Original article

Anopheles population belonging to Barbirostris complex in Narathiwat Province and its vectorial status for human and simian malaria

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Background: Besides well-perceived species of *Plasmodium* infecting humans, at least four simian malaria species including *P. knowlesi*, *P. cynomolgi*, *P. inui* and *P. fieldi* have been incriminated in human infections in Thailand. The vectors of simian malaria in this country have been recently identified as *Anopheles latens* and *An. introlatus*.
Objectives: To address whether mosquitoes in Barbirostris complex could be potential vectors of simian malaria and to analyze species composition of *Anopheles* in this complex from the sampled population.
Methods: During 2018 and 2019, a total of 115 female *Anopheles* collected by human-landing catches from Sukhirin and Waeng Districts in Narathiwat Province morphologically assigned to Barbirostris complex were included for analysis. Deoxyribonucleic acid (DNA) was extracted from salivary gland samples of individual mosquitoes. Molecular identification of mosquito members of Barbirostris complex was performed by polymerase chain reaction (PCR) amplification of the internal transcribed spacer 2 (*ITS2*) region and its partial flanking sequences of *Anopheles* ribosomal ribonucleic acid (*rRNA*) cistron, followed by sequencing. Analysis of individual salivary gland samples was performed by species-specific nested PCR targeting mitochondrial cytochrome oxidase I of four human *Plasmodium* species, *P. knowlesi*, *P. cynomolgi, P. inui* and *P. fieldi.*
Results: Two amplicon lengths comprising ~1.9 and ~1 kb fragments were observed in 72 and 43 mosquitoes, respectively. Sequence analysis of the 5' portion of *ITS2* from 21 randomly selected mosquitoes has shown two distinct phylogenetic clades. All 12 mosquitoes bearing 1.9-kb amplicons displayed perfect sequence identity across samples and were placed in a clade related to but distinct from *An. saeungae*. Nine specimens with 1-kb amplicons were clustered within *An. barbirostris* species A3. All salivary gland samples gave negative results for malarial DNA.
Conclusion: Two genetically distinct lineages have been identified in Barbirostris complex in Narathiwat Province. None of these carried malarial DNA in their salivary glands, suggesting that they might not be vectors of simian malaria.

Keywords: *Anopheles barbirostris*, internal transcribed spacer 2, malaria vector, primate malaria.

In malaria endemic areas, transmission of the disease requires occurrence and persistence of *Plasmodium*-infected humans and co-existence of

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Anopheles vectors where asexual multiplication and sporogonic development can be maintained in these respective hosts. It has been well perceived that malaria parasites incriminated in human infections belong to five species including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale wallikeri* and *P. ovale curtisi*, all of which have a wide geographic distribution in tropical regions.[1] Besides these plasmodia, some simian malaria species in Southeast Asia can cross transmit from macaque natural hosts to humans via

mosquito vectors, leading to a range of symptomatic illnesses.[2] Of these, at least six nonhuman primate malaria species have been identified to cause human infections in Thailand, comprising *P. knowlesi*, *P. cynomolgi*, *P. inui* and *P. fieldi*.[3-6] The distribution of simian malaria in humans seems to coincide with the natural habitats of the reservoir hosts including long-tailed (*Macaca fascicularis*) and pig-tailed (*M. nemestrina*) macaques.[7] Importantly, malaria caused by *P. knowlesi* may present with uncomplicated symptoms while severe and fatal infections have been reported in humans.[8, 9] Although the prevalence of human malaria in Thailand has dramatically declined since the turn of this century due to integrative control measures implemented across endemic areas, the occurrence of simian malaria in humans has challenged disease elimination of the country.[10] Meanwhile, during the past decade, an increased prevalence of malaria caused by *P. knowlesi* has been observed in diverse malaria endemic areas while more cases have been detected in the Southern provinces of the country.[5, 6] For instance, during the past five years the number of malaria patients in Narathiwat Province has been decreasing from 74 cases in 2018 to 23 cases in 2022 in which *P. vivax* remarkably exceeded *P. falciparum*, accounting for 64.3% to 93.3% and 5.4% to 21.4%, respectively. Importantly, sporadic cases of *P. knowlesi* infections have been observed in this province, contributing to 0.0% to 13.0% during these years.[10] Unlike other human malaria, simian *Plasmodium* infections seem to be more recalcitrant to control because natural infections with simian malaria in macaques are mainly asymptomatic while the infections can be prevalent and may spillover to humans.[7, 11] While control of simian malaria in macaque reservoir hosts remains to be elusive, other interventions are required such as vector control and preventive precaution to avoid disease acquisition from the infective mosquitoes.

