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First report of *Lipoptena axis* Maa, 1965, from captive cervids in Thailand, based on morphological and molecular data

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Deer louse flies (*Lipoptena* spp.) are hematophagous ectoparasites of cervids. The genus *Lipoptena* comprises 32 species, some of which are of veterinary importance as vectors of various pathogens, and are also known to attack human hosts. Recently, deer louse flies have been observed during annual checkups of captive cervids at Khon Kaen Zoo in Khon Kaen, Thailand. However, data on their specific identity and prevalence remain limited. This study aims to identify louse fly samples from captive cervids at Khon Kaen Zoo using morphological and molecular analyses. A total of 60 louse flies were collected from 17 captive cervids and identified based on their morphology. Major morphological characteristics, including mesothoracic bristle patterns, abdominal tergal plate bristles, and terminalia structure indicated that the Khon Kaen louse fly is *Lipoptena axis* Maa, 1965. Phylogenetic analysis of sequences from a portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene was performed, which confirmed that *L. axis* of this study belongs to the *cervi* group, which is distinct from other groups of *Lipoptena* species. This study represents the first report of *L.* axis in Thailand. We provide an updated taxonomic key for the identification of *Lipoptena* species in the *cervi* group.

Keywords *Lipoptena* spp., Deer louse flies, Deer keds, *Lipoptena axis*, Captive cervids, Zoo

Hippoboscid flies (Diptera: Hippoboscidae), commonly known as louse flies or keds, are hematophagous ectoparasites that have been overlooked for a long time. Most species of keds attack wildlife rather than domestic/farm animals. There are 213 species in the family in three main genera: *Melophagus*, *Lipoptena*, and *Hippobosca*[1]. *Lipoptena* spp., deer louse flies, are ectoparasites of cervids including the Japanese sika deer (*Cervus nippon*), the red deer (*Cervus elaphus*), European roe deer (*Capreolus capreolus*), and moose (*Alces alces*), and also colonize humans and birds[2–6]. About 32 species have been placed in the genus *Lipoptena*. Some species, including *L. cervi*, *L. capreoli*, *L. mazamae*, *L. depressa*, and *L. fortisetosa*, play an important role in veterinary medicine by causing alopecia and hair-loss syndrome that reduces the quality of host life[7–10]. Humans attacked by louse flies develop skin lesions, which are usually painless with pink erythematous papules, at the bite site[4,5,11,12]. The primary anatomical structure in *Lipoptena* spp. that contributes to lesion formation in their hosts is the mouthparts. The proboscis, which is long and needle-like to support its hematophagous habit, creates small wounds on the host's skin during feeding.

In addition, *Lipoptena* spp. are vectors of various zoonotic pathogens such as *Bartonella* spp., *Babesia* spp., *Coxiella* spp., *Borellia* spp., *Rickettsia* spp., *Theileria* spp. and *Anaplasma* spp[13–19]. To date, there have been no case reports of transmission of these zoonotic diseases to humans. However, the prevalence of this blood-

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sucking obligate ectoparasite should be monitored and medical practitioners should be aware of the potential risk of zoonotic disease.

The genus *Lipoptena* has been divided into five groups based on morphology: (a) *cervi*, (b) *pteropi*, (c) *capreoli*, (d) *sepiacea* and (e) *depressa* group[20]. There are 11 species in the *cervi* group, all of which have been reported in Asian countries[1,20,21]. Additionally, 2 of these 11 species can be found elsewhere: *L. cervi* in both Europe and North America, and *L. fortisetosa*, in Europe[2,16,21–23]. The *cervi* group is divided into two subgroups: *cervi* subgroup and *pauciseta* subgroup based on distribution of body bristles and female genitalia structure[24].

Owing to their louse-like appearance, these flies are often misidentified by veterinarians or zookeepers as lice or ticks. The original descriptions of *Lipoptena* species also suggested that some species were initially misidentified based on outdated taxonomic keys and limited specimens[24–27]. Confirming the morphological identification of *Lipoptena* species is difficult because there are no type specimens available. Thus, most species within this genus must be identified based on original descriptions or illustrations rather than by comparison with physical specimens. Taxonomic features, including head dimensions, bristle distribution on the mesothorax, abdominal tergal plate, and genital terminalia components have been recorded. Another challenge in the identification of *Lipoptena* species is the limited availability of DNA sequences in public databases: sequence data are available for only 5 species (Fig. 1), only 2 of them in the *cervi* group. In Thailand, 3 species of *Lipoptena* have been reported: *L. pteropi* Denny, 1843 (*pteropi* group), *L. pauciseta* Edwards, 1919, and *L. fortisetosa* Maa, 1965 (both in the *cervi* group), but sequence data are only available for putative *L. fortisetosa*, for which the sequences are listed as *Lipoptena* sp. in Genbank®[6,24,25]. The louse flies in this study were collected at Khon Kaen Zoo, Khon Kaen Province in Northeastern Thailand. In recent years, deer louse flies have been observed by veterinarians and zookeepers during annual checkups. However, there are limited data on their prevalence and their potential role in the transmission of zoonotic pathogens in this region.

Therefore, this study aimed to identify deer louse flies of captive cervids at Khon Kaen Zoo, Khon Kaen, Thailand by integrating morphological and molecular data. Furthermore, we aimed to provide an updated taxonomic key for the identification of *Lipoptena* in the *cervi* group. This is the first report of *L. axis* from Thailand.

Results

Sample collection

A total of 60 louse flies (31 females and 29 males) were manually collected from 17 anesthetized captive cervids, including nine barasingha (*Rucervus duvaucelii*), six Eld's deer (*Rucervus eldii*) and two chital deer (*Axis axis*).

Morphological characteristics of Khon Kaen louse fly

The morphological characteristics of 60 specimens of the Khon Kaen louse fly were examined using a stereomicroscope. The results of this examination were as follows:

Body flattened, dark brown in color. Total length of head and thorax of males 1.5–1.6 mm (1.58 ± 0.095 mm standard deviation) and of females 1.5–1.7 mm (1.64 ± 0.098 mm) (Fig. 2). Head ovoid, moderately narrowed

