Molecular detection of filarial nematode from Culicoides biting midges (Diptera: Ceratopogonidae) in northeastern Thailand

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INTRODUCTION

Biting midge species of the genus Culicoides Latreille are significant vectors of many disease causing agents including viruses, protozoa, and filarial nematodes (Mullen & Murphree, 2019). Examples of important diseases-causing pathogens transmitted by biting midges include Oropouche fever in humans, bluetongue disease and epizootic hemorrhagic disease in ruminants, African horse sickness in equines, and leucocytozoonosis in birds (Valkiūnas, 2005; Santiago-Alarcon et al., 2012; Mullen & Murphree, 2019). In addition, to animal and human welfare issues, involving biting midges vectors can cause serious problem for the livestock industry due to the direct loss of the livestock number and the international trade restriction or bans (Shults et al., 2021). For examples, the bluetongue virus (BTV) causing agent of bluetongue disease that is associated with at least seven biting midge species as vectors (C. fulvus Sen and Das Gupta, C. guilbenkiani Caéiro, C. imicola Kieffer, C. insignis Lutz, C. milnai Austen, C. obsoletus Meigen and C. sonorensis Wirth & Jones) (Mullen & Murphree, 2019) causes global economic loss of approximately 3 billion USD annually (Shults et al., 2021). Recently, there was an outbreak in Thailand of African horse sickness virus that has C. imicola as vector. This was the first time this disease had been reported in Southeast Asia, affecting 2,735 horses and causing >565 horses death (Bunpapong et al., 2021).

A total of 1,360 extant species of the genus Culicoides have been recorded globally (Borkent & Dominika, 2020; Borkent et al., 2022) and approximately 7% (100 species) occur in Thailand (Thepparat et al., 2015; Pramual et al., 2021a). However, it is highly possible that there are more species awaiting discovery in the country as recent molecular genetic investigations found cryptic diversity in many species (Jomkumsing et al., 2021; Gopurenko et al., 2022). At least ten species of the Culicoides in Thailand are known or suspected to be vectors of disease causing agents transmitted to human and other animals. C. breviviridis Kieffer, C. fulvus Sen and Das Gupta, C. imicola Kieffer, C. oxystoma Kieffer and C. peregrinus Kieffer have been reported as vectors of arbovirus (Mullen & Murphree, 2019). C. arakawae (Arakawa) and C. guttifer Meijere are vectors of avian haemosporidian parasite, Leucocytozoon caulleryi, a causative agent of leucocytozoonosis in chickens (Mullen & Murphree, 2019). The later species is also suspected as a vector of Trypanosoma sp. (Sunantaraporn et al., 2022). Recently, C. mahasarakhamense, a recent described species from a divergent genetic lineage of specimens formerly recognized as C. arakawae (Pramual et al., 2021a) and other five species (C. peregrinus Kieffer, C. oxystoma, C. hoffi Causey, C. fordae Lee and C. fulvus) were suspected as vectors of the flagellated protozoans, Leishmania martiniquensis (Sunantaraporn et al., 2021) and L. orientalis (Songumpai et al., 2022), the causing agents of Leishmaniases. C. mahasarakhamense

Keywords: Biting midge; Culicoides; filarial nematode; Thailand.
is also suspected to be a vector of avian haemosporidian parasites (Leucocytozoon sp., Plasmodium juxtanucleare, R. gallinaeum) (Pramual et al., 2021b). C. peregrinus, C. orientalis Kieffer, C. imicola, C. oxytoma, and C. fulvus are suspected as the vectors of the bluetongue virus in Thailand (Fujiwasa et al., 2021).