In Thailand, *Anopheles* vectors of human malaria have been identified and characterized by their vectorial capacity into three groups: 1) primary vectors consisting of *Anopheles dirus*, *An. maculatus* and *An. minimus*; 2) secondary vectors including *An. aconitus*, *An. sundaicus* and *An. pseudowillmori;* and 3) suspected vectors comprising *An. sawadwongporni*, *An. philippinensis*, *An. campestris* and *An. barbirostris*.[12, 13] Differential geographic distribution and bionomic differences occurred among these vectors that have implications

for control measures.[13] Our recent surveys have revealed that the vectors of simian malaria in Thailand have recently identified in Narathiwat Province in which *An. latens* and *An. introlatus*, members of the Leucosphyrus complex carried *P. knowlesi* in their salivary glands.[14] Importantly, feeding behaviors of these mosquitoes in the sampling areas were anthropophilic and preferentially occurred outdoor during the first quarter of the nights. This information has important implication for setting precautionary measures to avoid direct contact with the vectors.

During our mosquito surveys in Narathiwat Province, the predominant *Anopheles* mosquitoes caught by using human landing catches belonged to *An. maculatus* and *An. barbirostris*, accounting for 37.1% and 31.3% respectively.[14] Importantly, a recent survey of simian malaria vectors in Sarawak has identified *An. donaldi*, a member of Barbirostris Group, harboring *P. knowlesi* in their salivary glands, suggesting the potential role of this species as a simian malaria vector.[15] Besides *An. donaldi*, other mosquito members of Barbirostris Group that have been identified in Thailand include *An. hodgkini*, *An. pollicaris* and some species in Barbirostris complex.[13] Intriguingly, it remains unknown whether other members of Barbirostris Group could vector simian malaria.

This study aimed to analyze the vectorial potential of *An. barbirostris* in Narathiwat Province where both human and simian malaria in humans have been prevalent. Furthermore, species composition and phylogeny of *Anopheles* population belonging to Barbirostris complex were investigated by inferring from the internal transcribed spacer 2 (*ITS2*) sequences of the *rRNA* cistron. Due to a wide geographic distribution and differential abundance of mosquitoes in Barbirostris Group [16], this study could provide baseline information on species distribution and their potential to vector simian malaria in an area with relatively high prevalence of zoonotic malaria in Thailand.

Materials and methods
Ethical approval
This study was reviewed and approved by the Institutional Review Board in Human Research of Faculty of Medicine, Chulalongkorn University (IRB no. 272/61, COA no. 841/2019) and Naresuan University Institutional Review Board of Human Research (IRB no. 0614/62, COA no. 057/2020). Prior

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to serving as human baits for mosquito collection, written informed consent was obtained from all subjects. All procedures were performed in accordance with the relevant guidelines and regulations.

