สัญญาเลขที่ RSA5580010

รายงานวิจัยฉบับสมบูรณ์

โครงการ ชีววิทยาของแมลงวันหัวเขียว *Achoetandrus rufifacies*: การประยุกต์ใช้ในนิติเวชกีฏวิทยาและการควบคุม

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มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย,
มหาวิทยาลัยเชียงใหม่ และคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

กิตติกรรมประกาศ

ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย, คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่และมหาวิทยาลัยเชียงใหม่ ซึ่งเป็นผู้สนับสนุนการวิจัยในโครงการนี้ ขอขอบคุณ รองศาสตราจารย์ นายแพทย์นิเวศน์ นันทจิต อธิการบดีมหาวิทยาลัยเชียงใหม่, รองศาสตราจารย์ นายแพทย์วัฒนา นาวาเจริญ คณบดีคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่, รองศาสตราจารย์ ดร. ปรัชญา สมบูรณ์ หัวหน้าภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่อนุญาตและสนับสนุนการทำวิจัยครั้งนี้

ขอขอบคุณ ศาสตราจารย์ ดร. นายแพทย์ คม สุคนธสรรพ์ ที่เป็นผู้สนับสนุนการวิจัย ทำให้งานวิจัยครั้งนี้สำเร็จลุล่วงไปด้วยดี และคณะผู้ร่วมงานวิจัย Dr. Hiromu Kurahashi (Department of Medical Entomology, National Institute of Infectious Diseases, Japan), Dr. Jeffery Tomberlin (Department of Entomology, Texas A&M University, USA), คุณธันวดี คล่องแคล่ว, คุณนารินทร์ สนธิกันย์, คุณสงบ สนิท, คุณขวัญกมล ลิ้มโสภาธรรม, คุณจุฑารัตน์ เสมอใจ, คุณสุดธิดา สุวรรณยศ และเจ้าหน้าที่ทุกท่านของภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ

รหัสโครงการ RSA5580010

ชื่อโครงการ ชีววิทยาของแมลงวันหัวเขียว *Achoetandrus rufifacies*: การประยุกต์ใช้ในนิติเวชกีฏวิทยาและการควบคุม

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ระยะเวลาโครงการ 3 ปี (16 กรกฎาคม 2555 ถึง 16 กรกฎาคม 2558)

เนื้อหางานวิจัย

แมลงวันหัวเขียว *Achoetandrus rufifacies* เป็นแมลงวันที่มีความสำคัญทางการแพทย์ และพบมากในประเทศไทย ตัวเต็มวัยเป็นพาหะนำเชื้อโรคต่างๆมาสู่มนุษย์ ส่วนตัวอ่อนก่อให้เกิดโรคหนอนแมลงวันทั้งในมนุษย์และสัตว์ การหาวิธีควบคุมจำนวนประชากรแมลงวันจึงนับว่าเป็นสิ่งที่จำเป็น ซึ่งต้องอาศัยองค์ความรู้พื้นฐานทางชีววิทยาในด้านต่างๆของแมลงวันหัวเขียวชนิดนี้ การศึกษาครั้งนี้มีจุดประสงค์เพื่อ 1) ศึกษาการกระจายตัวเชิงพื้นที่ของแมลงวันหัวเขียวชนิดนี้ และหาความสัมพันธ์ระหว่างการแพร่กระจายตัวกับปัจจัยทางกายภาพ (เช่นอุณหภูมิ ความชื้น) ในเขตเมืองเชียงใหม่และศึกษาช่วงการบินในแต่ละฤดูกาลและในช่วงเวลาของวัน ตั้งแต่เดือนกรกฎาคม 2556-มิถุนายน 2557 โดยจับแมลงวัน 2 ครั้งต่อเดือน จับแมลงวันตัวเต็มวัยโดยใช้กรงดักแมลงวันอัตโนมัติ ณ ศูนย์วิจัยสาธิตและฝึกอบรมการเกษตรแม่เหียะ จังหวัดเชียงใหม่ โดยตั้งกรงในจุดศึกษา 3 จุด คือ สวนปาล์ม, พื้นที่ป่า, สวนลำไย ใช้เครื่องในวัวเน่า 1 วัน (300 กรัม) เป็นเหยื่อล่อ พบว่าจับแมลงวันชนิดนี้ได้ 55,988 ตัว พบมากที่สุดในป่า (53%) รองลงมาคือสวนปาล์ม (27%) และสวนลำไย (20%) จำนวนแมลงวันที่จับได้มีความสัมพันธ์เชิงบวกกับอุณหภูมิ แต่มีความสัมพันธ์เชิงลบกับความชื้น พบแมลงวันจำนวนมากในฤดูร้อนรองลงมาคือฤดูฝนและฤดูหนาว ช่วงที่จับแมลงวันได้มากที่สุดคือ 15.00 - 18.00 น. ส่วนการศึกษาชีววิทยาด้านการสืบพันธุ์ของแมลงวันชนิดนี้ในห้องปฏิบัติการ ทำการผ่าแมลงวันเพศเมียเพื่อศึกษาการเจริญของเซลล์ไข่ ตั้งแต่อายุ 1 วันถึง 9 วัน

จากที่ได้เสนอไว้ในโครงร่างทุนว่า หากมีตัวอย่างแมลงวันที่จับได้จากภาคสนามจากจุดประสงค์ที่ 1 จะทำการศึกษาชีววิทยาของแมลงวันดังกล่าวด้วย จึงได้ศึกษาช่วงการบินในแต่ละฤดูกาลและในช่วงเวลา ของแมลงวันหัวเขียว *Chrysomya megacephala* ในช่วงเวลาเดียวกับที่ศึกษาใน *A. rufifacies* พบแมลงวันจำนวนมากในฤดูร้อนรองลงมาคือฤดูฝนและฤดูหนาว ช่วงที่จับแมลงวันได้มากที่สุดคือ 15.00 - 18.00 น. ส่วนการศึกษาชีววิทยาด้านการสืบพันธุ์ของแมลงวันชนิดนี้ในห้องปฏิบัติการ พบว่าตัวเต็มวัยเพศเมียสามารถออกไข่ได้ถึง 7 ครั้งในช่วงชีวิต นอกจากนี้ยังจำแนกชนิดของแมลงวันหัวเขียวชนิดต่างๆที่มีความสำคัญทางการแพทย์ โดยวิธีอณูชีววิทยา โดยใช้ COI gene ผลจากการศึกษานี้ นับเป็นข้อมูลพื้นฐานสำคัญที่จะนำไปสู่การพัฒนาวิธีการควบคุมประชากรแมลงวันหัวเขียวต่อไปในอนาคต

คำหลัก แมลงวันหัวเขียว *Achoetandrus rufifacies*, *Chrysomya megacephala*, การควบคุม, การกระจายตัว, ชีววิทยา

Abstract

Project Code: RSA5580010

Project Title: Biology of the blow fly, *Achoetandrus rufifacies* (Macquart): Application in forensic entomology and control

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Project Period: 3 years (16 July 2013-16 July 2015)

Content

Achoetandrus rufifacies (Macquart) (Diptera: Calliphoridae) is the important blow fly species of medical concern in Thailand. The adults are mechanical vectors of various pathogens, while the larvae can cause myiasis. Regarding this, a method to control fly population is mandatory, with all aspects of the basic research of this fly species being needed. In this study, we investigated the response of this fly species to climatic and physio-environmental factors in Chiang Mai, northern Thailand. Adult fly surveys were carried out every two weeks from July 2013- June 2014 at study sites in Mae Hia Agricultural Research, Demonstrative and Training Center using automatic baited trap with 1-day tainted beef offal as bait. A total 55,988 adult *A. rufifacies* were captured, with peak densities being observed in summer, followed by rainy season and winter. Population density had positive correlation with temperature (r = 0.461), but negative correlation with relative humidity (r = 0.621). The daily activity showed major peak during 15.00 – 18.00 pm. Changes in the ovariole during egg development of this species were conducted from day 1 to day 9.

As has been proposed in the grant proposal, the extra researches were conducted for biological information of other medically important flies, if species were available from the field collections. In this regard, seasonal and daily activity of blow fly, *Chrysomya megacephala* were conducted, of which the experimental design was the same as that has been described in *A. rufifacies*. Peak population was found in summer, followed by rainy season and winter. The daily activity showed major peak during 15.00 – 18.00 pm. Biological investigation in the laboratory revealed that females of this species can oviposit upto 7 times in her lifetime. Moreover, molecular identification of blow flies of medical importance in Thailand was performed using COI gene. Information gained from this thorough study in both alimentary and reproductive systems established a basic database, which was useful in understanding their functional role and serving as knowledge to address a strategy to control this fly species in the future.

Keywords: *Achoetandrus rufifacies*, *Chrysomya megacephala*, control, distribution, biology

ชีววิทยาของแมลงวันหัวเขียว *Achoetandrus rufifacies*: การประยุกต์ใช้ในนิติเวชกีฏวิทยาและการควบคุม

PROBLEMS AND RESEARCH RATIONALE

The hairy maggot blow fly, *Achoetandrus rufifacies* (Macquart) is a medically important species worldwide. From a medical point of view, it has a positive aspect by acting as a forensically important species. Based on reports from many countries, e.g., Hawaii of the U.S.A., Malaysia and Thailand, the larvae of this fly have been collected from many human corpses, and thus used as entomological evidence in forensic investigations, particularly in estimating postmortem interval (PMI). In northern Thailand, this is one of the two most common fly larva species collected in corpses, along with *Chrysomya megacephala,* the Oriental latrine blow fly. Conversely, in the negative sense, the adult of this species is not only a mechanical carrier of various pathogens to humans (e.g., bacteria, virus and parasites), but its larva also carries the myiasis-producing agent as previously reported (Baumgartner 1993).

Ecologically, adult surveys in northern Thailand revealed that *A. rufifacies* presents in varied habitats, e.g., urban, suburban and forested areas, at altitudes from 25 to 1,000 metres above sea level. Flies are also collected as urban land use types, suggesting that their food source is associated with the human environment. In Thailand, this fly was recorded as the second most collected blow fly species in the fly survey while *C. megacephala* was the most common species (Sukontason et al. 2008). Although the biology, distribution and control of *C. megacephala* have been widely studied, but information for *A. rufifacies* is limited. Sukontason et al (2008) reported the morphology of the immature stage (egg, larva and puparium) and developmental rate of *A. rufifacies* larvae; while Byrd and Butler (1997) found that environmental temperature was as an important environmental abiotic factor for larval growth and development. In *C. megacephala*, other abiotic factors, such as relative humidity, solar radiation, wind velocity and rainfall, had influence on adult fly distribution and activities. However, the influence of these abiotic factors on the distribution of *A. rufifacies* has not been clarified. Therefore, fly distribution and the factors that affect fly population should be clarified in *A. rufifacies*. Hence, the first objective of this study was to determine the relationship between climatic factors (temperature, relative humidity, light intensity) and physical factors (land use types) that influence the distribution of *A. rufifacies* in central Chiang Mai province, northern Thailand. Using the field survey and Geographic Information System (GIS) methodology, the spatial and temporal distribution of *A. rufifacies* in urban and suburban areas of Chiang Mai province will be clarified. Association between population density of *A. rufifacies* and climatic factors was evaluated.

Information of fly behavior, especially activity patterns in nature, is important in each insect species for use in controlling its population. Daily and seasonal activity patterns have been investigated in many insects, but not for *A. rufifacies*. Therefore, the second objective of this study was to determine the daily and seasonal activity patterns of this fly under natural conditions in Chiang Mai using electronic timer control fly entrance traps. The last objective was to investigate the biology and behavior of *A. rufifacies* in the laboratory, which includes reproductive system of adult males and females, developmental rate of the egg, larva and pupa, etc. Data gaining from these experiments

were beneficial in understanding the biology of *A. rufifacies* in both laboratory and natural conditions. The inclusive knowledge of spatial and seasonal trends, activity patterns and biological laboratory data provide information that will be useful in applying where and when to implement effective control programs and for forensic entomology purpose.

OBJECTIVES

1. To determine the relationship between climatic factors (temperature, relative humidity, light intensity) and physical factors (land use types) that influent the distribution of *A. rufifacies* in central Chiang Mai province, northern Thailand.

2. To determine the daily and seasonal activity patterns of this fly under natural conditions in Chiang Mai using electronic timer control fly entrance traps.

3. To investigate the biology and behavior of *A. rufifacies* in the laboratory.

METHOD

Part I: Spatial and temporal distribution of *A. rufifacies* in urban and suburban areas of Chiang Mai province

Part II: Determination of the daily and seasonal activity patterns of *A. rufifacies* in Chiang Mai province

Experimental design (Parts I, II)

Study site: This study was conducted at the Mae Hia Agricultural Research, Demonstrative and Training Center, which is located on Canal road in Mueang Chiang Mai district. This training center is located in the area as wide as 2,068,800 sq.m. (1,293 Rai) which used to be a part of Doi Suthep-Pui National Park, then later Royal Forest Department gave permission to Chiang Mai University for use this area as a study and research center (http://web.agri.cmu.ac.th /maehia/index.php/background/information). The general description of this area, it is consisted of foothill slope, hill, and lowland. Furthermore, nine of water reservoirs were found within this area. It is surrounded by natural scenery. The elevation of this area is around 300 meter above sea level. Slope surface is about 0-15% of total area aligned from North to South.

Three different microenvironments were chosen as study plots included forested area, palm garden, and longan orchard.

- Forested area (N 18°46’01.08”, E 98°56’08.3”) is located at the altitude of 344 m above sea level. Fly traps were set at about 150 m far from the road at the foothill of mixed deciduous forest. This area was covered with Teak (*Tectona grandis*) and bushes. The study site was located close to rubber plantation. The grass-fed cows were also found in the pasture land located on the other side of the road opposite to the study site.

• Palm garden (N 18°45’27.841”, E 98°55’48.515”) is located at the altitude of 330 m above sea level. It is consisted with Tenera plam tree (*Elaeis guineensis* Jacq**.)** which is the oil palm with small nut and thin shell. The flower is produced in a dense cluster. The palm fruit is reddish and grow in large bunches. As the palm tree leaves are large, compound and arranged cluster at the top of the tree. When palm tree grow up, the leaves form in the dense cluster until the canopy closes. Palm garden is supplied with a large amount of water. It placed about 120 m far from the main research station road and opposite to the storage building.

• Longan orchard (N 18° 45’56.66”, E 98°55’40.13”) is located at the altitude of 347 m above sea level. The dominant species in this orchard is *Dimocarpus longan* Lour. Longan trees were planted in row. The flowering season of Longan in Thailand is during late December to late February and the harvesting season starts at late June to late August. The Longan orchard is 220 m far from the main road and placed at the foothill of the mountain.

Adult fly collection: By using automatic baited trap, invented by Kom Sukontason, fly trapping was performed every 2 weeks for 1 year (July 2013-June 2014). The traps consist of four basic parts: (1) an external metal case (40 x 40 x 60 cm), (2) a fly entrance module, which is made of transparent plastic board and controlled by an electronic timer mechanism, (3) a black fly net, and (4) an attractive bait (~300 g of tainted beef offal) put in a plastic container and placed at the base of the trap. To prevent the scavengers and rain, the automatic trap was placed inside a wire cage that half-length covered with transparent plastic sheet.

The operation of the automatic trap is controlled by timer which was adapted from the fish feeder that originated to feed fish at regular intervals. The timer was set to open and closed at the specific period of time. The timer was connected with electric relay, which is an electrically operated switch that used to control a circuit by a low-power signal. At the beginning of trap operation, timer rotated the food hopper axle at the setting intervals and triggered the relay. Then, electric power, from 12 voltage battery, was released to supply the CD-player tray and let it slided from the slot loading drive. As the tray was connected with fly entrance module by fishing net, therefore, the movement of fly entrance module was depending on the movement of the tray. When, the tray slide out of the slot pulled the fishing net, then fly entrance module was lifted up and let the flies to enter into the trap. By using one-day tainted beef offal (300 g) as bait, adult flies were lured to the attractive bait. As flies are phototaxis insects so when they enter inside the fly net, they generally fly upward to the light at the top of the trap and hold back within the fly capture net. When time reached, timer triggered the relay again and let power supplied CD-player tray. Then, the tray was slide back into the loading slot. The fishing net was released, let the entrance module slided down and closed fly entrance. Therefore, all captured flies were retained inside the fly net.

The adult fly collection was performed twice a month for data collection throughout the year. The traps were set to operate at the time intervals as showed in Table 1.

Table 1. The automatic trap operation time

	Open	Closed	Duration (h)
DAY TRAP	6.00	9.00	3
	9.00	12.00	3
	12.00	15.00	3
	15.00	18.00	3
NIGHT TRAP	18.00	6.00	12

Traps were emptied at 3 hours for day trap and 12 hours for night trap by removed fly net from the trap and installed the new one for the next loop of experiments. During the experimental time, hourly temperature ($^{\circ}$C) and relative humidity (%) were recorded using Temperature and Humidity loggers (Ebro EBI 20-TH1; ebro Electronic GmbH & Co. KG, Germany).

Fly identification: The collected flies were transported to the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. They were sacrificed by placing a fly net in a refrigerator set at 4°C for 2 hours. Then, all flies were identified individually into species under dissecting microscope using the taxonomic keys (Tumrasvin et al. 1979), sexed and counted.

RESULTS

A total of 55,988 *A. rififacies* was collected during July 2013 – June 2014. Among three microhabitats, the largest number of *A. rufifacies* was found in forested area (53%) followed by palm garden (27%) and longan orchard (20%) (Fig. 1).

In northern Thailand, three seasons are defined as summer, rainy season, and winter. Summer is the time during mid-February to the beginning of May, rainy season occurs from mid-May to the beginning of October, and winter starts from mid-October to the beginning of February. During July 2013 – June 2014: in summer, the weather was hot and dry. The temperature was high and relative humidity was low. Furthermore, the lowest relative humidity (41.14%) was recorded in summer (mid-March 2014). The rainy season was dominated by the southwest monsoon, during that time; rainfall was at its heaviest. Therefore, highest relative humidity (85.99 %) was recorded in this season (mid-June 2014) but temperature was lower when comparing with summer time. Interestingly, relative humidity was constant at the beginning of winter, and then it started to decrease in mid-December 2013 and continued drop through winter to the beginning of summer. From this study, temperature in winter was not low and the highest average temperature (34.87°C) was record in this season also (mid-October 2013). In contrast, the lowest average temperature (23.31°C) was recorded in rainy season (the beginning of September 2013) (Fig. 2). Pearson's correlation coefficient showed a significant relationship between the number of *A. rufifacies*, temperature (P =0.000) and relative

humidity. As the number of *A. rufifacies* was positively correlated with temperature(r=0.461), while negative correlation was found between the number of fly and relative humidity (r =0.621).

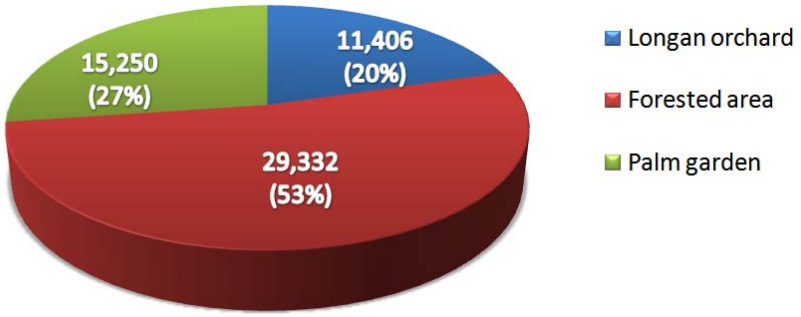

Figure 1. Total number of *A. rufifacies* captured from each microhabitat during July 2013 – June 2014.

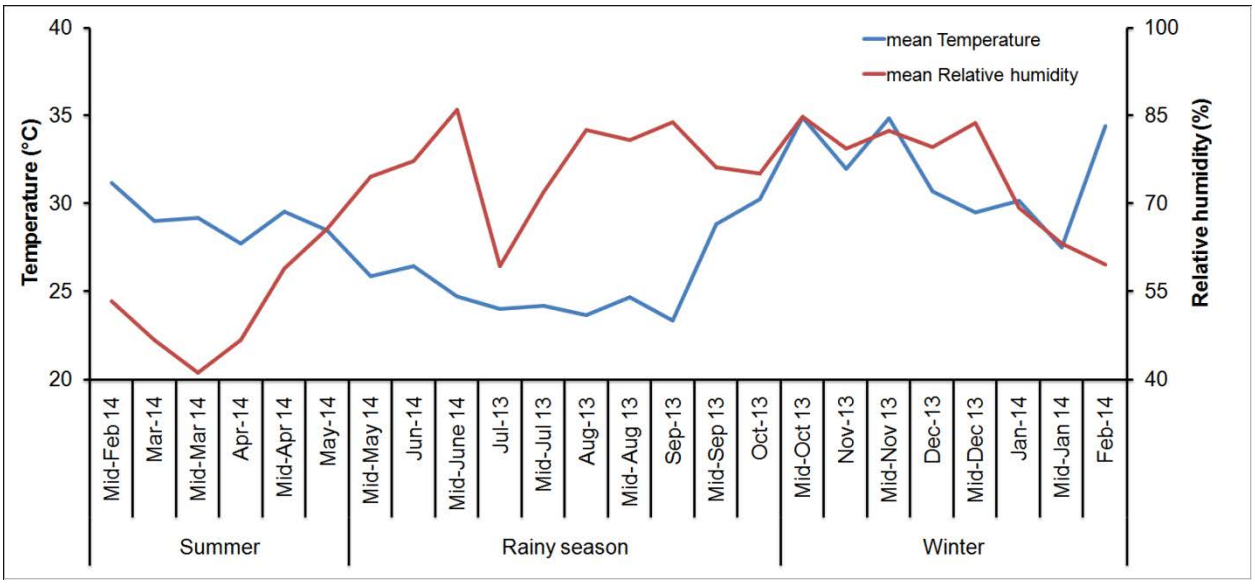

Figure 2. The fluctuation of average temperature (°C) and average relative humidity (%) recorded during July 2013 – June 2014.

According to this study, *A. rufifacies* was established throughout the year. High number of *A. rufifacies* was collected in summer (63.50% of collected *A. rufifacies*) and then the population was decreased during rainy season (25.71%) through the whole winter (10.79%). Peak population (8,669) was recorded in summer (the beginning of April 2014) and the lowest population density (132) was found in winter (the beginning of January 2014). Although high number of *A. rufifacies* was collected mainly in forested area, seasonal distribution patterns among those three microhabitats (forested area, palm garden, and longan orchard) were not different (Fig. 3).

High number of female (43,834 flies) was captured more than male. About eighty percents of them (35,247 flies) were non-gravid female. The highest ratio of male flies per one female (0.48) was

recorded at the end of summer (during mid-April 2014) suggested that high number of flies was emerged at that time, however the fluctuation of sex ratio did not show a clear pattern (Fig. 4). The average ratio between male per female was 0.26:1.

The daily activity of *A. rufifacies*, the major peak population was collected during 15.00 – 18.00, however; there was no significant different between the number of fly captured during 9.00-12.00, 12.00-15.00, and 15.00-18.00 ($P \geq 0.05$; One-way ANOVA, Dunnett's T3 post Hoc test). In summer, high number of *A. rufifacies* was capture during 15.00 -18.00. Interestingly, in rainy season, *A. rufifacies* showed peak population at 12.00-15.00 and similar pattern was also observed in winter (Fig. 5). Only small number of fly was captured during night time (18.00 -6.00). In summer and rainy season, the day length was longer than in winter, it was longer than 12 hours. High number of *A. rufifacies* was collected by the night time trap. On the contrary, when the day length was short (less than 12 hours) as in winter, only a few number of fly was collected at the night time (Fig. 6).

