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Culex (*Culiciomyia*) *sasai* (Diptera: Culicidae), senior synonym of *Cx. spiculothorax* and a new country record for Bhutan

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ABSTRACT

Culex (*Culiciomyia*) *spiculothorax* was described from Thailand based on the presence of spiculation on the thorax of larvae. Adult females are characterized but are indistinguishable from those of related species, such as *Cx. pallidothorax*. Phylogenetic analysis of mitochondrial oxidase subunit I (*COI*) sequences revealed that specimens identified as *Cx. spiculothorax* from Thailand, Japan and Bhutan form a single clade with *Cx. sasai* from Japan (Kimura 2-parameter genetic distances 0–0.9%) that is clearly distinct from clades comprised of other species of subgenus *Culiciomyia*. Attempts to collect *Cx. sasai* from several locations in Japan were unsuccessful – only larvae with thoracic vesicular-like spicules identified as *Cx. spiculothorax* were collected. Careful examination of specimens collected near the type locality of *Cx. sasai* revealed the presence of spicules on the thorax. Based on these findings, *Cx. spiculothorax* is formally synonymized with *Cx. sasai*, which replaces the former as the species present in Thailand and is a new country record for Bhutan.

1. Introduction

The subgenus *Culiciomyia* Theobald of the genus *Culex* Linnaeus (Diptera: Culicidae) consists of 55 mosquito species recorded from tropical areas of the Afrotropical, Oriental and Australasian Regions (Harbach, 2011). Sixteen species are recorded in Thailand (Rattanarithikul et al., 2005), including *Cx. bailyi* Barraud, *Cx. barrinus* Bram, *Cx. dispectus* Bram, *Cx. fragilis* Ludlow, *Cx. harrisoni* Sirivanakarn, *Cx. lampangensis* Sirivanakarn, *Cx. nigropunctatus* Edwards, *Cx. pallidothorax* Theobald, *Cx. papuensis* (Taylor), *Cx. sasai* Kano, Nitahara & Awaya, *Cx. scanloni* Bram, *Cx. spathifurca* (Edwards), *Cx. spiculothorax* Bram, *Cx. termi* Thurman, *Cx. thurmanorum* Bram and *Cx. viridiventer* Giles. *Culex nigropunctatus*, *Cx. pallidothorax* and *Cx. sasai* are also recorded from areas of the eastern Palaearctic Region (Japan, Korea and China) along with *Cx. kyotoensis* Yamaguti & LaCasse and *Cx. ryukyensis* Bohart (Tanaka et al., 1979).

The larvae of species of subgenus *Culiciomyia* are mostly distinct and have morphological features that are useful for identification. *Culex spiculothorax* was described from larvae found at high elevation on Doi Inthanon, the highest mountain in Thailand (Bram, 1967). Larvae are characterized by the presence of irregular rows of vesicular spicules on the dorsal surface of the thoracic integument. The species has also been recorded from China (Chau, 1982) and Malaysia (Ramalingam and Pillai, 1973). Identification of females is possible in some species but difficult in others due to morphological similarity. For example, the females of *Cx. bailyi*, *Cx. barrinus*, *Cx. harrisoni*, *Cx. lampangensis*, *Cx. pallidothorax*, *Cx. sasai*, *Cx. thurmanorum* and *Cx. viridiventer* are indistinguishable. The female of *Cx. spiculothorax* was unknown (Rattanarithikul et al., 2005) before the present study. Miyagi et al. (1986) reported the presence of *Cx. sasai* on Doi Inthanon but our repeated attempts to find this species failed. The larva of *Culex sasai* is morphologically similar to *Cx. spiculothorax* except the thoracic integument is “apparently smooth” (Tanaka et al., 1979). As attempts to find *Cx. sasai* on Doi Inthanon failed, we made collections in Japan where this nominal species was first discovered and described (Kano et al., 1954). A number of larvae collected in Japan that were initially identified as *Cx. sasai* proved to match the description of *Cx. spiculothorax* upon careful examination, particularly in having distinct vesicular spicules on the thoracic and abdominal integument, as described by Bram (1967) and Rattanarithikul et al. (2005). Collections

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were also carried out at high elevations in Bhutan, but no specimens identifiable as *Cx. sasai* were found. The larvae from Bhutan matched the larval description of *Cx. spiculothorax*. The present study provides evidence from larval morphology and *CO1* sequences for the conspecificity of *Cx. spiculothorax* and *Cx. sasai*, and a new record for the latter in Bhutan.