Mosquito samples

In total, 115 female anopheline mosquitoes morphologically identified as Barbirostris group from Sukhirin and Waeng Districts in Narathiwat Province were included in this study (Figure 1). These mosquitoes have been collected by human-landing catches for 12 hours per night from 6.00 PM to 6.00 AM for 40 nights in which detail of collection procedure has been described in our previous report.[14]

All mosquitoes assigned to *An. barbirostris* were based on morphological identification as follows: 1) entirely dark-scaled proboscis; 2) palpus with dark, numerous and erect scales; 3) pedicel containing dorsal and lateral scales; 4) clypeus without scales; 5) presence of three large dark spots on costa of wings; 6) veins R-R1 with costa lacking a presector pale spot and apex with two small fringe spots; 7) abdominal sterna with median patches of pale scales and rows of pale scales on lateral margins; and 8) VII-S with a tuft of dark scales (Figure 2). After morphological identification, the salivary glands of each mosquito were dissected under stereomicroscope and kept in absolute ethanol for preservation.

Figure 1. (A) Map of Thailand showing Narathiwat Province; and **(B)** sampling locations (black spots) in Sukhirin and Waeng Districts.
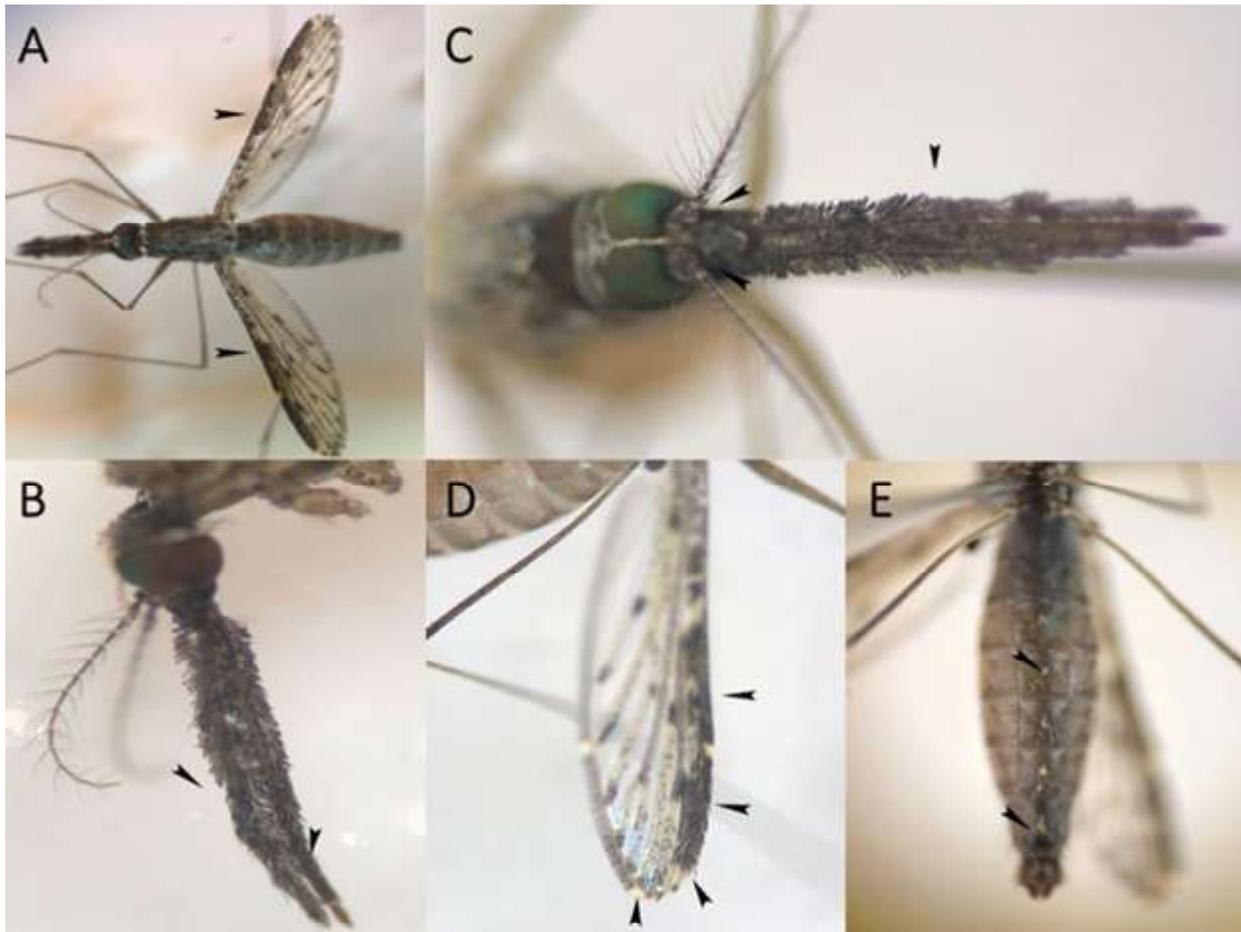
Figure 2. Adult female mosquito in Barbirostris complex: **(A)** dorsal view of the mosquito; **(B)** and **(C)** head with entirely dark-scaled proboscis; palpi with dark, numerous and erect scales; pedicel with dorsal and lateral scales; and clypeus without scales; **(D)** wing with three large dark spots on costa and veins R-R1; costa lacking a presector pale spot; and apex with two small fringe spots; and **(E)** abdomen containing sterna with median patches of pale scales and rows of pale scales on lateral margins; and VII-S with a tuft of dark scales.

