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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.

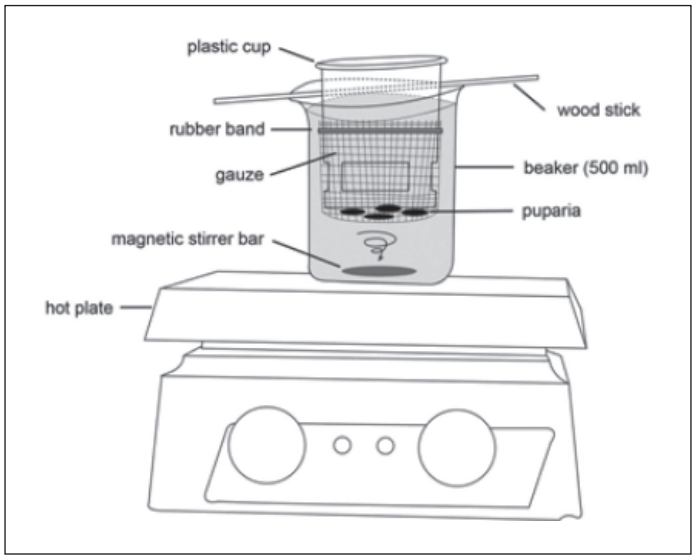

Figure 1. Illustration of apparatus for cleaning puparia (image not to scale)

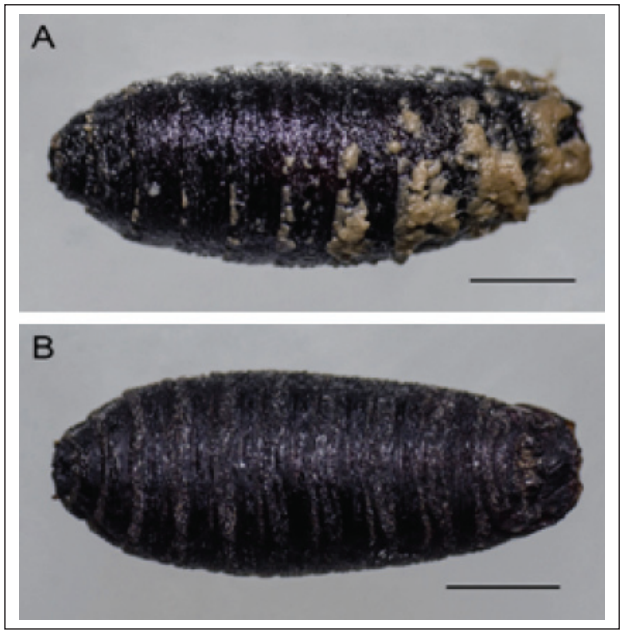

Figure 2. Puparia of *L. pattoni* before (A) and after (B) cleaning process. Bar = 2 mm

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)