2. Materials and methods

2.1. Mosquito collections and identification

Larvae identified as *Cx. spiculothorax* were collected in October 2014 from artificial containers at Ban Khun Klang (18°32′47.83″N; 98°30′57.49″E, elevation 1315 m), Doi Inthanon, Chiang Mai Province, Thailand, the type locality (Bram, 1967). In Japan, larvae were collected in Rinshino-mori Park, Tokyo (35°42′18.91″N; 139°43′11.54″E, elevation 18 m) in December 2014. Specimens from Bhutan were collected in Dechencholing market, Thimphu (27°29′23.79″N; 89°38′56.81″E, elevation 2415 m) in September 2016. Larvae were killed by briefly placing them in hot water (about 60–65 °C), and after identification they were preserved in 80% ethanol; some were also preserved in absolute ethanol for DNA analysis. Other larvae were reared to adults, and their associated larval and pupal exuviae preserved in 80% ethanol and later mounted on microscope slides in Hoyer's medium (Neo-shigaral, Shiga Konchu Fukyusha, Tokyo, Japan) or Euparal (Waldeck, Germany). Larval and adult mosquitoes were identified using the morphological keys of Bram (1967), Rattanarithikul et al. (2005) and Tanaka et al. (1979). The external morphology of adults was compared with reared adults of *Cx. pallidothorax*, a related species of the subgenus *Culiciomyia*, collected as larvae at Vachiratharn water fall (18° 32′30.62″N; 98°35′58.57″E, elevation 680 m), Doi Inthanon. The morphological terminology used herein is defined in the Anatomical Glossary of the Mosquito Taxonomic Inventory (http://mosquito-taxonomic-inventory.info/).

2.2. Bright field and scanning electron microscopy (SEM)

For examination of integumental spicules, the freshly killed larvae or alcohol-preserved larvae were placed on slides in distilled water and examined under a bright field microscope (Olympus CX31) using 10× and 40× objective lenses. Photographs were taken with a digital camera (Olympus E-330). The preparation of specimens for SEM was done following the procedures of Saeung et al. (2014). Alcohol-preserved larvae were fixed with 2.5% glutaraldehyde overnight, washed 2 times with phosphate buffer (pH 7.2), fixed with 1% osmium tetroxide, incubated in the dark for 1 h, washed 2 times with phosphate buffer (pH 7.2), dehydrated in a graded alcohol series, and dried in a critical point drier (Polaron, CPD 7501, Quorum Technologies, England). After being coated with gold using an ion sputter (JEOL JFC-1100E, Japan), the specimens were photographed to obtain digital images using a scanning electron microscope (JEOL JSM-6610LV, Japan).

2.3. DNA extraction, amplification and sequencing

Genomic DNA was extracted from the abdominal segments (I–VI) of larvae, and the remaining were retained for morphological confirmation. Extraction and amplification was accomplished as described by Wijit et al. 2013. The *COI* gene was amplified using the barcoding primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) of Folmer et al. (1994). PCR reactions were carried out in a 20-mL volume containing 0.4 U of Platinum®Taq DNA Polymerase, 1X of PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 1 mL of extracted DNA. The amplification profile comprised initial denaturation at 95 °C for 2 min, 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide. PCR products were purified using the illustra™ ExoProStar™ 1-Step (GE Healthcare Life Sciences, UK) and sequenced using a 23 ABI 3730XLs sequencer (Macrogen, South Korea). The *COI* sequences are deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers KY856950–KY856958.