DNA preparation

Prior to DNA extraction, ethanol was allowed to evaporate from mosquito's salivary glands. DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was deployed for DNA extraction and purification of each salivary gland sample as described by the manufacturer's recommendation. Salivary gland DNA from each mosquito was kept in a separate microtube and stored at - 40°C until use.

Amplification and sequencing of Anopheles ITS2

Polymerase chain reaction (PCR) primers for amplifications of the complete internal transcribed spacer 2 (*ITS2*) region of *Anopheles* RNA cistron were derived from the 3' portion of *5.8S rRNA* and the 5' portion of *28S rRNA*. The forward and reverse primers were AL58sF0 (5'-ATGAAGACCGCA GCTAAATGCG -3') and AL28sR0 (5'-ATGCTT AAATTTAGGGGGGTAGTC-3'), respectively.

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Amplification of the complete *Anopheles* ITS2 and its flanking regions was done in a total volume of 30 ml of the reaction mixture containing template DNA, 300 mM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.3 mM of each primer, 3 ml of 10X PCR buffer, and 1.25 units of ExTaq DNA polymerase (Takara, Seta, Japan). Thermal cycler profile contained preamplification denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 40 s, 58°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 4 min. Amplification was performed in a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). Amplicons were separated in 1% agarose electrophoresis with lambda/*Hin*d III marker as a size reference and visualized under an ultraviolet (UV) transilluminator. Prior to DNA sequencing, the amplicons were purified by using QIAquick PCR purification kit (Qiagen, Germany). Due to species-specific variation across the *ITS2* region of among members of Barbirostris complex, sequences of the 5' portion of the amplicons were determined from 21 randomly selected mosquito specimens from both districts. Sequencing was performed on an ABI3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) using the forward PCR primer for sequencing.

Identification of human and simian plasmodia

Each salivary gland DNA sample was analyzed by species-specific nested PCR assays targeting the mitochondrial cytochrome *c* oxidase subunit 1 of human and simian malaria parasites including *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi, P. cynomolgi, P. inui* and *P. fieldi*. Amplification reactions and thermal cycler profiles were essentially as reported.[6] Positive control DNA of these samples were from previously verified species of *Plasmodium* infecting humans and macaques as previously described.[5, 6, 17]

