Molecular Identification of Native Wolbachia Pipientis in Anopheles Minimus in a Low-Malaria Transmission Area of Umphang Valley Along the Thailand-Myanmar Border

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Research

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Abstract

Background

Wolbachia, obligate intracellular bacteria, infect the majority of arthropods, including many mosquito species of medical importance. Some *Wolbachia* strains interfere with the development of *Plasmodium* parasites in female *Anopheles*, a major vector of malaria. The use of Wolbachia as a means to block malaria transmission is an emerging vector control strategy in highly endemic areas. Hence, identification of native *Wolbachia* strains in areas where malaria transmission is low may uncover a particular *Wolbachia* strain capable of *Plasmodium* interference. This study aims to identify native *Wolbachia* strains in female *Anopheles* spp. that are predominant in a low-malaria transmission area in mainland Southeast Asia.

Methods

Following a two-year survey of malaria vectors in Umphang Valley of Tak Province, Thailand, DNA extracts of female *An. minimus*, *An. peditaeniatus*, *An. maculatus*, and *An. dirus* were subjected to amplification of the conserved region of the 16S rRNA-encoding gene. The DNA sequences of the amplicons were phylogenetically compared with those of known *Wolbachia* strains.

Results

Among four *Anopheles* spp., amplification was detected in only the DNA samples from *An. minimus*. The DNA sequencing of amplicons revealed 100% similarity to *Wolbachia pipientis*, confirming the specificity of amplification. The phylogenetic trees indicate a close relationship with *Wolbachia* strains in subgroup B.

Conclusion

To the best of our knowledge, the data presented herein provide the first molecular evidence of a *Wolbachia* strain in *An. minimus*, hereinafter named *w*Anmi, in a low-malaria transmission area in the Umphang Valley of western Thailand. Further biological characterization is required to examine its potential for malaria transmission control in the field.

Background

Malaria is a vector-borne parasitic disease caused by *Plasmodium* species. Ongoing malaria control programs have significantly decreased morbidity and mortality in Africa [1] and Asia [2] [3]; however, many parts of the border regions, especially in Southeast Asia, are still malaria-endemic areas [3]. Given that *Anopheles* mosquitoes are malaria vectors, one of the effective strategies to control malaria transmission relies on the use of insecticides, including indoor residual spraying and the use of

insecticide-treated mosquito nets. Accordingly, resistance to insecticides has emerged as a biological threat to malaria control and elimination efforts in endemic areas, including many regions located in forest-mountain landscapes along the Thailand-Myanmar [4] and Thailand-Laos PDR borders [5]. Widespread insecticide resistance has increased the malaria burden in many malaria-endemic regions [6], challenging global malaria eradication. Thus, an effective alternative to insecticides is needed.

Wolbachia is an intracellular bacterium that naturally infects the majority of insect species [7]. *Wolbachia* bacteria reside in the cytoplasmic vacuoles of various types of insect somatic and germ cells, allowing maternal transmission to their progeny. *Wolbachia* is capable of manipulating host reproduction through cytoplasmic incompatibility, in which *Wolbachia*-infected eggs form viable offspring and noninfected eggs do not, leading to widespread *Wolbachia* infection in an insect population, including mosquito species of medical importance. Given failures in the detection of *Wolbachia* in *Anopheles* mosquitoes, it was initially hypothesized that *Anopheles* mosquitoes are refractory to *Wolbachia* infection [8] [9] [10] [11]. However, a study demonstrated that the *Aedes albopictus*-specific *Wolbachia* strain AlbB (*w*AlbB) could infect laboratory-reared *An. stephensi* and suppress the development of *P. falciparum* within female *Anopheles* mosquitoes [12]. In concordance with the laboratory study, *Wolbachia* infections were observed in natural populations of *An. gambiae* and *An. coluzzii*, two major vectors in malaria-endemic regions of Burkina Faso [13] [14]. Interestingly, researchers phylogenetically identified *Anopheles*-infecting *Wolbachia* as a new arthropod-specific subgroup named *w*Anga [13]. Previous reports have shown evidence of natural *Wolbachia* endosymbiosis in other *Anopheles* species as well as its effects on *Plasmodium* development. In the high-malaria transmission area of Burkina Faso, a field study showed that natural infection with *Wolbachia* strain *w*Anga in blood-fed *An. coluzzii* females was negatively correlated with *Plasmodium* development [15]. Based on a mathematical model, natural *Wolbachia* infection potentially blocks malaria transmission from vector to human [15]. Further, infection by the *Wolbachia* strain *w*Anga-Mali in *An. gambiae* was associated with a reduced prevalence and intensity of sporozoite infection in field-collected females in Mali [16]. Altogether, studies strongly suggest that *Anopheles* mosquitoes are permissible to *Wolbachia* endosymbiosis and that some strains of *Wolbachia* are capable of interfering with the development of *Plasmodium* parasites in female *Anopheles*. Thus, the release of laboratory-reared, *Wolbachia*-infected *Anopheles* mosquitoes to replace the wild *Anopheles* population is a potential strategy to block malaria transmission. Hence, identification of native *Wolbachia* strains in areas where malaria transmission is low may uncover a particular *Wolbachia* strain capable of interfering with *Plasmodium* development in *Anopheles*. Considering the availability of DNA extracts from major *Anopheles* species obtained during a two-year survey of malaria vectors [17], this study aims to identify native *Wolbachia* strains in female *Anopheles* spp. that are predominant in a low-transmission area in Umphang Valley, located near the Thailand-Myanmar border of mainland Southeast Asia.

