BIOLOGICAL RESEARCH

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Sarcophaga (Liosarcophaga) dux (Diptera: Sarcophagidae): A flesh fly species of medical importance

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Abstract

Background: Although tropical climate of Thailand is suitably endowed with biodiversity of insects, flies of medical importance is not well investigated. Using information from literature search, fly survey approach and specialist's experience, we review database of Sarcophaga (Liosarcophaga) dux Thomson (Diptera: Sarcophagidae), one of the priorities flesh fly species of medical importance in Thailand.

Results: This review deals with morphology, bionomics and medical involvement. Important morphological characteristics of egg, larva, puparia and adult were highlighted with illustration and/or micrographs. Search pertaining to molecular analysis used for fly identification and developmental rate of larvae were included. Medical involvement of larvae was not only myiasis-producing agent in humans and animals, but associated with human death investigations.

Conclusions: This information will enable us to accurate identify this species and to emphasis the increase medically important scene in Thailand.

Keywords: *Sarcophaga dux*, Review literature, Thailand, Forensic entomology, Myiasis, Morphology, Adult, Immature stages

Background

Sarcophaga (Liosarcophaga) dux Thomson (= *exuberan* Pandellé) is a flesh fly (Diptera: Sarcophagidae) species of medical importance in many parts of the world [1]. Geographically, this fly prevails in many part of the world including, but not limited to, southern Europe [France] [2]; Oriental region [e.g., Thailand [3], Malaysia [4], India [5], Nepal [6], Saudi Arabia [7], Egypt [8], Myanmar, Philippines, Indonesia, Japan, Korea, Sri Lanka, Taiwan, China [6]; Australia; and Hawaii, USA [6]. This species is of medical importance as a myiasis-producing agent [9] as well as forensics as it is known to colonize decomposing human remains [1]. This paper reviews its adult morphology, bionomics and medical involvement.

Results and discussion

Morphology

Based on the fact that numerous flesh flies species exist in Thailand, information pertaining to morphology of flesh flies is significant for comparison into group and/or species level, particularly those of medical and forensic importance. Gathering information of all stages in *S. dux*'s life cycle would enable identification of this organism, leading to be applied in the primary step of forensic investigation. Much of the morphological traits come from LM and SEM observations.

Morphology of adults

Adults are dull gray with three longitudinal black strips on the mesonotum, while the abdomen possess checkered or spotted pattern (Figure 1). Body length of male *S. dux* is medium to large size (7–12 mm). Important morphological characteristics used for differentiated this species from other forensically important flesh flies are follows: third antennal segment fuscous black, palpus entirely blackish, Postsutural *ac* present, genital segment

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Figure 1 Adult male of *S. dux*. Online figure in color. Bar = 0.2 mm.

2 blackish or sometimes yellowish orange [10]. Notable features are located on the head including large compound eyes, antennae and a sponging mouthpart with prominent palps. To evaluate the number of ommatidia of medically important flies in Thailand, we designed a study by soaking the head in 20% potassium hydroxide solution in room temperature for 7 days, then the compound eye was dissected into six small parts which was placed onto a glass slide and flatted using a coverslip. Image of each part was manually counted by those printed part obtained using microscope from the computer [11]. Using this procedure, the average number of ommatidia in male compound eye was 6,032 ± 385 (left eye) and 6,032 ± 408 (right eye); whereas females had 6,073 ± 207 (left eye) and 6,100 ± 220 (right eye). Such great number of ommatidia would allow flies to perceive better visual resolution, and thereby implication for visual efficiency [12]. Our observation on the antennal sensilla using SEM revealed that antennal segment of both sex are endowed with several types of sensilla – sensilla chaetica, sensilla trichodea, sensilla basiconica, sensilla styloconica, sensilla coeloconica and sensory pits [13], allowing helpful in the perception various receptions (e.g., chemoreception, mechanoreception, olfactory). Similarly to antenna that endows several sensillae, our result displayed that sponging mouthpart of *S. dux* possess sensilla – sensilla trichodea, sensilla basiconica at labellar lobes; sensilla chaetica and sensilla basiconica at palpus (Figures 2A-D). The prestomal teeth are bifurcation at the tips (Figure 2A). Such features of sensilla observed over antenna, mouthpart and bifurcated prestomal teeth of *S. dux* were morphologically similar to previous published works of blow flies [12,13].

As one of the three priorities of flesh fly species of medical importance in Thailand, characteristic of adult for identification was of emphasis. For morphological investigations, research in the form of drawings, LM and SEM images are still needed. Characteristics of male genitalia of *S. dux* have been displayed using SEM by Chaiwong et al. [14]. Based on the terminology of Giroux et al. [15], the phallus is a short, broad structure that is formed by a tubular base connected to a trumpet-shaped, anteroventrally expanded vesica. The juxta is apically bifurcated (Figures 3 and 4). The pregonite and postgonite are slightly curved upward apically. The cerci are pointed and curved apically. Male genitalia *S. dux* was distinctively feature and much different from several flesh fly species observed with SEM [15]. Such information obtained from either LM or SEM provide relatively ease for identification into species. As for females, the ovipositor is shown in Figure 5. Kurahashi and Chaiwong [16] provides most recent taxonomic key checklists of male flesh flies in Thailand to include *S. dux*.

Information pertaining to morphology of internal organs of *S. dux* was very limited. Our dissection of reproductive organ in 7-d old female showed large ovaries, covered by an ovarian envelope comprising numerous ovariole which develop to be eggs with embryo inside (Figure 6A). The spermathecae has 3 lobes each being tubular in form (Figure 6B), of which this feature was distinctive with the globular structure that has been observed in blow fly, *Chrysomya megacephala* (F.) [17]. A pair of testis is elongated shape is present (Figure 7A). Accessory glands are tubular and proximally convoluted. The distal part of the gland form two distinct parts – long tubular structure lay above the long patch (Figure 7B).

Morphology of immature stages

S. dux can be oviparous. A morphological description of *S. dux* eggs was provided by Sukontason et al. [18]. They are elongated and slightly bean-shaped, measuring ~1.5 mm in length. The eggshell comprised of polygonal patterns externally, and sectioning displayed multiple layers of the eggshell: outermost exochorion, outer endochorion, transverse layer of pillars with aeropyles, inner endochorion, and the innermost chorionic layer. Interestingly, such features were comparable with those of blow flies [19]. However, no plastron region or median area was detected in *S. dux*.

Morphological information of immature *S. dux* revealed distinct morphological features of larvae. As with most sarcophagid species, larvae possessed posterior spiracles situated within a terminal concavity of the last abdominal segment. Larvae exhibit a light microscopic observation of the cephalopharyngeal skeleton of the first instar displayed apparent anterodorsal process; the anterior end terminally curved downward. The length of the dorsal cornua was slightly longer than the ventral

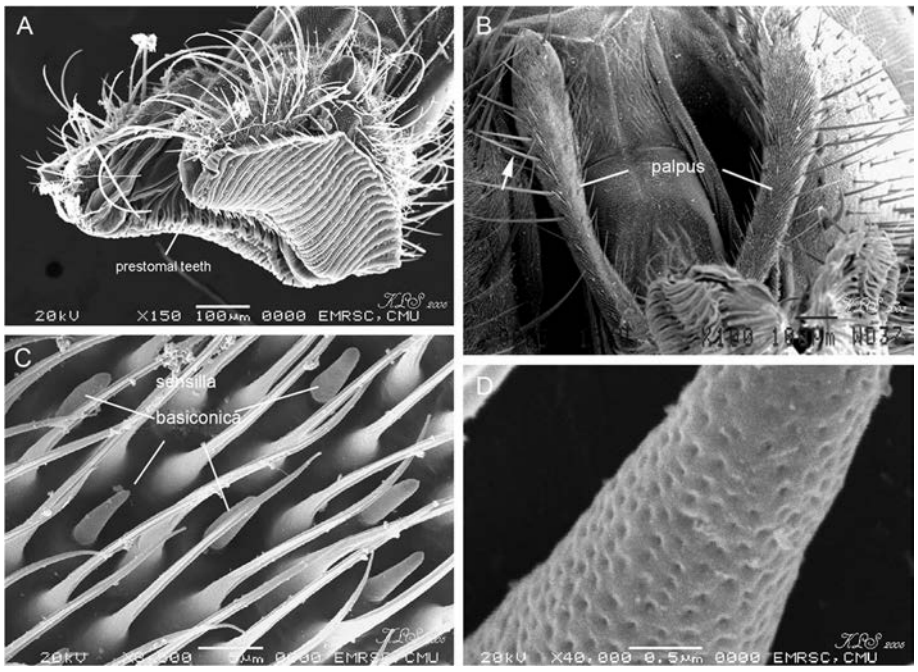

Figure 2 Scanning electron micrographs of adult *S. dux*. **A**: Sponging mouthpart showing everted labella lobes and numerous sensilla trichodea. Prestomal teeth are bifurcation at the tips. **B**: Palpus showing strong and long sensilla chaetica (arrow). **C**: Surface of palpus with amount of sensilla basiconica. **D**: Higher magnification of sensilla basiconica at palpus with perforation surface.

cornua, with the terminal end of the dorsal cornua projecting slightly downward. The dental sclerite was large, attaching the base of the hook part [10]. For the second instar, the dorsal cornua displayed a distinctive feature in having a narrow elongate window, and the length was much longer than the ventral cornua. The terminal end of the dorsal cornua slightly pointed upward. The dental sclerite became small. Regarding the third instar, small dental sclerite was observed. In contrast, the parastomal sclerite was apparent, being slightly curved apically upward. Comparing the third instar of *S. dux* with other forensically important species, although they are morphological similar in appearance and difficulty in identification, our preliminary study using LM revealed that the dorsal spines between the first and second thoracic segments are different from *Boettcherisca nathani* Lopes and *Lioproctia pattoni* (Senior-White). This investigation is on-going research. Besides LM, *S. dux* larvae has been described by SEM, highlighting the important characteristics of the cephalic region (terminal organs, dorsal organs and ventral organs), the ventrally curved mouth hooks, anterior and posterior spiracles [20]. The anterior spiracle located, at the lateral side of the prothorax, shows a single row of 14–17 papillae marginally. The posterior spiracle is D-shaped with an incomplete peritreme. An inner arc is quite pronounced. The distance between both posterior spiracles was narrow, separated by about one third of the spiracle's width [10]. Such features described for *S. dux* was distinctive with the other medically important flesh fly species, *Liopygia ruficornis* (Fabricius) and *Boettcherisca peregrina* (Robineau-Desvoidy), *B. nathani* and *L. pattoni*. Specifically on the anterior spiracle, the number and arrangement of papillae was morphological different; 10–15 papillae arrange in a single row of *L. ruficornis*; 21–27 papillae arrange in two rows in *B. nathani*; 24–26 papillae arrange in one or two rows in *B. peregrina*; 20–28 papillae papillae arrange in one or two rows in *L. pattoni* (unpublished data). This information was mandatory in using identification of these medically important sarcophagids.

Information pertaining to internal organs of sarcophagids was limited. Our dissection of the alimentary canal of mature third instar larvae revealed the large crop, a pair of tubular salivary glands, straight tube esophagus connected to bulb-like structure proventriculus (cardia). Posterior of the cardia is four tubular structure of gastric caecae (Figure 8A). Malpighian tubules form long chained of long tubule (Figure 8B) (unpublished data). Such features of these alimentary canal (e.g., crop, salivary glands, esophagus, proventriculus, gastric caecae, Malpighian tubules) are morphologically similar to those third instar of *C. megacephala* [21].