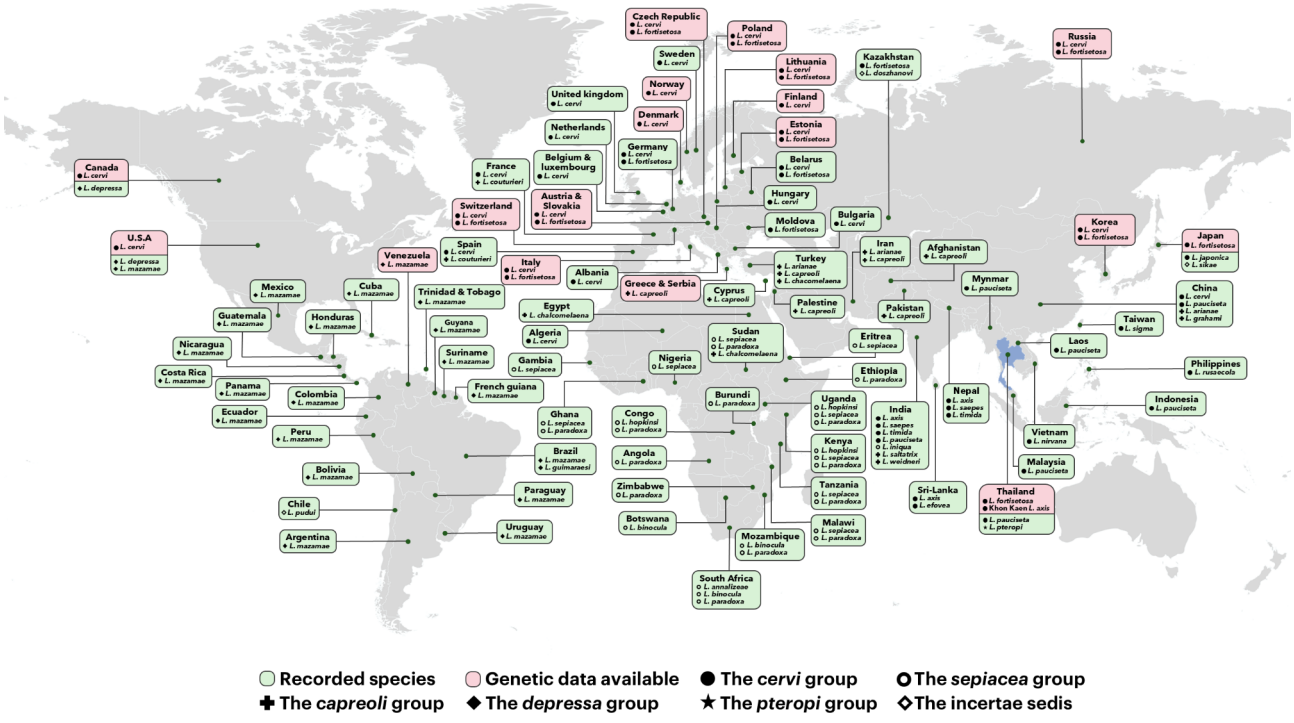

Fig. 1. Worldwide distribution of *Lipoptena* species[1,20,23–25,28].

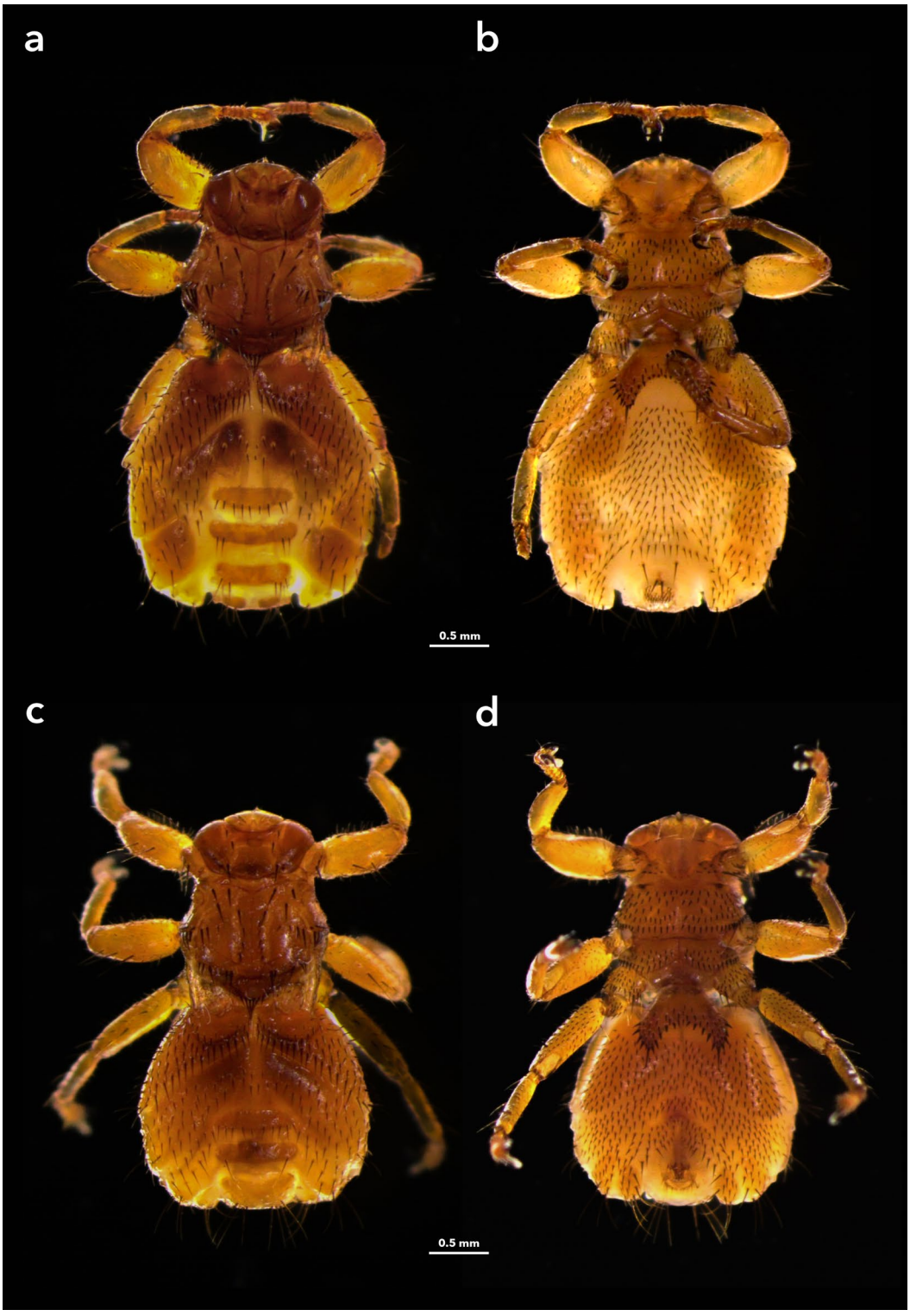

Fig. 2. Khon Kaen louse fly (*L. axis*). Dorsal and ventral view of female (**a**,**b**) and male (**c**,**d**).

behind eyes. Eyes large, elongated posteriorly, not extending to lateral margins of head (Figs. 3a and 4a). Inner orbit wider than outer orbit, bearing two frontal bristles, one vertical bristle, and 2–5 minute setae (Fig. 4c). Antennal pit comprises antennal pedicellum with pair of protruded aristae, 6–7 coeloconic sensillae, and one socketed mechanosensory bristle, scape poorly defined, flagellum embedded in hollows (Fig. 4d–f). Mediovertex nearly square, as long as elliptical fronto-clypeus and longer than postvertex (Fig. 4c). Preptilinal area distinct. Post-vertex prominent with triangular isogonal ocelli (Fig. 4c). Palp shorter than antennal pit (Fig. 4b,c). Thorax pentagonal, dorsum adorned with mesonotal bristles (Figs. 3b and 5a). Pronotum ribbon-like and distinctly