Methods

Biosafety for using biological samples of mosquitoes

The protocol for the use of DNA samples extracted from *Anopheles* mosquitoes was approved by the Siriraj Safety Risk Management Taskforce, Faculty of Medicine Siriraj Hospital, Mahidol University (SI2020-010). In accordance with the guidelines for ethics in animal use, this study submitted the DNA extraction protocol and sampling details to the Siriraj Animal Care and Use Committee, Faculty of Medicine Siriraj Hospital, Mahidol University (COA 012/2563) and received permission.

Collection and identification of Anopheles species

Since this study is an extension of a previously published report, we did not collect and identify *Anopheles* species. For detailed collection methods, we highly recommend reading the original article [17]. The collection site was located in Ban Nong Luang village (16°04'36.3"N 98°45'8.0"E), Umphang District of Tak Province, located in western Thailand (Fig. 1A). The village is located approximately 4 km from the border of Kayin state, Myanmar. Mosquitoes were captured for five consecutive nights every two months from February 2015 to December 2016. The standard mosquito landing collection procedure followed that in a previous report [18]. Mosquito capture methods included indoor human landing, outdoor human landing and cattle-baited outdoor collections. Mosquitoes were identified following a standard dichotomous key [19]. For molecular identification, DNA from individual mosquitoes was extracted from the head and thorax [20] and subjected to PCR.

Amplification of the Wolbachia-specific 16S rRNA coding region

To amplify a conserved region of the *Wolbachia* 16S rDNA-encoding gene, W-SpecF and W-SpecR primers were used in initial standard PCR, and 16SNF and 16SNR primers were used in the nested PCR, following a previous report [21] (Fig. 2A). Primer sequences are shown in Table 1. Samples were prepared with a total volume of 10 µL, which was composed of 0.5 µM of each primer, one µL of DNA template and AccuStart™ II Gel Track PCR SuperMix (Quantabio, Beverly, MA, USA). Amplification was performed with DNA template denaturation at 95 °C for 3 min; followed by 35 cycles of DNA denaturation at 95 °C for 15 sec, primer annealing at 50 °C for 25 sec, and DNA extension at 72 °C for 30 sec; and final extension at 72 °C for 5 min. To visualize the DNA bands, the PCR products were mixed with ViSafe Red (Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia) and subsequently electrophoresed in 2% agarose gel in 1x TAE buffer at a voltage of 100 V for 45–50 min. The ViSafe Red-intercalated, double-stranded DNA sequences were then exposed to UV light (Molecular Imager® Gel Doc™ XR System, Bio-Rad Laboratories, Inc., Hercules, California) for visualization. The length of the amplicon yielded from the initial PCR was approximately 438 bp in length. Subsequently, one µL of the initial PCR product was used as template for the nested PCR, in which 16SNF and 16SNR primers to bind to the internal sequence of W-SpecF and W-SpecR were added (Fig. 2A, lower panel). The thermal cycles included initial denaturation at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 15 sec, primer annealing at 60 °C for 25 sec and extension at 72 °C for 30 sec; with a final extension at 72 °C for 5 min. The length of the amplicon yielded from the nested PCR was approximately 412 bp in length. To confirm specificity, the 412-bp amplicons were purified from the agarose gel and sequenced by an ABI 3730XL DNA Analyzer

(Bionics, Seoul, South Korea). The 16SNF and 16SNR primers were used as DNA sequencing primers. DNA extracts of *Mansonia* mosquitoes were used as the positive control. PCR without DNA template was used as the negative control.

