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RESEARCH ARTICLE

First blood-meal record of *Simulium asakoae* (Diptera: Simuliidae) in Malaysia, with notes on its distribution in Asia and status as a potential vector

Izwan-Anas, N.[1], Ya'cob, Z.[1*], Low, V.L.[1], Lourdes, E.Y.[1], Teoh, B.-T.[1], Mansor, M.S.[2], Ramli, R.[3], Pramual, P.[4], Adler, P.H.[5], Takaoka, H.[1], AbuBakar, S.[1]

[1]Higher Institution Centre of Excellence (HICoE), Tropical Infectious Diseases Research & Education Centre (TIDREC), 50603, Universiti Malaya, Kuala Lumpur, Malaysia
[2]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[3]Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[4]Department of Biology, Faculty of Science, Mahasarakham University, Kantharawichai District, Maha Sarakham 44150, Thailand
[5]Department of Plant and Environmental Sciences, Clemson University, Clemson, SC, 29634, USA
*Corresponding author: zyacob@um.edu.my

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ABSTRACT

Simulium asakoae Takaoka and Davies has been confirmed to bite humans and has been incriminated as a vector of blood protozoan parasites of the genera *Leucocytozoon* and *Trypanosoma*, as well as an unknown filarial parasite in Thailand. However, its attraction to humans has remained uninvestigated in Malaysia. Recently, 27 black flies were collected in Pahang, Malaysia, of which 25 were captured in CO_2-baited Malaise traps and two were collected from humans during trapping activity. All specimens were morphologically identified as *S. asakoae*. Cytochrome c oxidase I sequences of the two specimens caught on humans showed 100% similarity with those of *S. asakoae* in the NCBI GenBank, confirming their morphological identity. Blood-meal analysis using a HAL·HAR^{TM} kit did not show the presence of domestic or wild animal DNA. However, human DNA was amplified from one engorged fly in the cytochrome b gene amplification assay, providing the first evidence of blood-feeding by *S. asakoae* in Malaysia.

Keywords: Public health; Malaise trap; infectious diseases; *Onchocerca* sp.; zoonosis.

INTRODUCTION

More than 25 species of the genus *Simulium* are vectors of *Onchocerca volvulus* (Leuckart), the sole causal agent of human onchocerciasis. This parasitic disease causes skin and eye symptoms and can lead to impaired vision and blindness. Over 20.9 million infections worldwide have been reported, with 14.6 million individuals experiencing epidermal problems and more than one million losing their vision (WHO, 2022). The disease is prevalent in sub-Saharan Africa and Yemen and in a few foci in Latin America. A reliable vaccination is still lacking. *Onchocerca* parasite transmission in affected areas is controlled with the microfilaricide ivermectin and vector suppression (Takaoka, 2015; WHO, 2022).

Zoonotic onchocerciasis, a human parasitic disease caused by *Onchocerca* spp. of animal origin and transmitted to humans via the bite of infected black flies, has become more common and is now a public health concern in affected countries (Cambra-Pelleja *et al.*, 2020). In Japan, a series of cases have been reported in which humans were infected with *O. japonica* Uni, Bain and Takaoka, a parasite that typically infects wild boars and is transmitted through the bites of infected females of *S. bidentatum* (Shiraki) (Takaoka *et al.*, 2012; Uni *et al.*, 2015; Fukuda *et al.*, 2019). Four other *Onchocerca* spp., namely *O. lupi, O. gutturosa, O. cervicalis*, and *O. jakutensis*, which are parasites of dogs, cattle, horses, and European deer, respectively, have been identified as the causative agents for zoonotic onchocerciasis in North America and Europe. However, their specific black fly vectors have not yet been identified (Koehsler *et al.*, 2007; Otranto *et al.*, 2011; Takaoka *et al.*, 2004). In Thailand, five black fly species, namely *S. asakoae, S. nodosum* Puri, *S. nigrogilvum* Summers, *S. khelangense* Takaoka, Srisuka and Saeung, and *S. chumpornense* Takaoka and Kuvangkadilok, are confirmed to transmit various parasites, such as blood protozoans of the genera *Leucocytozoon* and *Trypanosoma*, as well as filarial parasites including *Onchocerca* spp. (Takaoka *et al.*, 2014; Thaijarern *et al.*, 2019; Aupalee *et al.*, 2020; Pramual *et al.*, 2020a).

Malaysia is home to almost 100 named species of black flies (Adler, 2024). However, none of these species have been confirmed as vectors of disease-causing agents, despite their potential threat. *Simulium digrammicum* Edwards and *S. fuscopilosum* Edwards have been reported to ingest human blood in Malaysia (Takaoka & Davies, 1995), but scientific evidence is lacking. The elusive behavior of adult black flies in Malaysia, where they do not conspicuously swarm or suck blood, has made it difficult to investigate their roles as vectors, including vectorial capacity, bionomics, and parasite transmission. In the current study, two black fly adults, one of which was fully engorged, were accidentally collected from humans. The samples were analyzed for blood meals to evaluate possible hosts.

