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RESEARCH ARTICLE

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

Pramual, P.[1*], Khamluea, S.[1], Butlun, P.[1], Promdungdee, A.[1]

[1]Department of Biology, Faculty of Science, Mahasarakham University, Kantharawichai District, Maha Sarakham 44150, Thailand
*Corresponding author: pairot.p@msu.ac.th

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ABSTRACT

Culicoides Latreille biting midges are important blood feeding insects. Many species are pests and vectors of the disease causing agents including viruses, protozoa and filarial nematodes which can be transmitted to humans and other animals. However, knowledge of the role of *Culicoides* as vectors of filarial nematodes is limited, particular in Thailand, where at least 100 species of the genus *Culicoides* have been reported. In this study, a molecular approach using the 12S rRNA gene sequence was used to detecting the filarial nematode in four common biting midge species, *C. actoni* Smith, *C. oxystoma* Kieffer, *C. peregrinus* Kieffer and *C. mahasarakhamense* Pramual, Jomkumsing, Piraonapicha & Jumpato in animal shelters from northeastern Thailand. A total of 1,721 specimens were used for molecular screening. An unidentified Onchocercidae sp. was detected in a specimen of *C. mahasarakhamense* collected from Maha Sarakham province. This filarial species shows 93% sequence similarity with an unidentified Onchocercidae sp. isolated from *Culex* mosquitoes. Phylogenetic analyses revealed that Onchocercidae sp. from *C. mahasarakhamense* formed a clade with strong bootstrap support (100%) with filarial species detected in birds. Thus, it is very likely that the Onchocercidae sp. found in this study employes birds as vertebrate hosts which agrees with feeding behavior of *C. mahasarakhamense* which is known to feed on chicken. Further study is requiring to examine whether this biting midge species is a competent vector of this Onchocercidae sp.

Keywords: Biting midge; *Culicoides*; filarial nematode; Thailand.

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. gulbenkiani* Caeiro, *C. imicola* Kieffer, *C. insignis* Lutz, *C. milnei* 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 & Dominiak, 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. brevitarsis* 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. huffi* 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 Leishmaniasis. *C. mahasarakhamense*

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is also suspected to be a vector of avian haemosporidian parasites (*Leucocytozoon* sp., *Plasmodium juxtanucleare*, *P. gallinaceum*) (Pramual *et al*., 2021b). *C. peregrinus*, *C. orientalis* Kieffer, *C. imicola*, *C. oxystoma*, and *C. fulvus* are suspected as the vectors of the bluetongue virus in Thailand (Fujisawa *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 mansonellosis 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. oxystoma* 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 using a molecular approach to detect filarial DNA in four common cattle biting (*C. oxystoma*, *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. oxystoma*, *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 12SOvC (5'–TCGGCTATGCGTTTTAATTTT–3') and 12SOvB (5'–CAACTTACGCCCCTTTAGGC–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

Species	Location (code)	Latitude / Longitude	Collection date	Number of specimens (filarial detected)
C. oxystoma	Prangku, Sisaket (SK)	14°49'50"N/104°03'38"E	6 Mar 2021	15 pools (–)
	Kantharawichai, Maha Sarakham (MK1)	16°20'47"N/103°12'21"E	7 Feb 2021	15 pools (–)
	Nadun, Maha Sarakham (MK2)	15°40'59"N/103°14'09"E	20 Feb 2021	2 pools (–)
	Non Sang, Nong Bua Lampu (NL)	15°40'59"N/103°14'09"E	27 Feb 2021	18 pools (–)
	Waritchaphum, Sakon Nakhon(SN)	17°14'32"N/103°34'28"E	27 Mar 2021	10 pools (–)
	Phon Sawan, Nakhon Phanom (NP)	17°27'42"N/104°27'57"E	20 Nov 2020	15 pools (–)
	Loeng Nok Tha, Yasothon (YT)	16°15'40"N/104°31'22"E	16 Apr 2021	20 pools (–)
C. peregrinus	Prangku, Sisaket (SK)	14°49'50"N/104°03'38"E	6 Mar 2021	30 pools (–)
	Ban Wai, Kantharawichai, Maha Sarakham (MK3)	16°18'27"N/103°11'21"E	24 Feb 2021	18 pools (–)
	Tha Bo, Nong Khai (NK1)	17°52'36"N/102°34'20"E	30 Jul 2022	15 pools (–)
C. actoni	Si Chiang Mai, Nong Khai (NK2)	17°56'17"N/102°34'42"E	29 Jul 2022	1 pool (–)
C. mahasarakhamense	Ban Wai, Kantharawichai, Maha Sarakham (MK3)	16°18'27"N/103°11'21"E	24 Jan 2021	12 pools (–)
	Prangku, Sisaket (SK)	14°49'50"N/104°03'38"E	6 Mar 2021	5 specimens (–)
	Mahasarakham University, Kantharawichai, Maha Sarakham (MK4)	16°14'32"N/103°15'07"E	3 Feb 2020	6 specimens (1)
Total				**171 pools and 11 individuals (1)**