Puparia of *S. dux* are in the form of coarctate - cylindrical in shape - and composed of the hardened larval integument of the last third instar. They are measured 9.9 ± 0.3 mm in length and 3.8 ± 0.2 mm in width. Under SEM, the intersegmental spines between the prothorax

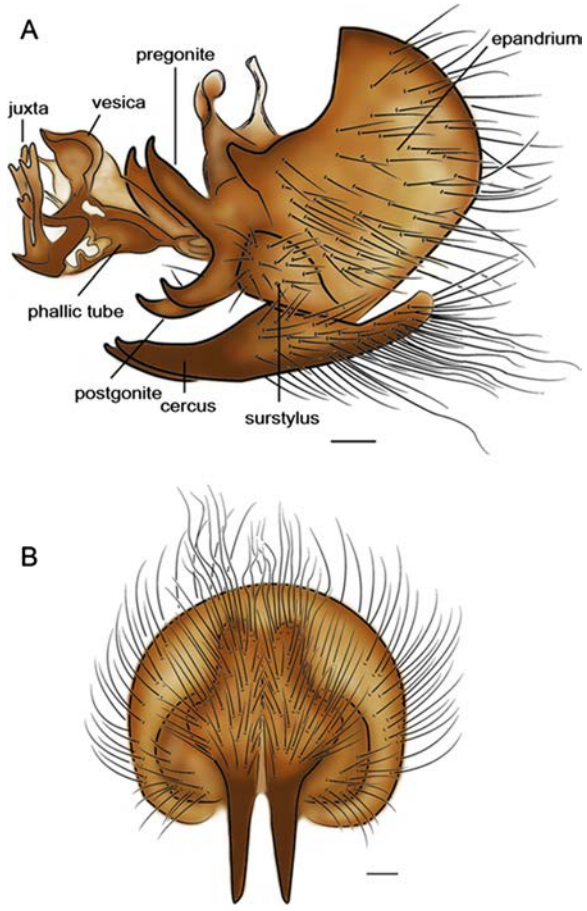

Figure 3 Illustrations of male genitalia *S. dux*. A: lateral view of genitalia displaying short, broad phallus, slightly curved upward apically of pregonite and postgonite, pointed and curved apically cerci and apically bifurcated juxta. **B**: Epandrium, cerci and surstyli, posterior. Online figure in color. Bar = 0.1 mm. Terminology followed Giroux et al. [15].

and mesothorax are broad and triangular (Figure 9A), which is resemble *L. ruficornis*; but different from those of *B. nathani* and *L. pattoni* (unpublished data). Based on such distinction, this feature was one of the characteristic used to differentiate among puparia of these four flesh fly species. No pupal respiratory horn was observed dorsolaterally in the first abdominal segment, and this was concordance with those observed in *L. ruficornis*, *B. nathani* and *L. pattoni*. Each posterior spiracular disc appears D shaped, with a pronounced medial projection and three vertically oriented long, narrow spiracular slits (Figure 9B) [22].

Molecular analysis

Molecular-level studies have addressed taxonomic status of organisms to distinguish morphologically similar species or even genera, including flesh flies. For example, molecular analysis using mitochondrial cytochrome oxidase gene subunits I and II (COI and COII) sequences of 17 Malaysian species of forensic importance successfully clustered into distinct clades and grouped accordingly: *peregrina, albiceps, dux, pattoni, princeps* and *ruficornis. S. dux* was classified as Clade C of the *dux*-group, comprising *S. dux* and *S. brevicornis* Ho [4]. Identification of forensically important sarcophagids from Egypt and China [*S. dux, Sarcophaga argyrostoma* (Robineau-Desvoidy), *Sarcophaga albiceps* (Meigen) and *Wohlfahrtia nuba* (Wiedemann)] was potentially assessed by using partial mitochondrial cytochrome oxidase I and II genes [23]. In four Chinese sarcophagid species including *S. dux*, the 289-bp fragment of the mitochondrial 16S rDNA gene and the 278-bp fragment of the mitochondrial COI gene of DNA method can be used as a supplemental

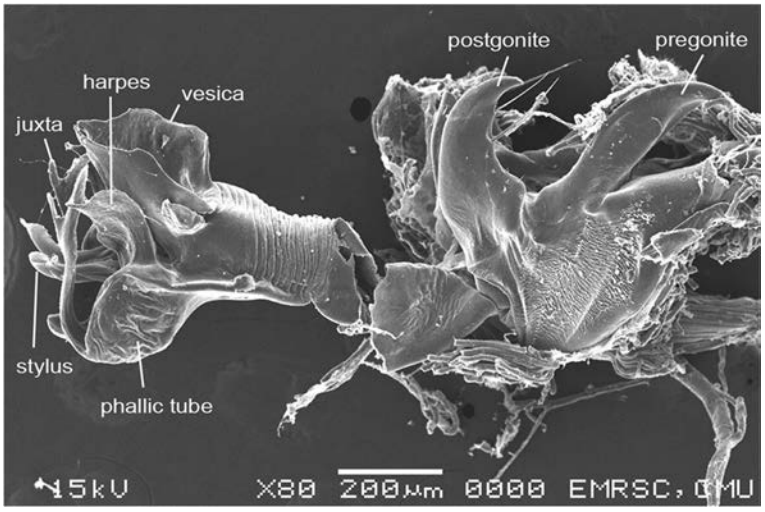

Figure 4 Scanning electron micrograph of male genitalia *S. dux* showing trumpet-shaped, anteroventrally expanded vesica, bifurcated juxta. Terminology followed Giroux et al. [15].

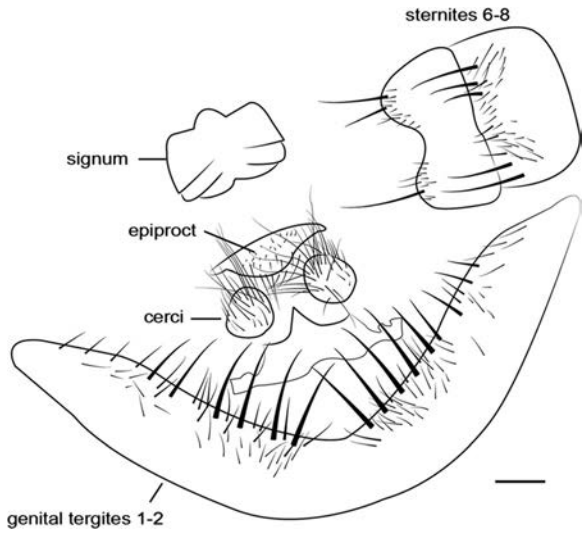

Figure 5 Illustration of ovipositor *S. dux*. Bar = 0.1 mm. Terminology followed Richet et al. [2].

means for morphological method in identification [24]. The genetic characterization of three flesh fly species has been compared [*S. dux*, *S. argyrostoma* (Robineau-Desvoidy) and *L. ruficornis*] using allozyme and RAPD-PCR markers, which indicated a very close relationship between these species [25].

Specifically for *S. dux*, molecular analysis using the sequencing of a 658-bp 'barcode' fragment of the mitochondrial COI gene to accurately identify adult sarcophagids from the Australian east coast demonstrated the intraspecific variation within the nonmonophyletic species of *S. dux*, as depicted from the NJ tree, indicating two distinct species which is portrayed graphically by separate clusters [26]. In addition, genetic variability of population has been analysed by electrophoretic profiles using allozymes at five enzyme loci [(Malic enzyme (ME), Acid phosphatase (ACPH), Alkaline phosphatase (APH), Lactate dehydrogenase (LDH) and Xanthine dehydrogenase (XDH)]. All enzymes were found to be encoded at a single locus. These profiles revealed that ME and XDH were monomorphic, whereas APH, LDH and ACPH displayed polymorphism for two electromorphs and three electrophoretic phenotypes, suggesting a low values of genetic variability with the deficiency of heterozygotes in two loci [27].

Bionomics

Although sarcophagids are commonly viviparous, depositing larvae directly onto a breeding medium; however, some species occasionally lay eggs. Examples of these species observed in laboratory conditions included *S. dux, L. ruficornis, B. nathani* and *L. pattoni* [10,28]. Habitats in Thailand colonized by *S. dux* are amphibiodotic (larviposition on both faeces and carrion), similar to *L. ruficornis* and *S. annandalei* Senior-White [29].

Developmental rate of fly larvae is mandatory to be applied to estimate the postmortem interval (PMI_{min}) in forensic investigations. For flies including *S. dux*, larval development (larviposition until pupariation) varied depending on time of year and location of study. In Thailand, development from the newly hatched larvae to pupariation of this species required 72 h during the summer months (March–June) with temperatures ranging between 27.1–29.8°C. In rainy season and winter, ~96 h were required [13]. In Guam 7 days at 29.5°C were required [30], while in South Africa (=*S. exuberans*) 8.2-10.2 days at 25°C were needed [31]. In Malaysia, the average developmental time of the second instar, third instar, post-feeding, pupa took 19 h, 40.5 h, 73 h and 91 h, respectively, based on fluctuation temperature of 28.9 ± 1.2°C and 64 ± 10% RH [32]. Research from Saudi Arabia indicated that development from first instar to adult emergence was 51.8, 33.0, 25.0, 16.4 and 15.1 d when reared at 16, 20, 24, 28, 32 and 36°C, respectively [33].

Although *S. dux* is prevails in a widespread regions, its bionomic was surprisingly rare. This fly species is a synanthropic [29]. Research conducted in northeast Thailand indicated that adult *S. dux* were collected in the customized reconstructable funnel fly traps baited with 250 g of

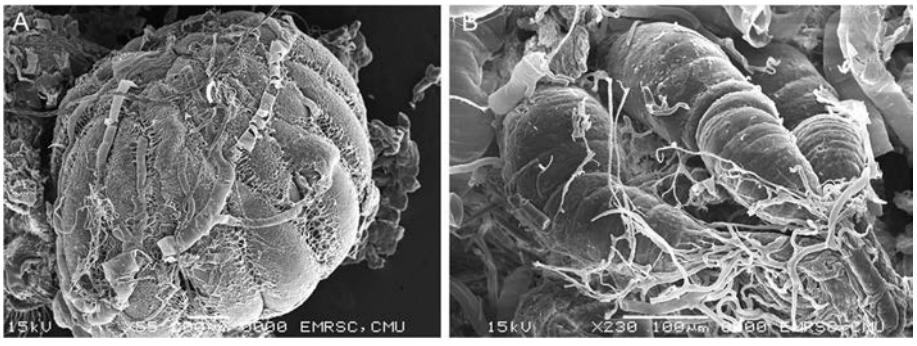

Figure 6 Scanning electron micrographs of 7-d-old female *S. dux*. A: Ovary. **B**: Spermathecae.

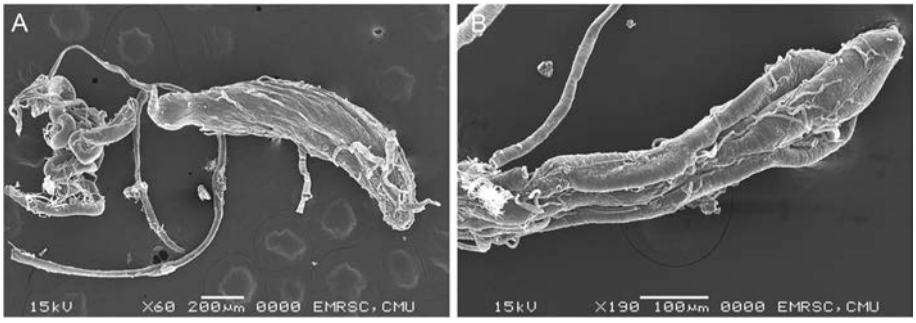

Figure 7 Scanning electron micrographs of male *S. dux*. A: testis and accessory gland. **B**: Higher magnification of distal part of accessory gland.