MATERIALS AND METHODS

Field collection

Sampling was conducted on 2 September 2020 at a highland tea plantation in Brinchang, Pahang, Malaysia (Table 1). Adult sampling was performed near a small stream, which had been identified as breeding habitat for *S. asakoae* Takaoka and Davies, *S. brinchangense* Takaoka, Sofian-Azirun and Hashim, and *S. aureohirtum* Brunetti (Ya'cob *et al.*, 2016a). A Malaise trap with a CO_2 source was set up at 1500 hours and operated for 24 hours. Briefly, the CO_2 was produced using baker's yeast mixed with sugar and water, following the protocols described by Guerenstein *et al.* (1995) and Smallegange *et al.* (2010). Samples collected in the Malaise trap, along with two flies accidentally collected when landed and bit on researchers during the trapping activity, (one biting near the ankle and another was landed but not biting), were fixed in 80% ethanol in a 1.5-mL microcentrifuge vial and stored in an icebox before being transported to the laboratory. The coordinates of the sampling sites were recorded using a handheld global positioning system instrument (Garmin International Inc., Olathe, KS, USA). Other ecological factors were also recorded, including temperature, vegetation, and canopy cover (Table 1).

Table 1. Ecological details of study sites in Cameron Highlands, Malaysia, 2 September 2020

Location	Brinchang, Pahang
GPS	N 4°28.738' E 101°22.979' and N 4°31.258' E 101°24.247'
Elevation (m asl)	1405–1431
Trapping duration	24 hours
Average temperature (°C)	19 to 21
Vegetation	Dispersed shrubs and tea plantation
Canopy cover	Open to partially closed
Wind speed (km/h)	3.2 to 3.3
Humidity (%)	85

Morphological and molecular identification

Specimens were morphologically identified using keys for Peninsular Malaysia and original species descriptions (Takaoka & Davies, 1995; Takaoka *et al.*, 2018a). Morphological identification was confirmed with molecular analysis through DNA barcoding. Genomic DNA was isolated individually using the G-spin Total DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Seongnam, South Korea). The samples, specifically one mid and hind legs, were subjected to COI barcoding using the primers of Folmer *et al.* (1994) and PCR protocol of Low *et al.* (2015). All PCR products were sent to Apical Scientific (Malaysia) for sequencing.

Sequence alignment and phylogenetic analysis

For molecular species identification, DNA sequences were manually edited using BioEdit version 7.2.5 (Hall, 2011) and compared in the GenBank database, using the Basic Local Alignment Search Tool (BLAST). Two newly generated sequences in this study were deposited in GenBank under accession numbers PP177910 and PP177911. All reference sequences of the *S. asakoae* species group in Malaysia were retrieved and included for comparison. *Simulium aziruni* Takaoka, Hashim and Chen of the *S. gombakense* species group was used as an outgroup. Neighbor Joining (NJ) analysis was performed using Kimura's two-parameter substitution model in MEGA 11 version 11.0.11 (Tamura *et al.*, 2021) with 1000 bootstrap replicates. Maximum likelihood (ML) analysis was performed using the IQ-TREE webserver (Trifinopoulos *et al.*, 2016) and an auto substitution model option and standard bootstrap analysis of 100 replicates. Bayesian analysis was run using MrBayes version 3.2.7a (Ronquist *et al.*, 2012) and the HKY + G substitution model was suggested as the best model by jModeltest2 (Darriba *et al.*, 2012). Bayesian analysis was performed on two million generations of Markov Chain Monte Carlo (MCMC) and the tree was sampled every 100th generation, with 25% of trees discarded as burn-in. All analyses were performed on the online server Cyber Infrastructure for Phylogenetic Research (CIPRES) (Miller *et al.*, 2011). All trees were visualized in FigTree v1.4.4 and edited in Interactive Tree of Life (iTOL) (Letunic & Bork, 2021) and Adobe Illustrator 2020. Pairwise genetic distance was calculated using the Kimura 2-parameter model in MEGA 11 version 11.0.11 (Tamura *et al.*, 2021).

Molecular identification of host blood meals

The extracted DNA was analyzed by two methods to identify possible blood meal sources. The first method used the mammalian cytochrome b gene and UNFOR403 and UNREV1025 primers, following the procedure of Kent and Douglas (2005), which generates a 623-bp product. PCR products were verified by gel electrophoresis and were sent for sequencing. The generated sequences were compared using BLAST analysis in GenBank to confirm the blood source.

The second method used the HAL·HAR^{TM} kit (TIDREC, Malaysia) to test whether the blood contained other non-human DNA according to specific band sizes, such as chicken (*Gallus* spp.) (207 bp), cow or buffalo (*Bos* spp. or *Bubalus* spp.) (298/299 bp), rabbit (*Oryctolagus* spp.) (417 bp), goat or sheep (*Capra* spp. or *Ovis* spp.) (504/505 bp), horse (*Equus* spp.) (639/640bp), pig (*Sus* spp.) (176 bp), cat (*Felis* spp.) (273 bp), frog (*Bufo* spp., *Fejervarya* spp., *Hoplobatrachus* spp., *Kaloula* spp., *Rana* spp.) (397/409 bp), dog (*Canis* spp.) (615 bp), and rat (*Rattus* spp.) (764/767 bp). In both analyses, two genomic DNA samples of black flies attracted to humans in Terengganu, Malaysia (Izwan-Anas *et al.*, 2021) were included to confirm any possibility of a blood source in the samples.

RESULTS AND DISCUSSIONS

Morphological and molecular species identification

A total of 27 flies were captured, including two individuals (7.4%) accidently collected from humans during trapping activity and 25 individuals (92.6%) from a Malaise trap. All specimens appeared morphologically identical and to belong to the subgenus *Gomphostilbia* based on the presence of a haired katepisternum. These flies were identified as *S. asakoae* based on the following characteristics: antennae each with nine flagellomeres, claws each with a large basal tooth, a yellow hair tuft at the base of the radial vein, hind tibia whitish on the basal half to two-thirds without a dark sub-basal spot, sensory vesicle less than 0.4 times the length of the third maxillary palpomere, and mandible with inner teeth and outer margin with five or six teeth (Takaoka & Davies 1995; Takaoka *et al*., 2017, 2018a).