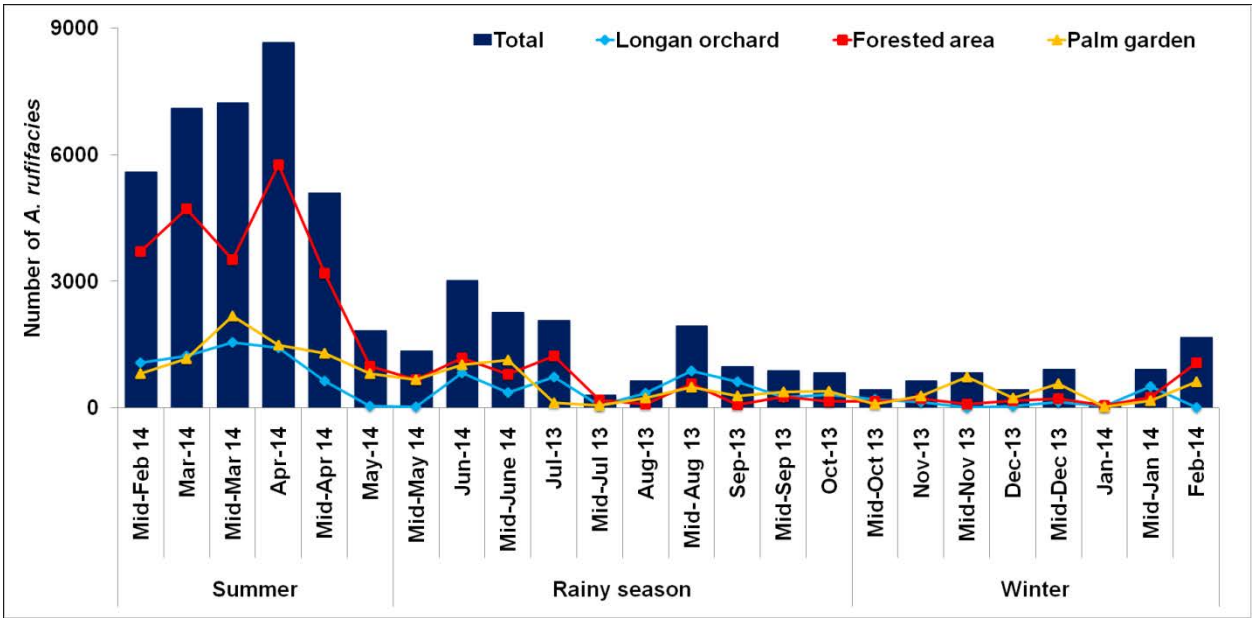

Figure 3. Seasonal fluctuation of population density of *A. rufifacies* collected during July 2013 – June 2014.

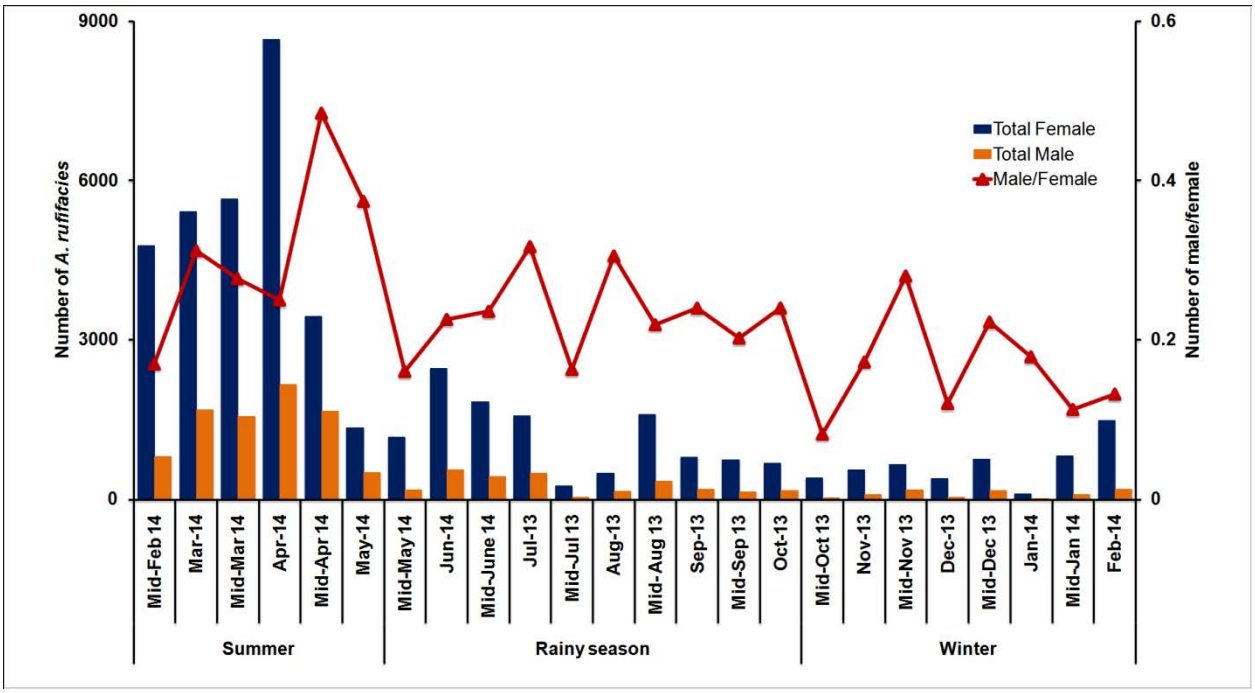

Figure 4. Sex ratio of *A. rufifacies* capture by the automatic trap during Jyly 2013 – June 2014.

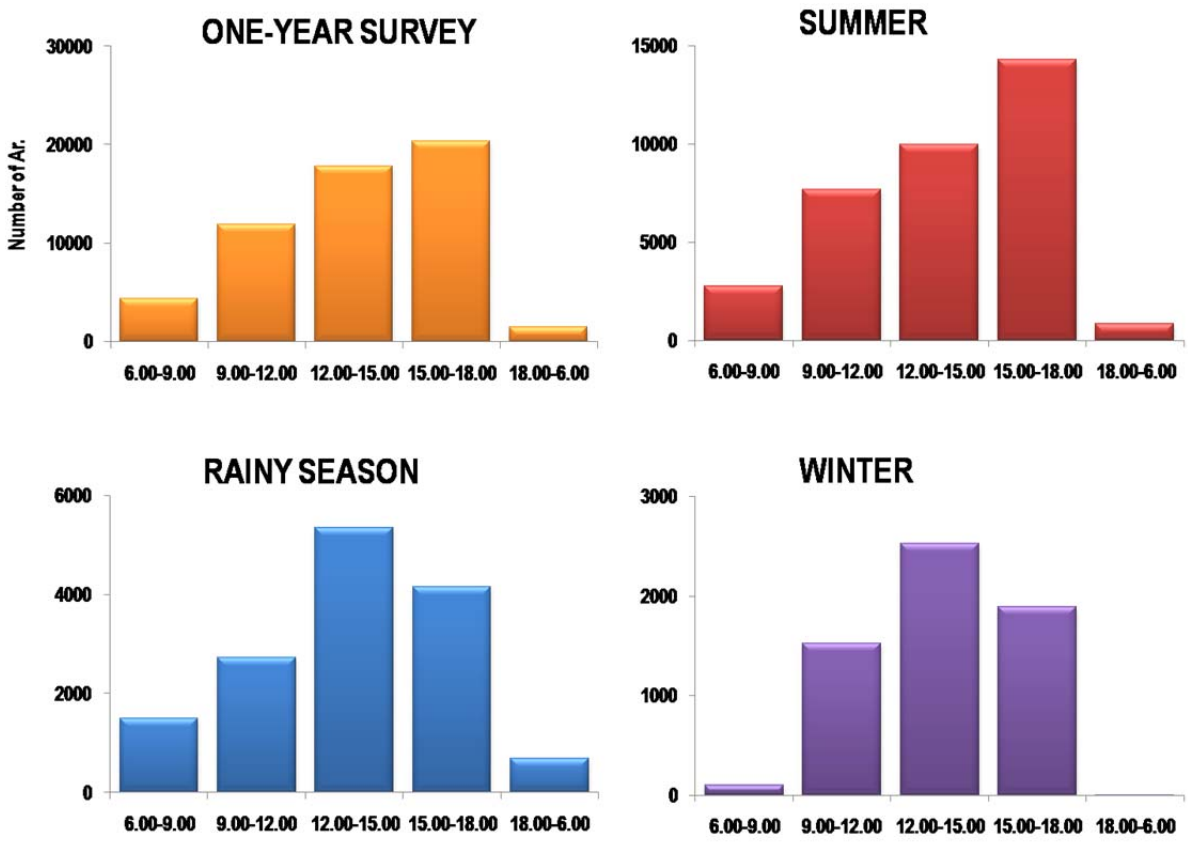

Figure 5. Daily activity pattern of *A. rufifacies* collected during July 2013 – June 2014)

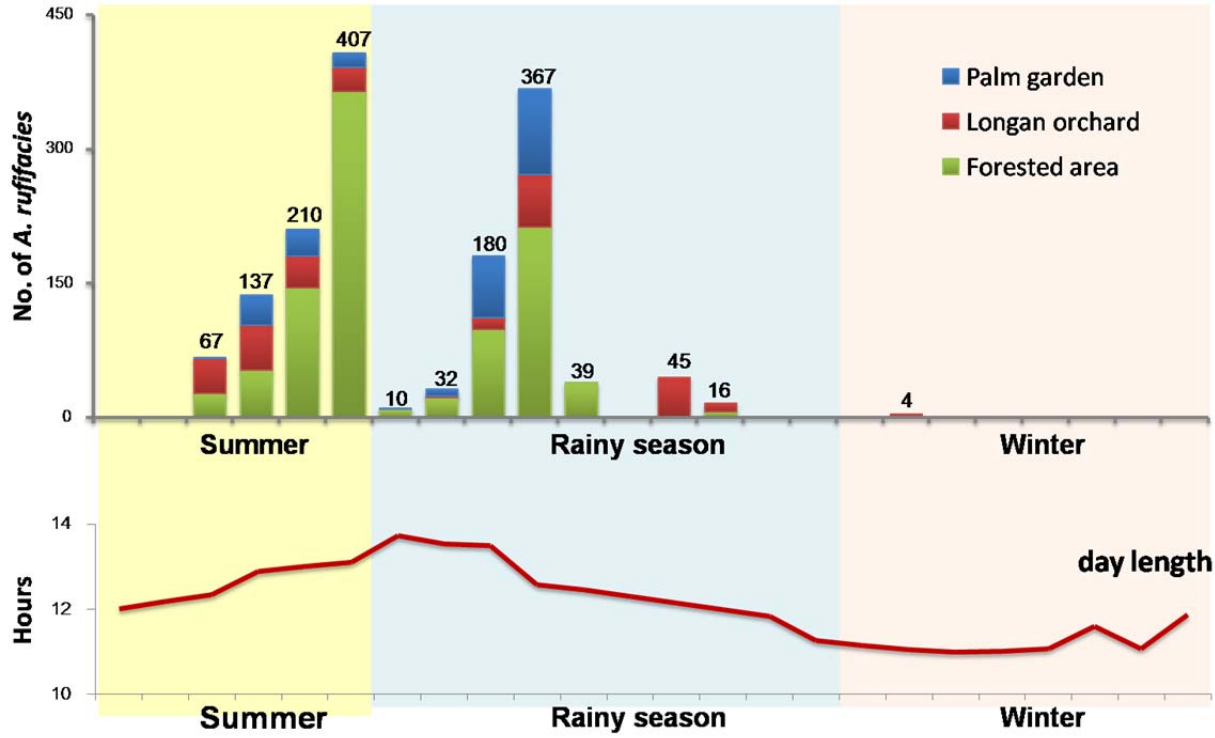

Figure 6. The number of *A. rufifacies* captured during the night time (6.00 p.m. – 6.00 a.m.) (above) and day length (below)

Part III: Determination of the biology of *A. rufifacies* in the laboratory

Dissection of female genitalia

The female internal reproductive organs of *A. rufifacies* consist of 2 ovaries, 2 lateral oviducts, a common oviduct, 3 spermathecae, vagina and 2 accessory glands.

Changes in the ovariole during egg development:

The stages described here for *A. rufifacies* form the basis of an aging technique that could be used to differentiate the ages of field collected female blowflies. In this experiment the ovarioles of *A. rufifacies* were observed for changes from day 0 (just after emergence) until 9-days-old under ambient temperature conditions of 18-27 $^{\circ}$C during the study period. The observations of the ovarian stages were conducted using fresh preparations in which the ovarioles were placed on a slide and covered with a cover slip. They were then examined and measured under an ordinary light microscope. The ovarioles of *A. rufifacies* are of the polytrophic ovariole type**.**

Day 1: The ovariole has a piriform germarium, which will develop to be oocyte. The follicles are not yet well differentiated from the germarium.

Day 2: The ovariole is enlarged. The follicle is nearly spherical and distinct from the germarium. Stalk cell is clearly observed. The cystocytes are clearly visible and situated in the basal region of the follicle which is surrounded by the follicular cells.

Day 3: The ovariole expanded into the globular shape. Nurse cells are more distinct. Oocyte is more developed, and the development of the 2nd follicle from the germarium cells was observed.

Day 4: The follicles are now oval in shape with yolk (oocyte) occupying up to one-third to one-half of the total follicle length. The epithelium around the oocyte has become columnar. Separation of the 2nd follicle from the germarium cell was apparent.

Day 5: The follicles are more develop, and have greatly increased in length and width. The yolk (oocyte) now accounts >two-third of the total follicle. The 2nd follicle showed enlargement, compared with the germarium cells.

Day 6-9: The eggs are full size with the oocyte occupying the entire follicle and the nurse cells have disappeared. Initiation of the 3rd follicle was apparent.

Part IV: Conduct biological experiments of other medically important flies (if specimens are available from field collections, e.g., investigation morphology using scanning electron microscope, study biology, etc.)

This part has been proposed in the grant proposal. In this regard, the extra researches were conducted for biological information of other medically important flies. Our team investigated the seasonal and daily activity of blow fly, *Chrysomya megacephala*, of which the experimental design was the same as that has been described in *A. rufifacies*. Result of seasonal activity was demonstrated in Fig. 7, while the daily activity was shown in Fig. 8. More female *C. megacephala* were collected than male (Fig. 9). Dissection of the reproductive status of female *C. megacephala* from the field collected revealed more non-gravid status than those gravid (Fig. 10).

The biological investigation of *C. megacephala* was conducted in the laboratory, focusing on the longevitiy of adult flies. Moreover, molecular identification of blow flies of medical importance in Thailand was performed.

Seasonal and daily activity of *C. megacephala*

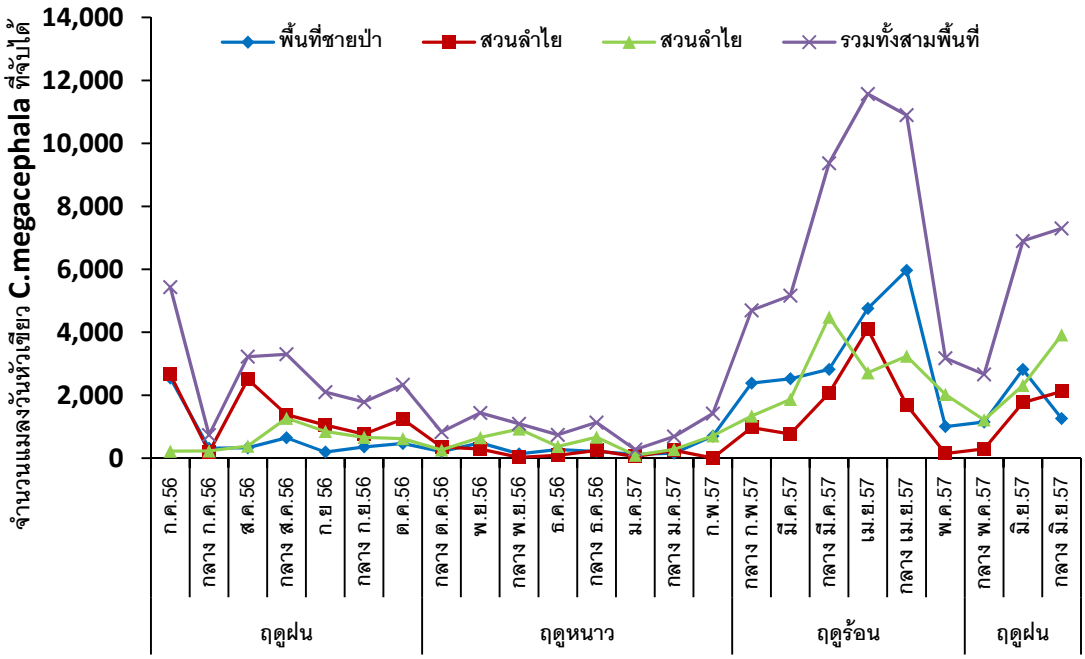

Figure 7. Seasonal activity of *C. megacephala* collected from 3 study areas. January was the month that collects the minimal number of this species, while the most collected flies was in April.

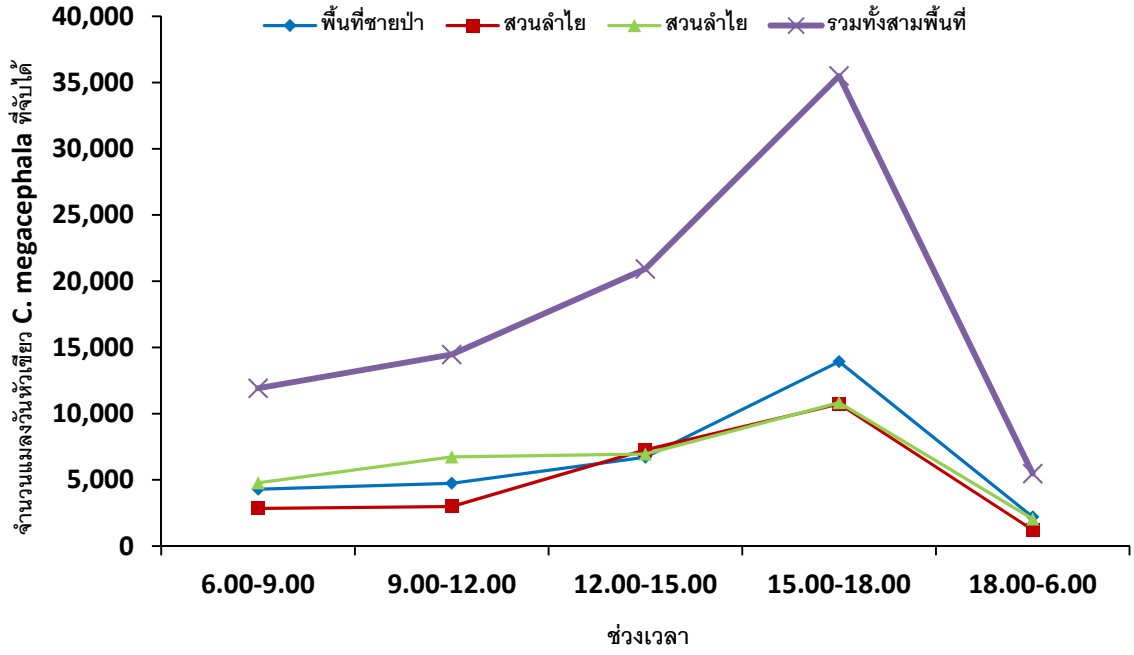

Figure 8. Dialy activity of *C. megacephala* in three study areas.

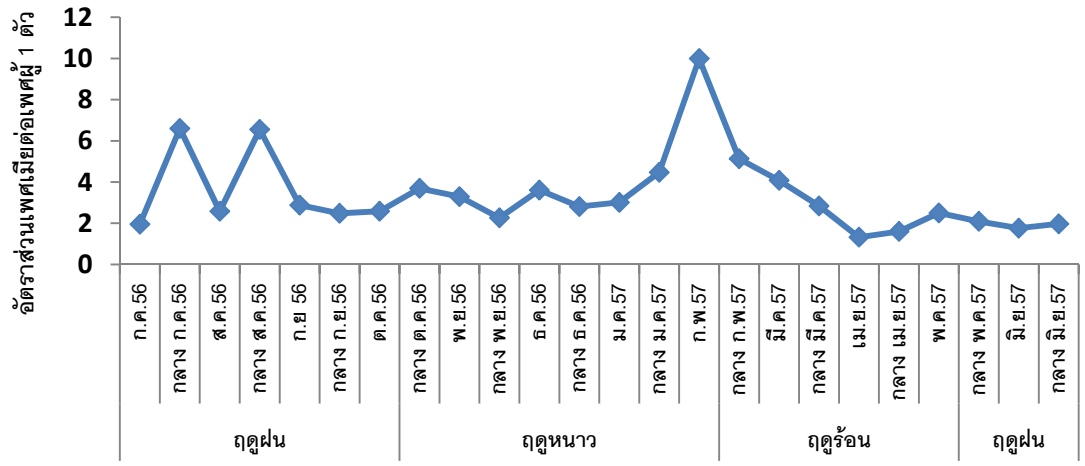

Figure 9. More female *C. megacephala* were collected than males during study period.

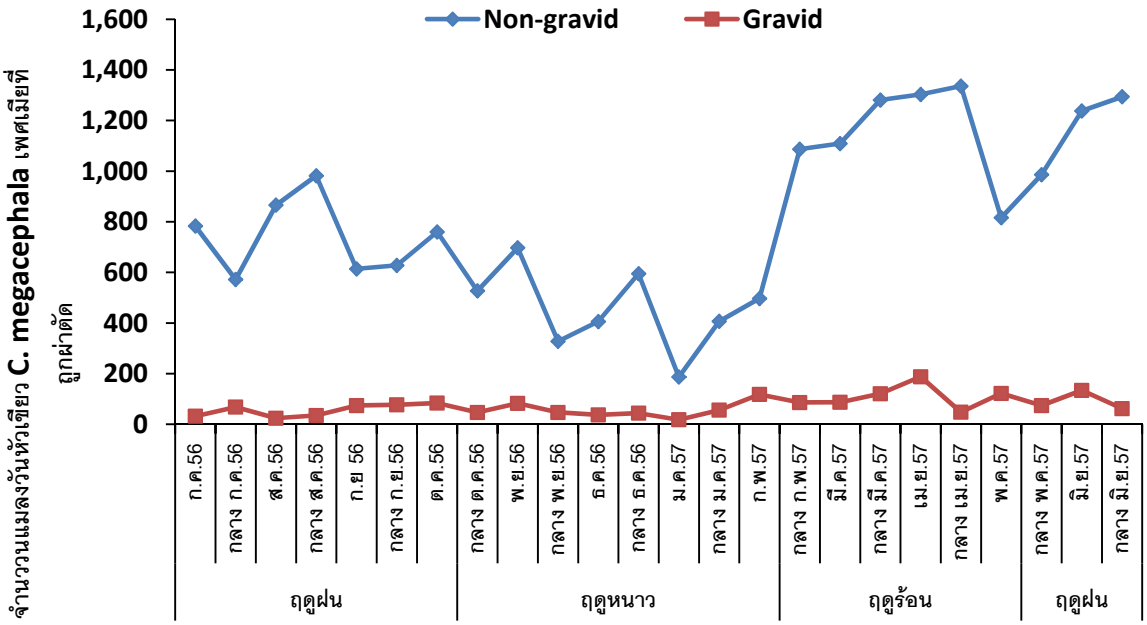

Figure 10. Reproductive status of female *C. megacephala* collected showing more non-gravid status than those gravid.

Longevity of adults *C. megacephala*

Adults were evaluated for their longevity in the laboratory condition. They were reared in a standard rearing cage and provided with a food source. One hundred males and one hundred females emerged from the same batch of egg were reared in the cage (30×30×30 cm) and fed with sugar and water. Fresh pork liver was provided as the protein source and oviposition site. Dead flies were counted daily. This experiment was replicated 3 times. Results indicated that in winter, males averaged 55.6 days in longevity, while 51.6 days in females.

Experiment of gonotrophic cycle in female *C. megacephala* was performed in the laboratory. After mating, gravid females were individually transferred into a small cage, and fed with sugar, water

and fresh pork liver. Once oviposit, the number of egg was counted. The number of oviposition was determined until death. The fresh pork liver was changed every 2 days. The experiment was conducted in 3 seasons. The result indicated that in winter, female can oviposit upto 7 times in her lifetime, and number of eggs in each batch was shown in Table 2.