1-day tainted beef located in the garbage piles and school cafeteria and not in rice paddy fields [3]. Based on this record, *S. dux* is likely to be endemic. In Thailand, adults were captured from both the flower of the *Bulbophyllum putidum* (Teijsm. & Binn.) plant, *Tectona grandis* L. and *Dimocarpus longan* Lour. fruit [29]. Adults *S. dux* were also associated with cow dung in Malaysia [34], and had some attraction to human excrement [35]. And, this species has been collected at altitudes of 2,000 m above sea level in Nepal [6], indicating the well-adapted to high altitude environments. Our survey assessment using an automatic trap in various land-used types (forested landscape, orchard environments and palm plantations) in Chiang Mai, northern Thailand, is ongoing conducted. This automatic trap, invented by one of authors (K. Sukontason), would help to clarify not only seasonal distribution, but also daily

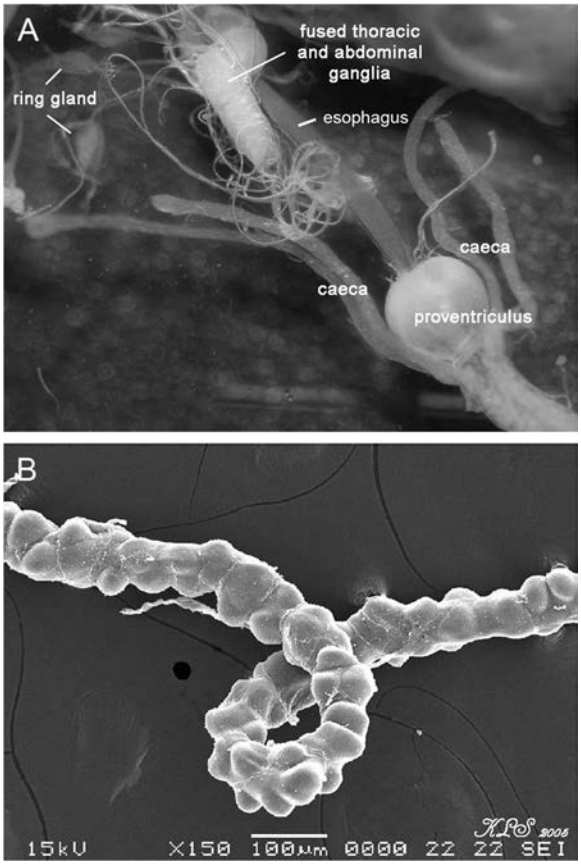

Figure 8 Micrographs of third instar *S. dux*. A: Anterior end displaying esophagus, proventriculus (cardia), gastric caecae. A pair of ring gland and fused thoracic and abdominal ganglia are apparent. **B**: Malpighian tubules.

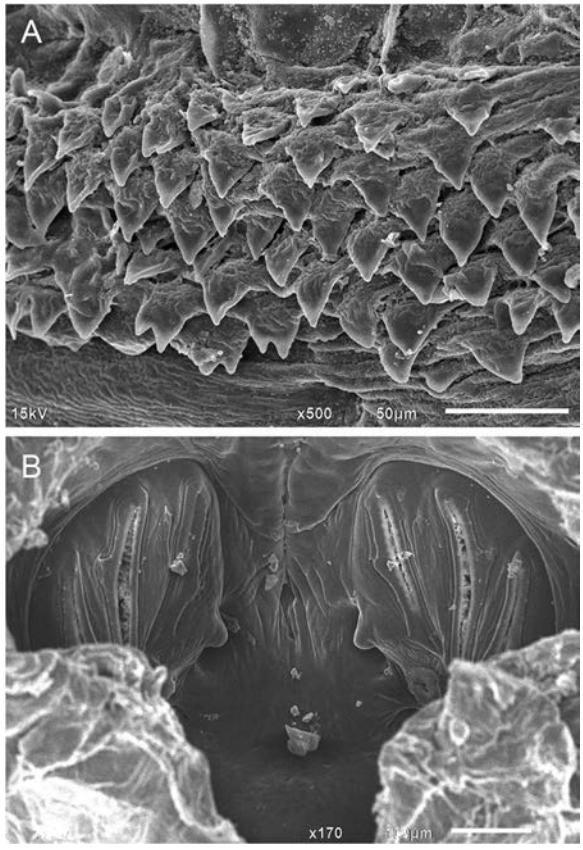

Figure 9 Scanning electron micrographs of puparium *S. dux*. A: Broad and triangular intersegmental spines between the prothorax and mesothorax. **B**: Posterior spiracle.

activity of this species and other medically important flies (e.g., blow flies, muscids). Yet, the knowledge of this view is still very limited. Scientific knowledge pertaining to this aspect is required in order to understand bionomics, distribution and richness for different local spots, thereby allowing to be used in forensic investigations, if specimens of *S. dux* are found in the human corpses.

Myiasis

There is a little published research on the myiasis in human caused by sarcophagids in Thailand. Myiasis cases caused by flesh flies may remain underreported, only that caused by *L. ruficornis* were recorded [36]. Although information regarding *S. dux* as a producing-myiasis agent in humans was very rare in the literature, we recently found that this fly cause aural myiasis in 5-day-old infant in Thailand. Identification of this species was accomplished through morphological characters of male genitalia of adult reared from the larvae recovered (unpublished data). This finding demonstrated rising of myiasis aspect caused by *S. dux*. Such recent case called for attention to the need for protection against flies closely associated with humans including this species. Regarding myiasis in animals, although records was also limited, with skin lesion of camels in India being documented [9]. It has been reported as parasitic on locust and cause bovine tissue myiasis [35].

Forensic entomology

Few human death investigations have recovered *S. dux*. However, a partial explanation for the lack of *S. dux* associated with human remains is the difficulty in identifying larvae or adults. However, this fly is recognized as forensic important in other regions the Iberian Peninsula [37] and in Switzerland where adult *S. dux* were found associating with human corpse [1].

Flies are the primary invertebrates to colonize animal carcasses and/or bait both on the ground and on the high-rise building. Investigation in Nan province of northern Thailand, *S. dux* adults were collected from domestic pig carcasses (*Sus scrofa* L.), in both suburban and forested areas during all seasons (summer, rainy season and winter) [38]. A study in Malaysia also found this species to be quite active larvae collected on chicken livers located on a rooftop (101.58 m from the ground) [39]. Immature and adult *S. dux* were also collected from rabbit carcasses during winter in Guangzhou, China [40].

Conclusions

Summarizing, despite *S. dux* prevails in many part of the world and is well recognized in medical view, information on this species being relatively limited. Despite its significance in public health seems likely nonentity, the role as myiasis-producing agent and forensic entomology increasingly brighten. There is a need to enhance study on various bionomic of this species. This would include developmental data in various temperature conditions, behavior, flight activity, seasonal prevalence and/or any research related to be applied in forensic entomology. Although such an investigation will require time, resources and expertise, efforts should be either maintained or initiated since it will not only be beneficial in Thailand, but several countries where this species exists.

Methods

To examine the morphology of *S. dux*, light microscopy (LM) and scanning electron microscopy (SEM) techniques was employed to observe the important characteristics each stage in its life cycle, except the egg which was published elsewhere [18]. To determine the anatomical feature of immature stages, focusing on the alimentary canal of third instar, mature larvae were dissected and examined under light microscopy, based on the procedure previously described [41]. To examine the morphology of adult, we focused on the genitalia, both ovipositor and male genitalia, which the latter is the most important characteristic used to differentiate flesh fly species. Flies were dissected to obtain their ovipositor or male genitalia by cutting their abdominal segments between 3rd and 4th segments on the clean glass slide using a sharp blade. The terminal tip of the posterior end was then transferring into a well containing 10% potassium hydroxide mixed with 95% ethanol for 3 days. The specimens were rinsed with distilled water before dissection in a centrical-well paraffin plate containing 0.85% NaCl. To dissect ovipositor, two fine long needles were used to cut the sternite. Once the ovipositor stretched, this part was transferred into a well containing 70% alcohol, 80% alcohol and 90% alcohol, each placement for 30 min. The specimens were transferred onto a clean glass slide. Alcohol was removed from the specimens using filter paper, and then xylene was added onto the specimens. A few drop of Permount was added onto the stretched ovipositor, and then cover with the coverslip. The ovipositor was then observed under light microsope and photographs were made using a digital camera (Pentax™, Japan). Illustration of ovipositor and male genitalia were performed using Adobe Illustrator CS4.

Regarding the SEM, puparia and adult of *S. dux* were processed. Puparia were firstly cleaned by washing process that they were placed in gauze and wrapped, and placed in a plastic cup, which was suspended in a beaker (500 ml) contained distilled water. The beaker which bore a magnetic stirrer bar at the bottom was placed onto a hotplate (Barnstead/Thermolyne, Model: SP46920-26, USA) for 3 h. The cleaned puparia were allowed to dry in small petri dish left at room temperature for 7 days, then attached to double-stick tape on an aluminum stub,

coated with gold in sputter-coating apparatus, and viewed under a JEOL-JSM6610LV scanning electron microscope. However, to view the posterior spiracle clearly, puparia were placed in 10% KOH for 1–2 days before being cut using sharp blade in the middle of the seventh abdominal segment. The cut part containing posterior spiracle was then attached to double-stick tape on aluminum stub. With respect to adult, flies were processed for SEM observation, as previous described [42].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KLS did the literature search, prepared SEM experiments and responsible for drafted and final versions of the manuscript. SN illustrated the figures. TK prepared SEM experiments. JKT was responsible for discussion and revision manuscript. KS provided further discussions in details and writing final version of the manuscript. All authors read and approved the final manuscript.

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Morphology of puparia of flesh flies in Thailand

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Abstract. Puparia of five flesh fly species were investigated for forensic study. *Boettcherisca nathani* (Lopes, 1961), *Boettcherisca peregrina* (Robineau-Desvoidy, 1830), *Lioproctia pattoni* (Senior-White, 1924), *Liopygia ruficornis* (Fabricius, 1794) and *Parasarcophaga* (*Liosarcophaga*) *dux* (Thomson, 1869) were examined with a scanning electron microscopy (SEM). Differences between species were found in the number and arrangement of papillae in the anterior spiracle, the shape of intersegmental spines between the prothorax and mesothorax and the pattern of spiracular tufts at the posterior spiracle. The anterior spiracle of *B. nathani* had two rows, comprising 21-27 papillae; while those of *B. peregrina* and *L. pattoni* had one or two irregular rows with 24-26 and 20-28 papillae, respectively. Anterior spiracle of *L. ruficornis* and *P. dux* had one row of 10-15 papillae. Intersegmental spines between the prothorax and mesothorax and pattern of spiracular tufts at the posterior spiracle are morphologically different. *L. ruficornis* and *P. dux* puparia are similar, but the position of the interslit plate between the inner and middle spiracular slits was found to be an important attribute to separate both species. Morphometric analysis on the length and width of puparia of these species revealed statistically different among them. The key for identifying puparia of forensically important flesh flies has been provided.

INTRODUCTION

In forensic entomology, identification of insect specimens found on the human corpse and/or death scenes is the primary step before using them as evidence in forensic investigations. Taxonomy of immature blow flies (Diptera: Calliphoridae) is well established and consequently are the most common arthropods used as evidence. In addition to blow flies, flesh fly (Diptera: Sarcophagidae) specimens are also frequently collected however, flesh fly larvae is too similar to be used for species identification.

Several flesh fly species have been identified in forensic cases. The sarcophagids species commonly associated with decomposing remains include *Liopygia argyrostoma* (Robineau-Desvoidy, 1830), *Robineauella caerulescens* (Zetterstedt, 1837) and *Parasarcophaga similis* (Meade, 1876) in Switzerland (Cherix *et al*., 2012), *L. argyrostoma* in Germany (Benecke, 1998; Amendt *et al*., 2000), *R. caerulescens* in Finland (Pohjoismaki *et al*., 2010), *L. ruficornis* in Thailand and Malaysia (Sukontason *et al*., 2007a; Kavitha *et al*., 2013); and *Bercaea africa* Wiedemann, 1824 and *B. peregrina* in Hawaiian Islands, USA (Goff & Odom, 1987). A study performed in Austria indicated *L. argyrostoma* from pig carcass (Grassberger & Frank, 2004). *Neobellieria bullata* (Parker, 1916) was found in rat carrion in South Carolina, USA (Tomberlin & Adler, 1998) and *Wohlfahrtia magnifica* (Schiner, 1862) in experimental studies conducted upon mummified rats in Egypt (Abdel-Maksoud *et al*., 2011). *Argoravinia rufiventris* (Wiedemann, 1830)

was collected from bear, deer and swine carcasses in Louisiana, USA (Watson & Carlton, 2003). *N. bullata* were reared from pig carcass in Michigan, USA (Pastula & Merritt, 2013), *B. peregrina* and *L. ruficornis* in Hawaii, USA (Davis & Goff, 2000), and *Parasarcophaga taenionata* (Wiedemann, 1819) in Guam (Jenson & Miller, 2001). In China, succession experiments using pig carcass included *Liopygia crassipalpis* (Macquart, 1839) (Ma *et al*., 1997), *L. ruficornis*, *Parasarcophaga albiceps* (Meigen, 1826) and *P. taenionota* (Wang *et al*., 2008); from rabbit carrion *P. similis*, *B. peregrina*, *Harpogophalla kempi* (Senior-White, 1924), *P. albiceps*, *Parasarcophaga kawayuensis* (Kano, 1950), *Parasarcophaga misera* (Walker, 1849) and *P. dux* (Shi *et al.*, 2009) were reported. In northern Thailand, *L. ruficornis*, *P. dux* and *B. peregrina* were commonly encountered (Vitta *et al.*, 2007; Sukjit, 2011). In this study, we elucidate puparia morphology for five Thai flesh flies: *B. nathani*, *L. pattoni*, *L. ruficornis P. dux* and *B. peregrina*. These observations can be directly applied to help identify these flesh fly species during forensic investigations.