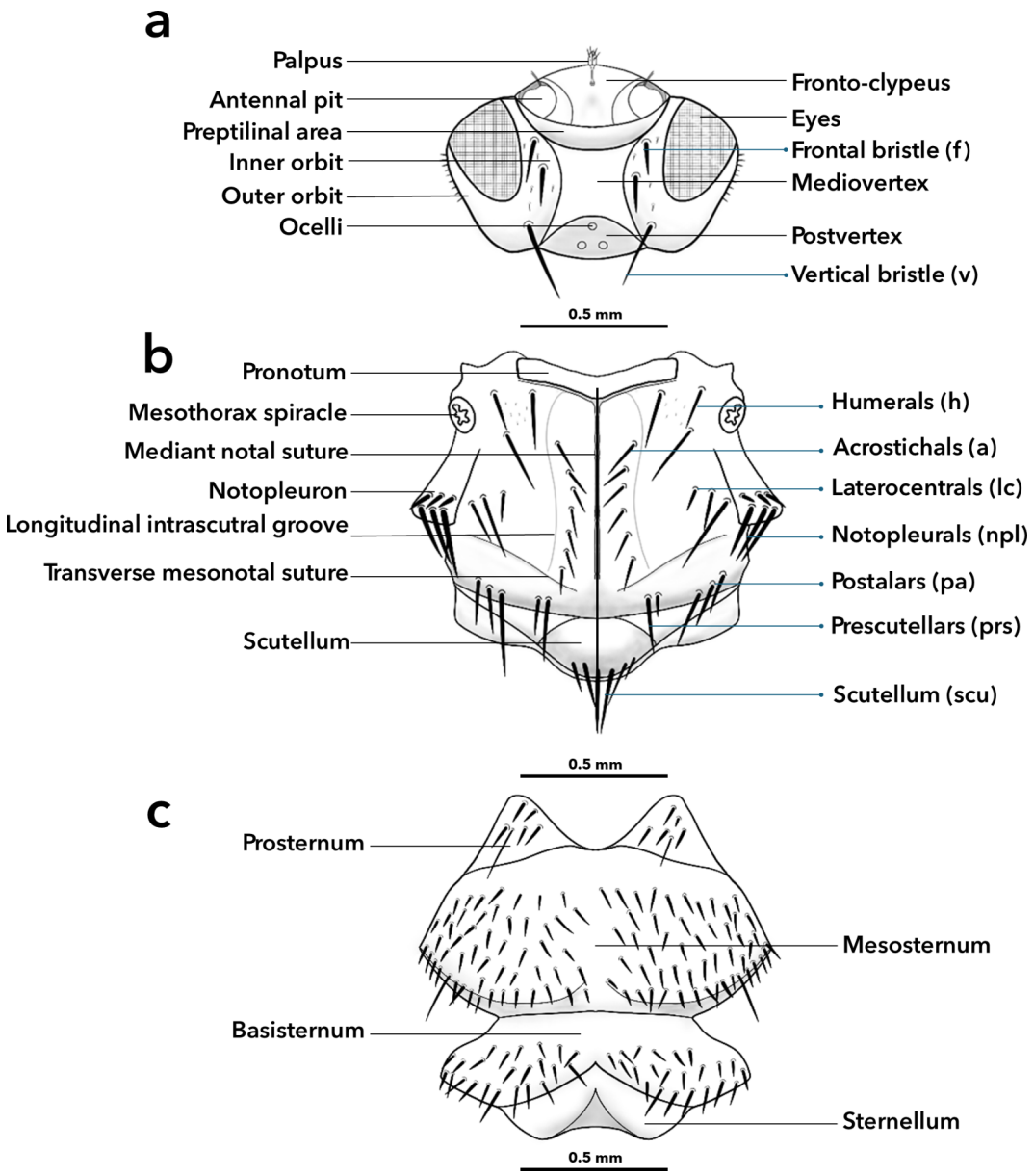

Fig. 3. Khon Kaen louse fly (*L. axis*), illustrating head (**a**), dorsal and ventral thorax (**b**,**c**).

angular at middle. Important suture lines, such as promesonotal suture, median notal suture, and transverse mesonotal suture well-defined (Fig. 5a). Longitudinal intrascutal groove well marked beside rows of acrostichals (Fig. 5a). Mesothoracic spiracles large and well-marked at latero-anterior edge of mesothorax (Fig. 5c). Post-humeral suture indistinct. Mesonotal bristles; 3 humerals with 5–6 coeloconic sensillae on humeral callosity (Fig. 5e), 5–6 acrostichals, 3 (sometimes 2 or 4) laterocentrals, 2–4 postalars, 2 pre-scutellars, 2 rows with 3–4 notopleurals each, posterior setae longer and thicker (Fig. 5a,c). Prosternum triangular in ventral view (Figs. 3c and 5b), shorter than wide, rounded anteriorly, anterior 2/3 scattered with 6 (occasionally 7) short setae and one long bristle (Fig. 5b,d,f). Mesosternum evenly covered with 5 or 6 rows of spines, of which posterior spines thicker and slightly longer than on anterior, interspersed with 1 long bristle on each side (Fig. 5b). Metabasisternum with 3 rows of spines, inner half of hindmost row slightly longer or equal in size to those on outer half, which approximately as stout as those on mesosternum. Spines on anterior rows finer and shorter than on mesosternum (Fig. 5b). Wings represented by wing stumps.

Female abdomen (Figs. 6a,c and 7a,b): large basal dorsal disk on 1st pleurite, divided from 2nd pleurite by row of setae (Fig. 6a). Well-defined 2nd to 5th pleurites, sclerotized. The second pleurite large, apically acute, finely bristled, straight margin with long bristles (Fig. 6a). Third pleurite large, separate from smaller 4th and 5th pleurites (Fig. 6a). Four discernible abdominal tergites; 3rd tergite indistinct. The fourth tergite large, elliptical, with 8–12 setae. The fifth and 4th tergites same size, 2–4 (rarely 5) setae each (Fig. 6a). The sixth tergite rectangular with rounded corners, larger than 4th and 5th tergites. The seventh tergite: pair of sclerites, each with 2–4 (rarely 5) long bristles (Fig. 6a). Ventral sternite deeply curved posteriorly, one apical bristle on each

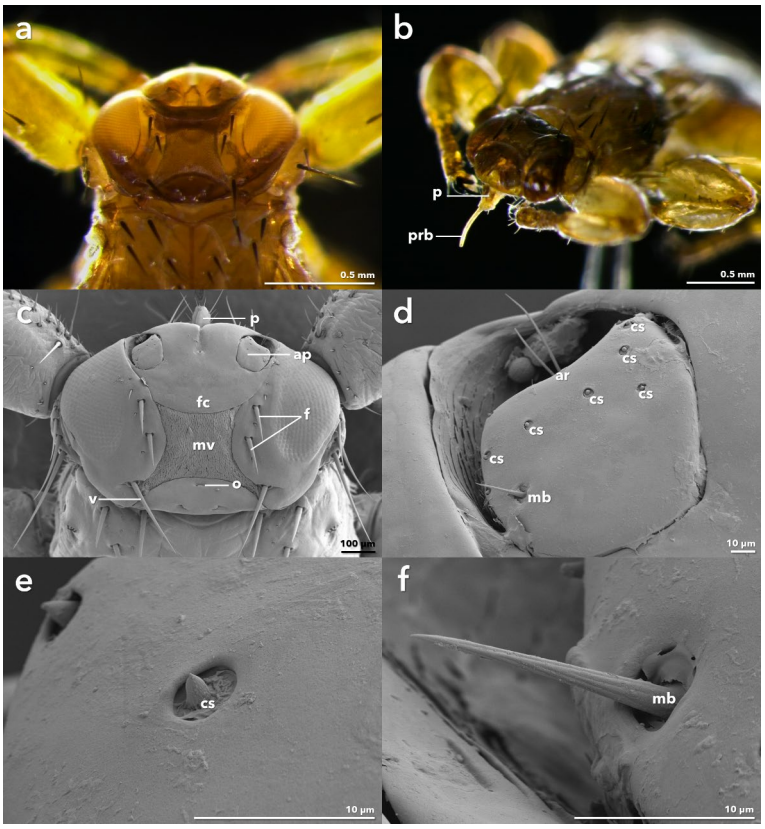

Fig. 4. Dorsal view of the head of female Khon Kaen louse flies (*L. axis*) (**a**) with the long proboscis (prb) protruding from the bilobed palps (p) (**b**). The head is ovoid, featuring well-marked elliptical frontoclypeus (fc) surrounded by three major orbital bristles and 5–6 minor ones (**c**). Within each antennal pit (**d**–**f**) is a single mechanosensory bristle (mb), a pair of aristae (ar) and seven coeloconic sensilla (cs) are situated on the antennal pedicellum (ap) (**d**–**f**).

lobe, outer margin convex with rows of setae, and spines (Figs. 6c and 7b). Female terminalia (Fig. 8a,c): three transverse pregenital sclerites, median sclerite triangular with 2 strong setae (rarely 1 extra) (Fig. 8c). Lateral sclerites one strong seta, rarely with minute extra one (Fig. 8c). Infra-anal plate sparsely covered posteriorly with stout and small setae (Fig. 8c). Basal ventral portion of pleurites sclerotized, covered with heavy setae, one or two longer at each hind corner (Fig. 7b). Setae on membranous area uniform in length and robustness, hindmost row slightly longer (Fig. 7b).