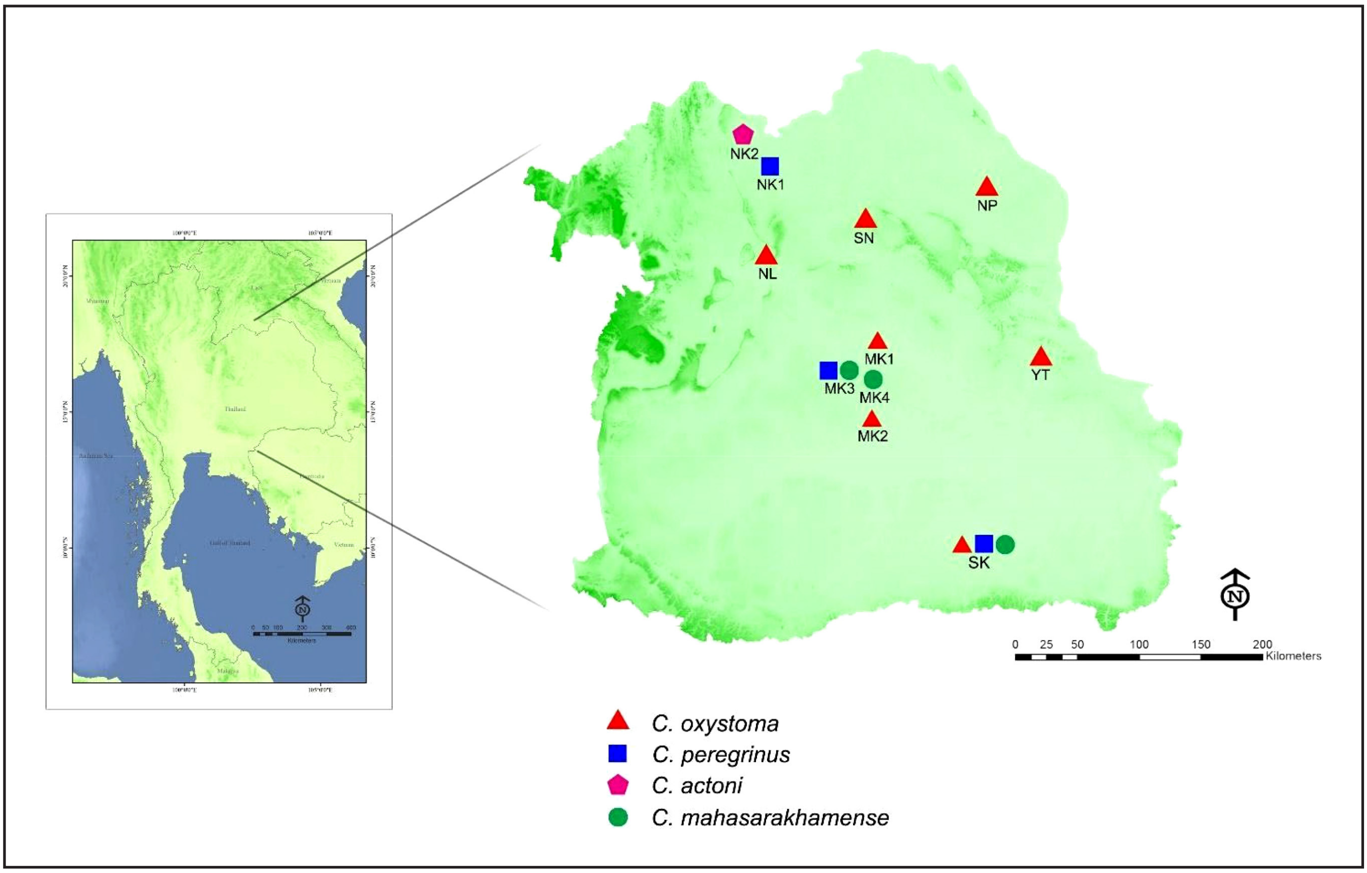

Figure 1. Map of Thailand showing the sampling locations of four *Culicoides* species from the northeastern region (enlarged) used in this study. Details of sampling locations are given in Table 1.

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 animal shelters 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 species of the Onchocercidae found in the *Culex* mosquitoes from Germany and Hungary, consistent with the BLAST results. Other sequences that belonged to this clade involved an unidentified filarial species isolated from northern saw-whet owl (*Aegolius acadicus*) from Canada (accession no. JX870434) and from falcated duck, *Mareca falcata* (MK424346) but with slightly lower sequence similarity (88%).