In addition to their role as vectors of the virus and protozoa transmitted to humans and other animals, biting midges are also vectors of filarial nematodes. At least eight species of the filarial nematodes are known to be transmitted by biting midges. Among these, three species of the genus (i.e. Mansonella ozzardi, M. perstans and M. streptocerca) cause mansoonellosis in humans. Other filarial species for which biting midges are vectors belong to the genus Onchocerca (O. cervicalis, O. gibsoni, O. gutturosa, O. reticulate, O. sweetae) that have horses, cattle, ponies and water buffalo as vertebrate hosts (Mullen & Murphree, 2019). Thus far, there is only one filarial species in cattle, O. gibsoni, known to occur in Southeast Asia (Malaysia) with C. oxytoma is suspected to be its vector (Wirth & Hubert, 1989; Mullen & Murphree, 2019). However, to the best of our knowledge, there have not been any reports on the possible role of Culicoides biting midge species in Thailand for transmission of the filarial nematode. Therefore, in this study, we are performing a molecular approach to detect filarial DNA in four common cattle biting (C. oxytoma, C. actoni, C. peregrinus) and chicken biting (C. mahasarakhamense) Culicoides species in northeastern Thailand (Jomkumsing et al., 2021). Although a molecular detection of the parasite DNA in the blood feeding insects does not necessary indicate that they are real biological vectors (Valkiūnas, 2011, Valkiūnas et al., 2013) the information provided will be useful to targeting species that are potentially competent vector for further investigation (Ferreira et al., 2020).

MATERIALS AND METHODS

Sample collection
Adult specimens of four common Culicoides biting midge species (C. actoni, C. oxytoma, C. peregrinus and C. mahasarakhamense) in the animal shelters were collected during February 2020 and July 2022 (Table 1 and Figure 1). Specimens were collected using sweep-net sweeping around or close to the animals. Specimen collections were conducted between 17:00 and 19:00 pm when the biting midges were actively searching for a host blood meal. Adult fly specimens were preserved in 80% ethanol and stored at ~20°C until use. Keys, descriptions and illustrations of Wirth and Hubert (1989), Dyce et al. (2007) and Pramual et al. (2021a) (for C. mahasarakhamense) were used for biting midge species identification.

Molecular detection of filarial nematode
The adult female biting midge specimens were checked for host blood in their abdomens before being used for DNA extraction. The blood-fed females were not used to avoid the possibility of detections of filarial nematode from the host blood. DNA was extract from a pool (10 individuals/pool) unless stated otherwise (Table 1). The GF-1 Tissue DNA Extraction Kit (Vivantis, Malaysia) was used for genomic DNA extraction. The primers 1250vC (5’–TCGGCTATGCCTTTAATTTT–3’) and 1250vB (5’–CAACCTACGCCTTTTAGCC–3’) (Morales-Hojas et al., 2006) were used to amplify a fragment of approximately 550 bp of the mitochondrial 12S rRNA gene. The PCR reaction was performed with a final volume of 25 µl using the conditions as described in Morales-Hojas et al. (2006). PCR products were checked with 1% agarose gel electrophoresis and purified using a PureDireX PCR CleanUp & Gel Extraction Kit (BioHelix, Taiwan). DNA sequencing was performed at ATCG Company Limited (Thailand Science Park (TSP), Pathumthani, Thailand) using the same primers as used in the PCR.

Data analysis
The 12S rRNA gene sequence of filarial nematode detected in this study was compared with those reported in NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine sequence similarity. To further examine genetic relationships between sequences that were similar to that of the filarial nematode detected in Culicoides in Thailand, phylogenetic analyses based on neighbor joining (NJ) and maximum likelihood (ML) methods were used. The first 100 most similar sequences based on BLAST result were included for phylogenetic analyses. Both NJ and ML analyses were performed in MEGA X (Kumar et al., 2018). Branch support was calculated using the bootstrapping method with 1,000 replications.