Male abdomen (Figs. 6b,d and 7d) similar to female in structure and chaetotaxy, Three abdominal tergites on male abdomen: 4th, 5th, 6th + 7th (Fig. 6b). The third tergite undefinable. The fourth tergite elliptical, with 8–11 setae. The fifth tergite similar size to 4th, with 1–2 setae each side. The sixth + seventh tergite largest, rounded rectangular with 3–4 setae each side (Fig. 7d). Male terminalia (Fig. 8b,d): cone-shaped aedeagus, ridge-shaped process (Fig. 8e). Bilobed external gonopods. Varied-sized cuticular depressions likely represent coeloconic sensillae. Surstyli well-developed pads with many long bristles (Fig. 8f). A summary of the anatomical characteristics of Khon Kaen louse flies, compared to similar species, is available in Supplementary Table S1.

To differentiate the Khon Kaen louse fly from other *Lipoptena* species in the *cervi* group, characteristics such as head and thorax size, number of bristles, mesothoracic bristles, and genital terminalia were analyzed. On the basis of its morphology, we identified the Khon Kaen louse fly as *L. axis* Maa, 1965.

Key to *Lipoptena* in the cervi group, and other Southeast Asian species

The keys by Bequaert, 1942 and Maa, 1965, 1969 are revised and updated here to distinguish species within the *cervi* group.

1 A: Average body size (head and thorax) less than 1.2 mm; primary hosts are tragulids (mouse deer) ……***pteropi* group** (1 species): ***L. pteropi***

B: Average body size (head and thorax) more than 1.2 mm; primary hosts are cervids ……***cervi* group**…… **2**

2 A: Average body size (head and thorax) more than 2 mm; body hairy …… …… ***cervi* subgroup**…..**3**

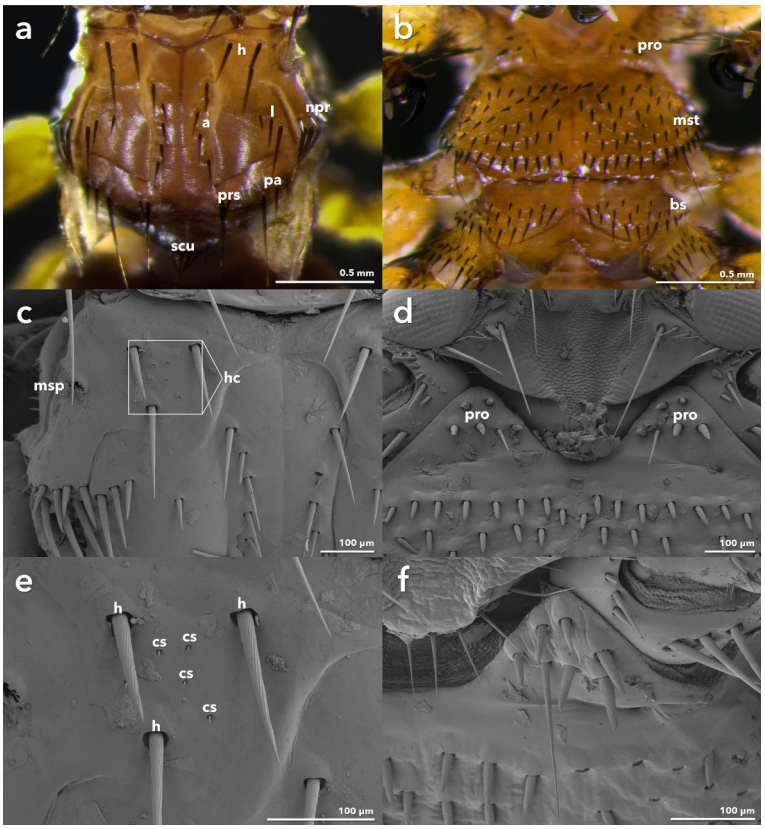

Fig. 5. Dorsal view of thorax of *L. axis* from Khon Kaen (**a**,**c**,**e**) illustrates the symmetrical mesothoracic bristles, including humerals (h), acrostichals (a), latero-centrals (l), postalars (pa), pre-scutellars (prs), notopleurals (npr), and scutellars (scu). Adjacent to the humeral callosity (hc) near the mesothoracic spiracle (msp), a few coeloconic sensilla (cs) are observed. Of taxonomic importance on the ventral thorax (**b**,**d**,**f**) is the arrangement of setae and bristles on the anteriorly rounded prosternum (pro) and the anterior 2/3 scattered with 6, sometimes 7 short setae and 1 bristle. mst; mesosternum, bs; basisternum.

B: Average body size (head and thorax) less than 2 mm; body less hairy or bare ……..……..……***pauciseta* subgroup**…..**8**

3 A: Inner orbit with 3 major frontal bristles and one long vertical bristle; mesothorax bears 10 acrostichals, about 28 laterocentrals; prosternum with more than 13 setae. ..…..…. ……..……***L. japonica***

B: Inner orbit with 2 major frontal bristles and one long vertical bristle; mesothorax bears fewer than 10 acrostichals, fewer than 20 laterocentrals; prosternum with fewer than 11 setae. ……. ……..…… **4**

4 A: Mesothorax bears 4–5 laterocentrals; male 4th and 5th abdominal tergites with 10 setae each. …… ……..……..……..…. ***L. efovea***

B: Mesothorax bears more than 5 laterocentrals; male 4th and 5th abdominal tergites with more than 10 setae each……..……..……..…**5**

5 A: Mesothorax bears 3 humerals, fewer than 7 laterocentrals; prosternum with 9–13 setae and one long bristle; male 4th and 5th abdominal tergites with more than 17 setae each….**6**

B: Mesothorax bears 5–8 humerals, more than 9 laterocentrals; prosternum with 5–10 setae and one long bristle; male 4th and 5th abdominal tergites with 12–15 setae each……**7**

6 A: First pleurite with 9 bristles on posterior and outer margins, and 12–13 small setae arranged near posterior margin. ……..……..……..…… ***L. nirvana***