2.4. Phylogenetic analysis

The new *COI* sequences obtained for putative *Cx. spiculothorax* (KY856950–KY856957) and *Cx. pallidothorax* (KY856958) were compared with those of related species of subgenus *Culiciomyia* available in GenBank using the Basic Local Alignment Search Tool (BLAST, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi): *Cx. sasai* (accession numbers AB690843.1, LC054491.1, LC054492.1, LC054493.1, LC054494.1, LC104332.1, LC104333.1), *Cx. kyotoensis* (LC104325.1, LC104327.1), *Cx. nigropunctatus* (AB738107.1, HQ398882.1), *Cx. pallidothorax* (LC054472.1, LC054475.1) and *Cx. ryukyensis* (AB738139.1, AB738156.1). *Culex quinquefasciatus* (AB738313.1, HQ398883.1) was used as the outgroup taxon. The *COI* sequences were aligned using the Clustal W algorithm implemented in MEGA v. 6.06. The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA software. The Kimura 2-parameter (K2P) model and bootstrap analysis with 1000 replicates were included. Genetic distances were calculated using the K2P model.

3. Results

The *Culex* specimens from Tokyo examined morphologically consisted of 33 whole larvae, 12 males and seven females with associated larval and pupal exuviae. Over 100 whole larvae and 30 males and females with associated exuviae from various localities in Bhutan were examined. The larval chaetotaxy and morphology of all specimens from Japan and Bhutan conform to the description of *Cx. spiculothorax* from Thailand (Bram, 1967). The vesicular spicules on the thoracic integument are distinct in most of the larvae examined (Fig. 1), very small indistinct spicules were observed in a few specimens. The pupal morphology is also similar (data not shown).

The females reared from larvae of *Cx. spiculothorax* collected in Thailand (Fig. 2) are similar to females reared from larvae collected in Japan and Bhutan. They are small to moderate in size; proboscis and maxillary palpus uniformly dark-scaled (Fig. 2a); decumbent scales of the vertex light brown and becoming lighter towards the orbital line, erect scales dark brown (Fig. 2b); integument of the thoracic pleura light brown, frequently tinged with green, with a distinctly darker brown pattern that stretches from the prespiracular area across the prealar area and terminates at the upper mesepimeron, another darker brown pattern is present on the upper area of the mesokatepisternum, 1 (rarely 2) strong lower mesepimeral seta is present (Fig. 2b); all legs are uniformly dark brown, but occasionally with pale scaling on the ventral and posterior surfaces of the femora (Fig. 2a); abdominal terga with rather broad, usually convex, basal pale bands (Fig. 2c); sterna uniformly pale-scaled. The males are in general as described for the female: maxillary palpus long, slender, exceeding the proboscis by the length of palpomere 5; antennal flagellum densely verticilate with long setae. The external morphology of the male and female of *Cx. spiculothorax* is indistinguishable from those of *Cx. pallidothorax*.

Phylogenetic analysis of *COI* sequences (560 bp) revealed that mosquitoes identified as *Cx. spiculothorax* from Thailand, Japan and Bhutan comprise a single clade with *Cx. sasai* from Japan (Fig. 3). K2P genetic distances within *Cx. spiculothorax* ranged from 0 to 0.3% and between *Cx. spiculothorax* and *Cx. sasai* from 0 to 0.9%. The latter value falls within the range of intraspecific variation seen in the other wide ranging taxa studied here: 0–0.8% K2P in *Cx. pallidothorax* and 1.1% in *Cx. nigropunctatus* from Thailand and Japan. The *COI* sequences of *Cx.*