Molecular analysis showed that the COI sequences of the two flies accidently collected from a human were 100% identical to those of *S. asakoae*, confirming their morphological identification. The genetic distance between the newly generated sequences and *S. asakoae* from the GenBank database also showed the highest similarity (genetic distance = 0.00%), whereas the next closest species were *S. tanahrataense* Takaoka, Sofian-Azirun and Ya'cob (6.68%) and *S. roslihashimi* Takaoka and Sofian-Azirun (6.82–7.33%). This finding was further supported by the DNA tree (Figure 1), which placed the specimens in the same lineage as *S. asakoae*, with a strong support value.

Blood meal analysis

Blood meal analysis using the HAL·HAR^{TM} kit revealed that none of the four black flies (two from Izwan-Anas *et al.*, 2021) had fed on non-human blood sources (Figure 2). The screening was then continued using mammal-specific primers, including human. One

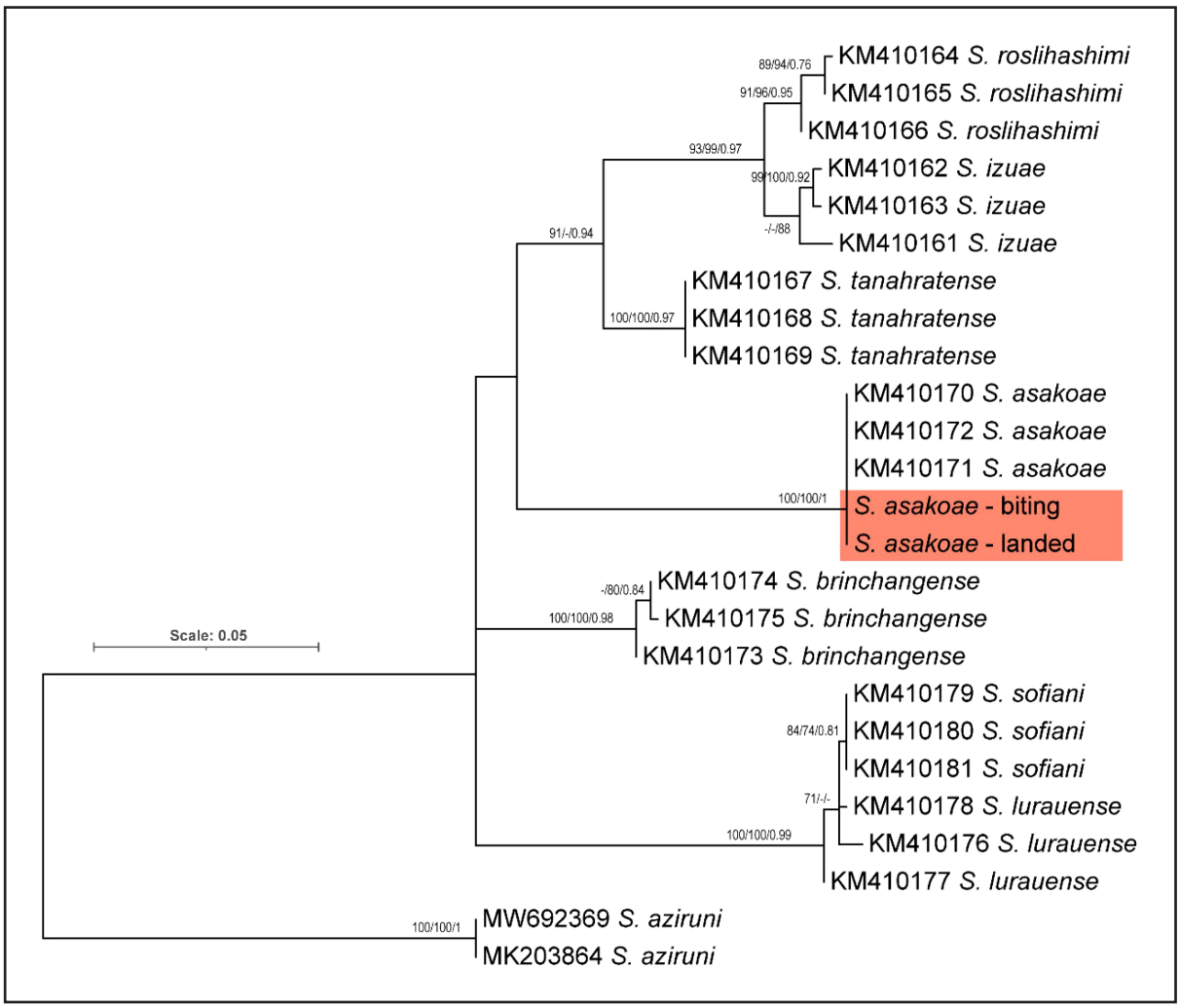

Figure 1. Maximum likelihood tree of *Simulium asakoae* species group in Malaysia, except *S. hoiseni*, inferred from mitochondrial COI gene sequences. The red rectangle is the newly generated sequence in this study. *Simulium aziruni* of the *S. gombakense* species group was used as an outgroup. Bootstrap values and posterior probability of more than 70% or 0.7 are shown on the branches (ML/NJ/BI).