MATERIALS AND METHODS

Fly colony

Puparia of *B. nathani*, *L. pattoni*, *L. ruficornis* and *P. dux* were obtained from laboratory colonies at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand. For rearing, adults were provided with sugar and water *ad libitum*; while fresh pork liver was provided *ad libitum* both as food and oviposition medium, and also to larvae as needed. Flies were maintained in the laboratory at ambient temperature, relative humidity and natural photoperiod. Puparia of *B. peregrina* was obtained from F_1 colony which originated from a single female collected from the field in 2006 using a sweep net. Adult males were used to identify species with available key (Tumrasvin & Kano, 1979). Taxonomy and terminology of the pupal stage followed Richet *et al*. (2011) and Kurahashi & Chaiwong (2013).

Morphometric measurement

Thirty to fifty puparia of five species were measured under a dissecting microscope using a vernier caliper (Soya Electronic Digital Caliper, Taiwan). Data were compared using the ANOVA test (SPSS, version 16, SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was considered significant.

Scanning electron microscopy preparation

Puparia of *B. nathani* and *L. pattoni*, were stored in 70% alcohol while those of *L. ruficornis* and *P. dux* were collected from the colony, were processed for cleaning their external surface. Initially, puparia from each species were placed in a small petri dish containing 70% alcohol, and cleaned using a fine brush. Then they were placed in gauze, wrapped and placed in a plastic cup which was holed in four lateral positions and was cut opened at the bottom (Fig. 1). The rubber band was used to secure this gauze with the plastic cup. The plastic cup was suspended hanging to the 1/3 mark in a beaker (500 ml) using a wooden dole as support. The beaker contained a stir bar and 50% alcohol and placed onto a hotplate for three hours (Barnstead/Thermolyne, Model: SP46920-26, USA). The next three washes consisted of dish detergent (linear alkylbenzene sulfonate 14% w/w + sodium lauryl ether sulphate 2.5% w/w) then distilled water and lastly 70% alcohol. Multiple rounds of wash were conducted in order to ensure detachment of debris from the puparial integument (Fig. 2). Finally, the cleaned puparia were allowed to dry in small petri dish left at room temperature (28-30ºC) for seven days. All specimens were then attached to double-stick tape on an aluminum stub, coated with gold in sputter-coating apparatus, and viewed under a JEOL-JSM6610LV scanning electron microscope (JEOL, Japan). To view the posterior spiracle clearly, puparia were placed in 10% KOH for 1-2 days before being cut using a sharp blade in the middle of the seventh abdominal segment. The cut part containing posterior spiracle was then attached to double-stick tape on aluminum stub.

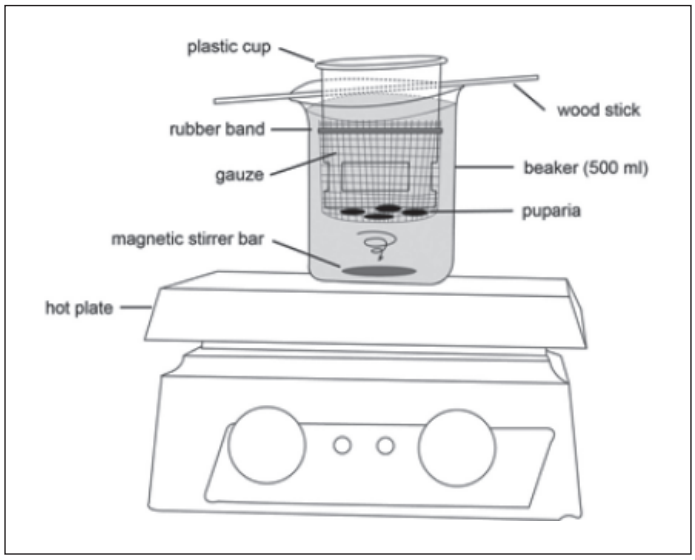

Figure 1. Illustration of apparatus for cleaning puparia (image not to scale)

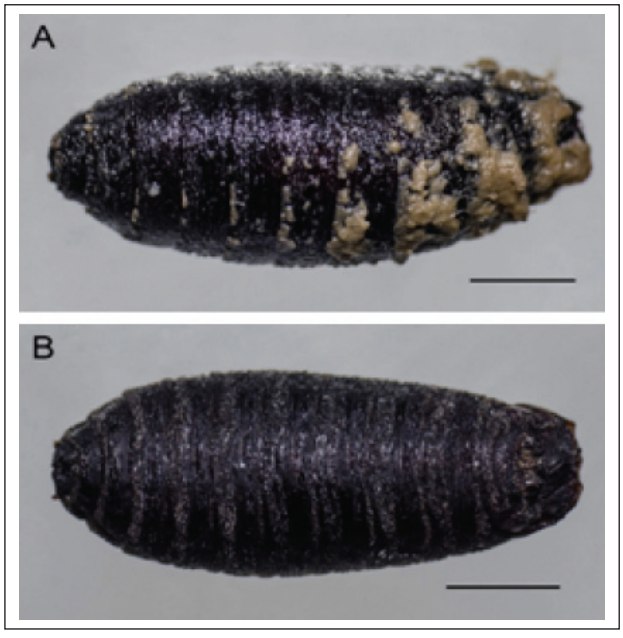

Figure 2. Puparia of *L. pattoni* before (A) and after (B) cleaning process. Bar = 2 mm

RESULTS

Morphometric analysis

Flesh flies have coarctate puparia with the first four anterior segments gradually rounded anteriorly, and the eighth abdominal segment being concave and containing a pair of posterior spiracles. As shown in Table 1, puparia of *L. pattoni* had the highest mean length (11.19 mm), followed by *L. ruficornis* (10.32 mm), *B. peregrina* (9.78 mm), *P. dux* (9.71 mm) and *B. nathani* (9.40 mm),

respectively. Analysis of puparia width indicated that *L. pattoni* had highest mean value, followed by *L. ruficornis*. The remaining species showed no significant difference in their width ($p > 0.05$, ANOVA).

SEM observation

Anterior spiracles are located laterally on the prothorax. Two irregular rows were observed on each anterior spiracle in *B. nathani* (number of papillae 21-27, n = 36, Fig. 3A) and *B. peregrina* (number of papillae 21, n = 1, data from this study [Fig. 1B]; number of papillae 24-26, n = unknown, data from Ishijima 1967). Papillae of *L. pattoni* were arranged either irregularly as one or two rows, bearing 20-28 (n = 56) (Fig. 3C). For *L. ruficornis* and *P. dux*, anterior spiracle bear one row of papillae, consisting 10-15 (n = 30) and 11-15 papillae (n = 33), respectively (Fig. 3D).

Table 1. Morphometric analysis of flesh fly puparia

Species	n	Length (mm) (mean ± SD)*	Width (mm) (mean ± SD)*
Boettcherisca nathani	50	9.40 ± 0.30^{d}	3.62 ± 0.14^{c}
Boettcherisca peregrina	50	9.78 ± 0.45^{c}	3.67 ± 0.16^{c}
Lioproctia pattoni	31	11.19 ± 0.68^{a}	4.44 ± 0.27^{a}
Liopygia ruficornis	50	10.32 ± 0.38^{b}	4.00 ± 0.27^{b}
Parasarcophaga dux	50	9.71 ± 0.38^{c}	3.64 ± 0.18^{c}

*Means within a column followed by different letter are significantly different ($p < 0.05$) (ANOVA)

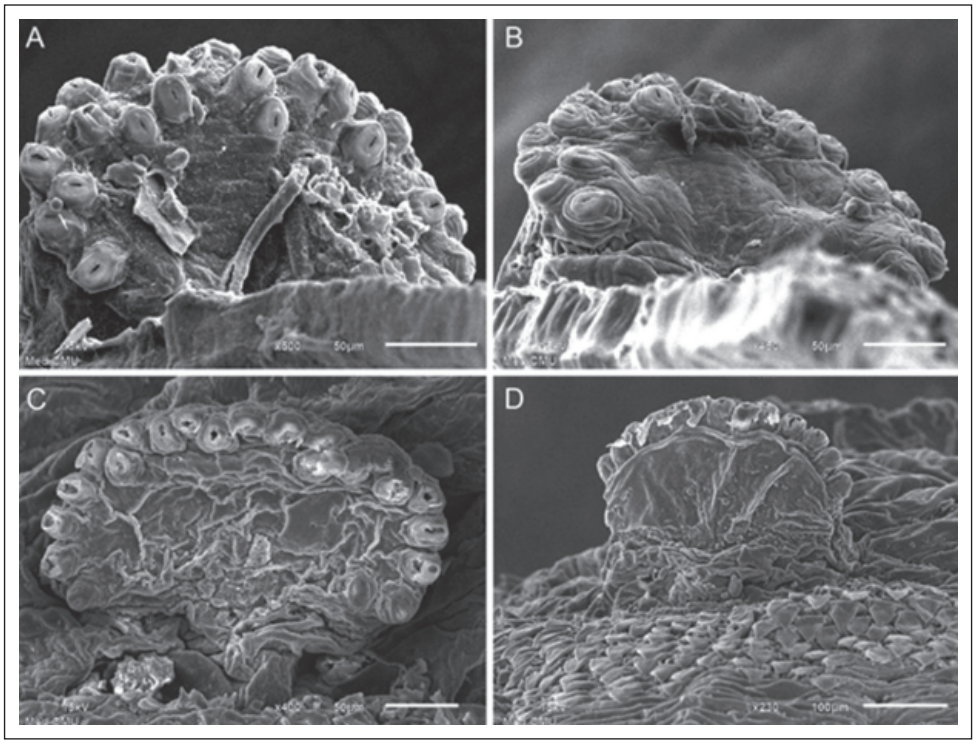

Figure 3. SEM micrographs of anterior spiracles of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*

Intersegmental spines between the prothorax and mesothorax of flesh fly puparia are markedly different between species when viewed with a SEM. Spines of *B. nathani* displayed slender triangular shape throughout (Fig. 4A); whereas *B. peregrina* displayed stout triangular shape at the upper part and slender at lower half (Fig. 4B). For *L. pattoni*, spines revealed moderate triangle either singly or adjoining as a small group at the upper part, while some displayed a few tips at the most lower part (Fig. 4C). Spines of *L. ruficornis* and *P. dux* are morphologically similar, displaying almost uniformly stout triangular shape throughout (Figs. 4D, 4E).

Posterior spiracles of the flesh flies examined revealed similarity in appearance. Those of *B. nathani*, *B. peregrina*, *L. pattoni*, *L. ruficornis* and *P. dux* were demonstrated in Figs. 5A, 5B, 5C, 5D and 5E, respectively. Button, an ecdysial scar located at the lower end of spiracular slits, is visible under SEM in all species examined.