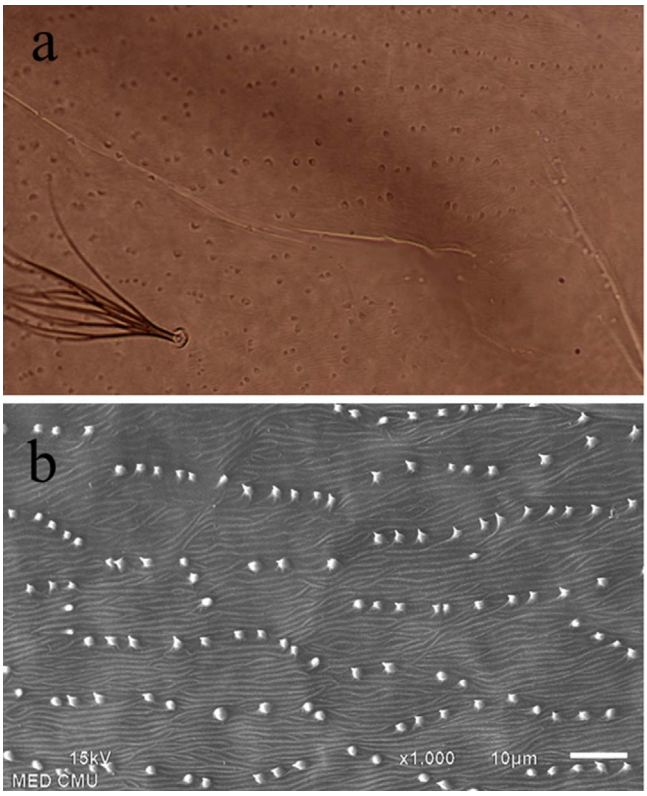

Fig. 1. Dorsal thoracic speculation of a larva from Japan identified as *Cx. spiculothorax*: a) bright field photograph and b) scanning electron micrograph.

spiculothorax and *Cx. sasai* are clearly distinct from the sequences of other species of subgenus *Culiciomyia* included in the analysis, i.e. *Cx. kyotoensis*, *Cx. nigropunctatus*, *Cx. pallidothorax* and *Cx. ryukyensis* (K2P genetic distances 1.6%–5.1%), of which *Cx. kyotoensis* is the most closely related to specimens in the *spiculothorax-sasai* clade.

4. Discussion

Based on *COI* sequences and larval morphology, including distinct vesicular spicules on the thoracic integument, our results provide evidence for the occurrence of specimens identifiable as *Cx. spiculothorax* in Japan and Bhutan. The females and males reared from these larvae are indistinguishable from the adults of *Cx. pallidothorax* and some other species of subgenus *Culiciomyia*, as mentioned above. The male genitalia have not been studied in detail, but according to Sirivanakarn (1977) they appear to be similar to those of some species of the subgenus. The larvae identified as *Cx. spiculothorax* closely resemble the larvae identified as *Cx. sasai*, except for the previously presumed absence of thoracic spicules in the latter. The spicules are difficult or impossible to see under low magnification (10x). In fact, we found that the spicules on larval exuviae and larvae mounted on microscope slides were sometimes unapparent, leading to misidentification. We recommend that the observations be made using water-mounted specimens under a magnification of 40 x to clearly view the spicules. Bram (1967) did not find larvae in Thailand identifiable as *Cx. sasai* (based on the absence of thoracic spicules) and described larvae with thoracic spicules as a new species, *Cx. spiculothorax*. In contrast, Miyagi et al. (1986) later reported finding *Cx. sasai* on Doi Inthanon and Doi Suthep in Chiang Mai Province. However, upon careful re-examination of slide-mounted larval specimens from Japan labelled *Cx. sasai* (kindly provided by I. Miyagi) we noted the presence of thoracic spicules. In recent years, we made several collections on Doi Inthanon and found many specimens identifiable as *Cx. spiculothorax*, but specimens identifiable as *Cx. sasai* have never been found. It should be noted that in the original description of *Cx. sasai*, Kano et al. (1954) did not mention the integument of the larva. Tanaka et al. (1979), however, reported that the thoracic integument of *Cx. sasai*, as well as that of *Cx. kyotoensis* and *Cx. pallidothorax*, is “apparently smooth”. In contrast, our specimens of *Cx. kyotoensis* and *Cx. pallidothorax* have minute vesicles or spicules on the thoracic integument, in agreement with Bram (1967) for *Cx. pallidothorax*. Tanaka et al. (1979) might have overlooked this character, which is sometimes unapparent or seemingly absent (Sirivanakarn, 1977). Furthermore, we examined larvae identi-