Table 2. Gonotrophic cycle of *C. megacephala* reared in the labortory

No. oviposit	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Average of ovariole development until oviposit (day)	13 (7-27)	6.8 (2-21)	5.4 (3-15)	4.7 (3-8)	9.0 (6-17)	8.3 (4-16)	6.0
Average of number of eggs in each oviposition	222.7 (121-370)	196.1 (78-330)	210.2 (60-339)	196.9 (100-300)	212.2 (99-305)	226.7 (165-284)	259.0
No. flies examined	63	51	28	15	6	3	1

Molecular identification of forensically important blow flies in Thailand

Specimen collection

Adults of blow fly species (*C. megacephala*, *Chrysomya chani, Chrysomya pinguis, Chrysomya thanomthini, Ceylonomyia nigripes, A. rufifacies, A. villeneuvi, Hemipyrellia ligurriens, Hemipyrellia pulchra, Hypopygiopsis infumata, Lucilia cuprina, Lucilia porphyrina, Lucilia papuensis* and *Lucilia sinensis*) were included in this study. All specimens were identified by the morphological identification method. The identified flies were pinned and some preserved in 70% ethanol, and stored at room temperature. All specimens used for molecular identification have been confirmed by a specialist (Dr. Hiromu Kurahashi). Besides, adults of *C. megacephala*, *A. rufifacies*, and *L. cuprina* from a laboratory colony will be used to compare with field captured specimens.

DNA extraction, amplification, and sequencing

DNA extraction and amplification was performed. Briefly, genomic DNA was extracted from the thorax of individual fly specimens using the NucleoSpin® Tissue Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions, while remaining parts of the flies was maintained as a voucher specimen. The extracted DNA was eluted in 200 µl of elution buffer, measured for concentration by the NanoDrop 8000 Spectrophotometer (Thermo Scientific, USA), and diluted to 50 ng/µl prior to the PCR amplification step. The mitochondrial COI regions were amplified using three pairs of primer sets. PCR reactions was performed in total volumes of 50 µl containing 100 ng of template DNA, 1 unit of Tag DNA polymerase (Promega®, USA), 1x PCR buffer (Promega), 1.5 mM of $MgCl_2$ (Promega), 200 µM of each dNTPs (Promega), and 0.4 µM of each forward and reverse primer (1st Base, Singapore). The thermal cycling conditions was carried out in a TPersonal Combi Thermo Cycler (Biometra, Göttingen, Germany) consisting of a pre-denaturation step at 94ºC for 5

min, followed by 35 cycles of (1) denaturation step at 94ºC for 1 min, (2) annealing step at 46ºC for TY-J-1460&C1-N-2800 primers, 58ºC for C1-J-2495&TK-N-3775 primers or 45ºC for C1-J-2495& C1-N-2800 primers for 1 min 30 sec, and (3) extension step at 72ºC for 2 min followed by a final extension period at 72ºC for 5 min. The amplified products were subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide. The PCR products were purified by using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL, Germany) and subsequently sent to Macrogen DNA Sequencing Services (Macrogen Inc., Seoul, Korea) for automatic sequencing. All specimens were sequenced in both directions using the same forward and reverse primers as those used in the PCR amplification.

Phylogenetic analysis

The complete sequence of COI gene was obtained by aligning the overlapping DNA segments using the CLUSTALX version 1.83 (Thompson et al. 1997). All sequences obtained from this study was compared with those on the NCBI website via BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for species identification, and submitted to the GenBank database for assigning the accession numbers. A phylogenetic tree was constructed by the Neighbour-joining method using Kimura's 2-parameter model (Kimura 1980) implemented in MEGA© version 4.0 (Tamura et al. 2007), with 1,000 bootstrap replications. The result was demonstrated in Fig. 11, Table 3.

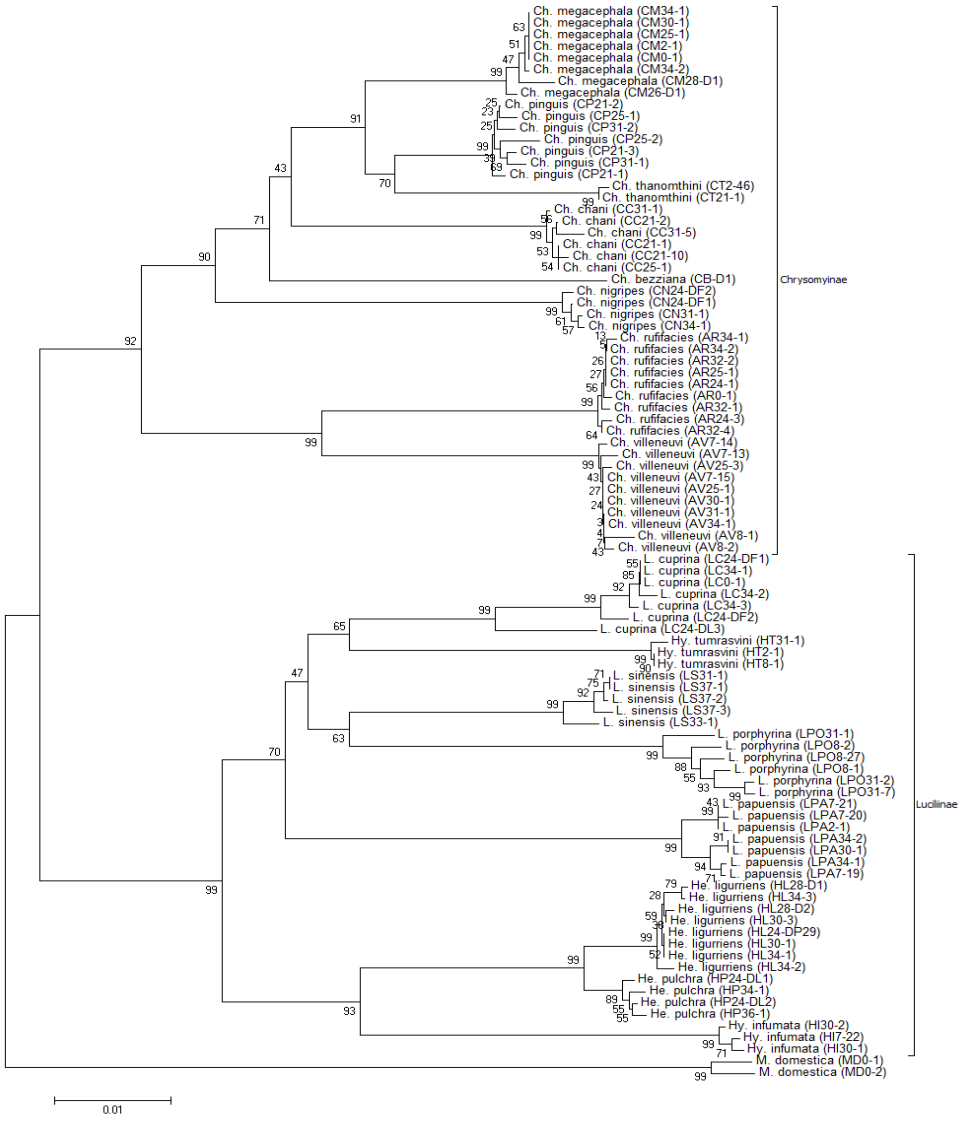

Figure 11. Neighbour-joining tree of Tamura-Nei substitution model for 92 COI sequences from all 16 species of forensically important blow flies and 1 species muscid outgroup. Number above branches refers to bootstrap proportions among 1000 replicates.

Table 3. Average of percentage of intraspecific divergences and interspecific divergences of COI gene (1247 bp) analyzed with neighbor-joining with Tamura-Nei model of substitubion

No.	Species	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Ch. bezziana*	1	**n/c**															
2	*Ch. megacephala*	8	5.0	**0.2**														
3	*Ch. chani*	6	5.7	4.6	**0.2**													
4	*Ch. pinguis*	7	5.4	2.7	4.4	**0.4**												
5	*Ch. thanomthini*	2	5.7	3.5	4.5	2.9	**0.1**											
6	*Ch. nigripes*	4	6.7	5.5	6.0	6.3	6.3	**0.2**										
7	*Ch. rufifacies*	9	8.3	7.1	6.9	8.0	8.1	8.1	**0.1**									
8	*Ch. villeneuvi*	10	7.8	6.8	7.6	8.0	7.6	7.8	4.9	**0.1**								
9	*L. cuprina*	7	9.8	8.9	8.0	9.8	9.1	9.8	10.5	10.3	**0.8**							
10	*L. papuensis*	7	10.8	10.9	8.5	10.9	10.3	10.1	11.0	10.2	6.8	**0.5**						
11	*L. porphyrina*	6	11.4	10.4	9.4	10.6	10.0	11.3	10.9	10.6	6.2	8.0	**0.7**					
12	*L. sinensis*	5	8.9	8.8	8.8	9.7	9.3	9.6	10.4	9.3	5.4	6.1	5.5	**0.4**				
13	*He. ligurriens*	8	9.9	9.2	9.1	10.1	9.8	10.1	10.8	10.3	6.2	8.8	7.6	6.2	**0.2**			
14	*He. pulchra*	4	9.6	9.1	8.6	10.1	9.6	9.9	10.4	9.9	6.2	8.5	7.4	6.0	1.2	**0.3**		
15	*Hy. infumata*	3	9.6	10.3	9.6	10.2	10.5	10.2	11.3	10.5	7.7	9.2	9.2	7.3	5.7	5.7	**0.2**	
16	*Hy. tumrasvini*	3	10.0	9.6	8.6	10.6	10.3	9.8	9.8	10.2	5.0	6.9	7.1	5.2	6.1	6.0	7.8	**0.1**

Number in bold indicate intraspecific divergens. The intraspecific divergence for *Ch. bezziana* is not available because there is only one sequence

Discussion

This study was the first to determine the population dynamics, seasonal and daily activity of *C. megacephala* and *A. rufifacies*, the two most common blow fly species in Thailand, over a full year period using automatic fly trap. Result of this study indicated that fly abundance was peaked in the summer, thus significantly influenced by temperature. Similar findings were reported in the previous investigations in Thailand (Ngoen-klan et al. 2011;Klong-klaew et al. 2014) and Australia, with fly density peak being in the summer months from December to February (Urech et al. 2012). Greater fly density coincided with the summer period, which was characterized by higher temperature and this would favor a rapid developmental rate (Zuha et al. 2012). Although peak population was collected recorded in the summer months (the beginning of April 2014), population was also collected in the winter time, but with the lowest population density (the beginning of January 2014). Experiment of Cammack and Nelder (2010) in the South Carolina, USA, showed that *A. rufifacies*is capable of developing below the lower threshold of 10.0 °C and adults are active as low as 9.0°C. To conclude, the total number of males and females of both blow fly species differed significantly among seasons.

As for other climatic factor such as relativel humidity, result of this study showed that *A. rufifacies* density was negatively correlated with relative humidity. Such phenomenon was correlated with previous investigations in India (Das et al. 1979), *C. megacephala* and *A. rufifacies* northern in

Thailand (Ngoen-klan et al. 2011), with *Achoetandrus albiceps*, and *C. megacephala* in Brazil (Azevedo and Kruger 2013).

More female *A. rufifacies* and *C. megacephala* were collected than males in this study. This information was consistent with other reports (e.g. Ngoen-klan et al. 2011; Klong-klaew et al. 2014). The explanation of the lower number of the male to female collected by our automatic traps is not easy. The following possible explanation may be that male need protein source as food, while beyond these two vital functions, females also need for food and oviposit. The potential for finding protein was from natural sources used for oviposition, food source and/or breeding places (Spradbery 1979).

Understanding the behavior and daily activity of flies of medical importance may help in their control management. This study was the first to determine the daily activity of *A. rufifacies* throughout the one-day period using automatic trap. The trapping number of this fly species in the course of a day had a major peak around 15.00 – 18.00 PM. Only small number of fly was captured during night time (18.00 - 6.00), correlated with other previous reports. Greenberg (1990) reported that three common and forensically important flies --*Calliphora vicina, P. sericata* and *Phormia regina*--oviposited during the dark hours of the night during the summers of 1988 and 1989. Moreover, the blow flies, *Phaenicia eximia* and *Cochliomyia macellaria*, oviposited nocturnally on ground beef under artificial lighting of at least 1,500 footcandles at temperatures 26°C or higher during onsets of low-atmospheric pressure at study sites near Snook, Texas, during 2003 (Kirkpatrick and Olson 2007). Work conducted in the US suggested that nocturnal oviposition of blow fly *Phormia regina* is rare in the natural environment (Berg and Benbow 2013). In a study reported from the USA, the probability of nocturnal oviposition of blow fly *Lucilia sericata* on pig carcasses in Michigan was extremely low to nonexistent (Zurawski et al. 2009). The findings from this study may help to improve the control strategy of both *A. rufifacies* and *C. megacephala* by identifying the best time for sampling and application of insecticides or other management strategies. Additional studies are needed to better understanding the ecological mechanisms governing blow fly oviposition important to forensic entomology.

While observing the reproductive potential performed in *C. megacephala*, the present study indicated the highly fecundity in this species. Females can lay egg upto 7 batches, much more time than other blow flies species. In *Calliphora vicina*, females laid a mean of 3 ± 0.3 batches of eggs (George et al. 2015). In addition, this work found that female has 51.6 days in their longevity, much longer than *C. vicina* that most females did not survive until day 38 (mean survival = 33 ± 1 day) (George et al. 2015). Our results presented here confirm that *C. megacephala* has a high reproductive potential in the environment of northern Thailand. Understanding reproductive potential in calliphorids and other Diptera provides important insights to future control strategy.

Identification of blow fly species is mandatory in the medical application in forensic investigation. As the conventional identification methods using external morphology has limitations for similarity, sibling and closely related species of blow fly. In this regard, molecular identification has

offered in the field of forensic entomology is species determination. Documents have been found for molecular identification in many groups of flies of forensic importance. Examples of these were provided in Malaysia of which mitochondrial cytochrome oxidase I (COI) and cytochrome oxidase II (COII) have been used for phylogenetic analyses of *C. megacephala, A. rufifacies* and *Chrysomya nigripes* (Kavitha et al. 2012). In China, forensically important blow flies were genetically analyzed using a 278-bp segment of the cytochrome oxidase subunit I gene. These species were *C. megacephala, A. rufifacies, Protophormia terraenovae, Lucilia caesar, Lucilia bazini, Lucilia porphyrina, Hemipyrellia ligurriens, Lucilia sericata* and *Calliphora vicina* (Liu et al. 2011). In the US, Wells and Williams (2007) identified blow fly of the subfamily Chrysomyinae (Diptera: Calliphoridae), which are often found to be associated with a human corpse in Canada or the USA. They used phylogenetic analysis of DNA sequence from a short segment of the mitochondrial gene for cytochrome oxidase one (COI) for *P. terraenovae, C. megacephala, Chrysomya bezziana, Chrysomya semimetallica, A. rufifacies, A. albiceps, Chrysomya putoria, Chrysomya norrisi, Chrysomya varipes, L. sericata, L. illustris, Eucalliphora latifrons, Protocalliphora sialia, Protophormia atriceps, P. regina, Cochliomyia macellaria* and *Cochliomyia hominivorax*. in Southeastern Nebraska, eight species of blow flies of forensic importance were genetically analysed in *Cochliomyia macellaria*, *Lucilia illustris*, *Lucilia sericata*, *Cynomya cadaverina*, *Lucilia silvarum*, *P. regina*, *C. vicina* and *A. rufifacies* using the utility of RFLP of the mitochondrial COI region (Samarakoon et al. 2013). In Taiwan, eight species have been genetically identified (*C. megacephala, Chrysomya pinguis, A. rufifacies, H. ligurriens, L. bazini, L. cuprina, Lucilia hainanensis* and *L. prophyrina*) (Chen et al. 2004). These authors used mitochondrial cytochrome oxidase subunit I (COI) DNA of the preceding blow fly species to study its application value for their differentiation, of which the cloning and sequencing of the COI gene was ≈1,588 base pairs. In Thailand, little information was found for investigation of such topic. Preativatanyou et al. (2010) documented that COI–COII sequence was useful for identification of *C. megacephala, A. rufifacies* and *L. cuprina*. Based on the literatures, result of the present study provided the strength molecular information in analyzing upto 14 blow fly species of forensically important in Thailand. Although *A. rufifacies* and *A. villeneuvi* are very closely related species with larvae that act as predator to other fly species, there is a detected difference in their genetic, as shown in the phylogenetic profile (see Fig. 11). Similarly, although the male genitalia of *C. megacephala, C. pinguis, C. chani* and *C. thanomthini* are morphologically similar, their genetic analysis usind COI is markedly divergence (see Fig. 11). Therefore, result of this molecular study provides a strong evidence for species identification and would augment the accurate identification of blow flies of forensical importance in Thailand.

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Suwannayod S, Sanit S, Sukontason K, **Sukontason KL.** *Parasarcophaga (Liopygia) ruficornis* (Diptera: Sarcophagidae): A flesh fly species of medical importance. Tropical Biomedicine 2013;30:174-80. (Impact Factor = 0.85)

1.2 Sanit S, Sukontason K, Klong-klaew T, Tomberlin JK, Limsopatham K, Samerjai C, Sontigun N, **Sukontason KL**. Ontogenesis and developmental rate of the blow fly, *Hypopygiopsis tumrasvini* Kurahashi (Diptera: Calliphoridae). Tropical Biomedicine 2014;31:760-8. (Impact Factor = 0.85)

1.3 Klong-klaew T, Sukontason K, Ngoen-klan R, Moophayak K, Irvine KN, Kurahashi H, Prangkio C, Sanit S, **Sukontason KL**. Impact of abiotic factor changes in blow fly, *Achoetandrus rufifacies* (Diptera: Calliphoridae), in northern Thailand. Parasitology Research 2014;113:1353-60. (Impact Factor = 2.098)

1.4 **Sukontason KL**, Sanit S, Klong-klaew T, Tomberlin JK, Sukontason K. *Sarcophaga (Liosarcophaga) dux* (Diptera: Sarcophagidae): A flesh fly species of medical importance. Biological Research 2014, 47, 14. (Impact Factor = 1.48)

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1.8 Chaiwong T, Kurahashi H, Sanit S, Moophayak K, Sukontason K, **Sukontason KL**. Three sarcophagid species (Diptera: Sarcophagidae) newly recorded in Thailand. Tropical Biomedicine 2015 (In press). (Impact Factor = 0.85)

หมายเหตุ Reprint ผลงานวิจัยเรื่องที่ 1-8 อยู่ในภาคผนวก

ผลงานวิจัยที่คาดว่าจะตีพิมพ์เพิ่มเติม

- Daily and seasonal activity of blow flies, *Chrysomya megacephala* and *Achoetandrus rufifacies* (Diptera: Calliphordae) in Chiang Mai, northern Thailand
- Molecular identification of medically important blow flies in Thailand
- Bionomic of of blow flies *Chrysomya megacephala* and *Achoetandrus rufifacies* (Diptera: Calliphordae) in Thailand
- Survey of medically important flies in Nan and Phitsanulok provinces, northern Thailand

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงสาธารณะ

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- Institute of Forensic Medicine, Goethe-Universität, Germany

- เชิงวิชาการ

มีการผลงานวิจัยไปพัฒนาการเรียนการสอน ในกระบวนวิชากีฏวิทยาการแพทย์ในระดับบัณฑิตศึกษา ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ และมีการสร้างนักวิจัยใหม่ จากโครงการนี้ โดยร่วมมือกับโครงการปริญญาเอกกาญจนาภิเษก คือ นส. ธันวดี คล่องแคล่ว, นส. นารินทร์ สนธิกันย์

3. การเสนอผลงานในที่ประชุมวิชาการ

- ได้รับเชิญให้เสนอผลงานวิจัยเรื่อง Forensic Entomology in Thailand ในการประชุมวิชาการนานาชาติ International Conference on Earth, Environment and Life Sciences เมืองดูไบ ประเทศสาธารณรัฐอาหรับเอมิเรตส์ วันที่ 23-24 ธันวาคม 2557

Tropical Biomedicine 30(2): 174–180 (2013)

Review Paper

Parasarcophaga (Liopygia) ruficornis (Diptera: Sarcophagidae): A flesh fly species of medical importance

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Abstract. *Parasarcophaga (Liopygia) ruficornis* is a well-known flesh fly species of medical importance, both as a myiasis-producing agent and fly seen in a forensic entomology context. This study performed a comprehensive literature review of this fly species, dealing with morphology, bionomics and medical involvement. Important characteristics used to identify *P. ruficornis* have been provided for both its third instar and adult for identification purpose in the future.

INTRODUCTION

The medical importance of the flesh fly species, *Parasarcophaga (Liopygia) ruficornis*, is of particular interest in many parts of the world, either as a myiasis-producing agent or fly seen in a forensic entomology context. Geographically, this species has been recorded as an Old World fly although it has invaded the New World. In the USA, it has been recorded from Hawaii (Davis & Goff, 2000), California, Florida, Massachusetts, New York, North Carolina, Pennsylvania and Washington D.C. (Alfred, 2011). In the Oriental region, *P. ruficornis* has been found in many countries such as Thailand (Sucharit *et al*., 1976), Malaysia (Kumara *et al*., 2012), Singapore (Sugiyama *et al*., 1990) and India (Sreevatsa *et al*., 1990).

The genetic characterization of *P. ruficornis* has been compared with that of *Parasarcophaga dux* and *Parasarcophaga argyrostoma* using allozyme and RAPD-PCR markers, which indicated a very close relationship between these species (Bajpai *et al*., 2011). However, based on the sequences of mitochondrial cytochrome oxidase gene *subunits I* and *II* (*COI* and *COII)*, the *ruficornis*-group was one of the six major clades of forensically important sarcophagids in Malaysia, with the other five being the *peregrina*-group, *albiceps*-group, *dux*-group, *pattoni*-group and *princeps*-group (Tan *et al*., 2010).

Morphology

In the forensic entomology aspect, identification of larval specimens that are found to be associated with a corpse is essential before they are used further in investigation [e.g., estimation of post-mortem interval (PMI_{min}) from the larvae collected]. Although flesh flies, including *P. ruficornis*, are commonly larviparous (larviposit the first instar), they also have been documented as oviparous in a laboratory colony. Sukhapanth *et al*. (1988) provided information of *P. ruficornis* eggs measuring 1.6±0.33 mm in length. Morphologically, the *P. ruficornis* larva, which is similar to other flesh fly larvae, exhibits a distinct morphological feature in having its posterior spiracle

situated within a terminal concavity of the last abdominal segment. The larva typically has a vermiform robust body, comprising a head region, three thoracic segments and eight abdominal segments (Figure 1A). The length of the first, second and third instar is 6.8±0.45 mm, 11.8±0.07 mm and 16.9±0.08 mm, respectively (Sukhapanth *et al.*, 1988). With the aid of light microscopy, the third instar of *P. ruficornis* has been documented by focusing on the main features used to differentiate from the other forensically important flesh fly species. On each lateral side of the prothorax, a pair of anterior spiracles having 11-15 papillae arranged in a single row (Figure 1B). The posterior spiracle is located in a terminal concavity of the last abdominal segment and is characterized by (1) the distinct inner projections between the spiracular slits (Figure 1C), (2) the prominent tail of the upper end of the peritreme, and (3) position of the peritreme at the base of the middle slit (Sukontason *et al.*, 2010). All larval instars of this fly species have been described by scanning electron micrograph (SEM) (Singh *et al.*, 2012), highlighting the sensory organs (dorsal, terminal and ventral organs) located on the cephalic segment as well as strong, slightly curved mouth hooks. In this review, the sharp bladed mouth hooks of the first instar are clearly demonstrated in Figure 2. Features of the internal cephalopharyngeal skeleton of all instars were also illustrated by Singh *et al.* (2012).

Puparia of *P. ruficornis* were measured as 11.7±0.14 mm in length (Sukhapanth *et al.*, 1988). A very short pupal respiratory horn was observed dorsolaterally in the first abdominal segment (Singh *et al.*, 2012). Regarding adults, males are slightly larger than females, measuring 13.8±0.16 mm and 13.2±0.01 mm, respectively (Sukhapanth *et al.*, 1988). The prime characteristics of *P. ruficornis* differ from two other forensically important species; *P. dux* and *P. peregrina*, which have yellowish orange third antenna and palpus (Figure 3). In adult flesh flies, only males could be identified, of which each species was endowed with distinct characteristics of terminalia (Figure 4). Regarding flesh flies of forensic importance, the key to identify adult males of the South American genera was published (de Carvalho & de Mello-Patiu, 2008), while that for identifying species including *P. ruficornis* in Thailand has been updated (Chaiwong *et al.*, 2009). Informative characteristics of male genitalia of this species, particularly the distiphallus, have been displayed using SEM (Giroux *et al.*, 2010).