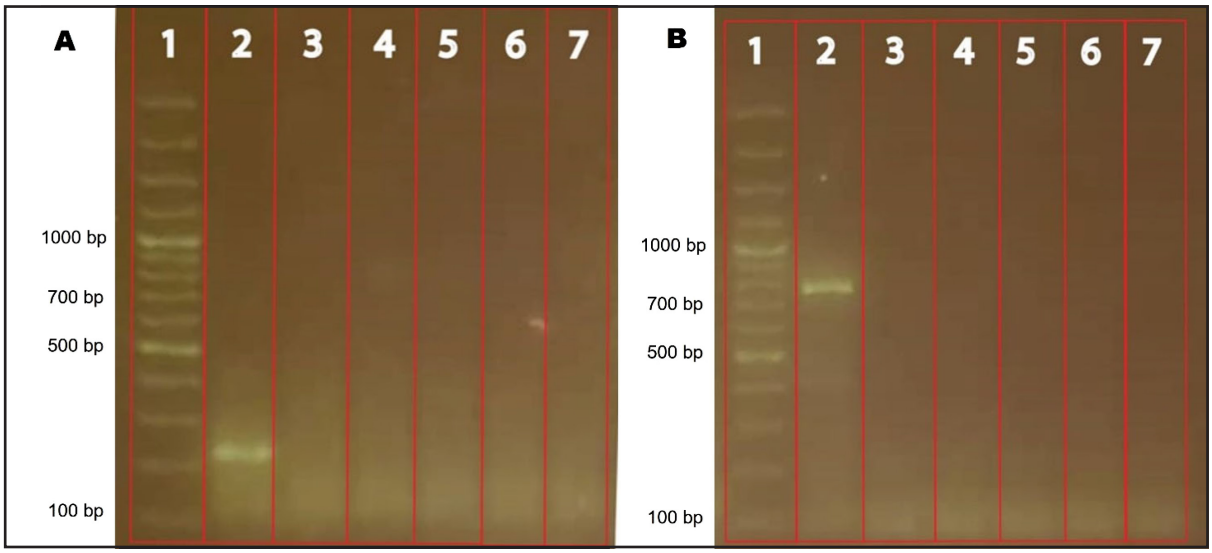

Figure 2. PCR amplification using a HAL- HAR™ kit to detect the possible blood source. **Set A:** 1) Ladder 100bp, 2) positive control (chicken), 3) negative control, 4) *Simulium aziruni* 1 (attracted to human, Izwan-Anas *et al*., 2021), 5) *Simulium aziruni* 2 (attracted to human, Izwan-Anas *et al*., 2021), 6) *Simulium asakoae* (biting human, this study), 7) *Simulium asakoae* (possibly biting human, this study). **Set B:** 1) Ladder 100bp, 2) Positive control (rat), 3) negative control, 4) *Simulium aziruni* 1 (attracted to human, Izwan-Anas *et al*., 2021), 5) *Simulium aziruni* 2 (attracted to human, Izwan-Anas *et al*., 2021), 6) *Simulium asakoae* (biting human, this study), 7) *Simulium asakoae* (possibly biting human, this study).

specimen collected in this study (Cameron Highlands) tested positive for human blood (Figure 3). BLAST analysis confirmed that the positive sample was human blood with 98% similarity, providing the first solid evidence of *S. asakoae* feeding on human blood in Malaysia.

Simulium asakoae was originally described from Malaysia and is a member of the *S. asakoae* species group with eight and 58 members from Malaysia and the Oriental Region, respectively (Adler, 2024). It has a wide geographical distribution: China (Hong Kong), Myanmar, Vietnam, Thailand, Taiwan, and Peninsular Malaysia (Takaoka *et al.*, 2021; Adler, 2024) (Figure 4).

Phylogenetic analysis has indicated that the Thai and Myanmar populations are genetically similar to typical *S. asakoae* from Malaysia, although the intraspecific variation is high in the Thai populations (Low *et al.*, 2020). The other closely related members described from Malaysia include *S. brinchangense*, *S. hoiseni* Takaoka, *S. izuae* Takaoka *et al.*, *S. lurauense* Takaoka *et al.*, *S. roslihashimi*, *S. sofiani* Takaoka and Hashim, and *S. tanahrataense* (Low *et al.*, 2015; Adler, 2024).

Simulium asakoae has received considerable attention due to its medical and veterinary importance (Ishii *et al.*, 2008; Thaijarern *et al.*, 2019; Aupalee *et al.*, 2020). The taxonomic and ecological status of most Malaysian black flies has been well-documented and characterized (Ya'cob *et al.*, 2016a, 2016b). *Simulium asakoae* inhabits middle to high elevations. Its larvae and pupae are found at water temperatures from 19°C to 21°C (Ya'cob *et al.*, 2016a). Similarly, in Thailand, *S. asakoae* is found in streams with water temperatures of 22°C and below (e"500 m asl) (Srisuka *et al.*, 2015).