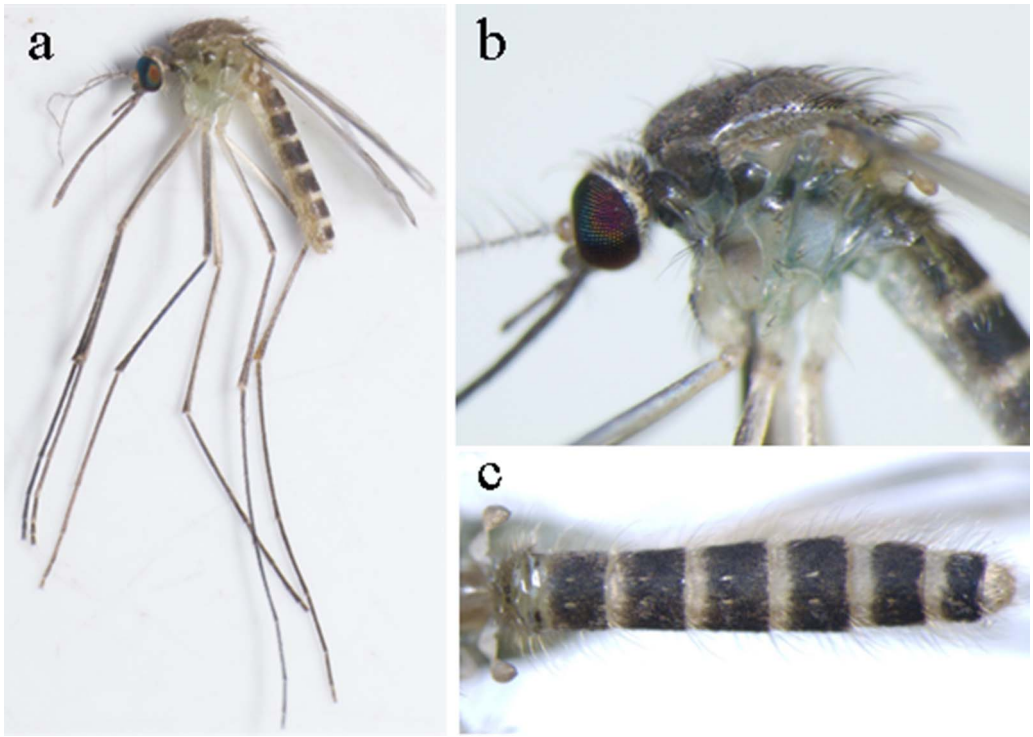

Fig. 2. A female reared from a larvae from Thailand identified as *Cx. spiculothorax*: a) lateral view, b) lateral (left) side of thorax, c) abdomen (tergal view).