Data analysis

Nucleotide sequences were aligned by using MUSCLE program.[18] As for *Anopheles ITS2* references, sequences of known species with the following GenBank accession nos. were included in the phylogenetic analysis: *An. vanderwulpi* (EU 812765, EU 812767 and EU 812768), *An. dissidens* (EU 812769, EU 812777 and EU 812786), *An. saeungae* (EU 812798-EU 812800), *An.*

wejchoochotei (EU812808 and EU812809), *An barbirostris* from Peninsular Malaysia (KJ 462237, KJ 462240 and KJ 462245), *An. barbirostris* species A3 (AB 362232-AB362234), *An. donaldi* (MT623054-MT863055), *An. korei* (MW546413 and MW546416) and *An. freyi* (KC808753 and KC808754). All sites at which the alignment postulated a gap were eliminated in pairwise comparisons of the analysis. Phylogenetic trees were constructed by using the maximum likelihood method with Kimura 2-parameter model and a discrete Gamma distribution to model evolutionary rate differences among sites as implemented in the MEGA version 6.0 program.[19] One thousand bootstrap pseudoreplicates were deployed to assess the confidence levels of the branching patterns in the phylogenetic tree.

Results

Abundance of mosquitoes in Barbirostris complex

Based on 40 nights of sample collection during 2018 and 2019, 85 (73.9%) and 30 (26.1%) mosquitoes belonged to *An. maculatus* s.l. and Barbirostris complex were obtained from Waeng and Sukhirin Districts, respectively. The number of *Anopheles* mosquitoes belonging to Barbirostris complex collected from Waeng District (n = 94, 81.7%) outnumbered that caught from Sukhirin District (n = 21, 18.3%). Since sample collection was performed during corresponding times and periods for both sampling sites, it was estimated that the biting rates of mosquitoes belonging to Barbirostris complex in Waeng and Sukhirin Districts were 0.106 and 0.038 mosquitoes collected per night per collector.

Amplicons and sequences of ITS2

Amplification of the *ITS2* fragment encompassing the 3' portion of *5.8S rRNA* and complete *ITS2* region and 5' portion of *28S rRNA* of 115 mosquitoes in Barbirostris complex has shown two distinct amplicon lengths comprising ~1.9 kb- and ~1-kb fragments, corresponding to ~1.8 kb and ~900 bp of *ITS2*, respectively. Of the 85 mosquitoes from Waeng District, 51 samples (60.0%) possessed ~1.9-kb amplicons whereas the remaining 34 specimens (40.0%) harbored ~1-kb PCR products. Meanwhile, both amplicon lengths of the *ITS2* fragments were detected among mosquitoes belonging to Barbirostris complex from Sukhirin District in which 21 (70.0%) and 9 (30.0%) samples contained ~1.9- and ~1-kb

PCR products, respectively (Figure 3). Based on randomly selected 21 DNA samples of these mosquitoes from Sukhirin (n = 10) and Waeng (n = 11), sequences spanning ~500 bp corresponding to the 5' portion of the *ITS2* region could be assigned into two types. All 13 specimens from the ~1.9-kb amplicons shared perfect sequence identity across nucleotide sites compared. Meanwhile, the remaining 8 samples from ~1-kb PCR fragments possessed identical sequences but distinct from those obtained from ~1.9-kb amplicons.

Phylogenetic tree

Maximum likelihood phylogenetic tree inferred from the 663-aligned nucleotide sites of the *ITS2*