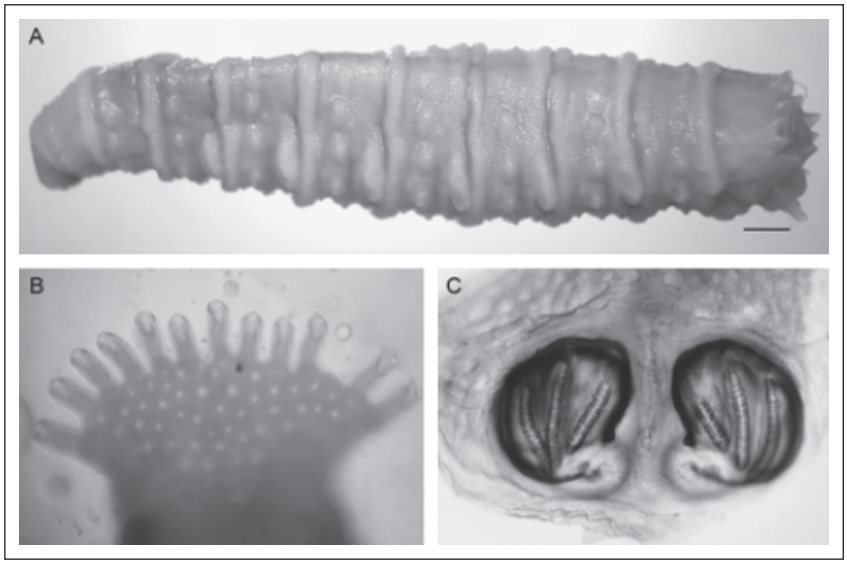

Figure 1. Third instar of *P. ruficornis*. (A) Lateral view. Bar = 1 mm. (B) Anterior spiracle. (C). Posterior spiracle. Online figure in color

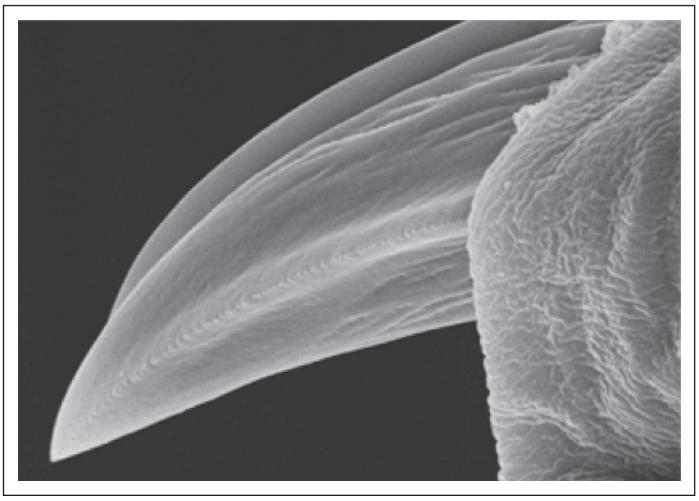

Figure 2. Scanning electron micrograph showing the sharp bladed mouth hook of the first instar of *P. ruficornis* (original picture of KL Sukontason)

Figure 3. Adult male of *P. ruficornis*. Inset displays important features of this species, yellowish orange of the third antenna (arrowhead) and palpus (arrow). Online figure in color

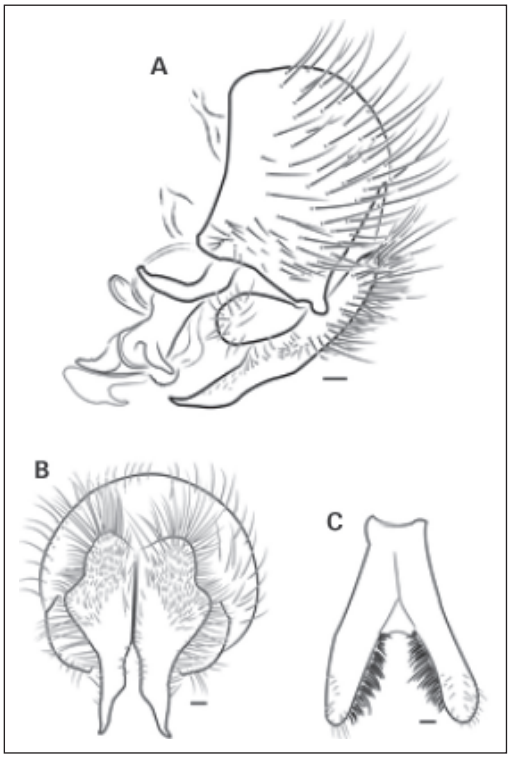

Figure 4. Male terminalia of *P. ruficornis*. (A) Lateral view. (B) Cercus, caudal view. (C) Sternite 5. Bar of all figures = 0.02 mm

Table 1. Documentation of human cases associated with larvae of *P. ruficornis*

Year	Country	Death scene	PMI_{min}	State of decay	Reference
2002	Thailand	indoor	ca. 3-4 d	bloat	(Sukontason *et al.*, 2007)
2006	Thailand	indoor, constant 25°C	unknown	mummified	(Sukontason *et al.*, 2007)
2011	Kuwait	indoor, 2nd floor (in air condition)	ca. 7.5-8.5 d	decomposed	(Al-Mesbah *et al.*, 2011)
2012	Malaysia	indoor	ND	ND	(Kumara *et al.*, 2012)

ND, no available data

Bionomics

Based on extensive study on the bionomics of sarcophagids in Thailand by Bänziger & Pape(2004), female *P. ruficornis* occasionally laid eggs on the mesh of boxes containing the breeding medium. Its laying habit is of an amphibiodotic type (larviposit on both faeces and carrion). Females typically behave in a larviparous manner by depositing 40-80 first instars (Verma & Ishikawa, 1984), but in special circumstance such as laboratory rearing, oviparous behavior also has been documented. This phenomenon was similar to that which occurred in *P. dux* in laboratory conditions (Sukontason *et al.*, 2005). Laboratory rearing conditions set at 27±4ºC and 78±4% RH demonstrated that female *P. ruficornis* laid eggs 5-7 days after emergence, with the number of eggs per batch being 19.1±11.1 (Sukhapanth *et al.*, 1988). Larval growth development (larviposition until pupariation) varied according to records. In Guam it took 7 days at 29.5ºC (Bohart & Gressitt, 1951); in Thailand 8.0±1.8 days at 27±4ºC (Sukhapanth *et al.*, 1988); and in Hawaii 7.5-10.8 days at 26ºC during experiments (Goff *et al.*, 1997). In Saudi Arabia, Amoudi *et al.* (1994) reared larvae at the constant temperatures of 13, 16, 19, 22, 25, 28, 31, 34 and 37ºC, and the larval development time took 31.5, 17.5, 10.5, 9.7, 8.5, 6.8, 6.0, 5.5 and 6.3 days, respectively. In Thailand, larvae were reared at 27±4ºC, and the pupal period was 11.7±0.14 days. Longevity of males and females ranged from 3.5 to 39 days and 2.0 to 31.8 days, respectively (Sukhapanth *et al.*, 1988).

This fly species is synanthropic, that is, it lives closely associated with the human environment. In Pakistan, adult *P. ruficornis* was trapped in residential areas, where flies fed and larviposited on rabbit, fish and chicken carcasses (Shazia *et al.*, 2006). In Thailand, adults were captured from the flowers of *Bulbophyllum putidum* and the fruit of *Dimocarpus longan* (Bänziger & Pape, 2004), as well as animal waste such as putrid fish (Sucharit *et al.*, 1976). Similarly, in northeast Thailand adult *P. ruficornis* was collected from restaurants and school cafeterias, but not from fresh-food markets, garbage piles or paddy fields (Chaiwong *et al.*, 2012). In southern India, this species was collected in several villages that were hit by the tsunami on 26th December, 2004 (Srinivasan *et al.*, 2006).

Myiasis

There are limited records of myiasis caused by *P. ruficornis* and it is either reported as the sole species responsible or co-infesting with other fly larvae. In Thailand, Sucharit *et al.* (1981) reported on cases in the vagina and in India patients suffering from leprosy have been documented as being infected (Sreevatsa *et al.*, 1990). A wound co-infested with the blow fly, *Chrysomya megacephala*, and house fly, *Musca domestica*, was recorded from the scalp of a man in Brazil (Ferraz *et al.*, 2010). Adult *P. ruficornis* were caught in the intensive care unit (ICU) of a hospital in Malaysia providing further evidence of the potential for the nosocomial infestation(Nazni *et al.*, 2011).

Forensic entomology

Although blow flies (Diptera: Calliphoridae) are often used in forensic investigations, several documented cases report the presence of flesh fly larvae, suggesting that there are other fly groups of forensic importance. Of these flesh fly species, specimens of *P. ruficornis* has been recorded to associate with human death scenes, as well as from pig carcasses (*Sus scrofa*); an animal experimental model in forensic entomology in Brazil (de Souza & Linhares, 1997; Barbosa *et al*., 2009), the USA (Oahu island of Hawaii) (Davis & Goff, 2000), and Thailand (Vitta *et al*., 2007; Sukjit, 2011). Investigation on the island of Oahu, Hawaii, U.S.A. indicated that this species was an early invader and insect colonizer of the death scene (Nolte *et al*., 1992). A document reporting from Kuwait demonstrated the significance of post feeding third instar *P. ruficornis*, which was collected from the blanket which the body remain was wrapped. Based on the age of *P. ruficornis* collected and the location of the body, ~7.5–8.5 days PMI_{min} was estimated (Al-Mesbah *et al*., 2011). Based on the literature, few number of human death scenes were found to be associated with this species, mostly indoor cadavers (Table 1). Difficulty in identification of flesh fly larvae and/or incorrect identification may lead to have lower number of cases infesting with *P. ruficornis*, than the actual cases. According to the indoor death scenes associated with *P. ruficornis*, this fly species might have special features in its biology that allow them to be colonized in houses or other dwelling. This phenomenon was similarly documented in other species, *Sarcophaga caerulescens* Zetterstedt, the sarcosaprophagous flesh fly species found in indoor death scene in southern Finland (Pohjoismaki *et al*., 2010). Such phenomenon is worthy of note in forensic investigation.

In addition, study pertaining to more precise estimation and/or analysis of death related to drugs has used specimens of *P. ruficornis*. Goff *et al*. (1994) reported the larval developmental rate of this fly species when fed on phencyclidine; a common drug abused in many country and legitimate veterinary tranquilizer, from the decomposing tissue of rabbit. No significant differences in larval growth rate were observed among rabbits administered with 3.66, 7.31, and 14.62 mg of phencyclidine via ear vein infusion. Pupariation period of *P. ruficornis* were longer for animals fed on tissues containing the drug.

In conclusion, despite information on all aspects of *P. ruficornis* being relatively limited; the significance of this fly species is increasing, particularly from a forensic entomology viewpoint. Based on the literature review, the indoor environment preferred by *P. ruficornis* is worthy of note in forensic investigation. Therefore, to increase worldwide reviews of human cases involving this fly species is mandatory, and their clarification would benefit forensic investigations.

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Ontogenensis and developmental rate of the blow fly, *Hypopygiopsis tumrasvini* Kurahashi (Diptera: Calliphoridae)

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Abstract. Blow flies of the genus *Hypopygiopsis* are considered forensically important. In Thailand, four *Hypopygiopsis* species coexist, i.e., *Hypopygiopsis fumipennis*, *Hypopygiopsis infumata*, *Hypopygiopsis violacea* and *Hypopygiopsis tumrasvini*. In this study, the ontogeny and developmental rate of *H. tumrasvini* eggs, larvae and pupae were determined in the laboratory chamber reared at 25.0±2.0°C and 80.0±5.0% RH. Larvae emerged from eggs 10-12 h after deposition. Mean length of the first, second, third (feeding phase), third (post-feeding phase) instars and puparia were 3.5±1.1, 7.2±1.1, 13.5±1.8, 12.5±0.5 and 9.0±0.7 mm, respectively. The median development time for first, second, third instar (feeding phase), third instar (post-feeding phase) and pupariation period was 8 h, 10 h, 34 h, 22 d and 9-10 d, respectively. Developmental curve of the larval length indicated the rapid progression from 0 until 40 h from the first instar until the feeding third instar. Video recording of pupariation revealed the development of pupal respiratory horn beneath the larval integument at 27.0 h; whereas it protruded through the orifice of the integument at 27.5 h.

INTRODUCTION

Blow flies (Diptera: Calliphoridae) are among the first arthropod colonizers of human remains (Greenberg & Kunich, 2002; Williams, 2008). Thus, their larvae can be used in forensic investigations to estimate the minimum postmortem interval (mPMI) of the decedent (Goff & Odom, 1987), determine the presence of toxins and potentially the manner of death (Gunatilake & Goff, 1989; Byrd & Castner, 2001). However, to utilize entomological evidence, it is important to identify the specimens correctly and then apply development data to determine their age. Such information can then be used to estimate the age of the larvae, and mPMI.

In Southeast Asia, blow flies in the genus *Chrysomya* are most often associated with human remains (Lee *et al.*, 2004; Sukontason *et al.*, 2007). However, cases have also been found with the genus *Hypopygiopsis* present (Ahmad Firdaus *et al.*, 2010). In Thailand, four species of *Hypopygiopsis* have been identified: *Hypopygiopsis fumipennis* Walker, *Hypopygiopsis infumata* Bigot, *Hypopygiopsis violacea* Macquart and *Hypopygiopsis tumrasvini* Kurahashi (Kurahashi & Bunchu, 2011). In Malaysia, Ahmad Firdaus *et al.* (2010) provided morphological descriptions of second and third instars of *H. violacea*; while Chen *et al.* (2011) investigated the immature growth rate of this species.

The *H. tumrasvini* species is distributed throughout Bangladesh, Cambodia, China (Hainan Is., Yunnan), India (Assam, Uttar Pradesh), Laos, Thailand and Vietnam (Verves, 2005). This species is mainly found in high elevations (349 – 1,700 m) in Asia,

and dense forested areas where human remains associated with criminal activity are often discovered (Moophayak *et al.*, 2014).

In Thailand, morphology of the *H. tumrasvini* larvae and puparia have been described using both light and scanning electron microscope (Moophayak *et al.*, 2011; Sanit *et al.*, 2012). The purpose of this study was to determine the ontogeny and developmental rate of *H. tumrasvini*.

MATERIALS AND METHODS

Fly colony

A colony of *H. tumrasvini* was obtained from the mixed deciduous forest area higher than 500 m above sea level, high relative humidity and low temperature at Suthep-Pui Mt. (18°48'20"N, 98°54'34"E, 952 m a.s.l.), Mueang Chiang Mai district, Chiang Mai province, northern Thailand. Bait consisted of 1-day-old, tainted beef. A sweep net was used to capture female flies arriving at the bait. Adult females of *H. tumrasvini* were identified according to identification key (Tumrasvin *et al.*, 1979). In the field, all gravid females were transferred into a transparent glass tube (8 cm height, 2.3 cm diameter) containing ~3 g of fresh beef and a small one piece of leaf as oviposition medium to imitate a species specific natural breeding site. Any kind of leaf around that collected area could be used for oviposition. The glass tube was covered with a layer of gauze for air ventilation, and transported to the laboratory at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, northern Thailand.

The laboratory rearing environment was designed to resemble the natural habitat. The adult rearing cage was built using a glass fish tank (25x50x30 cm) and covered the entrance with white muslin cloth (Fig. 1). The cage contained 6 plastic trays (20x40x6 cm) which were filled with water and pebble to maintain temperature and relative humidity. These glass tubes were observed every 2 h for oviposition on both beef and leaf settings. When females laid eggs, the tubes were not disturbed until the newly emerged larvae hatched from the eggs. These larvae were transferred using a wet fine brush and maintained in the transparent rearing plastic box (13x17x7 cm) of which ¾ of the lid covering with a fine muslin cloth to prevent entering of parasitoid and ventilation, containing fresh beef *ad libitum* and sawdust soaked with water (2:1) to increase relative humidity and simulate the natural breeding site. The larvae were reared under ambient temperature (25±2°C), relative humidity of 80±5% and a light/dark regime of 12:12 h in a rearing chamber (Fig. 1). Adults were fed with honey (serve as carbohydrate to provide energy), water and fresh beef (provided every 3 days) as a protein source for reproductive development and oviposition.

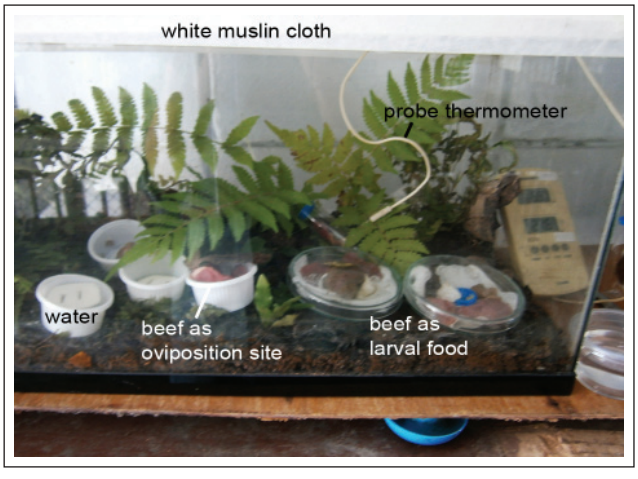

Figure 1. Rearing cage of *H. tumrasvini*, maintained at 25.0 ± 2.0°C and 80.0 ± 5.0% RH.

Developmental rate of egg

Eggs were divided into five groups of 24 and placed on a small piece of gauze (4.5x3.5 cm) located on a plastic tray (5.5x2.5 cm). A second piece of gauze soaked with water was placed over the eggs. These trays were then transferred into a plastic cage (13x17x7 cm) at 25.0±2.0°C, 80.0±5.0% RH and light/dark regime of 12:12 h. Eggs were observed every 2 h under a light microscope (Olympus®; CX31, Japan) until hatching. The experiment was replicated twice. Photographs were taken with a digital camera (Olympus®; VG-130, Japan). A video recorder function of a digital camera (Olympus®; VG-130, Japan) was also used to observe the hatching process.

Developmental rate of larvae

Larvae of *H. tumrasvini* were reared in a plastic container (13x17x7 cm) containing saw dust with water (2:1) at 25.0±2.0°C and 80.0±5.0% RH. Larvae were provided fresh beef *ad libitum*. Three larvae were sampled every 2 h from each egg batch, until the post-feeding stage when the third instar moves away from the beef toward the sawdust. Afterwards, two larvae (post feeding stage) were sampled until they entered the pupal stage.

Collected larva was sacrificed in 90°C water for 1 min. The length and width of each larva was measured using a digital caliper (Pittsburgh® 6 inch Digital calipers, model Soya, Taiwan). Larvae were then stored in 70% alcohol for preservation. As for puparia, each pupa was measured using digital caliper and then stored in 70% alcohol. Larval and pupal duration as well as time spent from newly hatched first instar to post-feeding stage was recorded.

Statistical analysis

Data were analyzed using the Mann-Whitney *U* test (SPSS version 17). A *P* value of less than 0.05 was considered significant.

RESULTS

Embryo development of *H. tumrasvini* is presented in Fig. 2. No obvious features of the embryos were observed during 0-6 h (Fig. 2A). One hundred fifty first instars occurred in each experiment, and two replications were conducted. Circular spinulation along the body was first observed at 8 h (Fig. 2B). Intense spinulation occurred during the 10 h period (Fig. 2C).

Each step of the hatching process was observed using a video recorder. During 10-12 h, the embryo was continuously active, as shown by changing in position of cuticular spines (Figs. 3A, 3B). The embryo shrunk vigorously prior to hatching, causing collapse of the latero-anterior margin of the eggshell (Fig. 3C), and later pushed out the most anterior region (Fig. 3D). Rupturing along the anterior hatching line allowed the first instar to eclose (Figs. 3E-G).

Morphometric measurement of larvae and puparia of *H. tumrasvini* was examined. The average length of the first, second, third (feeding phase), third (post-feeding phase) and puparia were measured: 3.5±1.1, 7.2±1.1, 13.5±1.8, 12.5±0.5 and 9.0±0.7 mm, respectively. Developmental period of immature *H. tumrasvini* reared in the laboratory at 25.0±2.0°C and 80.0±5.0% RH is shown in Table 2. The embryonation time (time for development of embryo inside the egg) was 10-12 h, while most of the first and second instars were rapid in development, taking 8 and 10 h, respectively. In contrast, the third instar development was

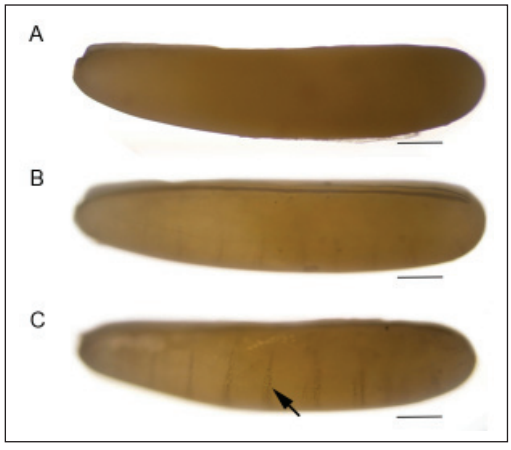

Figure 2. Embryo development of *H. tumrasvini*. A: 0-6 hr. B: 8 hr, showing circular spinulation of embryo. C: 10 hr, showing intense spinulation of embryo (arrow). Bar = 0.2 mm.

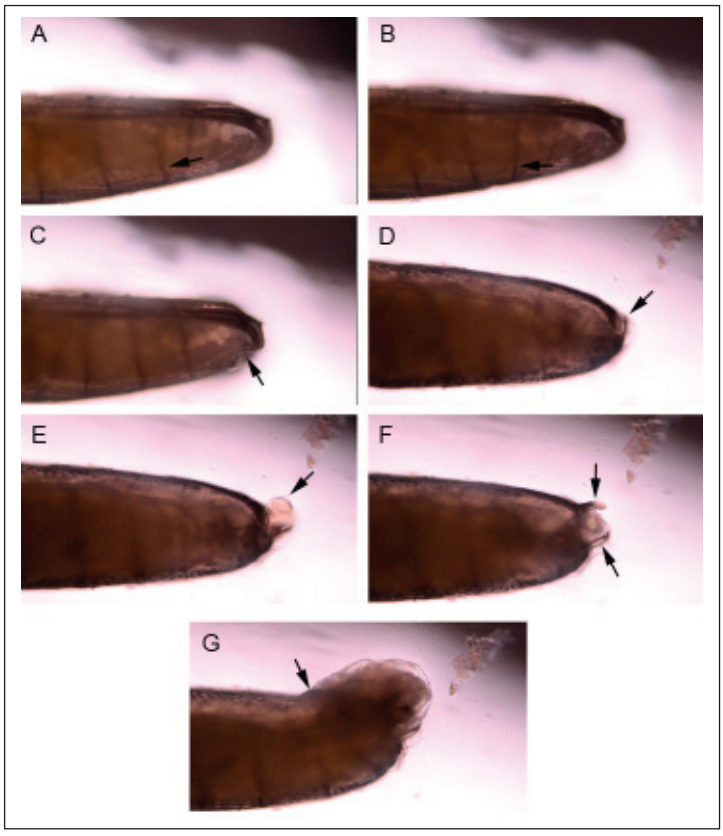

Figure 3. Hatching process of *H. tumrasvini* as demonstrated by a video recorder. A,B: Active movement of 10-12 hr embryo, as shown by changing in position of cuticular spines (arrows). C: Collapse of the latero-anterior margin of the eggshell due to shrunken embryo prior to hatching. D: Anterior straining by the larva. E,F,G: Rupturing along the anterior hatching line allowed the eclosion of first instar larva (arrows).