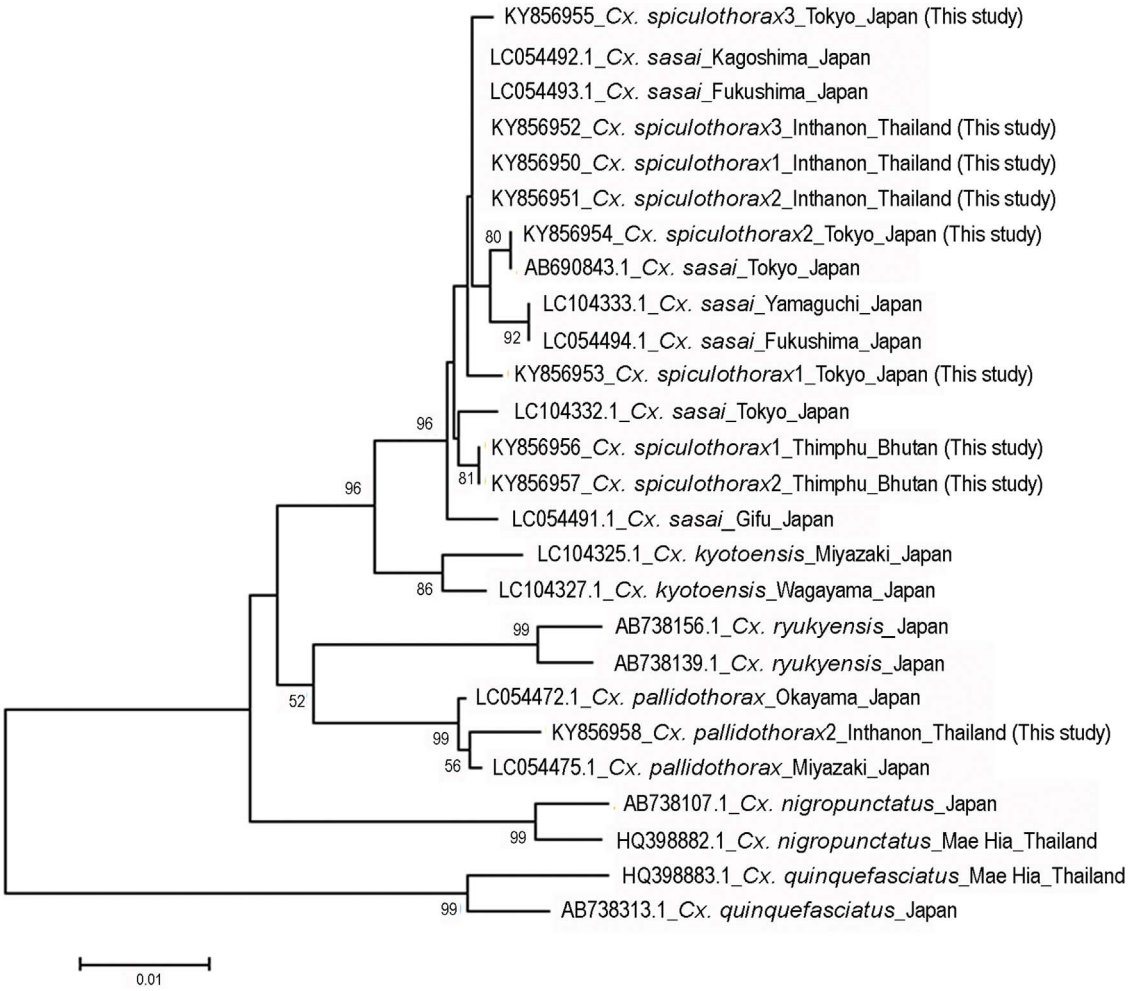

Fig. 3. Phylogenetic tree derived from *COI* sequences of specimens from Thailand, Japan and Bhutan identified as *Cx. spiculothorax*, including GenBank sequences of Japanese specimens identified as *Cx. sasai* and four other species of subgenus *Culiciomyia*, with *Cx. quinquefasciatus* as outgroup.

fied as *Cx. sasai* in the study of Tanaka et al. (1979) that were deposited in the Natural History Museum in London and found that minute, inconspicuous spicules are indeed present on the thorax.

In recent years, we attempted to find *Cx. sasai* in Rinshino-mori Park, Tokyo, but all specimens collected there were identified as *Cx. spiculothorax*. In addition, we collected over 200 larvae of species of subgenus *Culiciomyia* from four localities at the foot of Mt. Fuji (the type locality of *Cx. sasai*) in September and October 2015, but only larvae identifiable as *Cx. spiculothorax* were found. Our phylogenetic analysis of *COI* sequences clearly shows that *Cx. spiculothorax* collected in Japan (Tokyo) and Bhutan are conspecific with specimens of this nominal species in Thailand, with very low genetic distances between specimens, particularly those of *Cx. sasai* deposited in GenBank (AB690843.1, LC104332.1), which were obtained from specimens collected in the same park where we collected *Cx. spiculothorax* in Tokyo and elsewhere in Japan (Fig. 3). The *COI* barcoding region provides an alternative tool for identifying mosquito species, including species of subgenus *Culiciomyia* (Wang et al., 2012; Chan et al., 2014), keeping in mind that this identification method may not be able to distinguish members of species complexes, e.g. the *Culex pipiens* complex (Batovska et al., 2016). Therefore, we consider that all specimens from Tokyo and other areas in Japan identified as *Cx. sasai* in previous reports (e.g. Kim et al., 2009; Tsuda and Kim, 2010; Kuwata et al., 2012; Ejiri et al., 2014; Maekawa et al., 2016), including the GenBank submissions for *Cx. sasai*, actually refer to prevailing morphological concept of *Cx. spiculothorax*. Taken all together, the available evidence clearly indicates that the names *sasai* and *spiculothorax* were proposed for the same biological species, and are thus subjective synonyms. As *Cx. sasai* is the senior synonym, we hereby formally synonymize *Cx. spiculothorax* Bram, 1967 with *Cx. sasai* Kano et al., 1954. Thus, *Cx. spiculothorax* is hereby removed from the list of mosquito species in Thailand and is replaced by *Cx. sasai*. This species is also recorded in South Korea (Lee, 2003), China (Chau, 1982) and Taiwan (Chen, 1974), and the previous identification of this species in Thailand by Miyagi et al. (1986) is correct.

