DNA Barcoding for the Identification of the Malaria Secondary Vector, *Anopheles aconitus*, Collected in Western Thailand

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Abstract

Anopheles aconitus is widely distributed from Sri Lanka, India, and Nepal eastward to Hainan Island in China, and southward from southern China through Southeast Asia to Indonesia. In Thailand, this species is recognized as a secondary vector of malaria. This study aimed to confirm and identify the malaria secondary vector, *An. aconitus*, collected in Western Thailand using DNA barcoding. The BLAST results confirmed the identification of three *An. aconitus* samples collected from Western Thailand by comparing their DNA sequences with reference sequences in the GenBank database. All three samples, identified as IDs 001, 002, and 003, matched the *An. aconitus* references in the database. Furthermore, the phylogenetic tree elucidates the relationships among *Anopheles* species, showing that our *An. aconitus* samples from Ratchaburi (ID001, ID002, ID003) were grouped with other *An. aconitus* sequences from GenBank (MT434296 and MT753033), confirming they belong to the same species. These results provide guidelines for identifying malaria vectors and ultimately enhance the effectiveness of malaria surveillance.

Keywords: DNA barcoding, malaria vector, *Anopheles* mosquito, species identification

1. Introduction

Malaria is an infectious disease transmitted by *Anopheles* mosquitoes (Kar et al., 2014). The causative agent of malaria is a single-celled protozoan parasite from the genus *Plasmodium*. Five species within this genus are capable of causing disease in humans: *P. falciparum, P. vivax, P. ovale, P. malariae*, and *P. knowlesi* (Cox, 2010). This disease poses a major public health challenge in many tropical and subtropical countries. According to the World Health Organization, there were approximately 241 million cases of malaria and as many as 627,000 deaths globally in 2020 (World Health Organization, 2020). Malaria is endemic in Thailand's border areas, where dense forests provide suitable breeding grounds for *Anopheles* mosquitoes, the vectors of malaria (Kar et al., 2014). In Thailand, the *Anopheles* mosquito vectors are divided into three groups: (1) primary vectors, which include *An. minimus, An. dirus,* and *An. maculatus*; (2) secondary vectors, comprising *An. epiroticus, An. aconitus, An. sawadwongporni*, and *An. pseudowillmori*; and (3) suspected vectors, consisting of several species such as the *An. barbirostris* group, *An. nivipes, An. karwari, An. philippinensis, An. annularis* s.l., *An. tessellatus, An. kochi,* and *An. vagus* (Tainchum et al., 2014; Tainchum et al., 2015).

Effective malaria control planning necessitates comprehensive knowledge about the various species of *Anopheles* mosquitoes in an endemic area, as different species display distinct behaviors related to malaria transmission (Sumruayphol et al., 2020). However, a significant obstacle exists: standard morphological methods for identifying *Anopheles* mosquitoes are error-prone due to the similar morphological characteristics shared among many species (Chatpiyaphat et al., 2021). This similarity can lead to ineffective mosquito population control measures in the future. To address these identification errors, the adoption of new alternative methods, such as those based on molecular biology, is essential.

DNA barcoding is a molecular biology technique used as an alternative method to confirm mosquito species in many countries (Chan-Chable et al., 2019; Weeraratne et al., 2018), including Thailand (Chaiphongpachara et al., 2022). This technique is based on analyzing the nucleotide sequences of DNA fragments. These sequences exhibit low variability within the same species but high variability between species. Universal primers are used to amplify the DNA in the target region. The resulting DNA barcode is then compared with sequences in a universal DNA database to identify the species (Ratnasingham & Hebert, 2007). The cytochrome *c* oxidase subunit I (*COI*) gene is commonly employed as a universal barcode locus for the identification of mosquito species.

Therefore, in this study, this technique was applied to identify the malaria secondary vector, *Anopheles aconitus*, collected in Western Thailand. These results serves as a guideline for identifying malaria vectors, ultimately enhancing the effectiveness of malaria surveillance.

1.1 Research Objectives

This study aimed to confirm and identify the malaria secondary vector, *An. aconitus*, collected in Western Thailand using DNA barcoding.

2. Methodology

2.1 Mosquito collection

In August 2022, *Anopheles* mosquitoes were collected from Huai Nam Nak village in Ratchaburi province, Western Thailand (13°22'28.9"N, 99°16'29.4"E) using BG-Pro traps, CDC-style (BioGents, Regensburg, Germany). These traps were baited with dry ice as a source of carbon dioxide and equipped with BG-lure cartridges. They operated continuously from 6:00 p.m. to 6:00 a.m. over five consecutive days. The *Anopheles* samples were then transported to the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Campus, Thailand, for the morphological identification.