The only filarial worm species known to be transmitted by biting midges of the genus *Culicoides* in Southeast Asia is *Onchocerca gibsoni* (Mullen & Murphree, 2019). Based on BLAST results, the 12S rRNA gene sequence of the filarial nematode detected in *C. mahasarakhamense* had 86% similarity with those of *O. gibsoni* from Australia (accession nos. AY462912–13) (Morales-Hojas *et al*., 2006). Other filarial (*Mansonella ozzardi*, *M. perstans*, *O. gutturosa*) species that *Culicoides* spp. play roles as vectors are genetically very different (<85% sequence similarity). The 12S rRNA sequence from three other filarial worm species (*M. streptocerca*, *O. cervicalis*, *O. reticulata*) for which *Culicoides* biting midge species are known to be vectors, have not been reported in the public database (i.e. NCBI GenBank). Therefore, we cannot compare with the sequence obtained from *C. mahasarakhamense*.

According to the phylogenetic analyses, the filarial worm detected in *C. mahasarakhamense* is clustered with those of the 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

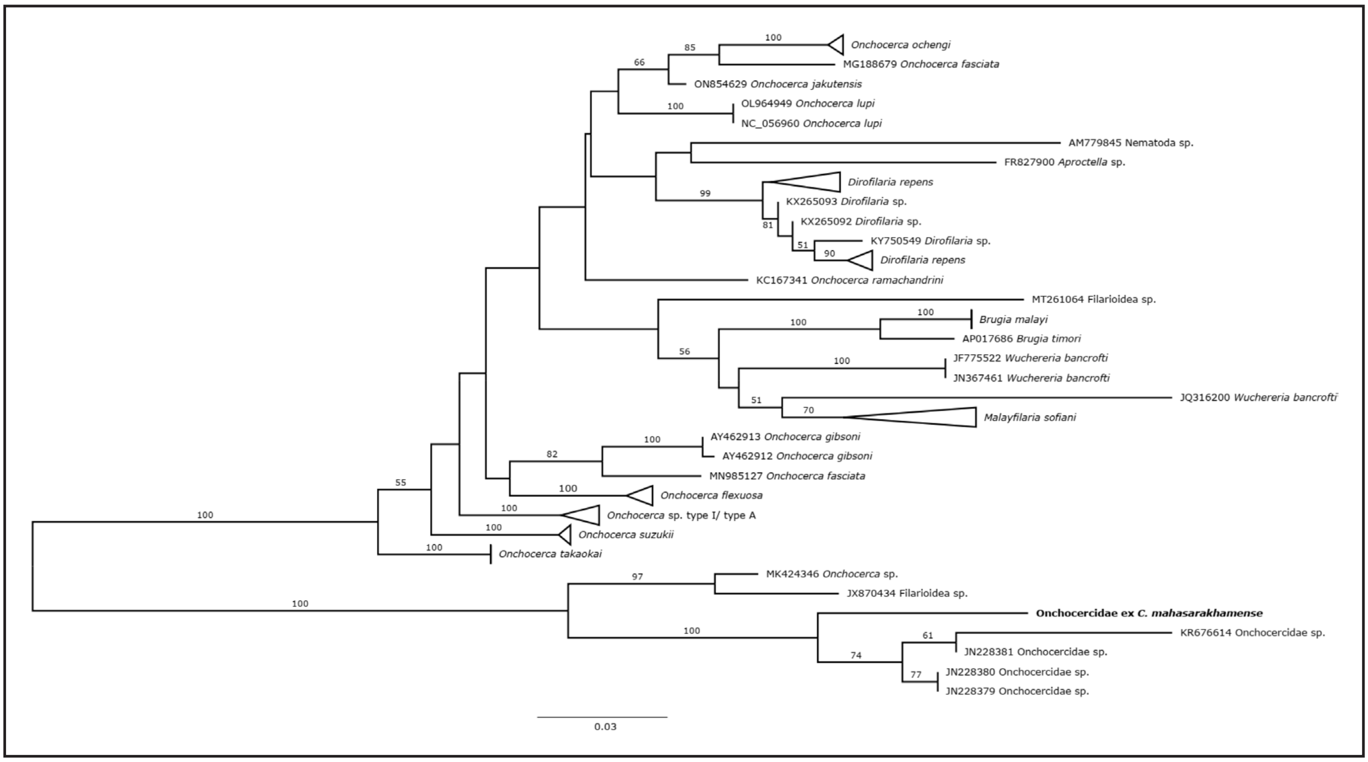

Figure 2. Neighbor joining tree based on the 12S rRNA sequences of the Onchocercidae sp. (in bold) detected in *Culicoides mahasarakhamense* from northeastern Thailand and the top 100 most similar sequences retrieved from NCBI GenBank. Bootstrap values based on 1,000 replications are shown above or near the branch.