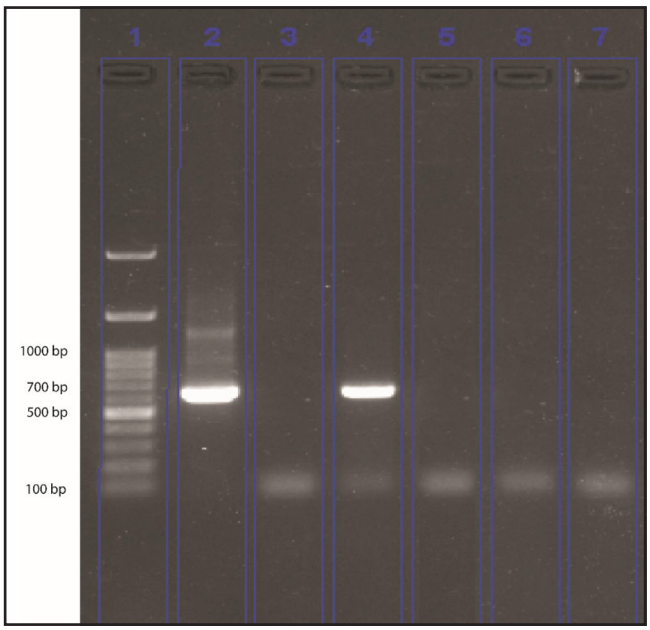

Figure 3. Positive PCR amplification of 623 bp using Cyt b. Lane 1: Ladder 100 bp, Lane 2: vertebrate DNA (positive control), Lane 3: negative control, Lane 4: *Simulium asakoae* – biting human (this study), Lane 5: *Simulium asakoae* – landed on human (this study), Lane 6: *Simulium aziruni* 1 (attracted to human, Izwan-Anas et al., 2021), Lane 7: *Simulium aziruni* 2 (attracted to human, Izwan-Anas *et al*., 2021).

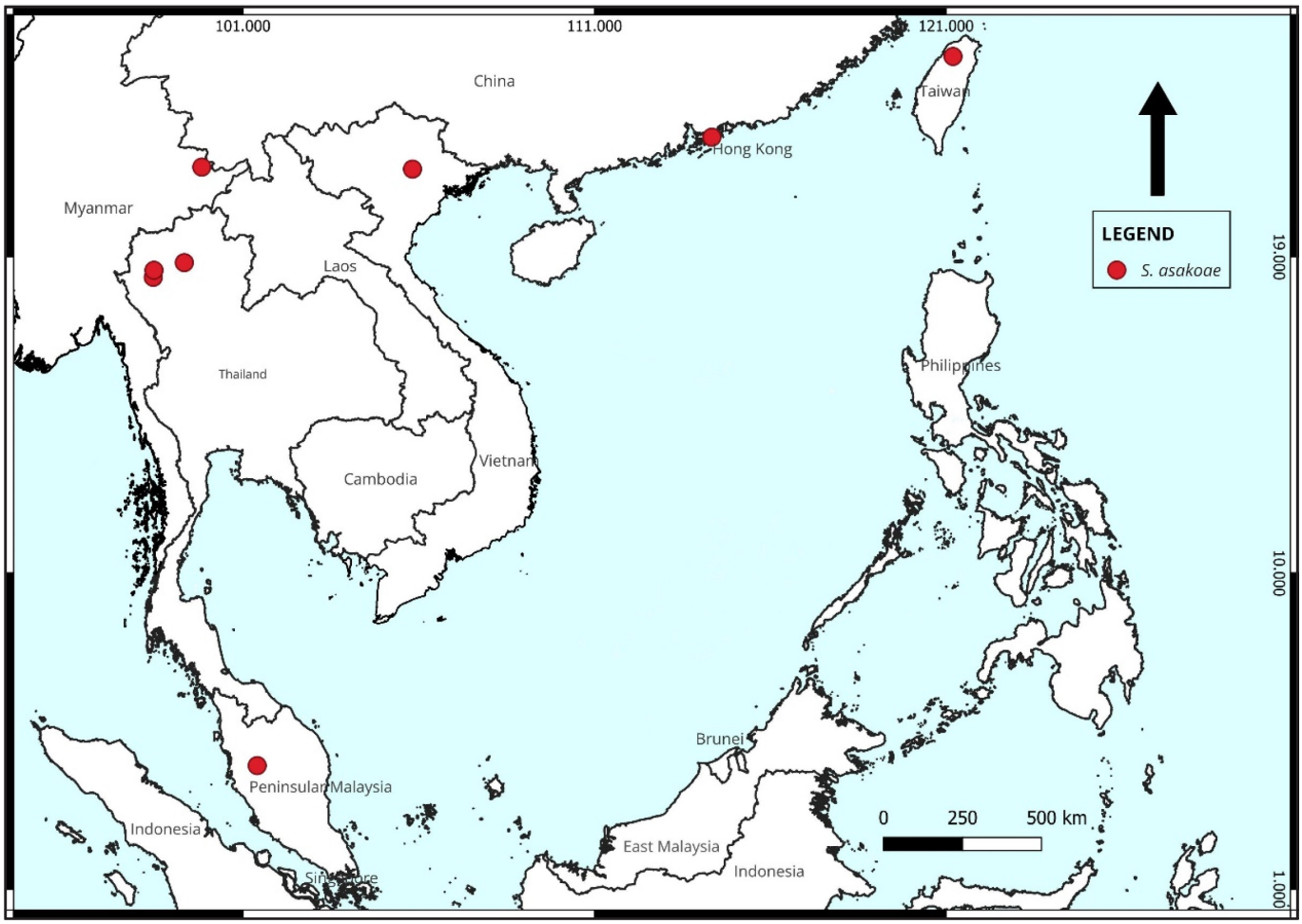

Figure 4. Distribution of *Simulium asakoae* in Asian countries: China (Hong Kong), Peninsular Malaysia, Myanmar, Taiwan, Thailand (dots represent studies on *S. asakoae* in Northern Thailand mostly related to biting activities), and Vietnam. Cameron Highlands of Peninsular Malaysia is the type locality of this species.