Table 1
Primers used in this study

Primer names	Primer sequence (5'-3')
W-SpecF	CATACCTATTCGAAGGGATAG
W-SpecR	AGCTTCGAGTGAAACCAATTC
16SNF	GAAGGGATAGGGTCGGTTCG
16SNR	CAATTCCCATGGCGTGACG

Bioinformatics

The obtained sequences of the *Wolbachia* 16S rRNA fragment were edited and assembled using BioEdit Sequence Alignment Editor (version 7.2.5). DNA sequences were deposited in GenBank (accession numbers MT449018 and MT449019). To identify similar sequences, the GenBank database was searched with BLASTN [22]. For sequence alignment, the following sequences of the 16S rRNA-encoding gene of *Wolbachia* subgroup B were obtained: *Wolbachia* strain *w*No from *Drosophila simulans* (CP003883.1), strain *w*AlbB from *Ae. albopictus* (KX155506.1) and *w*Pip from *Culex quinquefasciatus* (AM999887.1). Multiple sequence alignment was performed using MSAViewer.

Phylogenetic Analysis

To assess the relationship of wAnmi_UmpP21 and wAnmi_UmpP32 with other known *Wolbachia* strains, we assembled a phylogenetic tree to determine genetic similarity and heterogeneity based on the conserved region of the *Wolbachia* 16S rRNA-encoding gene (Fig. 2A and 2C), a heritable region in prokaryotes. To validate the output data, we employed rooted and unrooted phylogenetic inference methods. Based on an assumption of a common ancestral path, the rooted maximum likelihood phylogram illustrated that both *w*Anmi_UmpP21 and *w*Anmi_UmpP32 were genetically related to *Wolbachia* subgroup B (Fig. 3). *w*Anm_UmpP21 was in the same cluster as the *w*Pip strain from *Cx. quinquefasciatus* and the *w*AlbB strain from *Ae. albopictus*. In contrast, *w*Anmi_UmpP32 was closely related to *w*Anga isolated from *An. gambiae* in Burkina Faso (wAnga_BF) and *w*Anga isolated from *An. arabiensis* in Tanzania (wAnga_TZ140) (Fig. 3). To reveal the extent of genetic similarity between the *Wolbachia* members in subgroup B, we aligned the *Wolbachia* 16S rRNA-conserved regions of the *w*No, *w*AlbB, *w*Pip, *w*Anmi_UmpP21 and *w*Anmi_UmpP32 strains (Fig. 4). One hundred percent similarity was observed among the *w*AlbB, *w*Pip and *w*Anmi_UmpP21 strains. However, *w*Anmi_UmpP32 had two single nucleotide polymorphisms at two locations, implying genetic variation in *Wolbachia* in wild *An. minimus*

in Umphang Valley. In agreement with the rooted maximum likelihood phylogenetic tree, excluding the assumption of a common ancestor, the unrooted, bifurcating phylogenetic tree revealed that *w*Anmi_UmpP21 and *w*Anmi_UmpP32 clustered in the leaf node of *Wolbachia* subgroup B, confirming a close genetic relationship (Fig. 5).

Results

Number and type of Anopheles collected from the field

A total of 745 DNA samples were obtained from *An. minimus* (n = 401, 54%), *An. peditaeniatus* (n = 200, 13%), *An. maculatus* (n = 130, 17%) and *An. aconitus* (n = 14, 2%) (Fig. 1B). To pool the DNA from each mosquito species for analysis, two µL of DNA samples were mixed in the same tube. There were 40, 20, 13 and 2 DNA sample pools from *An. minimus*, *An. peditaeniatus, An. maculatus and An. aconitus*, respectively (Fig. 1B).