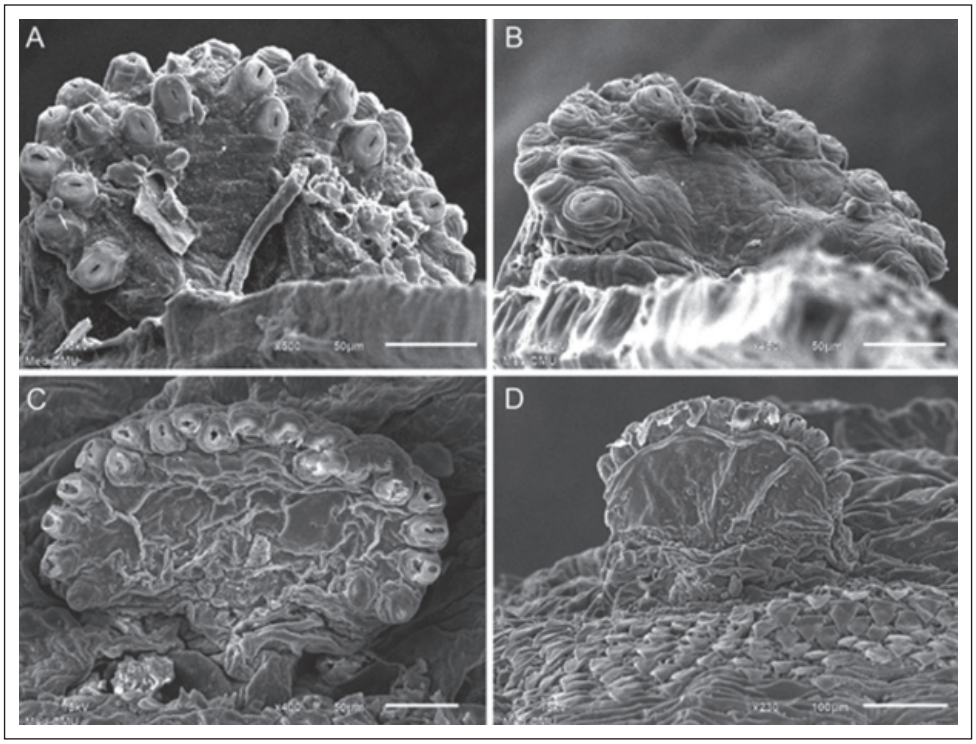

Figure 3. SEM micrographs of anterior spiracles of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*

