



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 (Rattanaarithikul 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 & Awaysa, *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 (Rattanaarithikul 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 Rattanaarithikul 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 *COI* 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 Fukuyusha, Tokyo, Japan) or Euparal (Waldeck, Germany). Larval and adult mosquitoes were identified using the morphological keys of Bram (1967), Rattananarithkul 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 *Culicomyia*, 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-μL volume containing 0.4 U of Platinum[®]Taq DNA Polymerase, 1X of PCR buffer, 1.5 mM of MgCl₂, 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 *Culicomyia* 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 verticillate 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.*

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