Table 2. Notes on the distribution and biological status of *Simulium asakoae* in Asian countries

Country	Distribution	Status	Reference
Malaysia	Cameron Highlands, Pahang	Confirmed as biting humans	Current study
Thailand	Chiang Mai Province (Doi Saket District)	Confirmed as biting humans and vector of unknown filaria parasites and avian blood protozoans *Trypanosoma* and *Leucocytozoon*	(Aupalee *et al.*, 2020; Fukuda *et al.*, 2003; Ishii *et al.*, 2008; Pramual *et al.*, 2020b; Thaijarern *et al.*, 2019)
Vietnam	Tam Dao National Park in Vinh Phuc Province	Not confirmed as biting humans, although collected while landed on a human	Takaoka *et al.* (2017); Takaoka *et al.* (2014)
Myanmar	Shan State in Northeastern	Not confirmed as biting humans	Takaoka *et al.* (2018b)
China	Hong Kong (Tai Po Kau, Sai Kung, Castle Peak, Lantau Island and Silver Mine Bay) and South China	Not confirmed as biting humans	Takaoka *et al.* (1995); Chen and An (2003)
Taiwan	Nantou County (Yuchih), Hsinchu County (Youlou River, Jiale Village, Jianshi Township)	Not confirmed as biting humans	Takaoka *et al.* (2021)

Simulium asakoae in Thailand has been confirmed as a natural vector of an unidentified filarial species and blood protozoan parasites of the genera *Leucocytozoon* and *Trypanosoma* (Fukuda *et al.*, 2003; Thaijarern *et al.*, 2019; Aupalee *et al.*, 2020; Pramual *et al.*, 2020b). The biting status and vectorial capacity of *S. asakoae* in other Asian countries, including Vietnam and Myanmar, remain unknown. Table 2 provides updated information on the distribution of *S. asakoae* and its status as an anthropophilic species and a vector of filaria in Asian countries.

The actual biting habits and vectorial roles of *S. asakoae* in the transmission of parasites and pathogens in Malaysia remain to be investigated. Females of most members of the *S. asakoae* species group are almost morphologically identical, sharing features such as mandibles with outer and inner teeth and claws each with a large basal tooth (Takaoka *et al.*, 2020). Female flies of this species group play a role in naturally transmitting various zoonotic parasites and other bacterial and viral pathogens. Further studies are warranted to elucidate their actual biting habits and potential for pathogen transmission.

Future investigation of *S. asakoae* should focus on the *Onchocerca* parasites to identify their potential to cause zoonotic onchocerciasis, including accurate species identification, prevalence, and the host-vector-parasite transmission cycle in Malaysia. A novel diagnostic tool, colorimetric loop-mediated isothermal amplification (LAMP) targeting specific DNA biomarkers of the genus *Onchocerca* (O-150), might be a good option for the mass screening of *Onchocerca* parasites due to its proven accuracy, sensitivity, and simplicity (Alhassan *et al.*, 2014). *Onchocerca* parasites of black flies are hypothesized to be highly diverse due to the abundance of various host animal species, such as wild boars in natural forests and marginalized areas and domestic animals, including those on cattle farms and deer ranches in Malaysia. Until recently, *O. dewittei* Bain, Ramachandran, Petter and Mak, 1977 was the only species of *Onchocerca* reported in Peninsular Malaysia (Bain, 2002). Another *Onchocerca* species, *O. borneensis* Uni, Mat-Udin and Takaoka, has been described from the bearded pig Sus *barbatus* M ller in Long Banga, Sarawak, Malaysia (Uni *et al.*, 2020). Therefore, it is recommended to conduct in-depth epidemiological surveillance on this potential zoonotic infectious disease by adopting the One Health investigative approach that includes human, animal, and environmental data.

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Conflict of interest statement

The authors declare that no competing interests exist.

REFERENCES

Adler, P.H. (2024). World blackflies (Diptera: Simuliidae): a comprehensive revision of the taxonomic and geographical inventory [2024]. https://biomia.sites.clemson.edu/pdfs/blackflyinventory.pdf. Accessed 27 May 2024.

Alhassan, A., Makepeace, B.L., LaCourse, E.J., Osei-Atweneboana, M.Y. & Carlow, C.K. (2014). A simple isothermal DNA amplification method to screen black flies for *Onchocerca volvulus* infection. *PLoS One* **9**: e108927. https://doi.org/10.1371/journal.pone.0108927

Aupalee, K., Saeung, A., Srisuka, W., Fukuda, M., Streit, A. & Takaoka, H. (2020). Seasonal filarial infections and their black fly vectors in Chiang Mai province, northern Thailand. *Pathogens* **9**: 512. https://doi.org/10.3390/pathogens9060512

Bain, O. (2002). Evolutionary relationships among filarial nematodes. In: The Filaria, Klei, T.R. & Rajan, T.V. (editors). Kluwer Academic Publisher, pp. 21-29.

Cambra-Pelleja, M., Gandasegui, J., Balana-Fouce, R., Munoz, J. & Martinez-Valladares, M. (2020). Zoonotic implications of *Onchocerca* species on human health. *Pathogens* **9**: 761. https://doi.org/10.3390/pathogens9090761

Chen, H.B. & An, J.Y. (2003). The Blackflies of China (Diptera: Simuliidae). Science, Press, Beijing.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772. https://doi.org/10.1038/nmeth.2109

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294-299.

