Natural infections with larvae of *Onchocerca* species type I in the human-biting black fly, *Simulium nigrogilvum* (Diptera: Simuliidae), in western Thailand

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ABSTRACT

Zoonotic onchocerciasis is a human infection caused by *Onchocerca* species of animal origins and transmitted by black fly vectors. The reported incidence of this disease has increased throughout the world. This study aims to clarify the vectorial roles of black fly species in zoonotic filarial transmission in Tak province, western Thailand. The integrated approach of morphological and DNA sequence-based analyses was used to identify species of both wild-caught female black flies and infective filarial larvae found in the infected black flies. All of 494 female black flies captured were identified as *S. nigrogilvum*, through scanning electron microscopy (SEM) and DNA sequence analyses based on the cytochrome *c* oxidase subunit I (*COI*) and subunit II (*COII*), and the fast-evolving nuclear elongation complex protein 1 (*ECP1*) genes. Four females of *S. nigrogilvum* harbored one to three third-stage larvae (infective larvae) in their thoraces, with an infection rate of 0.81% (4/494). All infective larvae were similar in morphology and size to one another, being identified as *Onchocerca* species type I (= *O. sp. type A*), a bovine filaria, originally reported from Japan, and also as *O. sp. found in S. nodosum* in Thailand, based on their body lengths and widths being 1,068–1,346 µm long by 25–28 µm wide, and morphological characters. Comparisons of cytochrome *c* oxidase subunit I (*COI*) and 12S rRNA sequences of mitochondrial DNA (mtDNA) and phylogenetic analyses with those of previous reports strongly supported that all larvae were *O. sp. type I*. This report is the first indicating the presence of *O. sp. type I* in Thailand and its vector being *S. nigrogilvum*.

1. Introduction

Black flies are small blood-feeding insects that belong to the family Simuliidae of the order Diptera. Some species serve as vectors of several pathogens to humans and animals, including filarioid nematodes, blood protozoa, viruses and bacteria (Crosskey, 1990; Adler et al., 2004). The bites of black flies can cause localized dermatitis in both humans and animals due to an IgE-mediated reaction to salivary gland proteins (Hempolchom et al., 2019a, 2019b). Additionally, zoonotic onchocerciasis, which is caused by the *Onchocerca* species of animal origins, has been reported from many countries. At least ten named *O. spp.*, including *O. cervicalis*, *O. dewittet japonica*, *O. eberhardi*, *O. jaktensis*, *O. gutturosa*, *O. lienalis*, *O. lupi*, *O. skrjabini*, *O. suzukii*, and *O. takaokai* and one unnamed species (*O. sp. type I*) have been recorded in animals (Otranto and Eberhard, 2011; Takaoka et al., 2012; Fukuda et al., 2019). For example, *O. gutturosa* and *O. cervicalis*, a common filarial parasite of cattle and horse, respectively, have been reported as suspected causes of cases identified in Europe and North
America (Takaoka et al., 2012). *Onchocerca lupi*, a parasite of canids, is responsible for ocular zoonotic onchocerciasis (Otranto et al., 2011). In Japan, the first case of zoonotic onchocerciasis occurred in Oita prefecture and three types (I, II and III) of *O. lupi* were found from three black fly species, i.e., *S. bidentatum, S. arakawae* and *S. aoki* which collected from cattle sheds (Takaoka and Bain, 1990).

To date, a total of 37 cases in humans have been reported worldwide, including the most recent case found in Fukushima, northeastern Honshu, Japan (Uni et al., 2001, 2010; Otranto and Eberhard, 2011; Otranto et al., 2011, Otranto et al., 2012; Takaoka et al., 2012; Eberhard et al., 2013; Fukuda et al., 2015a, Uni et al., 2015b, 2017; Caney et al., 2016; Fukuda et al., 2019). *Onchocerca dewittei japonica*, the parasite of wild boar (*Sus scrofa*), is the causative agent of zoonotic onchocerciasis in all Japanese cases and *S. bidentatum* is its vector in Oita, Japan (Takaoka et al., 2012; Fukuda et al., 2019).