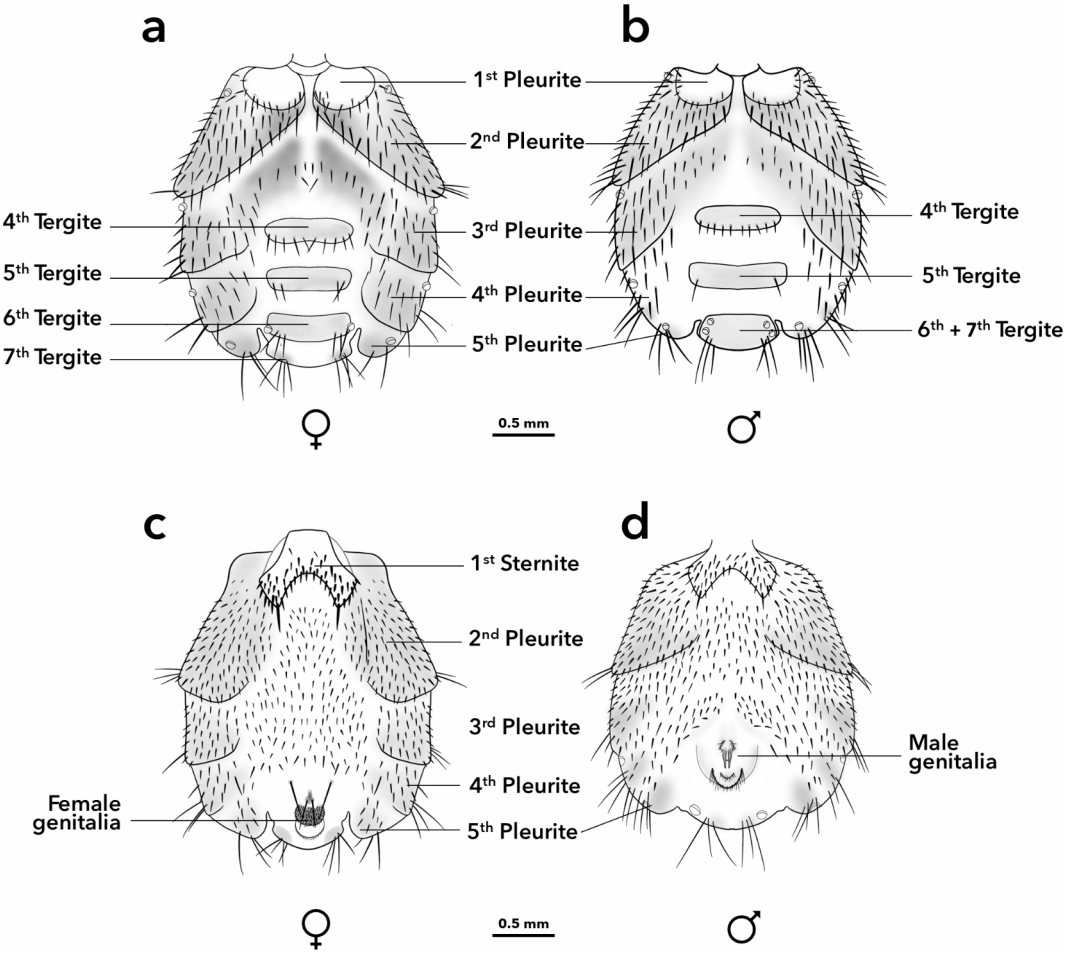

Fig. 6. Khon Kaen louse fly (*L. axis*), illustrating female dorsal (**a**) and ventral (**c**) and male dorsal (**b**) and ventral (**d**) abdomens.

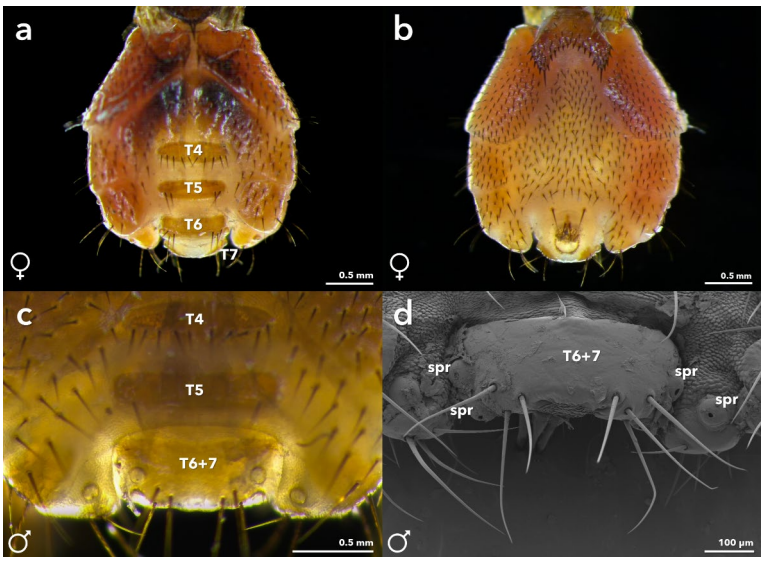

Fig. 7. The dorsal abdomen of Khon Kaen louse flies (*L. axis*) displays four detectable abdominal tergites in females (T4–T7; **a**) and only three in males (T4, T5, and T6 + 7; **c**,**d**). The genitalia are situated on the posterior part of the ventral abdomen (**b**). Upon high magnification, abdominal spiracles (spr) on the abdominal tergites are observed (**d**).

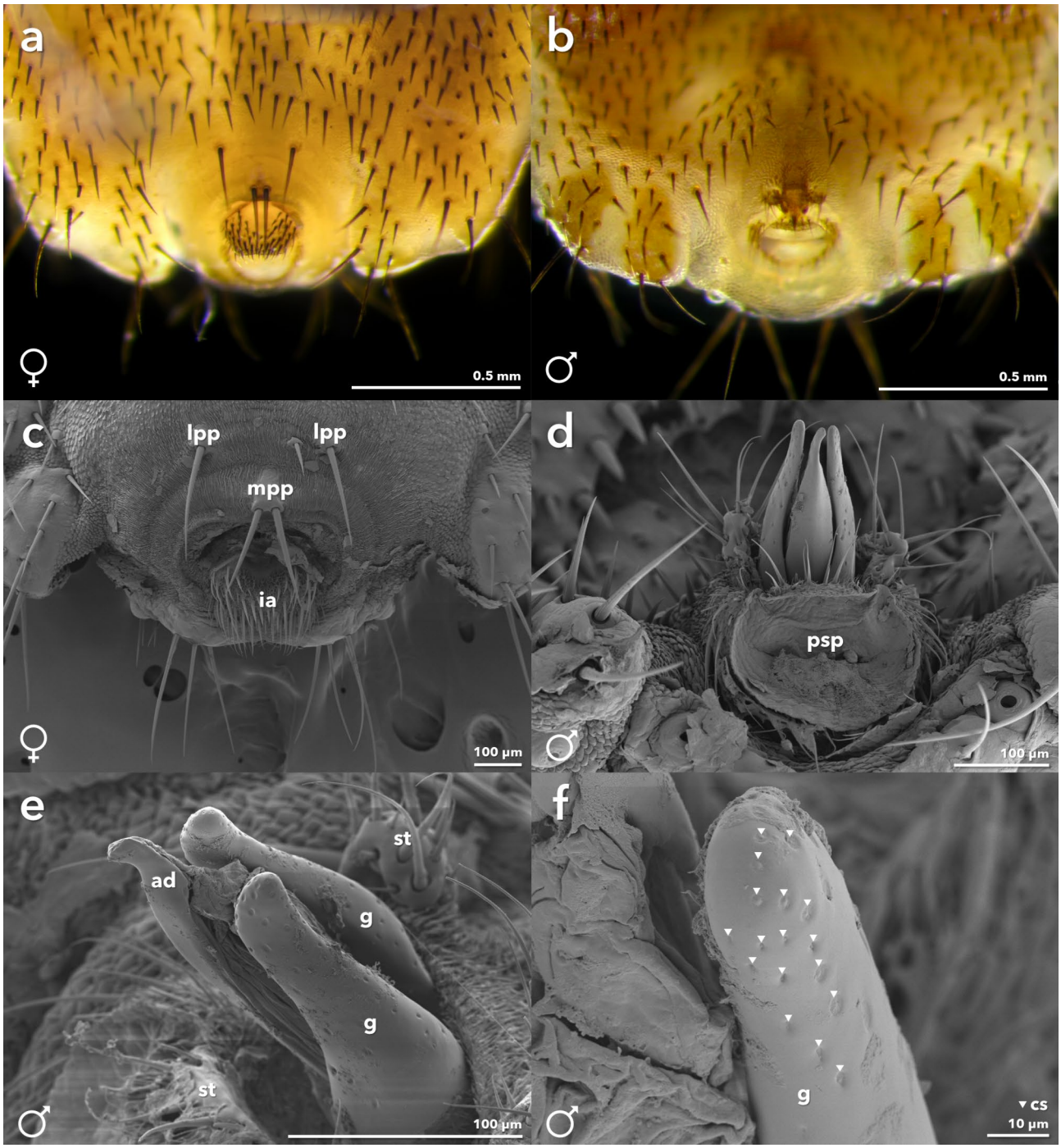

Fig. 8. The genital terminalia of Khon Kaen louse flies (*L. axis*). Female terminalia (**a**,**c**) consist of a transversed row of pregenital sclerites, two laterals (lpp) and a median (mpp) positioned anteriorly to the infra-anal plate (ia), which bares 3–4 rows of setae. Male terminalia (**b**,**d–f**), are characterized by a ridge-shaped process of the aedeagus surrounded by a pair of gonopods (**g**) and a well-developed surstyli pad (st) connected to post-genital plate (psp). Additionally, coeloconic sensilla (white triangles; cs) are distributed on the surface of the gonopods as cuticular depressions.

