kDA has been reported as apyrase, a protein that acts as an anticoagulant and platelet aggregation in *Aedes aegypti* [22] and *Glosssina* sp. [24].

One specific protein with a molecular weight of 99 kDa, which was only detected in *An. sundaicus* showed immunogenic activity. Furthermore, the negative result appeared in the reaction between individual neonates serum with SGE of *An. vagus* and *An. sundaicus.* The 99 kDa protein, which only appeared on SG of *An. sundaicus* is very likely to be a member of saglin. Nevertheless, this protein is not a species-specific protein. Saglin has been found in *An. gambiae* and *An. stephensi* [7, 25]. Saglin plays a role in the invasion of Plasmodium sporozoite in SG of *Anopheles* [7]. The difference in the profile and immunogenic protein between *An. vagus* and *An. sundaicus* is probably related to the vectorial capacity of Anopheles species. Saglin is commonly known as a receptor for malaria sporozoites related to the status of *An. sundaicus* as the primary vector of malaria in various regions in Indonesia [8].

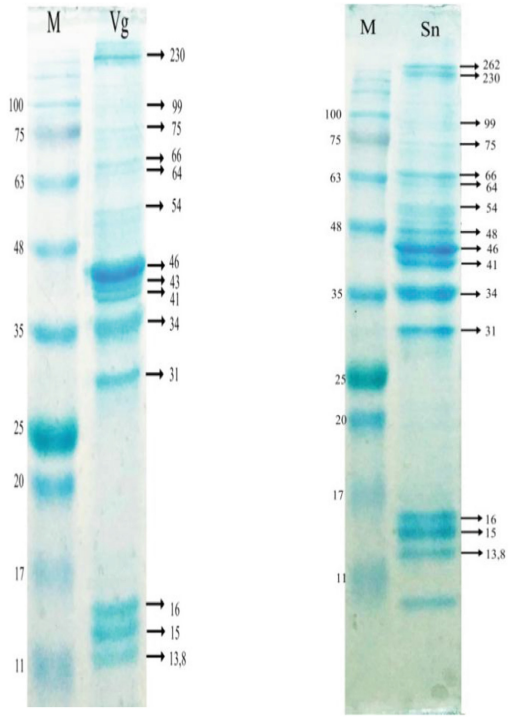

Fig. 1. Protein profile of salivary gland extract (sge) *An. vagus* (Vg) and *An. sundaicus* (Sn). Sample from landing collections, isolation of 10 pairs of salivary glands. M (marker).

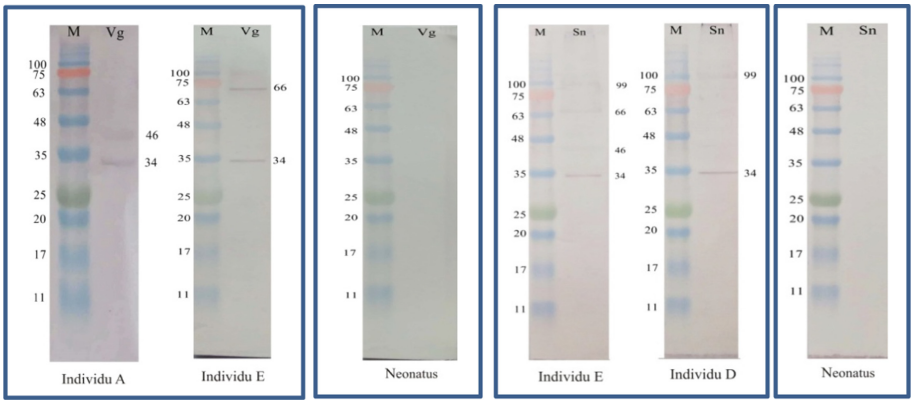

Fig. 2. Different patterns of immunogenic proteins of SGE *An. vagus* (Vg) and *An. sundaicus* (Sn) were identified by Western Blothing method, which were the result of cross reaction with individual human sera (Individu A-E). Three immunogenic proteins of *An. vagus* with molecular weight of 34, 44 and 66 kDA, whereas four immunogenic proteins were identified from *An. sundaicus* i.e 34, 46, 66 and 99 kDA. (M) marker, neonatus (Negative control).

These results could provide a novel target protein for future investigations aimed at malaria transmission, represent the first step toward the identification of immunogenic protein as molecular marker of exposure and vector-based vaccine development. Further analysis targeting the immunogenic proteins highly recommended such as protein identification by LC/MS analysis. But the methodology represented here appears very convenient for initial screening of immunogenic proteins. It provides qualitative data on the detection of immunogenic components of Anopheles.

This study highlights the differences in the protein and immunogenicity profile of two different species of *Anopheles*, *An. vagus* and *An. sundaicus* from Bangsring Banyuwangi. The protein profile of salivary gland protein extract (SGE) of *An. vagus* showed 14 dominant bands of protein, whereas *An. sundaicus* showed 15 dominant bands of protein. Three immunogenic proteins of 34, 46, and 66 kDa were identified from both species, whereas protein with a molecular weight of 99 kDA, which only appeared in SGE of *An. sundaicus* were able to cross-react with sera samples from the endemic area. Moreover, the result showed that two distinct species have different profiles and immunogenic proteins.

These present studies showed that two species were successfully identified morphologically and molecularly using ITS2 marker as *An. vagus* and *An. sundaicus*. Though these two species are in the same subgenus, the SDS-PAGE and immunoblotting results showed that distinct species are having different profile and immunogenic protein. These results indicate protein activity of Anopheles are likely related to the species identity.

Acknowledgments. This study was supported by "Master Grants" and KeRis (Kelompok Riset) of Vector Biology, University of Jember. We thank particularly Mujiono, researcher staff of The Centre for Research and Development of Disease Vectors and Reservoirs, (B2P2VRP) Salatiga, Indonesia for his input during this work.

Authors' Contributions. KS, IW and RO designed the experiments. IW and LNUH analysed the morphological and molecular identification of the Anopheles. IW and SW optimized the SDS-PAGE and Immunoblotting protocols. KS and IW analysed data, wrote and edited the manuscript with contributions from all authors. All authors read and approved the final manuscript.

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