Table 1. List of species and sampling location of Culicoides biting midge in Thailand used for molecular detection of filarial nematode

<table>
<thead>
<tr>
<th>Species</th>
<th>Location (code)</th>
<th>Latitude / Longitude</th>
<th>Collection date</th>
<th>Number of specimens (filarial detected)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. oxytoma</strong></td>
<td>Prangku, Sisaket (SK)</td>
<td>14°49'50&quot;N/104°03'38&quot;E</td>
<td>6 Mar 2021</td>
<td>15 pools (–)</td>
</tr>
<tr>
<td>Kantharawichai, Maha Sarakham (MK1)</td>
<td>16°20'47&quot;N/103°12'21&quot;E</td>
<td>7 Feb 2021</td>
<td>15 pools (–)</td>
<td></td>
</tr>
<tr>
<td>Nadun, Maha Sarakham (MK2)</td>
<td>15°40'59&quot;N/103°13'12&quot;E</td>
<td>20 Feb 2021</td>
<td>2 pools (–)</td>
<td></td>
</tr>
<tr>
<td>Non Sang, Nong Bua Lampu (NL)</td>
<td>17°52'36&quot;N/102°34'20&quot;E</td>
<td>27 Feb 2021</td>
<td>18 pools (–)</td>
<td></td>
</tr>
<tr>
<td>Waritchaphum, Sakon Nakhon (SN)</td>
<td>17°14'32&quot;N/103°34'28&quot;E</td>
<td>27 Mar 2021</td>
<td>10 pools (–)</td>
<td></td>
</tr>
<tr>
<td>Phon Sawan, Nakhon Phanom (NP)</td>
<td>17°27'42&quot;N/104°27'57&quot;E</td>
<td>20 Nov 2020</td>
<td>15 pools (–)</td>
<td></td>
</tr>
<tr>
<td>Loeng Noi Tha, Yasothon (YT)</td>
<td>15°40'54&quot;N/103°31'22&quot;E</td>
<td>16 Apr 2021</td>
<td>20 pools (–)</td>
<td></td>
</tr>
<tr>
<td><strong>C. peregrinus</strong></td>
<td>Prangku, Sisaket (SK)</td>
<td>14°49'50&quot;N/104°03'38&quot;E</td>
<td>6 Mar 2021</td>
<td>30 pools (–)</td>
</tr>
<tr>
<td>Ban Wai, Kantharawichai, Maha Sarakham (MK3)</td>
<td>16°28'17&quot;N/103°11'21&quot;E</td>
<td>24 Feb 2021</td>
<td>18 pools (–)</td>
<td></td>
</tr>
<tr>
<td>Tha Bo, Nong Khai (NK1)</td>
<td>17°52'36&quot;N/102°34'20&quot;E</td>
<td>30 Jul 2022</td>
<td>15 pools (–)</td>
<td></td>
</tr>
<tr>
<td><strong>C. actoni</strong></td>
<td>Si Chiang Mai, Nong Khai (NK2)</td>
<td>17°56'17&quot;N/102°34'42&quot;E</td>
<td>29 Jul 2022</td>
<td>1 pool (–)</td>
</tr>
<tr>
<td><strong>C. mahasarakhamense</strong></td>
<td>Ban Wai, Kantharawichai, Maha Sarakham (MK3)</td>
<td>16°18'27&quot;N/103°11'21&quot;E</td>
<td>24 Jan 2021</td>
<td>12 pools (–)</td>
</tr>
<tr>
<td>Prangku, Sisaket (SK)</td>
<td>14°49'50&quot;N/104°03'38&quot;E</td>
<td>6 Mar 2021</td>
<td>5 specimens (–)</td>
<td></td>
</tr>
<tr>
<td>Mahasarakham University, Kantharawichai, Maha Sarakham (MK4)</td>
<td>16°14'32&quot;N/103°15'07&quot;E</td>
<td>3 Feb 2020</td>
<td>6 specimens (1)</td>
<td></td>
</tr>
</tbody>
</table>