Table 1. Average length and width of immature *H. tumrasvini*[*]

Stage	*n*	Size (mean ± SD; mm)	
		Length	Width
First instar (L1)	30	3.5 ± 1.1	0.5 ± 0.2
Second instar (L2)	30	7.2 ± 1.1	1.1 ± 0.2
Third instar (L3, feeding phase)	102	13.5 ± 1.8	2.2 ± 0.3
Third instar (L3, post- feeding phase)	88	12.5 ± 0.5	2.3 ± 0.1
Puparia	40	9.0 ± 0.7	3.5 ± 0.1

[*]At 25±2°C and 80±5% relative humidity in laboratory.

considerably longer at 34 h in feeding phase; while 22 d in post-feeding period. Pupariation time took a longer period, being 9-10 d before emergence. The total developmental time of this species was 34-35 days. Figure 4 represents third instar length and width growth curves from the first until feeding phase. Both length and width median larval

Table 2. Developmental time of immature *H. tumarasvini*, with comparison to *H. violacea*

Stages	*H. tumrasvini*[*]		*H. violacea*[**]
	n	Duration (median)	
Egg	120	10-12 h	~6 h
First instar (L1)	30	8 h	12 h
Second instar (L2)	30	10 h	22 h
Third instar (L3, feeding phase)	102	34 h	16 h
Third instar (L3, post- feeding)	88	22 d	4 d 18 h
Puparia	40	9-10 d	5 d 18 h
First instar – pupariation		25 d	–
Total period (egg – emergence)		34.5-35.5 d	12 d 20 h

[*]At 25±2°C and 80±5% relative humidity in rearing chamber (present study)
[**]At 28±2°C and 70±5% relative humidity; 12 dark: 12 light (Chen *et al.*, 2011)

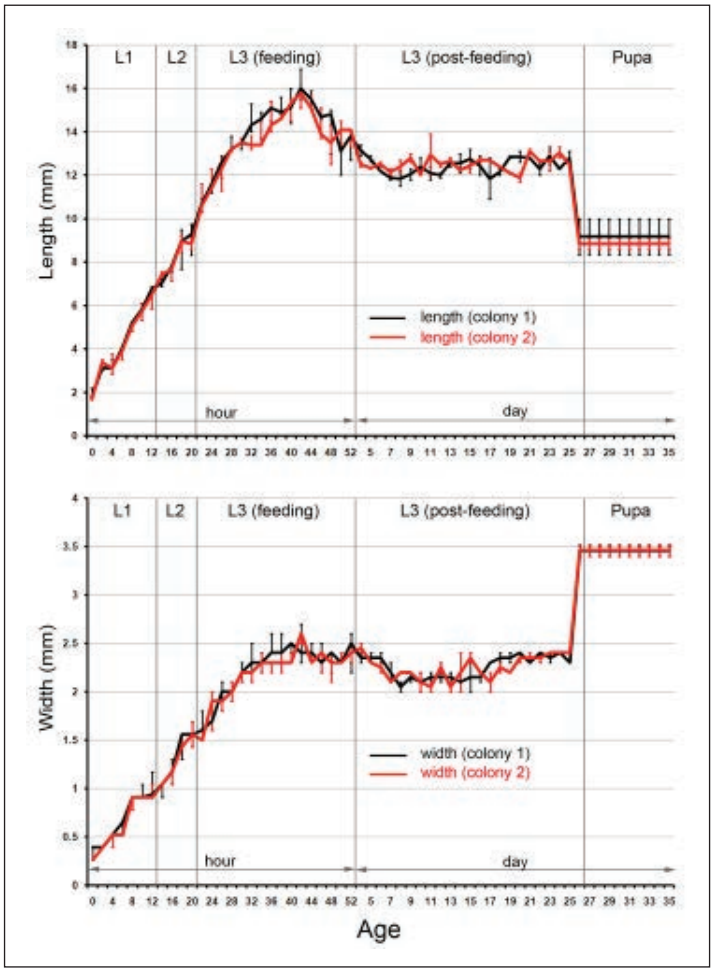

Figure 4. Developmental growth of *H. tumrasvini* showing median of larval length (upper) and width (lower). No significant differences between median of both colonies of their length and width (Mann-Whitney *U* test; $P>0.05$).

sizes were similar between colonies (Mann-Whitney *U* test; $P>0.05$).

Observation of the gradual coloration of *H. tumrasvini* puparia was displayed under stereomicroscope (Olympus SD30, Japan) (Fig. 5). At the first day of pupariation, the integument rapidly changed colors; from creamy-white (h 0) to orange, orange-brown, then light brown. Coloration of dark red-brown to almost black was observed from days 2-9. During this time, observation was conducted to examine development of the pupal respiratory horn, located at the dorso-lateral margin of the fifth segment (Fig. 6A-H). At h 0, a globular orifice appeared along the bubble membrane (Fig. 6A). The pupal respiratory horn began to emerge underneath the position of bubble membrane at 27 h thereafter. Rapidly the horn protruded through the orifice, at the center of bubble membrane with complete protrusion observed at 27.5 h (Figs. 6B-H). Microscopic observation also demonstrated that the position of the pupal respiratory horn is connected with the pharate adult (Fig. 7).

DISCUSSIONS

Species specific rates of development are the key information for estimating minimum PMI. Despite this potential utility in forensic investigations, ontogeny and developmental rate of *Hypopygiopsis* has not yet been established. Morphometric measurement in this study indicated that larvae of *H. tumrasvini* were relatively large, especially the third instar with lengths up to ~15.3 mm (range 11.7 – 15.3 mm). This size is comparable to the third instar stage of *H. violacea* (Ahmad Firdaus *et al.*, 2010).

Moreover, it is difficult to compare the developmental rate of immature stages of blow flies with that of other forensically important species (e.g., *Chrysomya megacephala* Fabricius, *Chrysomya rufifacies* Macquart, *Lucilia cuprina* Wiedemann, *Chrysomya nigripes* Aubertin, *Hemipyrellia ligurriens* (Wiedemann); or flesh flies, e.g., *Boettcherisca nathani* Lopes, *Lioproctia pattoni* (Senior-White), *Liopygia ruficornis* (Fabricius) and

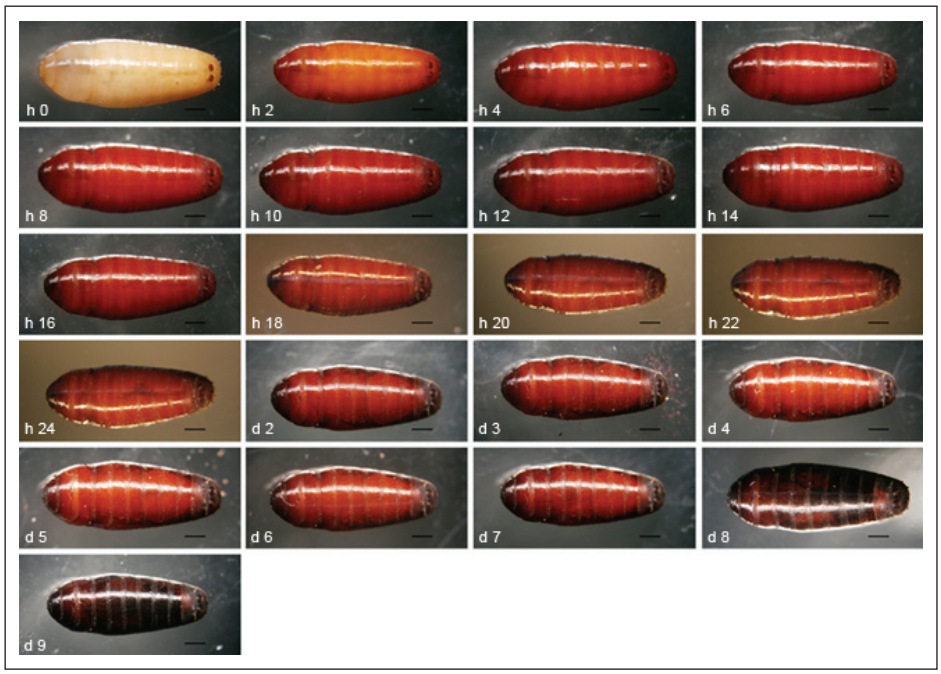

Figure 5. Gradual coloration change of puparia *H. tumrasvini* from h 0 to d 9. Bar = 1 mm for all figures.

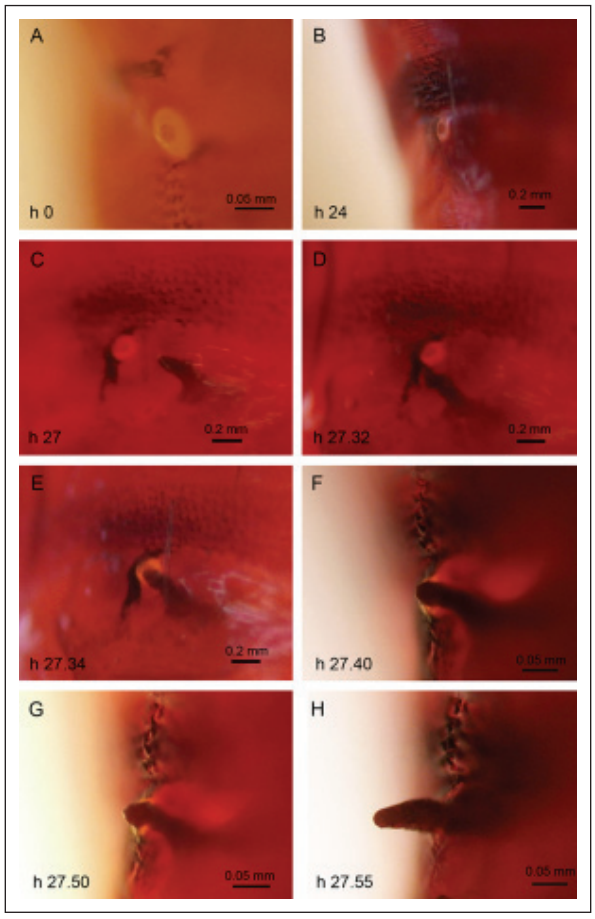

Figure 6. Development of pupal respiratory horn of *H. tumrasvini* as recorded via a video recorder. A-H: A remnant of the bubble membrane at h 0, and complete protrusion at h 27.55.

Figure 7. The connection between the pupal respiratory horn and anterior spiracle as shown in the pharate adult *H. tumrasvini*.

Parasarcophaga (*Liosarcophaga*) *dux* (Thomson) (Sukontason *et al*., 2008a; 2008b; 2010) due to a notably different biology. Results obtained from this study indicate the embryonation time of *H. tumrasvini*, reared at 25.0±2.0°C and 80.0±5.0% RH, was 10-12 h, which is consistent with embryonation of *C. rufifacies* (Sritavanich *et al*., 2009) but slower than that of *H. violacea* reared in ~6 h at 28.0±2.0°C and 70.0±5.0% RH (Chen *et al*., 2011). Also, the lengthy post feeding (~22 days), larval, and puparial periods of *H. tumrasvini* were longer than those of *H. violacea* (see Table 1), it is likely due to the lower temperature conditions (Chen *et al*., 2011).

First instar hatching from the eggshell was elucidated with the aid of a video recorder. In addition, the protrusion of pupal respiratory horns through puparial skin, at the position of bubble membrane was visualized to be completed at 27.5 h post-pupation. This protrusion of the pupal respiratory horn suggests oxygen supply requirements for the internal pharate adult as the structure observed in Fig. 7.

Furthermore, unique laboratory conditions are necessary for the blow fly development. This species exists in high altitude forests with low temperature and high humidity. Initially, our attempt to rear *H. tumrasvini* in ambient temperature (29-34°C and 42-74%RH) was ended up in failure. Rearing success occurred when we customized the cage at 25±2°C and 80±5% RH simulating a moist forest environment (see Fig. 1).

Mating conditions of *H. tumrasvini* were also distinct from that of other blow flies. Whereas other species of blow fly can reproduce freely while coexisting in a same cage, however, it was essential to separate newly emerged male and female *H. tumrasvini* and then reintroduce the both sexes for mating purposes in the ratio of 1:2, respectively. After successful mating, females produced eggs within the ovary (n = 107-312 eggs in single female), of which 80-218 eggs (75.3%; n = 845, hatching = 636) are able to hatch into first instar. This low fecundity limits the sampling in each interval and prohibits rearing experiments across multiple temperatures.

In conclusion, the present study expands the previous biological information of *H. tumrasvini* in describing the developmental rate of egg, larva and puparia. Such species-specific developmental data is imperative for future use in forensic investigations.

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ORIGINAL PAPER

Impact of abiotic factor changes in blowfly, *Achoetandrus rufifacies* (Diptera: Calliphoridae), in northern Thailand

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Abstract Understanding how medically important flies respond to abiotic factor changes is necessary for predicting their population dynamics. In this study, we investigated the geographical distribution of the medically important blowfly, *Achoetandrus rufifacies* (Macquart) (Diptera: Calliphoridae), and ascertained the response to climatic and physio-environmental factors in Chiang Mai, northern Thailand. Adult fly surveys were carried out every 2 weeks from May 2009 to May 2010 at 18 systematically randomized study sites in three districts of Chiang Mai province (Mueang Chiang Mai, Mae Rim, and Hang Dong), using reconstructable funnel traps with 1-day tainted beef offal as bait. During the study period, 8,861 adult *A. rufifacies* were captured, with peak densities being observed at the end of winter (i.e., late February) and throughout most of the summer (May to March). Population density had a weak but significant (α=0.05) positive correlation with temperature (r=0.329) and light intensity (r=0.231), and a weak but significant (α=0.05) negative correlation with relative humidity (r=−0.236). From the six ecological land use types (disturbed mixed deciduous forest, mixed deciduous forest, mixed orchard, lowland village, city town, and paddy field), greater fly densities were observed generally in the disturbed mixed deciduous forest and lowland village, but not in the paddy fields. In conclusion, *A. rufifacies* are abundant from the end of winter and throughout most of the summer in northern Thailand, with population density being weakly positively correlated with temperature and light intensity, but weakly negatively correlated with relative humidity. The greatest densities of this fly species were collected in disturbed mixed deciduous forest and lowland village land uses. The prediction of annual and season specific distributions of *A. rufifacies* were provided in each season and all-year patterns using a co-kriging approach (ArcGIS9.2).

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Introduction

The hairy maggot blowfly, *Achoetandrus rufifacies* (Macquart) (Diptera: Calliphoridae) is a medically important species commonly associated with anthropogenic activities. The adult fly acts as a mechanical carrier of pathogens that may cause diseases. For example, several species of bacteria were isolated from flies captured in Malaysia, e.g., *Escherichia coli*, *Klebsiella ozaenae*, *Proteus vulgaris*, *Pseudomonas fluorescens*, and *Burkholderia pseudomallei* (Sulaiman et al. 2000). Also, *A. rufifacies* were documented as a predominant mechanical carrier of helminth eggs (e.g., *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, *Taenia* spp., *Hymenolepis nana*) and protozoan cysts (e.g., *Entamoeba histolytica*/*dispar*, *Entamoeba coli*, *Cryptosporidium*, and *Giardia lamblia*) in Ethiopia (Fetene and Worku 2009). In Malaysia, the presence of *A. lumbricoides* and *T. trichiura*

eggs were detected in the gut lumen (Sulaiman et al. 1988). In addition to the fly larvae being a myiasis-producing agent in both humans and animals (Zumpt 1965), it plays another medically important role, since the larvae found in human corpses and/or death scenes can be used as entomological evidence in forensic investigations. Examples of such cases have been recorded in Europe (e.g., Benecke 2001), Thailand (Sukontason et al. 2007), Malaysia (Kavitha et al. 2013), India (Suri Babu et al. 2013), and Hawaii (USA) (Goff and Odom 1987). The presence of larvae in human corpses was reported at both outdoor and indoor death scenes (Sukontason et al. 2007; Kumara et al. 2012). In Australia, *A. rufifacies* was the second most abundant nuisance fly species collected in cattle feedlots, and it was noted that not only can such flies cause complaints from people living in the vicinity, but they can also contribute to production losses and animal welfare issues (Urech et al. 2012).

The geographical distribution of *A. rufifacies* has been expanding globally from Australasia/Oceania, Palaearctic, and Nearctic to Neotropical regions (Verves 2005). In the USA, flies are now found in Texas, Louisiana, Florida, California, Alabama, Arizona, Arkansas, Colorado, Georgia, Nebraska, Tennessee (Verves 2005), South Carolina (Cammack and Nelder 2010), and as far north as Wisconsin (Marche 2013). The species has also recently been identified in Canada (Rosati and VanLaerhoven 2008). It was the most abundant fly species collected from and near pig carcasses in Guam and Mexico (Jenson and Miller 2001; Valdes-Perezgasga et al. 2010) and the second most abundant species found in a regional survey of northern Thailand (Bunchu 2012).

A. rufifacies has a complete metamorphosis life cycle, comprising four distinct stages (egg, larvae or maggot, pupa, and adult) and their developmental rate is temperature dependent (Byrd and Butler 1997; Sukontason et al. 2008). The biology of this species has been reviewed in detail, i.e., taxonomy, ecological role, control strategy, and medical importance (myiasis, medico-legal importance) (Baumgartner 1993). Using applicability of the 304-bp cytochrome oxidase I gene fragment in molecular identification, *A. rufifacies* population displayed 0–0.8 % intraspecific variations in individuals collected from different locations in China (Aly and Wen 2013). This phenomenon was similar with work conducted in Australia (Harvey et al. 2003). Ecologically, although the seasonal distribution of this fly has been described in Australia (Vogt 1988; Urech et al. 2012), limited information exists for Thailand (Lertthamnongtham et al. 2003), especially with respect to abiotic factors (temperature, rainfall, and relative humidity). Hence, the objective of this study was to employ geographical information system (GIS) analytical techniques to investigate temporal population dynamics of this fly in relation to climatic factors and land use types in urban and suburban areas of Chiang Mai, northern Thailand.

Materials and methods

Study area

Three districts of Chiang Mai province were selected for this study: urban Mueang Chiang Mai (MU), suburban Mae Rim

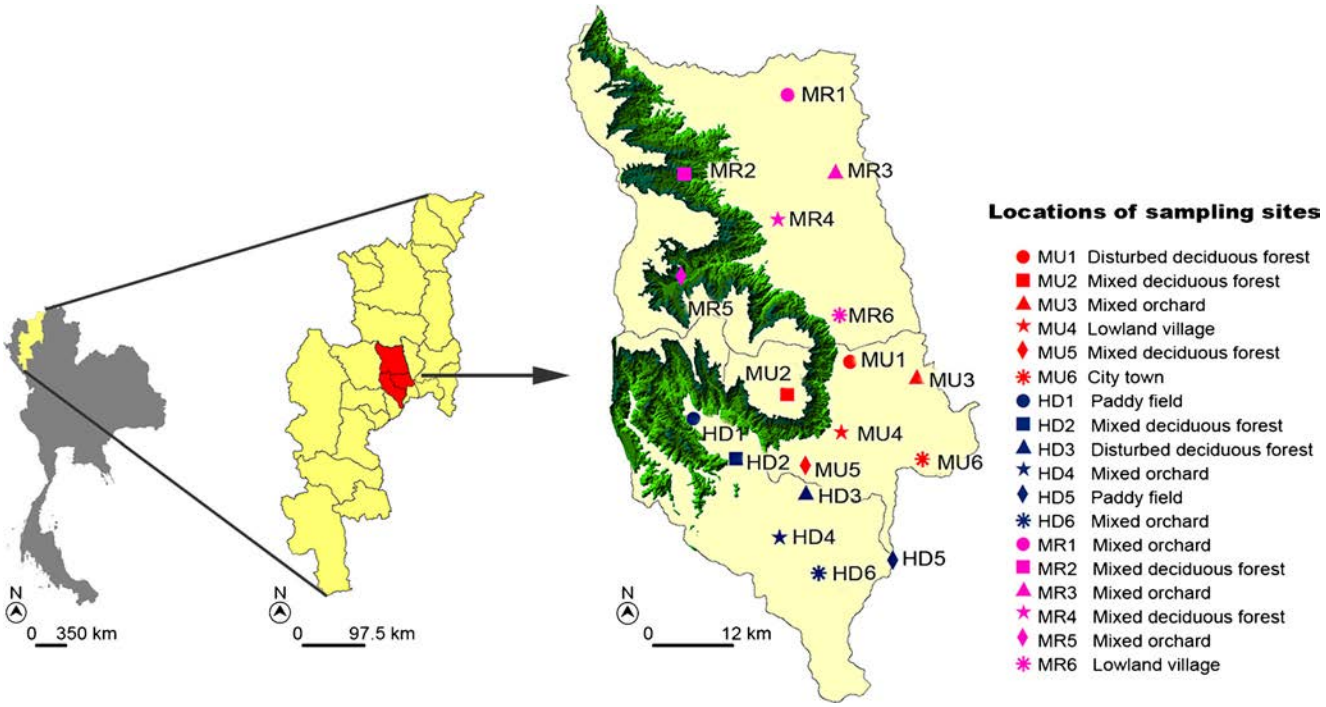

Fig. 1 Map of Thailand showing the three sample districts (Mueang Chiang Mai, *MU*; Mae Rim, *MR*; and Hang Dong, *HD*) of Chiang Mai province and the *A. rufifacies* sampling locations. *Green shade* represents mountainous area

Fig. 2 A reconstructable funnel trap used for adult fly collection

(MR), and Hang Dong (HD) to the north and south of MU, respectively (Fig. 1). Following a systematic random sampling method (Rogers and Williams 1993), the study area was plotted using the topographical maps of Chiang Mai (MapMagic™ scale 1: 150,000 with a UTM projection type, Everest Spheroid and the Indian 1975 Datum). A more detailed discussion of the sample site selection procedure is provided (Ngoen-klan et al. 2011). The land use categories were obtained from the Geo-Informatics and Space Technology Development Agency (Public Organization), Northern Region, Thailand.

Fly collection

Fly collection was performed every 2 weeks from May 2009 to May 2010 using an in-house prototype reconstructable funnel trap kit (Fig. 2). The collection procedure was described previously (Ngoen-klan et al. 2011). Two hundred and fifty grams of 1-day tainted beef offal (Bunchu et al. 2008) was used as bait positioned under the trap. The traps were exposed for a 1-h period between 9.30 a.m. and 12.00 noon. The following physical data was recorded for each study site: the GPS coordinates [Garmin™ eTrex Handheld GPS (China)], temperature (degree Celsius) and relative humidity (percentage) [Digital Hygro-Thermo Meter (DHT-1); Daeyoon Scale Industrial Co., Ltd (China)] and light intensity (lux) [LUX/FC light meter TM-204 Tenmars (Taiwan)].

Fig. 3 Ecological land use types sampled in this study. **a** Disturbed mixed deciduous forest. **b** Mixed deciduous forest. **c** Mixed orchard. **d** Lowland village. **e** City town. **f** Paddy field

The collected flies were transported to the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University within 1 h. Trapped specimens were sacrificed by placing them in a freezer set at 0 °C for 2 h, then counted individually, sexed, and identified.

Statistical analysis

For the statistical and geospatial analyses, all raw data were transformed using a logarithm base 10 ($\log_{10}$) transformation to improve approximation of normality. The relationship of climatic factors with fly populations was determined using bivariate correlation analysis and Pearson Product Moment Correlation (r), while the data for all land uses of these three regions were combined before analysis. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test were employed to investigate the relationship between land use types and mean total number of *A. rufifacies* using SPSS 12.0 for Windows (α=0.05). A co-kriging approach (using the Geostatistical Analyst tool of ArcGIS 9.2) was utilized to explore the spatial relationship between fly population and climate data (Ngoen-klan et al. 2011).

Results

A total of 18 study sites were selected for fly collection. Figure 3 shows the six ecological land use types sampled (disturbed mixed deciduous forest, mixed deciduous forest, mixed orchard, lowland village, city town, and paddy field). Collectively, a total of 8,861 adult *A. rufifacies* were captured during the 1-year study period. Trapped fly numbers varied seasonally with peak numbers occurring around late February coinciding with end of winter/early summer followed by the summer (April to June) (Fig. 4, upper). Fly numbers decreased during the rainy season, rapidly declining at the end of rainy season, and remained low throughout winter (September to January). A similar trend was observed in all three districts. There were more *A. rufifacies* females than males in all sample collections; the average ratio was 3 female:1 male (Fig. 4, lower). Neither nongravid nor gravid was examined in the captured *A. rufifacies*.

Pearson product moment correlation results indicated that fly population density had a weak positive correlation with temperature (r=0.329, P=0.000) (Fig. 5a) and light intensity (r=0.231, P=0.000) (Fig. 5c). However, a negative correlation was found between fly population and relative humidity (r=−0.236, P=0.000) (Fig. 5b). Based on land use types in central Chiang Mai (Table 1), it was observed that flies exhibited a preference for disturbed mixed deciduous forest and lowland village, followed by mixed deciduous forest and mixed orchard. A significantly lower abundance of flies was captured in the paddy field area (Bonferroni post hoc test, P<0.05).