In Thailand, a total of 114 black fly species belonging to six subgenera of the genus *Simulium* have been recorded (Takaoka et al., 2019; Thajirern et al., 2019a; Srisuka et al., 2019). Among these, seven species are the human-biting species: *S. asakoae* complex, *S. chamlongi*, *S. doipuiense* complex, *S. nigrogilvum*, *S. nodosum*, *S. tenebrosum* complex and *S. umphangense* (Chochoote et al., 2005; Pramual et al., 2016; Takaoka et al., 2017, 2019). *Simulium asakoae* complex and *S. chumpornense* have recently been reported as potential vectors to transmit blood protozoa of the genus *Leucocytozoon* (Jumapo et al., 2019) and *Trypanosoma* (Thajirern et al., 2019b) among birds and domestic chickens in Thailand. Furthermore, three different kinds of unidentified filarial parasites were recovered from three Thai black fly species, i.e., *S. asakoae* complex, *S. nigrogilvum* and *S. nodosum* (Fukuda et al., 2003; Ishii et al., 2008; Takaoka et al., 2003, 2019). Notably, filarial larvae found in *S. nodosum* were an unknown species of *Onchocerca* (Takaoka et al., 2003; Ishii et al., 2008). Thus, information about zoonotic filariae naturally transmitted by Thai simulids is limited to a few areas of Chiang Mai province, northern Thailand.

In this study, we attempted to examine wild-caught adult female black flies for filarial nematode infections and to identify filarial larvae in western Thailand.

2. Materials and methods

2.1. Study area and black fly collections

Collections of adult female black flies were carried out between May 2016 and August 2017 at the village in Ban Mae Klong Yai, Umphang district, Tak province, western Thailand (16°19′11.3″N; 99°01′05.6″E, elevation 1034 m). The cattle shed was situated within a 1-kilometer radius from the resident’s area. The wild-caught female black flies were collected from cattle sheds (Takaoka and Bain, 1990).

2.2. Morphological identification of female black flies under optical microscope and scanning electron microscope (SEM)

Adult female black flies were morphologically identified under an optical microscope following the checklist and keys for the black flies of Thailand (Takaoka et al., 2019). Additionally, the external morphology of the flies was examined using a scanning electron microscope to confirm the species identity of the closely related species. The heads of female black flies were excised under a stereomicroscope and rinsed three times in phosphate buffer (pH 7.4) to remove surface debris. The heads were then dehydrated through an ethanol series of 35, 70, 80% (10 min, two changes) and 95% (15 min, two changes), followed by absolute ethanol (10 min, two changes). Following dehydration, they were dried in a critical point dryer. The heads were mounted on aluminum stubs with double-sided carbon adhesive tape and sputter-coated with gold. The mouthparts of the heads were observed and photographed in a JEOL-JSM6610LV scanning electron microscope (JEOL, Japan) (Tasi et al., 2017).

2.3. Molecular identification of the infected black flies

Genomic DNA was extracted from individual black flies [four samples of *S. nigrogilvum* and three samples of *S. umphangense*] using the PureLink® Genomic DNA Mini Kit (Invitrogen, USA) according to the manufacturer’s instructions. Amplifications of the three genes (*COI, COII* and *ECP1*) were performed following the methods described previously (Conflitti et al., 2012; Low et al., 2014, 2016b). Primer pairs for amplifying each gene region are listed in Table 1. Briefly, PCR reactions were conducted in a total volume of 20 µl containing 1–2 µl of genomic DNA, 0.5 U of *Taq* DNA polymerase, 1.5–3 mM of MgCl₂, 0.25 mM dNTPs and 0.2 µM of each primer. The PCR cycling parameters followed those of Conflitti et al. (2012) for *COI*, Low et al. (2014) for *COII*, and Low et al. (2016b) for *ECP1*. The amplified products were electrophoresed on 1.5% agarose gels and stained with SYBR Safe (Invitrogen Corp., Carlsbad, CA, U.S.A.). The PCR products were purified and directly sequenced in both directions by 3130 genetic analyzers (Applied Biosystems, USA). DNA sequencing was performed at Macrogen (Seoul, Korea). DNA sequences of each three sample of *S. asakoae* complex and *S. nodosum* were also included in the phylogenetic analysis.