Amplification of the Wolbachia 16S rRNA-encoding gene from field-captured Anopheles species

In the initial PCR, the W-SpecF and W-SpecR primers specifically bound to a conserved region at the 3' end of the *Wolbachia* 16S rRNA-encoding gene, generating an amplicon with an approximate length of 438 bp (Fig. 2A, upper panel). A representative image of agarose gel electrophoresis shows low-intensity DNA bands of between 400 and 500 bp, amplified from DNA pools of *An. minimus*, *An. aconitus*, *An. maculatus* and *An. peditaeniatus* (Fig. 2B). Among 75 DNA sample pools, 15 pools yielded 438-bp amplicons: 10 pools of *An. minimus*, three pools of *An. peditaeniatus*, and one pool each of *An. maculatus* and *An. aconitus* (Supplementary information). Regardless of the results of the W-Spec-based PCR, the PCR product obtained from the 15 DNA sample pools from the initial runs were used as templates in the nested PCR (Fig. 1A, lower panel). The 16SNF and 16SNR primers yielded amplicons from *An. minimus* pool numbers 21 and 32 (Fig. 2C). As expected, the length of amplicons obtained from the nested PCR were relatively shorter than those obtained from the initial PCR. No amplification was observed in *An. peditaeniatus, An. maculatus* or *An. aconitus* in the nested PCR (Supplementary information).

To confirm whether the yielded amplicons were the conserved region of *Wolbachia* 16S rRNA, the PCR products derived from DNA pool numbers 21 (P21) and 32 (P32) from *An. minimus* were subjected to DNA sequencing using 16SNF and 16SNR primers. The GenBank database was searched for similar sequences using the BLASTN program, and P21 and P32 were 100% identical to the 16S rRNA sequence of *W. pipientis*. The BLASTN results are provided as supplemental files. Hereafter, we referred to the *Wolbachia* strain identified in *An. minimus* as *w*Anmi. The place and pool number were tagged; *w*Anmi_UmpP21 and *w*Anmi_UmpP32 represent *Wolbachia* from *An. minimus* isolated from Umphang Valley and from pool numbers 21 and 32, respectively.

Discussion

Here, the data presented herein are preliminary evidence of native *Wolbachia* in *An. minimus*, a major malaria vector in an endemic area of mainland Southeast Asia. The identified *Wolbachia* in *An. minimus*, herein called wAnmi, was phylogenetically clustered in subgroup B, similar to *w*AlbB, which has been associated with the suppression of development of *P. falciparum* in *An. stephensi* [12]. Along the Thailand-Myanmar border, malaria transmission continues in many areas [25] [26], including the Umphang District of Tak Province in western Thailand. Most collection sites were in the Ban Nong Luang Village of Umphang District located in a valley primarily surrounded by forested mountains. Since some villagers engage in hunting-gathering and agriculture in areas near the forest (Fig. 1A), the risk of malaria infection in individuals is high. In the Umphang Valley, there are reportedly seven putative malaria vectors: *An. minimus*, *An. dirus*, *An. baimaii*, *An. sawadwongporni*, *An. maculatus*, *An. pseudowillmori* and *An. aconitus* [17]. In the Thasongyang District northern Umphang Valley, female *An. minimus*, *An. maculatus*, *An. annularis*, and *An. barbirostris* have been shown to carry *P. vivax* sporozoites, confirming their role in malaria transmission [27]. Regarding the number of *Anopheles* in the Umphang Valley, *An. minimus* was the most abundant (> 50%), followed by the potential malaria vectors *An. peditaeniatus* (~ 20%) and *An. maculatus* (~ 10%) [17]. Hence, this study included samples from *An. minimus*, *An. peditaeniatus*, *An. maculatus* and *An. aconitus*, the major malaria vectors, for the detection of *Wolbachia*.

In Thailand, only one survey of *Wolbachia* in mosquitoes was conducted to amplify the *filamenting temperature-sensitive mutant Z* (*ftsz*) and *Wolbachia surface protein* (*wsp*) genes. All 23 mosquito species in the genera *Aedes*, *Culex*, and *Mansonia* were positive for the *ftsz* and *wsp* genes, whereas none of the 19 *Anopheles* species were positive [8]. Failure to detect *Wolbachia*-specific genes in *Anopheles* spp. was consistent with the results of studies in European, African, and American specimens [10] [9]. Nevertheless, detection of the *Wolbachia* 16S rRNA region was accomplished. The W-Spec primers were designed to specifically amplify a 438-bp sequence at the 3’ region of the 16S rRNA gene in *Wolbachia* [21]. The W-Spec primers allowed the detection of *Wolbachia* in temperate North American arthropods, including the family *Culicidae* but excluding other mosquito families. Subsequently, Baldini et al reported the first evidence of *Wolbachia* in the reproductive organs of male and female *An. gambiae*, a major malaria vector in sub-Saharan Africa. In the same DNA samples, the W-Spec primer-based PCR was able to amplify the 16S rRNA fragment, whereas *Wolbachia*-specific surface protein and fructose-biphosphate aldolase-based PCR failed [13], implying good sensitivity of the W-Spec primers. Moreover, Shaw et al. further improved the sensitivity of W-Spec primer-based PCR by using nested primers (16SNF and 16SNR). The use of nested PCR allowed the detection of *Wolbachia* in *An. coluzzii* [15], *An. gambiae* in Mali [16] and *An. arabiensis* in Tanzania [28]. Additional studies were able to amplify the *Wolbachia* 16S rRNA fragment in DNA samples extracted from head-thorax or thorax-abdomen, implying the possibility of *Wolbachia* infection in nonreproductive organs [29] [12]. Collectively, *Wolbachia* infection in somatic and germ cells can be detected using nested PCR, which amplifies the conserved region of the *Wolbachia* 16S rRNA gene.