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

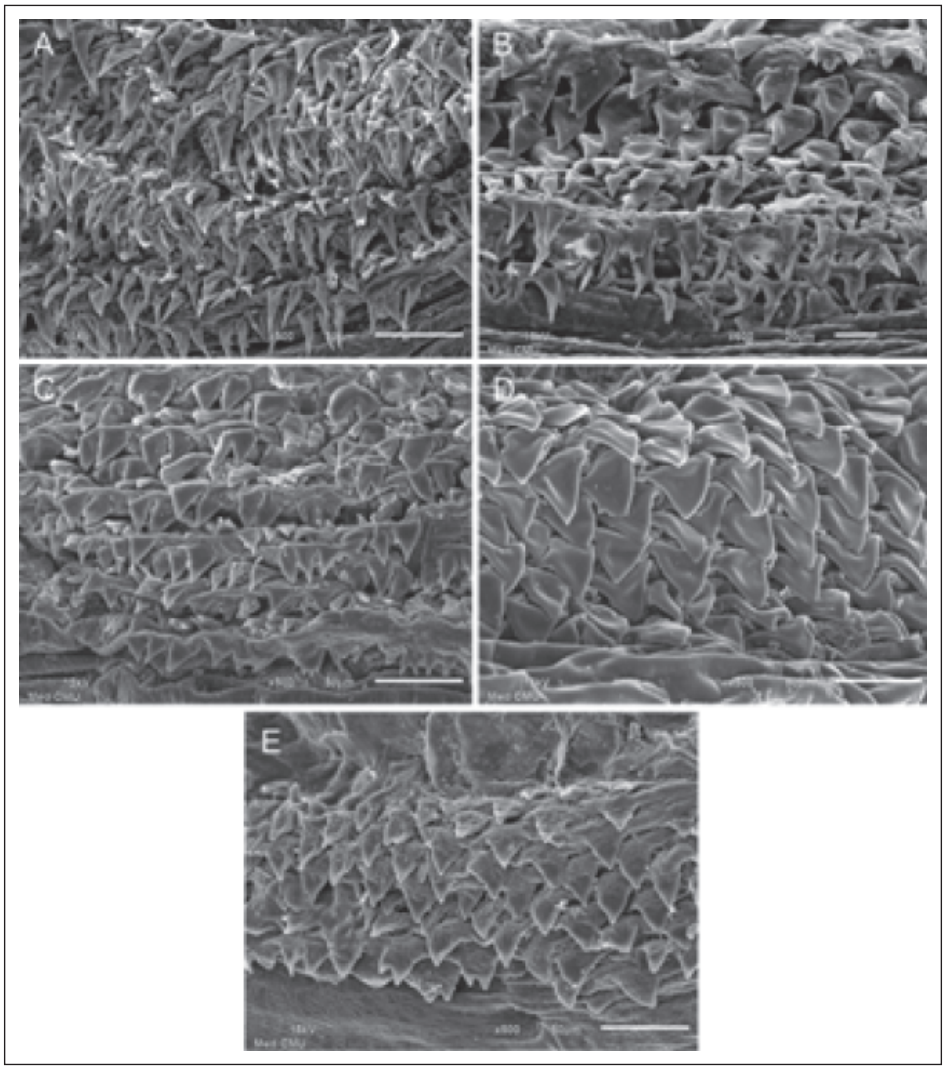

Figure 4. SEM micrographs of intersegmental spines between the prothorax and mesothorax of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*. **E:** *P. dux*

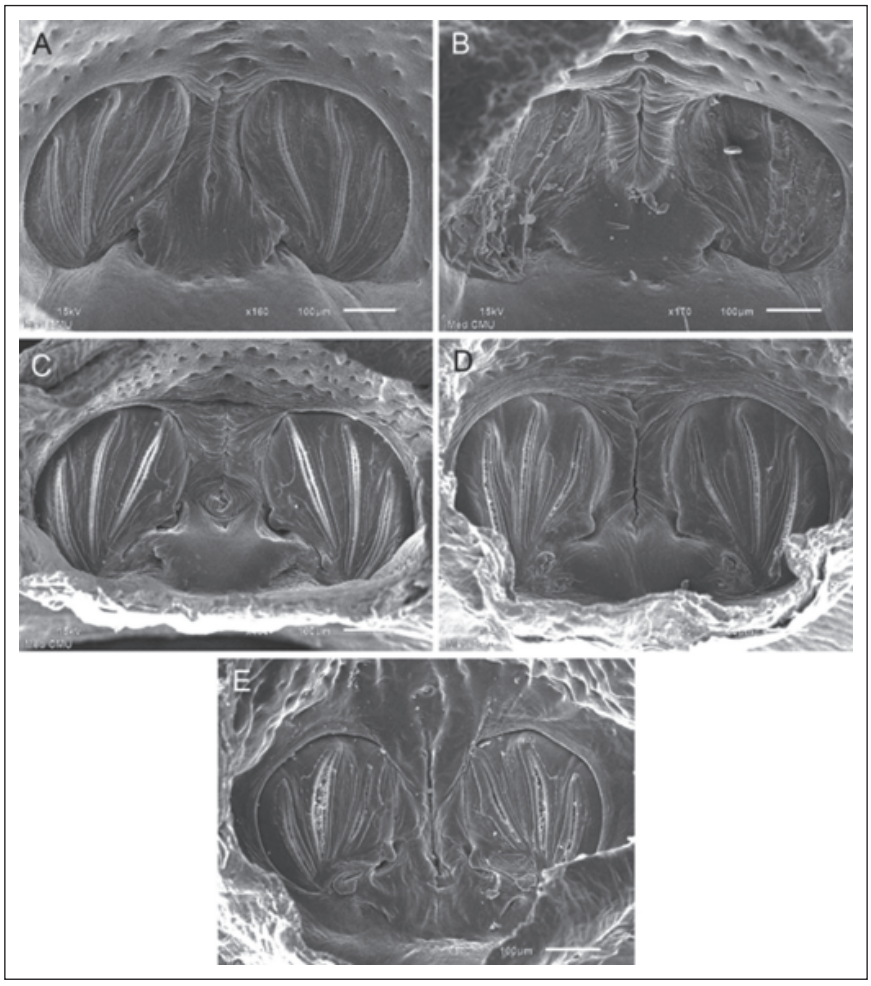

Figure 5. SEM micrographs of posterior spiracles of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*. **E:** *P. dux*