region has shown that the ~1.9-kb amplicons were placed together with those belonging to an unnamed member of Barbirostris complex from Peninsular Malaysia (GenBank accession nos. KJ462237, KJ462240 and KJ462245).[20] Eight sequences of mosquitoes bearing ~1-kb PCR products displayed perfect sequence identity and contained 2 nucleotide differences in the 5' portion of *ITS2* when compared with those of *An. barbirostris* species A3 from Kanchanaburi Province (AB362232-AB363234). Importantly, the estimated full length of the *ITS2* region of ~1-kb amplicons in this study was in good agreement with the corresponding lengths of *An. barbirostris* species A3 as displayed in Figure 4.
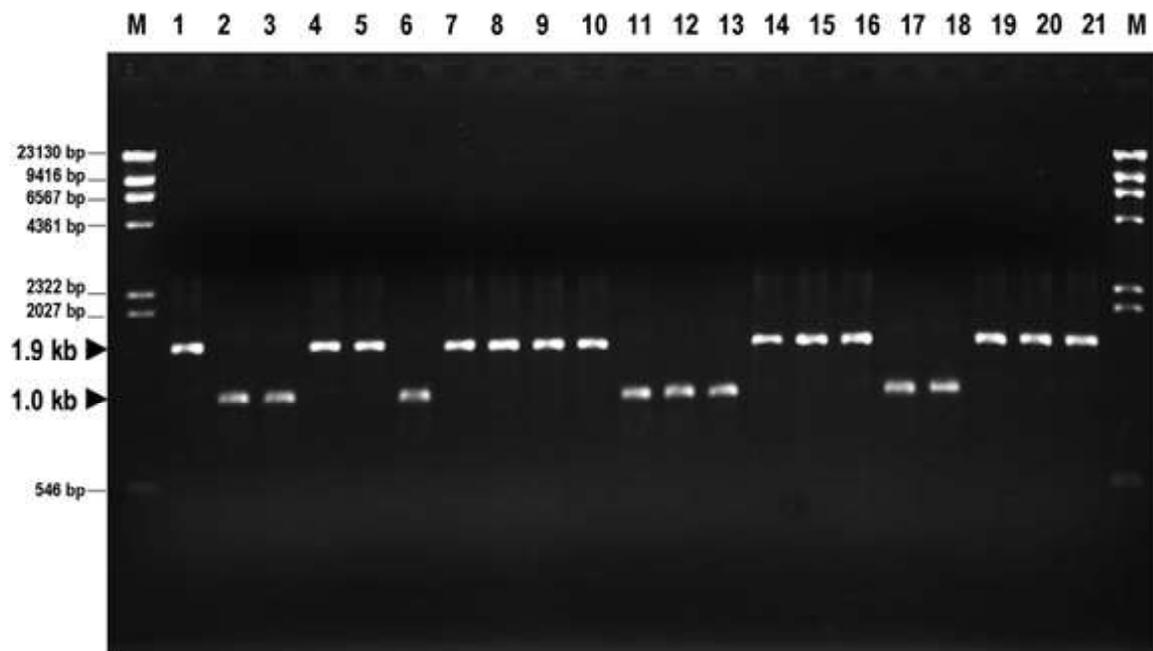
Figure 3. Agarose gel electrophoresis showing the amplicons encompassing ITS2 and its flanking regions of *Anopheles* mosquitoes in Barbirostris complex from Sukhirin (lanes nos. 1-10) and Waeng Districts (lane nos. 11-21). Lanes M are lambda/*Hin*d III markers.

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Figure 4. Maximum likelihood phylogenetic tree depicting mosquito species belonging to Barbirostris group/complex. GenBank accession numbers are in parentheses. Bootstrap values greater than 50.0% are shown along the branches. Scale bar represents number of nucleotide substitutions per site. Taxa with Sukhirin (square) and Waeng (triangles) initials denote mosquito samples from each district.

Malaria parasites in salivary gland samples

Because the *Anopheles ITS2* sequences could be efficiently amplified from all 115 mosquitos, the integrity of all DNA from salivary gland samples should be suitable for subsequent molecular detection of malaria parasites. Based on the PCR assay, the previously verified DNA samples of *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale, P. knowlesi, P. cynomolgi, P. inui* and *P. fieldi* gave positive results when using corresponding species-specific primers. However, none of 115 salivary gland samples gave positive test for these human and simian *Plasmodium* species in as shown Figure 5.