Nested PCR is regarded as a highly sensitive tool for detecting targets of interest that are present in very low amounts. We sometimes failed to amplify the 438-bp fragment using W-SpecF and W-SpecR in the initial PCR; however, there were 412-bp amplicons observed in the nested PCR [15], implying good

sensitivity of the nested PCR. As such, false-negative results may occur in cases of low-intensity *Wolbachia* infection because the quantity of the target of interest is below the limit of nested PCR. Given that DNA samples were extracted from the head and thorax of female *Anopheles*, detection failure in nested PCR is possibly due to low-intensity infection or the reproductive organ specificity of *Wolbachia*. Therefore, assays with high sensitivity, such as quantitative PCR, may aid in the detection of low-intensity *Wolbachia* infection [30] [31, 32]. Moreover, DNA preparation from the whole body of mosquitoes ensures the inclusion of *Wolbachia* strains that specifically infect germ cells.

The high sensitivity of nested PCR may cause low specificity, especially when primers bind to the conserved region of a common gene. Since *Wolbachia* is capable of infecting the majority of insect species [33], and the W-Spec primers amplify the conserved region of the *Wolbachia* 16S rRNA-coding gene, false-positive results due to environmental contamination from other insects may occur. In our study, 438-bp DNA amplicons with low-fluorescence intensity were present in the initial PCR, but we failed to reamplify these amplicons in the subsequent nested PCR using the 16SNF and 16SNR primers, suggesting the possibility of nonspecific amplification in the initial run. To minimize environmental contamination, PCR preparations were performed in a clean hood. Despite great care in the pre-PCR steps, we sometimes observed DNA bands in the negative control lane. Thus, DNA sequencing of the PCR product was necessary to confirm *Wolbachia*-specific amplification.

To the best of our knowledge, the present data are the first preliminary evidence of native *Wolbachia* in *An. minimus*. However, this study has limitations. First, the presence of the *Wolbachia* 16S rRNA gene in DNA samples from *Anopheles* is not direct evidence of natural *Wolbachia* infection because environmental contamination during mosquito capture, DNA extraction and PCR preparation is possible. Intracellular localization of *Wolbachia* in *Anopheles* spp. is a more definitive indicator of *Wolbachia* infection than sequencing. This could be done by using *in situ* hybridization [15] [34]. Moreover, given the availability of remaining DNA samples obtained during a previous two-year survey [17], this study included the DNA samples regardless of sample size. Thus, the prevalence of *Wolbachia* in *Anopheles* spp. in Umphang Valley could not be statistically estimated. Identification of the *Wolbachia* strain in *An. minimus* requires further confirmation using high-sensitivity assays and whole mosquitoes for DNA extraction.

Wolbachia has been under investigation for its potential application in blocking malaria transmission. As proof-of-concept, field trials in Australia demonstrated that the release of laboratory-reared mosquitoes infected with *Wolbachia* resulted in the rapid spread of *Wolbachia* among wild uninfected mosquito populations [35]. Population invasion by a particular *Wolbachia* strain depends on the level of cytoplasmic incompatibility, host fitness (survival, fecundity and fertility), and vertical transmission. Therefore, the following issues need to be assessed: the potential of the native *Wolbachia* identified in *An. minimus* to render resistance to *Plasmodium* parasites and interfere with malaria transmission, its ability to cause cytoplasmic incompatibility, and its effects on host fitness.

Conclusion

To the best of our knowledge, the data presented herein are the first molecular evidence of a *Wolbachia* strain in *An. minimus*, named *w*Anmi, in a low-malaria transmission area in the Umphang Valley of western Thailand. Further biological characterization is required to examine its potential as a malaria transmission control strategy in the field.