B: First pleurite with more than 9 bristles on posterior margins and more than 13 small setae arranged near posterior margin……. ***L. sigma***

7 A: Mesothorax bears 6–8 humerals, 15–18 laterocentrals; female 6th and 7th abdominal tergites with 6–7 setae and 4–6 setae on each side, respectively; female median pregenital plate with 5–6 long setae……. ……***L. cervi***

B: Mesothorax bears 5–6 humerals, 9–14 laterocentrals; female 6th and 7th abdominal tergites with 6–10 setae and 5–8 setae on each side, respectively; female median pregenital plate with 6–8 long setae. ..... ***L. saepes***

8 A: Discernible 3rd abdominal tergite or represented by a transverse row of setae **9**

B: Third abdominal tergite not discernible (Figs. 4a,b and 7a,c) **11**

9 A: Mesothorax bears 6 scutellars; prosternum with 6–7 setae with one long bristle; fourth abdominal tergite of male and female bearing 10–12 and 13–16 setae, respectively..... .....***L. fortisetosa***

B: Mesothorax bears 4 scutellars; prosternum with 4–5 setae with one long bristle; fourth abdominal tergite of male and female baring 5–8 setae each.....**10**

10 A: Third abdominal tergite with 2–4 setae; fifth abdominal tergite with 1–2 setae; female median pregenital plate with 4 long setae.....***L. rusaecola***

B: Third abdominal tergite with 5–6 setae; fifth abdominal tergite with 6–8 setae; female median pregenital plate with 2 long setae..... ***L. pauciseta***

11 A: Mesothorax bears 3–5 acrostichals (Figs. 3b, 4d, 6a and 7c).....***L. axis***

B: Mesothorax bears 7 acrostichals..... .....***L. timida***

Molecular identification, phylogenetics analysis and species delimitation analyses

Sixteen identical COI sequences of Khon Kaen louse flies (*L. axis*) were obtained. These have been submitted to the GenBank® database under accession numbers PQ428974–PQ428989. All sequences were trimmed to equal lengths prior to conducting the phylogenetic analysis, resulting in an alignment of 368 bp. The phylogenetic analysis revealed that *L. axis* (Khon Kaen sample) in this study is in the same clade as other *Lipoptena* spp. in the *cervi* group, *L. cervi* and *L. fortisetosa* and clearly distinct from the *depressa* group and the *capreoli* group (Fig. 9).

Two species-delimitation methods (ASAP and PTP) agreed in placing species boundaries in most cases, with the exception of the clade containing *L. fortisetosa* from a number of countries. Both methods agreed well with conclusions drawn from morphology in Khon Kaen louse flies. Within the clade of *Lipoptena* from Khon Kaen, both ASAP and PTP identified a single taxon (Fig. 9).

Discussion

All 11 species of the *cervi* group are found only in Asia, except *L. cervi* and *L. fortisetosa*, which have been reported in both Asia and other regions and are the most common species within the *cervi* group[2,9,20–22,28]. This is the first report of *L. axis*, a member of the *cervi* group, in Thailand. Three species of *Lipoptena* have been recorded previously in Thailand: *L. pteropi*, *L. pauciseta*, and *L. fortisetosa*[6,24,25,27]. The last two of these are also included in the *cervi* group. The average body size (total length of head and thorax) of Khon Kaen *L. axis* was greater than 1.2 mm, which differentiates them from the smaller *L. pteropi*. Similar to other *Lipoptena* species, *L. axis* has two sheaths formed by the maxillary palpi, which protrude from the anterior margin of the head. The palpi vary in size and in the number of setae among hippoboscid species, being long in *Melophagus* species but short or tiny in *Lipoptena* and *Neolipoptena* species[27]. SEM images show that the arrangement of sensilla and the terminalia of Khon Kaen *L. axis* differ from those of *L. fortisetosa.* The antennal pedicellum of Khon Kaen *L. axis* has a pair of linear aristae, seven coeloconic sensilla at the rim of the pedicellum, and one socketed sensillum. *Lipoptena fortisetosa*, exhibits a different pattern characterized by nine long sensory bristles arranged along the edge of the antennal pedicellum and jagged fan-shaped arista[2,29]. The sensilla arrangement pattern of Khon Kaen *L. axis* is similar to that of *L. cervi*, with the exception of the branched shape of the aristae, two trichoid socketed sensilla and one basiconic sensillum. The male terminalia of Khon Kaen *L. axis* exhibits a cone-shaped aedeagus with a ridge-shaped process at the end, as long as a surrounding pair of gonopods. The surstyli pads are distinct with several strong bristles. In contrast, the male terminalia of *L. fortisetosa* possesses a long bifurcate aedeagus and the reduced surstyli represented by a sclerotized area with strong bristles at the basal rim[2,29]. Based on terminology defined by Maa in 1969[24], female terminalia of Khon Kaen *L. axis* consist of three aligned pregenital plates, with one or two setae on each lateral plate and two setae on the median plate. Moreover, the infra-anal plate is covered by many stout small setae at the posterior curve. These characteristics are similar to those seen in *L. fortisetosa* that has a single seta on each lateral pregenital plate and two setae on the median plate while *L. cervi* has two or three setae on the lateral pregenital plates and four setae on the median plate[2,29].

The Khon Kaen *L. axis* is less hairy than *L. fortisetosa* and *L. cervi*, with distinct ocelli and eyes laterally almost touching the margin of head. The abdominal tergal plates are distributed across the dorsum, with indiscernible intersegmental folding of the tergites and pleurites. These characteristics indicate that Khon Kaen *L. axis* belongs to the *cervi* group. To differentiate the species within this group, the pattern of bristles on the mesothorax and the setae on the abdominal tergal plate were observed. The Khon Kaen *L. axis* shows 5 to 6 acrostichal bristles