Discussion

Accurate identification of *Anopheles* species responsible for malaria transmission has important implication for disease control measures. Furthermore, knowledge of temporal and spatial distribution of malaria vector species including their breeding places and bionomics is essential for prediction of the extent of malaria transmission across endemic areas and timely prevention when focal outbreaks occur. For example, misidentification of a zoophilic mosquito *An. varuna* as *An. minimus*, a highly anthropophilic species, resulted in misdirection of control measures as well as inefficient expenses for intervention activities in central Vietnam. [21] On the other hand,

Figure 5. Agarose gel electrophoresis showing species-specific nested PCR amplicons targeting the mitochondrial cytochrome *c* oxidase subunit 1 of human and simian malaria parasites. Positive control products of *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi, P. cynomolgi, P. inui* and *P. fieldi* are in lane nos. 1 - 8, respectively. Representative results from mosquito salivary gland DNA are in lane nos. 9 - 19. Lane M is a 100-bp ladder marker.

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accurate identification of vectors responsible for a resurgence of malaria cases in eastern Thailand has prompted active and directional interventions for disease control. [22, 23]

During the past decade, a remarkable decline in the number of malaria cases mainly due to *P. falciparum* and *P. vivax* has been observed in Thailand while sporadic infections caused by *P. knowlesi* have been more frequently identified. Although the reasons for potential resurgence of simian malaria in humans remain elusive, several possible factors could be considered such as: 1) changes in habitats of macaque reservoir hosts due to deforestation; 2) more ecotourism resulting in an increased risk of acquiring infection from natural forest-dwelling mosquito vectors and some of which might transmit simian malaria; 3) a decline in the number of malaria caused by human *Plasmodium* species could render simian malaria parasites propagate in a non-competitive environment within hosts; 4) an increased population of certain strains of simian plasmodia with adaptive capability in both humans and non-human primates; and 5) behavioral changes in vectors of simian malaria parasites from strict zoophily to combined zoophily and anthropophily.[24] Nevertheless, control of simian malaria in macaque natural hosts seems to be problematic due to their wide geographic distributions in Southeast Asia while infected macaques usually remain asymptomatic. On the other hand, vector control and prevention of mosquito bites could be an alternative measure.

The main vectors of *P. knowlesi* and other simian malaria parasites are mosquitoes in Leucosphyrus group. Of these, *P. knowlesi*, either sporozoites or its DNA, has been detected in salivary glands of *An. hackeri*, *An. dirus*, *An. introlatus*, *An. latens*, *An. balabacensis* and *An. cracens* in Peninsular Malaysia, Malaysian Borneo, Vietnam and the Philippines.[25] Our recent surveys of *Anopheles* mosquitoes in Narathiwat Province have identified *An. latens* and *An. introlatus* as vectors of *P. knowlesi* and other simian malaria parasites.[14] Consistently, *P. knowlesi*, *P. cynomolgi* and *P. inui* infections have been diagnosed in humans and macaques in southern Thailand including Yala and Narathiwat Provinces[4-6, 11]; thereby, the transmission cycles of these simian malarias have been maintained in the regions. Besides mosquitoes in Leucosphyrus group, other *Anopheles* species have been recently found to vector *P.*

knowlesi including *An. collessi* and *An. roperi* belonging to Letifer subgroup, *An. donaldi* in Barbirostris subgroup[15, 26, 27], and *An. sundaicus* in Sundaicus complex.[28] Therefore, it is possible that *P. knowlesi* and other simian malaria species could deploy a variety of *Anopheles* mosquitoes for transmission disease to vertebrate hosts. Interestingly, mosquitoes in Barbirostris complex seemed to be prevalent in Narathiwat Province while members of this group have been known to vector human malaria.