The biology of *Cx. sasai* is poorly known. In Thailand, this species is found at higher elevations, up to the summit of Doi Inthanon (elevation 2567 m) where temperatures drop to freezing during the winter. Similarly in Bhutan, this species has been collected only at high elevations, Thimphu, Bunakha, Dochula, Serbaythang, Simtokha, Nesergang, Khasarabchu and Paro. It may be widely distributed in other highland areas as well. In the cooler climate of Palaearctic Japan, this species has been found commonly from lowlands to mountainous regions (Tanaka et al., 1979). The larvae can survive freezing temperatures but develop to adulthood in an insectary at 25–27 °C. According to the current study and previous records, the larvae of *Cx. sasai* have been often found in high densities in various aquatic habitats, including marshy areas, stagnant water, rock pools, ground pools, artificial containers, used tyres, bamboo stumps, tree holes, plant and flower axils and bracts. Associated species include *Aedes albopictus* (Skuse), *Ae. flavopictus* Yamada, *Ae. gilli* (Barraud), *Ae. harveyi* (Barraud), *Ae. japonicus* (Theobald), *Ae. pulchriventer* (Giles), *Ae. reinerti* Rattanarithikul & Harrison, *Ae. shortti* (Barraud), *Anopheles baileyi* Edwards, *An. bengalensis* Puri, *An. lindesayi cameronensis* Edwards, *An. lindesayi*

lindesayi Giles, *Armigeres kesseli* Ramalingam, *Ar. subalbatus* (Coquillett), *Cx. kyotoensis*, *Cx. mimeticus* Noè, *Cx. pipiens* Linnaeus, *Cx. oresbius* Harbach & Rattanarithikul, *Cx. richei* Klein, *Cx. traubi* Colless, *Culiseta niveitaeniata* (Theobald), *Lutzia vorax* Edwards and *Tripteroides bambusa* (Yamada) (Tanaka et al., 1979; Miyagi et al., 1986; Rattanarithikul et al., 2005; Tsuda, 2012).

Kim et al. (2009) reported that *Cx. sasai* in Rinshino-mori Park fed primarily on avian hosts, and *Plasmodium* DNA was detected in wild caught females, suggesting its potential role in the transmission of avian malarial parasites. Ejiri et al. (2014) detected Koyama Hill virus (KHV), in blood-fed females collected in this park. Additionally, experimental inoculation with KHV revealed that females are highly susceptible to this virus, suggesting it is a potential vector of this virus. The biology and pathogenesis of KHV is poorly known.

In summary, *Cx. spiculothorax* is the junior synonym of *Cx. sasai* and we report for the first time the occurrence of *Cx. sasai* in Bhutan, based on the larval morphology and *COI* sequences. Further study is required to know the full distribution of this species and its role in transmission of pathogens.