2.2 DNA extraction, polymerase chain reaction, and DNA sequencing

After morphological identification, *An. aconitus* samples were prepared for DNA analysis. Initially, the samples were legged, then subjected to DNA extraction using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan), in accordance with the manufacturer's protocols. The *COI* gene of *An. aconitus* was amplified by PCR using two universal primers: the forward primer (5’-GGA TTT GGA AAT TGA TTA GTT CCT T-3’) and the reverse primer (5’-AAA AAT TTT AAT TCC AGT TGG AAC AGC-3’). The PCR reactions were conducted in 25 µl volumes per tube, which included 4 µl of DNA template, 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 1.5

U of Platinum Taq DNA Polymerase (Invitrogen, USA). The PCR cycling conditions commenced with an initial denaturation at 95°C for 5 min, followed by five cycles at 94°C for 40 s, 45°C for 1 min, and 72°C for 1 min. This was followed by 35 cycles at 94°C for 40 s, 54°C for 1 min, and 72°C for 1 min, ending with a final extension at 72°C for 10 min. Successfully amplified PCR products were then purified and sequenced by SolGent, Inc. (Daejeon, Korea).

2.3 Species confirmation based on genetic database comparison

Our forward and reverse sequences of *An. aconitus* were trimmed, edited, analyzed, and assembled to create a consensus sequence, which represents the integration of both sequences using BioEdit software version 7.2.5 (Hall, 1999). Each consensus sequence was then compared against sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST), a suite of commonly used algorithms for identifying matches between biological sequences, on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm species identity.

2.4 Phylogenetic analysis

Phylogenetic analysis was conducted to explore the relationships between *An. aconitus* and other *Anopheles* species. The Phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 11 (Tamura et al., 2021), and 1000 bootstrap re-samplings were performed to assess the reliability of this tree topology.

3. Results

3.1 DNA Barcoding

Three *An. aconitus* samples were collected in this study. The BLAST results confirmed the identification of these *An. aconitus* samples from Western Thailand by comparing their DNA sequences with reference sequences in the GenBank database, as detailed in Table 1. All three samples, identified as IDs 001, 002, and 003, matched with the *An. aconitus* references in the database. The percentage identity ranged from 97.46% to 100% across 16 matches for sample 001, from 98.17% to 99.43% across 9 matches for sample 002, and from 97.32% to 99.86% across 16 matches for sample 003. These high levels of sequence identity strongly support the accurate identification of the collected samples as *An. aconitus*.

Table 1. BLAST results of our An. aconitus samples collected in Western Thailand.

Species	ID in this study	Species match	% Identity (*n*)
An. aconitus	001	*An. aconitus*	97.46%–100% (16)
An. aconitus	002	*An. aconitus*	98.17%–99.43% (9)
An. aconitus	003	*An. aconitus*	97.32%–99.86% (16)

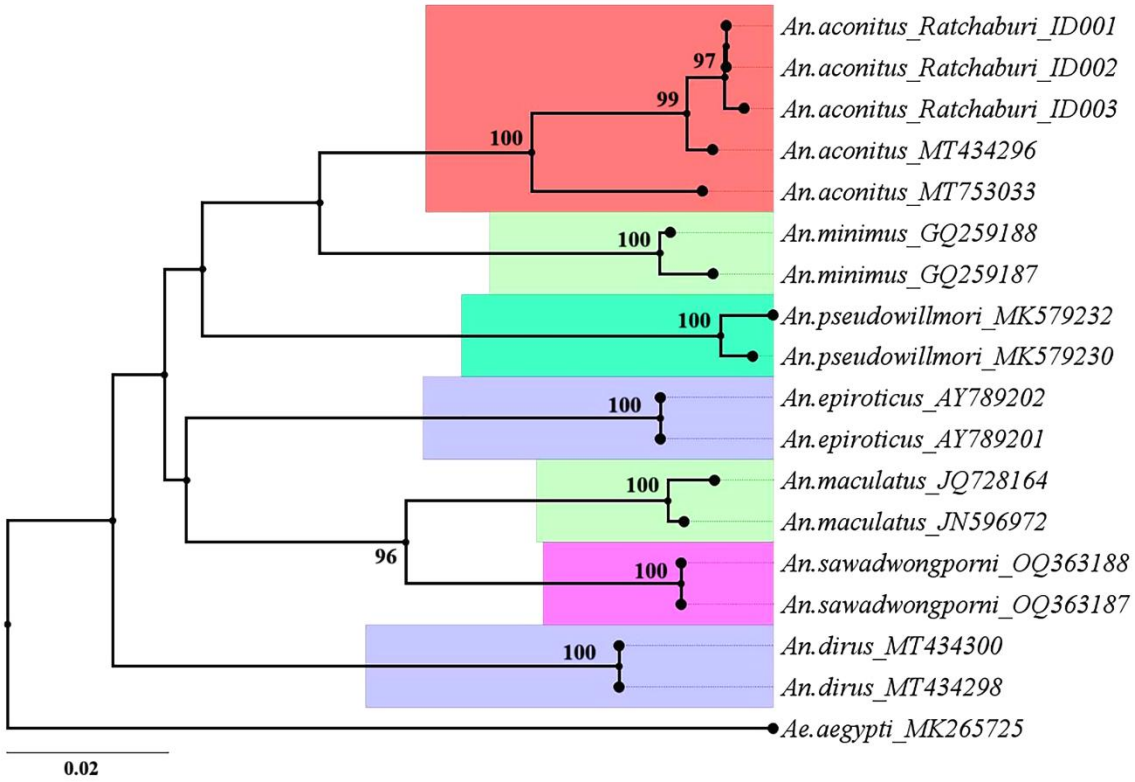
Only DNA reference sequences from the GenBank database with >90% query coverage were selected for comparison with our sequences.