Abbreviations

An.
Genus *Anopheles*
16S rRNA
small subunit 16 of ribosomal ribonucleic acid
16SNF
Small subunit 16 of ribosomal ribonucleic acid-nested forward primer
16SNR
Small subunit 16 of ribosomal ribonucleic acid-nested reverse primer
Ump
Umphang
W-SpecF
Wolbachia-specific forward primer
W-SpecR
Wolbachia-specific reverse primer

Declarations

Competing interests

The authors declare that they have no competing interests.

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Author contributions

NT performed the experiments and analyzed and interpreted the data; PR analyzed and interpreted the data and wrote the manuscript; CT and TC performed the experiments; KK designed the study, analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript

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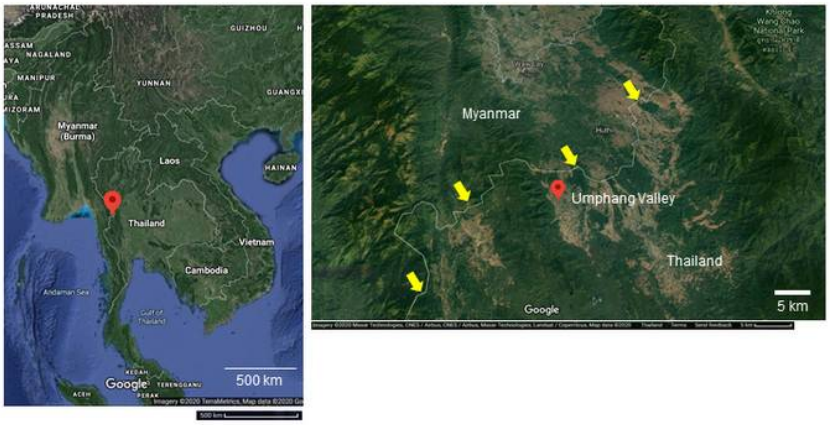
Figures

Fig. 1

A



B

Mosquito		Number (%)
Subgenus	Species	
Cellia	*An. minimus*	401 (54%)
	An. maculatus	130 (17%)
	An. aconitus	14 (2%)
Anopheles	*An. peditaeniatus*	200 (27%)
	Total	745

Figure 1

Collection site of Anopheles spp. A. Google map of mainland Southeast Asia (left) and the location in which the Anopheles spp. were collected (right side). The collection site was located at 16°04'36.3"N 98°45'8.0"E (red pins) in Umphang Valley of Tak Province in western Thailand. The yellow arrow indicates the Thailand-Myanmar border. Scale bars are 500 and 5 km. B. Anopheles spp. in the subgenera Cellia and Anopheles and the total number of DNA samples included in this study.