At the posterior spiracle, spiracular tufts located adjacent and/or between spiracular slits showed morphologically different characteristics among species. Particular

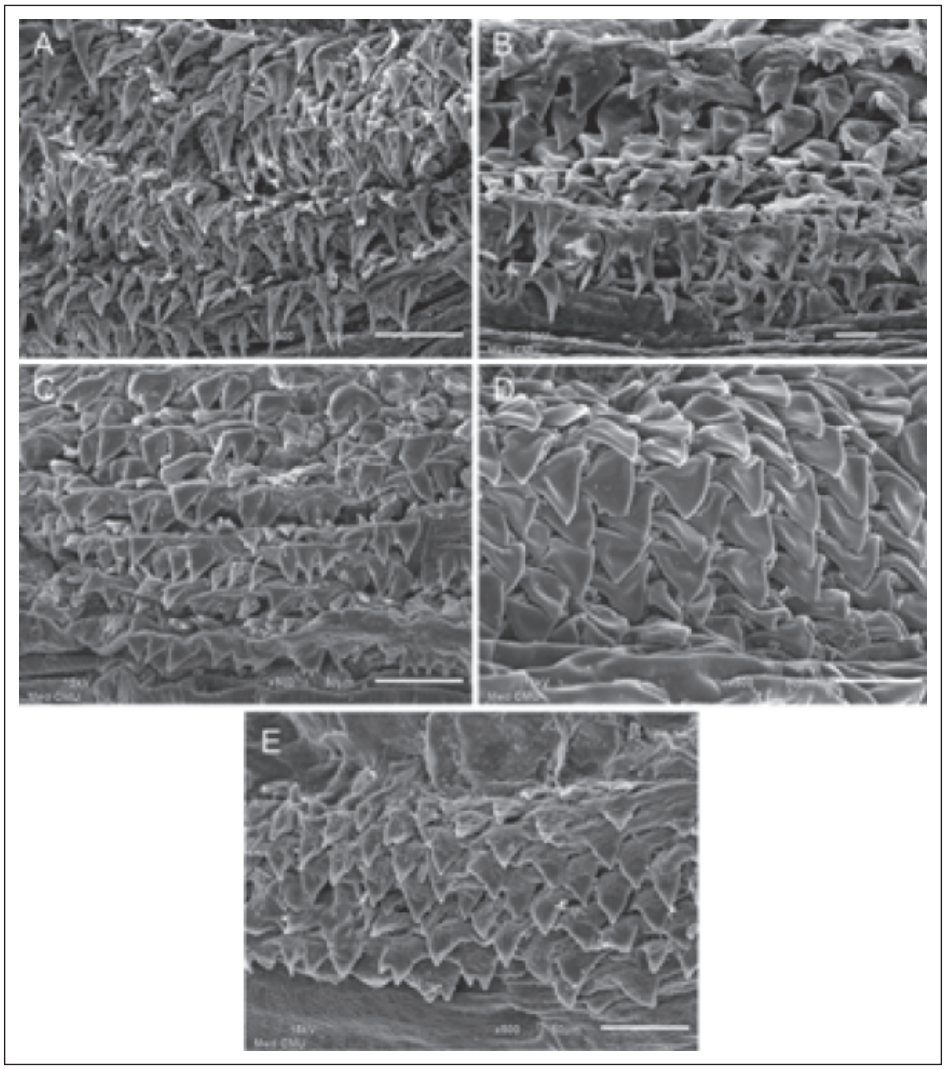

Figure 4. SEM micrographs of intersegmental spines between the prothorax and mesothorax of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*. **E:** *P. dux*

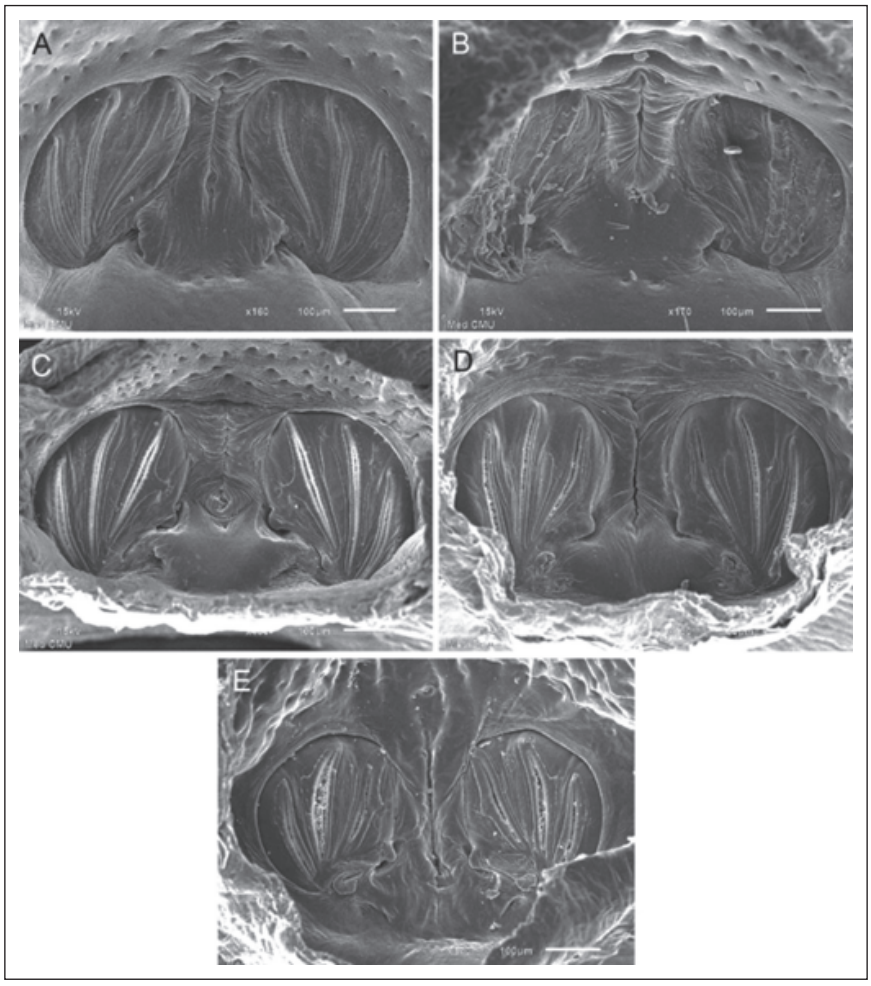

Figure 5. SEM micrographs of posterior spiracles of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*. **E:** *P. dux*

attention was given to the left spiracle as a position for comparison. The spiracular tufts of *B. nathani* are extensively branched at the area adjacent to the inner spiracular slit (Fig. 6A, black arrow), at the top of the plate between inner and middle spiracular slits (Fig. 6A, white arrowhead), and adjacent to the middle spiracular slit (Fig. 6A, white arrow). For *L. pattoni*, the spiracular tufts had fewer branches than the former species (Fig. 6B). On the other hand, those *L. ruficornis* and *P. dux* are similar in appearance and are having faint spiracular tufts in three areas (Figs. 6C, 6D, respectively). The obvious characteristic used to differentiate between *L. ruficornis* and *P. dux* was the interslit plate between inner and middle spiracular slits; *L. ruficornis* was much lower (Fig. 7A, long distance from peritreme) than *P. dux* as indicated by white double-headed arrow (Fig. 7B, short distance from peritreme).

DISCUSSION

Puparia are common remnants of necrophagous flies collected as part of forensic investigations involving decomposition (Mazzanti *et al.*, 2010). Additionally, these structures are commonly preserved in archaeological contexts in association with both human and animal remains (Huchet & Greenberg, 2010). Puparia

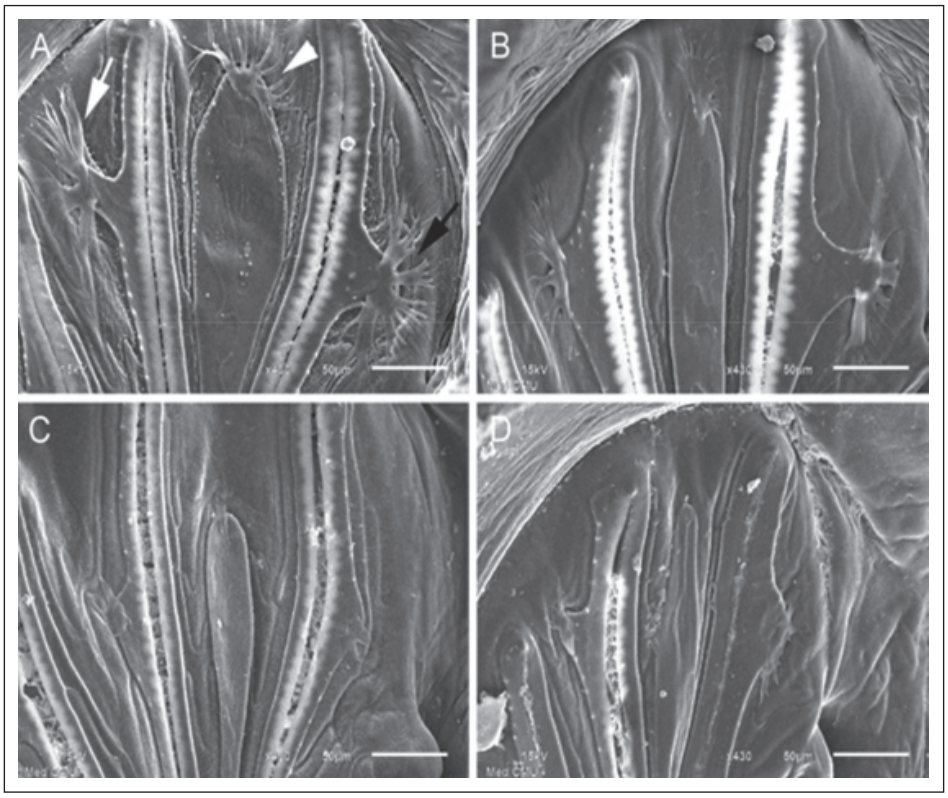

Figure 6. SEM micrographs of spiracular tufts on left spiracle of flesh fly puparia. **A:** *B. nathani* showing extensively branches, at the area adjacent to the inner spiracular slit (black arrow), at the top of the plate between inner and middle spiracular slits (white arrowhead) and adjacent to the middle spiracular slit (white arrow). **B:** *L. pattoni*. **C:** *L. ruficornis*. **D:** *P. dux*

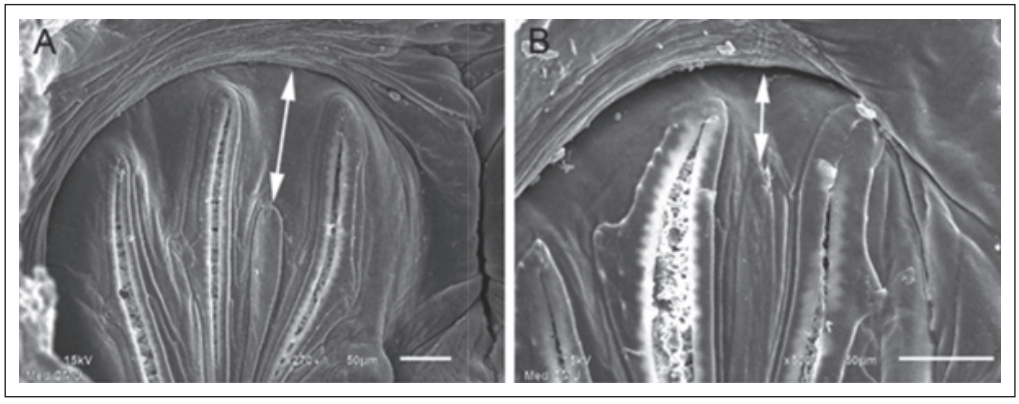

Figure 7. SEM micrographs of end up position of plate between inner and middle spiracular slits of posterior spiracle of flesh fly puparia. *L. ruficornis* showing much lower (long distance from peritreme) (A) than *P. dux* (short distance from peritreme) (B)

can be identified using various methods (e.g., morphology, molecular techniques, analysis using cuticular hydrocarbons) (Sukontason *et al.*, 2007b; Ye *et al.*, 2007; Mazzanti *et al.*, 2010). Morphological investigations using SEM *per se*, require a clean puparial surface for examination, especially for discerning unique and/or specific characteristics. In this

study, we developed a cleaning protocol to remove debris from the puparial integument using common laboratory equipment. This process can also be applied to puparia of other flies or insects. Once dry, the cleaned puparia can be placed onto double-stick tape on stubs, coated with gold, and viewed under SEM. Since the puparia integument is hard, there is no need for typical SEM processing chemical treatment, e.g., pre-fix 2.5% glutaraldehyde and post-fix 1% osmium tetroxide). Similarly, a blow fly [*Chrysomya (Achoetandrus) rufifacies* (Macquart, 1843), *Chrysomya (Achoetandrus) villeneuvi* (Patton, 1922) and *Chrysomya bezziana* (Villeneuve, 1914) (Diptera: Calliphoridae)] puparia wash step that includes a 20-30 minute shaking bath is sufficient for clear SEM images (Sukontason *et al*., 2006b; 2006c). In addition, our previous investigations demonstrated that puparia of flies, e.g., house fly, *Musca domestica* (L. 1758) (Diptera: Muscidae), blow fly, *Chrysomya megacephala* (Fabricius, 1794) or scuttle fly, *Megaselia scalaris* (Loew, 1866) (Diptera: Phoridae)], can be gently placed onto a stub after cleaning, and viewed clearly with SEM (Siriwattanarungsee *et al*., 2005; Sukontason *et al*., 2006a).