2.4. Dissections of adult black flies for filarial larvae and morphological identification of recovered larvae

After classification to species, individual adult female flies were microscopically dissected and examined for filarial larvae in a drop of 0.85% normal saline solution on a glass slide. Morphological identification of the infective larvae recovered followed Bain and

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Table 1

<table>
<thead>
<tr>
<th>Genes and primers</th>
<th>Sequences (5′–3′)</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Mitochondria</strong></td>
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<tr>
<td><em>COI</em></td>
<td>GGTCAAAACATCATAAAGATATTGG</td>
<td>Confitti et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>TAAACCTCAGGTTGACAAAAATCA</td>
<td></td>
</tr>
<tr>
<td><em>COII</em></td>
<td>ATTAGTGCAATTGTGCA</td>
<td>Low et al. (2014)</td>
</tr>
<tr>
<td>TL2-J-3034</td>
<td>GTTTAAGGACCACTTTG</td>
<td></td>
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<tr>
<td>TK-N-3785</td>
<td></td>
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<tr>
<td><strong>Nuclear</strong></td>
<td></td>
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<tr>
<td><em>ECP1</em></td>
<td>TGGCCCTCAATATGTGCA</td>
<td>Low et al. (2016b)</td>
</tr>
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<td></td>
<td>GGCTTCTCAATGTGCA</td>
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Chabaud (1986) and Takaoka and Bain (1990). Individual recovered larvae were preserved in 1.5 ml microcentrifuge tube containing 80% ethanol and kept at −20 °C until used for molecular study.

2.5. Molecular identification of filarial larvae

The filarial larvae were determined to species using partial mitochondrial COI and 12S rRNA genes. Genomic DNA of the individual infective larvae was extracted with the PureLink® Genomic DNA Mini Kit (Invitrogen, USA) according to the manufacturer's protocol. The partial mitochondrial COI gene was amplified using a primer set: COIintF (5′-TGA TTG GTG GTT TTG GTA A-3′) and COIintR (5′-ATA AGT ACG AGT ATC AAT ATC-3′) (Casiraghi et al., 2001) while the mitochondrial 12S rRNA gene was amplified with a primer set: 12SF (5′-GTA TTG GTG GTT TTG GTA A-3′) and 12SR (5′-ATT GAC GGA TG(A) TTT GTA CC-3′) (Casiraghi et al., 2004). PCR was performed in a final volume of 20 µl comprised 1–2 µl of genomic DNA, 0.5 U of Taq DNA polymerase, 2–3 mM of MgCl₂, 0.25 mM dNTPs and 0.2 µM of each primer under the thermal profile described previously by Fukuda et al., 2010b. After that, the amplified fragments were checked, purified and sequenced following the methods described above.

2.6. Data analysis

Both forward and reverse sequences were assembled and edited manually using MEGA Version 7.0 program (Kumar et al., 2016). All sequences were aligned using the ClustalW multiple alignment programs (Thompson et al., 1994). Gap sites were excluded from the following analyses. Genetic distances were estimated from the Kimura two-parameter (K2P) method (Kimura, 1980). Phylogenetic trees were constructed using neighbor-joining (NJ), maximum-parsimony (MP), Bayesian inference (BI) and maximum-likelihood (ML) methods. The NJ and ML trees were calculated in MEGA Version 7.0 program (Kumar et al., 2016). The MP tree was performed in PAUP 4.0b10 (Swofford, 2002) using the heuristic search option, 100 random sequences additions, tree bisection reconnection (TBR) branch swapping. BI was performed in MrBayes v.3.2 (Ronquist et al., 2012), after determining the appropriate substitution model by applying the Akaike information criterion (AIC) in MrModelTest 2.3 (Nylander, 2004). The analysis consisted of two runs, each with four MCMC chains running, and sampling every 500th generation. The average standard deviation of split frequencies (ASDSF) and the potential scale reduction factor (PSRF) were used to determine topology convergence between runs. The appropriate burn-in generations (25% for all analyses) were discarded based on convergence assessments of the ASDSF passing below 0.01. The remaining trees were summarized in a 50% majority-rule consensus tree. Branch support for all methods were calculated using bootstrap method with 1000 replications. The sequence of Prosimulium travisi was used as an outgroup in phylogenetic tree of infected black flies.