Fig. 2

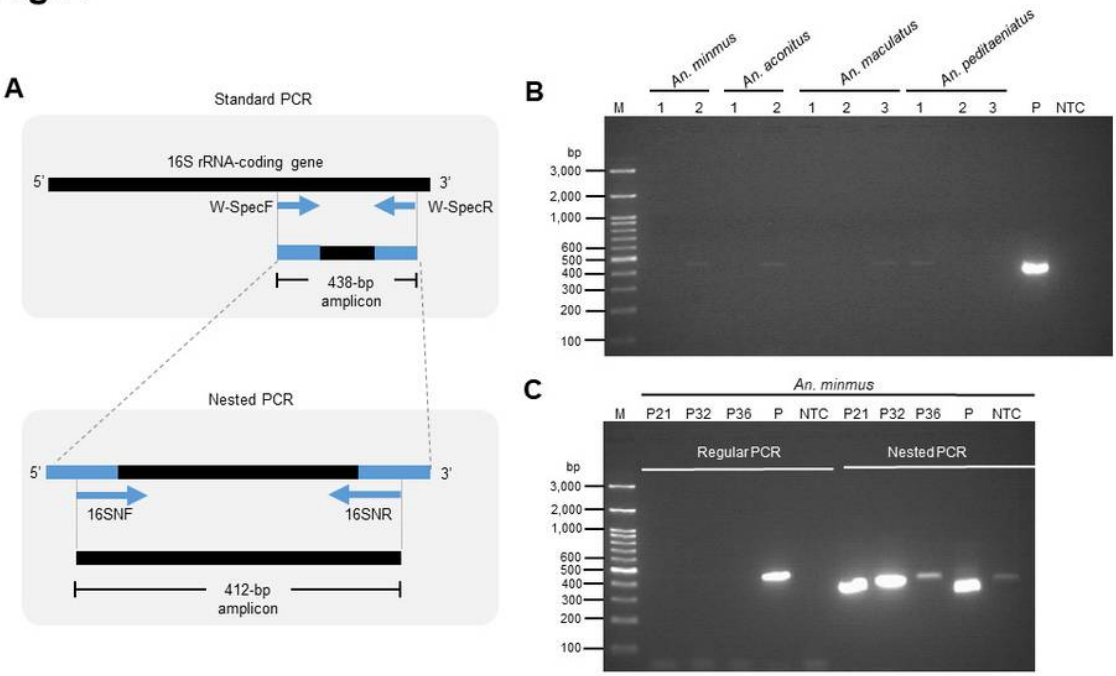


Figure 2

Amplification of the Wolbachia 16S rRNA-encoding gene. A. Schematic diagram showing two-step PCR, including standard and nested PCR. In the standard PCR, W-SpecF and W-SpecR primers (blue colored arrows in upper panel) attached to the 3' region of the Wolbachia 16S rRNA-encoding gene, amplifying a 438-bp fragment. In the nested PCR, the 438-bp amplicons generated from the regular PCR were used as templates. The 16SNF and 16SNR primers attached the internal sequence of the 438-bp fragment, generating a 412-bp PCR product. B. A representative image of the 438-bp amplicons obtained from the standard PCR. Two and three representative pools of DNA extracts of Anopheles are shown. C. A representative image of amplicons derived from the standard PCR (438 bp) and nested PCR (412 bp). For the standard PCR, templates were obtained from the DNA extracts from An. minimus pool numbers 21 (P21), 32 (P32) and 36 (P36). DNA from Mansonia spp. was used as the positive control (P), while absent template DNA was used as the negative control (NTC). In the nested PCR, all the samples from the standard PCR were used as templates. The PCR products were analyzed with electrophoresis in a 2% agarose gel. Lane M: the DNA ladder electrophoresed simultaneously with the PCR product to determine amplicon size.

Fig. 3

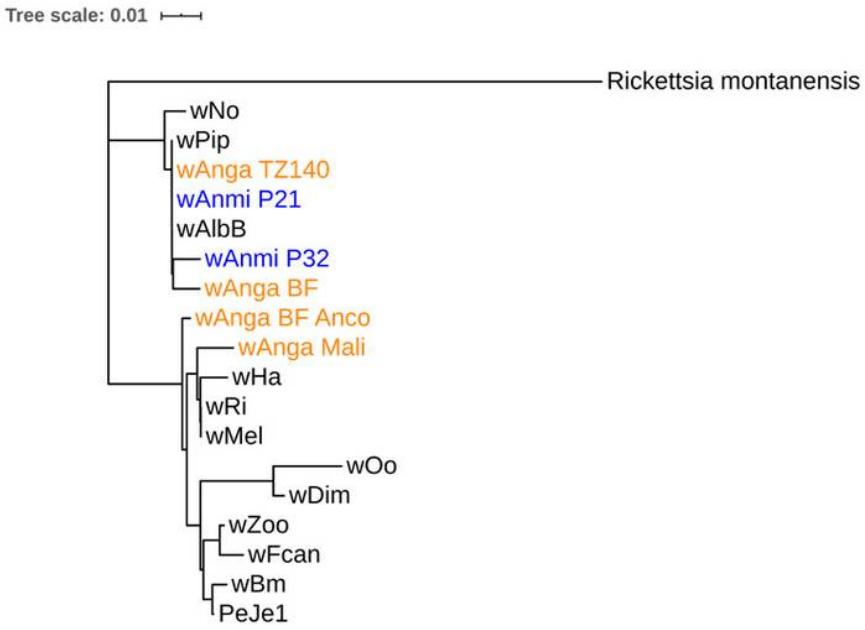


Figure 3

Rooted phylogenetic tree of the Wolbachia strain identified in An. minimus collected from Umphang Valley compared to other Wolbachia strains. The conserved region of the Wolbachia 16S rRNA-encoding DNA sequence obtained from the identified Wolbachia strains (blue letters) was phylogenetically compared with those in Wolbachia subgroups A, B, C, D, E, F, H (black letters) and Anopheles-specific subgroups (orange letters) using NGPhylogeny.fr. Rickettsia montanensis was used as the reference outgroup. A tree scale of 0.01 corresponds to inferred evolutionary changes. Details of the DNA sequences retrieved from GenBank are shown in Table 2.