In this study we also developed criteria for distinguishing flesh fly species based on puparia characteristics. Arrangement and number of larvae papillae and puparia were unique across species. Two rows of high papillae numbers were observed in *B. nathani* and *B. peregrina* puparia - similar to the third instar found in other flesh flies [*Boettcherisca septentrionalis* (Rohdendorf, 1937) (28-30 papillae), *P. misera* (28-34 papillae), *P. similis* (24-30 papillae), *Myorhina kagaensis* (Hori, 1954) (34-38 papillae) (Ishijima, 1967)]. Irregular rows of papillae on *L. pattoni* was similar to the third instar of the following flesh flies: *P. albiceps* (32-38 papillae), *Parasarcophaga harpax* (Pandellé, 1896) (40-44 papillae), *Parasarcophaga tsushimae* (Senior-White, 1924) (33-36 papillae), *Parasarcophaga kawayuensis* (Kano, 1950) (32-36 papillae), *Parasarcophaga shiritakaensis* (Hori, 1954) (46-49 papillae), *Parasarcophaga oshimensis* (Kano & Field, 1964) (38-46 papillae), *Kanoa okazakii* (Kano, 1953) (38-43 papillae), *Robineauella scoparia* (Pandellé, 1896) (48-54 papillae), *Sarcorohdendorfia antilope* (Böttcher, 1913) (46-52 papillae), *Sarcorohdendorfia mimobasalis* (Ma, 1964) (42-46 papillae) (Ishijima, 1967). Accordingly, when coupled with other flesh fly characteristics, these features can contribute to species identification.

Similar to other studies, we found that puparia intersegmental spines visualized by SEM can be a major differentiating characteristic among flesh flies. Previous examples were provided by Aspoas (1991) who used SEM to differentiate the micromorphology of spinulation of the third instar of some Afrotropica flesh flies – *P. dux*, *Parasarcophaga nodosa* (Engel, 1925), *B. africa* and *Parasarcophaga tibialis* (Macquart, 1851). Likewise, intersegmental spines between the prothorax and mesothorax of third instar of blow flies and muscids (Muscidae) are distinguishable, either determined by light and scanning electron microscopy (Erzinclioglu, 1987; Sukontason *et al*., 2004; 2010b).

Differentiation among flesh fly puparia using whole posterior spiracles is difficult due to their extreme similarities of incomplete peritreme and arrangement of posterior spiracular slits. The location of posterior spiracle within a deep cavity also hinders examination. In this study, puparia were kept in 10% KOH for 1-2 days before being cut and trimmed finely at the seventh abdominal segment using a sharp blade under compound microscope. Then puparia were attached to the stub and view clearly under SEM. Such cutting enables easy viewing of the entire posterior spiracle, in particular the pattern of spiracular tufts, which we discovered is morphologically different across species (see Fig. 6). Peculiarity of this feature is similar to what is found in the third instar of other flesh flies (Aspoas, 1991).

In the present study, several features were used to differentiate between *L. ruficornis* and *P. dux*, such as number of papillae in anterior spiracle, intersegmental spines between the prothorax and mesothorax and faint spiracular tufts.

Specifically, the end up position of interslit plate between inner and middle spiracular slits was found to be an important distinguishing attribute for puparia of *L. ruficornis* and *P. dux*. This feature was in agreement with the posterior spiracle of the third instar of both species, determined under light microscope (Sukontason *et al*., 2010a).

In summary, a key was created to identify puparia of five species of Thai sarcophagids as follows.

1. One row of papillae on anterior spiracle (Fig. 3D); number of papillae 10-15..... 2
   Irregular one or two rows of papillae on anterior spiracle (Figs. 3A-3C); number of papillae 20-28..... 3
2. A low end up position of interslit plate between inner and middle spiracular slits (Fig. 7A, long distance from peritreme) ..... *L. ruficornis*
   A high end up position of interslit plate between inner and middle spiracular slits (Fig. 7B, short distance from peritreme) ..... *P. dux*
3. Intersegmental spines between the prothorax and mesothorax slender triangular shape throughout (Fig. 4A) or moderate triangle either singly or adjoining as a small group at the upper part, while some displayed a few tips at the most lower part (Fig. 4C)..... 4
   Intersegmental spines between the prothorax and mesothorax stout triangular shape at the upper part and slender at lower half (Fig. 4B)..... ..... *B. peregrina*
4. Intersegmental spines between the prothorax and mesothorax slender triangular shape throughout (Fig. 4A); spiracular tufts extensive branched (Fig. 6A)..... *B. nathani*
   Intersegmental spines between the prothorax and mesothorax moderate triangle either singly or adjoining as a small group at the upper part, while some displayed a few tips at the most lower part (Fig. 4C); spiracular tufts not extensive branched (Fig. 6B)..... ..... *L. pattoni*

In conclusion, this study makes two notable contributions for helping utilize flesh fly evidence in forensic investigations. Firstly, we presented a protocol for cleaning the surface of flesh fly puparia for SEM, without a chemical treatment and dehydration process. This cleaning protocol may also be suitable for processing other insects with a hard integument. Secondly, we developed a key that may be used to identify puparia of five medically important flesh fly species, which may aid forensic investigation when these species are found on the corpse and/or death scenes.

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Aural myiasis caused by *Parasarcophaga (Liosarcophaga) dux* (Thomson) in Thailand

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Abstract. Herein is reported the first case in Thailand of aural myiasis caused by the flesh fly, *Parasarcophaga (Liosarcophaga) dux* (Thomson). A 5-day-old infant was taken to hospital with a slightly bloody ear. Two fly larvae exiting the ear and another recovered by a physician were alive, and confirmed as *P. dux* species from adult examination results. This case brought attention to the need for protection against synanthropic flies, particularly for infants and/or hearing impaired patients.

Although numerous myiasis producing fly species are indigenous to Thailand's fauna, myiasis cases might have been underreported for several reasons (e.g. personal reasons, not worth reporting, difficulty in fly identification etc.). The species reported to have caused myiasis in Thailand were *Chrysomya bezziana* (Papasarathorn & Piyarasana, 1962; Papasarathorn *et al.*, 1967; Koranantakul *et al.*, 1991; Nacapunchai & Laohavichit, 1999; Sukontason *et al.*, 2006), *Chrysomya megacephala*, *Achoetandrus rufifacies* (Sukontason *et al.*, 2005), *Oestrus ovis* (Nacapunchai *et al.*, 1998), *Eristalis tenax* (Siripoonya *et al.*, 1993), and *Dermatobia hominis* (Thanapatcharoen *et al.*, 2012). Only a single species of flesh fly; *Liopygia ruficornis*, was recorded (Sucharit *et al.*, 1981). Herein, we report a case of aural myiasis caused by the larva of another flesh fly species; *Parasarcophaga dux*.

Case History and Entomological Finding

A 5-day-old infant was taken to hospital with a slightly bloody ear, from which two second instar (each ~0.5 cm) were exiting and later died. Another invasive larva was removed with fine forceps by a physician during check up, thus revealing a stout-bodied flesh fly, recognized by its pair of posterior spiracles deep within a cavity. This larva was then placed in a small petri dish containing a tiny piece of beef (2x2 cm.) as a food source. The larva completed development and the adult was kept for identification (Figure 1). Species identification confirmed that the resulting adult male was a *P. dux* (Figure 2), based on the key of flesh flies in Thailand (Kurahashi & Chaiwong, 2013). The characteristic of the terminalia *P. dux* is presented in Figure 2A, the cercus normal in shape, not enlarged dorsally and without spines; ventralia composed of 1 lobe; lateral arm of juxta bifid at apex and Y-shaped of the fifth sternite (Figure 2B). The wound on the ear of the patient was treated with an antibiotic (amoxicillin 1.5 mg/ml) and ofloxacin ear drops (0.3%, 5 ml) according to the symptoms. Follow-up after treatment revealed complete resolution of the wound.

Figure 1. Male *P. dux* that emerged from reared puparium

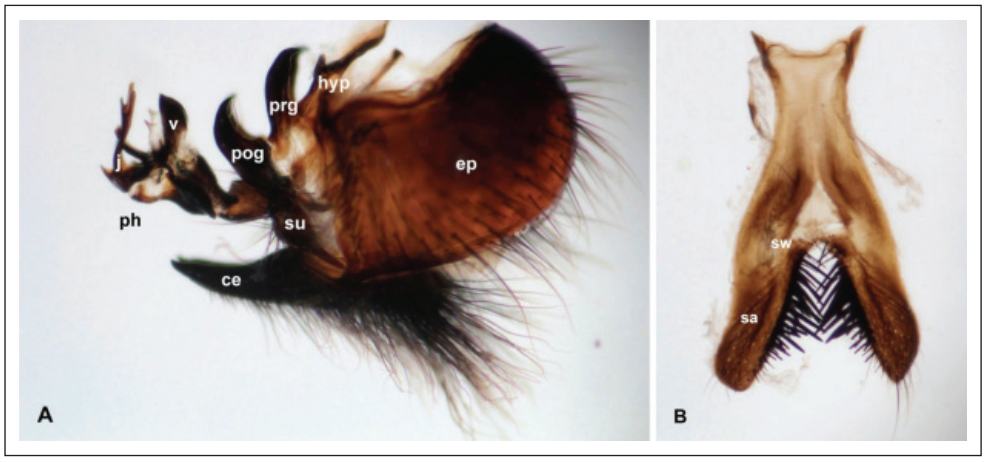

Figure 2. Light micrographs of the male genitalia. (A) Lateral view of the terminalia *P. dux* showing the cercus (ce) normal in shape, not enlarged dorsally and without spines; ventralia (v) composed of 1 lobe; lateral arm of juxta (j) bifid at apex. Ph, phallus; ep, epandrium; hyp, hypandrium; su, surstylus; prg, pregonite and pog, postgonite. (B) Ventral view of fifth sternite demonstrating Y-shaped sternite. Sa, sternal arms and sw, sternal window

DISCUSSION

To the authors' knowledge, this is the first report in Thailand of myiasis in humans caused by *P. dux*. A previous study reported myiasis caused by the flesh fly, *L. ruficornis* (Sucharit *et al.*, 1981). In Thailand, both these flesh fly species are synanthropic, thereby enhancing the risk that females could larviposit on humans, thus the risk of myiasis.

Biological knowledge of *P. dux* helps in understanding how humans may become an accidental host for this flesh fly species. In Thailand, female *P. dux* larviposit in both feces and carrion (Bänziger and Pape, 2004), and are found commonly in a synanthropic environment (Chaiwong *et al.*, 2012), thus allowing their involvement in human myiasis. According to the literature, *P. dux* causing human cases of myiasis is rare (James, 1947). In domestic animals, this species has been thought to be associated with flies in camels (Wernery and Kaaden, 2002).

Although reports of myiasis in infants are very rare in Thailand, this case exhibits the need for vigilant sanitation that may offer protection against synanthropic flies that dwell in human environments.