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References

Batovska, J., Blacket, M.J., Brown, K., Lynch, S.E., 2016. Molecular identification of mosquitoes (Diptera: Culicidae) in southeastern Australia. Ecol. Evol. 6 3301–3011.
Bram, R.A., 1967. Contributions to the mosquito fauna of Southeast Asia. II. The genus *Culex* in Thailand (Diptera: Culicidae). Contrib. Am. Entomol. Inst. 2 (1), 1–296.
Chan, A., Chiang, L.P., Hapuarachchi, H.C., Tan, C.H., Pang, S.C., Lee, R., et al., 2014. DNA barcoding: complementing morphological identification of mosquito species in Singapore. Parasit. Vectors 7, 569.
Chau, G.W., 1982. An Illustrated Guide to the Identification of the Mosquitoes of Hong Kong. Urban Council, Hong Kong.
Chen, C.Y., 1974. Studies on morphology of the cibarium in culicine mosquitos. II. Some *Culex* mosquitos of Taiwan belonging to subgenera *Lophoceraomyia*, *Eumelanomyia* and *Culiciomyia*. J. Form. Med. Assoc. 73, 511–525.
Ejiri, H., Kuwata, R., Tsuda, Y., Sasaki, T., Kobayashi, M., Sato, Y., et al., 2014. First isolation and characterization of a mosquito-borne arbivirus belonging to the species Umatilla virus in East Asia. Arch. Virol. 159, 2675–2685.
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. Mol. Mar. Biol. Biotechnol. 3, 294–297.
Harbach, R.E., 2011. Classification within the cosmopolitan genus *Culex* (Diptera: Culicidae): the foundation for molecular systematics and phylogenetic research. Acta Trop. 120, 1–14.
Kano, R., Nitahara, M., Awaya, J., 1954. Description of a new mosquito, *Culex* (*Culiciomyia*) *sasai* n. sp., collected in the southwestern part of Japan (Culicidae, Diptera) [in Japanese]. Jpn. J. Sanit. Zool. 5, 14–20.
Kim, K.S., Tsuda, Y., Sasaki, T., Kobayashi, M., Hirota, Y., 2009. Mosquito blood meal analysis for avian malaria study in wild bird communities: laboratory verification and application to *Culex sasai* (Diptera: Culicidae) collected in Tokyo, Japan. Parasitol. Res. 105, 1351–1357.
Kuwata, R., Hoshino, K., Isawa, H., Tsuda, Y., Tajima, S., Sasaki, T., et al., 2012. Establishment and characterization of a cell line from the mosquito *Culex tritaeniorhynchus* (Diptera: Culicidae). In Vitro Cell. Dev. Biol. Anim. 48, 369–376.
Lee, H.I., 2003. Taxonomic review and revised keys of the Korean mosquitoes (Diptera: Culicidae). Korean J. Entomol. 33, 39–52.
Maekawa, Y., Ogawa, K., Komagata, O., Tsuda, Y., Sawabe, K., 2016. DNA barcoding for molecular identification of Japanese mosquitoes [in Japanese]. Med. Entomol. Zool. 67, 183–198.
Miyagi, I., Toma, T., Tsukamoto, M., Horio, M., Mogi, M., Okazawa, T., et al., 1986. New distributions records of mosquitoes from Thailand with a collection list of 1983–1984 surveys. Trop. Biomed. 3, 181–192.
Ramalingam, S., Pillai, A.G., 1973. Ten new records of mosquitoes occurring in West Malaysia. Southeast Asian J. Trop. Med. Public Health 4, 271–272.
Rattanarithikul, R., Harbach, R.E., Harrison, B.A., Panthusiri, P., Jones, J.W., Coleman, R.E., 2005. Illustrated keys to the mosquitoes of Thailand: II. genera *Culex* and *Lutzia*. Southeast Asian J. Trop. Med. Public Health 36 (Suppl. 2), 1–97.
Saeung, A., Hempolchom, C., Yasanga, T., Otsuka, Y., Thongsahuan, S., Srisuka, W., et al., 2014. Scanning electron microscopy of *Anopheles hyrcanus* group (Diptera: Culicidae) eggs in Thailand and an ultrastructural key for species identification. Parasitol. Res. 113, 973–981.
Sirivanakarn, S., 1977. Redescription of four Oriental species of *Culex* (*Culiciomyia*) and the description of a new species from Thailand (Diptera: Culicidae). Mosq. Syst. 9, 93–111.
Tanaka, K., Mizusawa, K., Saugstad, E.S., 1979. A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). Contrib. Am. Entomol. Inst. 16 (vii +), 1–987.
Tsuda, Y., Kim, K.S., 2010. Prediapause migration and overwintering of *Culex tritaeniorhynchus* (Diptera: Culicidae) observed in a park in urban Tokyo during 2007–2009. Med. Entomol. Zool. 61, 69–78.
Tsuda, Y., 2012. Ecology of mosquitoes inhabiting a park in urban Tokyo, Japan: seasonal prevalence of larvae occurred in catch basins [in Japanese]. Med. Entomol. Zool. 63, 95–101.
Wang, G., Li, C., Guo, X., Xing, D., Dong, Y., Wang, Z., et al., 2012. Identifying the man mosquito species in China based on DNA barcoding. PLoS One 7, e47051.
Wijit, A., Saeung, A., Baimai, V., Otsuka, Y., Thongsahuan, S., Taai, K., et al., 2013. DNA barcoding for the identification of eight species members of the Thai Hyrcanus Group and investigation of their stenogamous behavior. C.R. Biol. 336, 449–456.