To assess the spatial distribution patterns of *A. rufifacies*, co-krigged seasonal maps were produced using fly population

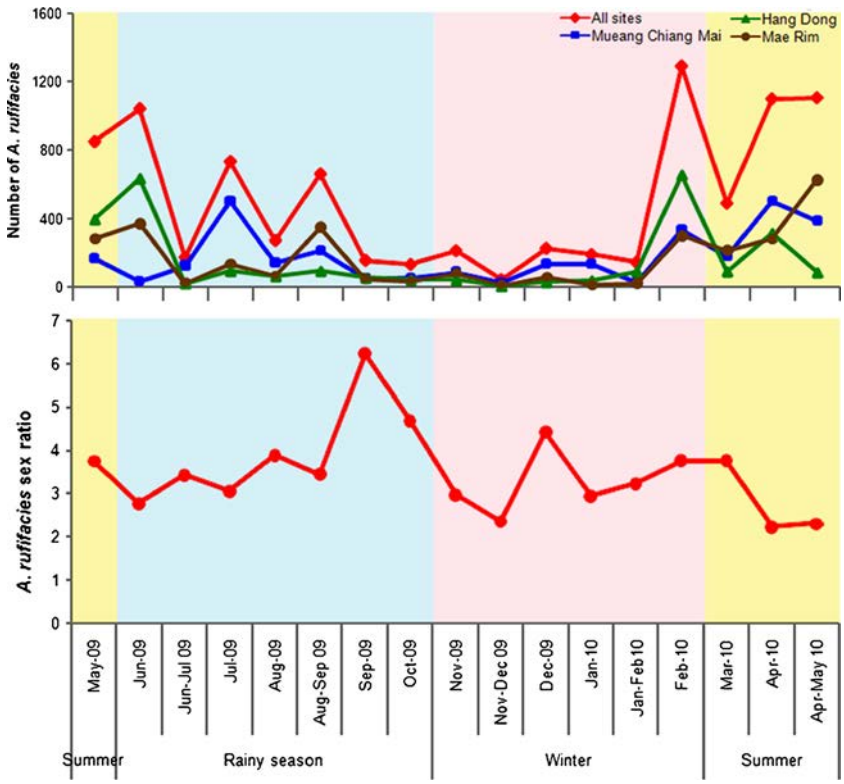

Fig. 4 Monthly fluctuations in population density of *A. rufifacies* determined using a reconstructable funnel trap baited with 1-day tainted beef offal in Chiang Mai, northern Thailand, May 2009 to May 2010

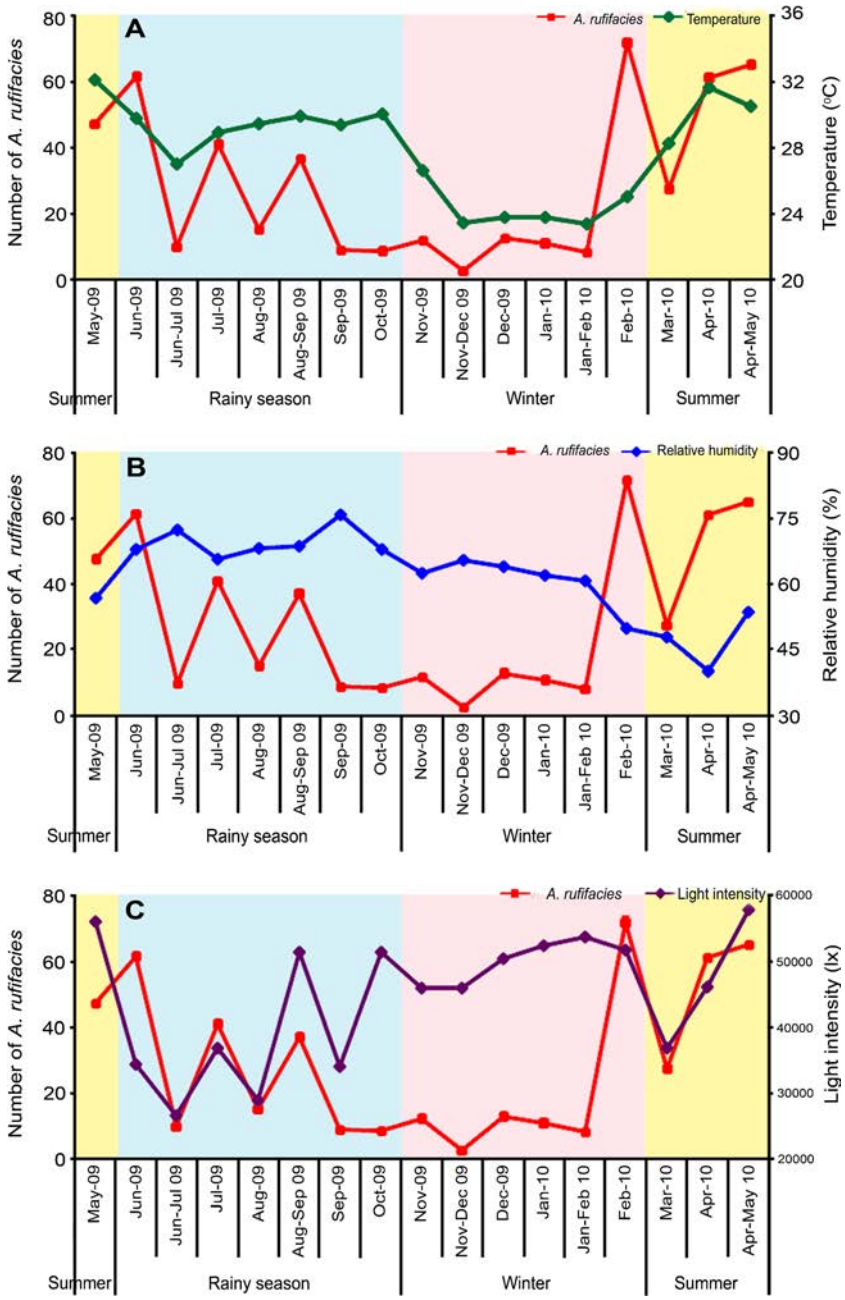

Fig. 5 Correlation of the overall *A. rufifacies* population density with climatic factors. **a** Weakly positive with temperature (r=0.329, P=0.000). **b** Negative correlation with relative humidity (r=−0.236, P=0.000). **c** Weakly positive with light intensity (r=0.231, P=0.000)

data, land use types, temperature, relative humidity, and light intensity. The total predicted seasonal abundance across the three districts is displayed in Fig. 6a–c, while the all-year pattern is shown in Fig. 6d. The red areas represent the highest population of *A. rufifacies* predicted by the analysis, while the blue areas represent the lowest population density predicted.

Discussion

Although the impact of *A. rufifacies* on humans and animals has been well documented, information pertaining to the fly's biology and ecology is scarce. Local surveys of flies of forensic importance, which include *A. rufifacies*, are critical in identifying what species are present in the various areas (Brundage et al. 2011). This study was the first to determine the population dynamics over a full year and to predict population density of *A. rufifacies* in Thailand using systematic random sampling and GIS.

Blowfly abundance was significantly influenced by climatic factors. Our results further indicate that although found year-round, *A. rufifacies* exhibited a bimodal peak with one maximum at the end of winter or early summer and another through the summer and shading into the

Table 1 The population density of *A. rufifacies* based on land use types in Chiang Mai, northern Thailand, using a reconstructable funnel trap

Land use types	Number	Log_{10} no. of AR[a]
Disturbed mixed deciduous forest	34	1.26±0.64a
Lowland village	34	1.26±0.79a
City town	16	1.09±0.54ab
Mixed orchard	101	0.81±0.67b
Mixed deciduous forest	84	0.79±0.78b
Paddy field	34	0.61±0.68b

Different lowercase letters indicate significant difference (Bonferroni post hoc test, $P<0.05$)

[a] Mean±SD of flies captured

rainy season and winter. Similar findings were reported in Australia, with fly density peak being in the summer months from December to February (Urech et al. 2012). Greater fly density coincided with the summer period which was characterized by higher temperature, and this would favor a rapid developmental rate (Zuha et al. 2012). In this context, the increasing temperature during early summer probably generates flight activity from breeding places. In Australia, it was found that flies did not enter the trap at temperatures <13 °C (Vogt 1988). Results from our study agree with this observation as only a few *A. rufifacies* were captured in November to December (see Fig. 4), when temperatures were lowest, around 18 °C. Also, *A. rufifacies* require a temperature >15.0 °C to pupate normally (O'Flynn 1983) which may further explain the temperature related to fly density results. Interestingly, adult *A. rufifacies* were still observed flying around deer carrion when the ambient temperature was ~9 °C in South Carolina of USA (Cammack and Nelder 2010).

In addition to temperature, our results clearly indicated that *A. rufifacies* density was negatively correlated with relative humidity, which is consistent with the findings in India (Das et al. 1979), *Chrysomya megacephala* (F.) in Thailand (Ngoen-klan et al. 2011), with *Achoetandrus albiceps* (Wiedemann), and *C. megacephala* in Brazil (Azevedo and Kruger 2013).

The weak positive correlation observed between *A. rufifacies* density and light intensity is in contrast with studies on field-captured *C. megacephala* that suggest no relationship ($P=0.420$) (Ngoen-klan et al. 2011). Similarly, density-related experiments using an I-box wind tunnel were also negatively associated with light intensity (Moophayak et al. 2013b). A greater attractive index to bait occurred in the afternoon (1–5 p.m.) as compared to the morning hours (9–12 a.m.). Perhaps, the increased attraction of *C. megacephala* to bait in higher light intensity is due to greater visibility for landing (Burkett and Butler 2005). More research is needed to confirm whether light intensity impacts *A. rufifacies* behavior similarly to *C. megacephala*. Currently, automatic trap-based field experiments are underway to capture *A. rufifacies* and *C. megacephala* behavior data at 3 h increments.

In this study, we found more *A. rufifacies* females than males in traps baited by tainted beef, a finding also noted by other studies (Mulieri et al. 2006; Bunchu et al. 2008; Ngoen-klan et al. 2011). The greater proportion of captured females may result from proteinaceous food requirements for oviposition, similar with *C. megacephala* (Bunchu et al. 2008). Recent experiments conducted in 2013 revealed that 80 % of total *A. rufifacies* collected were nongravid females ($n\sim6{,}000$ specimens) (KLS, unpublished data). Dissection of *A. rufifacies* revealed compact cells of ovary and densely supplied tracheoles, suggesting a newly emerged young female fly population.

In this study, *A. rufifacies* were densely localized in disturbed mixed deciduous forest and lowland village land use types. Similarly, earlier research conducted in urban Chiang Mai City identified *A. rufifacies* larvae frequently in human corpses from both environments (Sukontason et al. 2007). A survey in northern Thailand also found *A. rufifacies* in varying elevations from lowlands to 1,050 m above sea level, indicating an extensive geographical range for this species (Moophayak et al. 2013a). As for urban settings per se, although we found that lowland villages had higher fly densities, we did not target sampling to specific anthropogenic activities. In Ethiopia, high densities of this species were found at butchery sites, moderate numbers at defecating ground sites, and low amounts in rubbish bins at market places (Fetene and Worku 2009). Further studies of localized fly densities at specific anthropogenic activity sites could also be beneficial in Thailand.

In this study, the estimated annual distribution of *A. rufifacies* was similar to each of the three seasonal periods, with high populations consistently identified in disturbed mixed deciduous forest and lowland village. The mapping facilitates an understanding of the geographical distribution of this species in the study area, and offers the potential to identify approaches for management.

In conclusion, this study provides two important findings. First, population density of *A. rufifacies* correlates with several abiotic factors; positive with both temperature and light intensity, and negatively with relative humidity. Secondly, GIS co-kriging methods effectively visualized the relationship between fly population density and land use. Taken together, this data can contribute to baseline information for local control strategies and add value to forensic entomology approaches.

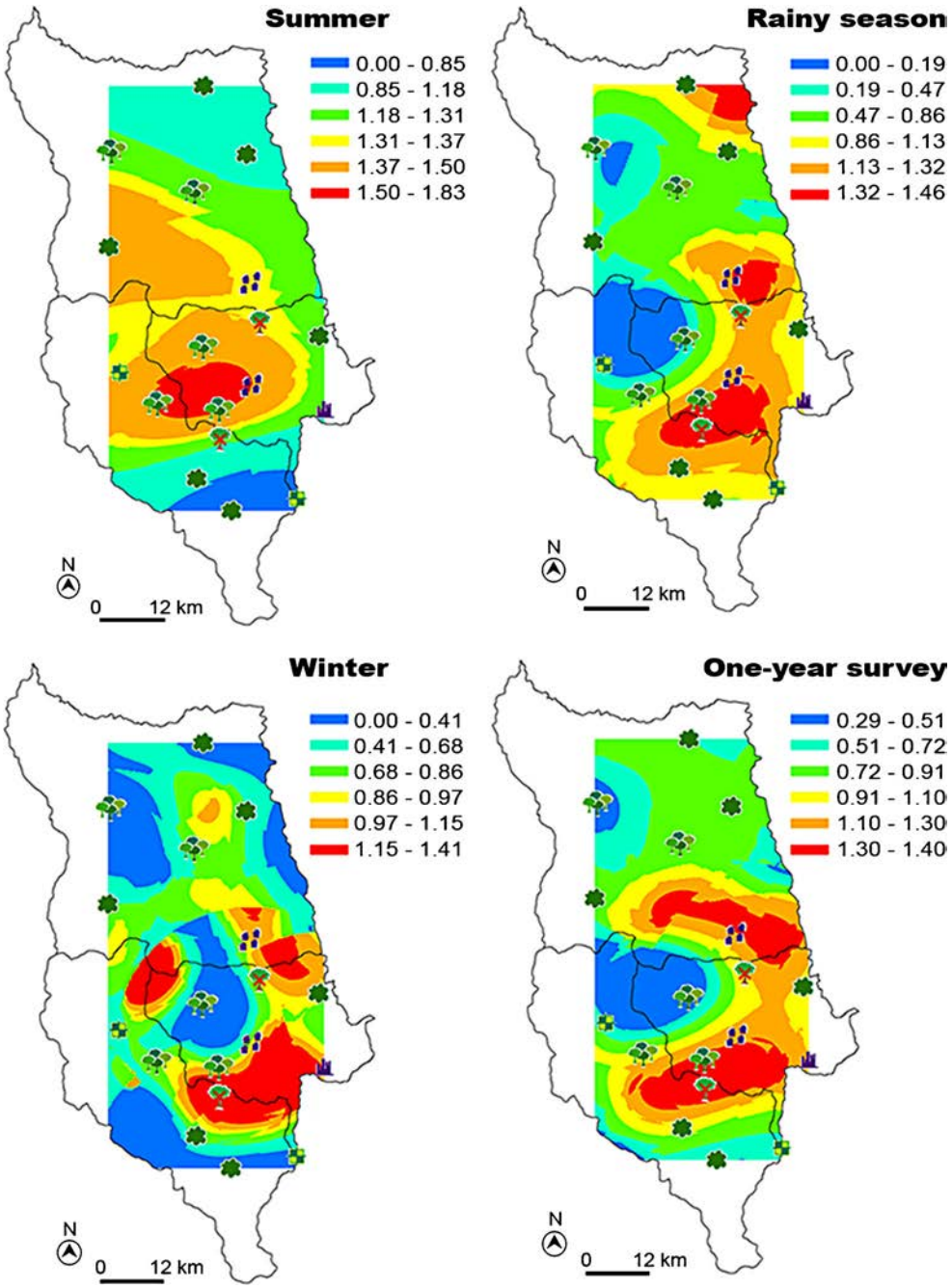

Fig. 6 Co-kriging to predict spatial distribution of *A. rufifacies* in three districts of Chiang Mai, northern Thailand. The *red areas* represent the highest population of *A. rufifacies* predicted by the analysis, while the *blue areas* represent the lowest population density predicted

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BIOLOGICAL RESEARCH

RESEARCH ARTICLE Open Access

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

Kabkaew L Sukontason[1*], Sangob Sanit[1], Tunwadee Klong-klaew[1], Jeffery K Tomberlin[2] and Kom Sukontason[1]

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

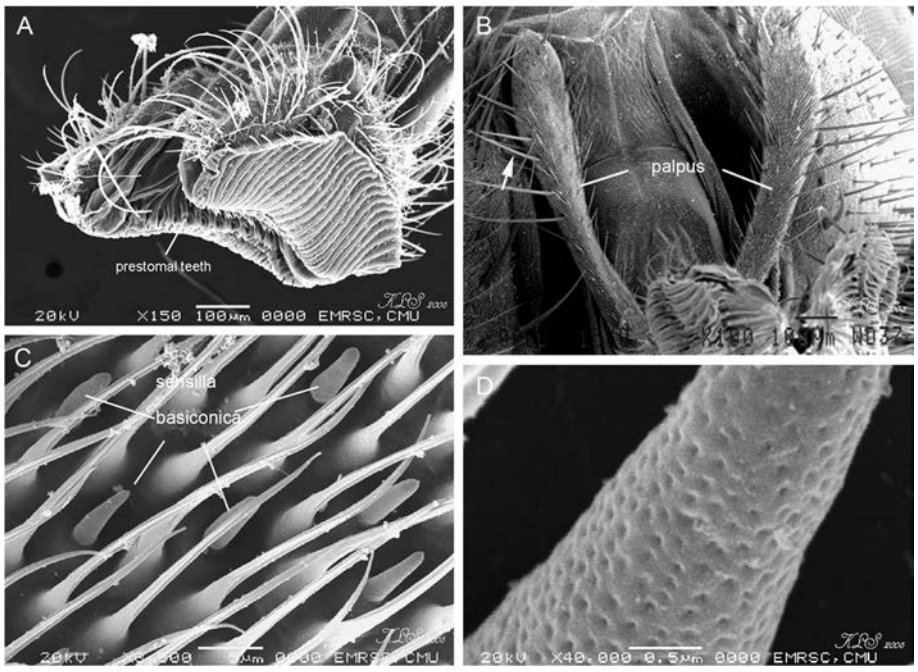

Figure 2 Scanning electron micrographs of adult *S. dux*. **A**: Sponging mouthpart showing everted labella lobes and numerous sensilla trichodea. Prestomal teeth are bifurcation at the tips. **B**: Palpus showing strong and long sensilla chaetica (arrow). **C**: Surface of palpus with amount of sensilla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

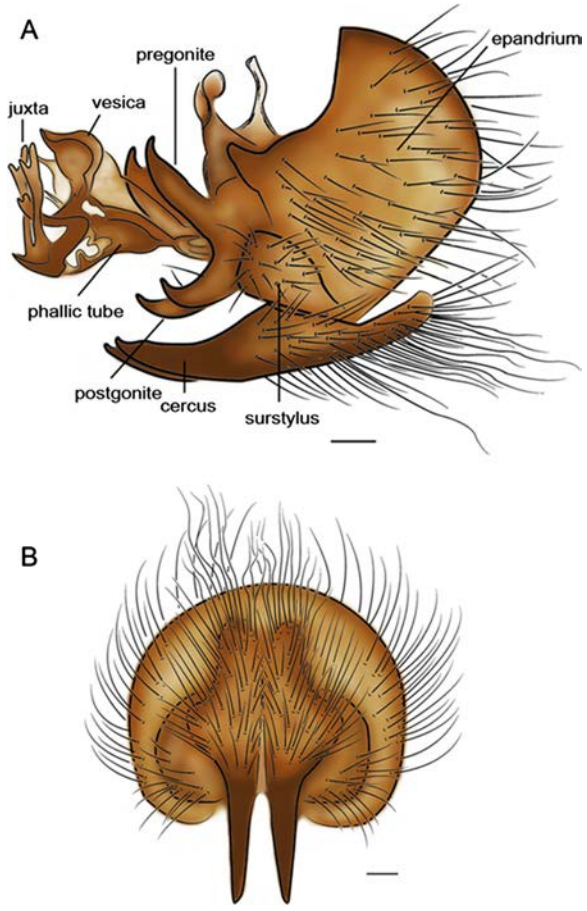

Figure 3 Illustrations of male genitalia *S. dux*. A: lateral view of genitalia displaying short, broad phallus, slightly curved upward apically of pregonite and postgonite, pointed and curved apically cerci and apically bifurcated juxta. **B**: Epandrium, cerci and surstyli, posterior. Online figure 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

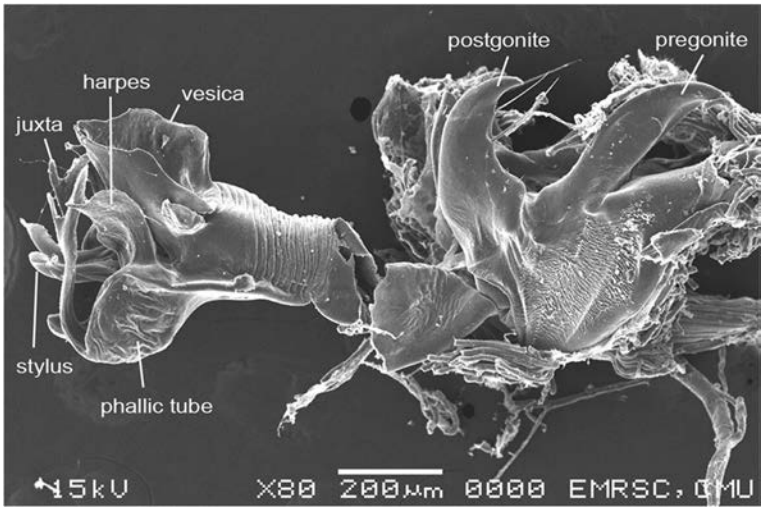

Figure 4 Scanning electron micrograph of male genitalia *S. dux* showing trumpet-shaped, anteroventrally expanded vesica, bifurcated juxta. Terminology followed Giroux et al. [15].

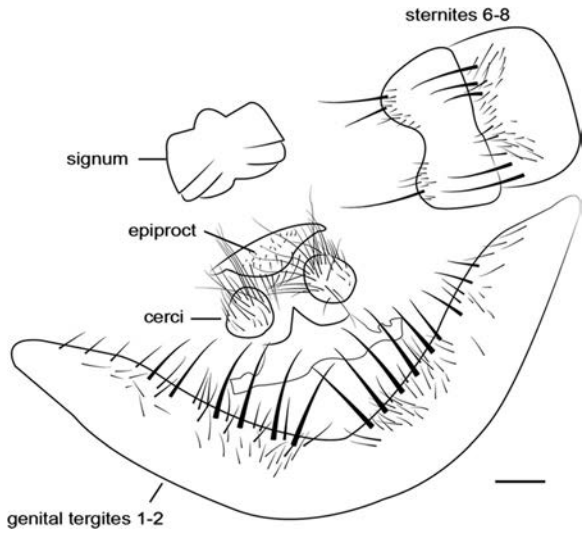

Figure 5 Illustration of ovipositor *S. dux*. Bar = 0.1 mm. Terminology followed Richet et al. [2].

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

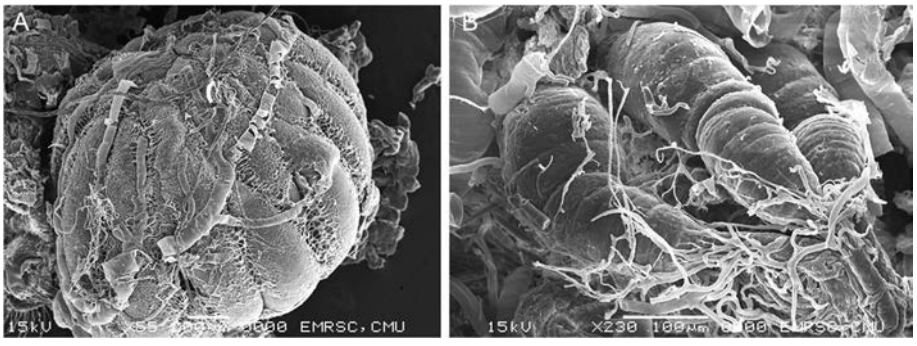

Figure 6 Scanning electron micrographs of 7-d-old female *S. dux*. A: Ovary. **B**: Spermathecae.

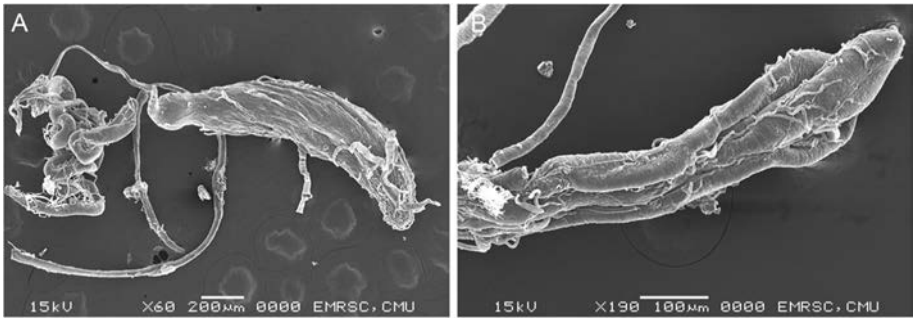

Figure 7 Scanning electron micrographs of male *S. dux*. A: testis and accessory gland. **B**: Higher magnification of distal part of accessory gland.