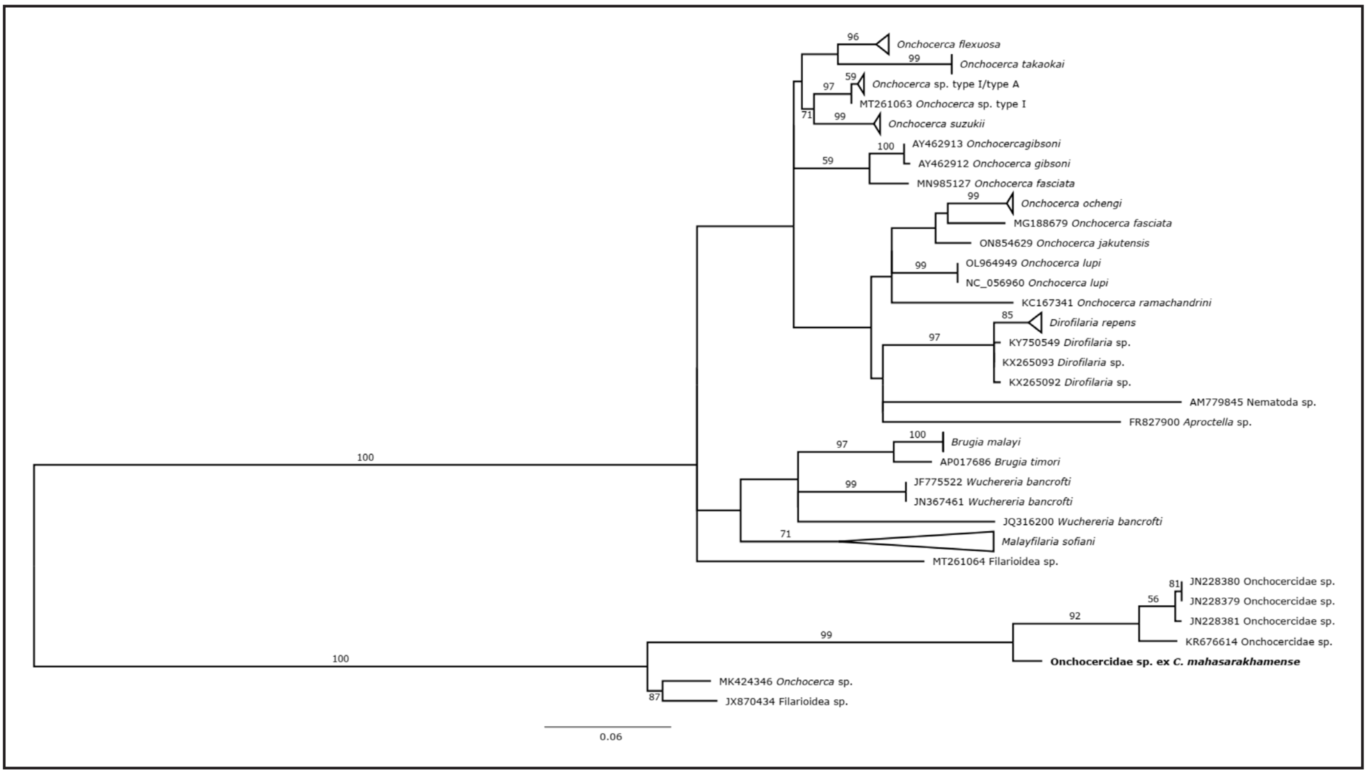

Figure 3. Maximum likelihood tree based on the 12S rRNA sequences of the Onchocercidae sp. (in bold) detected in *Culicoides mahasarakhamense* from northeastern Thailand and the top 100 most similar sequences retrieved from NCBI GenBank. Bootstrap values based on 1,000 replications are shown above or 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*., 2023)

In conclusion, we report here, for the first time of the detection of filarial worm in *Culicoides* biting midge in Thailand. Although the species of this nematode cannot be definitely determine, it is very likely to be a member of family Onchocerciadae transmitted among avian species. Detection of the filarial worm in *C. mahasarakhamense* increased the significance of this biting midge species as a possible vector of several parasitic disease agents. Previous reports found that this biting midge species is a possible vector of *Leucocytozoon* sp. and *Plasmodium juxtanucleare* and *P. gallinaceum* (Pramaul *et al*., 2021b). This species is also the possible vector of a flagellated protozoa, *Leishmania martiniquensis*, the causative agent of a neglected tropical human disease, leishmaniasis (Sunantaraporn *et al*., 2021; Songumpai *et al*., 2022). Further investigation on the vector competency of this *Culicoides* species with the pathogens molecularly detected will be useful.

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Conflict of interest
The authors declare that they have no conflict of interest.

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