Total 171 pools and 11 individuals (1)
RESULTS AND DISCUSSION

A total of 1,721 (171 pools and 11 individual specimens) representing four common biting midge species (C. peregrinus, C. actoni, C. oxystoma and C. mahasarakhamense) from northeastern Thailand were molecularly determined for filarial DNA. An unidentified filarial species was detected in a specimen of C. mahasarakhamense collected from Maha Sarakham province, northeastern Thailand (GenBank accession no. OQ658729). The NCBI BLAST analysis revealed that the 12S rRNA gene sequence obtained from this specimen was closest to the unidentified filarial species detected in mosquito (Culex pipiens, accession no. KR676614) from Hungary (Kemenesi et al., 2015) with 93% sequence similarity. The filarial DNA from C. mahasarakhamense was also similar to those of filarial nematodes of the family Onchocercidae detected in the mosquitoes of the genus Culex from Germany (accession no. JN228379–81) (Czajka et al., 2012) with a sequence similarity of 92%.

Phylogenetic analyses based on the NJ and ML methods revealed similar tree topologies (Figure 2 and 3). The filarial DNA detected in C. mahasarakhamense found in this study formed a clade with an unidentified filarial species isolated from avian species and with strong support (100%). Although the vertebrate host of the closest sequences (KR676614, JN228379–81) is unknown, those for the other two that were retrieved within the same clade (MK424346 and JX870434) were both isolated from avian species (northern saw-whet owl, Aegolius acadicus for JX870434 and falcated duck, Mareca falcata for MK424346 (Kim et al., 2022)). Therefore, it is very likely that the filarial worm detected in C. mahasarakhamense in the present study is occur in avian species. This is in agreement with a host blood meal analysis that found that C. mahasarakhamense feeds on domestic chicken (Jomkumsing et al., 2021). There are at least 160 filarioids species recorded from avian species (Bartlett, 2008). At least three avian filarial nematode species have been reported to be transmitted by two Culicoides species (C. stilobezzioides Foote and Pratt and Culicoides travisi Varga) (Bartlett & Anderson, 1980; Bartlett, 2008).

Although the detection of unidentified Onchocercidae sp. in this study indicates that C. mahasarakhamense is a potential vector species, detection of the parasite in blood-feeding insects based only on PCR does not necessarily indicate that they are competent vectors (Valkiūnas, 2011; Valkiūnas et al., 2013). Therefore, further
Figure 2. Neighbor joining tree based on the 12S rRNA sequences of the Onchocercidae sp. (in bold) detected in *Culicoides mahasarakhamense* from northeastern Thailand and the top 100 most similar sequences retrieved from NCBI GenBank. Bootstrap values based on 1,000 replications are shown above or near the branch.

Figure 3. Maximum likelihood tree based on the 12S rRNA sequences of the Onchocercidae sp. (in bold) detected in *Culicoides mahasarakhamense* from northeastern Thailand and the top 100 most similar sequences retrieved from NCBI GenBank. Bootstrap values based on 1,000 replications are shown above or near the branch.
investigation using molecular and microscopic approaches in both possible vertebrate hosts (e.g., chickens and birds) in the location where this filarial worm was detected, and in C. mahasarakhamense across a wider geographic area, as well as experimental studies on vector competence, will provide useful information on whether this biting midge species is a natural vector of this filarial worm or if it is accidentally acquiring the parasite via blood feeding. It is important to note that other blood-feeding insects in Thailand are also known as vectors of filarial nematodes of the family Onchocercidae. For example, black flies of the genus Simulium Latreille are potential vectors of Onchocerca sp. (Saeung et al., 2020), and several mosquito species of the genus Aedes Meigen, Anopheles Meigen, Armigeres Theobald, Culex Linnaeus, and Mansonia Blanchard are vectors of filarial nematodes of the genus Wuchereria, Brugia, Dirofilaria, and Setaria (Foster & Walker, 2019; Siriyasatien et al., 2021). Further investigation on the vector competency of this Culicoides species with the pathogens molecularly detected will be useful.

ACKNOWLEDGEMENTS

This research project was financially supported by Thailand Science Research and Innovation (TSRI). We would like to thank Adrian Plant for valuable comments on a previous version of the manuscript and Panya Jomkumsing for laboratory and specimen collection assistant.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES