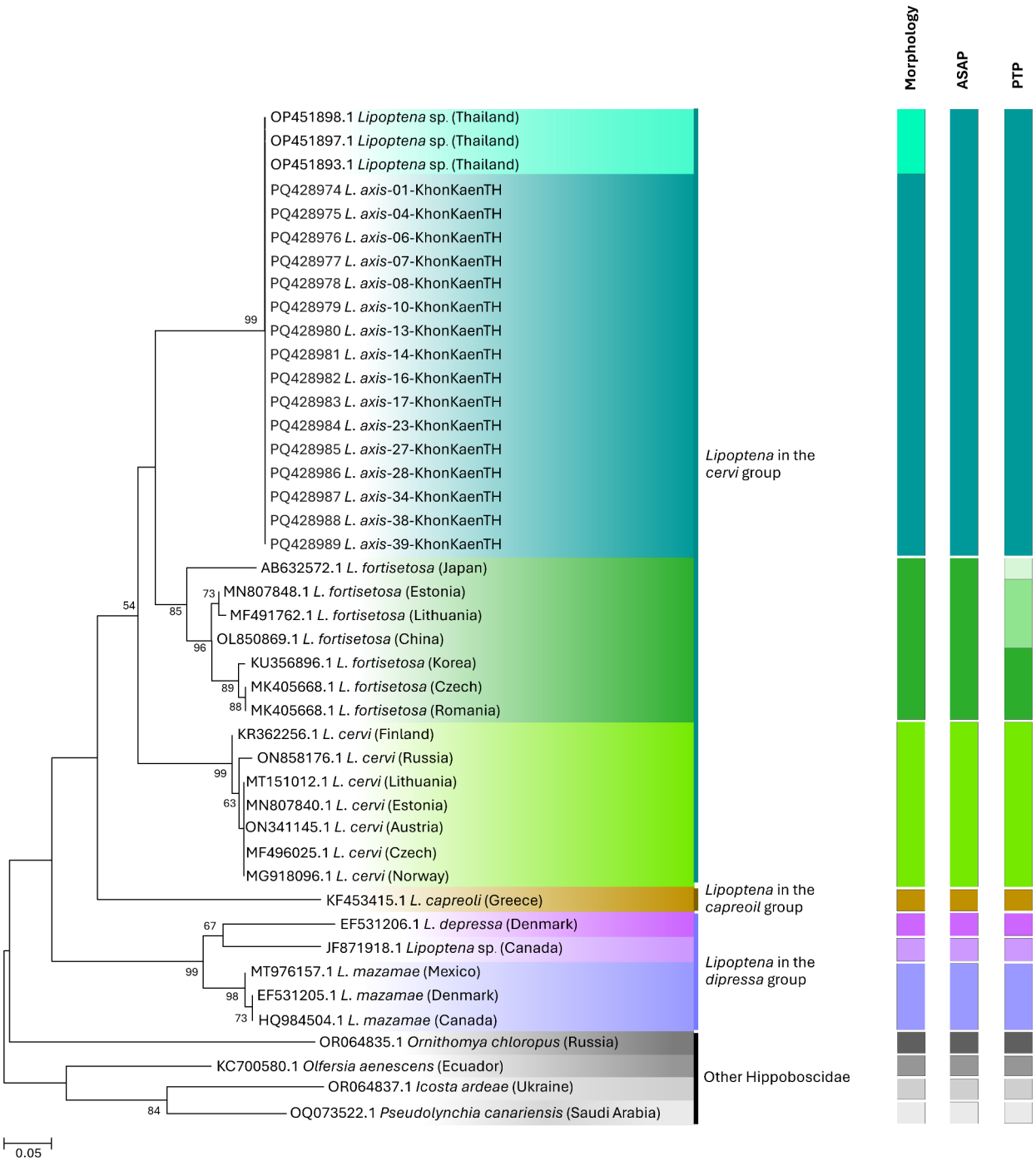

Fig. 9. The maximum-likelihood tree based on partial COI gene sequences using the general time-reversible model with gamma distribution and 1000 bootstrap replicates, implemented in MEGA X software. The Khon Kaen *L. axis* clusters within the same clade as *L. fortisetosa* and *L. cervi* (the *cervi* group). The number at each node is the bootstrap percentage support for that node. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bars on the right-hand side of the tree represent the results of species-delimitation methods, traditional morphology, ASAP (Assemble Species by Automatic Partitioning), and PTP (Poisson Tree Processes).

which is similar to *L. axis* from other countries that reportedly have 3 to 5 bristles[25]. Moreover, the setae on the abdominal tergal plates of Khon Kaen *L. axis* resemble those reported in *L. axis* except the 4th abdominal tergal plate. Male and female Khon Kaen *L. axis* have 8–11 and 8–12 setae on the 4th abdominal tergal plate, respectively, while male and female of *L. axis*[25] elsewhere have 4–8 setae and 6–11 setae, respectively. However, this difference

may reflect natural variation within hippoboscid species. For example, variation in chaetotaxy of the thorax was found in *L. fortisetosa* with greater variability observed in females than in males[30]. The species most similar to *L. axis* are *L. pauciseta*, previously recorded in Thailand, and *L. timida*, which is easily mistaken for *L. axis*[24,25]. The main difference between *L. axis* and *L. pauciseta* is the shape of the median pregenital plate, which is elongated and triangular in *L. axis* and short, obtriangular in *L. pauciseta*. In *L. timida*, the main differences from *L. axis* are the shorter palpi and the poorly developed plantar spines. However, comparisons are challenging due to the limited data and specimen availability. The only description of chaetotaxis of *L. timida* is based on a single male specimen and notes that the mesothorax bears 7 acrostichals[24]. Therefore, data on female bristles and genitalia for comparison are unavailable. According to all the mentioned characteristics, we concluded that the Khon Kaen louse fly is *L. axis*. Additionally, the molecular species delimitation methods were consistent with the morphological analysis of Khon Kaen *L. axis* samples. The PTP method agreed with morphology except for the clade containing *L. fortisetosa*, which included published sequences from various countries in Europe and Asia. For this clade, the PTP method proposed the existence of three MOTUs whereas the ASAP method recognized a single MOTU. Notably, the previously reported *Lipoptena* sp. from Thailand[6] had COI sequences identical to those of our *L. axis*, though no morphological information was published for these specimens. Discrepancies between the morphological and molecular data indicate a need for further analysis to resolve species boundaries. Therefore, additional samples of *Lipoptena* sp. from Thailand should be collected and systematically studied to verify the findings discussed above.

The phylogenetic analysis based on the COI gene confirmed that Khon Kaen *L. axis* belongs to the *cervi* group. The phylogenetic tree shows that louse flies in this study cluster within the same clade with *L. cervi* and *L. fortisetosa*. Additionally, the analysis revealed the distinct separation of the *cervi* group from the *depressa* group (*L. depressa* and *L. mazamae*), which is characterized by abdominal tergal plates crowded in the posterior third of the abdomen and an undefinable 3rd tergite. The tree also shows the *capreoli* group (*L. capreoli*) as distinct. This group consists of hairy flies usually lacking a pregenital plate[24]. However, a major challenge in this molecular analysis is the limited sequence data available in GenBank®. Although the genus comprises 32 species, genetic information is available for only five species. Moreover, the Thai specimens reported by Tiawsirisup and colleagues as *L. fortisetosa* were labeled as *Lipoptena* sp. without species-level identification in GenBank®[6]. Sequences from these specimens cluster within the same clade as Khon Kaen *L. axis*, highlighting the challenges of accurate species identification based primarily on morphology without the support of a comprehensive sequence database. Further investigations integrating both molecular and morphological approaches are essential to clarify the taxonomy and distribution of these flies in Thailand.

Lipoptena axis was initially documented in India from four-horned antelope (*Tetracerus quadricornis*) and was subsequently found on chital deer (*Axis axis*) in Nepal and Sri-Lanka[24,25]. In our study, the hosts include barasingha (*Rucervus duvaucelii*), Eld's deer (*Rucervus eldii*), and chital deer (*Axis axis*). Therefore, this is the first report of barasingha and Eld's deer serving as hosts for *L. axis*. The recorded hosts for *Lipoptena* species in Thailand are southern red muntjac (*Muntiacus muntjak*) for *L. pauciseta*, the Java mouse deer (*Tragulus javanicus*) for *L. pteropi*, and Eld's deer for *L. fortisetosa*[6,20,27]. Individual species of cervids can harbor multiple species of louse flies and the distribution of these flies may be facilitated by host migration. It is important to note that louse flies are weak fliers, typically capable of traveling only up to 50 m in search of a host, thus, movement over longer distances are likely mediated by the migration of their hosts[22,31]. Therefore, host transportation, as well as host migration should be considered in efforts to prevent the spread of louse flies and their associated pathogens into new environments. However, hippoboscids are restricted to a limited number of host species. Although they may accidentally infest various hosts while feeding, only certain species provide the necessary conditions for survival of the flies[29]. The new hosts reported in this study suggests that their role as the potential vector of pathogens needs to be noted.