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

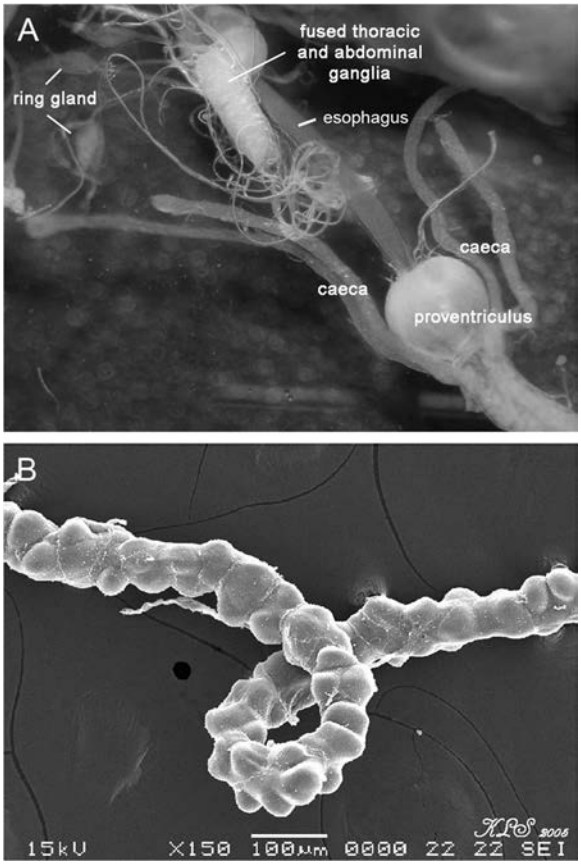

Figure 8 Micrographs of third instar *S. dux*. A: Anterior end displaying esophagus, proventriculus (cardia), gastric caecae. A pair of ring gland and fused thoracic and abdominal ganglia are apparent. **B**: Malpighian tubules.

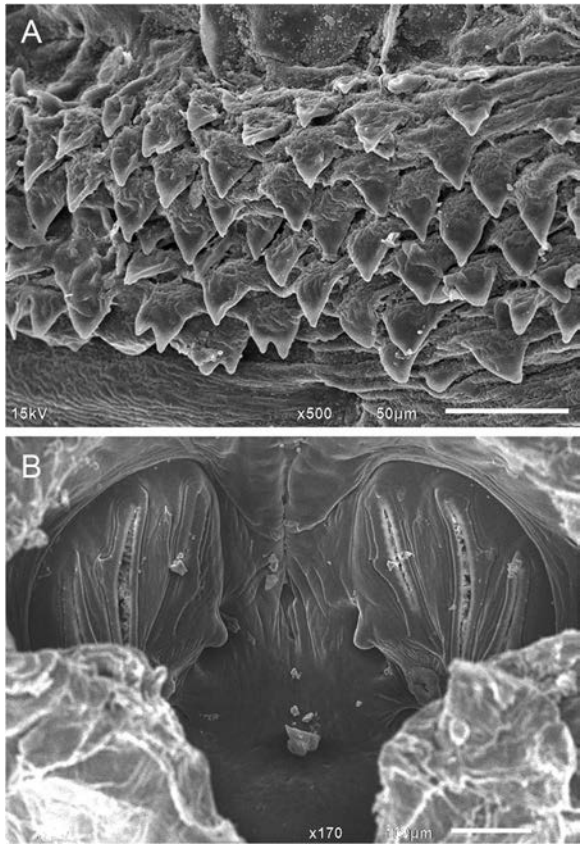

Figure 9 Scanning electron micrographs of puparium *S. dux*. A: Broad and triangular intersegmental spines between the prothorax and mesothorax. **B**: Posterior spiracle.

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.

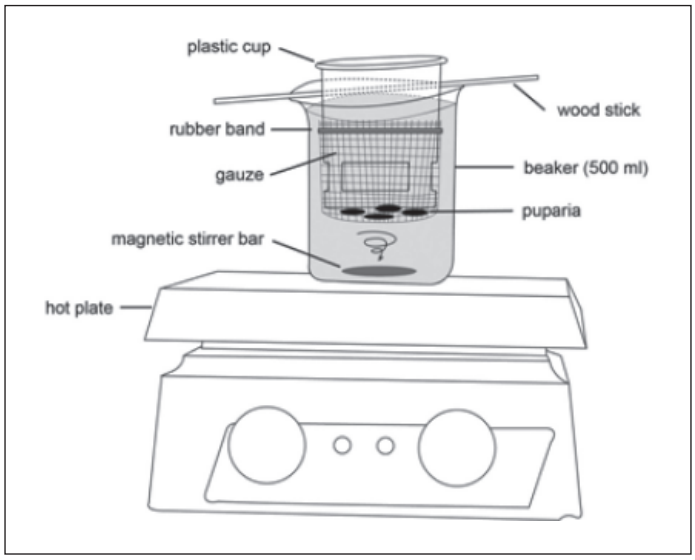

Figure 1. Illustration of apparatus for cleaning puparia (image not to scale)

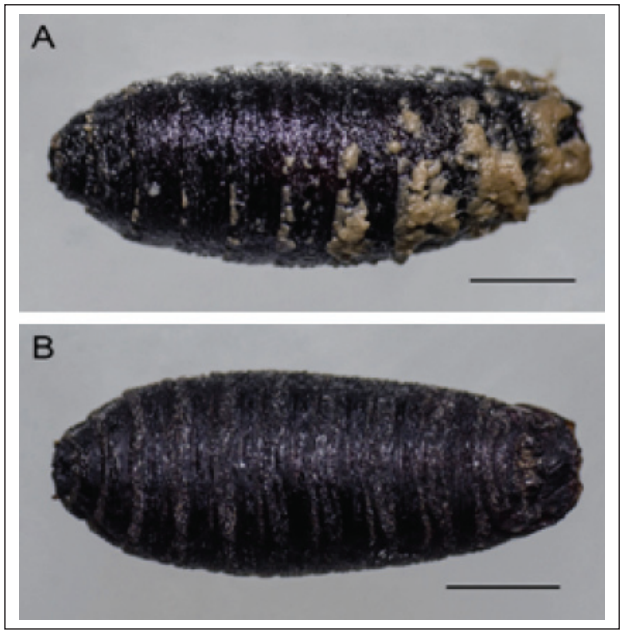

Figure 2. Puparia of *L. pattoni* before (A) and after (B) cleaning process. Bar = 2 mm

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)

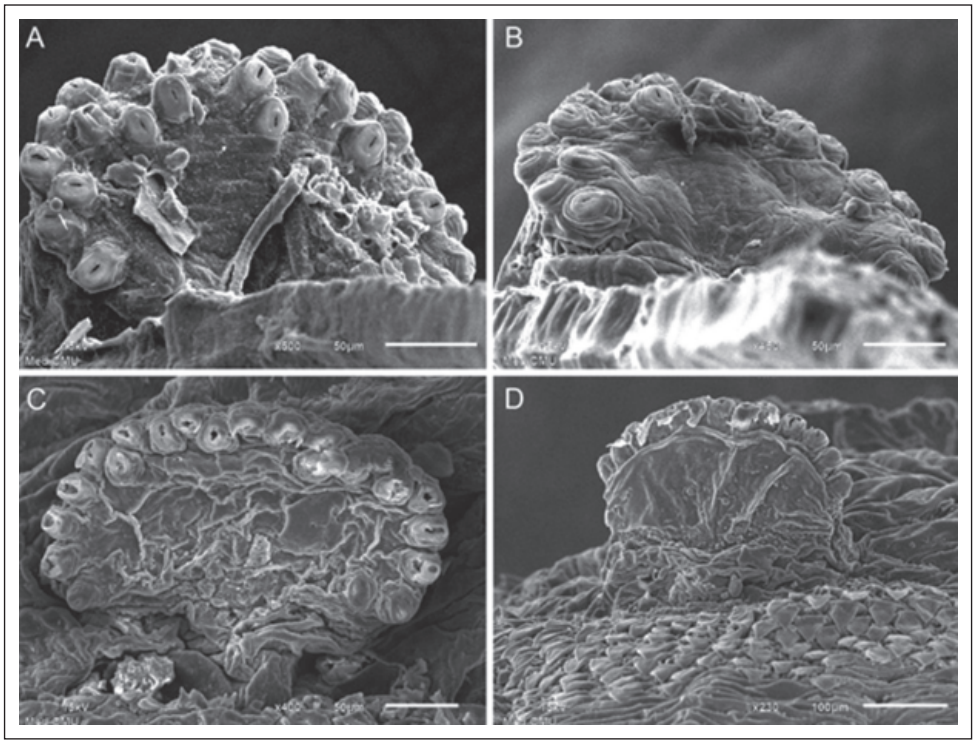

Figure 3. SEM micrographs of anterior spiracles of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*

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

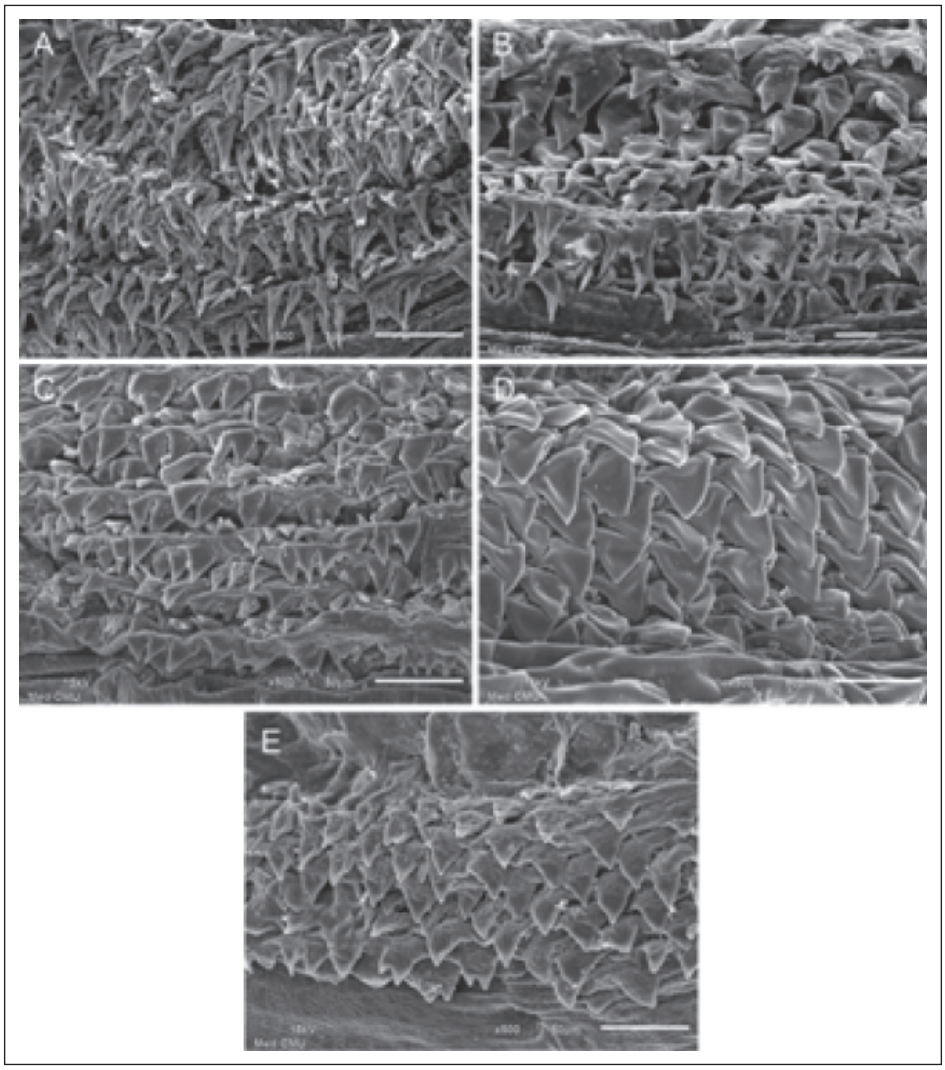

Figure 4. SEM micrographs of intersegmental spines between the prothorax and mesothorax of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*. **E:** *P. dux*

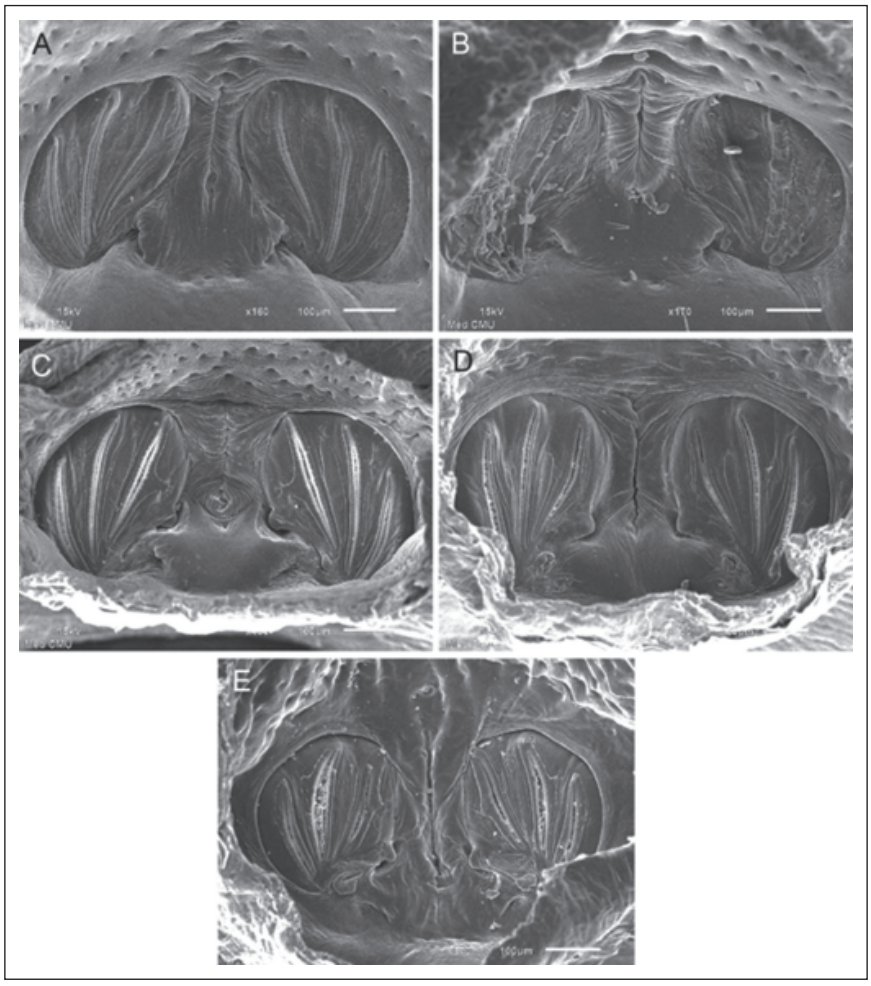

Figure 5. SEM micrographs of posterior spiracles of flesh fly puparia. **A:** *B. nathani*. **B:** *B. peregrina*. **C:** *L. pattoni*. **D:** *L. ruficornis*. **E:** *P. dux*

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

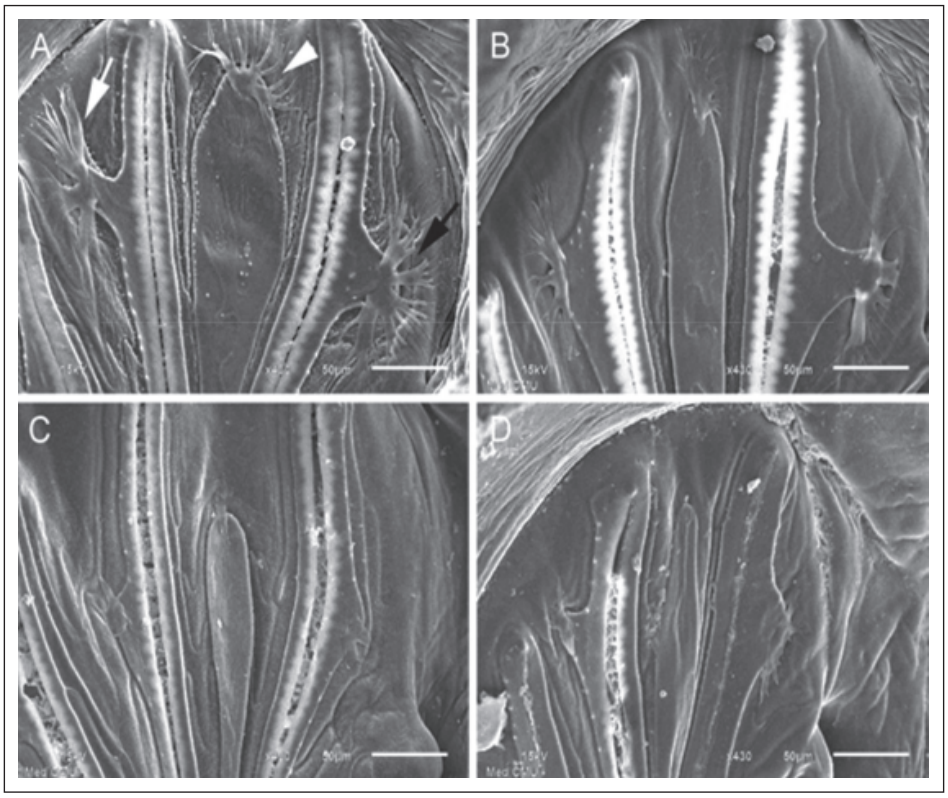

Figure 6. SEM micrographs of spiracular tufts on left spiracle of flesh fly puparia. **A:** *B. nathani* showing extensively branches, at the area adjacent to the inner spiracular slit (black arrow), at the top of the plate between inner and middle spiracular slits (white arrowhead) and adjacent to the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

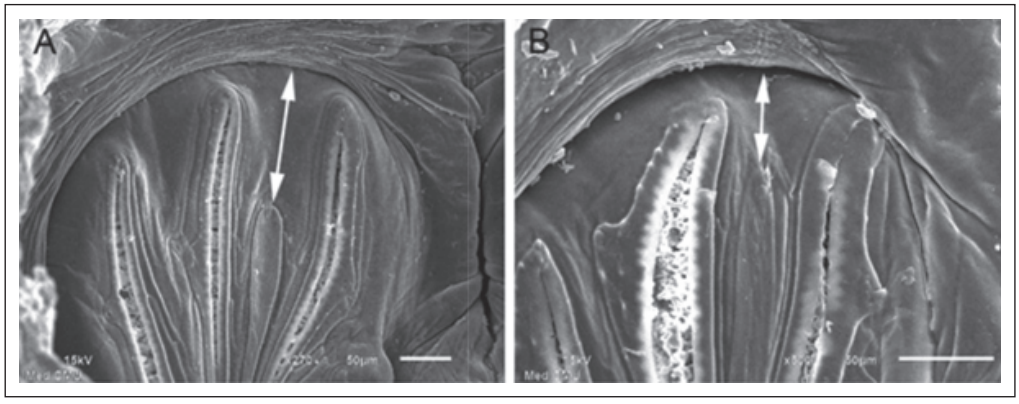

Figure 7. SEM micrographs of end up position of plate between inner and middle spiracular slits of posterior spiracle of flesh fly puparia. *L. ruficornis* showing much lower (long distance from peritreme) (A) than *P. dux* (short distance from peritreme) (B)

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

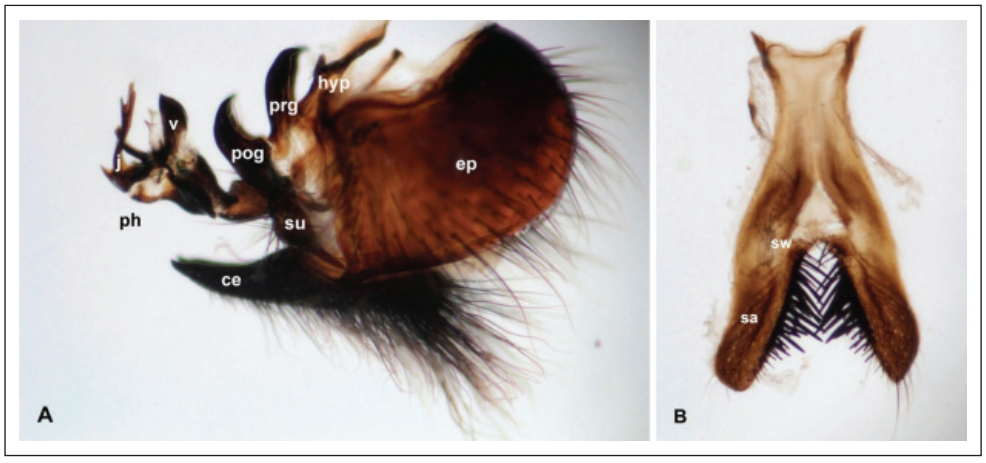

Figure 2. Light micrographs of the male genitalia. (A) Lateral view of the terminalia *P. dux* showing the cercus (ce) normal in shape, not enlarged dorsally and without spines; ventralia (v) composed of 1 lobe; lateral arm of juxta (j) bifid at apex. Ph, phallus; ep, epandrium; hyp, hypandrium; su, 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)

- CS5 setulose along anterior margin along more than basal 1/2, at most almost entirely; hind tibia with 2 *av*, without fringe on postero-ventral surface ..... *My. caudagalli* (Böttcher)

Key to the species *Iranihindia*

1. Tergite 3 with strong marginal bristles (*mb*); stylus long and blunt …….....*I. martellatoides* (Baranov)

- Tergite 3 without median *mb*; stylus short and pointed ..... *I. martellata* (Senior-White)

Discussion

Thailand is located in the tropics and contains various ecological niches and habitats, providing an ideal environment for variety of insects, including flesh flies. Based on the catalogue of Sarcophagidae of the World (Pape 1996), *Mi. tibita* is distributed in the Palaearctic region – China (Tibet). This study is the first record of this species in Thailand. *Mi. tibita* share a similar characteristic with another related species, *Miltogramma bimaculatum* Chao & Zhang. There is a noteworthy difference in the appearance between them, based on the illustrations of Fan (1992) (Figs. 1205e,1207e) in that *Mi. tibita* possesses fine long hairs on the dorsal surface of second to fourth tarsomere of the foreleg, whereas those of *Mi. bimaculatum* occur on the fourth tarsomere and are longer in length.

Regarding *I. martellata*, this species seems to be widely distributed in and around the Indian subcontinent of the Oriental region – India, Nepal, and Sri Lanka (Pape 1996), but it has been newly recorded in Malaysia recently (Tan *et al*. 2010). Habitats seem to be variable, such as a school cafeteria and restaurants (in this study) that are located in urban areas, gardens or bushes in residential areas and a sandy beach in Malaysia (Tan *et al.*, 2010), bushes and flowering plants (Nandi, 2002), or forests in India (Nandi, 1989).

In conclusion, *Mi. tibita*, *My. situliformis*, and *I. martellata* were newly recorded in Thailand, making a total of 86 species of sarcophagid flies in the country.