Phylogenetic trees of filarial larvae were constructed separately for each gene (COI and 12S rRNA) using NJ, MP, BI and ML methods. Accession numbers of the sequences retrieved from GenBank for analyses were shown in phylogenetic trees. The sequence of Thelazia callipea was used as an outgroup in all phylogenetic analyses. All the newly sequences generated from the present study were deposited in the National Center for Biotechnology Information (NCBI) GenBank database (accession numbers MN598519-MN598559).

2.7. Ethical approval

The protocol used in this study was approved by the Research Ethics Committee (Permit No. 46/2561), Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

3. Results

3.1. Morphological identification of adult female black flies under optical and SEM

All of 494 adult female black flies (Fig. 1A) collected were morphologically identified as S. nigrogilvum, which is distinguished from its allies of the S. indicum species-group, S. umphangense and S. vanellum, by the bicolored fore tibia (tb) (Fig. 1A) and mandible with distinct outer teeth, respectively. The mandible of S. nigrogilvum had 34 inner teeth (It) and six distinct outer teeth (Ot), as viewed with scanning electron microscopy (Fig. 1B).

3.2. Molecular identification of infected female black flies

The mean interspecific differentiation between S. nigrogilvum and its closely related species, S. umphangense, was 5.71% (5.52–5.90%), 7.51% (7.45–7.62%) and 3.80% (3.68–4.05%) for COI, COII and ECP1 genes respectively. The phylogenetic trees based on single genes (COI, COII and ECP1) revealed the similar tree topology in all phylogenetic methods, only NJ tree based on COI gene is shown (Fig. 2). The tree for COI separated S. nigrogilvum from S. asakoae complex, S. nodosum and S. umphangense with high bootstrap support (100). The level of genetic divergence and the phylogenetic analyses confirmed that all infected female black flies morphologically identified as S. nigrogilvum are indeed S. nigrogilvum.

3.3. Natural infections with filarial larvae

After dissections, four females of S. nigrogilvum harbored one to
three third-stage larvae (infective larvae) in their thoraces, with an infection rate of 0.81% (4/494). A total of six infective larvae were characterized by the measurement of their body size (1068–1346 µm long by 25–28 µm wide), and elongate esophagus, which was slightly shorter than the half of the body length (Fig. 3). The recovered infective larvae from *S. nigrogilvum* were almost similar in size and morphology to *O.* sp. type I and *O.* sp. type A, both found from *S. bidentatum* in Japan (Takaoka and Bain, 1990; Fukuda et al., 2008, Fukuda et al., 2010b) and *O.* sp. from *S. nodosum* in Thailand by Takaoka et al. (2003).

3.4. Molecular identification of filarial larvae

All infective larvae were used for the determination of the mitochondrial COI and 12S rRNA genes. All sequences were 649 bp long for the COI gene region and 471 bp for the 12S rRNA gene region. Five of the six infective larvae were successfully amplified and were identical to *O.* sp. type A (Figs. 4 and 5) with the nucleotide difference of 1.00–1.20% and 0.00% for COI and 12S rRNA genes, respectively. The *O.* sp. type I of the present study formed a monophyletic clade in all phylogenetic analyses with high bootstrap support (99–100) and clustered with *O.* sp. type A recovered from *S. bidentatum* in Japan. Sequence analyses of the COI and 12S rRNA genes of *O.* sp. type I of this study revealed 9.80–13.50% and 3.80–7.20% differences, respectively, compared with those of all other *O.* spp. The phylogenetic relationships based on COI and 12S rRNA gene sequences between *O.* sp. type I obtained from this study and other filarial nematodes are given in NJ trees (Figs. 4 and 5).