3.2 Phylogenetic tree

The phylogenetic tree elucidates the relationships among *Anopheles* species, showing that our *An. aconitus* samples from Ratchaburi (ID001, ID002, ID003) were grouped with other *An. aconitus* sequences from GenBank (MT434296 and MT753033), indicating they belong to the same species. The *An. aconitus* cluster forms a distinct clade, separate from primary vectors

such as *An. minimus* and *An. maculatus*, and from secondary vectors like *An. pseudowillmori*. This separation is supported by high bootstrap values of 100% at several nodes, confirming that these species are genetically distinct from *An. aconitus*. *Aedes aegypti* is positioned as an outgroup at the bottom of the tree, distinctly separate from all Anopheles species, consistent with its classification outside the *Anopheles* genus.

Figure 1. *Neighbor-Joining (NJ) tree based on COI gene sequences from primary vectors (An. minimus, An. dirus, and An. maculatus) and secondary vectors (An. epiroticus, An. aconitus, An. sawadwongporni, and An. pseudowillmori), which includes three An. aconitus samples collected for this study. Aedes aegypti served as an outgroup. Bootstrap support values of 90% or higher are indicated for significant branches.*



4. Discussion

Anopheles aconitus was first reported from Sumatra, Indonesia, and is believed to be widely distributed from Sri Lanka, India, and Nepal eastward to Hainan Island in China, and southward from southern China through Southeast Asia into Indonesia (Chen et al., 2012). It is considered the primary vector in Malaysia and Indonesia, while in Thailand, it is recognized as a secondary vector of malaria (Chen et al., 2012). Our results indicate that DNA barcoding techniques can efficiently aid in the identification of *An. aconitus*. This effective identification stems from the reference DNA sequences of the *COI* gene of this mosquito species, which are sufficient to serve as reference data for field comparisons (Ratnasingham & Hebert, 2007). In this study, only GenBank reference sequences with greater than 90% query coverage were used for comparison, ensuring the reliability of the species matching process. The consistent alignment of the sequences with a high number of references and percentage identity further validates the

genetic identification of the samples as *An. aconitus*. The results are consistent with previous research that reported this technique as effective in identifying different mosquitoes, such as *Armigeres* (Laojun et al., 2024), *Coquillettidia* (Hernández-Triana et al., 2019), *Culex* (Karthika et al., 2018), *Lutzia* (Somboon & Harbach, 2019), and *Mansonia* (Ruangsittichai et al., 2011) in Thailand. Similarly, the results of the phylogenetic tree support the identification by DNA barcoding, highlighting the genetic differences between *An. aconitus* and other mosquito vectors.

Although this technique is highly efficient, caution should be exercised as DNA sequences in the GenBank database are subject to species errors (Cheng et al., 2023). Therefore, we recommend that a large number of sequences be included for comparison to ensure accurate species identification.

5. Conclusions

The results of this study clearly confirm that the DNA barcoding can effectively help in identifying mosquito species. However, certain species, such as *An. dirus* and *An. baimaii*, cannot be differentiated based on previous studies. Additionally, several DNA sequences in the database have been misidentified. Therefore, it is recommended to include a large number of reference DNA sequences from databases to enhance the reliability of comparisons. Applying this technique to identify mosquito species will make surveillance of mosquito vectors in various areas more effective than relying solely on morphological classification, which is prone to errors.

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References

Chaiphongpachara, T., Changbunjong, T., Laojun, S., Nutepsu, T., Suwandittakul, N., Kuntawong, K., Sumruayphol, S., & Ruangsittichai, J. (2022). Mitochondrial DNA barcoding of mosquito species (Diptera: Culicidae) in Thailand. PloS One, 17(9), e0275090.

Chan-Chable, R. J., Martínez-Arce, A., Mis-Avila, P. C., & Ortega-Morales, A. I. (2019). DNA barcodes and evidence of cryptic diversity of anthropophagous mosquitoes in Quintana Roo, Mexico. Ecology and Evolution, 9(8), 4692–4705.