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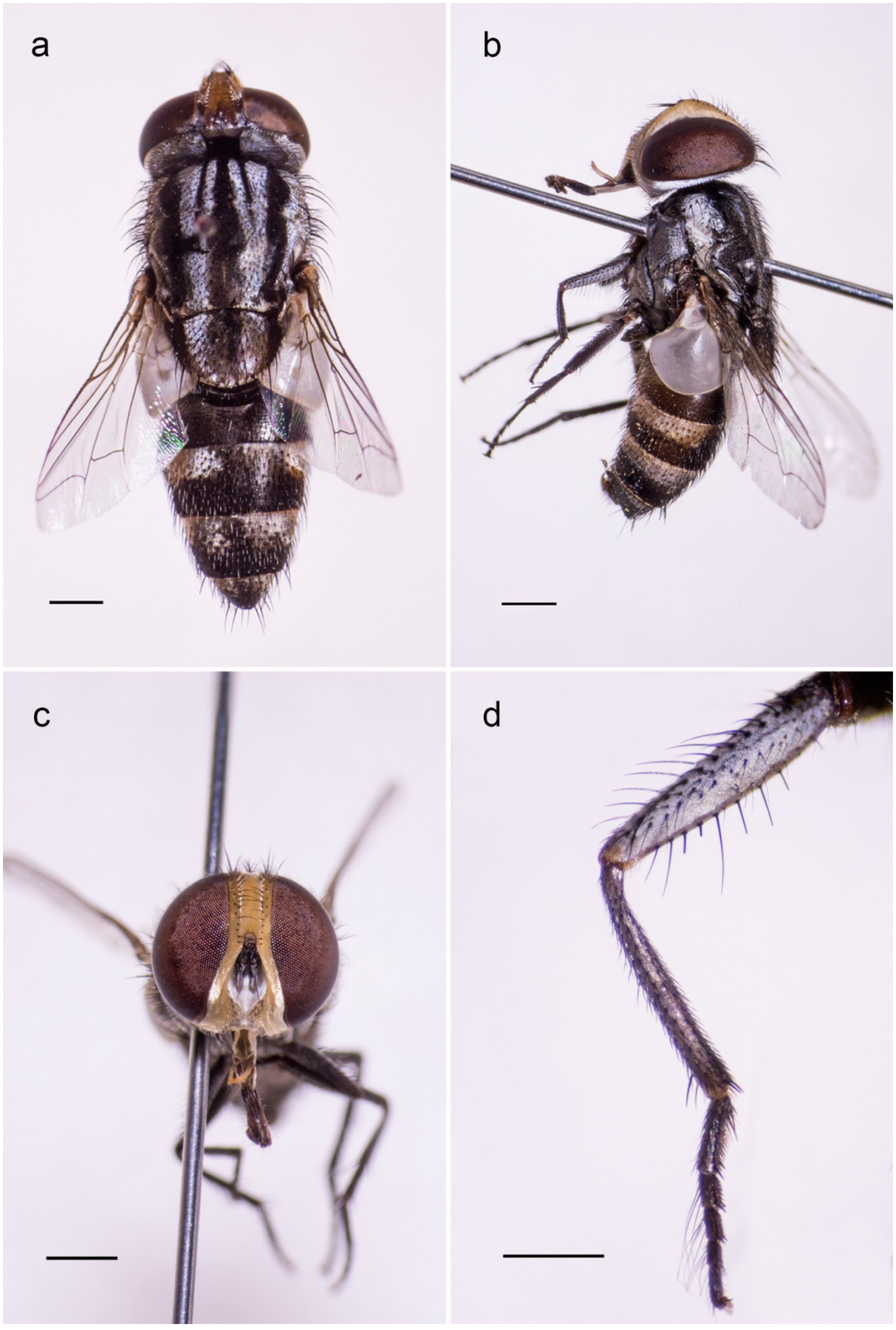

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309 **Fig. 1** Male *Mi. tibita*. (a) Dorsal view. Bar = 1 mm. (b) Lateral view. Bar = 1 mm. (c)
310 Anterior view of the head showing golden parafrontal and parafacial, blackish antennae.
311 Bar = 1 mm. (d) Second to fourth tarsal segment of foreleg showing long hairs on dorsal
312 surface. Bar = 0.5 mm.

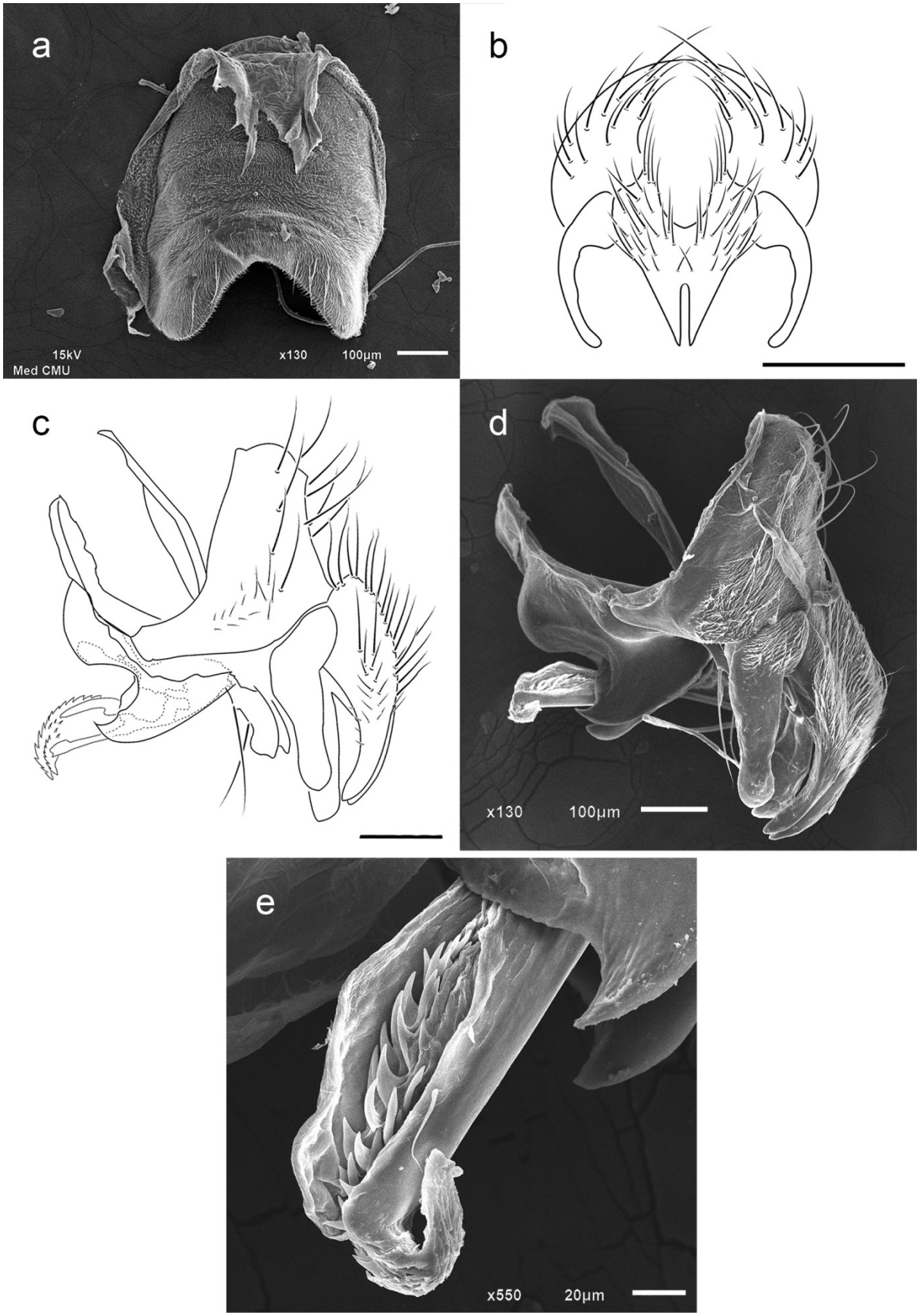

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Fig. 2 Male *Mi. tibita*. (a) SEM micrograph of fifth sternite. (b) Posterior view of cerci and surstyli. Bar = 0.1 mm. (c) Lateral view of male genitalia. Bar = 0.1 mm. (d) SEM micrograph of male genitalia. (e) SEM micrograph of aedeagus showing tubular juxta and membranous antero-lateral and serrated medially median stylus.

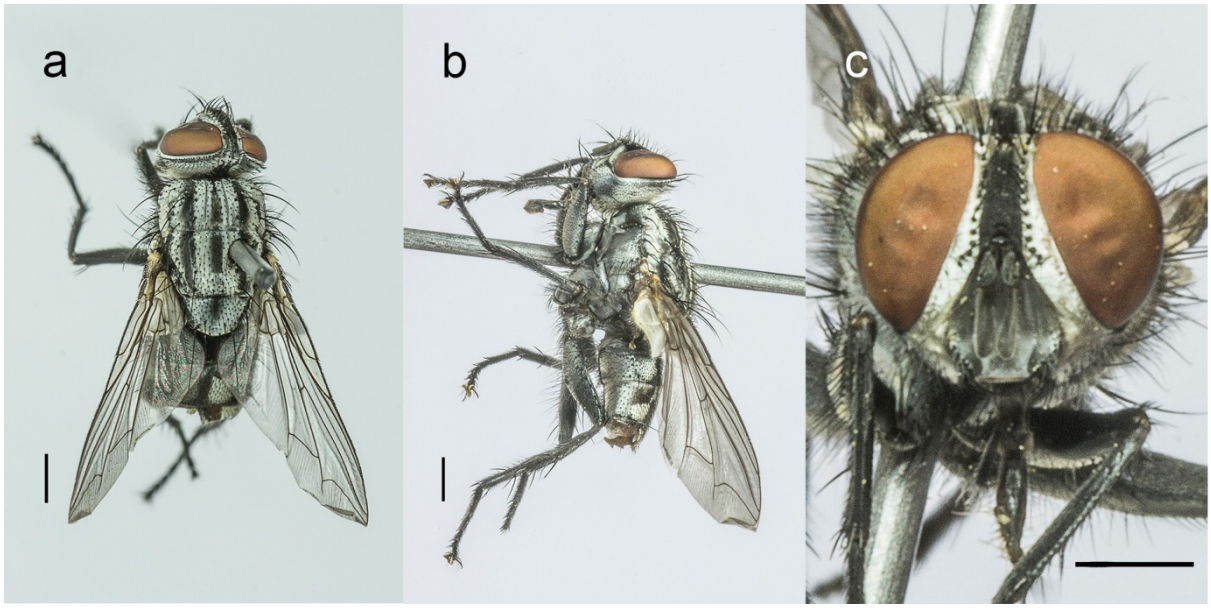
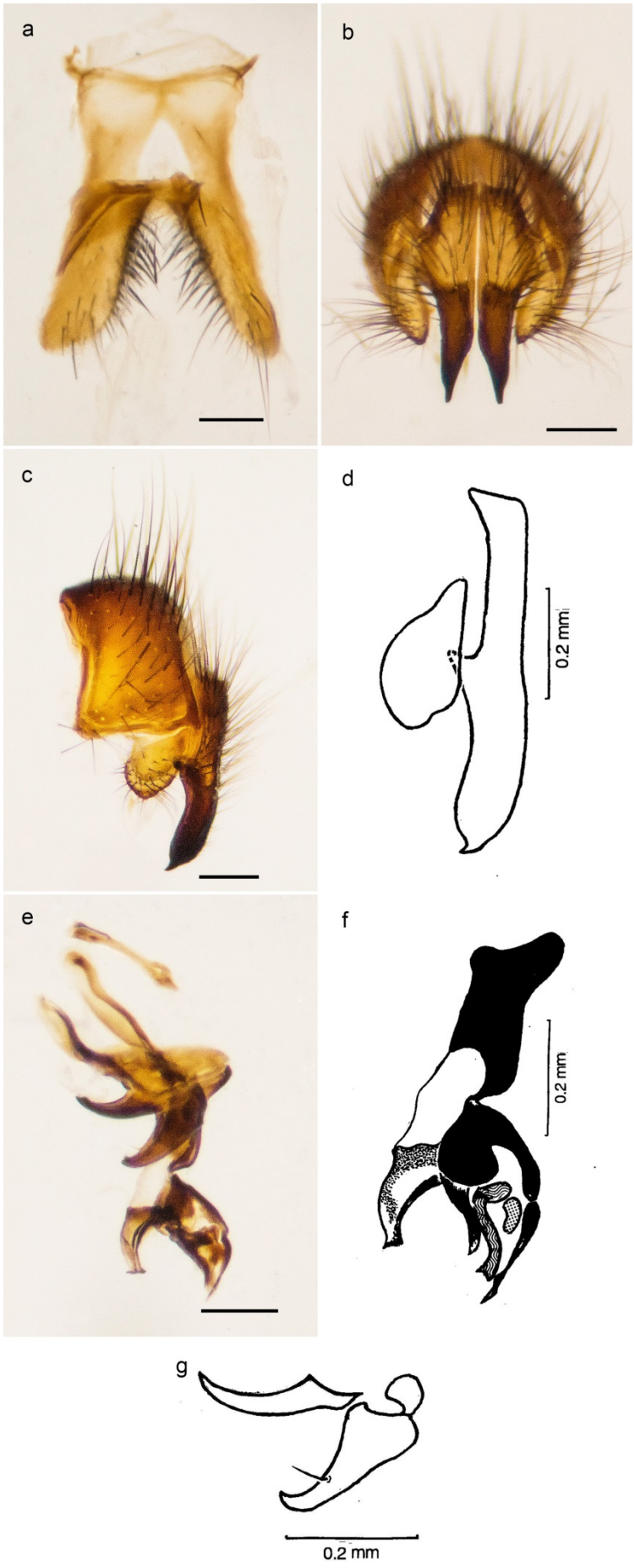


Fig. 3 Male *My. situliformis*. (a) Lateral view. Bar = 1 mm. (b) Dorsal view. Bar = 1 mm. (c) Anterior view of the head showing golden parafrontal and parafacial, blackish antennae. Bar = 1 mm.



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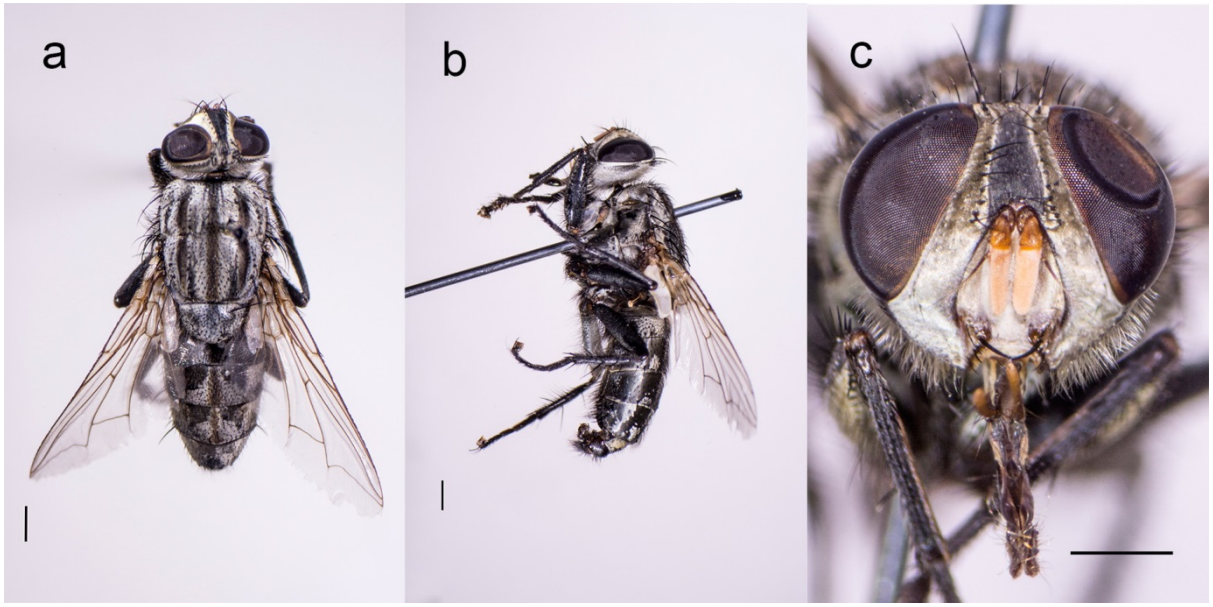
323 **Fig. 4** Male *My. situliformis.* (a) Light micrograph of fifth sternite. (b) Light micrograph of
324 cercus and surstylus, posterior view**.** (c) Lateral view of cercus and surstylus. (d)
325 Illustrations of cercus and surstylus, lateral view. (e) Light micrograph of aedeagus,
326 pregonite and postgonite, lateral view. (f) Illustrations of aedeagus, lateral view. (g)
327 Illustrations of pregonite and postgonite, lateral view.



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329 **Fig. 5** Male *I. martellata*. (a) Dorsal view. Bar = 1 mm. (b) Lateral view. Bar = 1 mm. (c)
330 Anterior view of the head showing yellowish-red to orange antennae. Bar = 1 mm.

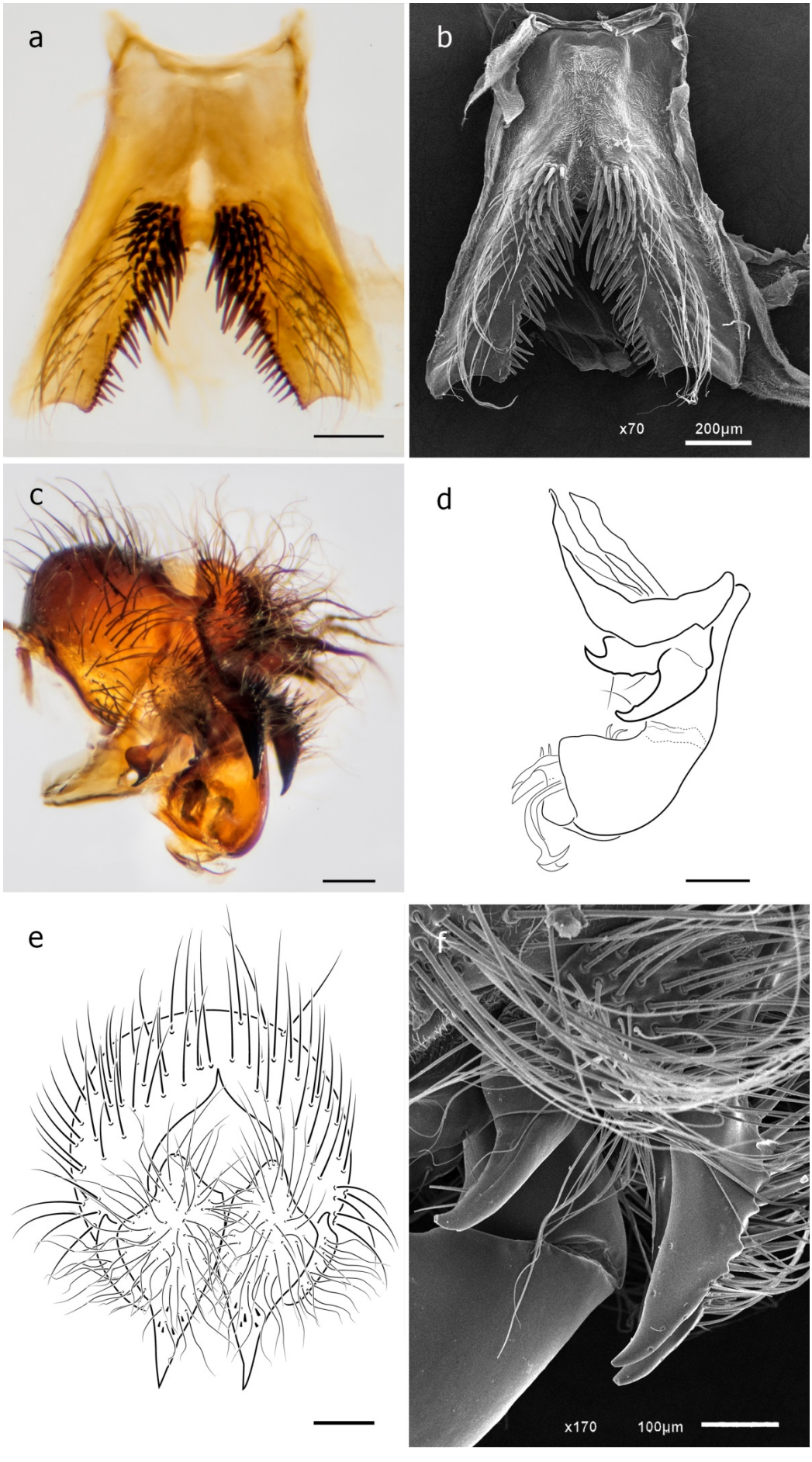

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Fig. 6 Male *I. martellata*. (a) Light micrograph of fifth sternite. Bar = 0.2 mm. (b) SEM micrograph of fifth sternite. (c) Light micrograph of male genitalia, lateral view. Bar = 0.2 mm. (d) Illustrations of male genitalia, posterior view. Bar = 0.2 mm. (e) Illustrations of cerci and surstyli. Bar = 0.1 mm. (f) SEM micrograph of cercus (C) and surstylus, lateral view.

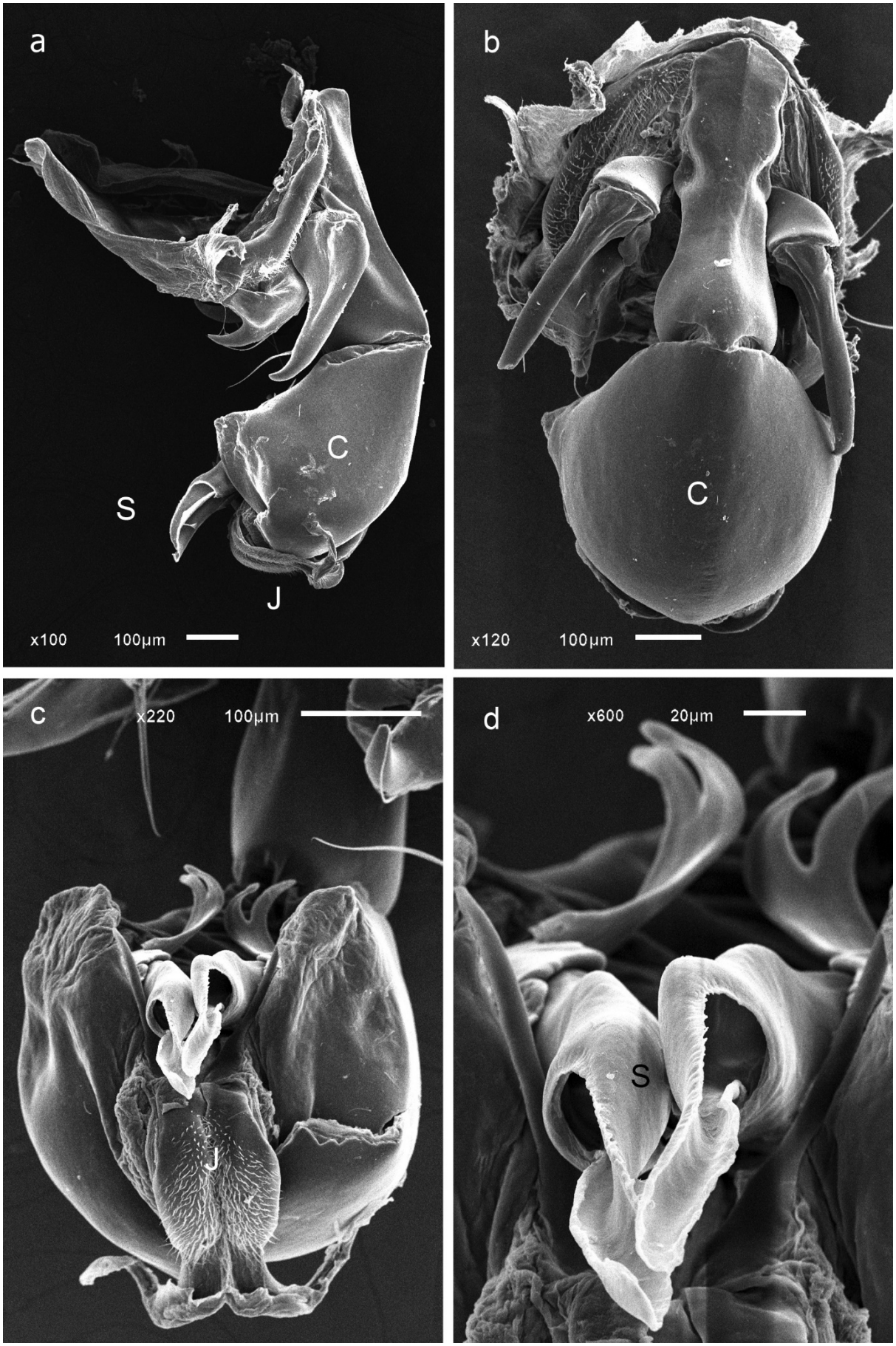

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Fig. 7 SEM micrographs of aedeagus *I. martellata*. (a) Lateral view showing large corpus (C). (b) Posterior view showing large rounded corpus (C). (c) Top view showing plough-shaped antero-lateral membranous juxta (J). (d) Higher magnification showing curved inwards and serrated stylus (S).