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CASE REPORT

BLOW FLY MAGGOTS (DIPTERA: CALLIPHORIDAE) FROM A HUMAN CORPSE IN A VEHICLE

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Abstract. Correct species identification and development data of insects associated with a cadaver can help estimate the time of colonization which could be used to infer a minimal post-mortem interval (minPMI) for forensic investigations. Human remains are found in a variety of locations ranging from open fields to inside automobiles. We report the investigation of blow fly larvae collected from a decomposing body located in the trunk of a car. There were two blow fly (Diptera: Calliphoridae) species: *Achoetandrus rufifacies* (Macquart) and *Chrysomya megacephala* (Fabricius). Blow flies can enter the vehicle and colonize human remains. Based on age estimations of third stage larvae of *A. rufifacies*, the minPMI was estimated to be 4-5 days, which was within the range of 3-5 days estimated by other forensically relevant information.

Keywords: forensic entomology, post-mortem interval, *Achoetandrus rufifacies*, blow flies, automobile

INTRODUCTION

Human remains can be discovered in a variety of environments ranging from open fields to concealed places such as automobiles. Although approximately 30 forensic entomology cases have been examined in northern Thailand (Sukontason *et al*, 2007), none have been discovered in an automobile that were recorded. Although such cases are rarely reported, entomological evidence coupled with the findings of the forensic autopsy are often jointly helpful to determine the time of death (Anderson, 2001; Hitosugi *et al*, 2007; Williams, 2008). Case studies can provide information about arthropod colonization of human remains providing guidance with future death investigations. We present here data regarding the use of entomological evidence from a forensic investigation in Thailand involving the remains of a woman located in an automobile. This case demonstrates the use of entomological evidence with other

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Table 1
Occurrence of *A. rufifacies* among human corpse in Thailand[a].

Species	Number of cases		PMI estimation[b]
	Indoor	Outdoor	
A. rufifacies		1	Yes
A. rufifacies + *C. megacephala*	1	3	No/Yes[c]
A. rufifacies + *C. megacephala* + *Chrysomya villeneuvi*		2	No
A. rufifacies + *C. megacephala* + *Lucilia cuprina*		1	No
A. rufifacies + *C. megacephala* + *Synthesiomyia nudiseta*	1		Yes
A. rufifacies + *C. megacephala* + *Lucilia cuprina* + *Sarcophaga* spp		2	No
A. rufifacies + *C. megacephala* + *Coelonomyia nigripes* + *Megaselia scalaris*		1	Yes
A. rufifacies + *Piophila casei* + *Hydrotaea spinigera* + *Sargus* spp + *Dermestes maculatus*		1	No
A. rufifacies + *C. megacephala* + *Sarcophaga* spp	1		No

[a]Sukontason *et al* (2007) from northern Thailand.
[b]Based on age of *A. rufifacies*
[c]Sritavanich *et al* (2009) from Khon Kaen Province, northeastern Thailand.

evidence to determine the minimum post-mortem interval (minPMI) of the deceased individual.

CASE REPORT

The nude body of a female was discovered in the trunk of a car (Honda, Japan) parked near a bamboo forest in Chiang Mai Province during the summer of 2013. The remains were severely bloated, blackened, with partial skin loosening on the arms and fingers. At autopsy done at the Department of Forensic Medicine, Chiang Mai University, maggots were found scattered along the body, especially on the face, neck and along the inside of the victim's thighs. The fly larvae were identified as third instar blow flies (Diptera: Calliphoridae): *Achoetandrus rufifacies* (Macquart) and *Chrysomya megacephala* (Fabricius) with *A. rufifacies* being the most developed. Therefore, age estimates of these larvae were 4-5 days. This estimate was within the range of 3-5 days provided by other forensic finding to determine the minPMI.

DISCUSSION

Little information is published regarding forensic cases involving the concealment of remains within a vehicle (Anderson, 2001). The case presented here illustrates that *A. rufifacies* and *C. megacephala* can successfully colonize human remains concealed in the trunk of a vehicle. The results generated from this case are in agreement with Anderson (2001) who observed a large number of blow flies in a vehicle in British Columbia. She concluded the flies gained access to the remains via many entrances, including drainage holes in the trunk. She suggested that the car itself did not provide much of an obstacle. Apparently, a number of

different species of fly larvae in British Columbia were collected from the remains in the car trunk, such as blow flies [*Lucilia* (*Phaenicia*) *sericata* (Meigen), *Phormia regina* (Meigen), *Protophormia terraenovae* Robineau-Desvoidy and *Calliphora vomitoria* (L.)]; flesh flies (Sarcophagidae *Liopygia argyrostoma* Robineau-Desvoidy); and skipper flies (Piophilidae) [*Stearibia nigriceps* (Meigen) and *Piophila casei* (L.)].

A. rufifacies is one of the most common species of blow flies found on dead bodies, often arriving within 10 minutes of death (Goff, 2000). In our case our estimate fell within the 3-5 days estimation developed in conjunction with other forensically relevant information. However, in similar circumstances of concealled death scenes, such as wrapping, delay in the arrival of flies to oviposit may be encountered. Voss *et al* (2008) determined blow fly colonization of vertebrate carrion was delayed within a car by 24-28 hours. Research in Malaysia found wrapping monkey carcasses (macaques) in rice sacks (made from plastic mesh) delayed the arrival of forensically important flies by 1 to 13 days depending on species (Ahmad *et al*, 2011). It was also reported in Malaysia that in an enclosed environment, fly arrival may be delayed by 1-3 days (Nazni *et al*, 2011). More research is needed to explore the insect arrival and oviposition in a vehicle death scene, so the most accurate estimate of minPMI can be made.

Our finding support a previous study that both *C. megacephala* and *A. rufifacies* are flies commonly associated with human death scenes both inside human dwellings and in open environments in urban, suburban, rural and high elevation areas in Thailand (Sukontason *et al*, 2007). This is also similar to forensic cases reported from Malaysia (Cheong *et al*, 1973; Lee *et al*, 2004; Kumara *et al*, 2012; Kavitha *et al*, 2013), Taiwan (Shiao and Yeh, 2008), Hawaii in the USA (Goff and Odom, 1987; Goff *et al*, 1988), and Colombia (Barreto *et al*, 2002). In Panama, both these species of blow flies are found along with *Cochliomyia macellaria* (Fabricius) from human remains (Sergio Bermudez and Pachar, 2010). These findings are also similar to a report from Chiang Mai, Thailand (Ngoen-klan *et al*, 2011). In Nakhon Sawan Province, Thailand, *A. rufifacies* was the most abundant species found on chicken remains and *C. megacephala* was the second most abundant (Dr Kittikhun Moophayak, unpublished).

This case demonstrates more than one species can colonize human remains concurrently in Thailand (Table 1). This finding is similar to reports from Malaysia (Lee *et al*, 2004; Kumara *et al*, 2012) and Panama (Sergio Bermudez and Pachar, 2010). Therefore, it is recommended investigators not assume single species occurrence. Care should be taken to identify all larvae sampled from human remains. This approach is important since different species may develop at different rates. If larvae are incorrectly assumed to be a single species, the estimate of the minPMI could be less accurate.

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Three Sarcophagid Species (Diptera: Sarcophagidae) Newly Recorded in Thailand

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Abstract. This study contributed new recordings of three flesh fly species (Diptera: Sarcophagidae) to the fauna of Thailand – *Miltogramma tibita* Chao & Zhang (subfamily Miltogrammatinae), *Myorhina situliformis* (Zhong, Wu & Fan, 1982), and *Iranihindia martellata* (Senior-White, 1924) (subfamily Sarcophaginae). Collections of these species were performed using a sweep net and one-day old beef offal as bait. *Mi. tibita* differs from other known *Miltogramma* by having a fine long seta on the dorsal surface of tarsomeres 2-4. With this new record, the number of species belonging to the genus *Miltogramma* known from Thailand has increased to three which includes *Mi. angustifrons* (Townsend, 1933) and *Mi. iberica* Villeneuve, 1912. The new record of *My. situliformis* makes a total of three species for *Myorhina* and these include *My. otiophalla* (Fan & Chen, 1981) and *My. caudagalli* (Böttcher, 1912). Regarding *Iranihindia*, the recording of *I. martellata* makes a total of two species, the other being *I. martellatoides* (Baranov, 1931). This study provides a revised key of each genus where these newly recorded species were recorded, with their re-descriptions, illustrations, photographs, and scanning electron micrographs focusing on the male genitalia. The findings of these newly recorded species means that is a total of 86 species of flesh flies have been recorded from Thailand.

INTRODUCTION

Flesh flies (Diptera: Sarcophagidae) are one of the medically and forensically important insect groups worldwide. According to Pape (1996), the catalogue of the Sarcophagidae of the world listed 2510 known species. In Thailand, the most recent list of flesh fly species consisted of 29 genera and 83 species (Kurahashi and Chaiwong 2013). Of these, two species of the genus *Miltogramma* [*Mi. angustifrons* (Townsend) and *Mi. iberica* Villeneuve], two species of the genus *Myorhina* [*My. otiophalla* (Fan & Chen) and *My. caudagalli* (Böttcher)], and one species of the genus *Iranihindia* [*I. martellatoides* (Senior-White)] were reported. Surveys of medically important flies conducted in several regions in Thailand revealed three species of sarcophagids that had not been recorded previously. This study recorded these three sarcophagids as new to Thailand, re-described the adult males in general morphology, presented revised keys to their identification, and documented the male genitalia using scanning electron micrographs.

Abbreviations for institutions housing specimens are as follows: BPBM, Bishop Museum, Honolulu; DPCM, Department of Parasitology, Chiang Mai University, Chiang Mai; CMPH, College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani; IDD, International Department of Dipterology, NSMT, National Museum of Nature and Science, Tokyo.

MATERIALS AND METHODS

Fly collections

Flies were collected from Lampang province (18º23’32.64°N, 99º12’29.03°E, 439 m), Nakhon Sawan province (15º34’56.5680°N, 100º08’48.1200°E, 81 m) and Ubon Ratchathani province (Muang Ubon Ratchathani,15°16'29.668°N, 104°49'27.471°E, 127 m and

Warinchamrap districts, 15°11'33.212°N, 104°50'6.756°E, 126 m), North-East Thailand using sweep nets with one-day beef offal as bait. The beef was left for 24 h at room temperature (≈25-30°C) before use. Identification using a key of Tumrasvin and Kano (1979) was performed and the male specimens of *Mi. tibita*, *My. situliformis* and *I. martellata* appeared as new records to Thailand.

Examination of external morphology and scanning electron microscopy

After pinning, the external morphology of flesh flies was examined under a dissecting microscope (Olympus, Japan). The length of the frons was measured using an ocular micrometer. Regarding the male genitalia, the last abdominal segment was dissected from pinned specimens under a dissecting microscope and soaked in 10% potassium hydroxide overnight. Specimens were then transferred to a compound microscope and photographed using a mounted digital camera. The specimens were cleaned of KOH by rinsing in normal saline solution and then kept in 70% ethanol. Illustrations were performed from the micrographs taken with the digital camera. For the scanning electron microscopy (SEM) process, specimens placed in 70% ethanol were left for 1 day at room temperature to let them dry completely. All specimens were then attached to double-sided sticky tape on an aluminum stub, coated with gold in sputter-coating apparatus, and viewed under a JEOL-JSM6610LV scanning electron microscope (JEOL, Japan, Faculty of Medicine, Chiang Mai University).

Terminology

The terminology related to fly taxonomy has been changed on several occasions. In this paper, the terminology of the general morphology follows McAlpine (1981) and Senior-

White *et al*. (1940). Terminology of male genitalia mainly follows Zumpt & Heinz (1950) and Giroux *et al*. (2010).