Chatpiyaphat, K., Sumruayphol, S., Dujardin, J. P., Samung, Y., Phayakkaphon, A., Cui, L., Ruangsittichai, J., Sungvornyothin, S., Sattabongkot, J., & Sriwichai, P. (2021). Geometric morphometrics to distinguish the cryptic species *Anopheles minimus* and *An. harrisoni* in malaria hot spot villages, western Thailand. Medical and Veterinary Entomology, 35(3), 293–301.

Chen, B., Harbach, R. E., Walton, C., He, Z., Zhong, D., Yan, G., & Butlin, R. K. (2012). Population genetics of the malaria vector Anopheles aconitus in China and Southeast Asia. Infection, Genetics and Evolution, 12(8), 1958–1967.

Cheng, Z., Li, Q., Deng, J., Liu, Q., & Huang, X. (2023). The devil is in the details: Problems in DNA barcoding practices indicated by systematic evaluation of insect barcodes. Frontiers in Ecology and Evolution, 11(March), 1–11.

Cox, F. E. (2010). History of the discovery of the malaria parasites and their vectors. Parasites & Vectors, 3(1), 5.

Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. Nucleic Acids Symposium Series, 41.

Hernández-Triana, L. M., Brugman, V. A., Nikolova, N. I., Ruiz-Arrondo, I., Barrero, E., Thorne, L., de Marco, M. F., Krüger, A., Lumley, S., Johnson, N., & Fooks, A. R. (2019). DNA barcoding of British mosquitoes (Diptera, Culicidae) to support species identification, discovery of cryptic genetic diversity and monitoring invasive species. ZooKeys.

Kar, N. P., Kumar, A., Singh, O. P., Carlton, J. M., & Nanda, N. (2014). A review of malaria transmission dynamics in forest ecosystems. Parasites & Vectors, 7, 265.

Karthika, P., Vadivalagan, C., Thirumurugan, D., Ravi, R., Murugan, K., Canale, A., & Benelli, G. (2018). DNA barcoding of five Japanese encephalitis mosquito vectors (*Culex fuscocephala*, *Culex gelidus*, *Culex tritaeniorhynchus*, *Culex pseudovishnui* and *Culex vishnui*). Acta Tropica, 183(April), 84–91.

Laojun, S., Changbunjong, T., Sumruayphol, S., & Pimsuka, S. (2024). Wing geometric morphometrics and DNA barcoding to distinguish three closely related species of *Armigeres* mosquitoes (Diptera: Culicidae) in Thailand. Veterinary Parasitology, 325, 110092.

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The Barcode of Life Data System: Barcoding. Molecular Ecology Notes. 7(3), 355–364.

Ruangsittichai, J., Apiwathnasorn, C., & Dujardin, J. P. (2011). Interspecific and sexual shape variation in the filariasis vectors *Mansonia dives* and *Ma. bonneae*. Infection, Genetics and Evolution, 11(8), 2089–2094.

Somboon, P., & Harbach, R. E. (2019). *Lutzia* (Metalutzia) *chiangmaiensis* n. sp. (Diptera: Culicidae), formal name for the Chiang Mai (CM) form of the genus *Lutzia* in Thailand. Journal of Medical Entomology, 56(5), 1270–1274.

Sumruayphol, S., Chaiphongpachara, T., Samung, Y., Ruangsittichai, J., Cui, L., Zhong, D., Sattabongkot, J., & Sriwichai, P. (2020). Seasonal dynamics and molecular differentiation of three natural *Anopheles* species (Diptera: Culicidae) of the Maculatus group (Neocellia series) in malaria hotspot villages of Thailand. Parasites & Vectors, 13(1), 1–11.

Tainchum, K., Kongmee, M., Manguin, S., Bangs, M. J., & Chareonviriyaphap, T. (2015). *Anopheles* species diversity and distribution of the malaria vectors of Thailand. Trends in Parasitology, 31(3), 109–119.

Tainchum, K., Ritthison, W., Chuaycharoensuk, T., Bangs, M. J., Manguin, S., & Chareonviriyaphap, T. (2014). Diversity of *Anopheles* species and trophic behavior of putative malaria vectors in two malaria endemic areas of northwestern Thailand. Journal of Vector Ecology, 39(December), 424–436.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution, 38(7), 3022–3027.

Weeraratne, T. C., Surendran, S. N., & Parakrama Karunaratne, S. H. P. (2018). DNA barcoding of morphologically characterized mosquitoes belonging to the subfamily Culicinae from Sri Lanka. Parasites & Vectors, 11(1), 266.

World Health Organization. (2020). World malaria report 2020. In WHO Regional Office for Africa.