Mosquitoes classified in Barbirostris group are prevalent and widely distributed from India, Southeast Asia to some Western Pacific Islands.[16] Despite morphological similarity, different members of this complex display differential vectorial capacity for malaria and filariasis.[16] Female mosquitoes in Barbirostris group are anthropophilic and outdoor feeders from dusk till dawn albeit having some zoophilic tendency toward bovids.[29-31] Their breeding places encompass a wide range of fresh water habitats such as animal footprints, rice fields, canals, rivers, marshes and lakes with some organic materials or vegetation. To date, at least 8 species of mosquitoes in Barbirostris group have been identified in Thailand including *An. donaldi*, *An. hodgkini*, *An. pollicaris*, *An. barbirostris* s.s., *An. campestris*, *An. dissidens*, *An. saeungae*, *An. wejchoochotei* and some unnamed species. Because of morphological similarity among members of Barbirostris complex, molecular analysis has enabled accurate species identification. The *ITS2* region of mosquitoes in this complex exhibited both sequence and length variation among species. Of 115 mosquitoes belonging to Barbirostris complex in this study, PCR amplification of the complete *ITS2* region has shown two amplicon sizes of ~1.9 and ~1 kb, corresponding to two unnamed species: 1) *An. barbirostris* species whose sequences were identical with mosquitoes from Peninsular Malaysia (e.g. GenBank accession nos. KJ462240 and KJ462245); and 2) *An. barbirostris* species A3 previously identified in Kanchanaburi Province (AB362232-AB362234). The occurrence of shared sequences or closely related sequences of these two *Anopheles* species/strains in this study with other mosquitoes from other geographic habitats has implied their adaptation to diverse environment. Previous surveys by others have shown that members of Barbirostris group caught in Narathiwat Province comprised *An. wejchoochotei, An. dissidens* and *An. saeungae* [32, 33] while *An. barbirostris* s.s. and

An. campestris have not been identified in this province. Our study has expanded the species composition of mosquitoes in Barbirostris complex in Narathiwat Province while cross-country distribution of an unnamed member of this complex (e.g. KJ462240 and KJ462245) has been observed.

All salivary gland samples of *Anopheles* in this study did not possess human and simian malarial DNA by PCR analysis. It is noteworthy that the presence of malarial DNA or sporozoites in mosquito's salivary glands may not directly indicate the vectorial status of the mosquitoes because some sporozoites may not be infective to their vertebrate hosts. However, repeated identification of specific malarial DNA or sporozoites from a given mosquito species can be highly suggestive for its vectorial capacity.[12 - 14] On the other hand, the absence of malarial DNA or sporozoites in mosquito salivary glands may occur when midgut oocysts have not completely developed to reach mature sporozoite stage. Therefore, further studies are required to exclude such possibility.[2]

The role of mosquitoes in Barbirostris group as *P. falciparum* and/or *P. vivax* vectors has been reported from diverse geographic areas in Thailand including Sakaeo, Prachuap Khiri Khan and Chumporn, Chanthaburi and Tak.[22, 34 - 37] Furthermore, this mosquito group has been incriminated in transmission of human malaria in Sri Lanka and Timore-Leste[37, 38] Although *Anopheles* in Leucosphyrus group has been identified to vector simian malaria in Narathiwat Province[14], none of the sympatric mosquitoes in Barbirostris group analyzed herein had detectable malarial DNA in their salivary glands. Therefore, evidence for these *Anopheles* mosquitoes to vector human and simian malaria in southern Thailand has not been confirmed in this study. Nevertheless, further investigation by including more *Anopheles* specimens and more diverse species would be required to document additional potential vectors of simian malaria in southern Thailand.

Conclusion

Two genetically distinct lineages have been identified in Barbirostris complex in Narathiwat Province. None of these carried malarial DNA in their salivary glands, suggesting that they might not vector simian malaria. However, further studies using more specimens and including other known human malaria vectors in other malaria endemic regions of the country would be important to unravel the roles of these mosquitoes in disease transmission.

Conflict of interest statement

Each of the authors has completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to content of this article.

Data sharing statement

The present review is based on the reference cited. Further details, opinions, and interpretation are available from the corresponding authors on reasonable request.

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