4. Discussion

In this study, we extend the knowledge of the zoonotic filarial infections in the western region of Thailand, and contribute to a natural vector, *S. nigrogilvum*, which can transmit the bovine filaria, *O.* sp. type I, in this area. The species identity of *S. nigrogilvum* and its closely related species, *S. umphangense*, was confirmed by comparing their morphology and DNA sequences as they are distributed in the same province (Takaoka et al., 2017), and also *S. vanellum*, which was recorded...
in Kanchanaburi province in Thailand (Huang et al., 2010). The results show that the mitochondria-encoded COI, COII and nuclear-encoded ECP1 genes were able to clearly distinguish S. nigrogilvum from S. umphangense. The large COI and COII genetic distances between S. nigrogilvum and S. umphangense would undoubtedly serve as the barcoding gap for species delimitation (Low et al., 2016a). Genetic divergence of 3% for the COI gene has been suggested as a general guide for recognizing distinct species (Ya’cob et al., 2017).

Herein, our findings reveal that all infective larvae recovered from S. nigrogilvum were morphologically and molecularly identified as O. sp. type I (= O. sp. type A sensu, Fukuda et al., 2010b) (Takaoka and Bain, 1990). The very low interspecific genetic divergences based on mitochondrial COI and 12S rRNA genes between our O. sp. type I and the type A larvae (Fukuda et al., 2010a,b) indicate that they are of the same species.

The morphology of these infective larvae from S. nigrogilvum appears to be the same as O. sp. found in S. nodosum in Thailand by Takaoka et al. (2003), suggesting that S. nodosum is also a vector of O. sp. type I. It is not unexpected if O. sp. type I is transmitted by two Thai black fly species belonging to the different species-groups (S. indicum and S. nobile species-groups), because its natural vectors in Japan include four black fly species of four different species-groups (S. argentinipes, S. malysschevi, S. variegatum, and S. venustum species-groups) (Takaoka et al., 2012).

On the other hand, O. sp. type I apparently differs at the subfamily level from the infective filarial larvae recorded from S. nigrogilvum in Thailand by Fukuda et al. (2003), indicating that S. nigrogilvum is vectors of two different filarial species in Thailand. Undoubtedly, this phenomenon is consistent with the findings of earlier study on black fly vectors in Japan (Takaoka et al., 2012). Simulium bidentatum has been reported as vectors of three different O. spp., i.e., O. japonica, O. takaokai, and O. sp. type I. Under these somewhat complicated relationships between Simulium spp. and filarial species, molecular identification of filarial larvae in black flies, in particular, those of O. sp. from S. nodosum and an unnamed filarial species from S. nigrogilvum, is strongly needed.

5. Conclusion

Our findings demonstrate that S. nigrogilvum plays a role as a natural vector in the transmission of zoonotic onchocerciasis in western Thailand. The exact species identity of this natural vector was confirmed based on morphological (optical and scanning electron microscope) and DNA-based approaches (multi-locus genes: COI, COII and ECP1 genes). Furthermore, the integrated approach of morphology and the DNA sequence analyses (COI and 12S rRNA genes) revealed that all recovered infective larvae found in S. nigrogilvum were O. sp. type I, a bovine filaria, originally found from S. bidentatum in Japan, and that further investigations are needed to explore the adult stage of O. sp. type I in cattle or water buffaloes.

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CRediT authorship contribution statement


Declaration of Competing Interest

All authors declare that we have no conflict of interest.

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References


Fig. 5. Neighbor-joining tree of Onchocerca spp. based on 12S rRNA gene sequences. Bootstrap values [NJ/MP/ML/BI] are shown above or near the branches. Scale bar represents 0.02 substitutions per nucleotide position. Red squares after accession number represent the specimens from this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)