RESULTS

Miltogramma tibita Chao & Zhang, 1988

(Figs. 1a, 1b)

Male (2 specimens). Body length 10.0 mm, width 3.0 mm. *Head.* Frons index 0.24 (*n* = 2), frontal stripe yellow brown, parafrontal and parafacial entirely golden pollinose (Fig. 1c), parafrontal slightly yellowish tinged, with about 10 frontal bristles (*ori*) and 0+1 fronto-orbital bristles (*ors*), outer vertical bristles (*ov*) shorter than 2/3 the length of inner vertical bristles (*iv*). Antennae entirely blackish (Fig. 1c), only second segment with yellow-brown apical margin, third segment more than three times as long as second segment, arista blackish. Gena silver-grey pollinose, with numerous white hairs except for several black ones below lower eye margin, postgena with numerous whitish hairs. Occiput with group of black postocular setae, on upper parts but only one row regular. Palpus slender, yellow brown. *Thorax.* Black, silver-grey pollinose, with 3 broad black longitudinal stripes; propleuron bare; mesothoracic spiracle yellowish; metathoracic spiracle creamy white. Chaetotaxy acrostichial (*ac*) 0+1 (prescutellar), *dc* 2+2, intraalars (*ia*) 0+1, humeral (*h*) 3 (strongly developed), posthumeral (*ph*) 1, presutural (*prs*) 1, supraalar (*sa*) 2, notopleural (*n*) 2 (strongly developed), postalar (*pa*) 2, *sc* 3+1. Wing entirely fuscous hyaline, epaulet blackish, basicosta creamy white, R_1 bare above, R_{4+5} with row of 9-10 setae extending nearly halfway from basal node to r-m above, CS 5 with short spines along less than basal one-third of anterior margin. Alar and thoracic squamae creamy white. Halter brownish. Legs black, fore femur with row of strong *pd*, *p* and *pv*, with row of long hair on postero-ventral surface; fore tibia with 1 short *ad* apically without *p*, male tarsal segment 2-4 of foreleg with several long hairs on dorsal surface (Fig. 1d); mid femur with row of strong hair, 2 *p*-*pd* apically, rows of *av*,

and *pv* apically; mid tibia with 3 *ad* (1 strong), 1 *pd* medially, with *p*, *pv* apically; hind femur with rows of strong *ad*, with 3 *pd* apically; hind tibia with 3-4 strong *ad*, row of *pd*. *Abdomen.* Clothed with yellowish-grey pollinose, more or less yellowish on lateral side of tergite 1+2 to tergite 3; tergite 4 with row of short erect marginal bristles; tergite 5 with row of erect marginal bristles. Sternite 5 U-shaped with fine setulae scattered on sternal arms (Fig. 2a). GS 1and GS 2 (epandrium) fuscous brown pollinose. Genitalia hypopygium: surstylus elongate when viewed from the rear (Fig. 2b). Cercus broad at base in lateral view, tapering to point at apex (Fig. 2c), with long hairs at basically 2/3, apical cercus pointed and sharp (Fig. 2d). Aedeagus small, juxta smooth tubular, median stylus membranous antero-lateral and serrated medially (Fig. 2e).

Material examined. 1 male, Chiang Mai Prov.,NW,Chiangdao,450m, 5-11.iv.1958,T.C.Maa (BPBM); 1 male 1 female, Lamphang Prov., Hang Chat Dist., Doi Khun Tan, 493m, rest area, 8.iii.2013, H. Kurahashi (IDD); 13 males 1 female >NSMT(gift); 2 males >DPCM (gift); 2 males > CMPH (gift).

Female. Unknown

Biology. Flies of the genus *Miltogramma* are commonly parasites brood of bee, as have been documented in the large Australian native bee by *Miltogramma rectangularis* (Alcock 2000). In the current collection, males *Mi. tibita* were collected around the rest of a wasp in the dense forested area of Lampang province, Northern Thailand.

Distribution. China (Tibet) and Thailand (Chiang Mai, Lampang)

Myorhina situliformis (Zhong, Wu & Fan, 1982)

(Figs. 3a, 3b)

Male (1 specimen). Body length 10.0 mm, width 3.0 mm. *Head.* Frons index 0.24 (*n* = 1), frontal stripe black, parafrontal and parafacial entirely silver-grey pollinose (Fig.3c),

parafrontal slightly blackish, with about 11 frontal bristles (*ori*) and 0+1 fronto-orbital bristles (*ors*), outer vertical bristles (*ov*) shorter than 2/3 the length of inner vertical bristles (*iv*). Antennae entirely blackish (Fig.3c), third segment more than three times as long as second segment, arista blackish. Gena silver-grey pollinose, with numerous black hairs, postgena with numerous whitish hairs. Occiput with group of black postocular setae, on upper parts but only one row regular. Palpus slender, entirely black. *Thorax.* Black, silver-grey pollinose, with 3 broad black longitudinal stripes; propleuron bare; mesothoracic spiracle brown; metathoracic spiracle brown. Chaetotaxy acrostichial (*ac*) 0+1 (prescutellar), *dc* 2+2, intraalars (*ia*) 0+1, humeral (*h*) 3 (strongly developed), posthumeral (*ph*) 1, presutural (*prs*) 1, supraalar (*sa*) 2, notopleural (*n*) 2 (strongly developed), postalar (*pa*) 2, *sc* 3+1. Wing entirely fuscous hyaline, epaulet blackish, basicosta creamy white, R_1 bare above, R_{4+5} with row of 9-10 setae extending nearly halfway from basal node to r-m above, Costal section 5 (CS5) with short spines along less than basal one-third of anterior margin. Alar and thoracic squamae creamy white. Halter brownish. Legs black, fore femur with row of strong *pd*, *p* and *pv*, with row of long hair on postero-ventral surface; fore tibia with 1 short *ad* apically without *p* (Fig. 3a); mid femur with row of strong hair, 2 *ad*, 1 *p-pd* apically, rows of *av*, and *pv* apically; mid tibia with 3 *ad* (1 strong), 1 *pd* medially, with *p*, *pv* apically; hind femur with rows of strong *ad*, with 3 *pd* apically; hind tibia with 3-4 strong *ad*, row of *pd*. *Abdomen.* Clothed with yellowish-grey pollinose, more or less yellowish on lateral. Sternite 5 V-shaped with fine setulae scattered on sternal arms (Fig.4a). GS 1and GS 2 (epandrium) fuscous brown pollinose. Hypopygium: surstylus elongate when viewed from the rear (Fig. 4b, 4c, 4d). Anterior part of cercus normal, tapering to point at apex (Fig. 4c, 4d), with long hairs at basically 2/3, apical cercus pointed and sharp (Fig. 4c, 4d). Phallus small, apical plate of juxta long, median stylus bifid anteriorly, ventraria curve-shaped (Fig.4e, 4f). Pregonite and Postgonite slender, pointed at end (Fig. 4e, 4g).

Material examined. THAILAND: 1 male, Nakhon Sawan, Pnayuhakiri, T. Khao Tong, 345 m, 28.xii.2012, K. Moophayak.

Distribution. China (Tibet), Nepal and Thailand (Nakhon Sawan)

Iranihindia martellata (Senior-White, 1924)

(Figs. 5a, 5b)

Male (*n* = 5). Body length 11-14 mm, width 3-4 mm. *Head.* Frons index 0.22 (*n*= 5), frontal stripe blackish, parafrontal and parafacial black, parafrontal slightly yellowish tinged, with about 10 frontal bristles (*ori*) and 0+1 fronto-orbital bristles (*ors*), outer vertical bristles (*ov*) shorter than ½ length of inner vertical bristles (*iv*). Antennae yellowish-red to orange (Fig. 5c), first segment brown, second segment yellowish red, third segment yellowish orange and about more than three times as long as second segment, arista plumose, brown, yellowish medially. Gena silver-grey pollinose, with numerous white hairs except for several black ones below lower eye margin, postgena with numerous whitish hairs. Occiput with group of black postocular setae, on upper parts but only one row regular. Palpus slender, entirely yellowish orange. *Thorax.* Black, silver-grey pollinose, with 3 broad black longitudinal stripes; propleuron bare; mesothoracic spiracle yellowish orange; metathoracic spiracle brown. Chaetotaxy acrostichal (*ac*) 0+1, *dc* 5+5, intraalars (*ia*) 1+2, humeral (*h*) 3, posthumeral (*ph*) 2, presutural (*prs*) 1, *sa* 3 (median 1 strongly developed), *n* 4 (2 strongly developed), postalar (*pa*) 2, *sc* 3. Wing entirely fuscous hyaline, epaulet blackish, basicosta creamy white, R_1 bare above, R_{4+5} with row of 8-9 short setae extending up to about three-fifths from basal node to r-m, Costal section 5 (CS5) with short spines along ½ anterior margin. Alar and thoracic squamae creamy white. Halter yellowish orange. Legs black, fore femur with row of strong

pd, *p* and *pv*; fore tibia with 2-3 short *ad* basally, 1 *p* at apical 1/3; mid femur with 3-4 short *a* medially, 2 *p-pd* apically, rows of strong *av*, and *pv* apically, without long hairs exceeding width of femur on postero-ventral surface; mid tibia with 1 *ad*, *pd* medially, with *p*, *pv* apically; hind femur with rows of strong *ad*, *a* and *av* of apical ½ , with 3 *pd* apically, 3 *av* medially, with low of long hair on postero-ventral surface; hind tibia with 3 strong *ad*, 2 *pd* medially. *Abdomen.* Black, silver-grey pollinose, tessellate; tergite 2-3 without median marginal bristle; tergite 3 with strong *marginal bristles (mb)*; tergite 4-5 with median and 2 strong lateral erect marginal bristles. Sternite 1-4with tuft of long hairs. Sternite 5 V-shaped with brush of spines laterally, fine setulae scattered on middle of arms, sternal arms with slightly curving inward apically (Figs. 6a,6b). GS1dark brown pollinose; GS2 (epandrium) blackish with long hairs. Genitalia hypopygium: cercus broad at base, tapering to point at apex (Figs. 6c,6d), with long hairs and a row of sort comb-like spines on the middle (Figs. 6e,6f); Pregonite bifurcated at distal end; Postgonite slender, pointed at end with hair anteriorly (Fig. 6f); Aedeagus large, corpus large, smooth, rounded (Figs. 7a,7b,7c), juxta with plough-shaped of antero-lateral membranous anteriorly (Figs. 7a,7c), stylus curved inwards and serrated (Figs. 7c,7d).

Material examined. THAILAND: 1 male, Ubon Ratchathani, Muang, school cafeteria, 15°16'29.668°N 104°49'27.471°E, 127 m, 12.iii.2011, T. Chaiwong (CMPH); 1 male, Ubon Ratchathani, Muang, school cafeteria, 15°16'29.668°N 104°49'27.471°E, 127 m, 22.v.2011, T. Chaiwong (CMPH); 1 male, Ubon Ratchathani, Muang, restaurant, 15°16'29.668°N 104°49'27.471°E, 132 m, 22.v.2011, T. Chaiwong (CMPH); 1male,Ubon Ratchathani, Muang, restaurant, 15°16'29.668°N 104°49'27.471°E, 132 m, 12.vi.2011, T. Chaiwong (CMPH); 1 male, Ubon Ratchathani, Warinchamrap, school cafeteria, 15°11'33.212°N 104°50'6.756°E, 126 m, 12.vi.2011, T. Chaiwong (CMPH).

Female. Unknown

Biology. Unknown except that males were collected from one-day beef offal (in this study) or two-day-old spoiled beef (Tan *et al*., 2010).

Distribution. India, Nepal, Sri Lanka (Pape, 1996), peninsular Malaysia (Tan et al. 2010) and Thailand (Ubon Ratchathani)

Key to the Thai species *Miltogramma* (Kurahashi and Chaiwong, 2013, revised to include newly recorded species)

1. AS3 largely orange yellow, especially on inner surface ..... *Mi. angustifrons* (Townsend)
- AS3 largely or entirely fuscous to black 2
2. Basicosta black or fuscous black, sometimes reddish-brown; male tarsomeres 2-4 of fore leg without long hairs at dorsal surface ..... *Mi. iberica* Villeneuve
- Basicosta yellow; male tarsomeres 2-4 of foreleg with long hairs at dorsal surface ..... *Mi. tibita* Chao & Zhang

Key to the Thai species *Myorhina* (Kurahashi and Chaiwong, 2013, revised to include newly recorded species)

1. Cell R5 closed at wing margin; fore tibia with 2 bristles on posterior surface (1 *p* and 1 *pv*) ..... *My. otiophalla* (Fan & Chen)
- Cell R5 open; fore tibia with 1 *p* 2
2. Costal section 5 (CS5) setulose along anterior margin on less than basal 1/2; hind tibia with 1 *av*, with fringe of several fine long hairs medially on antero- and postero-ventral surfaces ..... *My. situliformis* (Zhong, Wu & Fan)