Although captive animals in zoos are ecologically and territorially restricted, they interact with the surrounding environment through the daily movement of humans, free-roaming animals, and arthropods[32]. *Lipoptena mazamae*, *L. depressa*, *L. cervi*, and *L. fortisetosa*, have all been reported as the carriers of zoonotic pathogens. Previous studies have shown that *L. mazamae* and *L. depressa* are carriers of *Bartonella* spp. and *Anaplasma* spp., respectively[15,19]. Similarly, *L. cervi* is a carrier of various pathogens including *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Borrelia* spp., and *Theileria* spp[15,18,33,34]. The recently reported species in Thailand, *L. fortisetosa*, has been identified as a potential vector for *Theileria* spp., *Anaplasma* spp., and *Bartonella* spp. in Eld's deer at Khao Kheow Open Zoo, Chon Buri Province, eastern Thailand[6,33]. The Khao Kheow Open Zoo is located within a wildlife sanctuary where wild animals roam freely, allowing for frequent interaction between captive and wild animals, which can facilitate disease transmission[6]. While the Khon Kaen Zoo is not an open zoo, it is located near a village, which may contribute to the potential transmission of diseases to humans and domestic animals. Furthermore, in the exhibition zone, some animal species are free to roam and come into close contact with visitors, increasing the risk of ectoparasite bites. Although this study differs from previous reports in terms of region, zoo type, and louse fly species, the potential role of *L. axis* as a vector of zoonotic diseases, along with the distribution of louse flies in different regions of Thailand, should be closely monitored. Moreover, Thailand has a large population of cervids, including farmed, wild, and captive deer, which are endemic or imported. Therefore, there is potential for different species of *Lipoptena* spp. to be introduced to Thailand and this should be investigated.

In conclusion, this study presents the first report of *L. axis* from captive cervids in Thailand, confirmed by morphological characteristics and molecular techniques. Additionally, new host records for *L. axis*, including barasingha and Eld's deer, are reported. We also provide an updated taxonomic key for the *cervi* group of louse flies to aid in identification. The findings of this study will provide essential information for louse fly surveillance and the advancement of zoonotic disease control strategies.

Methods

Sample collection

The louse fly samples were collected during 2021–2022 from individual captive cervids in Khon Kaen Zoo, Khao Suan Kwang District, Khon Kaen, Northeastern Thailand (N 16° 50.730 E 102° 53.797). All studied cervids were under anesthesia for annual checkups. The ectoparasites were manually collected from the skin surface using forceps and were subsequently kept in 70% (v/v) ethanol until used. The collected louse flies were transported to the Biological Hazard Laboratory at the Faculty of Medicine of the Khon Kaen University in Khon Kaen for morphological identification. The study protocol was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Reference No.660301.6.1.2.2/62/65) and The Zoological Park Organization of Thailand (Code No.2301638).

Morphological identification

The louse flies were examined under a stereomicroscope (Olympus SZX10, Japan) for morphological identification. The species were identified based on keys and morphological descriptions available in Bequaert (1942), Maa (1965), and Maa (1969)[20,24,27]. Measurement and photo capture was performed using CellSens software v2.3.18987.0 (https://www.olympus-lifescience.com/en/software/cellsens/). Scanning electron microscopy (SEM) was used to illustrate the head and terminalia parts of the louse flies. Flies were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer before being dehydrated in ethanol and critical-point dried. The specimens were then coated with gold and observed using the SEM (Gemini, Zeiss, Germany).

DNA extraction

A total of 16 louse flies were selected from the 60 specimens for molecular analyses. Those chosen represented both sexes, all three host species, and were morphologically intact. The specimens were air-dried at room temperature for 30 min or until completely dry. Subsequently, each was sagittally sectioned into two parts, one was used for genomic DNA extraction and the other part was preserved in 70% (v/v) ethanol for another research project. For DNA extraction, one half of each louse fly was crushed with a sterile plastic grinding rod in a sterile microcentrifuge tube with 180 µl of PBS. Genomic DNA was extracted using DNeasy Blood and Tissue kit (QIAGEN, Germany) according to the manufacturer's protocol for purification of total DNA from insects and the DNA concentration was measured in the NanoDrop One Spectrophotometer (ThermoScientific, USA) and kept at −20 ℃ until used.

Polymerase chain reaction (PCR) and sequencing

The polymerase chain reaction was used as a first step to sequence DNA from the louse fly specimens. The primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HC02198 (5′-TAAACTTCAGGGTGA CCAAAAAATCA-3′) were used to amplify a portion of the mitochondrial cytochrome c oxidase gene subunit I (COI) with an expected size of 643 bp[35]. The PCR was performed in a final volume of 25 µL consisting of 12.5 µL 2× ViRed *Taq* Master Mix (Vivantis Technologies, Malaysia), 5 µL DNA template, 2 µL of 10 pM/µL of each primer, and 3.5 µL deionized water. Deionized water replaced DNA templates for the negative control. The amplification conditions included initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 s; annealing at 60 ℃ for 30 s; extension at 72 ℃ for 30 s and final extension at 68 ℃ for 5 min. The PCR products were subjected to gel electrophoresis on 3% agarose gel stained with Safe-Green™ (Applied Biological Materials Inc., Canada), and the gel was visualized in a UV transilluminator. Finally, the PCR products were submitted to a DNA sequencing service (Macrogen, Inc., South Korea) for bidirectional sequencing. The sequencing results were compared with the GenBank® sequence database using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

DNA sequences and phylogenetic analysis

All retrieved sequences were assembled and manually edited using the Geneious Prime software v11.0.18 (https://www.geneious.com/). BioEdit software v7.2.5 (https://bioedit.software.informer.com/) was used for multiple sequence alignment. The maximum-likelihood tree using the general time-reversible model with gamma distribution was constructed using MEGA X software v10.2.6 (https://www.megasoftware.net/)[36]. Sequences of other species of *Lipoptena* and other hippoboscid flies were retrieved from GenBank® for comparison with Khon Kaen louse fly sequences. The dataset consists of 16 sequences of Khon Kaen louse flies and 27 retrieved sequences, comprising 7 sequences of *L. fortsetosa* from Japan, China, Korea, Estonia, Czech and Lithuania; 7 sequences of *L. cervi* from Austria, Czech, Estonia, Finland, Lithuania, Norway and Russia; 3 sequences of *L. mazamae* from Canada, Denmark and Mexico, 3 sequences of *Lipoptena* sp. from Thailand (stated in reference to be *L. fortisetosa*)[6], as well as a sequence of *L. depressa* from Denmark, *L. capreoli* from Greece and *Lipoptena* sp. from Canada. Other species of hippoboscid flies were used as an outgroup.

Species delimitation analyses

Two species-delimitation methods were used to estimate the number of molecular operational taxonomic units (MOTUs) from DNA sequences. "Assemble Species by Automatic Partitioning" (ASAP)[37] is a tool for grouping sequences into putative species. The method was implemented online at https://bioinfo.mnhn.fr/abi/public/asap/using default settings with the Kimura (K80) (ts/tv: 2.0) model[37]. Similarly, "Poisson Tree Processes" (PTP)[38] is a species-delimitation method based on phylogenetic trees, which was also implemented online via https://mptp.h-its.org/#/tree using default settings with a model p-value threshold of 0.001[38].

Data availability

Sequence data of this study have been submitted to the GenBank® database under accession numbers PQ428974-PQ428989. All the morphological data have been submitted with this manuscript.

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Conceptualization: T.T., C.C., C.E., Data curation: T.T., O.P., N.H., C.E., Formal analysis: T.T., O.P., N.B., K.T., C.E., Funding acquisition: T.T., C.E., Resources: T.B., C.C., K.P., S.K., T.R., M.L.K., R.N., Methodology: T.T., O.P., N.H., N.B., C.E., Investigation: T.T., O.P., N.B., C.E., Project administration: C.E., Supervision: C.C., C.E., Writing-original draft: T.T., N.B., O.P., D.B., C.E., Writing-review and editing: T.T., O.P., N.H., T.B., N.B., C.C., T.R., M.L.K., R.N., D.B., C.E., All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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