Fukuda, M., Choochote, W., Bain, O., Aoki, C. & Takaoka, H. (2003). Natural infections with filarial larvae in two species of black flies (Diptera: Simuliidae) in northern Thailand. *Japanese Journal of Tropical Medicine and Hygiene* **31**: 99-102. https://doi.org/10.2149/tmh1973.31.99

Fukuda, M., Uni, S., Igari, T., Utsumi, Y., Otsuka, Y., Nakatani, J., Uga, S., Hara, T., Hasegawa, H. & Takaoka, H. (2019). Human case of *Onchocerca dewittei japonica* infection in Fukushima, Northeastern Honshu, Japan. *Parasitology International* **72**: 101943. https://doi.org/10.1016/j.parint.2019.101943

Guerenstein, P.G., Lorenzo, M.G., Nunez, J.A. & Lazzari, C.R. (1995). Baker's yeast, an attractant for baiting traps for Chagas' disease vectors. *Experientia* **51**: 834-837. https://doi.org/10.1007/BF01922439

Hall, T. (2011). BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences* **2**: 60-61.

Ishii, Y., Choochote, W., Bain, O., Fukuda, M., Otsuka, Y. & Takaoka, H. (2008). Seasonal and diurnal biting activities and zoonotic filarial infections of two *Simulium* species (Diptera: Simuliidae) in northern Thailand. *Parasite* **15**:121-129. https://doi.org/10.1051/parasite/2008152121

Izwan-Anas, N., Ya'cob, Z., Low, V.L., Lourdes, E.Y., Ramli, R., Bolongan, G. & Takaoka, H. (2021). *Simulium (Gomphostilbia) aziruni*: First record of a black fly (Diptera: Simuliidae) attracted to a human in Malaysia. *Acta Tropica* **218**: 105904. https://doi.org/10.1016/j.actatropica.2021.105904

Kent, R.J. & Douglas, N.E. (2005). Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome B. *American Journal of Tropical Medicine and Hygiene* **73**: 336-342. https://doi.org/10.4269/ajtmh.2005.73.336

Koehsler, M., Soleiman, A., Aspɤck, H., Auer, H. & Walochnik, J. (2007). *Onchocerca jakutensis* filariasis in humans. *Emerging Infectious Diseases* **13**: 1749-1752. https://doi.org/10.3201/eid1311.070017

Letunic, I. & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Research* **49**: W293-W296. https://doi.org/10.1093/nar/gkab301

Low, V.L., Srisuka, W., Saeung, A., Tan, T.K., Ya'cob, Z., Yeong, Y.S. & Takaoka, H. (2020). DNA Barcoding of *Simulium asakoae* (Diptera: Simuliidae) from Northern Thailand. *Journal of Medical Entomology* **57**: 1675-1678. https://doi.org/10.1093/jme/tjaa081

Low, V.L., Takaoka, H., Adler, P.H., Ya'cob, Z., Norma-Rashid, Y., Chen, C.D. & Sofian-Azirun, M. (2015). A multi-locus approach resolves the phylogenetic relationships of the *Simulium asakoae* and *Simulium ceylonicum* species groups in Malaysia: evidence for distinct evolutionary lineages. *Medical and Veterinary Entomology* **29**: 330-337. https://doi.org/10.1111/mve.12120

Miller, M.A., Pfeiffer, W. & Schwartz, T. (2011). The CIPRES Science Gateway: A Community Resource for Phylogenetic Analyses. Proceedings of the 2011 TeraGrid Conference: Extreme Digital Discovery. Association for Computing Machinery, New York, NY.

Otranto, D., Dantas-Torres, F., G rl , V.P., Sakru, N., Yakar, K., Bain, O., Lia, R.P. & Testini, G. (2011). First evidence of human zoonotic infection by *Onchocerca lupi* (Spirurida, Onchocercidae). *American Journal of Tropical Medicine and Hygiene* **84**: 55-58. https://doi.org/10.4269/ajtmh.2011.10-0465

Pramual, P., Tangkawanit, U., Kunprom, C., Vaisusuk, K., Chatan, W., Wongpakam, K. & Thongboonma, S. (2020a). Seasonal population dynamics and a role as natural vector of *Leucocytozoon* of black fly, *Simulium chumpornense* Takaoka & Kuvangkadilok. *Acta Tropica* **211**: 105617. https://doi.org/10.1016/j.actatropica.2020.105617

Pramual, P., Thaijarern, J., Tangkawanit, U. & Wongpakam, K. (2020b). Molecular identification of blood meal sources in black flies (Diptera: Simuliidae) suspected as *Leucocytozoon* vectors. *Acta Tropica* **205**: 105383. https://doi.org/10.1016/j.actatropica.2020.105383

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539-542. https://doi.org/10.1093/sysbio/sys029

Smallegange, R.C., Schmied, W.H., Van Roey, K.J., Verhulst, N.O., Spitzen, J., Mukabana, W.R. & Takken, W. (2010). Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito *Anopheles gambiae*. *Malaria Journal* **9**: 292. https://doi.org/10.1186/1475-2875-9-292

Srisuka, W., Takaoka, H., Otsuka, Y., Fukuda, M., Thongsahuan, S., Taai, K., Choochote, W. & Saeung, A. (2015). Seasonal biodiversity of black flies (Diptera: Simuliidae) and evaluation of ecological factors influencing species distribution at Doi Pha Hom Pok National Park, Thailand. *Acta Tropica* **149**: 212-219. https://doi.org/10.1016/j.actatropica.2015.05.024

Takaoka, H. (2015). Review of the biology and ecology of adult blackflies in relation to the transmission of onchocerciasis in Guatemala. *Tropical Medicine and Health* **43**(Suppl): 71-85. https://doi.org/10.2149/tmh.2015-S12

Takaoka, H. & Davies, D.M. (1995). The Black Flies (Diptera: Simuliidae) of West Malaysia. Kyushu University Press, Fukuoka, Japan.