Fig. 4

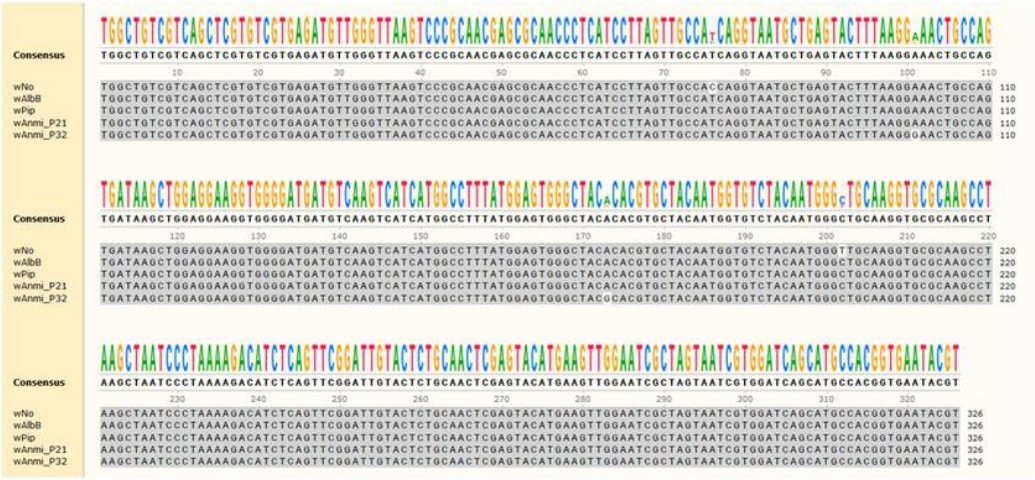


Figure 4

Multiple sequence alignment of the conserved region of the Wolbachia 16S rRNA gene. The sequence of the conserved region of the Wolbachia 16S rRNA gene was compared to those of wNo in Drosophila simulans (CP003883.1), wAlbB in Aedes albopictus (KX155506.1) and wPip in Culex quinquefasciatus (AM999887.1), members of subgroup B. DNA sequences were aligned with the wAnmi_UmpP21 and wAnmi_UmpP32 strains identified in the present study. The consensus sequence was illustrated using MSAViewer.

Fig. 5

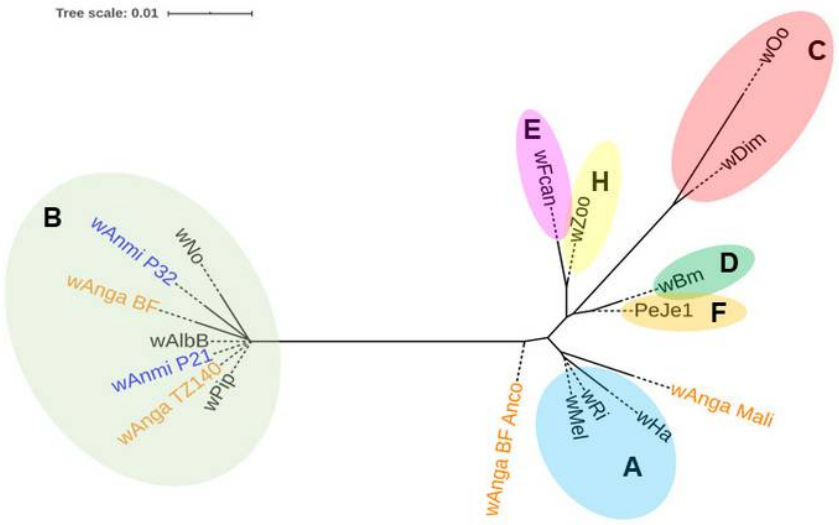


Figure 5

Unrooted phylogenetic tree of the Wolbachia strain identified in An. minimus collected from Umphang Valley compared to other Wolbachia strains. The conserved region of the Wolbachia 16S rRNA-encoding DNA sequence obtained from the identified Wolbachia strains (blue letters) was phylogenetically compared with those of Wolbachia subgroups A, B, C, D, E, F, H (black letters) and Anopheles-specific subgroups (orange letters) using NGPhylogeny.fr. Rickettsia montanensis was used as the reference outgroup. A tree scale of 0.01 corresponds to inferred evolutionary changes. Details of the DNA sequences retrieved from GenBank are shown in Table 2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- M9X6T45H0CB10450FE67AF7FD8EP.pdf
- SupplementaldataKulkeawK.doc