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

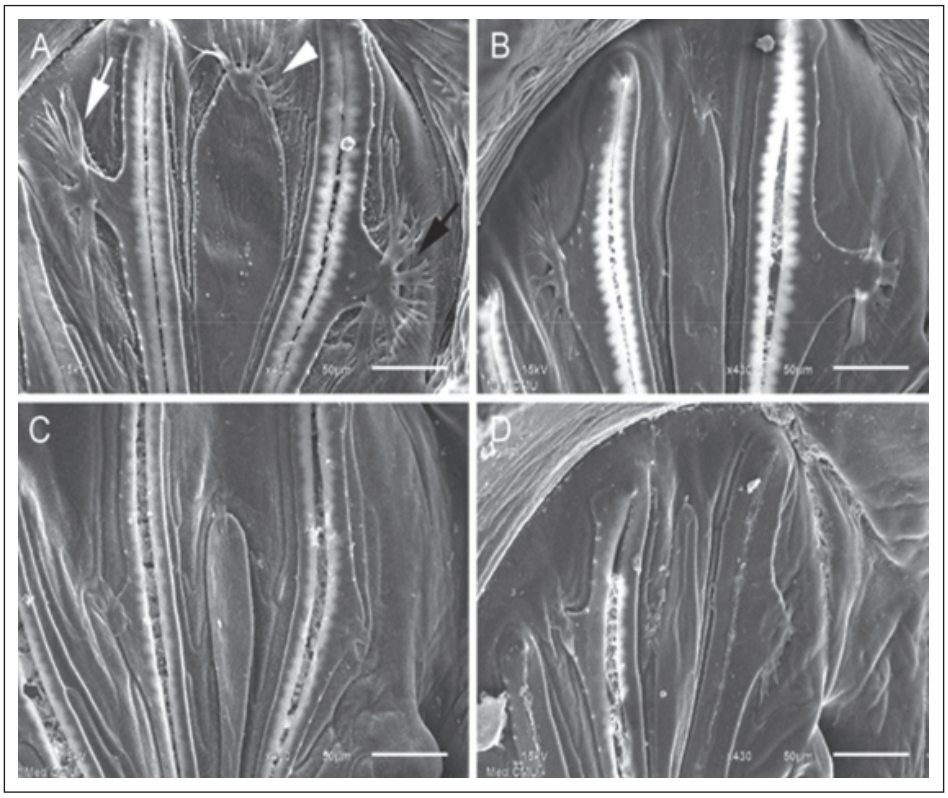

Figure 6. SEM micrographs of spiracular tufts on left spiracle of flesh fly puparia. **A:** *B. nathani* showing extensively branches, at the area adjacent to the inner spiracular slit (black arrow), at the top of the plate between inner and middle spiracular slits (white arrowhead) and adjacent to the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

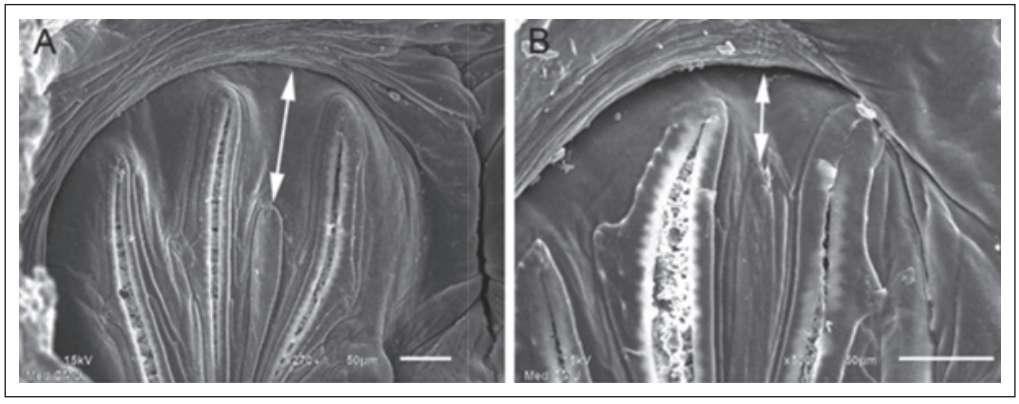

Figure 7. SEM micrographs of end up position of plate between inner and middle spiracular slits of posterior spiracle of flesh fly puparia. *L. ruficornis* showing much lower (long distance from peritreme) (A) than *P. dux* (short distance from peritreme) (B)

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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