Takaoka, H., Bain, O., Uni, S., Korenaga, M., Kozek, W.J., Shirasaka, C., Aoki, C., Otsuka, Y., Fukuda, M., Eshita, Y. *et al*. (2004). Zoonotic onchocerciasis caused by a parasite from wild boar in Oita, Japan. *Parasite* **11**: 285-292. https://doi.org/10.1051/parasite/2004113285

Takaoka, H., Davies, D.M. & Dudgeon, D. (1995). Black flies (Diptera: Simuliidae) from Hong Kong: taxonomic notes with descriptions of two new species. *Japanese Journal of Tropical Medicine and Hygiene* **23**: 189-196. https://doi.org/10.2149/tmh1973.23.189

Takaoka, H., Fukuda, M., Otsuka, Y., Aoki, C., Uni, S. & Bain, O. (2012). Blackfly vectors of zoonotic onchocerciasis in Japan. *Medical and Veterinary Entomology* **26**: 372-378. https://doi.org/10.1111/j.1365-2915.2012.01023.x

Takaoka, H., Low, V.L., Huang, Y.T., Fukuda, M. & Ya'cob, Z. (2021). Two new black fly species of the *Simulium (Gomphostilbia) asakoae* species-group (Diptera: Simuliidae) from Taiwan. *Acta Tropica* **222**: 106023. https://doi.org/10.1016/j.actatropica.2021.106023

Takaoka, H., Sofian-Azirun, M., Ya'cob, Z., Chen, C.D., Lau, K.W., Low, V.L., Pham, X.D. & Adler, P.H. (2017). The black flies (Diptera: Simuliidae) of Vietnam. *Zootaxa* **4261**: 1-165. https://doi.org/10.11646/zootaxa.4261.1.1

Takaoka, H., Sofian-Azirun, M., Ya'cob, Z., Chen, C.D., Lau, K.W. & Pham, H.T. (2014). Female black flies of *Simulium* (Diptera: Simuliidae) collected on humans in Tam Dao National Park, Vietnam: Description of a new species and notes on four species newly recorded from Vietnam. *Tropical Biomedicine* **31**: 742-748.

Takaoka, H., Srisuka, W., Fukuda, M. & Saeung, A. (2020). Twenty-one new species of the *Simulium* (*Gomphostilbia*) *asakoae* species group (Diptera, Simuliidae) in Thailand, with their genetic relationships. *ZooKeys* **950**: 51-152. https://doi.org/10.3897/zookeys.950.51298

Takaoka, H., Srisuka, W., Maleewong, W. & Saeung, A. (2018b). New records of 13 species of black flies (Diptera: Simuliidae) from Myanmar. *Acta Tropica* **178**: 281-289. https://doi.org/10.1016/j.actatropica.2017.12.009

Takaoka, H., Ya'cob, Z. & Sofian-Azirun, M. (2018a). Classification, annotated list and keys for the black flies (Diptera: Simuliidae) of Peninsular Malaysia. *Zootaxa* **4498**: 1-65. https://doi.org/10.11646/zootaxa.4498.1.1

Tamura, K., Stecher, G. & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology Evolution* **38**: 3022-3027. https://doi.org/10.1093/molbev/msab120

Thaijarern, J., Tangkawanit, U., Wongpakam, K. & Pramual, P. (2019). Molecular detection of *Trypanosoma* (Kinetoplastida: Trypanosomatidae) in black flies (Diptera: Simuliidae) from Thailand. *Acta Tropica* **200**: 105196. https://doi.org/10.1016/j.actatropica.2019.105196

Trifinopoulos, J., Nguyen, L.T., von Haeseler, A. & Minh, B.Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research* **44**: W232-235. https://doi.org/10.1093/nar/gkw256

Uni, S., Fukuda, M., Otsuka, Y., Hiramatsu, N., Yokobayashi, K., Takahashi, H., Murata, S., Kusatake, K., Morita, E., Maruyama, H. *et al*. (2015). New zoonotic cases of *Onchocerca dewittei japonica* (Nematoda: Onchocercidae) in Honshu, Japan. *Parasites & Vectors* **8**: 59. https://doi.org/10.1186/s13071-015-0655-2

Uni, S., Mat Udin, A.S., Agatsuma, T., Junker, K., Saijuntha, W., Bunchom, N., Fukuda, M., Martin, C., Lefoulon, E., Labat, A. *et al*. (2020). Description, molecular characteristics and *Wolbachia* endosymbionts of *Onchocerca borneensis* Uni, Mat Udin & Takaoka n. sp. (Nematoda: Filarioidea) from the Bornean bearded pig *Sus barbatus* Muller (Cetartiodactyla: Suidae) of Sarawak, Malaysia. *Parasites & Vectors* **13**: 50. https://doi.org/10.1186/s13071-020-3907-8

WHO (World Health Organization) (2022). Onchocerciasis. https://www.who.int/news-room/fact-sheets/detail/onchocerciasis. Accessed 26 January 2024.

Ya'cob, Z., Takaoka, H., Pramual, P., Low, V.L. & Sofian-Azirun, M. (2016a). Breeding habitat preference of preimaginal black flies (Diptera: Simuliidae) in Peninsular Malaysia. *Acta Tropica* **153**: 57-63. https://doi.org/10.1016/j.actatropica.2015.10.007

Ya'cob, Z., Takaoka, H., Pramual, P., Low, V.L. & Sofian-Azirun, M. (2016b). Distribution pattern of black fly (Diptera: Simuliidae) assemblages along an altitudinal gradient in Peninsular Malaysia. *Parasites & Vectors* **9**: 219. https://doi.org/10.1186/s13071-016-1492-7